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Abscisic acid (ABA, $7.5x10^{-5}M$), significantly induced freezing tolerance by -23C in bromegrass (Bromus inermis Leyss cv Manchar) cell suspension cultures after seven days at room temperature (23C). Metabolic changes were monitored over this treatment period. ABA reduced growth rates as a result of decreased cell number rather than cell size. The confounding factor of dry weight (DW) was removed in the standard expression of water content (% H_2O or g H_2O/g DW) by re-expressing water content as a function of cell number. On a cellular basis, the water content of control and hardy ABA-treated cells was found to be similar, but dry weight per cell significantly increased during ABA-induced frost hardiness. This finding differs from numerous prior reports on whole plants which express water content as a function of dry weight. The cell wall fraction accounted for most of the substantial change in dry weight and the increase in this fraction was highly correlated with increased frost tolerance. ABA also enhanced uptake of ^{14}C-(U)-sucrose that was partitioned into an insoluble fraction containing

cell wall components. In addition, metabolic activity increased as measured by CO_2 evolution. Ultrastructural changes observed during ABA-induced frost tolerance included increased cell wall thickness and density. Cytoplasmic alterations during hardiness development included increased numbers of lipid bodies, osmiophilic granules, starch grains, dictyosomes and vacuoles. Vacuoles and lipid bodies in ABA-treated cells became smaller as cells developed more frost tolerance. Both the cell walls and protoplasm of ABA-treated cells became strongly autofluorescent. The autofluorescing component(s) did not appear to be lignin, but was soluble in methanol/chloroform and thus may be lipidic in nature.

ABA markedly increased bromegrass cell tolerance to high hydrostatic pressure stress; to levels approaching the pressure resistance of deep sea aquatic microorganisms. The increasing resistance of cells to hydrostatic pressure closely parallelled their increase in frost tolerance over time. High pressure injury was not an immediate result of compression/decompression, but was cumulative over a 12 hour period, and did not involve cell rupture.

A new bromegrass cell culture was generated from the same cultivar and evaluated to establish whether the ABA-induced hardiness response was unique to the widely used old cell line, or a genetic artifact developed in that line over many generations in culture. The newly generated bromegrass culture acclimated to resist freezing stress in response to ABA treatment. The new culture expressed a greater rate of acclimation than the old culture, and normal green plants were produced only from cells of the new culture.

Abscisic Acid Induction of Environmental Stress Tolerance in Plant Cells

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1.0 INTRODUCTION

The essence of nature is the true harmony of its parts. It is a balance between action and reaction at all levels of organization. Perhaps nowhere is this balance more evident than in the interaction of a plant with its environment. The stationary form of plant existence demands effective adaptation for survival. It is not surprising therefore, that highly developed environmental sensor and response systems have evolved, and that the natural distribution of plant species reflects the degree of inherent plasticity and success of these adaptive systems.

Over 90 percent of the earth's land area is uncultivated because of insufficient plant adaptation to environmental stress (Weiser, 1970). Low temperature is one of the most important environmental selection pressures influencing world plant distribution (Sakai and Weiser, 1973). Unfortunately, the origins of most crop plants which sustain human civilization were centered in areas with more favourable (less stressful) climates than those which exist in many desired production areas. Although some crop species have been successfully expanded into temperate climates, limited genetic potential to acclimate (Chen and Gusta, 1987) and yield instability (Boyer, 1982) have largely prevented expansion of cultivated crop plants into harsher environments.

Unanswered questions about the mechanisms that permit some plants to adapt effectively to survive severe stress, and the desire to enhance the productivity of plants exposed frequently to sub-lethal stresses have stimulated much research. The strategies for improving frost survival are as diverse as the constraints, but essentially fall into three categories: genetic approaches involving classical breeding, molecular genetics, or both; elucidation of the underlying physiological mechanisms of low temperature acclimation and injury; and cultural and crop management practices.

The most direct methods for enhancing plant survival and productivity are often judicious applications of cultural and management practices, but there are limitations to those strategies including costly and unreliable dependency on stress avoidance, and inherent limits to the hardiness potential that can be achieved in many plants.

Classical breeding approaches have been moderately successful in selecting plants whose development is synchronized with annual climatic cycles and stresses, and in elevating plant hardiness levels. However, the threshold of hardiness has been reached with no transgressive segregation (Chen and Gusta, 1987). Recently, somaclonal variation (Chen et al., 1988) and chromasome substitution (Roberts, 1988, Galiba and Sutka, 1988) have increased hardiness levels and represent potentially fertile areas of research. Considerable research attention is also currently being devoted to identifying specific genes and proteins thought to confer stress resistance. Although this approach may prove

productive, resistance traits expressed at the morphological, physiological and biochemical levels are usually controlled by several genes. Moreover, stress acclimation appears to involve complex sequential and stepwise sensory and adaptive responses in plants (Weiser, 1970) of a nature that will not be readily amenable to single gene transfer approaches.

Fundamental to successful hardiness elevation under both cultural/management and breeding/genetic strategies is an understanding of the basic physiological mechanisms of plant acclimation.

However in the past 100 years, progress has been limited in understanding the mechanisms of plant tolerance to freezing stress. Progress has been slow, in part, because of the inability to separate confounding effects of coincident changes that occur during natural low temperature acclimation from the changes that play a causal role in acclimation. Low temperature exposure elicits many metabolic alterations in plants not directly related to frost tolerance which results in the induction of numerous responses (Kacperska, 1989).

In recent years, the natural plant growth hormone, abscisic acid (ABA), has been shown to significantly elevate plant cell hardiness levels at room temperature (Chen and Gusta, 1983; Chen et al., 1983). Since this ABA response bypasses the complicating effects of low temperature while rapidly inducing frost tolerance (Chen and Gusta, 1983, Keith and McKersie, 1986; Reaney and Gusta, 1987), it has become a powerful research tool for elucidating the nature of cold acclimation mechanisms in hardy plant species.

Furthermore, evidence that ABA can only elicit hardiness in cold hardy plant species (Chen and Gusta, 1983), and the observation of an endogenous rise in tissue ABA concentration just prior to a rise in protein synthesis and low temperature acclimation (Chen et al., 1983) suggests that ABA may play a pivotal role in hardy plant acclimation in nature.

The major research emphasis to date has been to compare the proteins/transcripts synthesized in response to low temperature and ABA treatments (Mohapatra et al., 1988; Robertson et al., 1986,1987; Tseng and Li, 1987). In spite of the significant frost hardiness induced by ABA, relatively little is known of the physiological mechanisms controlling this response (Reaney et al., 1989). To address this discrepancy, the thesis examined physiological alterations during acclimation that were elicited by ABA treatments. Acclimation is a metabolic and energy-dependent process (Levitt, 1980). Use of cell cultures provided a relatively homogeneous system that facilitated examination of the direct effects of ABA on carbohydrate partitioning and utilization.

Since the nature of ice formation plays a central role in freezing injury (Olien, 1967), a clear knowledge of the water status within the plant is important to understanding how plants acclimate. It was established that water content did not change during ABA-induced acclimation when the confounding effect of dry weight was eliminated by expressing water content on a per cell basis. This finding differs from previously published literature which suggested that low temperature-induced hardiness involves a decrease in cell water content in whole plants (Chen et al., 1976;

Chen and Gusta, 1978; McKenzie et al., 1974). Dry matter however, significantly increased per cell. By expressing values on a cellular basis, cellular components associated with dry matter could be expressed independently of this fraction. The cell wall component was determined to quantitatively represent the major portion of the dry matter accumulation and was highly correlated with the acquisition of frost tolerance. The importance of the cell wall in ABA-induced acclimation is also suggested by Reaney (1989). Its role in low temperature acclimation is supported by the findings of Tao et al. (1983), Olien (1977), Rajashekar and Burke (1982), Weiser (1989), and Griffiths et al. (1985). In addition, this thesis visually observed common ABA and low temperature-elicited metabolic alterations associated with hardiness in both the cytoplasmic and cell wall components.

The documented importance of ABA in the resistance of plants to several stresses (Addicott and Van Steveninck, 1983; Walton, 1980) may be extended to hydrostatic pressure. For the first time, ABA was demonstrated to induce plant cell tolerance to high hydrostatic pressures analogous to pressures found at the limits of ocean depth. Since tolerance to hydrostatic pressure followed the increments of ABA-induced tolerance to freezing stress over time, similar mechanisms of cellular resistance are suggested. Thus, hydrostatic pressure may represent a unique tool to address the mechanism of ABA-induced freezing tolerance. With reports of ABA detection in other organisms (Okamoto et al., 1988; Boyer and Dougherty, 1988; Chen et al., 1988), these results re-emphasize the possibility that ABA may play a central role in the

adaptational responses of terrestrial plants and marine organisms to a variety of environmental stresses.

The possibility existed that the ABA-induced hardiness response based on the twenty year old culture utilized in this thesis

and routinely used in other studies reflected a mutant cell line selected in culture over time. Thus, this traditional cell culture was compared with a new cell line generated from the same seed source. The response to ABA treatment of the twenty year old bromegrass cell cultures was determined not to be a result of mutation but appeared to reflect the characteristics of bromegrass. Furthermore, since the new cell line developed hardiness at a faster rate and was able to regenerate normal green plants, it has greater potential as a system in future studies elucidating the mechanism of ABA-induced frost acclimation.

The freezing point of water lies almost exactly at the centre of the temperature range which is associated with life on earth (Franks, 1985). The status of water both above and below this point represents the most important factor driving the evolution of organisms to resist environmental stress. ABA has been demonstrated to be associated with increased plant tolerance to both desiccation and freezing stress. This thesis indicates that ABA elicited physiological alterations in both the cytoplasmic and cell wall fraction that were analogous to those observed during natural low temperature-induced acclimation. In marine environments, hydrostatic pressure is the main factor influencing organismal distribution. Cell resistance to both hydrostatic

pressure and freezing stress during ABA treatment appeared to share similar mechanisms of response.

The beauty of nature is often expressed through elegantly simple control mechanisms of complex phenomena. This thesis lends additional support for a universal role of ABA in the adaptation of organisms to their environment.

The complex, physiological responses to low temperature acclimation reflect fundamental genetic regulation. ABA and hydrostatic pressure may offer a means to identify key acclimational changes and thereby provide a physiological basis for future molecular studies.

2.0 LITERATURE REVIEW

The consistency of plant response to seasonal change becomes more remarkable in light of the complexity of the plant itself. The effective regulation of such a system to react in a coordinated manner must clearly depend upon internal points of control. Hormones often represent those inherent controlling factors. Thus, hormone levels and activity are often tied to environmental parameters. Of all plant growth regulators, abscisic acid (ABA) has been most closely associated with eliciting plant responses to resist environmental stress.

ABA has been demonstrated to be associated with plant tolerance to several environmental stresses (Chen and Gusta, 1983; Ishikawa et al., 1985; Bray, 1988). Adaptation to one stress will often confer adaptation to another stress (Rikin et al., 1973). This cross-adaptation response may be a result of the similarity between stresses at the cellular level. Desiccation and hydrostatic pressure are two stresses that appear to share common elements of injury with freezing stress.

2.1 NATURE OF THE FREEZING STRESS

Freezing within plant tissues and cell suspensions involves the redistribution of water in terms of both its physical state and location. This pattern of redistribution is dependent on the rate of freeze/thaw and the type of tissue (Steponkus, 1984). Ice formation can exist either within the cells (intracellularly) or

outside the cell walls (extracellularly) (Siminovitch and Scarth, 1936).

Intracellular freezing is almost always fatal and thus the main mechanism of survival against this stress is complete avoidance of freezing. Avoidance may be achieved by absence of nucleating agents (Lindow, 1983), structural alterations (Chalker-Scott, 1988; Ashworth and Abeles, 1984; Ashworth and Rowse, 1982), avoidance of cavitation (Weiser and Wallner, 1988), or freezing point depression (Franks, 1985). Therefore, the approach adopted to enhance survival against intracellular freezing has been to identify and alter the potential factors involved. In nature, freezing avoidance is fairly widespread and includes plants with little freezing tolerance (Burke et al, 1976), flower buds that can deep supercool (Graham and Mullin, 1976; Quamme, 1978), and stems that deep supercool to temperatures below the homogeneous nucleation point (Gusta et al, 1983). Complete freezing avoidance can afford limited protection of less than -10C (Levitt, 1980). However, those plants that deep supercool are able to both tolerate extracellular freezing and avoid intracellular freezing by selective use of these strategies among tissues. Hence to optimize low temperature protection, the two survival strategies, avoidance and tolerance may not be mutually exclusive and can occur in different tissues within the same plant.

2.1.1 FREEZING PROCESS

Tolerant tissues can sustain extracellular freezing (Levitt, 1980) and in fact, avoid supercooling by initiating freezing outside the cell at temperatures above -2C (Ashworth and Davis, 1986). Nucleating agents are present at preferred sites and are either bacterial (Lindow, 1983) or non-bacterial (Gross et al., 1988) in nature. The class of nucleators appears to be tissue-specific (Proebsting and Gross, 1988). At the tissue level of organization, ice does not grow uniformly (Olien, 1981). The location of ice formation and accumulation is important in extraorgan freezing of vegetative buds (Sakai, 1978; Dereuddre, 1978) and stems (Ashworth, 1987). Further, Olien has extensively studied the significant dynamics and interactions of ice progression in intercellular spaces as well as its location, amount and size in relation to winter cereal hardiness (Kindel et al., 1989; Olien, 1981). In cell suspensions, cells often grow in large clumps (greater than 200 cells) and although a similar system of ice growth and dynamics may occur at this level, treatment of this topic will be based on a simple cell model.

This subject has been extensively addressed by Mazur (1969, 1970), Franks (1985), and Steponkus (1984). After nucleation in the extracellular space, latent heat of crystallization is released and serves to warm the system to a point below 0C, designated the freezing point. Water vapour pressure disequilibrium occurs due to cell compartmentalization and the inability of ice to penetrate the cell interior at slow cooling rates. Since the system tends to

equilibrium, water flows out of the cell to freeze in the extracellular spaces. As a result, the cell essentially desiccates. The cooling rate and permeability of the plasmamembrane determine this water flux. In nature, the cooling rate is usually relatively slow (less than 10C/hr) and equilibrium type freezing occurs. However, under conditions of rapid temperature change or decrease in membrane permeability, the system cannot attain equilibrium with subsequent intracellular freezing. Similarly, injury may occur if the rate of thaw is too high and the cell cannot re-absorb water quickly enough to maintain an equilibrium.

2.1.2 SITE OF INJURY

Since 1912 (Steponkus, 1984), the suggestion that the plasmamembrane represents the critical structure of direct or indirect injury after freezing stress has been supported with various lines of evidence. The flaccid, water-soaked appearance of leaves (Levitt, 1980) is the most obvious manifestation of injury and is a result of the alterations in the semipermeable state of the plasmamembrane. Moreover, many standard viability tests such as conductivity, fluorescein diacetate, and visual browning used to assess the extent of freezing injury (Palta, 1981), reflect the intactness of this membrane. Finally, perhaps the "test of time" is, in itself, the best supporting evidence that the plasmamembrane is the critical site of injury.

2.1.3 PLASMAMEMBRANE INJURY

Extracellular freezing stress to the plasmamembrane is predicated on two physical phenomena, low temperature and dehydration. The following summary is extracted from several sources (Steponkus, 1984; Graham et al., 1979; Levitt, 1980). Low temperature itself is not deleterious and injury is not observed in the absence of ice. However, freezing stress appears to result from the combination of both low temperature and dehydration stress. The manifestations of desiccation stress alone include cytoplasmic concentration, removal of water of hydration of macromolecules, volume and area contraction/expansion of the plasmamembrane and cell wall-induced plasmamembrane lesions. The combined impact of desiccation and low temperature creates an array of stresses on the plasmamembrane. Low temperature itself may cause a deleterious lipid membrane phase transition. Although this hypothesis was abandoned with evidence that the critical temperature of injury fell below the phase transition temperature, the possibility still exists that an interaction of phase transition and desiccation stress may cause membrane lesions.

Historically, technological advances and the incorporation of new knowledge have shaped the perception of the nature of stress. This perception will, in turn, direct the interpretation of the mechanism of injury.

2.1.4 MECHANISM OF INJURY

Current knowledge is a modification of historic observations. Unfortunately, since Molisch and others in the 1800's first described freezing injury through extensive observation with the light microscope, relatively little progress in understanding the mechanism of injury has been achieved (Steponkus, 1984). This may be partially due to the lack of appropriate tools to separate the confounding effects of natural low temperature acclimation. Abscisic acid (ABA), the natural plant growth regulator, confers resistance to freezing stress at room temperatures (Chen and Gusta, 1983) and is optimal at 27C (Gusta, pers. comm.). ABA thus appears to bypass the natural low temperature requirement and represents a potential tool to examine mechanisms of acclimation/injury.

2.2 ABA STRUCTURE AND ACTIVITY

The naturally occurring (+)-S-abscisic acid is the trivial name for the compound: (+)-(1'S,2Z,4E)-5-(1'-hydroxy-2',6',6'-trimethyl-4'-oxocyclohex-2'-enyl)-3-methylpenta-2,4-dienoic acid (Milborrow, 1983). Analogs have been extensively used to characterize ABA activity. The key sites include the carboxyl group, the tertiary hydroxyl group and the 2-cis and ring double bond (Milborrow, 1984). Over one hundred analogs have been produced and represent useful tools to elucidate ABA metabolism, transport and cellular control mechanisms (Gusta et al. 1990).

The natural material is optically active, rotating polarized light in the clockwise direction at the sodium D line and is therefore (+). Synthetic material consists of equal amounts of both (+) and (-). These optical isomers have different physiological activities and are metabolized in different ways and hence the nature of ABA used in experiments should be indicated (Milborrow, 1983). The active (1)-ABA, 2-cis form was employed in all the experiments of this thesis.

