The leaching of water soluble phenolic compounds from cold stressed Rhododendron leaves was found to be a reliable indicator of tissue damage. In sublethally stressed leaf tissues, more and novel phenolic compounds appeared and were hypothesized to be precursors of cell wall components such as lignin and suberin. Subsequent radiolabeling of sublethally cold stressed tissues showed a preferential incorporation of the phenolic precursor phenylalanine into cell walls. The appearance of more and new phenolic compounds in intact leaves was found in four varieties of artificially cold acclimated Rhododendron.

Phenolic polymers including suberin and lignin were also found in cold hardened Azalea (Rhododendron sp.) floral buds. Hardy Azalea floral buds appear to possess an ice nucleation barrier within bud tissues, which seems to prevent ice present in the stem and scales from nucleating water in the primordia. Non-hardy buds do not have such barriers. This physical barrier can be removed by a sublethal heat treatment of the buds; treated buds showed decreased hardiness as measured by differential thermal analysis (DTA), evidenced by a decrease in the number of low temperature exotherms (LTE's). Histochemical and photomicrographical studies suggest a lignified, suberized
barrier occurs at the base of bud scales and pedicels, and beneath the bud axis.
Relationships between Endogenous Phenolic Compounds of *Rhododendron* Tissues and Organs and Cold Hardiness Development

by

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Typed by Linda Chalker-Scott for Linda Chalker-Scott
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RELATIONSHIPS BETWEEN ENDOGENOUS PHENOLIC COMPOUNDS OF RHODODENDRON TISSUES AND ORGANS AND COLD HARDINESS DEVELOPMENT

I. INTRODUCTION

A reliable indicator of environmental stress is the appearance of browning in plant tissues; it has been used equally in tissue culture systems and field samples. The phenolic compounds responsible for this reaction are too often regarded as liabilities, as their presence interferes with protein and enzyme extraction and can induce senescence in culture systems. On a more practical basis, phenolic content can impart a bitterness or woodiness to edible crops, and cause hazing or cloudiness in beer and wine production. Until recently, the beneficial properties of phenolic compounds were explored only by pharmaceutical researchers and by the forest products industry in the manufacture of resins and adhesives. The intrinsic value of these substances to the plant itself has not been widely researched.

The microbial/fungal induction of phytoalexin synthesis is probably the best studied example of phenolic stress response. Similarly, changes in phenolic production and content have been observed in conjunction with wounding, nutrient and water deficit, radiation exposure, growth regulator and herbicide application, and temperature stresses. Little work has been done, however, on the role of phenolic compounds in response to long-term environmental effects, such as seasonal temperature changes.

Temperate plants can respond in two ways to the onset of winter and cold temperatures: they can adapt, or they can die. Those that die rely upon more resistant forms, such as seeds, rhizomes, etc., to begin growth in the spring. The plants that adapt have several mechanisms by which to protect sensitive organelles, cells, tissues, and
organisms from freeze-induced dehydration and disruption. Generally this is accomplished by tolerating or avoiding freeze conditions. Although these phenomena are fairly well documented, the physical characteristics of plants which allow such behavior are not understood.

A common ornamental shrub in the Pacific Northwest is the rhododendron. Many of these species are evergreen, so must have cold resistant leaves. All species, including deciduous azaleas, have cold hardy floral and vegetative buds and are highly prized by gardeners and landscapers. The tissues also contain high levels of phenolic compounds, whose appearance is manifested in any kind of physical disruption. The possibility that these phenolics might be involved in the acclimation of rhododendrons was explored in this thesis.

To rate objectively any kind of stress-induced damage is the reason for developing viability assays. The ubiquity of phenolic compounds in plant tissues made a phenolic leaching test desirable. The visual observation method uses the browning reaction of these compounds as an indicator, but it is time consuming and subjective in nature. A spectrophotometric assay was developed, which proved to be a reliable method. During its development, it was noticed that sublethally cold stressed tissues appeared to produce phenolics as a response to the stress. To trace the fate of these compounds, leaves which had been subjected to sublethally cold temperatures were allowed to assimilate labelled phenylalanine. After just a few hours, the sublethally stressed leaves incorporated a greater percentage into the cell walls than either the controls or the lethally stressed tissues. This suggested that the result of cold stress was to increase production of phenolic precursors and thus increase cell wall thickness.

The identity of the phenolic precursors was explored next using thin layer chromatography (TLC). There is great difficulty in performing analyses of small amounts of
phenolic materials, as isolation and purification methods often lead to low recoveries of sample. Therefore, partial characterizations were made for the numerous compounds isolated by TLC. The results suggest cold hardy Rhododendron leaves have an increase in condensed tannin content and in precursors for lignin and suberin synthesis compared to non-hardy tissues. As these types of leaves survive cold temperature by tolerating ice formation, a possible function of the compounds could be to make cell walls impervious to ice nucleation from the intercellular spaces. Thus, cell contents would remain unfrozen while ice formed extracellularly.

As mentioned previously, the floral and vegetative buds also are resistant to cold temperatures; their method of survival is different from that of leaves, however. Buds of Rhododendron are known to supercool, a process which allows bud water to cool several degrees below freezing before ice formation actually occurs. The presence of some kind of ice barrier within these buds had been previously conceptualized, but its nature was not known. As sublethal stresses of different sorts are found to break dormancy or reduce hardiness in many types of plants, the effect of a sublethal heat stress on the integrity of Azalea bud barriers was examined. The temperature and time requirements necessary for removing the barrier were established, and the quantitative loss of hardiness in treated buds was determined. Microscopic examination of hardy and treated buds allowed detection of histological differences between the two, and established the actual presence of a suberized, lignified barrier. It was determined that the presence of suberin and lignin in various places within the bud allowed these tissues to supercool, while heat treatment removed the barriers either physically or enzymatically.
CHAPTER II: LITERATURE REVIEW

I. ROLES OF PHENOLIC COMPOUNDS IN PLANT STRESS RESPONSES

A. Introduction

Plant phenolic compounds belong to a loosely-named group of products called secondary metabolites. Originally, secondary products were those regarded as either metabolic wastes or substances with no role in fundamental life processes; they were also thought to be unusual compounds limited in distribution among plants. Although such sweeping definitions are no longer used, the label remains and adds to the confusion associated with secondary products.

The evolution of phenolic compounds may have been in response to the lack of motility in plants. These compounds may have offered protection from predators and from ultraviolet radiation, which was much more intense when higher plants were evolving (Lowry et al., 1980). Phenolics in particular absorb far (40-280 nm) and midrange (280-320 nm) UV, which are damaging wavelengths to proteins and nucleic acids. They also act as radical quenchers of stress-created singlet oxygen (Knox & Dodge, 1985). It seems likely that any new secondary product that did not confer some kind of advantage to the plant would eventually have been lost through natural selection, unless it was closely associated on a chromosome with a highly favorable characteristic. Therefore, most of the so-called secondary products found in present day plants probably have functions associated with increasing survival, either by coping with unfavorable environmental conditions, including predation, or by regulating metabolic processes.

Loosely speaking, phenolic compounds are those containing a benzene ring with one or more hydroxyl groups;
more specifically, they are derivatives of the shikimic acid pathway which also produces phenylalanine and tyrosine. Phenolics are ubiquitous in all higher plants and occur in some lower plants as well. Usually phenolics are esterified with sugar moieties or are likewise detoxified in the intact plant; most of them are bound to the cell wall or are stored in the vacuole. However, disruption of the cell by disease, environmental stress, or by laboratory tissue homogenization releases these compounds, which then dissociate enzymatically from their ester linkages and will quickly modify cell components such as enzyme and proteins.

Chemical modification by phenolics of proteins may occur by any of four mechanisms as shown in Figure II-1:

1) **H-bonding**: The phenolic hydroxyl group(s) can hydrogen bond to the $\delta^{-}$ carbonyl oxygen of peptide bonds at low pH's. Hydrogen bonds can not be dissociated by dialysis or gel filtration methods typically used for protein and enzyme isolation (Loomis & Battaile, 1966; Nyman, 1985).

2) **Covalent coupling**: Phenolics can quickly oxidize to form quinones enzymatically or under high pH conditions (pH 7 and up). Quinones are powerful oxidants which readily polymerize and condense with sulfhydryl, amino, or imino groups (Laties, 1974; Loomis, 1974).

3) **Ionic interactions**: At high pH’s (pH 8 and up), phenolics can also form salt bridges with basic amino acid residues (Loomis, 1974, personal communication).

4) **Hydrophobic interactions**: The aromatic ring structure of phenolics will interact with hydrophobic protein regions (Loomis, 1974, personal communication).
1) Hydrogen bonding of a phenol to a peptide bond:

\[
\begin{align*}
\text{OH} & \quad \delta^+ \quad \delta^- \\
\text{R} & \quad \text{OH} \quad \text{-----} \quad \text{O} = \text{C} \quad \text{H} \\
\end{align*}
\]

2) Oxidative coupling of phenols:

\[
\begin{align*}
\text{OH} \quad \text{R} & \quad \text{OH} \quad \text{oxidation} \quad \rightarrow \quad \text{O} \quad \text{O} \quad \text{O} \\
\text{R} & \quad \text{OH} \quad \text{SH-protein} \\
\text{NH}_2-\text{protein} & \quad \text{HN-PROTEIN} \\
\end{align*}
\]

3) Phenolic salt bridge formation:

\[
\begin{align*}
\text{OH} \quad \text{R} & \quad \text{OH} \quad \text{O}^- \quad \text{-----} \quad \text{H}_3\text{N-lys-protein} \\
\end{align*}
\]

Figure II-1. Chemical modifications by reactive phenolic compounds
By these known mechanisms, phenolics and their oxidation products can effectively tie up proteins (Ezaki-Furuichi et al., 1987), modify enzymatic active sites and thus decrease activity, and even create artifactual enzymes by blocking ionic amino acid residues such as lysine.

B. Phenolic Biosynthesis

1. Metabolism

The first committed step in phenolic biosynthesis is the removal of ammonia from L-phenylalanine by phenylalanine ammonia-lyase (PAL, E.C. 4.3.1.5). The product, trans-cinnamic acid, is the precursor for the major plant phenolic components such as lignin, suberin, flavonoids, and cinnamate esters, and other compounds including tannins and coumarins as illustrated in Figure II-2. The regulation of PAL, then, is of critical importance in any metabolic activity in the plant which requires the synthesis of such compounds. (In monocots and a few dicots, tyrosine ammonia-lyase [TAL, E.C. 4.3.1] deammonifies L-tyrosine to form p-coumaric acid. In most higher plants, however, TAL is of secondary importance and may not exist at all [Jones, 1984].)

PAL is a phytochrome-controlled, tetrameric enzyme of molecular weight 333,000; it has no co-factor requirements (Rhodes & Wooltorton, 1978). It may occur as several isozenmes, each inhibited by a different phenolic, such as cinnamic or p-coumaric acid. Reportedly PAL exists in the lumen of endoplasmic reticulum membranes (Hrazdina & Wagner, 1985), in the cytosol and in the stroma of chloroplasts (Jones, 1984), and is subject to rapid turnover in vivo (Davies, 1972).

Although regulation of PAL de novo synthesis and activity has been the subject of extensive research, its precise regulatory function is "neither clear nor
Figure II-2. An outline of the biosynthesis of some selected plant phenolic compounds

PAL = phenylalanine ammonia-lyase
TAL = tyrosine ammonia-lyase
CH = cinnamate 4-hydroxylase
predictable" (Pascholati et al., 1985). The effect of visible light upon PAL is generally to increase synthesis and activity (Jones, 1984); similarly, UV-irradiation was seen to increase both PAL activity (Lercari et al., 1986) and mRNA for PAL (Schröder et al., 1979). Other stresses, such as wounding (Camm & Towers, 1973; Pascholati et al., 1986; Thorpe & Hall, 1984), thigmomorphogenesis (de Jaeger et al., 1985), ethylene treatment (Camm & Towers, 1973; Chalutz, 1973; Rivov, 1968), nutrient deficiencies (Gershenzon, 1983), viral (Jones, 1984), fungal (Bolwell et al., 1986; Thorpe & Hall, 1984), or insect attack (Cole, 1984), growth regulator (Camm & Towers, 1973; Jones, 1984) or herbicide treatment (Kömives & Casida, 1983), and low temperatures (Graham & Patterson, 1982; Podstolski et al., 1981) have also been shown to increase either PAL synthesis or activity in a variety of plants.

In contrast, stress events do not usually inhibit PAL synthesis or activity. Decreases in synthesis or activity occur by competitive substrate inhibition of L-phenylalanine (inhibitors include D-phenylalanine, 2-aminoxy-3-phenylpropionic acid [AOPP], IAA and tryptophan [Camm & Towers, 1973]), by irreversible inhibition via cyanide and sodium borohydride (Camm & Towers, 1973), and by feedback inhibition of product derivatives (including cinnamic acid and p-coumaric acid). Recent evidence suggests cinnamic acid may modify the dehydroalanine residue of PAL's active site, leading to enzymatic inactivation but not degradation (Bolwell et al., 1986).

The next enzyme in general phenolic biosynthesis is cinnamate-4-hydroxylase. It is an irreversible cytochrome P₄₅₀ oxygenase located on the surface or within (Hrazdina & Wagner, 1985) the membranes of the chloroplast and ER. The product, p-coumaric acid, may be subjected to enzymatic hydroxylation and/or methylation, which determines the direction of subsequent biosynthesis. For this reason it is
more convenient to examine each of the three major pathways separately.

Cinnamate Ester Biosynthesis

Cinnamate ester precursors will generally have 0-2 methyl or hydroxyl substitutions on the carbons adjacent to the original hydroxyl group of p-coumaric acid; the most common of these are 4-coumaric, caffeic, ferulic, and sinapic acid (Hahlbrock & Grisebach, 1979). These acids are usually activated by 4-coumarate:CoA ligase (E.C. 6.2.1.12) and esterified with hydroxy-containing compounds such as glucose, quinic acid, and shikimic acid (Hahlbrock & Grisebach, 1979). Typically these esters, which are probably formed in the endoplasmic reticulum cisternae, are packaged into small vesicles, fuse with the central vacuole, and are released (Bell, 1981; Zobel, 1986). Such chemical and physical compartmentalization as esterification and vacuolarization serve to protect the cell from otherwise reactive compounds (Conn, 1985). A widespread response in wounded plants is increased synthesis of these cinnamic acid derivatives (Rhodes & Wooltorton, 1978), which may or may not be esterified.

Lignin & Suberin Phenolic Biosynthesis

A well-characterized hydroxylase used in lignin and suberin biosynthesis is ferulic acid 5-hydroxylase. It is a cytochrome P₄₅₀ oxygenase and is thought to be located in the microsomal fraction (Grand, 1984). Methyl transferases, utilizing S-adenosyl methionine (SAM), are required for methylation of 4-coumaric, ferulic, and sinapic acids, which are then reduced to their respective alcohols via 4-coumarate:CoA ligase. These lignin precursors are formed in the endoplasmic reticulum, packaged into vesicles, and transported to the cell walls (Northcote, 1985).
Polymerization occurs within the wall and requires the presence of free radicals, which may be initiated on cell wall bound cinnamic acid moieties (Kubitzki, 1987), via the phenol oxidase/ascorbate system. The phenol oxidases responsible for incorporating phenolic precursors into lignin and suberin are laccase, peroxidase, and tyrosinase (Crawford, 1981); of the three, peroxidase (E.C. 1.11.1.7) has been the best characterized. It is located within the cell walls, where it attaches the phenolic alcohols by oxidizing NADH and forming peroxide (Mäder & Fössl, 1982). Not surprisingly, peroxidase activity is increased by thigmomorphogenesis and ethylene (de Jaegher & Boyer, 1987).

Polyphenol oxidase (PPO), which will link groups of phenolic acids, is also stimulated by ethylene (van Lelyveld & Nel, 1982) and fungal infection (Thorpe & Hall, 1984); it has been attributed to decreased water uptake and postharvest life of cut flowers (Paull & Guo, 1985). The activities of PPO and diphenyl oxidase (DPO) appear to be limited to wounded or diseased plants, however; both Duke (1985, & Vaughn, 1982) and Rhodes and Wooltorton (1978) reject any role for phenolase-mediated synthesis of phenolic compounds in healthy plants.

Catechin and Condensed Tannin Synthesis

The precursors of catechins, also known as flavan-3-ols, are activated by 4-coumarate:CoA ligase and subsequently joined with malonate units via flavanone synthase to form the flavanone naringenin. Although the succeeding enzymatic pathway is not yet known (Hemingway & Laks, 1984), it appears naringenin is hydroxylated to form dihydroquercitin, which is then reduced to catechin (Stafford & Lester, 1984). Catechin and its cohorts occur primarily in woody tissue and leaves, are easily transported within the cell, and are thought to have significant biological activity (Harborne, 1980). Through condensation
reactions, catechins and related oligomers form condensed tannins, which because of their high molecular weight are not transported within the cell. They are, however, chemically reactive and will readily "tan" proteins when cell tissues are disrupted, lending astringency to unripe fruits and acting as an intrinsic plant defense against herbivores. Catechins, incidentally, are the major phenolic component of tea leaves (Shipilova & Zaprometov, 1978), which accounts for the "dryness" of strongly brewed tea.

Strangely enough, catechin itself is thought to be have antioxidant and free-radical scavenging properties (Slater & Scott, 1981), and may even stabilize cytoplasmic and other cell membranes in animal systems (Perissoud et al., 1981). It would seem likely that a similar protective function could be found in plant tissues.

2. Phenolic Catabolism

Relatively little is known of the biodegradation of phenolic compounds, even though their natural synthesis, turnover and degradation in all plant tissues is widely accepted (Barz & Hosel, 1975; Harborne, 1980). Barz (1977) and Ellis (1974) report a state of constant turnover for secondary metabolites, including phenolics; the best known example is the loss of juvenile anthocyanin pigments in young vegetative tissues. Crawford (1981) suggests lignin degradation may be assisted by cellobiose dehydrogenase; however, he discounts the possibility of lignin degradation via phenol oxidase activity, pointing out the probability of further polymerization of the compounds rather than their degradation. Floral and leaf flavonoid pigments are known to turn over quickly via β-oxidation, decarboxylation, demethylation, and ring-cleavage (Ellis, 1974), finally converting to CO₂ (Harborne, 1980). Little more has been discovered about the nature of autodegradation of other
phenolic polymers, although fungal enzymes have been found to degrade phenolics.

3. Cell Wall Phenolics

One of the defensive barriers to environmental stresses in plant tissues is lignin, a random, amorphous cell-wall component of all vascular plants. Typically thought to be part of the secondary cell wall, simple phenolic acids such as p-coumarate (Burritt et al., 1984) and ferulic (Carpita, 1986, Knogge & Weissenböck, 1986) have been found in the primary wall of grasses, presumably serving as cross-linkages between cellulose fibers. Although considered to be a normal cell wall thickening process, lignification can also be induced by UV-irradiation (Zhakote et al., 1979), herbicide application (Barak et al., 1984), and thigmomorphogenesis (de Jaegher et al., 1985). Wounding (Bell, 1981; Biggs, 1986a; Rickard & Gahan, 1983; Thorpe & Hall, 1984), fungal infection (Beardmore et al., 1983; Friend, 1981; Ride, 1975; Thorpe & Hall, 1974; Zhou & Wang, 1984), and ethylene (de Jaegher et al., 1985; Fouad et al., 1979; Hillis, 1977; Rhodes & Wooltorton, 1980) had lignifying effects on several plant species. Cold temperatures can also induce lignification; this relationship will be discussed in detail later. It is interesting to note that lignin laid down in response to stresses such as wounding or infection differs slightly in structure and biosynthesis to native lignin (Rhodes & Wooltorton, 1978).

Although lignin is a part of secondary thickening of cells, it is the catechins and proanthocyanidins which contribute to the "woodiness" of a plant (Haslam, 1982). Like lignin, catechins and their products are synthesized in response to biotic and abiotic stresses (Hoque, 1982), such as drought (Pizzi & Cameron, 1986) and high intensity sunlight (Waterman et al., 1984). Disease resistant
varieties of tomato have been shown to contain more tannins than susceptible types (Bhatia et al., 1972). Recent research suggests condensed tannins have a structural role, much like that of lignin (Pizzi & Cameron, 1986); they have been found on the wall surfaces of Douglas fir (Ross & Corden, 1973). Cottle & Kolattukudy (1982) suggest condensed tannins may be involved in suberin structure as well. Because they are structurally similar to lignin and suberin phenolic precursors, it seems likely they could comprise part of the cell wall by oxidative polymerization.

Suberin, a polymer with both phenolic and polyester components, is yet another defensive barrier within plant tissues. Because suberin is deposited within the cell wall, it cannot be isolated so its exact structure and composition is not known (Kolattukudy, 1984a). Unlike cutin, another waxy polymer whose composition is less than 1% phenolic, the major portion of suberin is of phenolic nature (Kolattukudy, 1980b). In contrast to the lignin phenolics, suberin phenolics generally have fewer substitutions and fewer o-methoxy groups (Kolattukudy, 1981), and contain more condensed phenolics (Kolattukudy, 1984b). The polyester component of suberin appears to consist of ω-hydroxy fatty acids and dicarboxylic acids (Kolattukudy 1984a). Originally thought to occur only in plant roots, suberin serves to reduce moisture loss through the cell wall in vegetative tissues and imbibition by seeds (Espelie et al., 1976). It also acts as a barrier to solutes and microbes, and is formed as a general response to injury and physical or biological stress (Kolattukudy, 1984b).

Wound-induced suberin or its precursors have been found in mechanically injured jade leaves, tomatoes, and beans (Bell, 1981; Dean & Kolattukudy, 1976); potato tubers (Cottle & Kolattukudy, 1982); peach tree bark (Biggs, 1986a, 1984, & Miles, 1985) and twigs (Biggs & Northover, 1985). Similarly, suberin induction occurs in response to fungal elicitors (De Leeuw, 1985; Heale et al., 1982), magnesium
deficiency (Kolattukudy & Soliday, 1985), and cold temperatures (Johnson-Flanagan & Owens, 1985; Kolattukudy & Soliday, 1985). Cold temperatures generally induce cell wall thickening; Griffith ( & Brown, 1982, et al., 1985) noticed an increase in cell wall thickness towards the vascular bundles and leaf surfaces in cold acclimated winter rye. Associated with this was an increase in the production of lipids, hypothesized to be of suberin character. Paroschy et al. (1980) found the lack of adequate suberization to be the cause of frost damage to late-maturing grape varieties. Further discussion of suberization and its role in cold acclimation will occur later in this chapter.

C. Functions of Phenolic Compounds

1. Predator, Parasite, and Competitor Inhibitors

High phenolic content in plant tissues susceptible to grazing can often act as a deterrent to herbivores. Birch leaves with the highest foliage phenolic level were found to have the fewest herbivores (Haukioja et al., 1985); similarly, the increase in condensed tannins reduced the palatability of Barteria (Waterman et al., 1984), Machaerium (Waage et al., 1984), and Quercus (Feeny & Bostock, 1968; Scalbert & Haslam, 1987) leaves to their predators. Condensed tannins, which rapidly polymerize proteins, may inhibit predator ingestion (Reese et al., 1982) or assimilation of plant tissues (Feeny & Bostock, 1968).

Antipredation mechanisms may be active, such as the responsive increase in phenolic synthesis in lettuce resistant to root aphids (Cole, 1984), or passive, as in the normal storage of phenolics within the vacuole (Hendry, 1986). Because many phenolics readily react with functional groups of salivary proteins and enzymes, they provide a general chemical defense against predation; this may be a
result of coevolution of plant and animal relationships (Beart et al., 1985). Interestingly, there is no known process in animals for detoxification of tannins (Harborne, 1980).

Not only can phenolic compounds act as herbivore deterrents, they may also function as allelopathic agents. Seeds of many plants such as sugar beet (Battle & Whittington, 1969), wild oat (Chen et al., 1982), and wheat, sorghum, and corn (Guenzi & McCalla, 1966) contain such phenolics as p-coumaric acid and ferulic acid which can leach into the soil and effectively inhibit the germination of competitors. Similarly, the commonly occurring flavanols catechin and epicatechin also inhibit seed germination (Buta & Lusby, 1986). This allelopathic effect is exemplified by black walnut and oak trees, whose soil release of juglone and salicylic acid, respectively, prevents the establishment of possible competitors. The prevention of seedling growth and establishment can be particularly long lived; soil-deposited phenolic acids were still at inhibitory concentrations twelve years after a Picea stand had been removed from the site (Kuiters et al., 1987).

2. Growth Regulation

Phenolic compounds appear to be involved in the growth status of plants intrinsically as well. Seeds may be protected from premature germination by the phenolic growth inhibitors in their outer layers (Fadl et al., 1980; Cezard, 1973; Naqvi & Hanson, 1982), thus reducing the possibility of germination during environmentally unfavorable conditions. The mechanism by which germination is inhibited may involve the enzymatic formation of impermeable suberin (Espelie et al., 1980), condensed tannins (Aastrup et al., 1984), quinones (Werker et al., 1979), and lignins within the coat (Egley et al., 1985; Aparicio-Tejo et al., 1986), which would not only make the coat impervious to water, but
would also utilize any available atmospheric oxygen. The chemical inhibition of one of these enzymes, polyphenol oxidase, was shown to break the dormancy of barnyard grass seed (Shimizu & Ueki, 1972).

Generally, seed coat phenolics decrease during stratification (Chen et al., 1982; Fadl et al., 1980; Murphy & Noland, 1981), usually with no effect on seed germination. It might be concluded, therefore, that their function is not continuously associated with maintaining seed dormancy. Instead, because they are in the outer layer of the seed, it is possible that they initially prevent germination by restricting gas and water exchange through the seed, and also protect the embryo from environmental stresses and disease. For instance, fungally-infected strawberry seeds were seen to have accelerated germination, possibly due to the removal of phenolic inhibitors (Guttridge et al., 1984).