2.2.1 ABA STRUCTURE/ACTIVITY STUDIES

Several methods have been developed to detect ABA activity (Addicott et al., 1964; Cornforth et al., 1965; Aspinall et al., 1967; Jones and Mansfield, 1970). There are, however, inherent problems in determining biological activity of ABA which may originate from: rapid conversion of ABA and the dependency of biological activity on the specific bioassay system. The latter appears to be the result of compound permeability, metabolic enzyme activity, and active site structure between plants and plant tissues (Hirai, 1983). Since the bromegrass (Bromus inermis Leyss cv. Manchar) system used in this thesis and other studies seems to respond consistently to ABA, perhaps with defined growth conditions and freezing regimes, it has potential to be developed as a more appropriate bioassay in environmental stress studies. Reaney (1989) found S-ABA and R-ABA to be equally effective in increasing freezing tolerance in bromegrass cell suspensions. In

addition, treatment with the ABA metabolite, phaseic acid was ineffective in increasing freezing tolerance.

2.2.2 ABA, GENERAL EFFECTS

In addition to ABA's association with environmental stress, this hormone appears to both promote and inhibit growth, involved in abscission, seed dormancy, may or may not be associated with bud dormancy, parthenocarpy and flowering (for a review, see Zeevaart, 1988; Milborrow, 1984). In spite of the intricate nature of plant functions, regulation is apparently achieved by only five currently recognized hormones while animal systems possess upwards of forty such chemicals (Leopold, 1987). This situation predetermines the importance of hormonal ratios in that the function of individual hormones is increased when activity is dependent on a specific balance with other growth regulators. This information may help to explain some apparent contradictory effects of ABA where different concentrations in different systems may result in variable responses. The physiological status of the tissue will also significantly affect hormonal response Trewavas (1987). Trewavas (1987) has presented the concept of tissue hormonal sensitivity which serves to further enhance the levels and precision of control. These factors may in part, explain the contradictory nature of hormonal research. Thus, although still within physiological levels, the relatively high concentrations required to optimally induce freezing response in the bromegrass system (7.5×10^{-5}M, Chen and Gusta, 1983) may in

fact, be a natural plant mechanism to prevent constant initiation of acclimational changes that might be otherwise induced by lower concentrations of ABA.

2.2.3 ABA INDUCTION OF LOW TEMPERATURE TOLERANCE

Basically two lines of evidence infer involvement of ABA in cold acclimation. Exogenous application of physiological levels of ABA at ambient temperatures increase chilling and freezing resistance in a variety of plants including: cucumber seedlings (Rikin et al., 1976), cotton (Rikin et al., 1979; 1981), alfalfa seedlings (Waldman et al., 1975; Rikin et al., 1975), apple seedlings (Holubowicz and Boe, 1969), winter wheat, rye cell cultures (Chen and Gusta, 1983), winter wheat seedlings (Lalk and Dorffling, 1985), bromegrass cell suspension cultures (Chen and Gusta, 1983; Reaney and Gusta, 1987), birdsfoot trefoil cultures (Keith and McKersie, 1986); alfalfa cell cultures (Orr et al., 1985; Mohapatra et al., 1988), potato stem cultures (Chen et al., 1976; 1983), tobacco cultures (Bornman and Jansson, 1980), and Daucus callus (Nitzsche, 1978). Slight hardiness increases have been found in chrysanthemum rhizomes (Fayyaz et al., 1978). Endogenous levels of ABA increase during low temperature induction of cold acclimation in white bean leaves (Eamus and Wilson, 1983), potato stem cultures (Chen et al., 1983), and tomato (Daie and Campbell, 1981).

Although ABA appears to significantly and consistently elicit freezing tolerance in several hardy plant cell and tissue

culture systems, results from ABA treatment in whole plant studies have been less consistent (Gusta et al., 1982). This has been generally attributed to reduced uptake by whole plants compared with culture systems (Gusta et al., 1982) or rapid degradation (Milborrow, 1984). However, since ABA activity is highly dependent upon sufficient sucrose availability (Rikin et al., 1975), it is also possible that varying sucrose levels within the plant during the season may be a contributing factor to lack of response to ABA treatment.

The importance of ABA in the adaptation of plants against different environmental stresses is well documented (Addicott and Van Steveninck, 1983; Walton, 1980; Levitt, 1980). ABA has been demonstrated to be associated with salt (Bressan et al., 1985), heat (Ishikawa et al., 1985) and desiccation resistance (Bray, 1988; Walbot, 1988). Rikin and Rubin (1983) demonstrated an increased resistance of cotton cotyledon discs to herbicide by ABA pre-treatment.

The observation that pre-exposure to one stress with an associated rise in endogenous ABA concentrations will confer resistance to another stress is supporting evidence that ABA is a common stress hormone. Exposure to low temperatures was shown to cross-adapt barley and tobacco leaf tissue to drought (Rikin and Richmond, 1975). Rikin et al. (1973) had previously found that tobacco plants exposed to water stress, 5C temperatures, or mineral deprivation all improved resistance to sub-zero temperatures and the resistance was accompanied by a rise in ABA

content. Mizhrahi et al. (1972) concluded that resistance to lack of root aeration reflects adaptation to osmotic stress and was in part due to an increase in the content of leaf ABA. Boussiba et al. (1975) and Rikin et al. (1975) reported that tobacco plants pre-exposed to dehydration, mineral deprivation, salination, or BO_3^{-3} toxicity exhibited elevated ABA concentrations and resistance to low temperature and root anaerobic stress.

Endogenous ABA has been consistently found to increase under water stress and experiments have shown a pre-desiccation treatment results in elevated levels of chilling and freezing resistance comparable to that during low temperature acclimation. This desiccation to low temperature cross adaptation response has been demonstrated in Phaseolus vulgaris, Cucumis sativus, Gossypium hirsutum (Wilson, 1979); winter wheat and rye (Siminovitch, 1977; Cloutier and Siminovitch, 1982a,b,c; Cloutier and Andrews, 1984; Tyler et al., 1981); Cornus stolonifera (Chen et al., 1977); cabbage (Cox and Levitt, 1976); hydrated lettuce seeds (Keefe and Moore, 1981); and alfalfa (Stout, 1980).

2.2.4 PHYSIOLOGICAL MECHANISMS OF ABA-INDUCED FROST TOLERANCE

In spite of the growing evidence of ABA involvement in freezing tolerance, there is relatively little information on the physiological alterations induced by ABA during acclimation. Reaney et al. (1989) reported both physiological and morphological alterations during ABA-induced hardiness in bromegrass cell

suspension: a) Water content g H_2O/g DW decreased, cell sap of ABA -treated cells was greater than that of control cells, osmotic adjustment alone does not account for the ABA-increase in frost tolerance, synthesis of proteins that are both common with low temperature-induced acclimation and unique to ABA treatment were observed. b) Large central vacuole disappeared to form small vacuoles, lipid bodies and starch grains increased. Only control cells developed fluorescent endocytotic vesicles. The cell walls thickened dramatically and were more rigid than control cells during ABA treatment, and histochemical tests suggested an increase in cell wall phenolics.

There is however, ample information on specific physiological effects of ABA in other systems. ABA also elicits responses at the morphological, developmental, biochemical, physiological, and molecular level. Whenever relevant, literature has been incorporated into the individual papers of this thesis and the reader may refer there for further information and to Walton (1980), Addicott (1983), Zeevaart and Creelman (1988), and Creelman (1989) for reviews.

2.3 PHYSIOLOGICAL MECHANISMS OF LOW TEMPERATURE-INDUCED ACCLIMATION

In contrast with ABA-induced acclimation, literally thousands of citations on physiological alterations during low temperature-induced acclimation are available.

In short, every major component has been shown to alter during low temperature acclimation (Levitt, 1980). Identifying alterations directly associated with cold acclimation represents a consistent challenge. Temperature influences metabolism by its effect on chemical and thus enzymatically catalyzed reactions (Patterson and Graham, 1987). The multitude of interacting metabolic pathways and differential temperature sensitivity of these pathways will significantly influence carbon flow resulting in changes that may or may not be related to the acquisition of frost tolerance. Moreover, temperature will manifest its effects separately and interactively at the whole plant, tissue, and cell level.

However, alterations in water and dry matter status are two consistently observed low temperature-induced acclimational changes. Water plays an obvious and central role in freezing tolerance (Olien, 1967). The net accumulation of dry matter is dependent on excess synthesis over degradation. In acclimating plants, this normally translates into excess photosynthesis over respiration (Levitt, 1980). For a treatment of these subjects and other relevant low temperature-induced physiological changes, the reader is referred to the pertinent thesis papers.

2.3.1 METABOLISM OF SUCROSE DURING COLD ACCLIMATION

The possible role of sugars in acclimation has been broadly categorized (Sakai and Larcher, 1987): 1) The osmotic effect;

where the accumulation of sugars will decrease the cellular freezing point, 2) The metabolic effect; where the metabolism of sugars in the cytoplasm will induce energy or substances associated with hardiness, and 3) The cryoprotective effect: where sugars and sugar alcohols may provide direct protection of membranes. A fourth category might be suggested from information that sugars may indirectly alter metabolism through changes in osmotic potential (Laimins, 1981).

Of more direct interest here is the second category of sucrose metabolism. Low temperature appears to produce two types of metabolic adjustments in plants, a short term functional and longer term developmental response (Kacperska, 1989). For instance, ^{14}C incorporation revealed increased production of amino acids over sugars within 2 days of low temperature exposure although the response disappeared thereafter.

If low temperature is considered a stress, similarities are apparent between ^{14}C incorporation into photosynthetic metabolites under other stresses as heat (Chljustova and Tarchevsky, 1972), desiccation (Lawlor, 1976), and salinity (Passera and Albuzio, 1978). Based on these observations, Kacperska (1989) proposed a model in which stress conditions promote certain photosynthetic pathways that respond to ABA/GA ratios and depend on the availability of NADPH.

Several studies examining shifts in metabolic pathways during low temperature acclimation indicate the diversion of glucose-6-phosphate metabolism from glycolysis to the pentose

phosphate pathway (Sagisaka, 1974). Furthermore, it appears that reducing power utilizing reactions are favoured in cold acclimating cells (Sakai and Larcher, 1987).

Since a significant increase in ATP and adenylate charge was observed during cold acclimation of winter rape leaves (Kacperska, 1989), high levels of energy including NADPH were proposed necessary for cold acclimation (Kacperska-Palacz, 1978).

2.4 HYDROSTATIC PRESSURE (P)

All organisms which live below the sea's surface are subject to pressures in excess of 1 atm.

The static pressure P at a defined point in a liquid is given by the force F acting on a surface area A:

$$P = F/A \qquad (1)$$

If F is the weight of a water column with a specific weight g,
its value can be obtained:

$$F = gAd$$

Where A=horizontal area, and d=vertical depth of the water column. If the value for F is introduced into (1):

$$P = gd$$

Thus, the hydrostatic pressure is dependent on the density and vertical depth of the water column (Kinne, 1972).

'In view of the vast extent of the biosphere having a hydrostatic pressure higher than atmospheric, it seems paradoxical that so little is known about the effects of increased pressure on the structure, survival, growth, and biochemical activities of organisms' (Zobell, 1970).

In terms of hydrostatic pressure effects on plants, little has changed in the past twenty years. However, evidence based on bacterial systems have extended knowledge of mechanisms at the molecular level. Increasingly apparent are the similarities of adaptation/injury between hydrostatic pressure and low temperature stress. The reader is referred to chapter 6 for further information.

2.5 VIABILITY TESTS

Numerous viability tests have been developed to indicate plant survival after freezing stress (Palta et al., 1978). The 2,3,5-triphenyltetrazolium chloride (TTC) viability test has become one of the most well used cell viability assays in ABA-induced hardiness experiments (Towill and Mazur, 1975; Steponkus and Lanphear, 1967). However, there are apparent limitations of this method (Harber, 1989). The TTC reduction test appears to overestimate the viability of cells after freezing stress when compared with cell regrowth (Harber, 1989). This discrepancy may be due to ABA induction of NADPH which is closely tied to TTC reduction (Zhen, pers.comm.). In addition, the cell regrowth test selects for those cells that are uninjured and that have the

capacity to proliferate. The observed TTC increase of viability may thus be a reflection of the additional number of injured cells that are still capable of TTC reduction. However, until the mechanism of injury is understood, each viability test at the cellular level will have its own set of limitations and becomes a relative index of survival.

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3.0 Water Content During Abscisic Acid Induced Freezing Tolerance in Bromegrass Cells

3.1 ABSTRACT

Changes in water content and dry weight were determined in control cells and those induced to cold harden in response to abscisic acid (ABA) treatment (7.5×10^{-5} M). Bromus inermis Leyss cells grown in suspension at room temperature (23C) for seven days acclimated to -28C (LT_{50}) when treated with abscisic acid, or to -5C when untreated. ABA significantly reduced cell growth rates at 5 and 7 days after treatment. Growth reduction was due to a decrease in cell number rather than cell size. When the cell water content was expressed as percent water (% H_2O) or as grams water per gram dry weight (g H_2O/g DW), the water content of hardy ABA treated cells decreased from 85% to 77% or from 6.4 to 3.3 g H_2O/g DW in 7 days. Control cell water content remained static at approximately 87% and 7.5 g H_2O/g DW. However, cell water content, expressed as milligrams water per million cells (mg $H_2O/10^6$ cells), did not differ in ABA treated or control cells. The dry matter content of ABA treated cells, expressed as mg DW/10^6 cells increased to 3.3 mg/10^6 cells in 7 days whereas the dry weight of the control cells remained between 1.4 - 2.1 mg/10^6 cells. The osmotic potential of ABA treated cells decreased by the 5th day while that of control cells increased significantly and then decreased by day 7. Elevated osmotic potentials were not associated with enhanced ion uptake. In contrast to much published literature, these results suggest that cell water content does not decrease in ABA treated cells during the induction of freezing tolerance. Cell water content may be more

accurately expressed as a function of cell number when accompanying changes to cell dry matter occur.

3.2 INTRODUCTION

During cold acclimation plants become resistant to freezing stress. Freezing injury is influenced by the site, rate and extent of ice formation (12). Thus, a clear knowledge of the water status within the plant is important to understanding how plants acclimate. Numerous studies have shown that the water content of tissues declines as plants cold acclimate (3, 4, 11, 18). Also, plants accumulate dry matter under cold acclimating conditions (4, 5, 7). Despite the possibility that both water content and dry matter are changing during acclimation, technical difficulties particularly in whole plant measurements result in expression of water content that is not independent of dry matter accumulation: % H_2O: ((FW-DW)/FW) x100 (3, 11) or g H_2O/g DW: (FW-DW)/DW (6, 11, 18). Erroneous interpretations can occur when two changing variables are presented as dependent functions.

Although measurements of relative water content (RWC) and water deficit have been developed to account for this problem (18), changes associated with cold acclimation may alter the ability of cells to take up water to achieve full turgor. Confounding effects may result if full turgor is not attained.

ABA induction of cold acclimation in cell cultures provides a useful tool for examining water content during frost acclimation: 1) ABA can substitute for low temperature in inducing cold acclimation and thus avoid plant responses to cold that are not related to acclimation (2, 8, 13-17); 2) ABA induction of hardiness

is rapid and occurs in days rather than the weeks required for low temperature induced acclimation (2, 7, 8); 3) Cell cultures offer a relatively homogeneous system where barriers to hormone uptake are removed; and 4) Cell numbers can be readily estimated so data can be expressed as a function of cell number.

This study compared three methods of expressing cell water content in bromegrass suspension cultures and characterized the dry matter status and growth of these cells during ABA induced cold acclimation.

Abbreviations:
FW: Fresh Weight
DW: Dry Weight
RWC: Relative Water Content
LT_{50}: Lethal Temperature at which 50% of the cells are killed according to tetrazolium test (20)
PCV: Packed Cell Volume
ABA: Abscisic Acid
TTC: 2,3,5-triphenyltetrazolium chloride

3.3 MATERIALS AND METHODS

Cell Culture: a bromegrass cell suspension culture (BG-970) was grown in a modified Erickson's medium containing 0.5 mg/l 2,4-D (15). To reduce variability in all subsequent subcultures, similar inoculum sizes (1.75 ml packed cell volume following 20 minutes of settling in a graduated centrifuge tube) were transfered each two weeks into 50 ml media in 250 ml Erlenmeyer flasks and maintained on an Eberbach reciprocating shaker at 110 rpm at 23C in the dark. ABA at 7.5 x 10^{-5} M (Calbiochem) was added to the medium and autoclaved at 121C for 15 min. Cells were sampled and studied at 1, 3, 5 and 7 days after ABA treatment.

Freeze Test: Cells were harvested, and washed with 200 ml (about 20 volumes) dH_2O to remove the possible cryoprotective effects of the medium during freeze tests. Cells were frozen in 10x75 mm glass culture tubes. Preliminary tests indicated that excess extracellular water within cell clumps significantly affects freeze test results. To standardize the hydration level prior to freeze tests, excess extracellular water was removed by centrifuging cells in plastic 5 ml syringe barrels plugged with 2.5 cm^2 pieces of double-layered Kimwipe. Syringe barrels containing cells were suspended in 50 ml round bottom centrifuge tubes and spun at 2000 rpm (313 x g) at 4C in a Beckman JA-20 rotor in a model J2-21M centrifuge. Cells were spun until no further extracellular water was removed (10 min).

For the freeze test at least 200 mg cells fresh weight were placed in culture tubes, equilibrated in a Neslab LT-50 low temperature bath at -1C for 30 min and nucleated with ice crystals. Nucleated cells were held overnight at -1C. The next day the temperature was lowered at a rate of 2C/hr to -10C and at a rate of 3C/hr thereafter. At each freezing temperature, cells were sampled and placed at 4C for 24 hrs.