In contrast, the presence of phenolics in vegetative and reproductive parts of the plant may actively inhibit or stimulate growth and development. Depending on their potential reactivity with proteins and enzymes, phenolics are classified as either "growth inhibitory" or "growth stimulatory." The stimulatory o-dihydroxy and trihydroxyphenols such as caffeic acid have highly reactive hydroxyl groups in a conformation that can inactivate IAA-oxidase, thus inhibiting IAA decarboxylation. In addition, Beckman et al. (1974) claim the oxidation of dihydroxyphenolics leads to the formation of tryptophan and thus to IAA. Conversely, the monohydroxyphenolics including p-coumaric acid and the non-hydroxylated cinnamic acids do not have the obligatory adjacent hydroxyl groups, and are therefore not inimical to IAA-oxidase activity; hence, they are considered to be inhibitory to growth. Figure II-3 illustrates these common phenolic acids and their growth regulation designation. There is considerable confusion over these designations in the literature, primarily because phenolics which can regulate IAA-oxidase activity can also
GROWTH STIMULATORY PHENOLICS
(IAA-OXIDASE INHIBITORY)

DI- OR TRIHYDROXY PHENOLICS -

GROWTH INHIBITORY PHENOLICS
(IAA-OXIDASE STIMULATORY)

NON- & MONOHYDROXY PHENOLICS -

cinnamic acid
\[ R_1, R_2, R_3 = \text{H} \]

p-coumaric acid
\[ R_1, R_3 = \text{H} \]
\[ R_2 = \text{OH} \]

ferulic acid
\[ R_1 = \text{OCH}_3 \]
\[ R_2 = \text{OH} \]
\[ R_3 = \text{H} \]

sinapic acid
\[ R_1, R_3 = \text{OH} \]
\[ R_2 = \text{OCH}_3 \]
\[ R_2 = \text{OH} \]

DIHYDROXY FLAVONOIDS:

MONOHYDROXY FLAVONOIDS:

Figure II-3. Structures of some IAA-stimulatory and -inhibitory phenolic compounds
presumably regulate other enzymes as well, which may or may not inhibit growth.

Studies conducted on buds or other vegetative plant parts generally support the idea of phenolic participation in plant growth (Kefeli & Kutacek, 1977). Endogenous phenolics were found to be major determinants of IAA-oxidase activity in barley (Podstolski et al., 1981) and Douglas fir seedlings (Johnson & Carlson, 1979), tomato callus (Ranade & David, 1985), and Lens sp. (Dangar & Basu, 1984). Lavee et al. (1985, 1982) suggest that chlorogenic acid (a growth stimulatory phenolic) can replace IAA in olive callus cultures. It is likely the disappearance of growth inhibition in cold-stored chestnut cuttings (Vázquez & Gesto, 1982) is due either to degradation of IAA-oxidase inhibitory phenolics or to the biosynthesis of growth stimulatory phenolic compounds. More recently, Jacobs and Rubery (1988) suggested a regulatory role for flavonoids in auxin transport mechanisms.

Other mechanisms of phenolic influence in plant growth via ABA interactions have been postulated. Ray (1986, & Laloraya, 1984) found the phenolic acids t-cinnamic, chlorogenic and tannic were antagonistic to ABA action and would restore seedling growth in radish and Amaranthus. Likewise, the phenolic compounds t-cinnamic and p-coumaric acid were found to inhibit ABA-controlled abscission (Apte & Laloraya, 1982) and suppression of amylase activity (Sharma et al., 1986). In contrast, Rodriguez and Sanchez-Tames (1986) found total phenolics, especially the acid fraction, to enhance the effect of ABA in maintaining the dormancy of hazel buds.

Sharma and Singh (1987) recently described a stimulatory effect of caffeic and tannic acids on the Hill reaction in rice; caffeic acid is an o-dihydroxy phenol, and tannic acid consists of mixed gallic acid and galloyl-glucose esters. Another possible interaction between o-
dihydroxy groups was seen by Das et al. (1984). Glutathione-transferase activity was inhibited \textit{in vivo} best by phenolic acids with one or two o-dihydroxy rings, and was not affected by gallic acid (a trihydroxy phenolic). In neither case was the mechanism of phenolic regulation discovered.

The contradictory information surrounding the regulatory action of phenolic compounds upon growth was reviewed by Kefeli and Dashek (1984). They conclude that although endogenous phenolic regulators are found outside the vacuole in compartments where they may act upon metabolic pathways, it is questionable whether the \textit{in vivo} concentration of these compounds can become sufficiently elevated to function in a regulatory manner. Nevertheless, the amount of data compiled through \textit{in vitro} and \textit{in vivo} experimentation deem it likely for phenolics to act as non-hormonal stimulators and inhibitors of plant growth.

3. Disease Resistance

The interaction between plants and biotic elicitors (fungi, bacteria, viruses, nematodes) or abiotic elicitors (UV-irradiation, freezing, heating, wounding, chemicals) often results in the production of phytoalexins (Bailey, 1982; Darvill & Albersheim, 1984). Although phytoalexins were originally defined to be antimicrobial secondary metabolites which are synthesized in response to microbial infection, their elicitation by other factors demands that the definition be broadened. Furthermore, phytoalexin production is not the only method by which plants may resist disease. As there have been numerous reviews on phytoalexins and their role in plant defense (e.g. Darvill & Albersheim, 1984; Bailey & Mansfield, 1982; Friend, 1981), this discussion will focus primarily upon mechanisms of disease resistance.
The general nature of phytoalexin induction is suggested by the appearance of "reaction zones" in tree sapwood subjected to wounding injury or fungal attack (Kemp & Burden, 1986). Similar barriers of disease-resistant, phenolic-impregnated cell walls have been found in bean (Stumpf & Heath, 1985), soybean (Köhle et al., 1984), cucumber (Hammerschmidt et al., 1985), parsley (Jahnen & Hahlbrock, 1988), radish (Friend, 1981), tomato (De Leeuw, 1985), tobacco (Zhou & Wang, 1984), cotton (Mace et al., 1978; & Howell, 1974), wheat (Beardmore et al., 1983; Ride, 1975), beech (Ostrofsky et al., 1984), Prunus (Feucht et al., 1986), banana root (Beckman et al., 1974), and peach trees (Biggs, 1986b; Wisniewski et al., 1984) as a result of wounding and/or fungal infection.

In some cases, there appears to be an "immunization" against disease by a previous infection or wounding (Beckman et al., 1974; Kuc, 1982); this concept is illustrated in the work by Ostrofsky et al. (1984). Wound altered bark of fungus-resistant beech trees was found to have a higher phenolic content than that of susceptible beech; the healthy bark of both trees were equal in their phenolic content. Similarly, peach trees that had been wounded and then subjected to fungal infection showed an increase in phenolics over and above that due to the wounding response (Wisniewski et al., 1984). Herbicide exposure of potatoes increased lignification while reducing their susceptibility to fungal infection (Barak et al., 1984). Increases of these compounds may exemplify what Mace et al. (1978) call "agents of induced systemic resistance."

Naturally resistant plants may have inherently higher phenolic contents; for instance, the roots of rot-resistant species including pecan, persimmon, and passionfruit do have higher phenolic levels than susceptible species such as apple, almond, and peach (Sztejnberg et al., 1983). More tannins and their precursors are found in disease resistant tomatoes (Bhatia et al., 1972), roses (Conti et al., 1986)
and cotton (Mace et al., 1978; & Howell, 1974), compared to susceptible varieties. Such common phenolic compounds as ferulic, o-coumaric, and p-coumaric acids inhibit fungal growth in vitro (Perradin et al., 1983); similarly, onion bulbs containing catechol and protocatechuic acid are naturally fungal resistant (Deverall, 1982). Condensed tannins, such as those found in cotton (Mace et al., 1978; Waage et al., 1984), also show antibiotic properties.

The basis for resistance may be the activity of enzymes responsible for phenolic synthesis; the acceleration of enzymatic activity is often greater in resistant than susceptible hosts (Friend, 1981). PAL, PPO, DPO, and peroxidase activities have been implicated in disease resistance (Bell, 1981; Friend, 1981). Through these enzymatic activities, biologically reactive phenolic compounds, such as oxidized flavonols, are formed which can inhibit hydrolytic enzymes and pectinases (Mace et al., 1978), or render the plant tissues impenetrable (Mace & Howell, 1974). Additionally, treatments that reduce or inhibit PAL synthesis or activity will reduce resistance to pathogens (Darvill & Albersheim, 1984). A reduction or inhibition of phenolic-synthesizing enzymes may be the reason fungus-susceptible raspberries show a decrease in o-dihydroxyphenol levels after infection, while resistant varieties have constant levels of these phenolics (Czech-Kozlowski & Krzywanski, 1984).

4. General Stress Response

In addition to acting as protective agents against fungal stress, phenolic quantity and quality are affected by other types of stresses as well. If plants are not lethally damaged, they are able to regenerate destroyed tissue or protect themselves against parasitic attack by wound-healing (Barckhausen, 1978). In response to stress, oxidation of pre-formed phenolic compounds to quinones or other polymers
may occur; in other cases, monomeric phenols are synthesized and accumulated in greater proportions than in undamaged tissues (Rhodes & Wooltorton, 1978). The end result is the formation of either a physical barrier or antibiotically active compounds.

In general, any kind of mechanical wounding will lead to increased phenolic synthesis (de Jaegher et al., 1985; Thorpe & Hall, 1984; van Loon & Gerritsen, 1986) and accumulation (Baldwin & Schultz, 1983; Barker & Peterson, 1984; Biggs, 1986a, 1984; Feucht et al., 1986; Kimmerer, in press; Kolattukudy & Soliday, 1985; Tronchet, 1976, 1975), especially in the cell walls (Biddington, 1985; Biggs, 1986a; de Jaegher et al., 1985; Rickard & Gahan, 1983). Disruption of cell wall carbohydrates with such enzymes as cellulase (Geballe & Galston, 1982) and chitosan (Köhle et al., 1984) also results in increased phenolic production and lignification.

Nutrient stress also has a marked effect on phenolic levels in plant tissues. Deficiencies in nitrogen and phosphate lead to accumulation of phenolics (DiCosmo & Towers, 1983; Nobloch & Berlin, 1983) and lignification (Farmer, 1985). Shortages of potassium and sulfur are reported to increase phenolic concentrations (Gershenzon, 1983); water deficit has variable effects (Gershenzon, 1983), but has been seen to increase root suberization (Vartanian, 1981). Magnesium deficiency is also reported to increase suberization (Kolattukudy, 1984a), while iron deficiency inhibits the process (Kolattukudy & Soliday, 1985). Interestingly enough, high concentrations of glucose (Amorim et al., 1977) and sucrose (Phillips & Henshaw, 1977) will also stimulate phenolic production.

Other stresses found to increase phenolic accumulation include cadmium toxicity (Führer, 1982), heat shock (Akin et al., 1987; Stermer & Hammerschmidt, 1984; Tronchet, 1976, 1975), and UV-irradiation (Zhakote et al., 1979); this last stress may result in phenolic-mediated oxygen radical
quenching (Knox & Dodge, 1985). The effects of cold stress upon plant phenolic levels is considered later.

It is possible that phenolic synthesis and accumulation is a generalized stress response (Hoque, 1982), perhaps occurring at the membrane level. Walker-Simmons et al. (1984) found that potato and tomato leaves developed weakened cell membranes as a response to wounding. Fungal elicitors were seen to depolarize membranes by production of ethylene (Esquerré-Tugayé et al., 1985); similarly, Glass and Dunlop (1974) found membranes were depolarized by the addition of phenolics to cell cultures, rapidly inhibiting K⁺ uptake (Glass, 1974). In both cases, membranes were made more permeable to ions. Herbicide treatment of crops also increased membrane permeability, while simultaneously increasing PAL activity and generating ethylene (Kömives & Casida, 1983). The release of either ethylene or phenolics could act as a general elicitor to the cell’s defense mechanisms.

5. Interactions with Ethylene

There exists in the literature a great deal of evidence that ethylene production and phenolic synthesis are closely related. Mapson (1970) thought p-coumaric acid to be a necessary co-factor in the synthesis of ethylene during post-harvest fruit development; later Wardale (1973) expanded upon this hypothesis by adding naringenin as an ethylene inducer. He hypothesized that these two phenolic compounds are substrates for peroxidase activity leading to ethylene production, and that other endogenous phenolics were inhibitory. Similarly, Yang (1967) found peroxidase catalysis of monohydroxy phenolics (such as p-coumaric acid) did lead to the formation of ethylene, but dihydroxy phenolics were inhibitory to its biosynthesis. An unknown phenolic is reported to inhibit the synthesis of ethylene from ACC in carnation petals as well (Itzhaki et al., 1984).
More recent information confirming these hypotheses is provided by Billot (1983) on the post-harvest physiology of pears. He observed that the ratio of monohydroxy to dihydroxy phenolics reached a maximum as ethylene synthesis increased during ripening. As mentioned previously, monohydroxy phenolics are considered to be IAA-oxidase sparing; therefore this ripening period would also have maximal IAA-oxidase activity. This observation correlates well with the results of Vioque et al. (1981), who postulated that the splitting of the ACC ring structure to form ethylene is dependent upon IAA-oxidase-generated hydrogen peroxide.

There is convincing evidence, however, that ethylene can induce the production of phenolic compounds as well. Ethylene has been seen to increase PAL activity and/or synthesis in carrot (Chalutz, 1973), lettuce (Hyodo et al., 1978), and in various fruit and vegetable tissue (Rhodes & Wooltorton, 1980). It also increases the activity of other enzymes in the phenolic pathway including peroxidase (de Jaegher et al., 1985), polyphenol oxidase (van Lelyveld & Nel, 1982), and cinnamate 4-hydroxylase:p-coumarate CoA ligase (Rhodes & Wooltorton, 1980). Lignification has been reported to increase as a result of ethylene stimulation (de Jaegher et al., 1985; Fouad et al., 1979; Hillis, 1977; Rhodes & Wooltorton, 1980). Hillis (1975) also suggests ethylene plays a role in the formation of monomeric and polymeric phenols in *Eucalyptus* exudates.

Stress conditions, such as the presence of cadmium, can induce the synthesis of ethylene and activate phenolic enzymes (Führer, 1982). In wounding studies performed on cottonwood (Kimmerer, in press) and poplar leaves (Baldwin & Schultz, 1983), increases in phenolics were seen not only in the wounded trees, but in undamaged neighboring trees as well. The presence of an airborne, transmitted signal is suggested; this signal could conceivably be ethylene.
To add to the confusion about whether ethylene stimulates phenolic production or vice versa, there is also evidence discounting ethylene's role in phenolic response. In the cadmium study mentioned earlier, the increase in phenolics was not dependent upon ethylene (Fuhrer, 1982). In another study (Engelsma & van Bruggen, 1971), no correlation was found between ethylene production and PAL synthesis or activity in intact tissues; an increase in activity was only seen in excised materials. This distinction between complete and detached tissues may help to determine the interaction, if any, between ethylene and phenolic production. It seems likely both compounds are synthesized in response to stress; however, it may be only coincidental and not a cause-and-effect mechanism.

6. Cold Stress Response

There is evidence in the literature which supports the concept of phenolic involvement in cold stress response and acclimation. Jaffe and Biro (1977), noting a general increase in cold hardiness after non-freezing stress, showed mechanically perturbed tissues were more frost-resistant than non-stressed tissues. Coincidentally, mechanically perturbed tissues also tend to have higher phenolic levels than control tissues as was discussed previously. The synthesis of PAL is seen to increase following chilling of barley seedlings (Podstolski et al., 1981), apple fruit and potatoes (Graham & Patterson, 1982), leading to an increase in flavonoids and other phenolics; a similar accumulation was noted in chestnut cuttings stored at -10°C (Vázquez & Gesto, 1982). Winter-hardy grapevines are reported to contain more tannins than do frost-susceptible varieties (Kezeli & Beridze, 1986). In addition, apple tree adaptation to cold climates was found to be associated with high levels of chlorogenic acid (Streltsina, 1980).
It has been concluded that lignification serves to increase frost tolerance of grapevine (Hubackova, 1982; Kur’yata, 1978), apple trees (Huang & Wang, 1982), and sugarcane (Sharma et al., 1981). Similarly, suberin deposition is also shown to increase resistance to cold temperatures. Suberization of white spruce roots occurred following cold temperature regimes (Johnson-Flanagan & Owens, 1985), which presumably would increase hardiness. During cold acclimation of winter rye, Griffith et al. (1985) noticed thickened cell walls and increased production of suberin-type lipids. Frost-hardened pine seedlings were also seen to increase their lipid synthesis (Selstam & Oquist, 1985). The lack of cold hardiness in late maturing grape was also attributed to inadequate suberization (Paroschy et al., 1980).

A mechanism by which suberin and lignin may protect plants from freeze damage has been suggested by Griffith et al. (1985) and Paroschy (1980): the deposition of cell wall materials could maintain the water status of tissues by acting as a water barrier. As plants acclimate, their water content decreases; if they have no mechanism by which to keep their freezable water below critical levels during thaws, they can be killed by subsequent frosts. In addition, effective water barriers would prevent desiccation injury by reducing evapotranspiration from extracellular freezing. This may account for the increased thickening seen in cell walls next to vascular bundles and towards leaf surfaces in cold acclimated winter rye (Griffith & Brown, 1982).

The importance of cell-wall associated phenolic substances in the process of cold acclimation is further enhanced by research by Bartolo and Wallner (1986). They noted that cold acclimation increases the resistance to cellulase in leaf tissues, as does wounding; wounding also induces cold hardiness in the tissues. If an increase in cold-induced phenolic synthesis also occurs, it could
facilitate a change at the cell wall-membrane interface, resulting in increased membrane adhesion to the cell wall and prevention of cell collapse during freeze-induced dehydration.

In Figure II-4, a stylized cell wall is shown, similar to one proposed by Kolattukudy and Soliday (1985). It is quite likely that the outer layers of the cell are not as distinct as once was assumed. The primary cell wall, consisting of cellulose and other sugars, has been recently found to contain cinnamate (Fry, 1984), ferulic acid (Carpita, 1986), and p-coumaric acid (Taiz, 1984) which are normally associated with the secondary cell wall. They could serve as insoluble linkages between cellulose and secondary cell wall materials. Beardmore et al. (1983) suggest wall-associated phenolic acid-protein or -carbohydrate complexes could act as the initial matrix for lignin deposition. Suberin, which is already known to be deposited between the cell wall and the plasma membrane (Kolattukudy, 1980a), would provide phenolic attachments towards the outer layers, and lipid attachments towards the membranes. This orientation could well account for the speculated increased membrane-cell wall adhesion suggested by Bartolo and Wallner (1986).

II. THE PHENOMENA OF FREEZE TOLERANCE AND AVOIDANCE IN COLD HARDY PLANTS

A. Introduction

Extensive damage to plants of commercial value occurs each year as a result of freezing temperatures. Such damage, which includes bud and blossom death, winter leaf burn, and xylem blackheart, may result from mechanical injury upon cells from ice crystal formation, or from cell dehydration as liquid water leaves the cell and becomes ice (Burke & Stushnoff, 1979). In either case, cytoplasmic and
Figure II-4. Stylized cross-section depicting cell wall layers of a hypothetical freeze-resistant plant
other membranes are damaged, leading to solute leakage and phenolic oxidative browning. In response to potential low temperature injury, temperate plant species have evolved mechanisms for cold tolerance.

A plant's ability to withstand freezing conditions has been variously termed cold tolerance, acclimation, hardiness, or resistance. By definition, induction of hardiness is via exposure to increasingly lower temperatures, associated with shorter photoperiod (Levitt, 1980); possibly there is a genetic component which also controls hardiness induction (Siminovitch, 1982). Environmental factors other than low temperature can lead to cold hardiness; Miller (1938) concluded "any treatment that checks the growth of the plant increases its resistance to cold." He cites such early examples as salt, drought, or severe pruning stress as inducers of hardiness in plants (Miller, 1938). Increases in cold hardiness have been seen more recently in plants subjected to thigmomorphogenesis (Jaffe & Biro, 1977), wounding (R. Weiser & S. Wallner, personal communication), and nutrient (Tyler et al., 1981) or water depletion (Dhawan et al., 1986). The application of growth regulators and other substances has given variable results ranging from no effect to increased or even decreased hardiness (Levitt, 1980).

Several physiological changes are associated with increasing cold hardiness. These include increases in carbohydrates, some amino acids (especially proline), soluble proteins, nucleic acids, and lipids (Franks, 1985; Levitt, 1980); the antioxidant glutathione (Guy & Carter, 1982); and lignin (Huang & Wang, 1982; Hubackova, 1982), suberin (Griffith et al., 1985; Johnson-Flanagan & Owens, 1985), and tannins (Kezeli & Beridze, 1986). In contrast, water content decreases as acclimation proceeds. These changes may also be indicative of dormancy.

While the phenomena of cold hardiness and dormancy frequently occur together, they are not necessarily mutually
dependent. In his review on environmental stresses, Levitt (1980) states that plants may be dormant but not frost tolerant, or frost tolerant but not dormant. In some cases, plant tissues may be the least resistant when they are dormant (Levitt, 1980). Generally speaking, temperate species which are capable of becoming dormant do so after sensing photoperiodic shortening; if they are able to acclimate, hardening will occur after air temperatures begin to drop. Such sequencing usually means plants become dormant before they become hardy. Similarly, these plants often break dormancy while still maintaining or even increasing their hardiness levels. Although they still do not grow under natural conditions, these plants are no longer under internal growth inhibition; instead, environmental factors (e.g. low temperatures) maintain them in a quiescent state. If brought into a warm environment, they will quickly resume growth. Therefore, non-dormant tissues can still retain their ability to resist cold stress even though dormancy is technically completed.

B. Ice Nucleation

In the absence of extrinsic or intrinsic nucleators, liquid water can reach temperatures as low as $-38^\circ C$ before freezing (Burke & Stushnoff, 1979). At this temperature, also known as the homogeneous ice nucleation temperature, spontaneous ice nucleation occurs (George et al., 1982). With the addition of solutes, this freezing point can be further depressed to $-47^\circ C$; this occurs in several woody plant species (Burke et al., 1976). Many plants, however, freeze long before this point due to the presence of ice nucleators. An ice nucleator is a substance which allows liquid water to form around it in a conformation favorable to crystal formation. Once water has begun to freeze, it quickly organizes other water molecules around it, and ice growth progresses.
Characterization of external nucleators, especially epiphytic bacteria, has been a subject of recent interest. Lindow (1983) has extensively reviewed the literature regarding freezing injury of plant tissues caused by surface bacteria acting as nucleating sites. The identity of intrinsic ice nucleators has proven to be more elusive; Ashworth et al. (1985) speculate a stable component of mature wood acts as an ice nucleator in peach trees. Presumably this nucleator would occur extracellularly on walls or within spaces, as these areas are more tolerant to ice formation.

C. Freeze Tolerance

The ability of cold hardy plants to withstand freezing temperatures can be expressed by tolerance or avoidance of ice formation within tissues. Frost tolerance, which may be linked to dormancy (Larcher, 1982), is characterized by extracellular ice formation at relatively warmer temperatures than occur in frost avoiding tissue. During this process, extracellular ice forms, creating a vapor pressure deficit; intracellular water then migrates outside the cell wall and freezes in the extracellular spaces (George et al., 1982). Atmospheric loss of this extracellular water by evaporation during repeated freeze-thaw cycles could lead to freeze dehydration, which might account for the wilted appearance of evergreen tissues during winter months.

Sakai (1982) includes the phenomena of extratissue and extraorgan freezing as freeze tolerance mechanisms. In Rhododendron buds subjected to freezing temperatures, water is apparently drawn from the tender primordia, which avoid freezing, into the outer scales where it freezes (Ishikawa & Sakai, 1982). Hence "extraorgan" freezing occurs, as the freezing takes place on the outside of and away from the organ in question (in this case the bud). Although Kaku et
al. (1982) disagree with the theory of water migration, citing instead initially low bud water content, they concur that the scales freeze long before the rest of the bud tissues.

D. Freeze Avoidance

1. Supercooling of Tissues

In contrast to tolerance, frost avoidance is a phenomenon by which water within plant cells supercools to temperatures as low as \(-41^\circ\)C before freezing (Burke & Stushnoff, 1979), either in the absence of ice nucleators or in the presence of ice growth barriers (Burke et al., 1976). This phenomenon is termed deep supercooling and appears to be confined primarily to woody plants (George & Burke, 1984) and seeds (Juntilla & Stushnoff, 1977). Olien (1965) found water-soluble cell wall polymers of rye to interact with the ice-liquid interface and thus interfere with the freezing process, resulting in small, imperfect ice crystals; such crystals appear to be non-injurious to the cell (Burke et al., 1976) and perhaps cannot act as nucleators. The tissues most often associated with deep supercooling or undercooling are xylem ray parenchyma cells and dormant flower buds; examples of the latter include cherry, peach, apricot, plum, blueberry, grape, and rhododendron species (Biermann et al., 1979; Graham & Mullin, 1976; Quamme, 1974; Wolpert & Howell, 1984). Interestingly, there are reports of leaf tissues exhibiting supercooling behavior as well (Ishikawa, 1984; Rada et al., 1987).

A convenient method of observing supercooling in plant tissues is through differential thermal analysis (DTA). The method records freezing events within tissues based on the heat of fusion (exotherm) associated with water phase transitions. By correlation with other viability tests, such as electrical conductivity and triphenyl tetrazolium
chloride (TTC) reduction, (Rajashekar et al., 1982), visible browning (Hong et al., 1980; Rajashekar et al., 1982) or vital staining (Hong et al., 1980), these exotherms may be associated with the freezing of specific tissues or water fractions within the test plant. Flower bud tissues (e.g. Rhododendron spp.) which exhibit supercooling will have an initial exotherm associated with the freezing of bulk water in the xylem and bud scales between -4 and -10°C. As the temperature continues to decrease, distinct exothermic peaks appear, each correlating to the freezing of water within a floret and death of that tissue (Graham & Mullin, 1976a; Quamme, 1978, 1974).