Viability Test: Cell samples (50 mg) were placed into shell vials and cell viability was estimated by the TTC reduction method (20). All samples were sealed with a serum stopper and incubated in the dark for 24 hrs. TTC was then removed with a pasteur pipet placed flat on the bottom of the shell vial. TTC was extracted with 3 ml of 95% ethanol per vial for at least three days. After extraction, 300 ul samples were transferred to ELISA plates and absorbance read at 495 nm. with a Biotek Microplate Autoreader EL 309. Three samples were analyzed for each temperature and treatment. Each experiment was repeated six times. Viability was expressed as LT_{50}, the temperature at which 50% of the cells were killed as determined by the TTC test.

Cell Number Determination: Cells occurring in clumps were separated by suspending samples of 0.2 ml of packed cell volume (PCV) in 2 N HCl solution (3 ml 2 N HCl) at 80C for 45-90 minutes. followed by vigorous vortexing. Cell lysis was minimal. ABA treated cells required higher HCl treatments to disperse than control cells. One thousand cells per sample were counted on a

hemacytometer. Three replications were analyzed for each treatment and each experiment was performed twice.

Fresh Weight/Dry Weight: Fresh samples of 0.7 to 1.0 ml PCV were weighed after centrifugation for 5 min. at 2000 rpm as described for the freeze tests. Samples were weighed after 24 hrs of drying at 80C. No weight loss was observed after 24 hrs of drying. Percent water was calculated by dry weight (DW) subtraction from fresh weight (FW). The result was expressed as a function of FW. Grams of water/g DW was calculated by subtracting DW from FW and expressing this amount as a function of DW.

Osmotic Potential: Osmotic potential was measured with a Wescor osmometer after excess intercellular water was removed by centrifugation as described for the freeze tests. Sap was obtained by manually squashing cells in flexible teflon tubes of 3 mm diam sealed at one end (1).

3.4 RESULTS

Growth and Hardiness

Both ABA treated cells and control cells continued to grow over the 7 day test period but ABA treated cells had significantly reduced rates of growth by days 5 and 7 (Fig 3.1A). Since there was no significant difference in cell number per ml packed cell volume, the growth reduction was apparently not due to a decrease in cell size but rather to a reduction in cell number (Fig 3.1B) .

ABA (7.5×10^{-5} M) treatment produced significant frost hardiness in bromegrass cells compared to controls, after 1, 3, 5 and 7 days of treatment (Fig 3.2). Control cell hardiness remained constant with an LT_{50} of about -5C, while ABA induced hardiness to -10C after 1 day of treatment, and to -28C by 7 days. Between 3 and 7 days of treatment the rate of increase induced by ABA was 3.8C/day.

Water Status

When water content was expressed as a function of fresh weight, the water content of control cells remained unchanged throughout the 7 day study period at 87% and 7.5 g H_2O/g DW. In contrast, the water content of ABA treated cells decreased significantly during the treatment period, from 86% to 77% (Fig 3.3A), and from 6.4 to 3.3 g H_2O/g DW (Fig 3.3B) when calculations were based on fresh and dry weights.

When water content was expressed on a per cell basis however, treatments did not differ (Fig 3.3C). Both control and ABA cells maintained an average water content of 11 mg/10^6 cells throughout the seven day monitoring period.

Dry Matter Accumulation

Control cell dry weight remained between 1.4 and 2.1 mg/10^6 cells. The dry weight of ABA treated cells increased after treatment (Fig 3.4) and by 7 days the dry weight reached 3.3 mg/10^6 cells.

Osmotic Potential

The osmotic potentials of ABA treated cells were consistently higher than those of control cells (Fig 3.5). At day 1 control cell osmotic potential (-.89 MPa) was higher than that of ABA cells, but subsequently increased to about -.57 MPa on days 3 and 5 before decreasing to -.73 MPa on day 7. The osmotic potentials of ABA treated cells decreased slightly but steadily from -.78 MPa on day 1 to -.87 MPa on day 7.

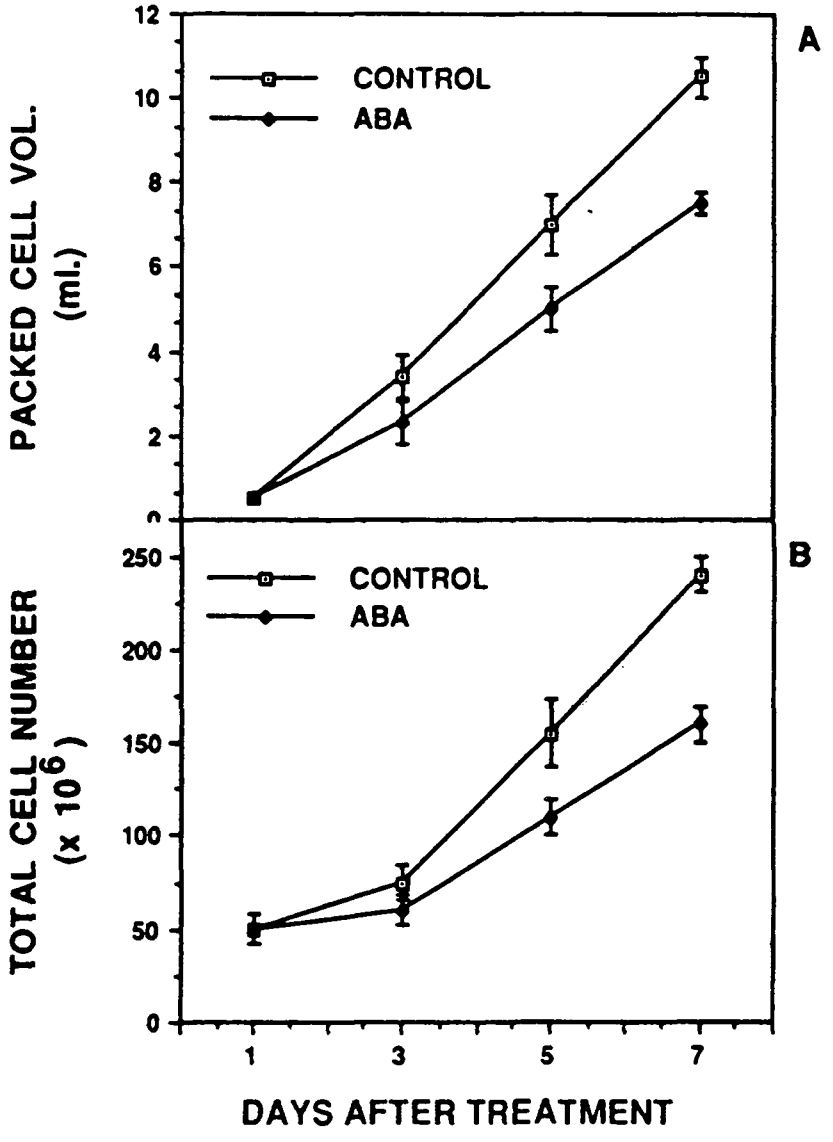

Figure 3.1 Growth of bromegrass cell suspension cultures with and without 7.5 x 10^{-5} M ABA treatment. A) Total packed cell volume (ml). Values represent means ±S.D. of five separate experiments. B) Total cell numbers in the packed cell volumes. Cell number values represent the means ±S.D. of two experiments of 1000 cells per replication, three replications per sampling date for each treatment and each experiment.

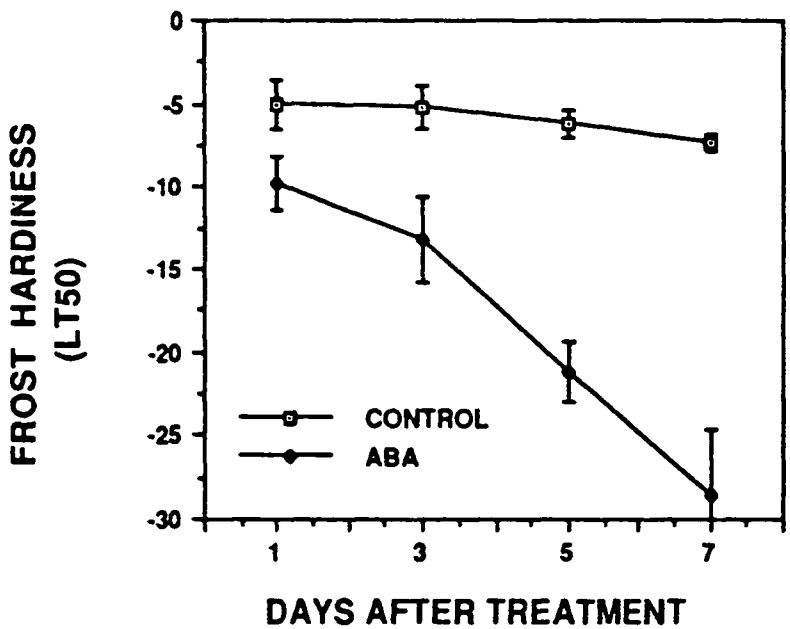

Figure 3.2 Development of freezing resistance in bromegrass cells (LT_{50}) with and without 7.5 x 10^{-5} M ABA treatment, estimated by TTC reduction. Means ±S.D. represent results of six separate experiments.

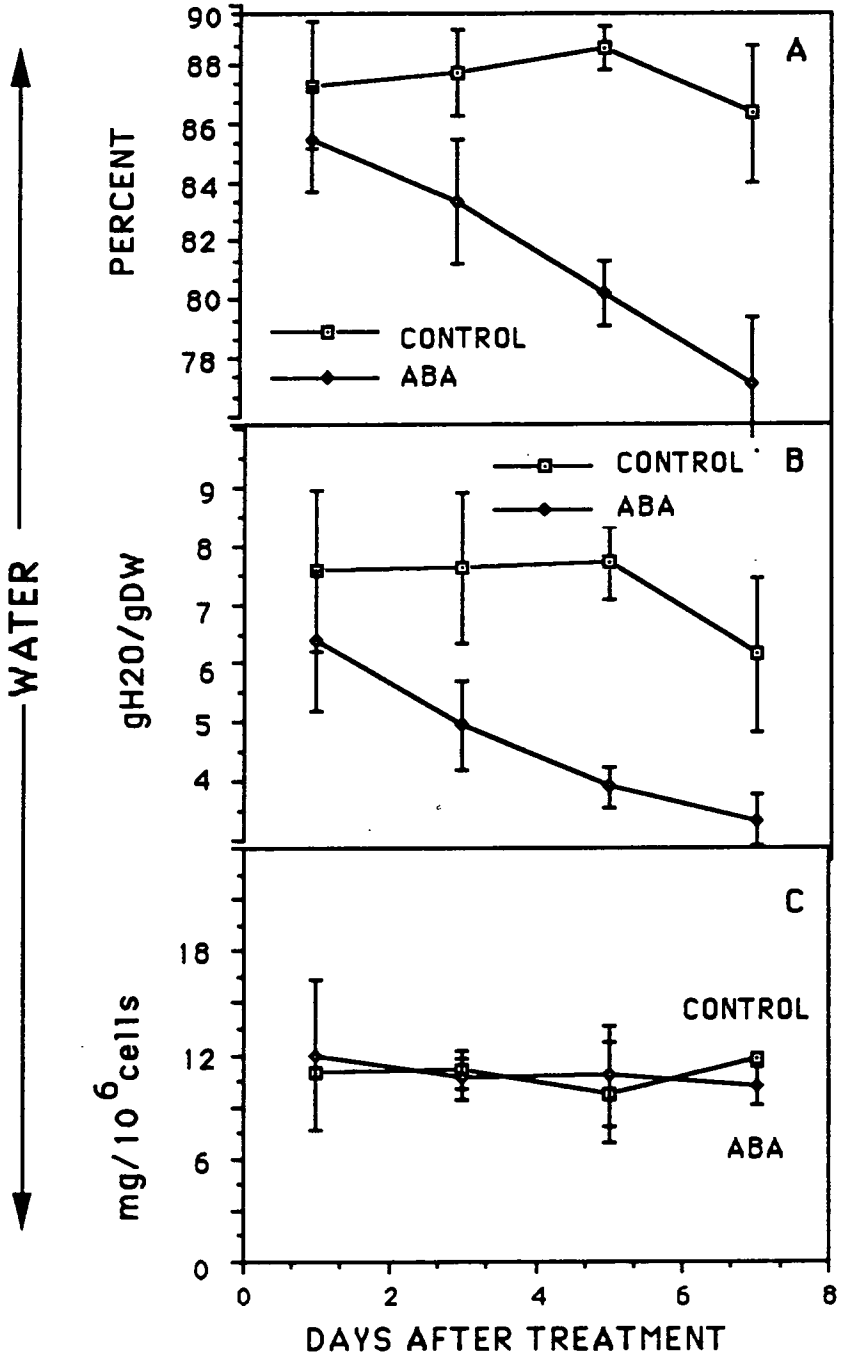

Figure 3.3 Cell water content of bromegrass cells with and without 7.5 x 10^{-5} M ABA treatment. A) Water content expressed as % cell water (FW-DW)/FW x 100. Means±S.D. represent analysis for six separate experiments. B) Water content expressed as gH_2O/gDW. Means±S.D. represent analysis for six separate experiments. C) Water content expressed as mg $H_2O/10^6$ cells. Cell numbers estimated for two separate experiments of 1000 cells per replication, three replications for each treatment in each experiment.

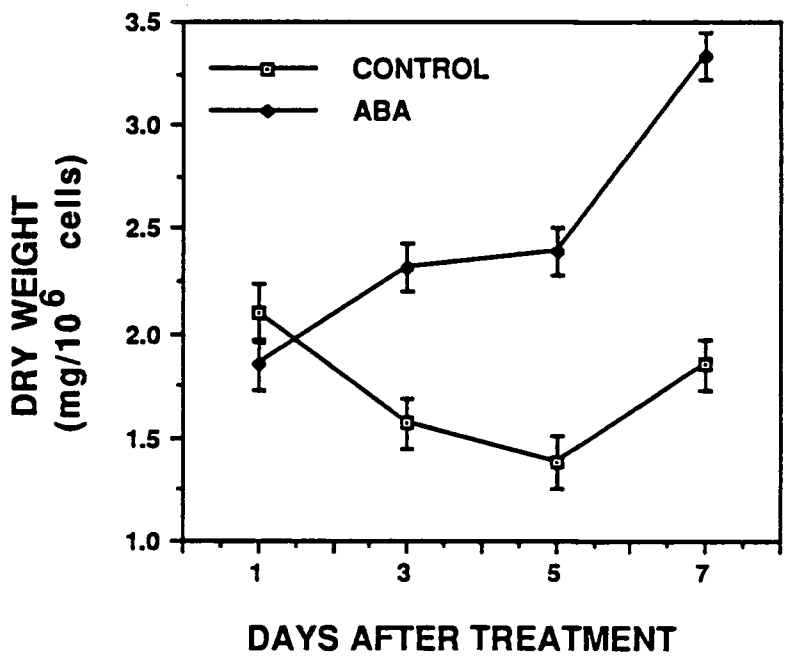

Figure 3.4 Dry matter accumulation in bromegrass cells with and without 7.5 x 10^{-5} M ABA treatment. Means ±S.D. represent results of six separate experiments.

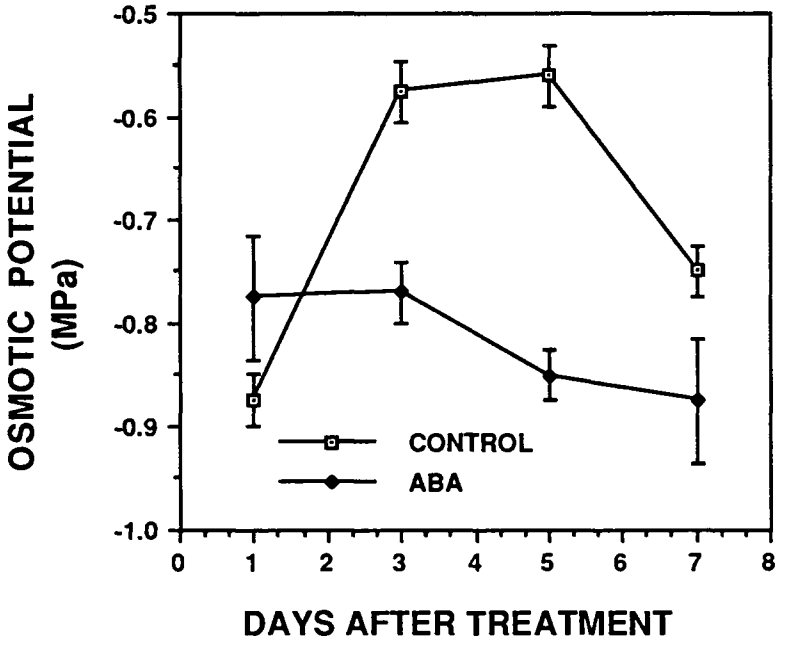

Figure 3.5 Cell osmotic potential with and without 7.5 x 10^{-5} M ABA treatment. Means ±S.D. represent results of two separate experiments.

3.5 DISCUSSION

Results of this study show that conventional methods for expressing cell hydration, in terms of fresh and dry weight measurements, may be inappropriate and misleading.

Two consistent plant responses at the cellular level during cold acclimation are dry matter accumulation (5, 7) and a decrease in H_2O content expressed as a percent (3, 11) or g H_2O/g DW (6, 11, 18). Percent water content is routinely expressed as either a fraction of the fresh or dry weight of the sample. Since dry weight is often changing during acclimation, percent water content and g H_2O/g DW are not an appropriate methods to express the water status of the plants or plant cells. In this study dry matter accumulation was 69 percent in ABA treated cells and 12 percent in control cells. Any method that expresses two interdependent and changing variables as a function of the other will yield misleading results.

Cellular water content of control and ABA treatments did not differ when water content was expressed as a function of cell number. The observed decrease in percent water content thus reflects an increase in cell dry matter rather than a decrease in water content.

Chen and Gusta (4) found that water potentials of wheat crown cells did not change during acclimation and suggested that the reduction in crown water content might be attributed to an exclusion of water from the cell by accumulation of dry matter and not merely a result of restricted water uptake by hardy crowns.

Reaney and Gusta (14) reported that the ratio of dry weight to fresh weight increased in ABA treated bromegrass cells over time. If water content remained constant, this increase could be attributable to dry matter accumulation. Robertson et al. (17) using the same bromegrass cultures also found an increase in dry weight with ABA treatment, but the increase was less than in control cells, and may have reflected an ABA induced reduction in growth. Fowler et al. (5) reported that dry weight was among the few factors correlating with field survival in winter wheat. We found that both dry matter accumulation and increased levels of hardiness induced by ABA exhibited similar patterns throughout the 7 day treatment period.

In terms of osmotic potential our results showed a significant difference between control and ABA treated cells. ABA treated cells appear to osmotically adjust as has been observed in ABA-induced cell adaptation to salt stress (10). Osmotic adjustment did not appear to be mediated by ion movement since media analysis on 15 elements did not reveal differences between controls and the ABA treatment over time, while pH measurements indicated that the ABA treatment inhibited H^+ extrusion from cells into the media (19). Total sugar accumulation paralleled the increase in osmotic potential.

The observed increase in osmotic potentials of control cells (Fig 5) has been reported in bromegrass cell cultures (15). The absence of a corresponding increase in water content (Fig 3) implies that osmotically active agents in cells were the primary factors regulating osmotic potential. Similarly, if cell water

content is constant, as this study suggests, then the decrease in osmotic potential induced by ABA in 7 days cannot be accounted for by water loss. Since dry matter accumulation did occur in this study, it is possible that the decreased osmotic potential was due to a decreased volume of space within the cells available for free water.

Tissues containing a large fraction of free water are highly susceptible to freezing injury (3, 4, 11). Although the regulation of water content by whole plants may be different than the regulation of water content by cultured cells, water content does not appear to be changing during ABA-induced frost acclimation.

Results of this study show that: 1) Water content expressed in terms of cell numbers does not vary during ABA treatment of bromegrass cell cultures, 2) Changes in cell dry matter during ABA treatment introduces a variable that may preclude using % H_2O or g H_2O/g DW to express cell hydration, 3) ABA significantly reduces growth rates of cells and appeared to be associated with a decrease in cell number rather than with cell size.

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4.0 Cellular Alterations Associated With Abscisic Acid-Induced Frost Hardiness

I. Biochemical Changes

4.1 ABSTRACT

Metabolic alterations associated with the induction of freezing tolerance by ABA were characterized by chemical analysis and by ^{14}C-(U)-sucrose partitioning into cellular constituents in bromegrass (Bromus inermis Leyss) cell suspension cultures. In contrast to low temperature induced acclimation, ABA increased metabolic activity which was reflected by enhanced CO_2 evolution and ^{14}C-sucrose uptake. However, in several other respects, changes during ABA induced acclimation were similar to changes during acclimation induced by low temperature. ABA caused a significant increase in dry matter accumulation, particularly the component insoluble in 85% ethanol that was correlated with frost tolerance. Differences between ABA treated and control cells became evident after 1 day of treatment. Cell walls, the largest component of that insoluble fraction by dry weight, increased significantly with frost tolerance throughout the ABA treatment period. ABA enhanced total ^{14}C-sucrose uptake by cells from 7% on day 1 to 97% on day 7 compared to control cell uptake. Partitioning studies detected a significant increase in ^{14}C-sucrose incorporation into the CO_2 fraction at 3, 5 and 7 days after treatment and into the ethanol insoluble component at 5 and 7 days. Organic acid depletion in ABA-treated cells was also highly correlated with hardiness increases. The concentration

of total sugars was higher in ABA-treated cells 5 days after treatment.

4.2 INTRODUCTION

Progress in understanding the mechanisms of cold acclimation is hindered by the induction of a multitude of metabolic alterations that are not directly related to frost tolerance (Kacperska, 1989). Nevertheless, two consistent responses observed during cold acclimation under both field and controlled environment conditions were: 1) a significant decrease in cellular water content (Chen et al., 1976; Chen and Gusta, 1978; McKenzie et al., 1974), and 2) dry matter accumulation (Chen and Gusta, 1978; Fowler et al., 1981; McIntyre et al., 1988; Sugawara and Sakai, 1978). In most experimental systems, cellular water content can only be expressed as a function of dry matter. When expressed independently, on a per cell basis using cell cultures, the cellular water content remained constant while the dry matter significantly increased per cell during ABA-induced frost acclimation (Tanino, 1989). ABA treatments have been observed to enhance dry matter accumulation in several plant systems (King, 1976; Goldbach and Michael, 1976; Neskovic et al., 1977; McLaren and Smith, 1976; Tanino, 1989). The components that account for this dry matter increase have not been well characterized.

Carbohydrates contribute a significant fraction of dry matter accumulation. More emphasis in acclimation studies has been placed on examining soluble rather than on the insoluble dry matter fractions. Although carbohydrate reserves are important as an energy source in winter survival (Levitt, 1980), quantitative carbohydrate increases may also be directly related to hardiness.

Total dry matter determinations have been largely utilized to normalize other measurements.

ABA induces cold acclimation of cell cultures at room temperatures (Chen et al., 1983; Keith and McKersie, 1986; Reaney et al., 1989). It thus provides researchers with another means of studying changes associated with acclimation. The non-photosynthetic cell culture system also permits examination of the direct effect of ABA on carbohydrate partitioning and utilization.

Both low temperature and ABA-induced frost acclimation are metabolic processes requiring energy (Levitt, 1980; Chen et al., 1983). In cell cultures, sucrose provides the major energy source for dry matter accumulation. The objectives of this study were to characterize: 1) biochemical changes that occur during ABA-induced acclimation; 2) partitioning of ^{14}C-sucrose within the ABA-treated cells; and 3) insoluble dry matter fractions associated with ABA-induced frost tolerance.

4.3 MATERIALS AND METHODS

Cell Culture A bromegrass cell suspension culture (BG-970) was grown in 250 ml erlenmeyer flasks containing 50 ml of a modified Erickson's media with 0.5 mg/l 2,4-D (Chen and Gusta, 1983). A 1.75 ml packed cell volume (PCV) inoculum size (measured following 20 minutes of settling in a graduated centrifuge tube) was chosen to yield linear growth throughout the treatment period. The suspension culture was maintained on an Eberbach reciprocating shaker at 110 rpm at 23C in the dark and routinely transferred every two weeks. Abscisic acid (ABA, Calbiochem) at 7.5×10^{-5} M was added to the media and autoclaved at 121C for 15 min. Cells were sampled and cellular alterations analyzed at 1, 3, 5 and 7 days after treatment.

Freezing Test Cells were harvested onto Kimwipes, and washed with 200 ml ddH_2O (about 20 volumes) to remove possible cryoprotective media residues before test freezing. Cells of 200 mg fresh weight were frozen in 10x75 mm glass culture tubes. Since preliminary tests indicated that the amount of external moisture significantly affected freezing tolerance, a consistent amount of extracellular water was frozen with the cells. Extracellular water was normalized by centrifugation. Cells were placed into plastic 5 ml syringe barrels plugged with a 2.5 cm^2 piece of double-layered Kimwipe. Syringes containing cells were then suspended in 50 ml round bottom centrifuge tubes and spun at

2000 rpm (313xg) at 4C in a Beckman JA-20 rotor in a model J2-21M centrifuge for 10 min.

In the freezing test, samples were equilibrated in a Neslab LT-50 low temperature bath at -1C and nucleated with ice crystals. Frozen cells were maintained overnight at -1C. The next day the temperature was lowered at a rate of 2C/hr to -10C and and at a rate of 3C/hr thereafter. One cell sample tube was removed each hour between -3 and -33C, and thawed at 4C for 24hrs. Three 50 mg subsamples per tube were assessed for viability. The experiment was repeated six times.

Viability Test Cell viability was estimated by the TTC reduction method modified from Towill and Mazur (1974). Fifty mg fresh weight of sample was incubated in a 1 ml TTC solution (0.5 g/100 ml) in a 5 ml shell vial sealed with a serum stopper for 24 hr in the dark. Excess TTC was discarded and the residual TTC extracted for about 3 days with 3 ml of 95% ethanol per vial. Three hundred ul of the ethanol extractant were transferred to Eliza plates and read at 495 nm. with a Biotek Microplate Autoreader EL 309. Viability was expressed as LT_{50}, the temperature at which 50% TTC reduction occurred.

Cell Number Determination Cells (0.2 ml PCV) occurring in clumps were separated by suspending the cells in 2N HCl solution (3ml 2N HCl) at 80C for 45-90 minutes followed by vigorous vortexing. ABA treated cells required longer treatments to disperse than control cells. Cell lysis was minimal. One thousand

cells per sample were counted on a hemacytometer. Three replications were analyzed for each treatment and the study was performed twice.

Fresh Weight/Dry Weight Cell samples of 0.7 to 1.0 ml PCV were weighed after centrifugation for 5 min. at 2000 rpm as described for the freeze tests. Dry weights were then determined after 24 hr of drying at 80C when no further weight loss occurred. Percent water was calculated by dry weight (DW) subtraction from fresh weight (FW), divided by FW x 100. Grams of water/g DW was calculated by subtracting DW from FW and expressing this amount as a function of DW.

^{14}C-(U)-Sucrose One ml of cells (PCV) were harvested at 1, 3, 5 and 7 days from control and ABA treatments, inoculated with 10$_u$Ci of ^{14}C(U)-sucrose (560 mCi/mmole sp. act., ICN) and shaken for 3 hrs. at 22C. The media was then separated from the cells by centrifugation at 10,000g for 10 min at 4C. The pellet was sonicated for 20 sec. in 85% ethanol. The insoluble fraction was separated by centrifugation at 10,000 g for 5 min. The pellet was resuspended in 9 ml sodium phosphate buffer (100 mmol, pH 7) and 2 x 0.2 ml. aliquots were sampled for counting. The soluble fraction was partitioned as follows:

a) Lipids: the total soluble fraction was evaporated in a rotovaporator in a 100 ml boiling flask until 1ml liquid remained. The residue was extracted twice with 5 ml of 1:1 ether/ethanol and

aspirated through a 0.45 um membrane and 2 x 0.1 ml aliquots sampled for radioactivity counting.

b) Water soluble fraction: was solubilized with 9 ml dH_2O in the boiling flask, transferred to a test tube where 1 ml 0.1N HCl was added. The extract was passed through 10g Dowex 50/6g Dowex 1 cation/anion exchange columns (1.1 cm. diameter) at a constant rate of 1ml/min. to separate total amino acids, sugars and organic acids. Total amino acids were extracted from the Dowex 50 column with 2 x 10 ml 4N NaOH and 2 x 0.1 ml sampled for counting. Total sugars were eluted from the Dowex 1 column with 2 x 10 ml dH2O and 2 x 0.1 ml taken for counts. Thereafter, organic acids were eluted from the Dowex 1 column with 2 x 10 ml 2N acetic acid and 2 x 0.1 ml were counted.

A CO_2 trap, 0.9 cm dia. x 3 cm high glass tube fixed to the center floor of the flask, was constructed within a 25 ml Erlenmeyer flask. A 2 x 5 cm Whatman number 1 filter paper fan folded and saturated with 500 ml of 1 N NaOH was placed into the tube. After 3 hr incubation, the filter paper was placed in 10 ml scintillation fluid (Readi-Solv, Beckman) and counted directly. For other counts, 100 ul sample was placed into 2 ml scintillation fluid in 3 ml shell vials and counted with a Beckman LS 7000 scintillation counter for 2 min./sample. Recovery was 85%. Percent incorporation is expressed on a basis of total soluble and insoluble fractions.

Biochemical Assays

Quantities of cellular constituents are expressed on a per cell basis to avoid the possible confounding effects of dry weight accumulation.

i) The total sugars contained in the media and cell samples extracted under the radiolabelling experiment were assayed using the anthrone method (Smith et al.,1964).

ii) The insoluble fraction was separated as described above. Fifty mg lyophilized insoluble samples were analyzed in a micro mortar and pestle system designed to break open the cells. Cells were placed into a 1.5 ml microfuge tube and a fitted stainless steel pestle. Purified washed sand and acetate buffer (.1 M, pH 4.5) were employed to aid cell breakage. Microscopic observations of control cells indicated at least 60% of the cell were broken following treatment.

iii) Fifty mg of lyophilized ethanol insoluble samples were assayed for starch content. The samples were ground with the system described above. Starch was initially degraded with amyloglucosidase (10,0000 units/gm) into glucose units and assayed according to the Sigma #510-A glucose test kit (Wang and Breen, 1986). Preliminary tests on cellobiose showed no breakdown after amyloglucosidase treatment and thus the assay did not reflect cellulose degradation.

iv) Total proteins were extracted on fresh samples after cell breakage with the system as previously described. Proteins were extracted according to Robertson et al., (1987) at 25C overnight on the 110 rpm reciprocating shaker.

v) The quantity of cell wall was determined on the pellet remaining after the total starch and proteins were removed. The pellet was washed three times with three volumes of 0.1M sodium acetate buffer pH 4.5, and dry weight determined.

Statistical and Design Analysis: A randomized complete block design was used with three replications per block. Linear and quadratic components were assessed through an analysis of variance. Correlations were tested between total dry weight and LT_{50}.

4.4 RESULTS

A. Non-Labelling Studies

Hardiness

ABA (7.5×10^{-5} M) significantly increased the frost hardiness of bromegrass cells in suspension culture compared to control samples (Fig. 1). The hardiness of control cells remained constant with an LT_{50} of approximately -5C. ABA treated cells increased in hardiness from -10C after 1 day to -28C after 7 days of treatment.

Dry Matter Accumulation

ABA treatment significantly elevated total dry matter accumulation over control samples (Fig. 4.2A). Regression analysis of dry matter accumulation (Fig. 4.2B, R^2 = .03 and .54, for ln dry weight = a + bX for control and ABA samples of Fig. 4.2A, respectively) indicated accumulation initiated from 1 day after ABA treatment. Slopes were significantly different at the 1% level. Dry weight of control cells remained between 1.4 and 2.1 mg/10^6 cells while ABA treated cells continued to accumulate dry matter from 1 to 7 days. The largest dry weight increase occurred between 5 and 7 days. The 0.63 correlation coefficient between dry matter accumulation and LT_{50} was significant at the 1% level.

Insoluble Fraction

The ethanol insoluble fraction in both control and ABA treated cells constituted about 70% of the observed total dry matter

accumulation (Table 4.1). The weight of the insoluble fraction remained relatively constant in control samples whereas ABA elevated insoluble weight following 5 and 7 days of treatment (Fig. 4.3). Regression performed on the ln insoluble dry weight vs time indicated that the dry weight increase of the insoluble fraction occurred within 1 day after treatment. A correlation coefficient of 0.94 between total dry weight and the dry weight of the insoluble fraction was significant at the 1% level. The cell wall-containing fraction contributed over 80% of the total insoluble dry weight. Total starch and protein together accounted for less than 3% of the insoluble weight and the chloroform:methanol (2:1) fraction accounted for the balance of dry weight increase. Expressing the data on a cell number basis indicated that the cell wall fraction significantly increased during ABA treatment and attained a plateau after 5 days (Fig. 4.4). The 0.91 correlation coefficent between the dry weight of the insoluble component and cell wall fraction was significant at the 1% level.

Total Sugars

Concentrations of total cellular sugar increased by 5 days after ABA treatment (Fig. 4.5). Conversely, cellular sugar concentrations of control samples decreased until day 5 and increased slightly by 7 days.

B. Radiolabelling Studies

Sucrose Uptake

ABA treatment caused an increase in total ^{14}C-sucrose uptake. At 1 day following ABA treatment, total uptake increased by 7% over control cells. By 7 days, there was an average of 97% more uptake in ABA than in control samples. After 3 hr incubation, most of ^{14}C-sucrose incorporation was associated with the soluble fraction in both control and ABA samples. The insoluble fraction represented between 15 to 20% of the total label. ABA-stimulated uptake reflected enhanced radiolabel incorporation into both the ethanol soluble and insoluble fractions.

Respiration

ABA treatment significantly increased the CO_2 evolution over control cells at from cells between 3, 5 and 7 days after treatment (Table 4.2).

Insoluble Fraction

ABA significantly stimulated the incorporation of ^{14}C-sucrose into the insoluble cell fraction over control levels at 5 and 7 days of treatment (Table 4.2). At 7 days, ABA elevated radiolabelled sucrose incorporation into insoluble fractions by 40% over control samples.

Soluble Fraction

ABA treated cells had significantly less radiolabel incorporation into the % organic acid fraction compared to the controls after 3, 5 and 7 days (Table 4.2). Control samples maintained slightly elevated incorporation into the % organic acid fraction from 3 to 7 days. The correlation coefficient between % organic acid incorporation and hardiness (LT_{50}) was 0.77, significant at the 1% level. No significant difference was observed in distribution of ^{14}C-sucrose into the amino acid, polar lipid and total sugar fractions between control and ABA treated cells (Table 2).