2. Barriers to Ice Nucleation

When ice formation is initiated via a nucleating event, the growth of ice can be quite rapid and will continue through tissues unless barred in some way. Since many cold hardened floral buds are able to delay ice formation long after the bulk water in the scales and stem of the bud has frozen, researchers have speculated about the presence of a barrier which would prevent nucleation of the supercooled bud water. Several theories have been advanced describing the nature of such a barrier.

One such theory, as presented by Quamme (1978), employs a two-barrier mechanism for the supercooling ability of peach buds. Noting the formation of ice in the scales and axes, but not primordia, of hardy peach buds, he proposed a cuticular barrier to prevent surface nucleation. Such a barrier would also help protect leaves and other above-ground tissues from environmental stress. In addition, he theorized that ice nucleation was prevented within the bud by the presence of a dry region below the primordia, which would cause a discontinuity between the frozen stem water and the supercooled water of the bud itself (Quamme, 1978). The concept of a dry region beneath the bud is supported by
Cary (1985), who reported the presence of a vapor barrier formed within the liquid phase of water beneath peach buds, causing a discontinuity of flow.

A mechanism to explain such theories states cell wall pore size is the limiting factor in the spread of ice within cold hardy plants (George & Burke, 1977). Recently, however, Wisniewski et al. (1987) discounted the importance of cell wall pore size, suggesting instead the pore size in the pit membrane may be the critical element in water movement and ice spread.

Ashworth (1982) suggests the barrier in Prunus buds is physical or thermodynamic in character. Low temperature supercooling was prevented in buds killed by heat or freezing, or in buds pierced below the bud primordium (Ashworth, 1982). The reported lack of xylem traces into the primordium in hardy Prunus buds (Ashworth, 1984; Hanson & Breen, 1985), associated with the inability of such buds to take up dye (Ashworth, 1982), led Ashworth to propose a physical, developmental barrier to ice nucleation from the stem into the flower bud.

There has been some skepticism expressed regarding the idea of a tangible barrier within cold hardy flower buds. Krasavtsev et al. (1983) conclude slow dehydration of supercooled flower primordia is due to resistance of water movement inside the primordium, not to some hypothetical barrier. They admit, however, that supercooled water within tissues does not act in this manner, since these tissues avoid dehydration stress. It seems reasonable to suppose a physical barrier to ice nucleation could exist within such primordia. The presence of a barrier is further strengthened by George et al. (1982, 1974), who found excised Azalea primordia to freeze independently of the freezing rate. In contrast, non-excised primordia froze in a dependent manner; from this information it seems clear the point of attachment of the primordium is important in supercooling activity.
Literature Cited


CHAPTER III

Cellular and Plant Physiology

SPECTROPHOTOMETRIC MEASUREMENT OF LEACHED PHENOLIC COMPOUNDS
AS AN INDICATOR OF FREEZE DAMAGE

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Additional index words. Hardiness, stress, viability test.

Abstract. Leaf discs of Rhododendron (L.) (cv. 'Jean Marie de Montague') release water-soluble phenolic compounds when subjected to lethal freezing stress. Following low temperature exposure, the levels of phenolic compounds leached from the discs were assessed by spectrophotometric measurement (260 nm). The increase in phenolics was highly correlated with other viability test results; electrolyte leakage, visual browning, ethane production, and TTC reduction had r values of .99, .99, .95, and -.88, respectively.

[Chemical names used: polyvinylpolypyrrolidone (PVPP), 2,3,5-triphenyl tetrazolium chloride (TTC)].

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Introduction

Several established methods of measuring plant viability are commonly used in assessing tissue injury resulting from environmental stress. These include TTC reduction (Towill & Mazur, 1975), electrical conductivity (Dexter \textit{et al.}, 1932; Ketchie \textit{et al.}, 1972; Wilner, 1960), visual discoloration (Stergios \& Howell, 1973; Yadava \textit{et al.}, 1984), ethylene/ethane evolution (Harber \& Fuchigami, 1986; Li \textit{et al.}, 1982), and amino acid leakage (Siminovitch \textit{et al.}, 1964). Generally, these tests measure intrinsic factors such as membrane integrity or enzymatic activity. Interpretive difficulties arise when one test is used exclusively for viability assessment: the more valid test, regrowth, is time consuming, while the more rapid methods, such as TTC reduction, can give conflicting results (Stergios \& Howell, 1973). Because there is no single absolute measurement of viability, two or more tests are often correlated to increase the reliability of the results (Blazich \textit{et al.}, 1974; Burr \textit{et al.}, 1985; Palta \textit{et al.}, 1978; Yadava \textit{et al.}, 1978). Quantitative and non-destructive viability assays are preferred since the same tissue can be used to make multiple measurements.

The objective of this study was to assess freezing stress damage of rhododendron leaf tissue by measuring phenolic leakage and simultaneously comparing this with four other viability tests. The method involves the quantitative detection of phenolic compounds, which absorb UV radiation. A similar method has been used recently to quantify salt injury in leaf tissue (Redmann \textit{et al.}, 1986).

Materials and Methods

Rhododendron (cv. 'Jean Marie de Montague') leaves were removed September 5, 1985; January 24, 1986; and April 15, 1986 from mature plants in Corvallis, Oregon and rinsed with
distilled water. Leaf discs of 10 mm were cut between major veins, moistened with distilled, deionized water and cooled 2°C/hour to the following test temperatures: 25°C (room temperature), 0, -5, -10, and -15°C. After one hour at each test temperature, the discs were thawed at 4°C for one hour and subjected to one of the following procedures:

1) Phenolic Leaching and Conductivity. Ten discs from each test temperature were individually placed in plastic scintillation vials containing 10 ml distilled, deionized water (pH < 5.5), which were then capped with rubber serum stoppers. The vials were placed on an Eberbach shaker and shaken for 24 hours at 105 rpm under a cool white fluorescent light bank (284 μmol m⁻² sec⁻¹, 16 hr light:8 hr dark) at 23.5°C. Aliquots from each vial were subsequently measured at 260 nm using a Bausch and Lomb 2000 Spectrophotometer. Immediately after these readings, electrical conductivity measurements were made on the same material using a Marksen 4403 Digital ElectroMark Analyzer pH/conductivity meter. Following these initial measurements, the vials and their complete contents were frozen at -60°C for 12 hours to kill the tissue. After thawing, the samples were shaken again for 24 hours under the light bank at 23.5°C. Final spectrophotometric and conductivity measurements were made after this time. Percent conductivity and % phenolic leakage were calculated for each sample using the ratio of the initial to the final measurements.

2) Ethane Evolution. Ten leaf discs were placed into each of four vials for each experimental temperature and were incubated in the dark at 25°C. After 8 hours, 1 ml aliquots of gas were taken from each vial and analyzed on a Carle 210 gas chromatograph with an alumina column (90°C) to determine ethane concentration as described previously (Harber & Fuchigami, 1986).

3) TTC Reduction. Twenty leaf discs from each treatment were divided equally among four plastic
scintillation vials containing .1% TTC (pH = 7.4, .05 M Na$_2$PO$_4$ buffer) and vacuum infiltrated and incubated for 12 hours. After removal of the remaining TTC solution, 10 ml of 95% ethanol were added. The vials were capped and frozen at -60°C for 12 hours, then thawed and shaken at 23.5°C for 24 hours. Spectrophotometric measurements at 485 nm were made to determine TTC reduction to the red-colored formazan (Stergios & Howell, 1973).

4) Visual Browning. Ten leaf discs from each temperature treatment were placed into petri dishes containing filter paper moistened with distilled water. Tissues were incubated for one week at 23.5°C under a cool white fluorescent light bank (284 μmol m$^{-2}$ sec$^{-1}$, 16 hr light:8 hr dark). The discs were visually rated on a scale of 1 to 5: 1) no browning, 2) <25% browning, 3) 25-50% browning, 4) 50-75% browning, or 5) 75-100% browning.

Statistical analyses were performed to determine correlation coefficients (r) between phenolic leakage and other viability tests. Calculated t-values determined the significance of each r-value.

Results and Discussion

High positive correlations were found between % phenolic leakage and % conductivity, visual browning, and ethane production with r-values of .99, .99, and .95, respectively (Figure III-1, Table III-1). TTC reduction shows a significant negative correlation (r = -.88) with % phenolic leakage. Tissues were injured between -5°C and -10°C, as evidenced by the slight increase in browning, ethane production, and phenolic leakage (Figure III-1). By -10°C, the tissues were completely killed; ethane production, % conductivity, % phenolic leakage and browning all increased dramatically, while TTC reduction declined. Corresponding LT$_{50}$ values were as follows: TTC, -3.2°C;
Figure III-1. Comparison of phenolic leachate method of analyzing freeze-induced tissue damage with visual browning, ethane production, TTC reduction, and electrical conductivity...
Table III-1. Correlation coefficient between % phenolic leakage and other methods of freeze-damage evaluation, and their corresponding t-values (data shown from September 5, 1985 experiment)

<table>
<thead>
<tr>
<th>Method</th>
<th>Correlation coefficient (r)</th>
<th>t-value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Conductivity</td>
<td>.99</td>
<td>12.15</td>
<td>**</td>
</tr>
<tr>
<td>Visual browning</td>
<td>-99</td>
<td>12.15</td>
<td>**</td>
</tr>
<tr>
<td>Ethane production</td>
<td>.95</td>
<td>5.27</td>
<td>*</td>
</tr>
<tr>
<td>TTC reduction</td>
<td>-.88</td>
<td>-3.34</td>
<td>*</td>
</tr>
</tbody>
</table>

**Significant at 1%

*Significant at 5%

\[ r = \frac{\sum x_i y_i - \frac{\sum x_i y_i}{n}}{\sqrt{\sum (x_i^2 - \frac{(\sum x_i)^2}{n}) (y_i^2 - \frac{(\sum y_i)^2}{n})}} \]

\[ t = \frac{r \sqrt{n-2}}{\sqrt{1-r^2}} \text{; df = n-2} \]

\( n \) = number of observations
visual browning, -7.2°C, % phenolic leakage, -7.3°C; ethane and % conductivity, -7.4°C.

The inconsistency of TTC as a viability indicator has been noted previously (Stergios & Howell, 1973). As seen in Figure III-1, there is no clear-cut point of tissue injury. This may be partially explained by the presence of pigments such as chlorophyll which were seen in the ethanol extract. Since the chlorophyll pigments have absorption maxima near 450 nm in ethanol, it is possible that the presence of these and other photosynthetic pigments would interfere in the spectrophotometric measurement of reduced TTC at 485 nm. [In an independent experiment, chemical changes in the TTC test were made in an effort to reduce inconsistency; the results are reported in Appendix 1].

In preliminary studies (data not presented), tissues containing large veins generally leached more phenolics than those with smaller veins. Holt and Pellett (1981) previously reported that the interveinal tissue of rhododendron leaves was the most hardy. By using leaf tissue without large veins, variability was decreased both in relative hardiness levels and leakage. Variability was also reduced by rinsing whole leaves thoroughly with distilled water before cutting the sample discs. In the UV wavelengths of interest (260-280 nm), the presence of contaminants such as bacteria could introduce artifacts into the absorbance readings. Shaking time can also be reduced; time course studies show that significant phenolic leakage can be detected after 8 hours (Figure III-2).

Measurement of phenolic leachate can be used as a reliable viability indicator throughout the year. Leaf discs from actively growing and dormant rhododendrons subjected to cold stress were assayed for damage by various indices. As shown in Table III-2, % phenolic leakage was significantly correlated with other methods of freeze-damage evaluation.
Figure III-2. Time course of phenolic leakage in cold stressed *Rhododendron* leaves as a percentage of the total leakage at 24 hours.
Table III-2. Correlation coefficients between % phenolic leakage and other methods of freeze-damage evaluation at different times of the year

<table>
<thead>
<tr>
<th>Date</th>
<th>% Conductivity</th>
<th>Browning</th>
<th>Ethane</th>
<th>TTC Red.</th>
</tr>
</thead>
<tbody>
<tr>
<td>September 85</td>
<td>.99**</td>
<td>.99**</td>
<td>.95*</td>
<td>-.88*</td>
</tr>
<tr>
<td>January 86</td>
<td>.99**</td>
<td>.99**</td>
<td>.96*</td>
<td>---</td>
</tr>
<tr>
<td>April 86</td>
<td>.99**</td>
<td>.99**</td>
<td>.99*</td>
<td>---</td>
</tr>
</tbody>
</table>

**Significant at 1%
*Significant at 5%

(Significance determined by using t-values where:
\[ t = \frac{r\sqrt{n-2}}{\sqrt{1-r^2}} \])
The UV absorption spectrum performed on the leachate of rhododendron leaf discs subjected to cold stress can also be used as an indicator of freeze damage (Figure III-3). A large difference in absorbance was found between leachates from non-lethal cold treatments (C, 0°C, and -5°C) and those from lethal cold temperatures (-10° and -15°C). The characteristic "hump" between 260 and 280 nm was considered to result from the water-soluble phenolic compounds, which have conjugated ring structures that absorb somewhere in this range. Additionally, we have found this increase in absorbance between 260 and 280 nm in freeze-damaged Douglas fir, Ponderosa pine, and noble fir needles (data not presented).

Since other viability tests rely on using compounds that also absorb in the 260-280 nm range, such as amino acids (Siminovitch et al., 1964), phenolic-binding PVPP (20% w/v) was added to the leachates from a -60°C killed treatment after the initial absorbance measurements were determined. As Figure III-4a shows, the absorbance peak between 260-280 nm disappears after treatment with PVPP, confirming that the compounds absorbing in this range are not amino acids, but PVPP-binding phenolic substances. Because of the low pH (≤ 5.5) of the leachate, phenolics were not able to bind significantly with proteins or amino acids (Loomis, 1974).

The possibility that other UV-absorbing compounds, such as soluble proteins or nucleic acid derivatives, could be contributing significantly to leachate absorbance at 260-280 nm was investigated by the addition of TCA. Figure III-4b depicts the absorption spectra of leachate from killed tissue before and after the addition of 10% TCA. As the peak between 260-280 nm changes so little, it seems apparent that the majority of UV-absorbing substances in the leachate are probably phenolic in nature. This claim is substantiated by Loomis (1974), who reports that most 260 nm absorbing materials in plants are phenolic compounds; amino
Figure III-3. Absorption spectra of water-soluble leachate from temperature-stressed _Rhododendron_ leaf discs
Figure III-4a. Absorption spectra of leachate from leaf discs killed at -60°C before and after addition of 20% (w/v) PVPP
Figure III-4b. Absorption spectra of leachate from leaf discs killed at -60°C before and after addition of 10% TCA
acid content is only 1/200 the concentration of phenolics in tissues such as apple protoplasts (Yamaki, 1984).

The measurement of phenolic leakage from injured plant tissue is rapid, reliable, and convenient. Since it is a non-destructive method as well, the same tissues can be used concurrently in other viability testing to increase the reliability of experimental results.
Literature Cited


CHAPTER IV

INCREASES OF PHENOLIC COMPOUNDS IN RHODODENDRON
LEAVES FOLLOWING COLD STRESS<sup>1</sup>

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Additional index words.  Acclimation, hardiness, resistance

Abstract. Exposure of Rhododendron (L.) (cv. "Jean Marie de Montague") leaf discs to sublethally cold temperatures causes increased synthesis of water-soluble phenolic compounds within 24 hours after treatment. Increases of these phenolics is shown both spectrophotometrically ($A_{260}$) and chromatographically. Two dimensional thin layer chromatography of all extracts reveals the presence of new phenolic compounds in sublethally stressed leaves, which may be precursors for lignin, suberin, or other cell wall associated phenolics.

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Introduction

**Phenolic Synthesis in Response to Stress:**

The quantity and quality of plant phenolic compounds and their synthesis have been seen to change in response to stresses such as wounding (de Jaegher et al., 1985), nutrition (DiCosmo & Towers, 1983), ultraviolet exposure (Zhakote et al., 1979), and fungal infection (Friend, 1981). Similar changes have been noted in various plant tissues exposed to cold temperatures (Streltsina, 1980; Vázquez & Gesto, 1982). The result of many of these environmental stresses may be to increase the plant's cold hardiness; Miller (1938) concluded "any treatment that checks the growth of the plant typically increases its resistance to cold." In woody plants, cold hardiness increases as a direct result of cold temperatures associated with short photoperiods (Levitt, 1980); however, increases in cold hardiness have also been seen in plants subjected to thigmomorphogenesis (Jaffe & Biro, 1977), wounding (R. Weiser & S. Wallner, personal communication), and nutrient (Tyler et al., 1981) or water depletion (Dhawan et al., 1986).

The synthesis of phenylalanine ammonia-lyase (PAL, E.C. 4.3.1.5), a regulatory phenolic enzyme, is seen to increase following chilling of barley seedlings (Podstolski et al., 1981), apple fruit and potatoes (Graham & Patterson, 1982), leading to an increase in flavonoids and other phenolics; a similar accumulation was noted in chestnut cuttings stored at -10°C (Vázquez & Gesto, 1982). In addition, apple tree adaptation to cold climates was found to be associated with high levels of chlorogenic acid (Streltsina, 1980).

In temperate species, foliar phenolics such as anthocyanins will increase with the onset of cold weather (McClure, 1975). Qualitative changes, such as the appearance of new glucosidic phenolics, often occur in plant materials subjected to cold temperatures (McClure, 1975);
similar differences in phenolic content were noted between young and old coffee leaves (Amorim et al., 1978). Presumably, older leaves would be more resistant to environmental stress than young, tender leaves. Tronchet (1976, 1975) previously reported the appearance and disappearance of various phenolic compounds in leaf and other tissues subjected to low temperature stress.

Phenolic substances, including lignin and suberin, are also involved in cell wall modifications. It has been concluded that lignification serves to increase frost tolerance of grapevine (Hubackova, 1982; Kur’yata, 1978), apple trees (Huang & Wang, 1982), and sugarcane (Sharma et al., 1981). Similarly, suberin deposition is also shown to increase resistance to cold temperatures. Suberization of white spruce roots occurred following cold temperature regimes (Johnson-Flanagan & Owens, 1985), which presumably would increase hardiness. During cold acclimation of winter rye, Griffith et al. (1985) noticed thickened cell walls and increased production of suberin-type lipids. The lack of cold hardiness in late maturing grape was also attributed to inadequate suberization (Paroschy et al., 1980).

Many Rhododendron species in the Pacific Northwest retain their leaves throughout the winter, in spite of freezing and sub-freezing temperatures. Leaves of these varieties apparently survive freezing damage by tolerating extracellular freezing, in which ice forms in the extracellular spaces (George et al., 1982). Previously, an increase in water-soluble phenolic compounds was seen in sublethally cold treated Rhododendron leaves (unpublished observations), which are known to be especially rich in proanthocyanidins and other phenolics (Haslam, 1982). The objective of this study was to qualify further the increase in phenolic substances and to investigate the appearance of novel compounds in sublethally cold stressed Rhododendron leaf material via thin layer chromatography (TLC).
Materials and Methods

Leaves were collected from *Rhododendron* variety "Jean Marie de Montague" during the months of September through November. The leaves were rinsed in distilled water, patted dry, and cut with a #5 cork borer between major veins to form 10 mm discs. Discs were wrapped in foil packets, placed in a Kelvinator Ultracold Freezer (Series 500), and subjected to increasingly cold temperatures at a rate of 20°C/hour as described previously (Chalker-Scott et al., 1988); test temperatures ranged from 0°C to -15°C. After each test temperature had been reached, the packets were removed and allowed to thaw at 4°C for one hour before further use.

Leaf discs from each test temperature were used either for viability testing or for phenolic extraction. The effect of the temperature treatments on viability was determined visually and by % phenolic leakage detected spectrophotometrically at A260. Three sets of five discs from each test temperature were placed in polyethylene scintillation vials containing 10 ml distilled, deionized water (pH ≤ 5.5), capped, and shaken for 24 hours as described previously (Chalker-Scott et al., 1988). Aliquots from each vial were subsequently measured at 260 nm using a Bausch and Lomb 2000 Spectrophotometer. Following these initial measurements, the vials and their complete contents were frozen, thawed, and shaken again for 24 hours (Chalker-Scott et al., 1988). After making final spectrophotometric measurements, % phenolic leakage was calculated for each sample using the ratio of the initial to the final measurements.

Tissue damage was further assayed by visual browning. Ten leaf discs from each temperature treatment were placed into petri dishes containing filter paper moistened with distilled water. After incubation for one week at 23.5°C
(Chalker-Scott et al., 1988), the discs were visually rated on a scale of 1 to 5: 1) no browning, 2) ≤25% browning, 3) 25-50% browning, 4) 50-75% browning, or 5) 75-100% browning.

Other similarly treated samples were thinly sliced and homogenized in 70% acetone:distilled water with a Tekmar Tissumizer (Model SDT-1810). Extracts were centrifuged for 10 minutes and the supernatant was decanted. The acetone extract was frozen in capped glass vials until further characterization by TLC could be performed.

**Thin Layer Chromatography:**

TLC was performed in two dimensions to separate phenolic components for further characterization. The frozen solutions were thawed and each was extracted three times with equal portions of hexane to remove chlorophyll and other large, hydrophobic molecules (Markham, 1975). The remaining material was roto-evaporated (Büchi Rotavapor RE 111) at 35°C to approximately one ml liquid (primarily water), and one ml methanol was added to each sample. TLC-Ready-Foil (Schleicher & Schuell) plates, coated with cellulose and a luminescent compound, and measuring 20 cm x 20 cm, were each cut into 4 equivalent plates measuring 10 cm x 10 cm. Samples were applied in 15 μl quantities by calibrated micropipets to the lower left-hand corner of each plate, approximately .75 mm from each edge. After the plates were completely dried, they were rotated 90° so the sample was at the bottom right hand corner, and placed two at a time into chromatography chambers (1000 ml beakers covered with aluminum foil) containing a 5 mm deep layer of TBA (3 parts t-butanol:1 part acetic acid:1 part water). Plates remained in the covered chambers until the solvent front had moved to 5 mm from the top edge of the plate. The plates were removed and dried completely at room temperature (20°C), and the chamber solution was discarded and fresh TBA was added for the subsequent plates.
Chromatography chambers containing a 5 mm layer of 6% acetic acid were used for running the plates in the second dimension. The dried plates from the TBA solution were rotated 90° back to their initial positions, with the origin at the bottom left hand corner. The plates were placed in the solution, two at a time, and the chambers were covered until the solvent front reached to 5 mm from the top edge of each plate. Plates were removed and dried, and the solution was discarded and replaced with new acetic acid for subsequent plates. All chromatograms were run at an angle to the solvent of approximately 80°, and at a temperature of 22°C.

Identification of Phenolics:

Before spray reagents were used on the completed TLC plates, they were placed in a Chromato-vue cabinet (Ultra-violet Products, Inc., San Gabriel, CA) and observed under long (366 nm max.) wavelength UV. Visible spots were outlined in pencil and the characteristics of each were noted. The plates were then subjected to ammonia fumes and color changes of the spots under visible and UV conditions were observed (Markham, 1982); these methods are all non-destructive and the plates could be reused with indicator sprays.

Two spray reagents were utilized for further characterization of the compounds; the first was a 1:1 solution of 2% ferric chloride: 2% potassium ferricyanide with a drop of potassium permanganate. Following the spraying of this solution, the chromatograms were rinsed in .1N HCl, then distilled water, and air dried. All easily oxidized phenolics, such as o-di- and tri-hydroxyphenols, turn bright blue after such treatment (Haslam, 1966). A solution of 2 g vanillin and 1 g toluene-p-sulfonylic acid in 100 ml absolute ethanol was sprayed on a second set of chromatograms, which were then heated with a blow dryer; proanthocyanidins react positively to this test by turning
bright pink (Roux & Maihs, 1960). Individual phenolic compounds and classes of compounds were identified based on comparison of these observations with those of other researchers (L.Y. Foo, personal communication; Thompson et al., 1972). (For a discussion of thin layer chromatography and phenolic identification, see Appendix 2).

Results and Discussion

Cold Hardiness of Leaf Tissues:

Leaf discs subjected to freezing stress survived \(-5^\circ\text{C}\) (Figure IV-1) as indicated by visual browning and % phenolic leakage tests. Damage occurred between \(-5\) and \(-10^\circ\text{C}\); tissues were almost completely dead by \(-10^\circ\text{C}\). Since little browning or leakage occurred at \(-5^\circ\text{C}\), it was considered to be the point of sublethal cold stress.

The differences between initial and final absorbances of the % phenolic leakage test confirmed the results of the above tests (Figure IV-2). As had previously been noted (unpublished data), there is a tendency for the sublethally cold-stressed discs to produce proportionally more soluble, UV-absorbing substances during the 24 hour incubation period (Figure IV-3). Since the bulk of the water soluble, UV-absorbing compounds are removed by the addition of polyvinylpolypyrrolidone, a specific phenolic binder (Chalker-Scott et al., 1988), the compounds are considered to be phenolic, rather than proteins, nucleic acids, or nucleotides.

Phenolic Identification:

Initial classification of phenolic compounds is invariably based on UV absorption, TLC behavior, and color reactions; for more complete identification, methods such as mass spectroscopy and nuclear magnetic resonance are necessary (Haslam, 1982). These methods often require larger amounts of pure compounds than was feasible to
Figure IV-1. Freeze injury of *Rhododendron* leaf discs as measured by visual browning and % phenolic leakage.
Figure IV-2. Differential accumulation of water-soluble phenolics in freeze-stressed leaf discs.
Figure IV-3. Representative spectrophotometric scans depicting accumulation of water-soluble phenolics in sublethally stressed leaf discs compared to control tissues.
collect in this study. Since the scope of this research was to document overall differences in phenolic content of cold stressed leaves, positive identification of phenolics was not attempted in most cases.