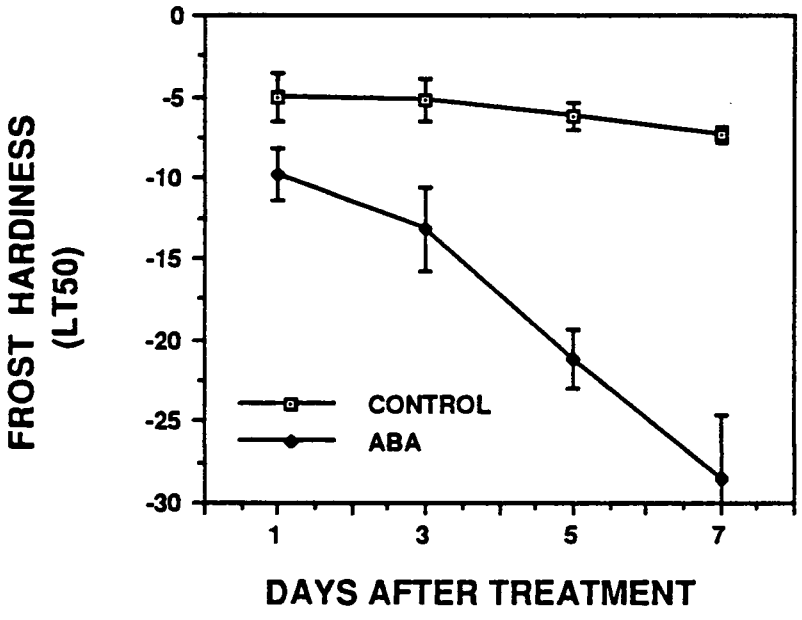

Figure 4.1 Frost hardiness of bromegrass cell suspension cultures with and without 7.5 x 10^{-5} M ABA treatment, as estimated by LT_{50} using TTC. Values represent mean ±S.D. of 6 separate experiments.

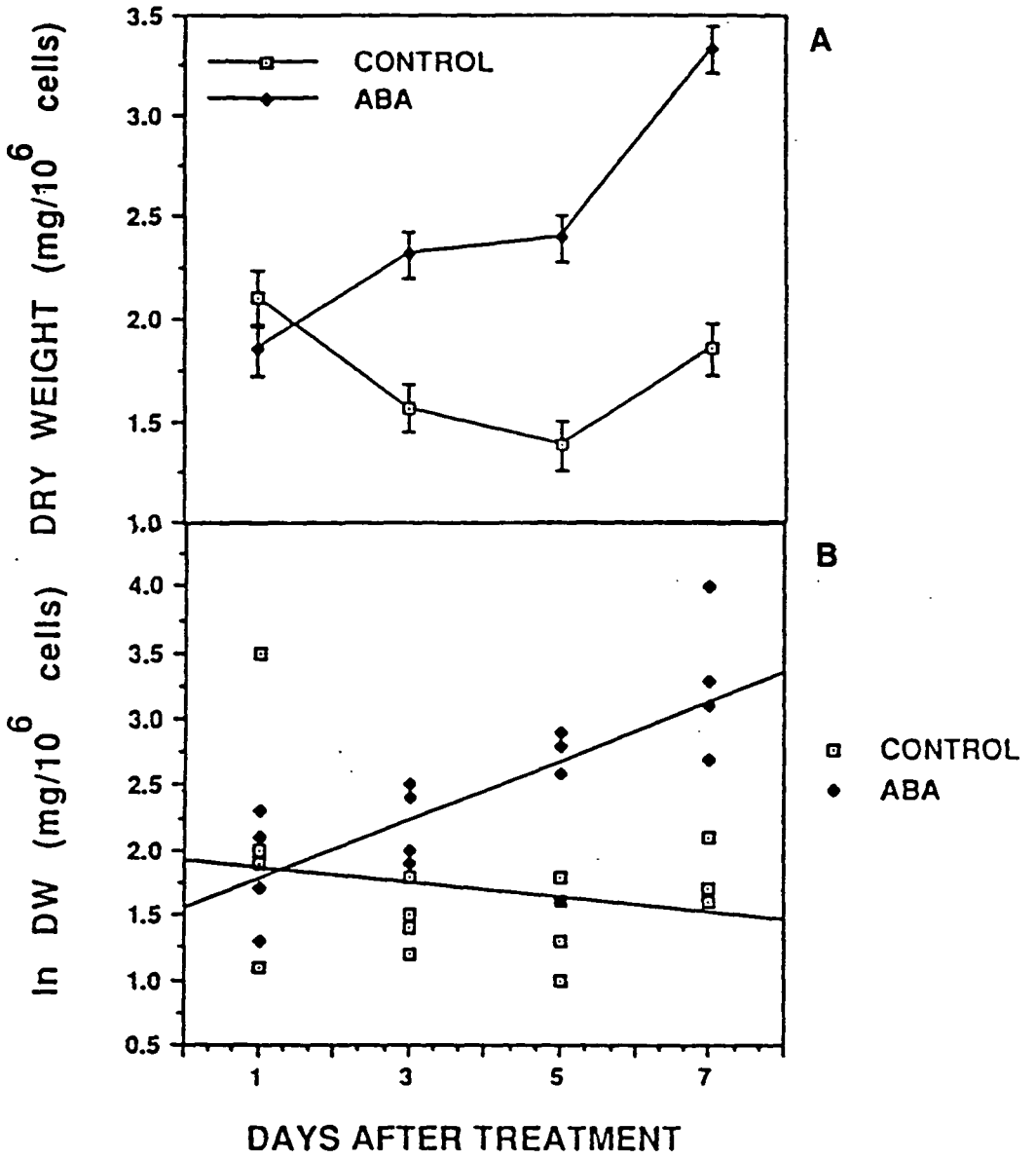

Figure 4.2 A. Bromegrass cell dry weight with and without 7.5 x 10^{-5} M ABA treatment. Values represent the mean±S.D. of 6 experiments. B. Linear regression of ln of A. Slopes were significantly different at P = 0.01.

Table 4.1 Fraction of ethanol insoluble dry weight to total dry matter with and without 7.5 x 10^{-5} M ABA treatment of bromegrass cell suspensions. Values represent the mean ± S.D. of 2 separate experiments with 2 subsamples per run.

	DAYS AFTER TREATMENT			
	1	3	5	7
control	70±6	76±2	78±15	71±1
ABA	67±1	74±8	70±19	79±8

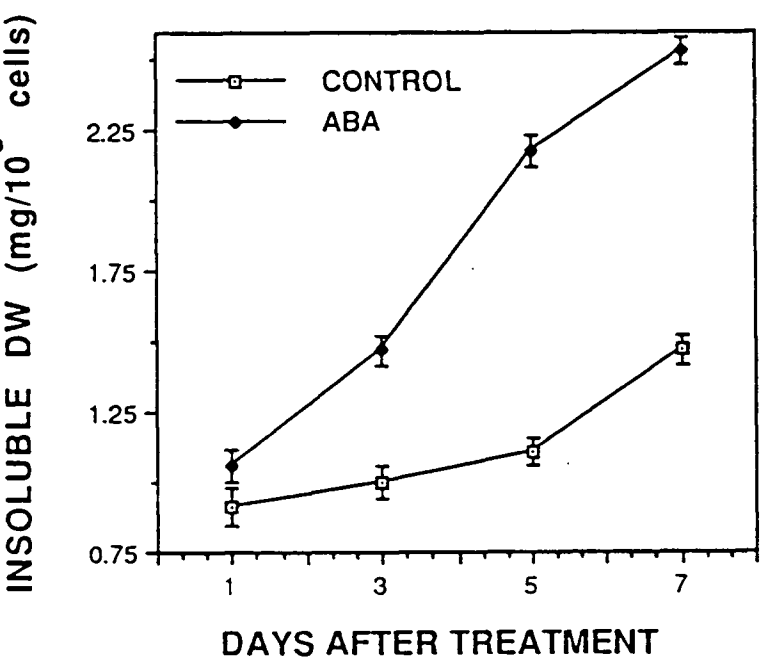

Figure 4.3 Total ethanol insoluble fraction after 1, 3, 5 and 7 days with and without 7.5 x 10^{-5} M ABA treatment. Values represent the mean ±S.D. of 3 separate experiments.

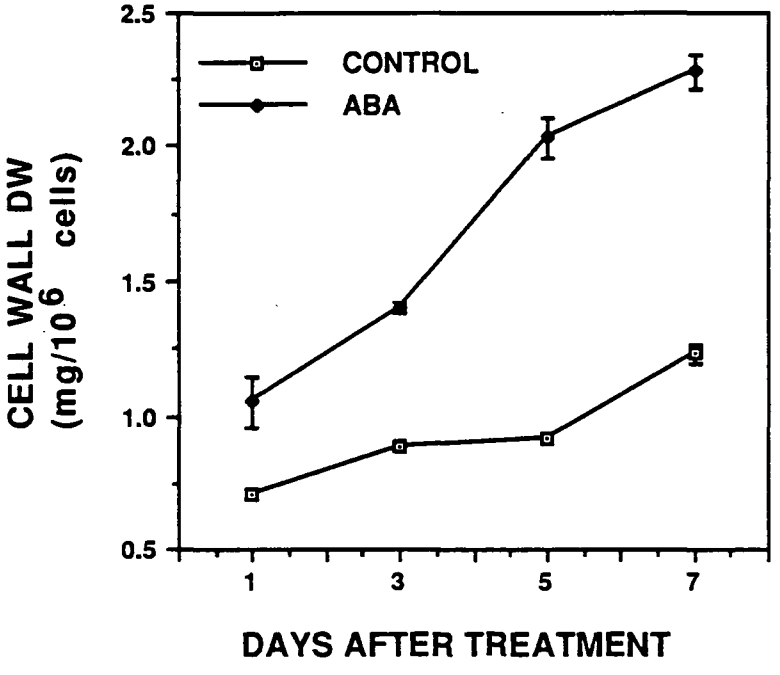

Figure 4.4 Cell wall-containing fraction after 1, 3, 5 and 7 days with and without 7.5 x 10^{-5} M ABA treatment. Values represent the mean±S.D. of 2 separate experiments.

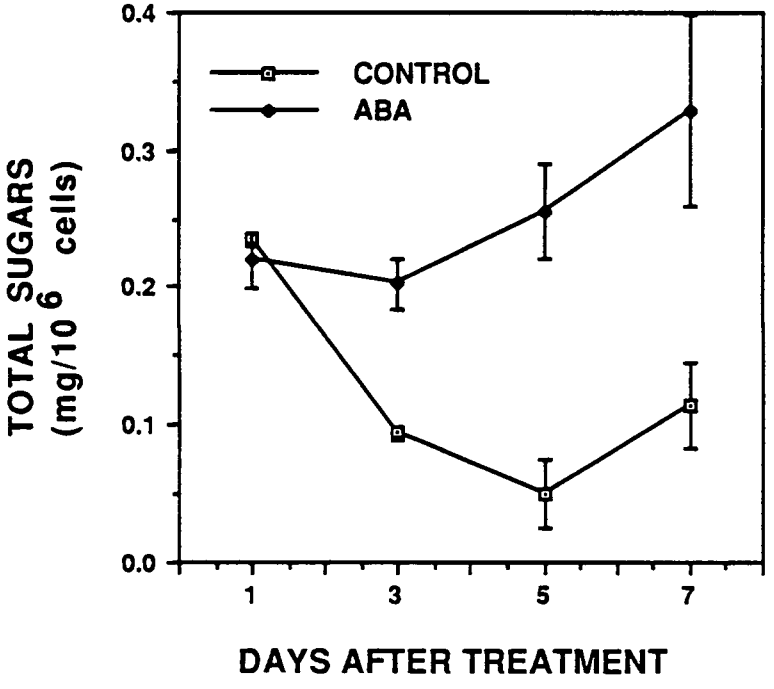

Figure 4.5 Total cellular sugar content with and without 7.5×10^{-5} M ABA treatment. Values represent the mean±S.D. of 2 separate experiments.

Table 4.2 Incorporation of ^{14}C-sucrose into the ethanol insoluble, soluble and CO_2 fractions after 7.5 x 10^{-5} M ABA treatment of bromegrass cell suspension. Values are expressed as a fraction of total counts and represent mean±S.D. of 2 or more separate experiments.

PERCENT INCORPORATION OF 14C-SUCROSE								
DAYS AFTER TREATMENT								
FRACTION	1		3		5		7	
	CONTROL	ABA	CONTROL	ABA	CONTROL	ABA	CONTROL	ABA
INSOLUBLE	17.5 ± 2	18.9 ± 1.7	15.5 ± 2	18 ± 3.5	15.5 ± 1.7	19.4 ± 0	15.9 ± 1	22.1 ± 1
CO2	6.7 ± 4	8 ± 4	5.3 ± 1.7	9.5 ± 0	3.8 ± .5	6.3 ± 1.5	4.2 ± 1	9 ± 2.5
ORGANIC ACID	38.9 ± 2.5	39.1 ± 1.5	40 ± 2.2	35 ± 1	41.2 ± 1	35.3 ± 1.4	41.1 ± .25	30 ± .5
AMINO ACID	7.9 ± 5.5	9.5 ± 3	9 ± 3	9 ± 4	10 ± 3	10.5 ± 1	7.3 ± 1.5	11 ± 3.5
TOTAL SUGAR	18 ± 5	14.5 ± 10	23.2 ± 3	22 ± 2	23 ± 2	22.5 ± 1	26 ± 2	19.9 ± 1
LIPID	11 ± 1	10 ± 3	7 ± 1	6.5 ± 1.5	6.5 ± .5	6 ± 1	5.5 ± 2	8 ± 1

4.5 DISCUSSION

ABA-induced frost hardiness of bromegrass cell cultures was significantly associated with altered carbon utilization. ABA significantly stimulated ^{14}C-sucrose uptake into the cells and increased partitioning of radiolabel into the insoluble and CO_2 fractions. Cell wall components were identified as the major cell fraction contributing to dry matter accumulation. Changes in the cell wall fraction were associated with increased freezing tolerance throughout the 7 day treatment period. A decreased distribution of label into the organic acid components occurred as the hardiness levels of cells increased.

ABA elicited significant dry matter accumulation expressed on a cell basis. The additional carbon source required for dry matter accumulation is derived from enhanced cell uptake of ^{14}C-sucrose from the media. The ABA-stimulated ^{14}C-sucrose uptake that occurred throughout the treatment period is consistent with observations in other systems including strawberry explants (Archbold, 1988), soybean reproductive structures (Ackerson, 1985), sugar beet root discs (Saftner and Wyse, 1984), and apple fruits (Beruter, 1983).

Total carbon usage can be partitioned into growth, maintenance (turnover and repair), and storage (Cheeseman, 1988). Our previous study indicates that ABA significantly reduces bromegrass cell population growth (Tanino, 1989). Under these conditions, assimilate partitioning into storage would normally be

favoured. This dry matter accumulation does not appear to result from a decrease in metabolic usage commonly associated with storage, but rather from the synthesis and accumulation of cell wall components. ABA stimulated incorporation of ^{14}C-sucrose into the cell wall-containing fraction and supports observations by Dick and ap Rees (1976) that newly translocated sucrose is the likely source of carbon for cell wall synthesis. Ultrastructural studies on ABA treated bromegrass cell suspension cultures indicated increased thickness of the cell wall (Tanino, 1989; Reaney, 1989). Furthermore, plant responses to low temperature acclimation often include cell wall augmentation. After acclimation, pea epicotyl cell wall weight increased by 40 percent (Weiser, 1989). In addition, the hardier Acaule potato species were found to have thicker cell walls than the less frost tolerant Tuberosum species (Chen et al., 1977; Li and Palta, 1978).

Analysis of a bromegrass cell suspension culture revealed that the major components in the cell wall by weight were arabinose (29.3%) and xylose (27.5%) (Burke et al., 1974). The specific compounds contributing to the ABA-induced increase in cell wall dry weight observed in this study were not identified. However, evidence from low temperature acclimation studies indicate that there is a significant suberin deposition in Puma rye (Griffith et al., 1985).

We observed increased dry matter content in bromegrass cells during ABA-induced frost acclimation. However during low temperature acclimation, cell culture fresh or dry weight did not change in bromegrass (Robertson et al., 1987) and alfalfa

(Borochov et al., 1989). ABA has stimulated significantly greater increases in hardiness than low temperature-induced acclimation in both bromegrass (Chen and Gusta, 1983) and alfalfa (Keith and McKersie, 1986) over the same time period, and ABA increased hardiness above the maximum level attained by low temperature alone in bromegrass suspension culture (Chen and Gusta, 1982).

Low temperature acclimation of cell cultures is usually achieved by exposing cells directly to temperatures between 2 and 4C. Conversely, natural whole plant acclimation occurs in discrete stages (Weiser, 1970) over a longer period of time, at variable and often higher temperatures that may permit accumulation of dry matter. The optimal low temperature for eliciting bromegrass cell hardiness was actually determined to be 9C (Reaney, pers. comm.). The net accumulation of dry matter for any one system will reflect a unique balance between anabolic and catabolic events. It is conceivable that full hardiness expression may not be achieved in response to low temperature treatment because of limited dry matter production, be specifically, the inability to augment cell wall dry matter components at temperatures too low for their production.

The bromegrass control cells in this study averaged 1.6 mg/10^6 cells. However, Kurz and Constabel (1981) reported a dry weight of 0.67 mg/10^6 cells in a chemostat culture of Acer pseudoplatanus cells. This difference may be genetic or could result from the highly clumped nature of bromegrass cell suspensions in which thousands of cells characteristically adhere together. In contrast, chemostat conditions permit growth of

single cells or small cell groups. Ultrastructural observations of untreated bromegrass cells (Zhang and Willison, 1986; Tanino, 1989) revealed that material is deposited extracellularly in intercellular spaces within cell clumps. Measurements on transmission micrographs of ABA-treated cells indicated significant increased thickness of the combined cell wall and intercellular space (Tanino, 1989). Thus, the role of the middle lamella in extracellular synthesis or extrusion of cellular constituents may represent an important factor in the observed dry matter increase during ABA treatment in bromegrass cell suspension cultures.