In leaf tissues homogenized immediately following cold stress, there were no discernible differences among the treatments; it has been noted elsewhere (Chalker-Scott & Fuchigami, Chapter V) that lethally stressed *Rhododendron* leaf discs do not die immediately and can still carry on enzymatic processes. After a 24 hour incubation, however, distinct differences were seen between control, sublethal, and lethal treatments.

Reproductions of sample chromatograms are shown in Figures IV-4 through IV-6. Catechin, epicatechin and some of their dimers (B-1, B-2) could be positively identified based on their staining properties and relative positions on the TLC plate (L.Y. Foo, personal communication). Other phenolic compounds could be partially characterized (Figure IV-7) based on these same properties (Markham, 1982).

Chromatograms made from control tissue extracts immediately and 24 hours after experimental treatment did not differ from one another. Chromatograms made from lethally stressed tissues (-10° and -15°C) 24 hours after treatment showed little separation of components, as they had been oxidized following freeze-induced tissue disruption. Sublethally stressed tissues, however, contained new compounds not seen either in the chromatograms made immediately following treatment, or in the controls.

These new compounds did not react with ammonia fumes or with vanillin, but did turn blue with ferric chloride. Based on their relative placement on the plate and their color reactions, it is possible they are simple phenolics, perhaps modified by glycosylation or methylation. Since they move relatively well in both solvent systems, it is unlikely they are large molecules.
Figure IV-4. Two-dimensional thin layer chromatograms (TLC's) of phenolic compounds extracted from control tissues stained with vanillin reagent (a) and ferric chloride (b).

P = stained pink with vanillin
L = visible under longwave UV
Figure IV-5. Two-dimensional thin layer chromatograms (TLC's) of phenolic compounds extracted from sublethally stressed tissues stained with vanillin (a) and ferric chloride (b).

P = stained pink with vanillin
L = visible under longwave UV
Figure IV-6. Two-dimensional thin layer chromatograms (TLC's) of phenolic compounds extracted from lethally stressed tissues stained with vanillin reagent (a) and ferric chloride (b).

P = stained pink with vanillin
L = visible under longwave UV
Figure IV-7. Partial identification of phenolic compounds based on UV absorption, ammonia reactions, staining properties, and relative location on TLC plates.
The appearance of new compounds after sublethal cold stress implies an active enzymatic response; cold has previously been shown to increase PAL activity (Graham & Patterson, 1982; Podstolski et al., 1981), as well as phenolic content (Vázquez & Gesto, 1982). Previous work (Chalker-Scott & Fuchigami, Chapter V) has noted an increase in cell wall incorporation of phenylalanine, the precursor for all phenolics, in response to sublethal cold stress. It is a logical conclusion that the new compounds seen by TLC in tissues subjected to the same sublethal cold stress could be precursors for new cell wall modifications. The possibility that these compounds are methylated increases the likelihood they will be used in cell wall modifications, as methylated phenolic alcohols are the building blocks of lignin and suberin, whose role in cold hardiness is just beginning to be understood.
Literature Cited


CHAPTER V

PREFERENTIAL UPTAKE OF $^{14}$C-L-PHENYLALANINE BY CELL WALLS OF SUBLETHALY COLD-STRESSED RHODODENDRON LEAVES

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Additional index words. Acclimation, lignin, resistance, suberin

Abstract. Leaf discs of Rhododendron cv. "Jean Marie de Montague" which were sublethally cold stressed incorporated proportionally more $^{14}$C-L-phenylalanine into their cell walls compared to control or lethally stressed tissues. The increases seen in total phenolic compounds and in relative incorporation of phenolic substrate into the cell wall fraction may indicate that the synthesis of cell wall component precursors is one of the plant's responses to cold temperatures.

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Introduction

A plant's ability to withstand freezing conditions and injury has been variously termed cold tolerance, acclimation, hardiness, or resistance. By definition, induction of hardiness is via exposure to increasingly lower temperatures, associated with shorter photoperiod (Levitt, 1980). Environmental factors other than low temperature can lead to cold hardiness; Miller (1938) concluded "any treatment that checks the growth of the plant increases its resistance to cold." He cites such early examples as salt, drought, or severe pruning stress as inducers of hardiness in plants (Miller, 1938). Increases in cold hardiness have been seen more recently in plants subjected to thigmomorphogenesis (Jaffe & Biro, 1977), wounding (R. Weiser & S. Wallner, personal communication), nutrient deficiency (Tyler et al., 1981), and water depletion (Dhawan et al., 1986).

Several physiological changes are associated with increasing cold hardiness. These include increases in carbohydrates, some amino acids (especially proline), soluble proteins, nucleic acids, and lipids (Franks, 1985; Levitt, 1980); the antioxidant glutathione (Guy & Carter, 1982); and lignin (Huang & Wang, 1982; Hubackova, 1982) and suberin (Griffith et al., 1985; Johnson-Flanagan & Owens, 1985). In contrast, water content decreases as acclimation proceeds.

When exposed to various stress regimes, hardy plant material will often modify cell walls by lignification (Biggs, 1986; de Jaegher et al., 1985), suberization (Biggs, 1986; Kolattukudy, 1984), or other phenolic-related processes (Hoque, 1982). Cold stress has been found to enhance thickening of cell walls (Griffith et al., 1985), presumably by increasing suberization (Johnson-Flanagan & Owens, 1985) and lignification (Sharma et al., 1981). Previous observations indicated *Rhododendron* leaves
synthesized UV-absorbing, water soluble phenolic compounds in response to sublethal cold temperatures (Chalker-Scott & Fuchigami, Chap. IV). To test whether phenolic compounds were induced by sublethal cold stress, *Rhododendron* leaves were incorporated with radiolabelled phenylalanine after exposure to various levels of cold temperatures. Subsequent cell fractionation and analysis permitted the tracing of the label within the leaf tissues.

Materials and Methods

The current season’s leaves from mature rhododendrons (cv. 'Jean Marie de Montague') were randomly collected in September 1986. After the leaves were washed in distilled water, 10 mm discs were excised using a cork borer. The discs were pooled and divided into three temperature treatments: control (4°C), sublethal cold stress (-4°C), and lethal cold stress (-8°C). Preliminary tests had established the killing temperature to be between -6°C and -8°C. Stressed leaf discs were cooled at a rate of 2°C/hour, and held at the test temperature for one hour, in a Kelvinator Ultracold Series 500 Freezer controlled by Omega Series CN-2010 Programmable Temperature Controller. Control discs were kept at a constant 4°C in a temperature-controlled room. After each freezing regime had been completed, leaves were thawed for one hour at 4°C and all treatments were then divided into three further treatment groups:

1) **Freeze damage:** To assess initial frost damage to leaf discs, % phenolic leakage, % conductivity, ethane evolution and visual browning tests were performed:

Three replicates of 5 discs from each test temperature were placed in plastic scintillation vials containing 10 ml distilled, deionized water (pH ≤ 5.5), then capped and shaken for 24 hours as described previously (Chalker-Scott *et al.*, 1988). Aliquots from each vial were subsequently
measured for absorbance at 260 nm using a Bausch and Lomb 2000 Spectrophotometer, and for conductivity with a Marksen 4403 Digital ElectroMark Analyzer pH/conductivity meter. Following these initial measurements, the vials and their complete contents were frozen at -60°C, thawed, shaken again for 24 hours, and measured for final spectrophotometric and conductivity values (Chalker-Scott et al., 1988). Percent conductivity and % phenolic leakage were calculated for each sample using the ratio of the initial to the final measurements.

Three replicates of 20 discs for each test temperature were placed in stoppered glass vials and used to determine ethane concentrations. After incubation in the dark at 25°C for 8 hours, 1 ml aliquots of gas from each vial were analyzed on a Carle 210 gas chromatograph with an alumina column (90°C) to determine ethane concentration as described previously (Harber & Fuchigami, 1986).

Discs used in ethane determinations were also used for the visual assay. Discs were placed into petri dishes containing filter paper moistened with distilled water. After incubation under lights for one week at 23.5°C, discs were visually rated on a scale of 1 to 5: 1) no browning, 2) <25% browning, 3) 25-50% browning, 4) 50-75% browning, or 5) 75-100% browning (Chalker-Scott et al., 1988).

2) Radiolabel uptake: Two uptake experiments were performed: a time course and a delayed uptake study. In the time course study, 5 leaf discs from each temperature treatment were placed in 5 ml of uptake solution (Carpita, 1986) containing 2 μCi of 14C-L-phenylalanine and were incubated under cool white fluorescent lights (284 μmol m⁻² sec⁻¹, 16 hr light:8 hr dark) at 23.5°C. During the light period at 1, 2, 4, and 12 hours, discs were removed, rinsed with distilled water three times and frozen in plastic scintillation vials in liquid nitrogen for later fractionation.
Delayed uptake of labelled phenylalanine was performed on discs from each temperature treatment stored at 4°C under cool white fluorescent light banks (284 μmol m⁻² sec⁻¹, 16 hr light:8 hr dark). After 24 and 48 hour periods of incubation at 4°C, groups of five discs were placed in 5 ml of the uptake solution containing 2 μCi of label, incubated as above for 12 hours, then rinsed and frozen as described above.

Fractionation of frozen leaf discs was based on methods described by Carpita (1982). Because of the reactivity of most phenolic compounds, care was taken in the initial steps to inhibit enzymatic or spontaneous oxidation of these materials. Leaves were ground with a frozen mortar and pestle in liquid nitrogen until a powder was formed as described by Sandstrom and Loomis (in press). Liquid nitrogen homogenation is thought to be the best method for opening the cell wall envelope, as the low temperature prevents protein modification by blocking chemical reactions (Harris, 1983; Sandstrom & Loomis, in press). The powder was scraped into tubes containing 5 ml of .05 M TES, 10 mM ascorbate, and 1% Triton-100X. The contents of the tubes were mixed, then centrifuged at 12,000g at 4°C for 5 minutes. The supernatant was decanted and reserved, and the precipitate was extracted again using 5 ml of the TES-ascorbate-Triton solution. After centrifugation and decantation, the precipitate was subjected to the following sequence of washes (using 5 ml solution each time), followed by centrifugation and decantation:

1) .5 M KH₂PO₄
2) distilled water
3) 70% ethanol at 30°C (2 hours)
4) 1:1 chloroform:methanol
5) acetone
6) 50:50 30% H$_2$O$_2$:70% perchlorate in an agitated 60°C bath (until precipitate was dissolved and uncolored; approx. 21 hours.)

The fractionation series used is an amalgam of procedures described by Carpita (1986, 1982) Giaquinta (1977), and Cottle and Kolattukudy (1982). The first extraction in TES/ascorbate/Triton X-100 will remove most soluble metabolites, soluble and some membrane bound proteins, loosely associated cytoplasmic contaminants (Carpita, 1982), and some suberin phenolics (Cottle & Kolattukudy, 1982). Washing in the phosphate buffer extracted ionically-bound proteins (Harris, 1983), and the chloroform/methanol wash removed membrane fragments (including glycolipids and phospholipids) (Carpita, 1982). By the time of the last extraction with perchlorate and hydrogen peroxide, the precipitate theoretically contained pure cell wall fragments. According to Giaquinta (1977), complete digestion of leaf material occurs under these conditions with no loss of label. The supernatants from each step were mixed with Beckman Ready-Solv MP fluor in scintillation vials and measured in a Beckman LS 7000 Microprocessor-controlled scintillation counter for 10 minutes or 10,000 counts. Resulting values were quench-corrected.

Results and Discussion

1) Initial freeze damage: The three viability assays indicate that rhododendron leaf discs subjected to the control temperature (4°C) or sublethal cold treatment (-4°C) showed little or no injury; in contrast, the -8°C treatment was severely injured or killed (Figure V-1). Phenolic production (measured as final - initial absorbance readings) at the sublethal cold temperature was greater than either the control or the lethal temperature treatment (Figure V-
Figure V-1. Freeze injury of Rhododendron leaf discs as measured by visual browning, ethane production, and % phenolic leakage.
Figure V-2. Differential accumulation of water-soluble phenolics in control, sublethally freeze-stressed, and lethally stressed leaf discs
2). Although the difference between these treatments is not highly significant (0.1 > p > 0.5), the trend of increased production of water-soluble phenolic compounds is in agreement with the results found on other occasions (Chalker-Scott & Fuchigami, Chap. IV).

2) Radiolabel uptake: In most cases during the time course study, the sublethally cold stressed tissues incorporated a greater percentage of labelled phenylalanine into its cell walls than either the control or the lethally stressed tissues (Figure V-3). In contrast, 4 hours after the stress treatment the lethally stressed tissues incorporated the greatest percentage of radiolabel. Plant tissues subjected to freezing stress do not 'die' immediately, and it is not surprising to see the enzymes of these tissues still functional at this time, perhaps involved in repair activity. In a related study, Beardmore et al. (1983) found increased incorporation of phenylalanine and cinnamic acid into the cell walls of resistant wheat following fungal infection. Similarly, Grand and Rossignol (1982) reported enhanced incorporation of phenylalanine into lignin monomers in fungally-resistant muskmelons.

Maximum % incorporation in sublethally stressed tissues decreases progressively over time, as it does in the other treatments (Figure V-4). Although the -4°C treatment incorporates relatively more than the control immediately following low temperature treatment, this trend reverses itself 12 and 24 hours after the temperature treatment. Cottle and Kolattukudy (1982) saw the greatest incorporation of labelled phenylalanine in potato discs within a day after wounding stress; similarly, Fry (1984) reports spinach cells take up and incorporate labelled cinnamate within 20 minutes. It is possible the sublethally stressed tissue more rapidly incorporates cell wall phenolics during the first 24 hours after stress, then decreasing after deposition is complete. In contrast, the control tissue may
Figure V-3. Time course of incorporation of $^{14}$C-phenylalanine into leaf discs following cold stress.
Figure V-4. Incorporation of $^{14}$C-phenylalanine into leaf discs following 0, 12, or 24 hours lag time after cold stress.
sense a 'stress' of sorts several hours after being subjected to 4°C, and could respond by maintaining constant cell wall incorporation for two days. The tissue exposed to -8°C shows a marked decrease in % cell wall incorporation after the first day, demonstrating that the cells are indeed dead.

Although this study has not determined whether the labelled cell wall components are lignin (Grand & Rossignol, 1982), suberin (Cottle & Kolattukudy, 1982), or perhaps catechin in nature (Devchoudhury & Bajaj, 1979), it is apparent that sublethally stressed rhododendron leaf discs preferentially incorporate phenylalanine into cell wall components, which may serve to increase the cold hardiness of the tissue.
Literature Cited


CHAPTER VI

CONSTITUTIVE CHANGES IN PHENOLIC COMPOUNDS
OF RHODODENDRON VARIETIES DURING COLD ACCLIMATION

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Additional index words. Resistance, stress

Abstract. Non-hardy specimens of "Holden", "Pawhuska", "Virginia Richards", and "Vulcan" varieties of Rhododendron (L.) were subjected to a cold hardening regime. Resultant leaf hardiness levels were determined throughout a 5 month period and their viabilities analyzed via visual browning and % phenolic leakage. On the dates of the freezing tests, identical plant samples were homogenized and analyzed for phenolic compounds by two dimensional thin layer chromatography (TLC). Hardiness of all varieties significantly increased one month following initiation of low temperature exposure; similarly, phenolic quantity and diversity increased during the same period.

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Introduction

Extensive damage to plants of commercial value occurs each year as a result of freezing temperatures. Such damage, which includes bud and blossom death, winter leaf burn, and xylem blackheart, may result from mechanical injury upon cells from ice crystal formation, or from cell dehydration as liquid water becomes ice (Burke & Stushnoff, 1979). In either case, cytoplasmic and other membranes are damaged, leading to solute leakage and phenolic oxidative browning. In response to potential low temperature injury, temperate plant species have evolved mechanisms for cold hardiness.

By definition, induction of hardiness is via exposure to increasingly lower temperatures, associated with shorter photoperiod (Levitt, 1980); possibly there is a genetic component which also controls hardiness induction (Siminovitch, 1982). Environmental factors other than low temperature can lead to cold hardiness; Miller (1938) concluded "any treatment that checks the growth of the plant increases its resistance to cold." He cites such early examples as salt, drought, or severe pruning stress as inducers of hardiness in plants (Miller, 1938). Increases in cold hardiness have been seen more recently in plants subjected to thigmomorphogenesis (Jaffe & Biro, 1977), wounding (R. Weiser & S. Wallner, personal communication), and nutrient (Tyler et al., 1981) or water depletion (Dhawan et al., 1986).

Several physiological changes are associated with increasing cold hardiness. These include increases in carbohydrates, some amino acids (especially proline), soluble proteins, nucleic acids, and lipids (Franks, 1985; Levitt, 1980); the antioxidant glutathione (Guy & Carter, 1982); and lignin (Huang & Wang, 1982; Hubackova, 1982) and suberin (Griffith et al., 1985; Johnson-Flanagan & Owens,
1985). In contrast, water content decreases as acclimation proceeds.

There is evidence in the literature which supports the concept of phenolic involvement in cold stress response and acclimation. Jaffe and Biro (1977), noting a general increase in cold hardiness after non-freezing stress, showed mechanically perturbed tissues were more frost-resistant than non-stressed tissues. The synthesis of phenylalanine ammonia-lyase (PAL E.C. 4.3.1.5) is seen to increase following chilling of barley seedlings (Podstolski et al., 1981), apple fruit and potatoes (Graham & Patterson, 1982), leading to an increase in flavonoids and other phenolics; a similar accumulation was noted in chestnut cuttings stored at -10°C (Vázquez & Gesto, 1982). In addition, apple tree adaptation to cold climates was found to be associated with high levels of chlorogenic acid (Streltsina, 1980).

It has been concluded that lignification serves to increase frost tolerance of grapevine (Hubackova, 1982; Kur’yata, 1978), apple trees (Huang & Wang, 1982), and sugarcane (Sharma et al., 1981). Similarly, suberin deposition is also shown to increase resistance to cold temperatures. Suberization of white spruce roots occurred following cold temperature regimes (Johnson-Flanagan & Owens, 1985), which presumably would increase hardiness. During cold acclimation of winter rye, Griffith et al. (1985) noticed thickened cell walls and increased production of suberin-type lipids. Frost-hardened pine seedlings were also seen to increase their lipid synthesis (Selstam & Oquist, 1985). The lack of cold hardiness in late maturing grape was also attributed to inadequate suberization (Paroschy et al., 1980).

A mechanism by which suberin and lignin may protect plants from freeze damage has been suggested by Griffith et al. (1985) and Paroschy (1980): the deposition of cell wall materials could maintain the water status of tissues by acting as a water barrier. As plants acclimate, their water
content decreases; if they have no mechanism by which to keep their freezable water below critical levels during thaws, they can be killed by subsequent frosts. In addition, effective water barriers would prevent desiccation injury by reducing evapotranspiration from extracellular freezing. This may account for the increased thickening seen in cell walls next to vascular bundles and towards leaf surfaces in cold acclimated winter rye (Griffith & Brown, 1982).

The importance of cell-wall associated phenolic substances in the process of cold acclimation is further enhanced by research by Bartolo and Wallner (1986). They noted that cold acclimation increases the resistance to cellulase in leaf tissues, as does wounding; wounding also induces cold hardiness in the tissues. If an increase in cold-induced phenolic synthesis also occurs, it could facilitate a change at the cell wall-membrane interface, resulting in increased membrane adhesion to the cell wall and prevention of cell collapse during freeze-induced dehydration. A similar role is postulated for catechin and proanthocyanidins naturally occurring in xerophytic plants (Pizzi & Cameron, 1986).

Within the genus *Rhododendron* there exists a wide range of cold hardiness levels, which limits the northern and altitudinal distribution of many varieties (Kaku et al., 1982). The leaves of those species which are evergreen are known to survive damage by tolerating freezing, probably by extracellular ice formation (George et al., 1982). Previous work with sublethally cold stressed rhododendron leaves has shown an increase in cell wall-incorporated phenolics (Chalker-Scott & Fuchigami, Chap. 5) and the appearance of new phenolic substances (Chalker-Scott & Fuchigami, Chap. 4). Accumulation of phenolics as a result of cold temperatures has been previously noted (Vázquez & Gesto, 1982; Podstolski et al., 1981). In addition, qualitative differences in phenolic compounds have been noted between
young and older coffee (Amorim et al., 1978) and oak leaves; older leaves would presumably be hardier than young, tender leaves. This research examines the seasonal diversity of phenolic constituents in the leaves of four varieties of evergreen Rhododendron subjected to warm and cold temperatures.

Materials and Methods

Two and three year old plants of Rhododendron varieties 'Holden', 'Pawhuska', 'Virginia Richards', and 'Vulcan' were grown in a lathhouse from January 1986 to June 1986. In June, 12 or more of these plants were moved to growth chambers (Controlled Environments Model PGW36), which were set at 22°C/18°C and illuminated with Sylvania cool white and incandescent bulbs on 10 hr light/14 hr dark schedules (210 μmol sec⁻¹ m⁻² as measured by a Li-Cor Model Li-185 Light Meter). Six weeks later, the temperature was decreased to 10°C/5°C with the same light/dark schedule. These parameters were maintained until the end of December 1986. Relative humidity was held continuously at approximately 60%.

The current year's leaves were harvested monthly from June to August, and twice a month from September to December. On each sampling date, ten leaves from each variety were harvested, washed with distilled water and blotted dry. A cork borer was used to cut 10 mm discs from the leaves, avoiding major veins. Ten discs from each variety were placed in glass test tubes containing 5 ml of 70% acetone, homogenized using a Tekmar Tissumizer (Model SDT-1810), and centrifuged with a tabletop centrifuge at 4700 rpm for 10 minutes. The supernatant was decanted into capped vials and frozen at -20°C until thin layer chromatography was performed.

The remaining discs were used for hardiness determination via freezing tests and analysis of phenolic
leakage and visual browning as described previously (Chalker-Scott et al., 1988). Leaf discs were cooled in 2-4 degree increments from 0°C to -20°C; control leaves were maintained at 4°C. After one hour at each test temperature, the discs were thawed at 4°C for one hour. Freezing rates were maintained at 2-4°C/hour via a ramp-and-soak program using Omega Series CN-2010 Programmable Temperature Controller in conjunction with a Kelvinator Ultracold Series 500 Freezer.

Three sets of five discs from each test temperature were placed in plastic scintillation vials containing 10 ml distilled, deionized water (pH < 5.5), which were then capped and shaken for 24 hours as described previously (Chalker-Scott et al., 1988). Aliquots from each vial were subsequently measured at 260 nm using a Bausch and Lomb 2000 Spectrophotometer. Following these initial measurements, the vials and their complete contents were frozen to -60°C, thawed, and shaken again for 24 hours (Chalker-Scott et al., 1988). Final spectrophotometric measurements were determined, and % phenolic leakage was calculated for each sample using the ratio of the initial to the final measurements. To measure visual browning, ten leaf discs from each temperature treatment were placed into petri dishes containing filter paper moistened with distilled water. Tissues were incubated for one week under a cool white fluorescent light bank (284 µmol m⁻² sec⁻¹, 16 hr light:8 hr dark) at 23.5°C and visually rated on a scale of 1 to 5: 1) no browning, 2) ≤25% browning, 3) 25-50% browning, 4) 50-75% browning, or 5) 75-100% browning (Chalker-Scott et al., 1988).

Thin Layer Chromatography

As described previously (Chalker-Scott & Fuchigami, Chapter IV), two dimensional thin layer chromatography (TLC) was performed to separate phenolic components for further characterization. The frozen solutions were thawed and each
was extracted three times with equal portions of hexane, then roto-evaporated to approximately 1.0 ml liquid. One ml methanol was added to each sample. TLC-Ready-Foil (Schleicher & Schuell) cellulose plates, measuring 6.67 cm x 6.67 cm, were spotted with five μl quantities of sample. Plates were placed into chromatography chambers containing a 5 mm deep layer of TBA (3 parts t-butanol:1 part acetic acid:1 part water) for separation in the first dimension, and then into chambers containing a 5 mm layer of 6% acetic acid for separation in the second dimension as described previously (Chalker-Scott & Fuchigami, Chapter 4). Identification of Phenolic Compounds

Before spray reagents were used on the completed TLC plates, they were placed in a Chromato-vue cabinet (Ultra-violet Products, Inc., San Gabriel, CA) and observed under long UV (366 nm max.) wavelength. Visible spots were outlined in pencil and the characteristics of each were noted. The plates were then subjected to ammonia fumes and color changes of the spots under visible and UV conditions were observed (Markham, 1982); these methods are non-destructive and the plates could be reused with indicator sprays.

Two spray reagents were utilized for further characterization of the compounds; the first was a 1:1 solution of 2% ferric chloride: 2% potassium ferricyanide with a drop of potassium permanganate. Following the spraying of this solution, the chromatograms were rinsed in .1N HCl, then distilled water, and air dried. All easily oxidized phenolics, such as o-di- and tri-hydroxyphenols, turn bright blue after such treatment (Haslam, 1966). A solution of 2 g vanillin and 1 g toluene-p-sulfonic acid in 100 ml absolute ethanol was sprayed on a second set of chromatograms, which were then heated with a blow dryer; proanthocyanidins react positively to this test by turning bright pink (Roux & Maihs, 1960). Individual phenolic compounds and classes of compounds were identified based on
comparison of these observations with those of other researchers (L.Y. Foo, personal communication; Thompson et al., 1972). Quantitative estimates were also made by comparing spot size of identical compounds (Randerath, 1966).

Results and Discussion

Changes in Hardiness Levels:

At the initial sampling time in July, none of the four varieties of Rhododendron were hardy past -4°C; injury occurred at 0°C for Pawhuska and Virginia Richards, -2°C for Holden, and -4°C for Vulcan tissues (Figure VI-1) as determined by visible browning. After decreasing the growth chamber temperature, hardiness significantly increased in all varieties by August (Figure VI-1, Table VI-1) and continued to increase through September. After this time, hardiness levels remained fairly constant throughout the remainder of the study.