Maximum hardiness at 7 days after ABA treatment is accompanied by a combination of factors. Ultrastructural studies (Tanino, 1989) showed the presence of high levels of starch grains and lipid bodies by 7 days after ABA treatment. The increased partitioning of label into the insoluble fraction may result from enhanced synthesis of these two components. By 7 days total sugar content and osmotic potential (Tanino, 1989) were elevated above control levels. Absolute counts of polar lipid and amino acids are also enhanced at this time. In addition, increases in sugars, osmotic potential, amino acids as well as polar and nonpolar lipid during low temperature acclimation has been well documented (Levitt, 1980).

The observed ABA-induced alterations necessitates a shift in carbon metabolism. The significant rise in CO_2 evolution under ABA treatment may suggest 1) enhanced generation of ATP and reducing power through a faster turnover of the Krebs cycle and/or

2) a higher turnover of the pentose phosphate pathway. The reduction in ^{14}C-(U)-sucrose incorporation into organic acids after ABA treatment suggests either a pathway diversion away from the Krebs cycle or increased utilization of organic acids. Partitioning studies did not indicate elevated amino acid synthesis. ABA appears to increase NADPH (Zhen, pers. comm.) and reducing power (Harber, 1989) in bromegrass cell suspension cultures. Further, NADPH would be required for synthesis of lipid bodies and osmiophilic granules observed to significantly increase during ABA treatment (Tanino, 1989). Sagisaka (1974) reported a low temperature acclimation-induced shift in metabolism to the pentose phosphate pathway. Finally, Dooner (1985) found that ABA vp1 mutants showed decreased activity of glucose-6-phosphate dehydrogenase. Thus, in addition to the role of the Krebs cycle in acclimation, accumulating evidence also implicates ABA in directing carbon flow into the pentose phosphate pathway.

In summary: 1) ABA elicited a large increase in bromegrass cell frost tolerance. Enhanced incorporation of carbon into the cell wall-containing fraction was closely correlated with this hardiness increase at 1, 3 and 5 days after treatment. 2) ABA enhanced the uptake and incorporation of ^{14}C-sucrose into the total insoluble and CO_2 fraction. The ethanol insoluble fraction is the major contributing factor to the observed dry matter accumulation under ABA-induced frost tolerance. Cell wall components constitute the largest portion of this insoluble fraction. 3) ABA significantly depressed incorporation of ^{14}C-

sucrose into organic acids from 3-7 days after treatment. 4) By 7 days, total sugar content significantly increased.

Low temperature acclimation is the result of a series of independent, metabolic changes that appear to be additive in nature. Low temperature slows metabolism. Conversely, ABA stimulates metabolism as is reflected in the higher levels of CO_2 evolution and sucrose uptake observed. Interestingly, the resultant metabolic alterations elicited by ABA share common features with those changes correlated with low temperature acclimation and involve both cytoplasmic and cell wall factors. These ABA-induced alterations are correlated with the additive increments of frost hardiness.

This study describes metabolic changes associated with the acquisition of frost tolerance, and will hopefully be useful in future elucidation of specific roles of proteins/enzymes induced by ABA during acclimation.

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5.0 Cellular Alterations Associated With Abscisic Acid-Induced Frost Hardiness

II. Ultrastructural Changes

5.1 ABSTRACT

Ultrastructural changes in bromegrass (Bromus inermis Leyss cv Manchar) cell suspension cultures were characterized at different hardiness levels using fluorescence and transmission electron microscopy. After one day, when abscisic acid (ABA) treated cells were 5C hardier than control cells, the hardier cells exhibited slightly greater numbers of lipid bodies and starch grains. However, no changes were observed in cell wall thickness or vacuole number. Vacuole size in ABA treated cells were equally distributed among three size classes while control cells had a higher proportion of large vacuoles. Conversely, the lipid bodies in cells exposed to ABA treatment were consistently smaller than those in control cells. After seven treatment days when hardiness had increased to -28C, the walls of ABA treated cells were twice as thick and starch grain more than doubled compared to control cells. There was no change in the size of treated or untreated cells, but differences observed in lipid bodies after one day were accentuated in the hardier ABA treated cells after seven days. The size of lipid bodies decreased and their numbers increased by a factor of ten. Vacuoles decreased in size and on average doubled in number. Osmiophilic granules and golgi apparati became more prevalent near the plasmamembrane in the more frost tolerant cells. No differences in the lignin content of treated and control cells were detected but cell wall and protoplasm autofluorescence increased in response to ABA. These results show that ABA treatments at room temperature elicit ultrastructural changes

associated with hardiness that are similar to those reported after low temperature acclimation.

5.2 INTRODUCTION

Electron microscopic studies of cytological changes associated with frost hardiness were first described by Siminovitch et al. (1968). Subsequent investigations have described ultrastructural alterations during low temperature induced cold acclimation in woody plants (Pomeroy and Siminovitch, 1971; Niki and Sakai, 1981, 1983; Wisniewski and Ashworth, 1986; Wisniewski et al., 1987a,b; Senser and Beck, 1982). Low temperature acclimation may produce similar modifications in herbaceous plants (Chen et al., 1977; Li and Palta, 1978; Steponkus et al., 1977; Toivio-Kinnucan et al., 1981; Griffith et al., 1985; Singh, 1979; Chalker-Scott, 1988). Ultrastructural changes in algae (Hatano et al., 1982) and plant cell culture (Borochov et al., 1989) have been observed as cells develop tolerances to low temperature. However, there has been only limited examination of ultrastructural changes in cells induced to freeze acclimate in response to abscisic acid (ABA) treatment at room temperature (Reaney and Gusta, 1989).

ABA-induced hardiness of cell cultures provides a useful tool for examining ultrastructural alterations associated with cold acclimation because it can substitute for low temperature-induced tolerance to freezing stress. This eliminates the complication of plant responses to cold that are not directly related to acclimation (Chen et al., 1983; Keith and McKersie, 1986; Reaney and Gusta, 1987; Orr et al., 1985). Furthermore, ABA induction of hardiness is rapid. Hardiness develops within a few days compared to weeks

often required for inducing hardiness with low temperature treatments (Chen et al., 1983; Reaney and Gusta, 1987). Cell cultures of hardy plant species are very responsive to ABA treatment, and provide a relatively simple system for observing ultrastructural changes.

A companion study of ABA-induced biochemical alterations in bromegrass cells in suspension culture revealed significant shifts in carbon utilization (Tanino, 1989). Metabolic activity increased and total sucrose uptake was stimulated in cells exposed to ABA. As frost tolerance of cells markedly increased there was a major elevation in dry matter content and the cell wall fraction accounted for the majority of this dry matter accumulation. In addition, frost hardiness induced by ABA was associated with enhanced CO_2 evolution and organic acid depletion. This parallel study identified ultrastuctural changes in bromegrass cells that occurred concurrently with the biochemical alterations associated with ABA-induced frost tolerance.

5.3 MATERIALS AND METHODS

Cell Culture. A bromegrass cell suspension culture (BG-970) was grown in a modified Erickson's media containing 0.5 mg/l 2,4-D (Chen and Gusta, 1983). To reduce variability in all subsequent subcultures, similar inoculum sizes (1.75 ml packed cell volume following 20 minutes of settling in a graduated centrifuge tube) were transferred every two weeks into 50ml media in 250ml Erlenmeyer flasks. The suspension culture was maintained on an Eberbach reciprocating shaker at 110 rpm at 23C in the dark. Abscisic acid (ABA, Calbiochem) at 7.5 x 10^{-5} M was added to the media and autoclaved at 121C for 15min. Cellular alterations and freezing resistance were monitored at 1 and 7 days after ABA treatment.

Transmission Electron Microscopy. Cells were fixed in microfuge tubes with an addition of 2.5% glutaraldehyde to the culture media for 2 hrs. under a vacuum of 29 inches of mercury at 25C. Cells were then spun at 12,000 rpm in a microfuge for 10 min. and cells washed with media to remove glutaraldehyde. Cells were held in fresh media at 4C for 7 days or less before sectioning. Cells were stained and post-fixed with a 1% osmium tetroxide solution in sample buffer and observed with a Philips 300 transmission electron microscope operated at 60Kv.

All cell cross-sectional areas were estimated by the average of two radii measurements. Lipid body size classes were partitioned as: class I, less than .35 um; class II, 0.35 - 0.7 um;

class III, 0.7 - 1.4 um; and class IV, greater than 1.4 um. Vacuole size classes were divided into: class I, less than 0.7 um; class II, 0.7 - 1.4 um; and class III greater than 1.4 um. Total organelle counts were based on a minimum of eight cells for each treatment and time period.

Fluorescence Microscopy. Isolated cells were observed under UV-excitation using a 50W Mercury Vapour Lamp fitted with a DAPI 02 blue filter. Photographs were taken using Kodak tri X pan film with a 10 sec exposure.

5.4 RESULTS

After 1 day and 7 days of treatment ABA induced frost hardiness levels to -5C and -28C respectively (Table 5.1).

Electron micrographs (Fig. 5.1) documented ABA-induced changes in protoplasmic and cell wall components. Protoplasmic alterations in ABA treated cells on day 1 and day 7 of treatment included: increased numbers of lipid bodies and vacuoles compared to control cells (Fig. 5.2); a shift in these components toward smaller size classes between day 1 and day 7 (Figs. 5.3 and 5.4); and increased numbers of starch grain (Fig.5.5). Osmiophilic granules and dictyosomes located along the plasmamembrane were observed only in the hardiest cells (-28C) after 7 days. Cell size remained constant with no detectable differences between ABA-treated and control cells (Fig. 5.6).

Cell wall thickness (Fig. 5.7) significantly increased with hardiness levels during ABA treatment. No secondary wall thickening was observed. Both control and ABA treated cells autofluoresced, but ABA treatment greatly enhanced cell autofluorescence (Fig. 5.8). Autofluorescence originated from both the cell perimeter and protoplasm. Phoroglucinol staining did not detect differences in lignin distribution between ABA and control cells. The autofluorescence was quenched after methanol: chloroform extraction.

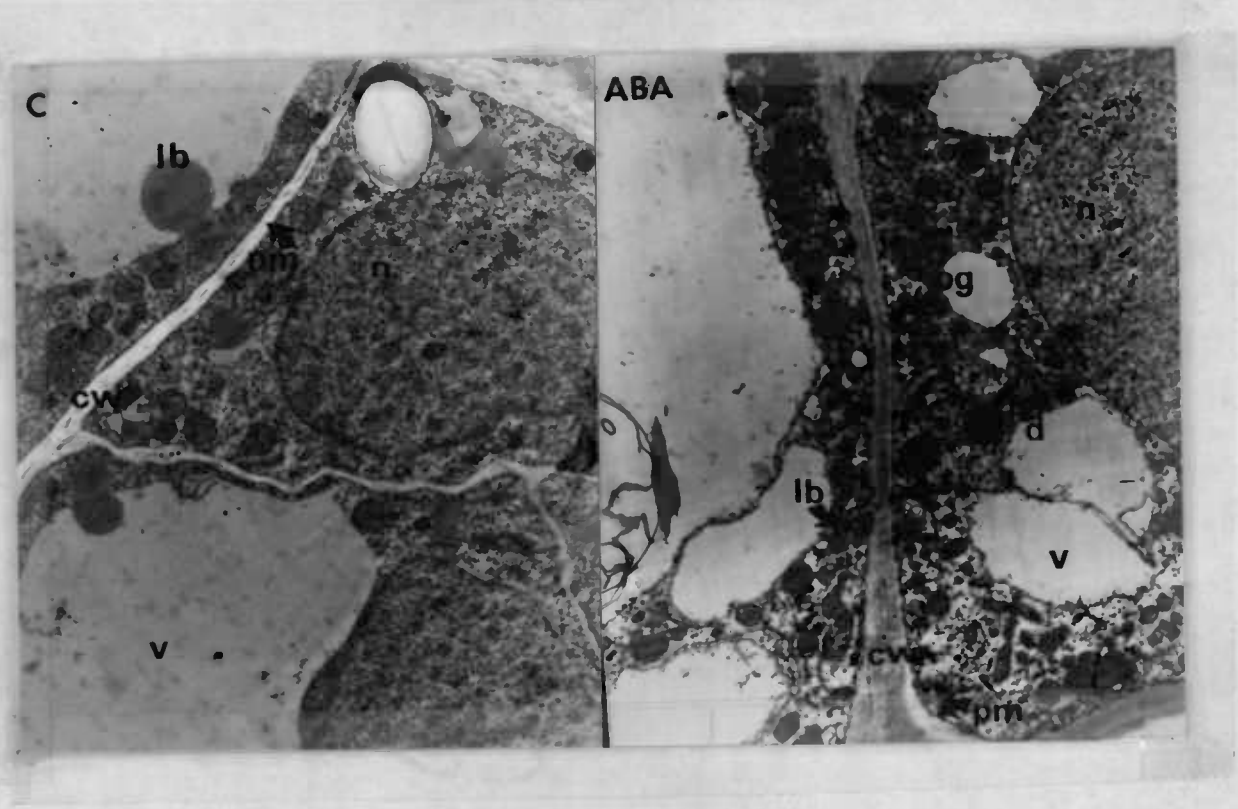
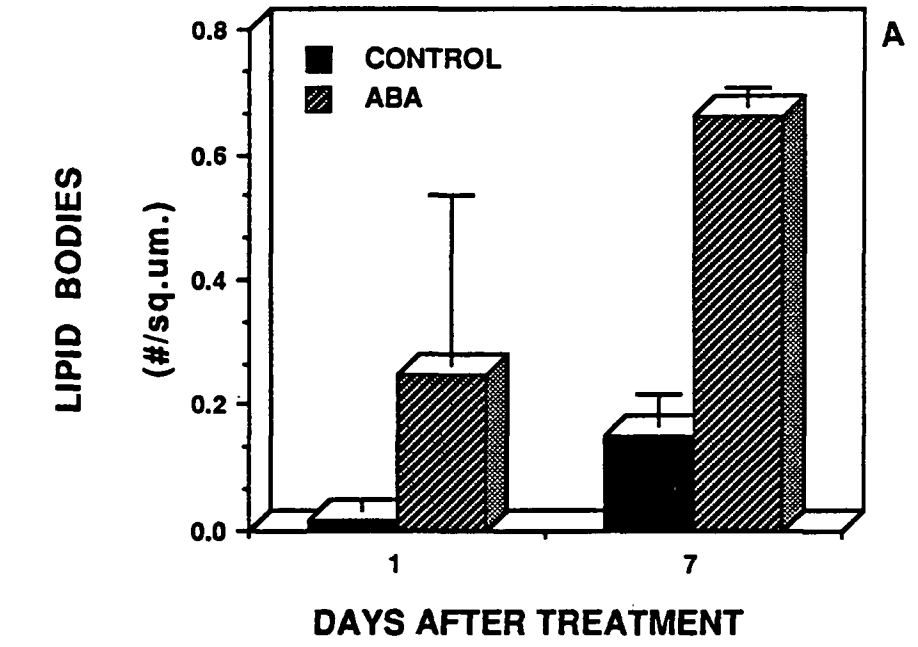


Figure 5.1 Transmission electron micrographs of typical control and ABA treated cells after 7 days of treatment. cw = cell wall; lb = lipid body; og = osmiophilic granules; v = vacuole; d = dictyosome; pm = plasmamembrane. Magnification: 4700X, 1mm=212nm

Table 5.1 Hardiness of bromegrass cells with and without 7.5 x 10^{-5}M ABA at 1 and 7 days of treatment. Hardiness estimated by TTC reduction and values represent means ±S.D. of 6 separate experiments.

TREATMENT	HARDINESS LT50 (C)
CONTROL	
1 day	-5
7 days	-5
ABA	
1 day	-10
7 days	-28



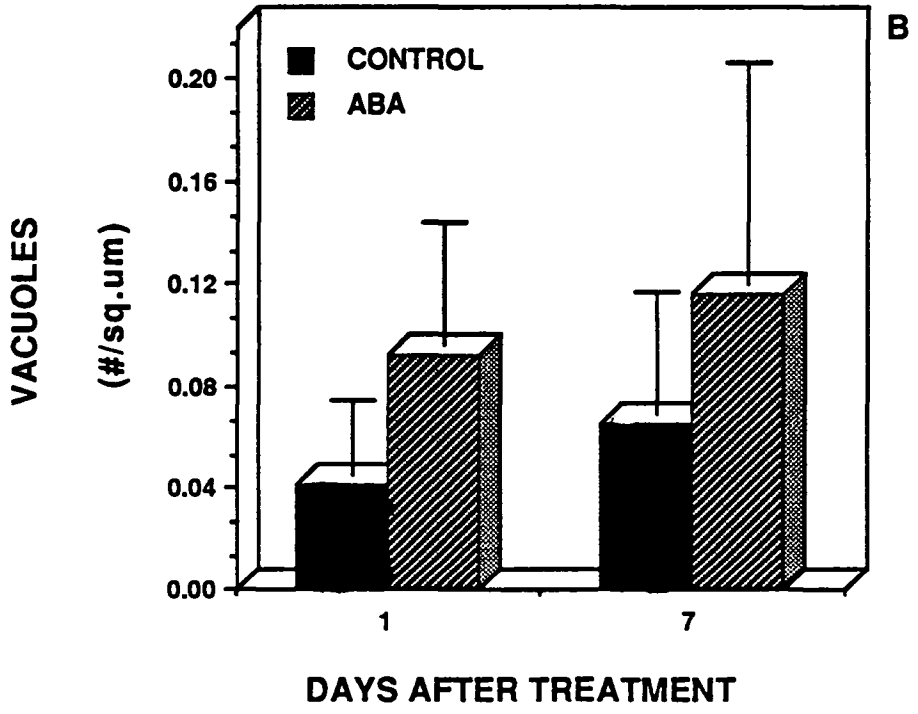


Figure 5.2 A. Numbers of lipid bodies expressed per um^2 with and without 7.5 x 10^{-5}M ABA. Values represent the average counts of 8 cells for each treatment. Depending on the cell size and treatment, mean numbers of lipid bodies ranged from 2 to 420 per cell. B. Numbers of vacuoles expressed as described in A.