Viability curves as determined by % phenolic leakage for three sampling dates (July 23, August 26, and November 14) for each variety (Figures VI-2 through 5) compared favorably to those obtained by visual observation (Figure VI-1).

Changes in Endogenous Phenolic Constituents:

The relative quantity and diversity of phenolics observed in TLC’s correspond positively to increased hardiness in all four varieties. TLC plates from the three sampling dates used previously (Figures VI-2 through 5) show quantitative and qualitative increases in phenolics as tissues progress from non-hardy to hardy states (Figures VI-6 through 9). The greatest difference is seen to occur between the July and August sampling dates, when the ambient temperature was decreased. Compounds which were positively
*damage must be greater than 1.1  
(one leaf out of 10 may have up  
to 25% browning)

Figure VI-1. Seasonal changes in hardiness of four Rhododendron varieties ("Holden",  
"Pawhuska", "Virginia Richards", and "Vulcan") as measured by visual  
browning
Table VI-1. Comparison of means from 7/23/86 and 8/26/86 hardiness testing as determined by visual browning (vb) and % phenolic leakage (%p); * denotes significant difference between dates for comparable temperatures and tests.

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Figure VI-2. Seasonal changes in hardiness of "Holden" as measured by % phenolic leakage. The three lines represent non-hardy, semi-hardy, and hardy leaf tissues.
Figure VI-3. Seasonal changes in hardiness of "Pawhuska" as measured by % phenolic leakage. The three lines represent non-hardy, semi-hardy, and hardy leaf tissues.
Figure VI-4. Seasonal changes in hardiness of "Virginia Richards" as measured by % phenolic leakage. The three lines represent non-hardy, semi-hardy, and hardy leaf tissues.
Figure VI-5. Seasonal changes in hardiness of "Vulcan" as measured by % phenolic leakage. The three lines represent non-hardy, semi-hardy, and hardy leaf tissues.
Figure VI-6. Two-dimensional thin layer chromatograms (TLC's) of phenolic compounds extracted from non-hardy, semi-hardy, and hardy "Holden" leaf tissues

- t-butanol : acetic acid : water (3:1:1)
- 6% acetic acid
- a= 7/23/86 (non-hardy)
b= 8/26/86 (semi-hardy)
c= 11/14/86 (hardy)
P = stained pink with vanillin
L = visible under longwave UV
f = stained blue with ferric chloride
a = 7/23/86 (non-hardy)
b = 8/26/86 (semi-hardy)
c = 11/14/86 (hardy)

Figure VI-7. Two-dimensional thin layer chromatograms (TLC's) of phenolic compounds extracted from non-hardy, semi-hardy, and hardy "Pawhuska" leaf tissues
Figure VI-8. Two-dimensional thin layer chromatograms (TLC's) of phenolic compounds extracted from non-hardy, semi-hardy, and hardy "Virginia Richards" leaf tissues.

P = stained pink with vanillin
L = visible under longwave UV
f = stained blue with ferric chloride

a= 7/23/86 (non-hardy)
b= 8/26/86 (semi-hardy)
c= 11/14/86 (hardy)
Figure VI-9. Two-dimensional thin layer chromatograms (TLC's) of phenolic compounds extracted from non-hardy, semi-hardy, and hardy "Vulcan" leaf tissues.
or partially identified via UV-absorption and selective staining are listed in Figure VI-10. There were no great differences in final leaf hardiness of the four varieties, even though they do differ in their listed hardiness levels (hardiness increases from 'Virginia Richards' to 'Vulcan' to 'Holden' to 'Pawhuska').

In all varieties, an increase was found in total phenolics and notably in proanthocyanidins (condensed tannins). Condensed tannins, especially catechin, are common chemical defenses against herbivores (Feeny & Bostock, 1968) and disease (Mace et al., 1978). These compounds may also be a structural component of the cell wall, associated with either lignin (Pizzi & Cameron, 1986) or suberin (Cottle & Kolattukudy, 1982). Both lignification (Huang & Wang, 1982; Hubackova, 1982; Kur’yata, 1978; Sharma et al., 1981) and suberization (Griffith et al. 1985; Johnson-Flanagan & Owens, 1985; Paroschy et al., 1980) are implicated in maintaining or increasing cold hardiness.

In addition to serving as a cell wall structural component, catechin may also function as a general stress resistance compound, as suggested by its ability to stabilize membranes (Perissoud et al., 1981), and its antioxidant and free radical-scavenging capacities (Slater & Scott, 1981). The effect of many stresses is the generation of free radicals; the increased presence of catechins and related phenolic compounds in the leaves of Rhododendron species could serve to protect the tissues from injury due to cold stress.
Figure VI-10. Partial characterization of phenolic compounds based on UV absorption, ammonia reactions, staining properties, and relative location on TLC plates.
Literature Cited


CHAPTER VII

AN ICE-NUCLEATION BARRIER IN COLD HARDY AZALEA BUDS:
A. THE EFFECT OF SUBLETHAL HEAT TREATMENT UPON
BARRIER INTEGRITY AND RESULTANT BUD HARDINESS

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Additional index words. Acclimation, phenolics, resistance

Abstract. Hardy buds of Azalea spp. were subjected to a
sublethal heat stress of 45°C for 2 hours. Two hours after
treatment the low temperature exotherms (LTE’s) of the
flowers were determined by differential thermal analysis
(DTA). The LTE’s of buds exposed to heat stress were either
fewer in number and/or occurred at higher temperatures than
in controls. Visual assay of the flowers following DTA
confirmed that treated flowers were killed at higher
temperatures than control flowers. Cold hardy buds which
have been pretreated as described also have lower resultant
hardiness compared to controls as evidenced by controlled
freezing experiments. Similar results were found in other
plant materials (Prunus sp.). These studies suggest the
presence of an ice barrier within the buds of the plants
which avoid freezing by supercooling, and that the barrier
is partially overcome by sublethal heat stress.

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Introduction

Supercooling of Floral Buds

Extensive damage to plants of commercial value occurs each year as a result of freezing temperatures. Such damage may result from mechanical injury upon cells from ice crystal formation, or from cell dehydration as liquid water becomes ice (Burke & Stushnoff, 1979). In response to potential low temperature injury, temperate plant species have evolved mechanisms for cold tolerance.

The ability of cold hardy plants to withstand freezing temperatures can be expressed by tolerance or avoidance of ice formation within tissues. Frost tolerant plants undergo extracellular freezing, which occurs at relatively warm temperatures. During this process, extracellular ice forms, creating a vapor pressure deficit; intracellular water then migrates outside the cell wall and freezes in the extracellular spaces (George et al., 1982). In contrast, frost avoidance is a phenomenon by which water within plant cells supercools to temperatures as low as \(-47^\circ\text{C}\) before freezing (Burke & Stushnoff, 1979). This phenomenon is termed deep supercooling and appears to be confined primarily to woody plants (George & Burke, 1984) and seeds (Juntilla & Stushnoff, 1977), perhaps because cell wall polymers modify the freezing process to form small, imperfect, (extracellular) ice crystals (Olien, 1977) which are non-injurious to the cell (Burke et al., 1976) and perhaps cannot act as ice nucleators. The tissues most often associated with deep supercooling or undercooling are xylem ray parenchyma cells and dormant flower buds; examples of the latter include cherry, peach, apricot, plum, blueberry, grape, and rhododendron species (Biermann et al., 1979; Graham & Mullin, 1976a; Quamme, 1974; Wolpert & Howell, 1984).
A convenient method of observing supercooling in plant tissues is through differential thermal analysis (DTA). The method records freezing events within tissues based on the heat of fusion (exotherm) associated with water phase transitions. By correlation with other viability tests, such as electrical conductivity and triphenyl tetrazolium chloride (TTC) reduction, (Rajashekar et al., 1982), visible browning (Hong et al., 1980; Rajashekar et al., 1982) or vital staining (Hong et al., 1980), these exotherms may then be associated with the freezing of specific tissues or water fractions within the test plant. Flower bud tissues which exhibit supercooling will have an initial exotherm associated with the freezing of bulk water in the xylem and bud scales between -4 and -10°C. As the temperature continues to decrease, distinct low temperature exotherms (LTE’s) appear, each correlating to the freezing of water within a flower and death of that tissue (Graham & Mullin, 1976a; Quamme, 1978, 1974).

A Hypothetical Ice Nucleation Barrier

When ice formation is initiated via a nucleating event, the growth of ice can be quite rapid and will continue through tissues unless barred in some way. Many floral buds are able to delay ice formation long after the bulk water in the scales and stem of the bud has frozen by a process termed extraorgan freezing (Ishikawa and Sakai, 1982). This phenomenon is thought to involve the presence of an ice barrier, whose nature has been characterized through several theories summarized below.

One such theory, as presented by Quamme (1978), employs a two-barrier mechanism for the supercooling ability of peach buds. Noting the formation of ice in the scales and axes, but not primordia, of hardy peach buds, he proposed a cuticular barrier to prevent surface nucleation. In addition, he theorized that ice nucleation was prevented
within the bud by the presence of a dry region below the primordia, which would cause a discontinuity between the frozen stem water and the supercooled water of the bud itself (Quamme, 1978). The concept of a dry region beneath the bud is supported by Cary (1985), who reported the presence of a vapor barrier formed within the liquid phase of water beneath peach buds, causing a discontinuity of flow.

A mechanism to explain such theories states cell wall pore diameter is the limiting factor in the spread of ice within cold hardy plants (George & Burke, 1977). Recently, however, Wisniewski et al. (1987) discounted the importance of cell wall pore size, suggesting instead the pore size in the pit membrane may be the critical element in water movement and ice spread.

Ashworth (1982) suggests the barrier in Prunus buds is physical or thermodynamic in character. Low temperature supercooling was prevented in buds killed by heat or freezing, or in buds pierced below the bud primordium (Ashworth, 1982). The reported lack of xylem traces into the primordium in hardy Prunus buds (Ashworth, 1984; Hanson & Breen, 1985), associated with the inability of such buds to take up dye (Ashworth, 1982), led Ashworth to propose a physical, developmental barrier to ice nucleation from the stem into the flower bud.

There has been some skepticism expressed regarding the idea of a tangible barrier within cold hardy flower buds. Krasavtsev et al. (1983) conclude slow dehydration of supercooled flower primordia is due to resistance of water movement inside the primordium, not to some hypothetical barrier. They admit, however, that supercooled water within tissues does not act in this manner, since these tissues avoid dehydration stress. It seems reasonable to suppose a physical barrier to ice nucleation could exist within such primordia. The presence of a barrier is further strengthened by George et al. (1982, 1974), who found
excised azalea primordia to freeze independently of the freezing rate. In contrast, non-excised primordia froze in a dependent manner; from this information it seems clear the point of attachment of the primordium is important in supercooling activity.

Supercooling of Rhododendron and Azalea Buds

Cold-hardy flower buds of the genus Rhododendron, which include those of the subgenus Azalea, also undergo supercooling (George et al., 1982; Graham & Mullin, 1976a and b; Ishikawa & Sakai, 1981; Iwaya-Inoue & Kaku, 1983; Kaku et al., 1980; Sakai et al., 1986; Sakai & Malla, 1981); they are the most frost-sensitive above-ground organs of the plant (Sakai et al., 1986). In natural habitats, the degree of cold hardiness will increase with increasingly cold winters (Sakai & Malla, 1981), although there must be a theoretical limit. Kaku et al. (1982) report the main factor in determining northern latitude or high altitude distribution of Rhododendron is hardiness of the flower buds.

It is unclear whether Rhododendron buds undergo slow dehydration by water migration into the scales during supercooling; Ishikawa and Sakai (1982) maintain this is true, although Kaku et al. (1982) found no significant water loss in buds or water gain in scales of Azalea during low temperature exposure. This lack of dehydration could be due to the absence of transpiration in dormant, cold-hardy buds. It may also explain the reports of non-functional or incomplete xylem in Prunus buds (Ashworth, 1984; Hanson & Breen, 1985), since non-transpiring buds would have little xylem activity.

1 This statement appears to be in dispute, as some researchers claim the leaves to be less hardy than the flower buds (Iwaya-Inoue & Kaku, 1983).
Although the phenomena of dormancy and cold acclimation in rhododendron species appear to be independent of one another (Chalker-Scott, personal observations; Kaku et al., 1983; Levitt, 1980), both can be overcome by heat treatments (Kaku et al., 1983; Pemberton & Wilkins, 1980). Azaleas have been seen to break dormancy (Pemberton & Wilkins, 1980) and deharden (Kaku et al., 1983) following high temperature exposures. The use of relatively higher "heat shock" temperatures on plant material results in cell wall related changes similar to those found in response to other stresses.

Short exposure of cucumbers to 50°C temperatures has been shown to increase ethylene (Stermer & Hammerschmidt, 1985) and cell wall-associated peroxidase activity (Stermer & Hammerschmidt, 1984). Interestingly, such heat shock both causes fungus-resistant seedlings to become susceptible, and makes susceptible strains more resistant (Stermer & Hammerschmidt, 1984). Obviously, some kind of change is occurring at the cell wall level, both forming and destroying fungal barriers. It seems reasonable to assume that cold hardy azalea buds, subjected to sublethally warm "heat shock" temperatures, could lose their supercooling ability by a mechanism which removes the ice nucleation barrier.

In the following experiments, we exposed cold hardy Azalea buds to a previously determined (Chalker-Scott & Fuchigami, Chapter VIII) sublethal heat stress. Our objectives were:

1. to confirm the presence of a physical ice nucleation barrier in cold hardy buds;
2. to establish the reliability of DTA as a measure of bud hardiness;
3. to observe supercooling abilities of flowers killed by heat and cold treatments;
4. to determine the effect of sublethal heat stress on barrier integrity and resultant bud hardiness;
5. to investigate removal of the barrier by sublethal heat treatment in other genera.

Materials and Methods

Floral buds were excised from mature, naturally-growing, cold hardy Azalea species in Corvallis, Oregon, December through February (1986-1988). Tissues were utilized for the following experiments:

Establishment of the presence of a physical ice nucleation barrier in Azalea buds by DTA:

During January through April of 1987, several hardy and non-hardy buds with approximately .5 cm of stem tissue were placed in aluminum foil cylinders and attached to AWG #30 copper-constantin thermocouples. One thermocouple in each experiment was attached to a piece of dried wood for reference temperature measurements. The tissues and thermocouple head were attached to an aluminum chamber with holes corresponding to each of the test tissues. The entire unit was cooled at a rate of 4 to 8°C per hour in an Ultracold Kelvinator Series 500 freezer. Exothermic events resulting from the freezing of water from various bud tissues were measured on a Honeywell Electronik 196 recorder. Graphical data from the recorder were used to confirm the presence or absence of LTE’s in bud tissue.

To establish the physical nature of the barrier, some hardy buds, having been analyzed by DTA, were pierced with a 36 gauge syringe needle through the stem into the area beneath the flowers of the bud. Following tissue disruption, DTA was performed again, and graphs from both DTA’s were compared.

Relationship of DTA-generated LTE’s with flower mortality:

During February 1988, ten heat treated and ten control buds were prepared and subjected to DTA as described above;
freezing rates were increased to $30^\circ C$/hour. The temperature of the freezer was visually monitored, and when it registered $-10^\circ C$, the experiment was halted and tissues were thawed and incubated in distilled water at $25^\circ C$ for 4 days. Flowers were then excised from the buds and were rated visually as living (green) or dead (brown); these figures were compared with the corresponding DTA graph for each bud.

**Supercooling abilities of lethally stressed buds:**

Ten cold hardy buds on approximately 2 cm stems were used for each of three treatments. Control and heat killed buds were placed into 150 x 25 mm glass test tubes containing 3 ml of distilled water; controls were stored in a refrigerator at $4^\circ C$, while the heat treatment tube was placed in a water bath at $80^\circ$ for 5 minutes. Freeze killed buds were placed directly in a liquid nitrogen bath ($-196^\circ$) for 5 minutes. Half of the treated and control tissues were then analyzed by DTA, and the presence of low temperature exotherms (LTE) was reported. The cut ends of the remaining tissues were placed in distilled water, incubated at $25^\circ$ for 2 weeks, and visually assayed for damage.

Limitations in the aforementioned DTA equipment restricted analysis to one bud per freeze treatment. To increase efficiency and obtain statistically valid DTA results, the following experiments were performed at the National Clonal Germplasm Repository in Corvallis, Oregon. Bud stems were trimmed to .5 cm, and each bud was placed in an aluminum foil cylinder and attached to 36 gauge chromel-constantan thermocouples. The samples were placed in small wells drilled into an aluminum block, corresponding to each thermocouple attachment. For reference temperature measurements, each sample thermocouple was paired with a similar thermocouple attached to a piece of dried wood. This entire apparatus was placed in a Revco Ultralow Freezer (ULT-1735 AMA) set at $-50^\circ C$. Although the best supercooling can be seen at slow freezing rates of $1^\circ$/hour (Kaku et al., 1980), the freeze rates used for these DTA were
approximately \(30^\circ\)\text{/hour}; this serves merely to raise the freezing temperature of the primordia (George et al., 1982). The DTA graphs made previously at slower cooling rates (4-8\(^{0}\)/hr) were used for comparison, so the differences can be better distinguished between control and heat treated buds; this also verified the results of the fast freezing procedure.

Attached to the thermocouple board was a CR-7 Data Logger (Campbell Scientific, Inc.), which compiled sample thermocouple information obtained during freezing tests onto a cassette tape (Montano et al., 1987). After completion of each freeze, the cassette tape information was transposed to computer disk via Campbell Scientific's C20 Cassette Interface. Manipulation of these data using dBase program 'shuffle' allowed the consecutive use of Lotus programs 1-2-3 and Printgraph to view the data graphically. The number and temperatures of sample freezing events were obtained from these figures. (For a more detailed explanation of these procedures, see Appendix 2.)

After DTA was performed, the buds were removed from the aluminum block and dissected so the number of flowers per bud could be counted; this information was used to determine the percentage of flowers per bud which had LTE’s. All LTE temperatures were determined and analyzed from the DTA graphs for both treatments.

**Sublethal heat treatment and bud barrier integrity:**

In each of four separate experiments performed December 1987 - February 1988, 20 intact buds on approximately 2 cm stems were placed in each of two 150 x 25mm glass test tubes containing three ml of distilled water. Control materials were stored in a refrigerator at \(4^\circ\)C, while treatments were immersed in a water bath at \(45^\circ\). All materials were incubated at temperature for 2 hours, then maintained at \(25^\circ\) for 2 hours before DTA was performed.

Other control and heat-treated buds not used for DTA were left at \(25^\circ\)C in water for several days to insure they
had not been irreparably damaged by experimental methods, and so signs of bud break could be observed.

**Resultant hardiness of sublethally-stressed Azalea buds:**

Ten cold hardy Azalea flower buds were collected in February 1988 and subjected to sublethal temperatures of 45°C for 2 hours as previously described; an equal number of buds were held at 4°C for 2 hours as a control. Following a 2 hour recovery period at 25°C, some of the tissues were analyzed by DTA at the National Clonal Germplasm Repository as described above. Other treated and control tissues were placed in glass test tubes containing 2 ml distilled water and held at 0°C for .5 hour in a Cryomed Model 990C Freezer (microcomputer controlled, Model 1010). The tissues were then cooled at a rate of -8°/hour to -20°C; sample tissues were removed at 2°C intervals. After freezing, the tissues were allowed to thaw slowly at 4°C, and were then held at 25°C for four days. The buds were dissected and the number and position of viable flowers were recorded.

**Removal of the ice nucleation barrier by sublethal heat stress of hardy peach floral buds:**

In February 1988, 5 buds of 'Criterion' apple, 'Comice' pear, 'Lambert' cherry, 'Greengage' plum, and 'Curless' peach collected from the resident instruction plot of Oregon State University's Lewis-Brown farm (Corvallis, OR) were examined for the presence of LTE's by DTA. Peach buds (*Prunus* sp.), which exhibited LTE's, were selected as the test material for sublethal heat treatment. Buds with a small portion of stem attached were treated at 45°C in the same manner as previously described. Treated and control tissues were then analyzed by DTA, and the presence of LTE's was reported.

Means of sample populations within each of the above described experiments were statistically compared, and significance was determined via t-tests.
Results and Discussion

Evidence of a physical ice nucleation barrier in cold hardy Azalea buds:

Two representative DTA tracings of a hardy and a non-hardy Azalea bud are shown in Figure VII-1 and VII-2, respectively. Each distinct exothermic peak seen after the initial freezing of bulk water indicates the freezing of a single flower. The freezing of the flowers with the bulk water in the non-hardy bud (Figure VII-2) can be assumed based on the absence of distinct LTE’s for these flowers (M.J. Burke, personal communication).

Earlier, Ashworth (1982) had demonstrated that piercing of cold hardy peach buds with a fine gauge needle resulted in loss of supercooling ability. Similarly, Azalea buds with LTE’s such as those illustrated in Figure VII-1, which were subjected to piercing, also lost their LTE’s, as illustrated in Figure VII-3. This suggests the presence of a physical barrier to ice penetration through the bud axis into the flowers, which can be mechanically altered as noted previously by Ashworth in peach buds (1982).

Reliability of DTA as a measure of cold hardiness:

During a partial freeze to -10°C, only 38% of the cold hardy Azalea flowers were killed as evidenced by visual browning (Table VII-1). These results were verified by DTA, as evidenced by the number of LTE’s compared to the number of dead flowers in control tissues. The reliability of DTA as a measure of plant hardiness had been previously established for Rhododendron tissues (George et al., 1974; Graham & Mullin, 1976b; Iwaya-Inoue & Kaku, 1983). These researchers also found the number of floral primordia to be similar to the number of LTE’s recorded.

The relative position of the flower within the bud has no relationship to hardiness (Table VII-2). This indicates the freezing of the flowers is entirely random in Azalea...
Figure VII-1. Differential thermal analysis (DTA) of a cold hardy Azalea bud
Figure VII-2. Differential thermal analysis (DTA) of a non-hardy *Azalea* bud
DECREASING TEMPERATURE (°C)

Figure VII-3. Differential thermal analysis (DTA) of a cold hardy Azalea bud following piercing of bud axis with a fine gauge needle
Table VII-1. Comparison of % flower mortality at $-10^\circ C$ between control and heat-treated Azalea buds as determined by visual browning

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<td>37.96 ± 23.76</td>
<td>90.71 ± 19.66</td>
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</tbody>
</table>

Comparison of means: $t = 5.13$ (18 df)

$p < .001$

where $t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{sp^2 \left( \frac{1}{n_1} + \frac{1}{n_2} \right)}}$
Table VII-2. Percentage of flowers from 10 control and 10 heat treated Azalea buds killed at $-10^\circ C$ relative to position within bud

<table>
<thead>
<tr>
<th>Flower position*</th>
<th>% flowers killed by $-10^\circ C$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>62.5</td>
</tr>
<tr>
<td>3</td>
<td>82.5</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>33</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>82.5</td>
</tr>
<tr>
<td>8</td>
<td>51</td>
</tr>
<tr>
<td>9</td>
<td>---</td>
</tr>
</tbody>
</table>

*1 = basal position
buds, as opposed to the greater hardiness of basal flowers of blueberry as reported by Biermann et al. (1979).

Supercooling abilities of lethally stressed cold hardy buds:

The question of whether dead flowers have LTE's has been debated by researchers; Biermann et al. (1979) claim dead blueberry buds have no LTE's while George et al. (1974) find dead flowers of Azalea do have LTE's. Table VII-3 lists the mean percentages of flowers with LTE's in control, heat-killed, and frost-killed tissues. The highly significant results indicate heat-killed buds have no LTE's, while control buds and those killed in liquid nitrogen do have LTE's. This may indicate a chemical change occurring in heat-treated and heat-killed buds, resulting in the loss of the ice nucleation barrier. It may also help explain discrepancies in the literature regarding LTE's for dead buds, as the means of death may affect supercooling behavior.

Effect on bud barrier integrity following sublethal heat stress:

It had been established previously that a 2 hour, 45°C treatment was not lethal to cold hardy Azalea buds; (Chalker-Scott & Fuchigami, Chapter VIII). Buds left at room temperature following 45°C treatment and observed several days later substantiate this claim, as none showed damage and after December occasionally broke dormancy faster than the controls (data not presented).

A 2 hour, 45°C heat stress reduced the supercooling ability of hardy Azalea buds (Table VII-4). The percentage of flowers with LTE's decreased significantly as a result of heat treatment; the other flowers froze with the bulk water in the stem and scales (M.J. Burke, personal communication). In those cases where LTE's did not disappear, they occurred at warmer temperatures relative to control tissues (Figure VII-4).
Table VII-3. Comparison of percentage of flowers with LTE's among control, heat killed, and frost-killed Azalea buds

<table>
<thead>
<tr>
<th></th>
<th>4°C</th>
<th>80°C</th>
<th>-196°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>93.8±8.5</td>
<td>12.34±11.5</td>
<td>80.72±23.6</td>
</tr>
</tbody>
</table>

Comparison of means:

- **4°C : 80°C**: 12.73 ***
- **4°C : -196°C**: 1.17 NS

*** p < .001

\[ t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{sp^2 \left( \frac{1}{n_1} + \frac{1}{n_2} \right)}} \]
Table VII-4. Hardiness of cold hardy *Azalea* buds subjected to 45°C for 2 hours as measured by the appearance of LTE's from DTA

<table>
<thead>
<tr>
<th>Date</th>
<th>4°C</th>
<th>45°C</th>
<th>4°C</th>
<th>45°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>12/9/87</td>
<td>77 ± 25</td>
<td>32 ± 12</td>
<td>-12 ± 1.7</td>
<td>-11 ± 1.7 **</td>
</tr>
<tr>
<td>12/22/87</td>
<td>77 ± 17</td>
<td>41 ± 9</td>
<td>-12 ± 1.9</td>
<td>-10 ± 1.9 ****</td>
</tr>
<tr>
<td>1/14/88</td>
<td>88 ± 20</td>
<td>52 ± 14</td>
<td>-12 ± 1.5</td>
<td>-11 ± 1.7 *</td>
</tr>
<tr>
<td>2/4/88</td>
<td>90 ± 15</td>
<td>49 ± 19</td>
<td>-11 ± 2.0</td>
<td>-9 ± 1.7 ***</td>
</tr>
</tbody>
</table>

* .05 > p > .025  (Statistics done via t-test comparison of means, where: *
** .025 > p > .01
*** .005 > p > .001 \( t = \frac{x_1 - x_2}{\sqrt{sp^2 \left( \frac{1}{n_1} + \frac{1}{n_2} \right)}} \)
**** p < .001
Figure VII-4. DTA's of a cold hardy Azalea bud (——) and a cold hardy Azalea bud subjected to 45°C for 2 hours (----)
The percentage of flowers with LTE's increases seasonally in the control tissues (Table VII-4); there is a significant difference between late December and early February percentages (.025 > p > .01). A similar seasonal increase in flower hardiness has been noted previously in blackberry buds (Warmund et al., 1988). The heat-treated tissues also show an increase in flowers with LTE's as winter progresses, although the difference between means of sampling periods is not significant due to the large variance. Mean LTE temperatures are also recorded for each sampling date (Table VII-4). Most differences between means were highly significant as evidenced by the p-values.

**Resultant hardiness of heat-treated buds:**

Buds which had been subjected to sublethal heat stress were found to be less hardy than the controls as evidenced by % flower survival. In the test performed February 1988 (Table VII-4, February data), the control buds had a significantly higher number of supercooling flowers than do the buds treated with sublethal temperatures. The result of this decrease is manifested in the resulting hardiness of the buds.

Figure VII-5 graphically depicts the % surviving flowers at each temperature for control and heat treated buds. There is no difference in survival through -8°C; below this point, however, the heat treated buds begin to die, with an LT_{50} of -12.8°C. In contrast, 100% of the control buds survived past -12°C, and have an LT_{50} of approximately -15.4°C. Table VII-5 compares the mean survival of the flowers for the two treatments between -10°C and -20°C; a highly significant difference in mean survival occurred at -14°C. No difference between the two treatments were found from -20°C on, as all flowers are dead by this time. The difference at -12°C and -16°C is not significant due to the large variability within the treatment and control tissues.
Figure VII-5. Resultant hardiness of cold hardy *Azalea* buds subjected to 45°C for 2 hours compared to controls as determined by visual assay.
Table VII-5. Comparison of mean survival between control and heat treated Azalea buds at specific temperatures

<table>
<thead>
<tr>
<th>Temperature</th>
<th>-10</th>
<th>-12</th>
<th>-14</th>
<th>-16</th>
<th>-18</th>
<th>-20</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C</td>
<td>100 ± 0</td>
<td>100 ± 0</td>
<td>95.0 ± 11.2</td>
<td>28.0 ± 43.8</td>
<td>0 ± 0</td>
<td>20.0 ± 44.7</td>
</tr>
<tr>
<td>45°C</td>
<td>83.9 ± 24.6</td>
<td>81.0 ± 32.5</td>
<td>3.3 ± 7.5</td>
<td>17.3 ± 28.9</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

Comparison between means of 4°C and 45°C pretreatments at the same freezing temperature:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>t-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>-10</td>
<td>1.464 NS</td>
</tr>
<tr>
<td>-12</td>
<td>1.308 NS</td>
</tr>
<tr>
<td>-14</td>
<td>15.212 ***</td>
</tr>
<tr>
<td>-16</td>
<td>.456 NS</td>
</tr>
<tr>
<td>-20</td>
<td>1.000 NS</td>
</tr>
</tbody>
</table>

*** p < .001

\[
t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{sp^2 \left( \frac{1}{n_1} + \frac{1}{n_2} \right)}}
\]
Effect of sublethal heat stress on cold hardy peach buds:

Heat treatment also decreased the cold hardiness in buds of peach (Prunus sp.). Initially, 'Criterion' apple, 'Comice' pear, 'Lambert' cherry, 'Greengage' plum, and 'Curless' peach buds were examined for the presence of LTE's by DTA. In agreement with Quamme's work (1974), apple and pear flower buds had no low temperature exotherms. Cherry, plum and peach floral tissues all exhibited LTE's; peach buds were then subjected to sublethal heat stress as described previously for Azalea, and the resultant DTA indicate only 22% of the treated buds had exotherms, in comparison with 66% of the controls (Table VII-6). Because peach buds only have one flower, a large variability is seen; the number of flowers with exotherms can either be 100% or 0%. Even with the large variability, the difference between the two populations is still significant (.05 > p > .025), and indicates removal of cold hardiness by sublethal heat treatment is not limited to Azalea species.

Andrews et al. (1983) suggest cold hardy buds of cherry and peach flowers have four stages of hardiness; dormant and cold hardy, transitionally hardy during early bud swell where LTE's occur at higher temperatures and eventually disappear, frost tolerant, and frost sensitive. Azalea buds have been observed to have LTE's at higher temperatures during early spring as well (George et al., 1974). The effect of a sublethal heat treatment could be to force the bud from stage one to stage two, possibly due to the removal of the ice barrier, as evidenced by the appearance of LTE's at higher temperatures than in the control.

There is an apparent discrepancy in the effect of heat shock upon the hardiness of plant tissues. While the effect of such shock was seen to increase membrane permeability and vacuolation in rice (Thompson et al., 1987), and increase disease resistance in cucumbers (Stermer & Hammerschmidt, 1984), it is also seen to remove or inhibit the ice barriers
Table VII-6. Hardiness of cold hardy peach buds subjected to 45°C for 2 hours as measured by appearance of LTE's from DTA

<table>
<thead>
<tr>
<th></th>
<th>4°C</th>
<th>45°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>66.67 ± 50</td>
<td>23.22 ± 44.10</td>
</tr>
</tbody>
</table>

Comparison of means: \( t = 2.00 \) (16 df)
\[ .05 > p > .025 \]

where \( t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{sp^2 \left( \frac{1}{n_1} + \frac{1}{n_2} \right)}} \)
in already hardy *Azalea* buds and to inhibit lignification in cucumbers (Stermer & Hammerschmit, 1982). As Stermer and Hammerschmidt (1985) state, "The common denominator in all these effects of heat shock ... appears to be the temporary halt of many active processes." Therefore, the developmental stage in which a plant exists may determine how the tissues will react to heat stress; if they are not hardy, it may induce hardiness, while the opposite may be true in cold hardy plants.

In cold hardy *Azalea* flower buds, there is an ice nucleation barrier which inhibits freezing of the primordia. The presence of this barrier is substantiated by the appearance of LTE's during DTA; its nature appears to be physical as it can be removed by mechanical disruption or high temperature stress. This barrier may exist on more than one level (i.e. within the bud axis and below each flower), since LTE's do not always completely disappear following sublethal heat stress. The removal of the barrier leads to reduced bud hardiness as evidenced by survival testing and the disappearance of LTE's. This removal is extremely rapid, as a 5 minute treatment of 80°C is enough to prevent all supercooling of flowers; it is doubtful this occurs merely by leaching as has been suggested previously (Washmon *et al.*, 1982). The general success of a sublethal heat treatment in partially or completely removing the barrier was shown in cold hardy peach buds as well.
Literature Cited


CHAPTER VIII

AN ICE-NUCLEATION BARRIER IN COLD HARDY AZALEA BUDS:

B. ESTABLISHMENT OF AN OPTIMUM TEMPERATURE TREATMENT FOR BARRIER REMOVAL

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Department of Horticulture, Oregon State University, Corvallis, OR 97331

Additional index words. Acclimation, resistance, stress

Abstract. The time and temperature requirements necessary for removal of the ice nucleation barrier of cold hardy Azalea buds were studied. Previous work (Chalker-Scott & Fuchigami, Chapter VII) determined a sublethal heat treatment would remove the barrier in buds as measured by differential thermal analysis (DTA). Temperatures increasing from 35°C to 45°C lead to progressively fewer flowers exhibiting LTE’s, and/or more flowers exhibiting LTE’s at higher temperatures. A 50°C treatment was lethal to the tissues. Similarly, the optimum exposure time and post-treatment recovery time were found to be 2 hours each; in other words, times greater than these did not give significantly lower % LTE values.

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Introduction

In response to potential injury caused by unseasonably cold weather, temperate plant species have evolved mechanisms for frost tolerance. One such mechanism is the phenomena of supercooling, by which tender tissues are protected from ice formation by the presence of some barrier (George et al., 1982). The tissues most often associated with deep supercooling or undercooling are xylem ray parenchyma cells and dormant flower buds; examples of the latter include cherry, peach, apricot, plum, blueberry, grape, and rhododendron species (Biermann et al., 1979; Graham & Mullin, 1976a; Quamme, 1974; Wolpert & Howell, 1984).

Ashworth (1982) suggests the ice nucleation barrier in Prunus buds is physical or thermodynamic in character. Low temperature supercooling was prevented in buds killed by heat or freezing, or in buds pierced below the bud primordium (Ashworth, 1982). The reported lack of xylem traces into the primordium in hardy Prunus buds (Ashworth, 1984; Hanson & Breen, 1985), associated with the inability of such buds to take up dye (Ashworth, 1982), led Ashworth to propose a physical, developmental barrier to ice nucleation from the stem into the flower bud.

In cold hardy Rhododendron and Azalea flower buds, there is a physical ice nucleation barrier which inhibits freezing of the primordia (George et al., 1982; Graham & Mullin, 1976a and b; Ishikawa & Sakai, 1981; Iwaya-Inoue & Kaku, 1983; Kaku et al., 1980; Sakai et al., 1986; Sakai & Malla, 1981). The presence of this barrier is substantiated by the appearance of low temperature exotherms (LTE’s) during differential thermal analysis (DTA); its removal by sublethal heat stress leads to reduced bud hardiness as evidenced by viability testing and the disappearance of LTE’s (Chalker-Scott & Fuchigami, Chapter VII).
Andrews et al. (1983) suggest cold hardy buds of cherry and peach flowers have four stages of hardiness; dormant and cold hardy, transitionally hardy during early bud swell where LTE's occur at higher temperatures and eventually disappear, frost tolerant, and frost sensitive. The effect of a sublethal heat treatment could be to force the bud from stage one to stage two, possibly due to the removal of the ice barrier, as evidenced by the appearance of LTE's at higher temperatures than in the control.

Although Azalea buds are known to deharden (Kaku et al., 1983) in response to high temperature exposure, the optimum conditions have yet to be established. Experiments such as those mentioned above generally use lower temperatures and extended time periods, in contrast to 'heat shock' experiments, which may use treatments of 50°C for 40-50 seconds (Stermer & Hammerschmidt, 1984). Previous research (Chalker-Scott & Fuchigami, Chapter VII) has established sublethal heat treatment as a means to remove partially or completely the ice nucleation barrier in cold hardy Azalea buds. The sets of experiments described herein examine the time and temperature requirements necessary to initiate this phenomenon.

Materials and Methods

The following methods had been previously shown to be desirable for determining the hardiness levels of Azalea buds (Chalker-Scott & Fuchigami, Chapter VII). Cold hardy Azalea species buds were collected during December 1987 through February 1988. In each experiment, intact buds on approximately 2 cm stems were placed in 150 x 25 mm glass test tubes containing 3 ml of distilled water. Control materials were stored in a refrigerator at 4°C, while treatments were immersed in water baths at various temperatures. Following the experimental regime, some materials were transported to the National Clonal Germplasm
Repository in Corvallis, Oregon for differential thermal analysis (DTA).

For DTA, bud stems were trimmed to .5 cm, attached to 36 gauge chromel-constantan thermocouples, paired with a similar reference thermocouple, and placed in an aluminum block. The apparatus was placed in a Revco Ultralow Freezer (ULT-1735 AMA) set at -50°C; freeze rates were approximately 30°C/hour. A CR-7 Data Logger (Campbell Scientific, Inc.), compiled sample thermocouple information obtained during freezing tests onto a cassette tape (Montano et al., 1987), which was transposed to computer disk via Campbell Scientific’s C20 Cassette Interface. Following data manipulation using dBase program 'shuffle', Lotus programs 1-2-3 and Printgraph were used to view the data graphically. The number and temperatures of sample freezing events were obtained from these figures. (For a more detailed explanation of these procedures, see Appendix 2.)

After DTA was performed, the buds were removed and dissected so the number of flowers per bud could be counted; this information was used to determine the percentage of flowers per bud which had low temperature exotherms (LTE’s). All LTE temperatures were determined and analyzed from the DTA graphs for both treatments.

Other control and heat-treated buds not used for DTA were left at 25°C in water for several days to insure they had not been irreparably damaged by experimental methods, and so signs of bud break could be observed.

Establishment of Test Temperature: Eight floral buds were exposed to each of five temperatures (4°C, 35°C, 40°C, 45°C, and 50°C) in either a refrigerator or water baths for two hours, and allowed to recover for two hours at 25°C. Four of the buds were examined for LTE’s using DTA, and four were left in test tubes with distilled water at 25°C for one week as indicators of temperature damage. These buds were dissected and flowers were judged as ‘live’ (green) or ‘dead’ (brown).
The experiment was repeated four times over the sampling period.

**Establishment of Temperature Exposure Time:** Sets of eight floral buds were exposed to 45°C for either 0, 1, 2, 3, or 6 hours, followed by two hours recovery at 25°C as described above. LTE's were found for half the buds via DTA, while the remaining buds were left at 25°C for one week to be visually assayed for damage as before. The experiment was repeated twice during the sampling period.

**Establishment of Recovery Time:** Sets of eight to ten floral buds were exposed to either 4°C or 45°C for two hours, followed by 1, 2, 3, 6, or 24 hours of recovery time at 25°C. Half the buds were analyzed for LTE's by DTA and half were left as viability indices as before. The experiment was repeated twice during the sampling period.

The population means for each treatment within an experiment were compared to each other and p-values were computed based on the statistical t-test.

**Results and Discussion**

The reliability of DTA as a measure of plant hardiness has been previously established for *Rhododendron* tissues (George *et al*., 1974; Graham & Mullin, 1976b; Iwaya-Inoue & Kaku, 1983). These researchers also found the number of floral primordia to be similar to the number of LTE's recorded. We have also established that the percentage of flowers exhibiting LTE's is a reliable indicator of bud cold hardiness (Chalker-Scott & Fuchigami, Chapter VII). A decrease or absence of LTE's indicates some or all of the flowers froze with the bulk water (M.J. Burke, personal communication).
Test Temperature: Control tissues had a significantly greater percentage of flowers with LTE's than the other high temperature exposure treatments (Table VIII-1). The 35°C treatment was also significantly different from the other heat treatments, as it retained a higher percentage of LTE's; there was no difference among the 40°C, 45°C, or 50°C treatments. The results of these tests indicated 40°C and 45°C were better than the other temperature treatments, as they were significantly different from either the controls or the 35°C treatment and were not lethal to the buds. Buds which were left at 25°C for a week following heat treatment were uniformly undamaged, except for the 50°C treatment which was completely killed.

Temperature Exposure Time: In general, the 45°C treatment differed significantly from the controls (Table VIII-2). One hour at 45°C was significantly different from the 2 and 6 hour treatments; the 2, 3, and 6 hour treatments at 45°C did not differ from one another. Visual observation of buds left at 25°C for one week revealed no damage to any of the tissues. Because the 2 hour treatment was not significantly different from the longer exposure times, it was used in subsequent studies to remove the ice barrier.

Recovery Time: Control buds had significantly greater % LTE values compared to all other treatments (Table VIII-3). The LTE's of buds allowed to recover for 1 hour were not significantly different from those left for 2 or 3 hours, but were significantly different from those left 6 or 24 hours. With increasing incubation time, the % LTE's of tissues progressively decreased, although no significant difference was found among the other recovery times of 2, 3, 6, and 24 hours. These results indicate incubating buds for 2 hours at 25°C following a 2 hour treatment at 45°C is sufficient time to overcome the ice barrier. It is important to note there is no loss of hardiness in control
Table VIII-1. Comparison of % flowers exhibiting LTE’s as measured by DTA in cold hardy Azalea buds after 2 hours treatment at $4^\circ$, $35^\circ$, $40^\circ$, $45^\circ$, and $50^\circ$

<table>
<thead>
<tr>
<th></th>
<th>$4^\circ$</th>
<th>$35^\circ$</th>
<th>$40^\circ$</th>
<th>$45^\circ$</th>
<th>$50^\circ$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Value</td>
<td>92.7±8.6</td>
<td>64.48±16.71</td>
<td>35.58±6.09</td>
<td>33.95±14.52</td>
<td>36.40±9.05</td>
</tr>
</tbody>
</table>

Comparison of means:

<table>
<thead>
<tr>
<th>Comparison</th>
<th>$t$-value$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$4^\circ$ : $35^\circ$</td>
<td>3.00 **</td>
</tr>
<tr>
<td>$4^\circ$ : $40^\circ$</td>
<td>10.84 ***</td>
</tr>
<tr>
<td>$4^\circ$ : $45^\circ$</td>
<td>6.96 ***</td>
</tr>
<tr>
<td>$4^\circ$ : $50^\circ$</td>
<td>8.99 ***</td>
</tr>
<tr>
<td>$35^\circ$ : $40^\circ$</td>
<td>3.25 **</td>
</tr>
<tr>
<td>$35^\circ$ : $45^\circ$</td>
<td>2.76 *</td>
</tr>
<tr>
<td>$35^\circ$ : $50^\circ$</td>
<td>2.95 *</td>
</tr>
<tr>
<td>$40^\circ$ : $45^\circ$</td>
<td>NS</td>
</tr>
<tr>
<td>$40^\circ$ : $50^\circ$</td>
<td>NS</td>
</tr>
<tr>
<td>$45^\circ$ : $50^\circ$</td>
<td>NS</td>
</tr>
</tbody>
</table>

$^*$ $p < .01$
$**$ $.025 > p > .01$
$***$ $.001 > p > .025$

$\bar{x}_i - \bar{x}_j$ where $t = \frac{\bar{x}_1 - \bar{x}_2}{s_p^2 \left( \frac{1}{n_1} + \frac{1}{n_2} \right)^{1/2}}$
Table VIII-2. Comparison of % flowers exhibiting LTE’s as measured by DTA in cold hardy Azalea buds after 0, 1, 2, 3, or 6 hours treatment at 45°C

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>93.75±12.50</td>
<td>53.5±4.04</td>
<td>30.83±21.67</td>
<td>32.15±23.69</td>
<td>20.1±18.31</td>
</tr>
</tbody>
</table>

Comparison of means:  

<table>
<thead>
<tr>
<th>Comparison of means:</th>
<th>t-value$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 : 1</td>
<td>6.128 ****</td>
</tr>
<tr>
<td>0 : 2</td>
<td>5.030 ***</td>
</tr>
<tr>
<td>0 : 3</td>
<td>4.600 ***</td>
</tr>
<tr>
<td>0 : 6</td>
<td>6.641 ****</td>
</tr>
<tr>
<td>1 : 2</td>
<td>2.057 *</td>
</tr>
<tr>
<td>1 : 3</td>
<td>1.776 NS</td>
</tr>
<tr>
<td>1 : 6</td>
<td>3.561 **</td>
</tr>
<tr>
<td>2 : 3</td>
<td>.082 NS</td>
</tr>
<tr>
<td>2 : 6</td>
<td>.756 NS</td>
</tr>
<tr>
<td>3 : 6</td>
<td>.805 NS</td>
</tr>
</tbody>
</table>

$^1$where $t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{sp^2 (\frac{1}{n_1} + \frac{1}{n_2})}}$

**** $p < .001$
*** $.0025 > p > .001$
** $.005 > p > .0025$
* $.05 > p > .025$
Table VIII-3. Comparison of % flowers exhibiting LTE's as measured by DTA in cold hardy Azalea buds after 0, 1, 2, 3, 6, or 24 hours at 25°C following 2 hours treatment at 4° or 45°

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>6</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>92.55 ± 8.50</td>
<td>62.71 ± 21.42</td>
<td>55.04 ± 24.13</td>
<td>47.04 ± 22.59</td>
<td>34.74 ± 24.24</td>
<td>31.16 ± 25.10</td>
</tr>
</tbody>
</table>

Comparison of means: $t$-values\(^1\)

<table>
<thead>
<tr>
<th></th>
<th>0 : 1</th>
<th>0 : 2</th>
<th>0 : 3</th>
<th>0 : 6</th>
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<th>1 : 2</th>
<th>1 : 3</th>
<th>1 : 6</th>
<th>1 : 24</th>
<th>2 : 3</th>
<th>2 : 6</th>
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<th>3 : 24</th>
<th>6 : 24</th>
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<tbody>
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\(^1\) where $t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{sp^2 \left( \frac{1}{n_1} + \frac{1}{n_2} \right)}}$

*** $p < .001$

* 0.025 < $p < .01$
tissues, even after incubation in water for several hours. This contrasts sharply with Washmon et al. (1982), who report loss of cold hardiness in peach and apple buds following 'leaching' in water for as few as three hours. The probability that such leaching occurs is decreased further by previous data (Chalker-Scott & Fuchigami, Chapter VII) which showed the barrier could be removed in only 5 minutes by an 80°C treatment.

The loss of the barrier at warm temperatures, compared to no loss at a lethally cold temperature (Chalker-Scott & Fuchigami, Chapter VII), suggests the barrier could be either "melted" physically or degraded enzymatically. The need for a recovery time following sublethal heat stress points to the later explanation; an inactive enzyme already present in the tissues could be activated by the increased temperature and begin to break down the barrier. These processes would require a lag time. In contrast, the rapid loss of the barrier at lethally high temperatures could indicate a physical change in the barrier is also occurring non-enzymatically.

It appears from these experiments cold hardy Azalea buds will lose most of their hardiness after only 2 hours of 45°C treatment, in contrast with days or weeks of exposure at lower temperatures (Kaku et al., 1983; Pemberton & Wilkins, 1980). This statement agrees with Kaku et al. (1983), who find hardiness is lost as a result of long exposure time coupled with lower temperatures (16 weeks at 5°C), or higher temperatures and lower exposure times (4 weeks at 17°C). It is also critical to use temperatures below those of 'heat shock' level (i.e. 50°C [Stermer & Hammerschmidt, 1984]), as prolonged exposure to these temperatures are fatal to the tissues.

There is an apparent discrepancy in the effect of heat stress upon the hardiness of plant tissues. While the effect of such shock was seen to increase membrane permeability and vacuolation in rice (Thompson et al.,
1987), and increase disease resistance in cucumbers (Stermer & Hammerschmidt, 1984), it is also seen to remove or inhibit the ice barriers in already hardy Azalea buds and to inhibit lignification in cucumbers (Stermer & Hammerschmidt, 1982). Obviously, some kind of change is occurring at the cell wall level, either forming or destroying barriers. As Stermer and Hammerschmidt (1985) state, "The common denominator in all these effects of heat shock ... appears to be the temporary halt of many active processes." Therefore, the developmental stage in which a plant exists may determine how the tissues will react to heat stress; if they are not hardy, it may induce hardiness, while the opposite may be true in cold hardy plants. It seems reasonable to assume that cold hardy Azalea buds, subjected to sublethally warm 'heat shock' temperatures, could lose their supercooling ability by a mechanism which removes the ice nucleation barrier.
Literature Cited


CHAPTER IX

AN ICE-NUCLEATION BARRIER IN COLD HARDY AZALEA BUDS:
C. HISTOCHEMICAL CHARACTERIZATION OF THE BARRIER

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Abstract. Histochemical studies were conducted in an attempt to characterize the ice barrier which enables flower buds of cold hardy Azalea spp. to supercool and thus avoid freezing. Autofluorescence microscopy indicated the presence of a barrier in three places within the bud tissues. First, a heavily suberized, lignified area beneath the bud scales may serve as a barrier to penetration by ice from the scale tissue into the bud axis. A second heavily lignified zone occurs radially through the bud at the point where bud scales begin; this zone could prevent ice nucleation from the stem into the primordia. Finally, the cell walls of the pedicel of the flowers appears to be suberized; the presence of this hydrophobic barrier could partially explain the pattern of low temperature exotherm formation seen within a given bud. Condensation of the hydrophobic suberin lining as a result of heat stress may allow ice to penetrate from previously frozen tissues into the flower during freezing conditions.

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Introduction

Supercooling ability of hardy bud tissues:

The phenomenon of supercooling as a mechanism of frost resistance appears to be confined primarily to woody plants (George & Burke, 1984) and seeds (Juntilla & Stushnoff, 1977). Tissues which are most often associated with this mechanism are xylem ray parenchyma cells and dormant flower buds; examples of the latter include cherry, peach, apricot, plum, blueberry, grape, and Rhododendron (Biermann et al., 1979; Graham & Mullin, 1976; Quamme, 1974; Wolpert & Howell, 1984). When ice formation is initiated via a nucleating event, the growth of ice can be quite rapid and will continue through tissues unless barred in some way. Since many floral buds are able to delay ice formation long after the bulk water in the scales and stem of the bud has frozen, there must be some barrier to prevent nucleation of the supercooled bud water.

Previous work has established the presence of an ice-nucleation barrier within cold-hardy Azalea buds which can apparently be removed by sublethal heat treatment (Chalker-Scott & Fuchigami, Chapters VII & VIII). Although there has been speculation as to the nature and occurrence of the barrier in the buds of this and other plant species (Biermann et al., 1979; Graham & Mullin, 1976; Quamme, 1974; Wolpert & Howell, 1984), its composition has not been determined.

Ashworth (1982) suggests the barrier in Prunus buds is physical or thermodynamic in character. Low temperature supercooling was prevented in buds killed by heat or freezing, or in buds pierced below the bud primordium (Ashworth, 1982). The reported lack of xylem traces into the primordium in hardy Prunus buds (Ashworth, 1984; Hanson & Breen, 1985), associated with the inability of such buds to take up dye (Ashworth, 1982), led Ashworth to propose a
physical, developmental barrier to ice nucleation from the stem into the flower bud.