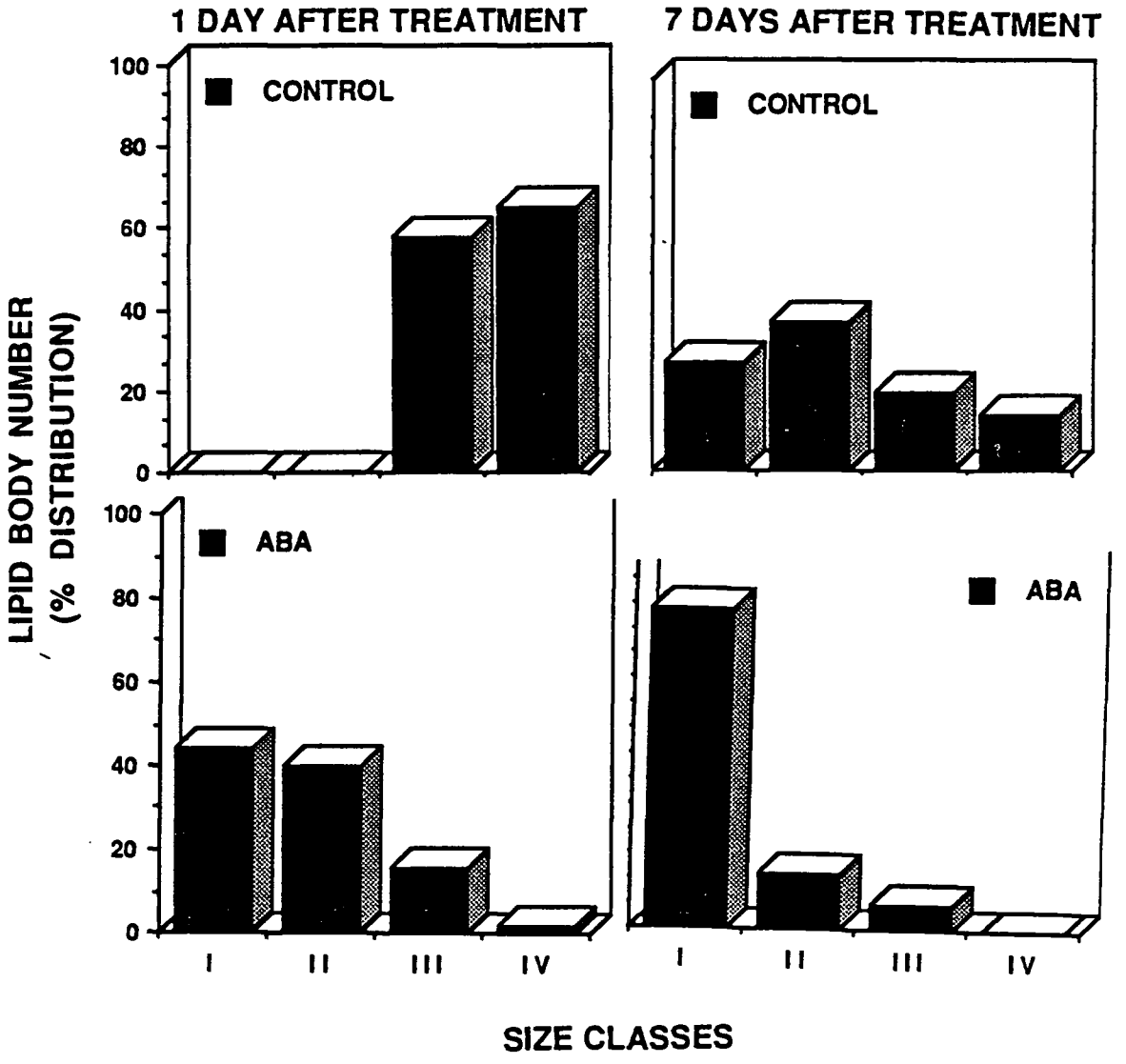


Figure 5.3. Distribution of lipid bodies by size class I (< .35um); II (.35-.7 um); III (.7-1.4 um); and IV (> 1.4 um) with and without 1 and 7 days of 7.5 x 10^{-5} M ABA treatment. Values represent means of 4 cells per treatment and sampling day. Depending on cell size and treatment, quantities of lipid bodies in each class ranged from 0 to 450 per cell.

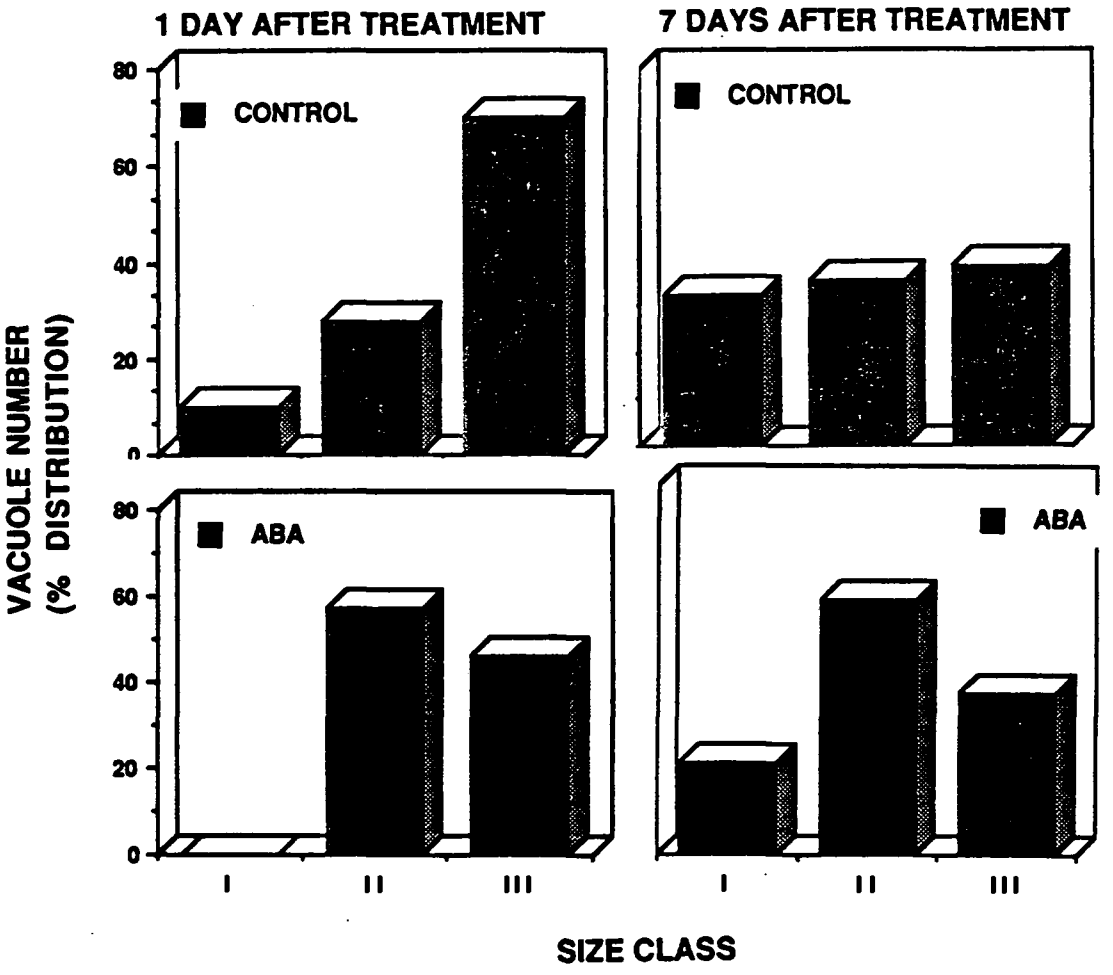


Figure 5.4 Distribution of vacuoles by size class I (< .7 um); II (.7-1.4 um); and III (> 1.4 um) with and without 1 and 7 days of 7.5×10^{-5} M ABA treatment. Values represent means of 4 cells per treatment and sampling day. Depending on cell size and treatment, quantities of vacuoles in each size class ranged from 0 to 13 per cell.

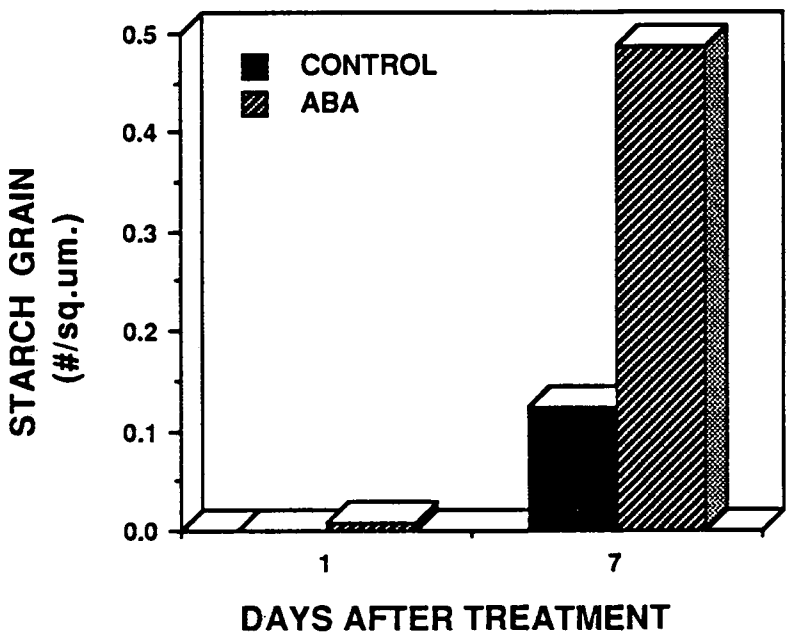


Figure 5.5 Numbers of starch grains with and without 1 and 7 days of 7.5 x 10^{-5} M ABA treatment. Values expressed per um^2 and represent means of eight cells for each treatment and sampling day.

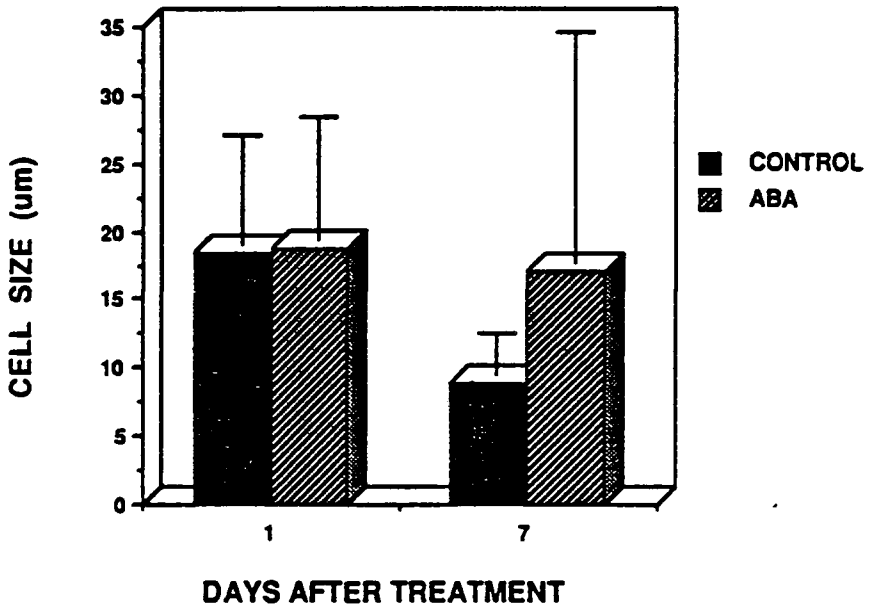


Figure 5.6 Average cell size with and without 1 and 7 days of 7.5 x 10^{-5} M ABA treatment. Calculations as outlined in text. Values represent the means ±S.D. of 8 cells for each treatment on each sampling date.

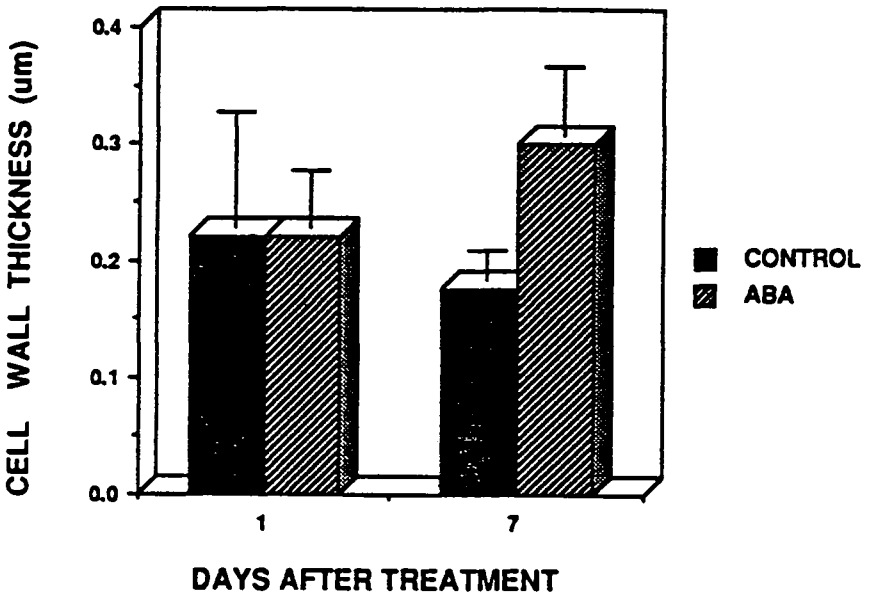


Figure 5.7 Cell wall thickness (um) with and without 1 and 7 days of 7.5 x 10^{-5} M ABA treatment. Values represent means ±S.D. of twenty measurements for each treatment and sampling date.

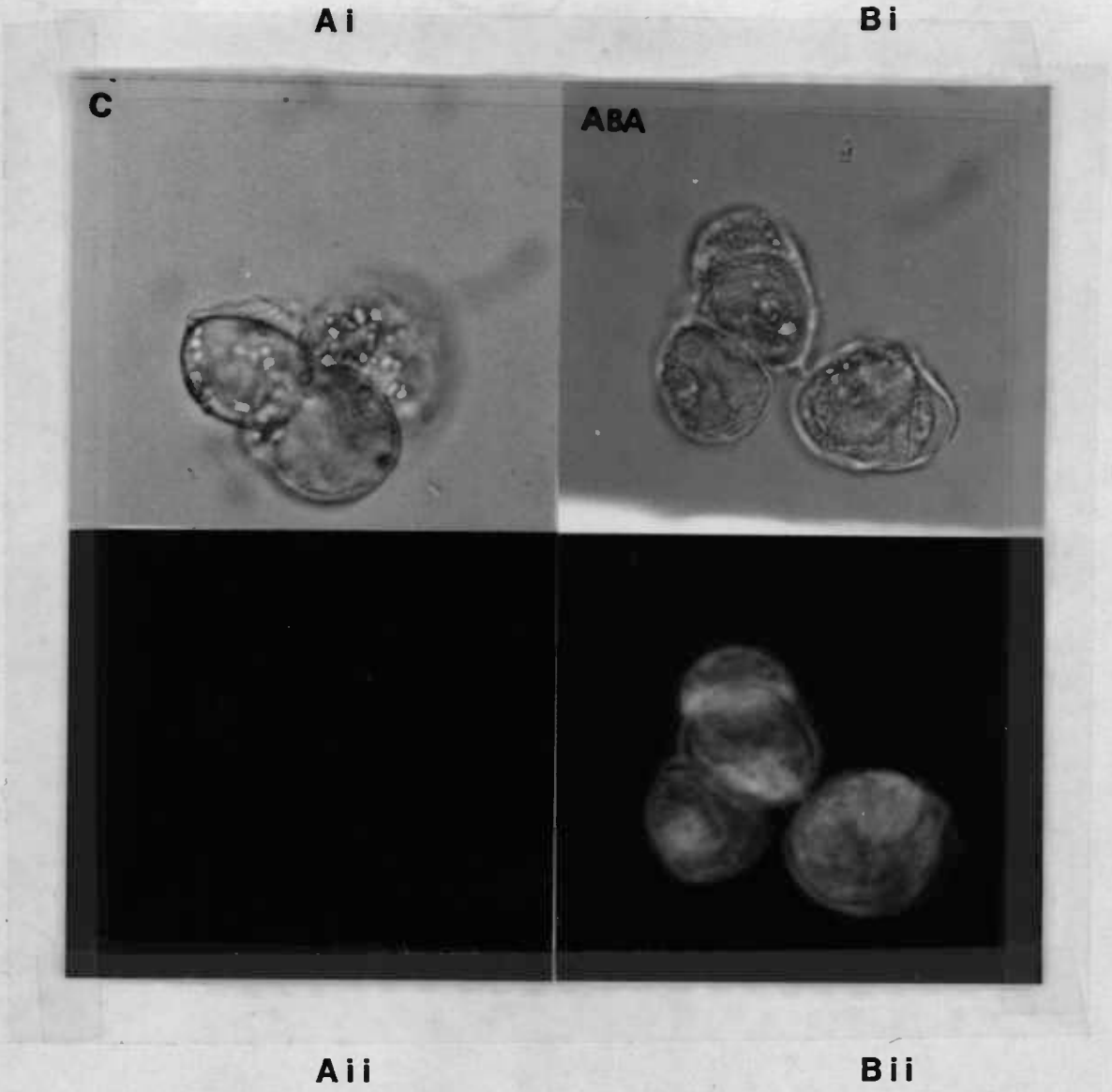


Figure 5.8 Bright field (400x) (i) and autofluorescence (ii) of the same bromegrass cells. Cells minus (A) and plus (B) 7.5 x 10^{-5}M ABA were sampled at 7 days after treatment.