**Cell wall alterations in response to stress:**

Friend (1981) describes cell wall alterations as being physical and chemical barriers in impeding disease organisms and protecting polysaccharides from cell wall-digesting enzymes. Such alterations are known to include lignification and suberization of outer cell walls and intracellular spaces (Bell, 1981).

Other phenolic compounds may accumulate in cells as general defense chemicals and could possibly act in cold hardiness as well. Condensed tannins and their precursors occur in the phloem rays and ray initials of *Prunus* species; (Feucht *et al.*, 1986). These tissues are also associated with supercooling abilities as mentioned earlier. Condensed tannins also accumulate on the wall surfaces of Douglas fir (Ross & Corden, 1973), where they could modify ice crystals (Olien, 1965) and render them non-injurious to the cell (Burke *et al.*, 1976).

There is evidence in the literature which supports the concept of phenolic involvement in cold stress response and acclimation. Jaffe and Biro (1977), noting a general increase in cold hardiness after non-freezing stress, showed mechanically perturbed tissues were more frost-resistant than non-stressed tissues. The synthesis of phenylalanine ammonia-lyase (PAL, E.C. 4.3.1.5) is seen to increase following chilling of barley seedlings (Podstolski *et al.*, 1981), apple fruit and potatoes (Graham & Patterson, 1982), leading to an increase in flavonoids and other phenolics; a similar accumulation was noted in chestnut cuttings stored at -10°C (Vázquez & Gesto, 1982). In addition, apple tree adaptation to cold climates was found to be associated with high levels of chlorogenic acid (Streltsina, 1980).

It has been concluded that lignification serves to increase frost tolerance of grapevine (Hubackova, 1982;
Kur'yata, 1978), apple trees (Huang & Wang, 1982), and sugarcane (Sharma et al., 1981). Similarly, suberin deposition is also shown to increase resistance to cold temperatures. Currently, suberin is thought to be deposited within the cell walls in response to developmental or physiological signals in all parts of the plant (Kolattukudy, 1984). Suberization of white spruce roots occurred following cold temperature regimes (Johnson-Flanagan & Owens, 1985), which presumably would increase hardiness. During cold acclimation of winter rye, Griffith et al. (1985) noticed thickened cell walls and increased production of suberin-type lipids. Frost-hardened pine seedlings were also seen to increase their lipid synthesis (Selstam & Oquist, 1985). The lack of cold hardiness in late maturing grape was also attributed to inadequate suberization (Paroschy et al., 1980).

A mechanism by which suberin and lignin may protect plants from freeze damage has been suggested by Griffith et al. (1985) and Paroschy et al. (1980): the deposition of cell wall materials could maintain the water status of tissues by acting as a water barrier. As plants acclimate, their water content decreases; if they have no mechanism by which to keep their freezable water below critical levels during thaws, they can be killed by subsequent frosts. In addition, effective water barriers would prevent desiccation injury by reducing evapotranspiration from extracellular freezing. This may account for the increased thickening seen in cell walls next to vascular bundles and towards leaf surfaces in cold acclimated winter rye (Griffith & Brown, 1982).

The importance of cell-wall associated phenolic substances in the process of cold acclimation is further enhanced by research by Bartolo and Wallner (1986). They noted that cold acclimation increases the resistance to cellulase in leaf tissues, as does wounding; wounding also induces cold hardiness in the tissues. If an increase in
cold-induced phenolic synthesis also occurs, it could facilitate a change at the cell wall-membrane interface, resulting in increased membrane adhesion to the cell wall and prevention of cell collapse during freeze-induced dehydration. A similar mechanism has also been postulated for cell wall-associated proanthocyanidins in xerophytic plants (Pizzi & Cameron, 1986). Additionally, extracellular cell wall polymers could modify the freezing process to form small, imperfect ice crystals (Olien, 1977) which are non-injurious to the cell (Burke et al., 1976) and perhaps cannot act as ice nucleators.

Lignin, suberin, and other phenolic polymers can function as barriers to bacterial and fungal invasion (Feucht et al., 1986), herbivory (Haukioja et al., 1985), evapotranspiration (Griffith et al., 1985; Paroschy et al., 1980), and other environmental stresses. Because they can be impervious to water (Aastrup et al., 1984; Egley et al., 1985; Espelie et al., 1980), it is likely such compounds could form barriers to ice nucleation. Therefore, the idea of a suberized and/or lignified barrier to ice nucleation within cold hardy flower buds is an attractive one.

**Fluorescence microscopy and cell wall structure:**

Because of the autofluorescent nature of phenolic compounds such as suberin and lignin, fluorescent microscopy has been a valuable tool in analyzing the deposition of phenolic materials within cell walls. Vascular bundles are known to fluoresce (Führer, 1982; Knogge & Weissenböck, 1986), primarily due to the intense lignification of the xylem elements. Autofluorescence has also been used to detect increases in cell wall phenolics in the hypersensitive response of resistant wheat to rust infection (Beardmore et al., 1983). Similar depositions of suberin were seen using fluorescence microscopy in fungally infected tomatoes (De Leeuw, 1985).
One of the most interesting reports of fluorescence is from Biggs (1984a) in his work with wounded peach tissues. He describes a 'zone of imperviousness', which is 2-4 cell layers thick, and is associated with the deposition of a thin lining of suberin on the inner cell wall. Montano and Proebsting (1988) describe a similar protective layer beneath the ascission zone of Douglas fir needles; this layer was determined to be heavily lignified and suberized.

Materials and Methods

Vegetative and reproductive buds from cold hardy Azalea plants were collected December through February (1986-1988) and subjected to a variety of treatments as described previously (Chalker-Scott & Fuchigami, Chapters VII & VIII). Briefly, intact buds on approximately 2 cm stems were placed in each of two 150 x 25mm glass test tubes containing three ml of distilled water. Control materials were stored in a refrigerator at 4°C, while treatments were immersed in a water bath at 45°C. All materials were maintained at temperature for 2 hours after reaching equilibrium with the environment. Following the 2 hour experimental regime, all materials were held at 25°C for 2 hours before tissue fixation and differential thermal analysis (DTA) was performed.

For DTA, bud stems were trimmed to .5 cm, attached to 36 gauge chromel-constantan thermocouples, paired with a similar reference thermocouple, and placed in an aluminum block. The apparatus was placed in a Revco Ultralow Freezer (ULT-1735 AMA) set at -50°C; freeze rates were approximately 30°C/hour. A CR-7 Data Logger (Campbell Scientific, Inc.), compiled sample thermocouple information obtained during freezing tests onto a cassette tape, which was transposed to computer disk via Campbell Scientific's C20 Cassette Interface. Following data manipulation using dBase program 'shuffle', Lotus programs 1-2-3 and Printgraph were used to
view the data graphically. The number and temperatures of sample freezing events were obtained from these figures.
(For a more detailed explanation of these procedures, see Appendix 2.)

The buds were fixed in FAA, dehydrated, and embedded in Paraplast (Appendix 4). Control buds were likewise preserved, as were buds that had broken dormancy several days following heat treatment and buds which had been killed by an 80°C heat treatment (Chalker-Scott & Fuchigami, Chapter VII). After sectioning to 8 μ thicknesses, the tissues were attached to slides using a general aqueous adhesive as described by Fink (1987), and either mounted under glycerin or stained with lignin and suberin specific solutions (see Appendix 4 for further methodology):

Toluidine Blue O (TBO) was used for the staining of lignin; under brightfield microscopy lignin appears blue, while TBO will quench lignin autofluorescence under UV-excitation (Biggs, 1984b). Two suberin stains were employed: Sudan IV and Sudan Black B (after Pearse, 1968). Sudan Black B gives suberin a blue-black color under brightfield microscopy, but quenches its autofluorescence under UV-excitation. Sudan IV stains suberin a red color under brightfield microscopy, but will enhance suberin autofluorescence under fluorescence microscopy, giving suberin a bright red color against a yellow-green background (Biggs, 1985).

The sections were observed under UV-excitation conditions on a Zeiss Axioskop fitted with a DAPI 02 blue filter. Another Zeiss fluorescing microscope, outfitted with a FITC filter, was also used for some photography. Photomicrographs were taken using Ektachrome and Kodacolor ASA 400 35 mm film.
Results and Discussion

The presence of 1-3 layer(s) of lignified and suberized cells between bud scales and axis is visible in non-heated control buds (Figure IX-1a-c). Autofluorescence of an unstained section (Figure IX-1a) under UV conditions suggests a phenolic component within the cell walls. Subsequent staining with TBO (Figure IX-1b) and Sudan Black B (Figure IX-1c), which quench autofluorescence of lignin and suberin, respectively, indicate the presence of these two compounds within the walls of the cell layer. The presence of a suberin and lignin barrier between the scales and the bud may serve to prevent ice nucleation proceeding from the frozen water within the scales into the bud primordium. The possibility this is an abscission layer is refuted by Biggs and Northover (1985), who find the protective layer of suberin and lignin to form after abscission in peach leaves. The suberin and lignin layer could be the manner by which bud scales protect the flowers of Azalea from external ice crystals, in addition to acting as an ice sink (Ishikawa & Sakai, 1981). The scales themselves are heavily lignified and suberized on their inner surface (Fig. IX-2); such tissue would not freeze easily and may serve as an additional insulative barrier between the external environment and the bud itself.

A barrier similar in composition to that seen in Figure IX-1 also occurs underneath hardy vegetative buds associated with the floral bud tissue (Figures IX-3a-b). The existence of LTE's in vegetative buds of Azalea species has not been previously established; therefore DTA was performed on vegetative buds. The DTA graph of a vegetative bud clearly shows the presence of one LTE at -170°C (Figure IX-4), in agreement with Rajashekar et al. (1982) who found vegetative buds of Prunus to have LTE's. Because vegetative buds of Azalea appear to supercool, it is likely the barrier seen in
IX-1a. Photomicrograph of a longitudinally sectioned, cold hardy *Azalea* flower bud seen under autofluorescence. Arrows indicate presence of phenolic layer in walls of cells located between base of bud scale and bud axis. 200x
IX-1b. Photomicrograph of a longitudinally sectioned, cold hardy Azalea flower bud. As seen in Figure 1a, but stained with Toluidine Blue O to quench lignin autofluorescence. Residual fluorescence is due to the presence of suberin. 200x
IX-1c. Photomicrograph of a longitudinally sectioned, cold hardy *Azalea* flower bud. As seen in previous figures, but stained with Sudan Black B to quench suberin autofluorescence. Residual fluorescence is due to the presence of lignin. 200x
IX-2. Photomicrograph of a longitudinally sectioned, cold hardy Azalea flower bud. Heavily lignified and suberized inner layer of bud scale, seen under autofluorescence. 200x
IX-3a. Photomicrograph of a longitudinally sectioned, cold hardy *Azalea* vegetative bud under autofluorescence. Arrows designate phenolic layer seen between bud and stem axis. 256x
IX-3b. Photomicrograph of a longitudinally sectioned, cold hardy *Azalea* vegetative bud as shown in Figure 3a, but stained with Sudan IV for suberin. Residual fluorescence is due to the presence of lignin. 256x
Figure IX-4. Differential thermal analysis (DTA) of a cold hardy *Azalea* vegetative bud
Figure IX-3 would prevent the nucleation of ice from the stem into the bud primordium.

Given the presence of the barriers seen beneath the scales, the only route available for ice nucleation would be from the stem into the flower primordia. At the juncture where bud scales begin, the stem of the bud undergoes a transition from heavily-lignified cells to non-lignified cells (Figures IX-5a-c). When unstained material (Figure IX-5a) is treated with Sudan Black B, the residual fluorescence can be attributed to lignified tissue (Figure IX-5b). These fluorescing, lignified cells, when quenched with TBO as seen in Figure IX-5c, also contain a thin fluorescent intercellular lining which is possibly suberin in nature. Such a hydrophobic barrier would inhibit ice growth from proceeding up the stem into the flowers.

A third barrier similar in nature to that found in Figure IX-5 may also exist in the pedicel tissues of each flower. Hardy tissues stained with TBO have the same intercellular fluorescent linings as seen in Figure IX-5b (Figure IX-6a), which must be non-lignin in nature. These linings, when subjected to a sublethal heat stress of 45°C for 2 hours, appear to have condensed into "spots" (Figure IX-6b). When stained with TBO as above, these "spots" still fluoresce, indicating the presence of suberin or some other non-lignin phenolic compound (Figure IX-6c). As suggested earlier, these linings would inhibit nucleation of cell water from external ice crystals. It appears a sublethal heat treatment condenses this lining into the "spots" seen in Figures IX-6b and 6c, possibly rendering the tissues less cold hardy (Chalker-Scott & Fuchigami, Chapter VII) by permitting ice penetration and death of the flower. This conclusion is substantiated by the appearance of LTE's at much warmer temperatures in heat treated buds as compared to control tissues during DTA (Figure IX-7, from Chapter VII).

As mentioned earlier, a general response to environmental stresses often includes cell wall
IX-5a. Photomicrograph of a longitudinally sectioned, cold hardy Azalea flower bud. Heavily lignified/suberized area is apparent beneath flower axis. 200x
IX-5b. Photomicrograph of a longitudinally sectioned, cold hardy Azalea flower bud. As seen in Figure 5a, but stained with Sudan Black B to quench suberin autofluorescence. Residual fluorescence is due to the presence of lignin. 200x
IX-5c. Photomicrograph of a longitudinally sectioned, cold hardy *Azalea* flower bud. As seen in Figures 5a and b, but stained with Toluidine Blue O to quench lignin autofluorescence. Thin intercellular linings are marked with arrows, indicating the presence of suberin. 200x
IX-6a. Photomicrograph of a longitudinally sectioned, cold hardy *Azalea* flower bud. Flower pedicel is stained with Toluidine Blue O to quench lignin. Thin autofluorescent intercellular lining is visible, indicating the presence of suberin. 200x
IX-6b. Photomicrograph of a longitudinally sectioned, cold hardy Azalea flower bud after 2 hours at $45^\circ$C. Autofluorescent spots in flower pedicel suggest condensation of suberin lining found in hardy tissues. 100x
IX-6c. Photomicrograph of a longitudinally sectioned, cold hardy Azalea flower bud after 2 hours at 45°C. As seen in Figure 6b, but stained with Toluidine Blue O to quench lignin autofluorescence. Residual fluorescence indicates condensed suberin lining. 200x
Figure IX-7. DTA's of a cold hardy Azalea bud (---) and a cold hardy Azalea bud subjected to 45°C for 2 hours (----). (From Chalker-Scott & Fuchigami, Chapter VII, Figure VII-4)
modifications such as increased incorporation of phenolic materials. Autofluorescence of phenolics including suberin and lignin have been seen in fungally stressed wheat (Beardmore et al., 1983) and tomatoes (De Leeuw, 1985). Golgi bodies may increase in activity in stressed tissues (Barckhausen, 1978), and fuse with the plasmalemma, leading to increased cell wall thickness in dormant *Rhododendron* (Lynch & Rivera, 1981). Hrsel (1966) also observed the preponderance of organelles and endoplasmic reticulum next to the plasmalemma in plants under xerophytic conditions. Hardened rye cells have been reported to be multivacuolate (Griffith et al., 1985), while rice protoplasts given a 15 minute heat shock at 45°C also showed an increase in vacuolation and membrane permeability (Thompson et al., 1987). These observations correlate well with the known mechanisms of lignin and suberin biosynthesis. The precursors for lignin (and presumably for the phenolic component of suberin) are formed in the endoplasmic reticulum, where they are packaged into vesicles and transported to the cell walls for incorporation (Northcote, 1985).

Although the autofluorescence in the bud tissues examined in this research is of phenolic origin, the actual compounds that can fluoresce are not limited to lignin and suberin. Wall-bound ferulic acid esters can also autofluoresce (Fry, 1983), as can various flavonoid compounds (Markham, 1982) and other phenolics. UV-absorbing material in the bordered-pit membranes of hemlock were determined to be procyanidin in character, rather than lignin (Gray et al., 1983). The 'specific' stains used by histochemists may not be as specific as once believed; TBO is thought to stain only lignin in plants, but its chromatasy is not well understood (O'Brien et al., 1964). Possibly it could stain other phenolics such as condensed tannins, which are known to be structural cell wall
There is an apparent discrepancy in the effect of heat stress upon the hardiness of plant tissues. While the effect of such shock was seen to increase membrane permeability and vacuolation in rice (Thompson et al., 1987), and increase disease resistance in cucumbers (Stermer & Hammerschmidt, 1984), it is also seen to remove or inhibit the ice barriers in already hardy Azalea buds (Chalker-Scott & Fuchigami, Chapters VII & VIII), and to inhibit lignification in cucumbers (Stermer & Hammerschmidt, 1982). As Stermer and Hammerschmidt (1985) state, "The common denominator in all these effects of heat shock ... appears to be the temporary halt of many active processes." Therefore, the developmental stage in which a plant exists may determine how the tissues will react to heat stress; if they are not hardy, it may induce hardiness, while the opposite may be true in cold hardy plants.
Literature Cited


BIBLIOGRAPHY


APPENDIX 1

AN IMPROVED TTC VIABILITY TEST FOR WOODY TISSUES:
PRELIMINARY EXPERIMENTS

Originally developed as a method for determining seed viability, the TTC (triphenyl tetrazolium chloride) reduction test has been applied to other plant tissues as well. Reduction of TTC to the red-colored formazan product occurs via several dehydrogenases, including NAD- and NADP-linked dehydrogenases (Jensen, 1962). Ideally, once a tissue has been fatally damaged it should show little reductive activity. Though TTC has successfully been used to determine injury in some plant systems (Towill & Mazur, 1975), others report the overestimation of cold hardiness by several degrees in bromegrass suspension cultures (Ishikawa et al., 1986). The TTC viability test has been unsuccessful as a measure of heat injury (Wu & Wallner, 1983), as it overestimates heat tolerance as well (Ishikawa et al., 1986).

In our studies on woody tissues (those containing high amounts of condensed tannins and other phenolics) and cell cultures, the test shows considerable variance. Stergios and Howell (1973) report a similar lack of reliability with TTC reduction in cherry, raspberry and strawberry tissues. Yadava et al. (1978) found poor correlation between TTC reduction and electrical conductivity in measuring cold injury in peach twigs; additionally, they reported greater TTC values in tender tissues in comparison with hardy tissues. The possibility of non-specific TTC reduction in fresh tissues was mentioned by Steponkus (1971) as an explanation for some of the variation; it could be inferred that TTC reduction is somehow impaired by substances present in hardy, woody tissues.

A critical part of a successful TTC reduction test is the maintenance of pH. Maximum reduction occurs at pH
values between 6.5 and 7.5, with little reduction occurring below pH 5.0 (Roberts, 1951). Other plant enzymes, however, including phenol oxidases like tyrosinase (Barrett, 1984), have the same pH optima. The activity of phenolic oxidases, and the reactivity of their products with proteins and enzymes leads to the familiar oxidative browning seen in injured plant tissues. It seems likely these phenolics could interfere directly with TTC reduction or with the spectrophotometric analysis of the end-product, formazan.

In their review of plant viability assays, Stergios & Howell (1973) emphasize the use of specific techniques for specific tissues to evaluate tissue injury successfully. With this suggestion in mind, the objective of this study was to determine the reasons for variability in the TTC test and to try to reduce this variability in woody plant tissues.

Materials and Methods

Freezing tests were performed during late winter 1987 using stem tissues from various deciduous and coniferous trees. The first experiment was made using cold-hardy stem tissues of *Malus domestica* "Starking", *Quercus garryana*, and *Pyrus communis*. Stem sections approximately 15 cm long were placed in 25 x 150 mm glass test tubes containing 2 ml distilled water and cooled to -3°C in a refrigerated circulating bath (Neslab Endocal LT-50DD). Ice crystals were added to each tube to promote nucleation of stem water, and the bath temperature was lowered to -6°C overnight. Freezing test temperatures were set at -10°C, -20°C, -30°C, -40°C and -45°C. The low temperature bath was held at each temperature for 1.5 hours, after which a sample tube of each plant tissues was removed. In addition, a sample of each material was left at 4°C as a control, and another sample was immersed in liquid nitrogen to kill the tissue completely.
After all samples were thawed at 4°C, they were incubated in the tubes at 4°C for 0, 1, and 4 days. Following incubation, some tissues were reserved for visual observations; 1 cm segments were cut from the remaining stem tissues, debarked, halved lengthwise, and placed individually into small test tubes containing 2 mls of each solution listed below:

1. .05 M Na₂HPO₄ buffer, pH = 7.4
2. .05% TTC in .05 M phosphate buffer, pH = 7.4
3. .05% TTC in .05 M phosphate buffer, pH = 6.4
4. .05% TTC in .05 M phosphate buffer, pH = 5.4
5. Solution #2 + 10 mM glutathione
6. Solution #2 + 1 M glutathione
7. Solution #2 + .5% sodium hypochlorite following TTC incubation
8. Solution #2 + .1 mM germanium dioxide

The tissues were vacuum-infiltrated with these solutions at 30 mm Hg for 10 minutes; the vacuum was removed very slowly to prevent air bubbles from entering the tissues and displacing solution. The vials and their contents were stored for 24 hours at 25°C under dark conditions, after which the remaining solution was decanted, tissues were rinsed with distilled water, and 2 ml 95% ethanol were added to each tube to extract the formazan. In the case of solution #7, tissues were soaked in 2 ml of .5% sodium hypochlorite for 5 minutes after TTC removal, then rinsed with distilled water and extracted with ethanol. All tissues were incubated in the dark for 24 hours, and the extract from each sample was measured at 485 nm on a Bausch & Lomb 2000 Spectrophotometer; some samples were also spectrophotometrically scanned from 200 to 550 nm. Tissues were then oven-dried and their dry weights were determined; results are calculated as absorbance₄₈₅ units/gram dry weight. Damage to all tissues was determined visually as
well; a discoloration of the wood was rated "injured", while brown tissues were rated "killed."

The second experiment used *Malus* "Starking" wood and bark tissues. Freezing tests and other experimental procedures were performed as in the first experiment, except incubation time and temperature before TTC addition was changed, and the following solutions were used:

1. \(0.05 \text{ M Na}_2\text{HPO}_4\) buffer, pH = 7.4
2. \(.5\% \text{ TTC in } 0.05 \text{ M phosphate buffer, pH = 7.25}\)
3. \(.5\% \text{ TTC in } 0.05 \text{ M phosphate buffer, pH = 6.5}\)
4. \(.5\% \text{ TTC in } 0.05 \text{ M phosphate buffer, pH = 6.0}\)
5. Solution #2 + 1 mM ascorbate
6. Solution #2 + 10 mM ascorbate
7. Solution #2 + 10 mM glutathione
8. Solution #2 + 1 mM germanium dioxide
9. Solution #2 + 10 mM germanium dioxide
10. Solution #3 + 10 mM germanium dioxide
11. Solution #4 + 10 mM germanium dioxide
12. Solution #9 + 10 mM glutathione
13. Solution #10 + 10 mM glutathione
14. Solution #11 + 10 mM glutathione
15. Solution #12 + 10 mM ascorbate
16. Solution #13 + 10 mM ascorbate
17. Solution #14 + 10 mM ascorbate

The third experiment utilized only bark tissues from "Starking", *Pyrus communis*, and *Tsuga heterophylla* (hemlock) stems. Again, experimental procedures were as in previous experiments, except for the following changes in the solutions:

1. \(0.05 \text{ M Na}_2\text{HPO}_4\) buffer, pH = 7.4
2. \(.5\% \text{ TTC in } 0.05 \text{ M phosphate buffer, pH = 7.4}\)
3. \(.5\% \text{ TTC in } 0.05 \text{ M phosphate buffer, pH = 6.0 + 50 mM germanium dioxide}\)
4. .5% TTC in .05 M phosphate buffer, pH = 5.5 + 50 mM germanium dioxide

Malus floribunda 'Red Splendor' crabapple and Pyrus ussuriensis 'Hang Pa-Li' pear were used in the final experiment; both bark and wood tissues were tested. No freezing test was run per se; half the tissues were held at 40°C as live controls, and the other half was killed at -60°C prior to TTC testing. Other experimental procedures were the same as above except for the following changes in TTC solutions:

1. .05 M Na₂HPO₄ buffer, pH = 7.4
2. .5% TTC in .05 M phosphate buffer, pH = 7.4
3. .5% TTC in .05 M phosphate buffer, pH = 6.5
4. .5% TTC in .05 M phosphate buffer, pH = 6.0
5. .5% TTC in .05 M phosphate buffer, pH = 5.5
6. Solution #1 + 10, 25 or 50 mM germanium dioxide
7. Solution #2 + 10, 25 or 50 mM germanium dioxide
8. Solution #3 + 10, 25 or 50 mM germanium dioxide
9. Solution #4 + 10, 25 or 50 mM germanium dioxide
10. Solution #5 + 10, 25 or 50 mM germanium dioxide

Results and Discussion

Woody plants typically have large amounts of proanthocyanidins, especially catechin, within their tissues. When injured by frost or other stresses, these compounds are released into the cytoplasm. Because many of the proanthocyanidins are red in color, their absorption could interfere with the measurement of reduced TTC at 485 nm. Therefore, solutions of catechin at pH 7.5 (typical for TTC test solutions) and at pH 6.0 were made and their absorption spectra recorded (Figure 1). At the more acidic pH, catechin has one major peak at 285 nm; however, more alkaline pH's cause catechin to polymerize, resulting in
Figure Al-1. Absorption spectra of catechin in distilled water at pH 6.0 (1 x 10^{-2} M) and pH 7.5 (2.725 x 10^{-4} M)
three major peaks at 285, 425, and 500 nm. There is an obvious absorption at 485 nm, which would be classified as formazan presence during a TTC reduction test. The effect of pH was studied closely as a result of this preliminary observation.

The solutions used in the first experiment were chosen on the basis of previous research and by speculation. A solution of .05% TTC is normally used at a pH of 7.4, but this pH may allow other enzymatic activity to interfere with the reduction reaction and cause proanthocyanidin interference; therefore other pH values were introduced. Glutathione is thought to have reducing capabilities and might have an inhibitory effect on interfering phenolic oxidative activity. Sodium hypochlorite is often used in the TTC test to "keep browning from interfering with the red color." This statement (attributed to Larcher & Eggarter, 1960) is difficult to understand, as the high pH of a sodium hypochlorite solution would quickly oxidize any phenolics present and therefore magnify the problem. Finally, germanium dioxide was added because it forms a strong complex with o-dihydroxyphenols (Loomis, 1974), which cause most of the browning behavior seen in damaged plants. It also is a competitive inhibitor of dihydroxyphenol oxidase (Loomis, 1974) and various dehydrogenases (W.D. Loomis, personal communication); because it could interfere with the reductase involved in the TTC to formazan reaction, the concentration was initially low.

In the first experiment, an incubation time of four days at 4°C before addition of TTC to Malus domestica "Starking", Pyrus communis, and Quercus garryana tissues gave more reliable results shorter incubation times. Little reductive activity was seen in any of these tissues if TTC was added immediately or after one day, which may have been due to too low a concentration of TTC, or interference from compounds released from dying cells.
When TTC was added after four days, pH values of 5.4 gave the most reliable results in all three genera (Figures 2-4). An increase in absorbance is seen in *Pyrus* tissues (Figure 2) without TTC after lethal cold temperatures are reached; obviously, leached phenolic compounds do interfere with the test. An increase in reductive activity is found at the point where tissues are injured but not killed; in other words, sublethal stress apparently causes increased reduction of TTC, perhaps as a result of repair activity. The curves for -TTC and TTC at pH 7.4 mirror each other well in *Quercus* tissues without distinguishing living or dead tissues (Figure 3). In contrast, the 5.4 pH treatment clearly shows the point of injury or death at -30°C.

"Starking" tissues (Figure 4) were similar to the *Quercus* results for -TTC, 7.4 pH, and 5.4 pH treatments; they also responded well to germanium and glutathione treatments.

Based on the above results, the TTC and germanium concentrations were increased to .5% and 1 or 10 mM, respectively. Two concentrations (1 and 10 mM) of ascorbate were included to maintain reduced conditions; ascorbate is a known inhibitor of quinone-forming diphenyl oxidase (Walker, 1980). Unfortunately, ascorbate also causes reduction of TTC to formazan; stock solutions turned red in less than 12 hours. The inclusion of the bark with the wood (using "Starking" tissues) may have caused the results (Figure 5) to be even more variable than in the previous study (Figures 2-4); however, the tissues pre-incubated for five days following freezing and then treated with TTC at a pH of 6.5 tended to be more reliable. An interesting rise in reduction was observed at the point where bark tissues are injured but not killed; this could be interpreted as an increase in repair activity as mentioned previously.

Because germanium did not appear to interfere with reductive activity, its concentration was increased for the next experiment with *Tsuga heterophylla*, "Starking", and *P. communis*. *Pyrus* showed the best results with TTC plus 50 mM
Figure A1-2. Absorbance$_{485}$ of ethanol extractives from cold-stressed Pyrus stems incubated in various TTC solutions
Figure A1-3. Absorbance of ethanol extractives from cold-stressed Quercus stems incubated in various TTC solutions.
Figure A1-4. Absorbance at 485 nm of ethanol extractives from cold-stressed Malus "Starking" stems incubated in various TTC solutions.
Figure A1-5a. Absorbance \(485\) of ethanol extractives from cold-stressed Malus "Starking" stems and bark incubated in various TTC solutions.
MALUS DAY 5

Figure Al-5b. Absorbance of ethanol extractives from cold-stressed Malus "Starking" stems and bark incubated in various TTC solutions.

Temperature (°C)

Absorbance (485 nm)/gm dry weight

- bark injury
- all killed
- wood injury

- pH 6.5, 10 mM ger., 10 mM GSH
- pH 6.0, 10 mM ger., 10 mM GSH
- pH 7.5, 10 mM ger., 10 mM GSH, 10 mM ascorbate
germanium at pH = 5.5 and 6.0 (Figure 6). "Starking" tissues with or without TTC at pH 7.4 (Figure 7) produced similar curves as noticed previously; lower pH's gave better results. In both genera, there is a rise in reduction at the point of bark injury, which is dampened at pH 5.5. In contrast, hemlock was best at pH 7.4 without germanium (Figure 8); chemical differences between gymnosperms and angiosperms could account for this.

In the last experiment, germanium was not found to interfere with living tissue reduction of the pear ("Hang Pa-Li"); it did, however, greatly limit 'reduction' by dead tissues at all pH levels (Figures 9a and b). This may be due to germanium's ability to inhibit phenolic oxidation (Loomis, 1974). This was supported by the minimal colorimetric interference at pH levels below 7.4. The results from crabapple tissue were similar, although the overall levels of TTC reduction were lower (Figures 10a and b).

In general, it was observed that large tissues reduce less TTC than smaller tissues. This may be due to differences in surface area available to the TTC solution, the relative frequency of active cells, and oxygen content; in any case, maintaining consistent sample sizes is important in decreasing variability in TTC reduction.

The addition of reducing agents was not as helpful to reducing variability as was the addition of germanium. Ascorbate caused TTC reduction independently, and glutathione had little effect; in contrast, germanium at fairly high levels (e.g. 25 and 50 mM) depressed the apparent 'reduction' of TTC by dead tissues, presumably by inhibiting phenolic polymerization. Adding sodium hypochlorite to tissues did not enhance differences between living and dead tissues (data not shown).

It is obvious that woody tissues contain compounds which interfere with TTC reduction or absorb near 485 nm. It is also important to realize sublethally stressed tissues
Figure A1-6. Absorbance at 485 nm of ethanol extractives from cold-stressed Pyrus bark incubated in various TTC solutions
Figure A1-7. Absorbance of ethanol extractives from cold-stressed Malus "Starking" bark incubated in various TTC solutions
Figure A1-8. Absorbance of ethanol extractives from cold-stressed hemlock bark incubated in various TTC solutions.
Figure Al-9a. Absorbance of ethanol extractives from *Pyrus* "Hang Pa-Li" stems as influenced by germanium concentration and pH of TTC solutions.
Figure A1-9b. Absorbance at 485 nm of ethanol extractives from dead Pyrus "Hang Pa-Li" stems as influenced by germanium concentration and pH of TTC solutions.
Figure Al-10a. Absorbance of ethanol extractives from live *Malus* "Red Splendour" stems as influenced by germanium concentration and pH of TTC solutions.
Figure Al-10b. Absorbance₄₈₅ of ethanol extractives from dead Malus "Red Splendour" stems as influence by germanium concentration and pH of TTC solutions.
may actually reduce more TTC as compensatory processes are activated, resulting in more active turnover. These studies suggest several ways of improving the TTC reduction test as a viability assay for woody angiosperm tissues: 1) by allowing tissues to recover or regenerate for several days following freezing and then test their viability with TTC, as suggested by Wu and Wallner (1983); 2) by increasing to .5% the TTC in solution to provide more substrate for the vital reduction; 3) by lowering the pH to values below 7.4, preferably near 6.0; 4) by adding phenolic oxidative inhibitors such as germanium; and 5) by controlling sample parameters such as size, age, location, etc. Utilizing two or more viability tests for increased accuracy, as suggested by Palta et al. (19), would also be advisable. Nevertheless, better controlled and repeated experiments should be conducted before the validity of the TTC viability test can be assessed for woody tissues.

Literature Cited


APPENDIX 2

THIN LAYER CHROMATOGRAPHY (TLC)
AND PHENOLIC IDENTIFICATION

TLC has replaced paper chromatography in many instances due to the relative versatility, speed and sensitivity of the former method (Harborne, 1984); it is an excellent method of analyzing mixtures of compounds. Cellulose coated plates are ideal for the separation of hydrophilic substances, since they contain many free hydroxyl groups to bind with water and alcohol (Fried & Sherma, 1982). Many prepared plates have an added fluorescence indicator to enhance detection of compounds which absorb 366nm UV radiation; this includes the majority of phenolic compounds. These spots are sometimes "identified" by computing $R_f$ values, which measure the distance a compound has travelled on a chromatogram relative to the solvent. Although they are often used as identifying characteristics, $R_f$ values are not readily reproducible as they are affected by temperature, humidity and solvent differences which are hard to control (Ribereau-Gayon, 1974). It is therefore hard to compare experimental $R_f$ values with those found in the literature. Some generalizations can be made, however, regarding the relative position of unknown compounds on a chromatogram.

Figure 1 illustrates a typical 2-D TLC plate, split into four quadrants, with each axis representing a different solvent system (X-axis = 3 parts t-butyl alcohol:1 part acetic acid:1 part water (TBA); Y-axis = 6% acetic acid). This is a typical 2-D TLC solvent system for phenolic separation, which is based on differential absorption to cellulose and solvent solubility (Haslam, 1982). In general, aglycone flavonoids (those without sugar moieties) will be found along the bottom axis, as they move well in non-polar solvent systems such as TBA but not in polar
Figure A2-1. Relative movements of a hypothetical phenolic compound on 2-D TLC after various chemical modifications.

1. Hydroxylation (-OH)
2. Glycosylation
3. Methylation of hydroxyl (-O-CH₃)
4. Unmodified compound
solvents (Markham, 1975). These compounds tend to be planar molecules whose low acetic acid movement is due to hydroxyl attachment with the cellulose plates, as well as poor water solubility (Haslam, 1982). The addition of sugar moieties will disrupt the planarity of the molecule and thus increase movement in the polar solvent. In contrast, large tannin complexes are found along the y-axis, as they move best in polar solvents and poorly in non-polar solvents. Certain chemical modifications will also affect movement in the solvents (Ribereau-Gayon, 1972); an increased number of hydroxyl groups lowers the $R_f$ values in aqueous and alcohol phases, while methylation of these hydroxyls will increase the values. Glycosidation will increase the value in aqueous solvents and decrease the $R_f$ value in non-aqueous solvent types. The result of these modifications is shown in Figure 1. It should be noted these are not the only modifications possible.

Literature Cited


APPENDIX 3

DIFFERENTIAL THERMAL ANALYSIS AND DATA MANIPULATION USING EQUIPMENT AT THE NATIONAL CLONAL GERMPLASM REPOSITORY

These procedures are adapted from directions compiled by Jose Montano in June 1987. They have been modified here as used during completion of the preceding research.

NOTE: When performing a time course study, allow yourself 20-30 minutes to prepare the samples and set up the equipment. In other words, if a test must begin in two hours following experimental treatment, begin your set-up at 1.5 hours post-treatment. Once you have become comfortable with the equipment, the preparation time will decrease.

Preparation of samples: Before any tests can be run, prepare aluminum foil cylinders and modified rubber bands for your sample tissues. Aluminum foil cylinders can be made by taking strips of foil approximately 2 cm x 5 cm and wrapping them lengthwise around the eraser end of a pencil. Allow about .5 cm to extend past the eraser; this excess is folded over the end of the cylinder after it has been rolled. Gently slip the completed cylinder off the pencil and continue making cylinders until you have at least 20. Make additional cylinders for later runs as time permits. To make miniature rubber bands, cut narrow rubber bands into about 3 cm sections. Wrap the band once around your finger or another object of about the same diameter, and tie a strong double knot. These small bands are vital to your experiment, as larger ones are difficult to work with and will not fit well in the aluminum block. These are reusable and about 25 or so are needed.
Attach your sample to the thermocouple board, which consists of 20 sample thermocouples and 20 reference thermocouples (already covered with aluminum foil). The thermocouple wires are very delicate; if broken, nonsense data will result and several hours will be spent repairing the thermocouple. Use the utmost care when touching any of the wires. Record what samples will be placed on which thermocouples; it is best to alternate treatments on the board, i.e. odd numbers = controls, and even numbers = treatments. Slip each sample into an aluminum foil chamber, lower it over its corresponding thermocouple wire (but not the rod next to it), and attach it to the rod with one of your small rubber bands. It should be tight enough to hold it firmly, but don’t force it. Continue until all 20 samples are attached. Carefully invert and lower the board onto the aluminum block with its corresponding holes, making sure not to smash any of the samples between the board and the block. This is a process that will take some time to learn to do correctly, so be very slow and careful. After it is firmly seated, the block, the CR-7 data logger, and the cassette recorder can be moved onto a cart (watch out for the thermocouple wire bundle!), and the entire set-up is transported to the freezer. Gently set the aluminum block inside the freezer and shut the door gently over the wires without bending them sharply. It will take 15-30 minutes for the samples to reach 0°C; while you wait, you can set up the cassette recorder and the data logger.

Only use unused tapes for data recordings. Set the tape number at zero and be sure the tape speed switch is on "off". Activate the record button and vocal record the date and your experiment into the microphone; then turn it off. Hook up the jacks from the data logger into the power and microphone ports of the cassette recorder. To start recording, press the record button on the recorder. The tape moves forward only when receiving data from the logger,
which is about every 4-5 minutes. Now set up the programs for the data logger.

Use of the CR-7 Data Logger: Before using this equipment, be sure to read the owner’s manual, located in the research offices at the NCGR. This manual will explain in detail the operations of the machine. Once you are comfortable with the data logger, the following instructions can be used to run differential thermal analyses of azalea buds.

Begin by clearing the data logger’s memory. If this is not done, you will get an "E-9" reading on the screen when you begin your experimental run. Press the keys *A6000A0000A7072A1688*0. Now press *1AA; this allows you access to the programming of the logger. Listed below are the commands you enter and what the screen will read. For an explanation of what each command means, please see Jose’s instructions. (NOTE: A=advance, B=backup, D=decimal, and P=program.)

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After entering these programs, you can check them by pressing "B", which will backspace step by step to the beginning. Then press "A" to return to the last step. When you are sure the programming is correct, press *0. The screen will read :LOG. To begin recording temperatures, press *1A*0; the screen will then read :LOG1. The numbers to be filled in the blank correspond to your desired sampling rate in seconds. In DTA runs with azalea buds, where temperatures below -25°C are not needed, an ideal rate is 10 seconds. If the freezing rate is slower, or if lower temperatures are required, 15 seconds is used instead.

To monitor the temperature of any thermocouple, such as number 9, press *610A. The sample thermocouples are at locations 2 through 21 on the logger, so to monitor number 9, ask for location 10. Push "A" to advance through the thermocouples, and "B" to backspace. Keep an eye on the temperatures, as you will want to start recording when they are around +1 or 0°C. There is no reason to record data.
before this, as it will clutter your disk and make your data manipulation more difficult.

During the run, notice the colon before LOG1 blinking at regular intervals. This indicates the recorder is sampling the temperature at each thermocouple at your selected sampling rate. When your run is finished, stop the sampling by pressing *1A0A*0. The colon will stop flashing; however, there is still some data left in the logger's memory. To transfer this to the cassette tape, press *83A3A. Now unhook the cassette recorder from the CR-7.

Manipulation of data: Before rewinding the cassette tape, be sure to note the ending position on the tape counter. You will need to know its location later; ideally, it should read anywhere up to 40. If you've gone much past 40, you will not be able to fit all of your data onto one disk. Get the cassette jacks from the desk drawer holding the cassette recorder, along with a blank, formatted floppy disk and the hard disk marked "DTA data and programs." You will have to use one of the computers at the NCGR; Kim Hummer will show you how to set up the programs for your analysis.

Attach the jacks to the remote and monitor outlets in the recorder and the C-20 cassette interface (found in the computer area). Plug the C-20 into a power source, and attach the IBM PC cable to Port A of the C-20. Push "reset" on the C-20. Put the DTA hard disk into drive D.

Using either a floppy disk copy of PC Talk III in drive A, or the computer's hard disk (if it has a copy), type talk128 and press return on the keyboard (or pc-talk if using the hard disk). When the program has loaded, press alt P; this allows you to reset the parameters listed under choice #3. Press alt R and type in D:DTA1.txt. It is critical you type this in exactly or the shuffle program used later will not work. D signifies that the data will feed into drive D, where your hard disk has been inserted.
When you then press return, the screen will tell you it is receiving file DTA.txt in drive D. Push the "play" button on the recorder, and watch the C-20 interface; the "data" light should be lit, and then the "transmit" lights should also go on. Now check the computer screen; it should begin registering data in sets of 6 lines beginning with 01---- and ending with 42----. The entire process of dumping the cassette data will take anywhere from 15-30 minutes, depending on how much data you recorded. When the cassette recorder reaches the end number, wait for the screen to stop registering data. At this point, you can turn off the recorder, and press alt R. This terminates the file. Press alt X to leave PC Talk. Insert your blank, formatted diskette into drive A (removing PC Talk if you used a floppy copy).

Get into drive D and type "necws" to get into the Wordstar program on your hard disk. Push "n" to open a non-document file, and retrieve "DTA1.txt" to edit. Using the delete commands, remove all lines from the top of the file that do not have numbers (i.e. there are x’s in some spots) or that do not begin with the 01---- reading. There should be 5 complete and 1 partial row for each complete set of readings. After the top of the file is clean, press control Q to get into the quick commands. Press C to get to the bottom of the file; once reached, all blank and incomplete lines and spaces must be removed using the delete commands. THIS INCLUDES THE BLANK SPACES TO THE RIGHT OF YOUR LAST DATA ENTRY (i.e. 42----). When this is done, press control Q and then R to get to the top of your file; you can now save your file and exit the system by pressing control K, then X.

When you are back into the regular system, type "dBase" to enter this system. Next type "do shuffle", and this program will begin. Shuffle is a program developed by Mark Rebuhn in 1986, but the copy on your hard disk has been modified by Kim Hummer to be more useful for the azalea bud
DTA work. Press any key after the "greetings" message has come onto the screen, and the program will begin. This will take from .5-1 hour, depending on the amount of data you have compiled. BE SURE YOUR BLANK DISK IS IN DRIVE A! The shuffle program will convert machine language to temperature readings, and then match the experimental with the reference thermocouple readings. Then all of your data is transferred onto the disk in drive A, in four print (prn) files labelled DTA11, 12, 13, and 14. After you have ascertained the files have been transferred to your blank disk, label the disk with your name, date, and description of the experiment, and erase these files from the hard disk in drive D. To do this, type "dir dta*.*". The directory should list dta files from that day's work. Then type "erase dta*.*" to remove them. If you do not erase them, the next time you do a transfer of data from the cassette recorder, it will be added onto the data from the previous run. Be sure not to erase anything else from this disk.

Before you leave the NCGR, be sure to replace the C-20 interface where it belongs, as well as the hard disk and the cassette recorder and jacks. The rest of your data analysis can be done at OSU using the Lotus 1-2-3 and PrintGraph programs available in the Horticulture department. Instructions for these programs are found in detail elsewhere and are not included here.
APPENDIX 4

A RELIABLE METHOD OF FIXING, SECTIONING, AND STAINING WOODY TISSUES

Although a great deal of literature exists regarding the fixation of plant tissues, the majority of the techniques assume the use of tender material. When these methods are applied to more lignified tissues, such as woody stems and buds, problems often arise in the fixation or sectioning procedures as was described by Bell and Facey (1937). The methods outlined below have been adapted from typical histochemical preparations for use with cold hardy Azalea buds; they should prove helpful with tissues from other woody species. Such chemical fixation will not affect either lignin or suberin within the tissues (Biggs, 1984).

Tissue Fixation: Use a preparation of FAA (9 parts 70% ethanol, .5 part 37% formalin, .5 part glacial acetic acid) for killing of tissues. For quickest penetration of FAA into the tissue, cut the proximal tip off the bud before submersing it into the solution. Vacuum-infiltrate (27-30 mm Hg) the bud in solution for 24 hours; release the vacuum slowly to prevent air bubbles from being forced back into the bud. Air bubbles will interfere with sectioning. The buds may remain in tightly capped jars of FAA indefinitely.

The dehydration series is adapted from Rickson (1983) for Paraplast embedding; at each step make sure the tissue is completely covered by solution:

1) Pour off FAA and add TBA I; immerse 8 hours minimum.
2) Pour off TBA I, add TBA II; immerse 8 hours min.
3) Pour off TBA II, add TBA III; immerse 8 hours min.
4) Pour off TBA III, add TBA IV; immerse 8 hours min.
5) Pour off TBA IV, add TBA V; immerse exactly 8 hours as longer will cause brittleness. Do not use eosin to color the TBA V, as this will fluoresce under UV microscopy (Pearse, 1980).

6) Pour off TBA V, add warm (25-35°C) 100% TBA and store in a warm (25-35°C) place for 4 hours. Repeat this step.

7) Pour off TBA and add a warm mixture of equal parts 100% TBA and paraffin oil. Store in a 60°C oven for at least 8 hours.

8) Place tissue and solution on 2 cm cold Paraplast in a glass vial. Return to the oven.

9) When the Paraplast has melted, pour off liquid portion and add new melted Paraplast. Let sit in oven at least 12 hours.

10) Pour off old and add new Paraplast. Let buds remain in melted Paraplast in the oven for at least 1 week. It is critical to allow the Paraplast to infiltrate the bud scales and flowers completely to prevent formation of air bubbles.

**Embedding, storage, and sectioning of tissues:** Typical embedding techniques, such as those described by Rickson (1983), can be used with the woody tissues. After embedding and mounting tissues on wooden blocks, they may be stored in a refrigerator indefinitely. Before sectioning such tissues, trim off the hardened Paraplast with a razor blade down to a level where the tissue is exposed. Place these sliced blocks in a 10% solution of glycerol for at least 24 hours (Biggs, 1984). This will soften tissues to permit sectioning with the rotary microtome at 8-10µ thicknesses; if tissues become brittle during sectioning, return them to 10% glycerol for further softening. During sectioning, a steady stream of dry ice vapor over the microtome knife and Paraplast block helps maintain ribbon quality.
Mounting of sections: Haupt's adhesive is typically used for mounting soft tissue sections, but is not as reliable with woody tissues. Instead, a general aqueous adhesive as described by Fink (1987) gave better results. This organic adhesive prevented the loss of sections from slides during staining and interfered little with later fluorescence microscopy.

Staining for Lignin and Suberin: For the staining of lignin, a procedure as described and modified by Biggs (personal communication) was followed. The Toluidine Blue O procedure is outlined below for use with paraffin sections, and works well in quenching lignin autofluorescence under UV-excitation (Biggs, 1984).

Toluidine Blue:

1. Xylene 5 min
2. Xylene 5 min
3. Xylene + 100% ethanol 5 min
4. 95% ethanol 5 min
5. 70% ethanol 5 min
6. 50% ethanol 5 min
7. 30% ethanol 5 min
8. Distilled water 5 min
9. Aqueous solution of 0.1% Toluidine Blue O 2 min
10. Distilled water rinse, blot dry 5 min
11. Mount with Fluoromount or other non-fluorescing, non-aqueous media

A blue color indicates the presence of lignin under brightfield microscopy.
Two suberin stains were employed: Sudan IV and Sudan Black B. Sudan Black B will quench autofluorescence of suberin under UV-excitation, while Sudan IV will enhance suberin autofluorescence (Biggs, 1985).

Sudan Black B (after Pearse, 1968):

1. Xylene 5 min
2. Xylene 5 min
3. Xylene + 100% ethanol 5 min
4. 95% ethanol 5 min
5. 70% ethanol 5 min
6. Saturated solution of Sudan Black B in 70% ethanol 15-20 min
7. 95% ethanol * 5 min
8. Sudan solution in step 6 15-20 min
9. 70% ethanol 1 min
10. Distilled water rinse 5 min
11. Mount in 90% glycerin

* Decolorization and Sudan treated tissues with 95% ethanol and subsequent recolorization distinguishes true Sudanophilia from nonspecific staining (Biggs, 1985).

A blue-black color indicates the presence of suberin under brightfield microscopy.

Sudan IV:

1. Xylene 5 min
2. Xylene 5 min
3. Xylene + 100% ethanol 5 min
4. 95% ethanol 5 min
5. 70% ethanol 5 min
6. 50% ethanol 5 min
7. Saturated solution of Sudan IV in 95% ethanol:glycerin (1:1) 15-20 min
8. 95% ethanol * 5 min
9. Sudan IV solution as in step 7 15-20 min
10. 50% ethanol 1 min
11. Distilled water rinse 5 min
12. Mount in 90% glycerin

* Decolorization and Sudan treated tissues with 95% ethanol and subsequent recolorization distinguishes true Sudanophilia from nonspecific staining (Biggs, 1985).

A red color indicates the presence of suberin under brightfield microscopy. Under fluorescence microscopy, suberin appears bright red against a yellow-green background (Biggs, 1985).

For observation of autofluorescence of tissues in the absence of any stains, paraffin sections were treated in the following manner:

1. Xylene 5 min
2. Xylene 5 min
3. Xylene + 100% ethanol 5 min
4. 95% ethanol 5 min
5. 70% ethanol 5 min
6. 50% ethanol 5 min
7. 30% ethanol 5 min
8. Distilled water 5 min
9. Mount in 90% glycerin

Under fluorescence microscopy, lignin and suberin appear bright blue.
Photomicroscopy: A microscope with a mercury lamp for UV-emission is necessary for fluorescence microscopy. Two Zeiss microscopes, one with an FITC filter and one with a DAPI filter, were used in these studies. Both filters allow excitation in the UV range. The FITC filter tends to give micrographs a greenish tinge, while the DAPI filter allows a bright blue fluorescence, which is also much brighter. The latter is preferred for plant autofluorescence, as the exposure time for photography is much shorter because of the brighter fluorescence. Ektachrome ASA 400 film made good quality photomicrographs, although slower film (such as 200) can be used for clearer results if exposure time is not excessive.

Literature Cited