5.5 DISCUSSION

This study shows that ABA elicited ultrastructural changes similar to those observed during low temperature acclimation. Fluorescence and transmission electron microscopy confirmed results of a companion study of biochemical changes that determined cell wall augmentation to be associated with ABA-induced frost hardiness (Tanino, 1989).

Two plant acclimation responses that are consistently observed are a decrease in cell water content, expressed as percent water, (Chen et al., 1976; McKenzie et al., 1974) and an increase in total dry matter accumulation (Fowler and Gusta, 1977; McIntyre et al., 1988). Cell wall accounted for most of the dry matter increase in ABA treated bromegrass cells (Tanino, 1989).

Reaney and Gusta (1989) also noted cell wall thickening after ABA treatment, and Pilet (1972) reported that abundant cell wall mucilage formed in response to ABA in Rubus cell cultures. Our results confirm that ABA stimulates cell wall thickening, and indicates that the additional deposition of cell wall material is uniformly distributed. Cellular responses to low temperature induced cold acclimation also characteristically involve cell wall augmentation. The epidermal and mestome sheath cell walls in hardened Puma rye leaves became significantly thicker (Griffith et al., 1985). After acclimation, pea epicotyl cell wall weight increased by 40 percent (Weiser, 1989). The hardy S. acaule possessed thicker cell walls than S. tuberosum potato species

which does not tolerate frost (Chen et al., 1977; Li and Palta, 1978).

Accumulating evidence indicates that augmentation may be also a result of qualitative cell wall alterations (Griffith et al., 1985; Bartolo and Wallner, 1986; Chalker-Scott, 1988; Johnson-Flanagan and Owens, 1985; Paroschy et al., 1980). The enhanced autofluorescence of ABA-treated cells observed in this study also suggests that cell wall compositional changes are occurring. Phenolic cell wall components such as suberin and lignin are potential sources of autofluorescence (Biggs, 1985). In contrast to Reaney and Gusta's results (1989), ABA did not appear to alter lignin levels in this study. Zhang and Willison (1986) proposed that the autofluorescence observed in bromegrass control cells were lipidic in origin. Our results supports their work since methanol:chloroform extraction, which solubilizes lipids, decreased the ABA-enhanced autofluorescence.

There is growing evidence that the cell wall has a role in freezing injury/resistance (Tao et al., 1983; Olien, 1977; Paroschy et al., 1980; Griffith and Brown, 1982; Griffith et al., 1985; Bartolo and Wallner, 1986, 1987; Jaffe and Biro, 1979; Rajashekar and Burke, 1982; Reaney, 1989; Chalker-Scott, 1988; Singh and Johnson-Flanagan, 1987). Negative turgor measurements (Rajashekar and Burke, 1982) have prompted some researchers to suggest that the rigidity of cell walls influences the cold hardiness on woody plants (Rajashekar and Burke, 1982; George and Burke, 1984) and herbaceous plants (Anderson et al., 1983; Reaney, 1989).

Cell wall rigidity could influence hardiness by decreasing the degree of cell volume reduction during extracellular freeze dehydration. The cell wall augmentation we observed in response to ABA could also alter wall elasticity. Reaney (1989) determined that ABA increased the rigidity of bromegrass cell walls. In maize coleoptiles, Kutschera and Schopfer (1986) reported a reduction in cell wall extensibility by ABA treatment. In pea plants, Weiser (1989) found that increased freezing tolerance was always associated with elevated levels of cell wall extensin, a cell wall glycoprotein. Extensin is thought to crosslink cellulose microfibrils, thereby adding rigidity and strength to cell walls (Wilson and Fry, 1986). Although wounding and ethylene treatments elevated extensin levels without a concomitent rise in hardiness (Weiser, 1989), this does not exclude the possibility that extensin has a role in hardiness, since acclimation may require several cellular alterations. Extensin glycoproteins are generally associated with dicots (Tierney and Varner, 1987). However, it is interesting that ABA significantly elevated the accumulation of glycoproteins in bromegrass cell culture media (Gusta, pers. comm.).

Freezing injury invariably results in cell lysis and leakage and the plasmamembrane is thought to be the site of freezing injury (Steponkus, 1984). Therefore, the interaction between cell wall and plasmamembranes are of particular interest. One postulated mechanism of membrane damage is the physical tearing that may occur when the cell wall separates from the plasmalemma at low temperatures during freeze-dehydration.

Addition of material typically occurs to the inner face of the cell wall. Thus, a less obvious though potentially important consequence of cell wall thickening is a reduction in protoplast volume and an increase in the surface/volume ratio. The greater the specific surface area, the less volume strain there will be per unit surface at any degree of cell contraction (Levitt, 1980). Bartolo and Wallner (1986) speculate that increased cell wall/plasmamembrane adhesion will elevate resistance to cell collapse and increase dehydration resistance. In pear cell suspension cultures, Wallner et al. (1986) determined that cold-induced callose deposition occurs at the plasmamembrane/cell wall interface (Weiser, 1989). Furthermore, Johnson-Flanagan and Singh (1986) demonstrated that the number of membrane attachments to the cell wall increased during cold acclimation in alfalfa cell cultures. If ABA-enhanced cell wall autofluorescence is indeed lipidic in nature, it is possible that the cell wall component, which increases during acclimation, may be a suberin compound. Although information is limited, ABA has been demonstrated to stimulate suberization in potato tissue cultures (Cottle and Kolattukudy, 1982). The chemical composition of suberin permits attachments to both cell wall and membrane components and thus may serve to increase plasmamembrane/cell wall adhesion (Chalker-Scott, 1988).

Several hardiness-related cytoplasmic modifications were also observed in biochemical (Tanino, 1989) and ultrastructural studies of ABA treated bromegrass cells. Similar changes have been reported during low temperature acclimation. As noted by

Reaney and Gusta (1989) in bromegrass cell cultures, the number of lipid bodies and starch grains increase while vacuole size decreases. Our measurements show that cell size remained constant, and support their observations that lipid body size decreased and vacuole numbers increased during ABA-stimulated frost hardiness treatments. Further, carrot cell suspension cultures that during 5 days of 7.5 x 10^{-5} M ABA treatment only increased hardiness by 3C over control samples, also did not exhibit lipid body, vacuole or osmiophilic granule proliferation (Tanino, 1989). Additionally, no differences in cell wall thickening were observed in the carrot cells.

Early studies (Rosa, 1921; Levitt and Scarth, 1936) indicated that small cell size was associated with cold hardiness. However, subsequent investigations have consistently shown that small cell size is not a basis for frost hardiness (Levitt, 1956; Chen et al., 1977; Palta and Li, 1979; Huner et al., 1981). The results of this study further indicate that cell size is not a criterion for ABA induced hardiness development.

The accumulation of starch observed in this study contrasts with reports of starch degradation during low temperature acclimation (Sakai and Larcher, 1987). Amylase activity is optimal at 4C and hence low temperature-stimulated starch degradation may reflect this result. Starch depletion was also observed after ABA treatment in leaves (Mittelheuser and Van Steveninck, 1971a,b) and tissue culture (Pilet, 1972). The ABA effect on starch degradation in whole plants was suggested to be an indirect result of ABA inhibition on the rate of photosynthesis

(Mittelheuser and Van Steveninck, 1971b). Growth rates of the bromegrass cell cultures used in this study were linear (Tanino, 1989) and thus sucrose substrate appeared to be non-limiting. Since ABA also enhances sucrose uptake (Tanino, 1989), ABA may be shifting excess cellular sucrose to starch.

This study indicates that ABA treatment stimulates osmiophilic granule formation at room temperature and under non-injurious conditions. The high affinity of osmium tetroxide for unsaturated lipids suggests that the observed densely staining osmiophilic granules may be rich in unsaturated lipids. Osmiophilic granules have been associated with low temperature induced cold acclimation (Li and Palta, 1978; Chen et al., 1977; Siminovitch et al., 1968), with cell injury caused by osmotic stress (Singh, 1979), leaf senescence (Mittelheuser and Steveninck, 1971a) and intracellular freezing damage (Pearce and McDonald, 1977). Additionally, the typical lipid proliferation observed to occur during low temperature acclimating conditions (Sakai and Larcher, 1987) has also been reported at room temperature in response to ABA treatment in this and other studies (Mittelheuser and Van Steveninck, 1971a,b) .

Postulating definitive roles for the observed cytoplasmic alterations in frost hardiness is beyond the scope of this study. But an interesting consequence of ABA-induced acclimational changes emerge. If water content during ABA-induced acclimation remains relatively constant (Tanino, 1989), the free water fraction must be lowered in some way to avoid lethal intracellular freezing. Previous observations (Tanino, 1989) indicated that ABA

stimulated sucrose uptake and elevated osmotic potential. Since dry matter also accumulated, it seemed plausible that osmotic potential increased as a result of the decreased space within cells available for free water. Recently, Koster et al., (1989) determined that the sucrose and raffinose that accumulated during cold acclimation, was compartmentalized in regions outside of the vacuole. Furthermore, the greater number of smaller vacuoles observed in this study during ABA-induced acclimation will serve to decrease large areas of free water contained in larger vacuoles of control cells. In addition, the observed shift toward greater numbers of lipid bodies and vacuoles of smaller size during acclimation effectively increases the surface/volume ratio of these components. A unit membrane surrounds each of these structures with polar hydrophilic surfaces exposed to the cytoplasm. During extracellular freeze dehydration, the interaction between these surfaces and the remaining free water within the cell will increase and may prevent dehydration injury.

In conclusion, ABA appears to share ultrastructural alterations with those observed during low temperature acclimation which are associated with frost hardiness. Acclimational changes protecting the plasmamembrane may originate at both the cell wall and cytoplasmic levels. For attainment of optimum hardiness, a combination of several cytoplasmic and cell wall changes is likely required.

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6.0 Abscisic Acid Increases Plant Cell Resistance to Hydrostatic Pressure

6.1 ABSTRACT

This study demonstrated, for the first time, that the natural plant growth hormone, abscisic acid (ABA), induces plant cells to increase markedly in their ability to resist hydrostatic pressure. ABA has been shown to elevate plant tolerance to several environmental stresses including freezing, chilling, salt, and desiccation. In deep sea organisms, evidence is also accumulating for a common cellular response to both low temperature and hydrostatic pressure stress. Although bromegrass is not naturally adapted to withstand the hydrostatic pressure environment of aquatic organisms, after ABA treatment, cells survived pressures corresponding to near the limits of ocean depth. At 1000 atm pressure, ABA substantially elevated tolerance within one day and increased tolerance 90 fold over untreated cells after 7 days. Hydrostatic pressure tolerance developed in bromgrass cell culture with increasing exposure to ABA throughout 7 days of treatment, in parallel with the marked elevation of cell tolerance to freezing stress. Injury caused by high hydrostatic pressure did not result from a rapid compression/decompression but increased gradually and cumulatively over a 12 hour stress test period. Cell rupture was not observed. Hydrostatic pressure resistance studies may provide a useful means for unravelling the mechanism(s) of ABA-induced tolerance to freezing and other stresses.

6.2 INTRODUCTION

Organism distribution in terrestrial and aquatic environments is mainly determined by temperature, water, and pressure (Bernhardt et al., 1988). Organisms are often required to co-adapt to more than one environmental stress and thus must develop resistance mechanisms to several stresses.

The importance of the natural plant growth regulator, abscisic acid (ABA), in the adaptation of land plants to a variety of environmental stresses is well documented (Addicott and Van Steveninck, 1983; Walton, 1980). It has been observed that pre-exposure of plants to one stress elicits a rise in the level of endogenous ABA and can confer resistance to a different stress. This suggests that ABA may function as a general stress hormone in plants.

Exposure to low temperatures was shown to cross-adapt barley and tobacco leaf tissue to drought (Rikin and Richmond, 1975). Rikin et al. (1973) had previously found that endogenous ABA levels rose in tobacco plants exposed to water stress, chilling at 5C, or mineral deprivation, and that these stressed plants also became more resistent to sub-zero temperatures. In addition, exogenous applications of ABA have been demonstrated to increase the resistance of land plants to chilling and freezing temperatures (Chen and Gusta, 1983; Rikin et al., 1975).

The site of freezing injury in plants is considered to be the plasmamembrane (Steponkus, 1984). High hydrostatic pressures induce deleterious membrane responses similar to those observed

in plant cells exposed to low temperatures. A 1000 atm pressure will cause ordering of membrane bilayers to a level equivalent to that caused by a 15 to 25C drop in temperature (Chong and Cossins, 1983; Lakowicz and Thompson, 1983). The ordering of membrane bilayers reulsts in a phase transition of the lipid bilayer from a sol to a gel state (MacDonald, 1984). This phenomenon has been observed in goldfish brain (Chong and Cossins, 1983), bacteria (Braganza and Worcester, 1986, DeLong and Yayanos), deep sea fish (Cossins and MacDonald, 1984), and in cell free systems (Wong and Mantsch, 1985; Braganza and Worcester, 1986).

Evidence indicates a homeoviscous adaptation to hydrostatic pressure stress where the proportion of unsaturated fatty acids in membrane phospholipids increases to optimize membrane fluidity in deep sea bacteria (DeLong and Yayanos, 1985) and fish (Cossins and MacDonald, 1984). This adaptational strategy is analogous to membrane alterations during low temperature acclimation in several organisms including plants, bacteria, and poikilothermic animals (DeLong and Yayanos, 1985). Hydrostatic pressure is of ecological significance to organisms in aquatic environments. However, there are no reports of inducing pressure tolerance, or pressure acclimation in biology.

ABA significantly increases freezing resistance in bromegrass cell suspension cultures (Chen and Gusta, 1983; Reaney et al., 1989; Tanino, 1989). Since freezing and hydrostatic pressure both appear to produce similar stress effects, this study examined whether ABA treatment would also increase tolerance to

hydrostatic pressure in bromegrass cells which are not naturally adapted to high pressure conditions.

6.3 MATERIALS AND METHODS

Cell Culture A bromegrass (Bromus inermis Leyss cv Manchar) cell suspension culture (BG-970) was grown in modified Erickson's media containing 0.5 mg/l 2,4-D (Chen and Gusta, 1983). A 1.75 ml packed cell volume inoculum size was used to achieve more linear growth throughout the treatment period (Tanino, 1989). The suspension culture was maintained on an Eberbach reciprocating shaker at 110 rpm at 23C in the dark and routinely transferred every two weeks. Abscisic acid (ABA, Calbiochem) at 7.5×10^{-5} M was added to the media and autoclaved at 121C for 15 min. This ABA concentration is known to induce a rapid increase in freezing resistance in the bromegrass cell culture (Chen and Gusta, 1983). Cells were sampled, observed microscopically and tested for pressure and freezing resistance at 1, 3, 5 and 7 days after ABA treatment.

Freezing Test Cells were harvested, and washed with 200 ml ddH_2O (about 20 volumes) to remove the possible cryoprotective effects of the media residue during freezing. Cell samples of 200 mg fresh weight were frozen in 10x75mm glass culture tubes after removal of excess water (Tanino, 1989). Samples were equilibrated in a Neslab LT-50 low temperature bath at -1C and nucleated with ice crystals. Frozen cells were maintained overnight at -1C and the next day temperature lowered at a rate of 2C/hr to -10C and 3C/hr thereafter. Cell sample tubes were removed each hour from -3 to

-33C and thawed at 4C for 24hrs.

Viability Test Cell viability was estimated by the 2,3,5-triphentetrazolium chloride (TTC) reduction method modified from Towill and Mazur (1974), and by the fluorescein diacetate method described by Widholm (1974). **TTC**: Each 50 mg sample was incubated in a 1 ml solution of 0.5 g/100 ml TTC for 24 hr in the dark and sealed with a serum stopper. Excess TTC was then removed and extracted with 3 ml of 95% ethanol per vial. After full extraction (about 3 days), 300 ul of the ethanol extractant were transferred to Eliza plates and read at 495 nm. with a Biotek Microplate Autoreader EL 309. In each experiment, three samples were analyzed for each treatment and the experiment was repeated 6 times. Viability was expressed as LT_{50}, the temperature at which 50% of the cells were killed, as determined by the TTC test. **Fluorescein diacetate:** A 5 mg/ml (in acetone) fluorescein diacetate stock solution was diluted to 0.01% (v:v) in culture media. Although a gradient of fluorescence was observed, only the cells emitting the highest fluorescence intensity were considered viable. Cells were isolated with a 30 um sieve. At least 100 cells were counted within each sample.

Hydrostatic Pressure To prepare cells and cell clumps for exposure to hydrostatic pressure, samples of ABA treated and control cells were placed into a 1 ml disposable syringe with a 23 gauge needle. Excess air bubbles were removed and the needle was plugged with a size 00 rubber stopper. Syringes containing cell

samples were placed into stainless steel pressure cylinders. Hydrostatic pressures were applied for various periods of time (3, 6, 9 and 12 hours) with a barokam unit described by (Morita, 1970). Compression and decompression to and from treatment pressures were attained within minutes. Pressure stress experiments were performed at 23C.

Cell Rupture Decreased isolated cell counts and light microscopic observations of cell rupture at 400X magnification after hydrostatic pressure treatment were used to evaluate whether cells had ruptured. Cells were counted with a hemacytometer in a .1 mm^3 volume. At least 200 cells were counted in each replicate and three replications performed.

6.5 RESULTS AND DISCUSSION

Seven days of ABA treatment increased bromegrass cell tolerance to hydrostatic pressure stress at 500, 750, and 1000 atm as determined by tetrazolium chloride reduction (TTC) and fluorescein diacetate (FDA) viability tests (Table 1). At 1000 atm, the 27% viability observed for ABA treated cells was below the 50% viability standard used in this study, but still significantly higher than the 3% viability observed for control cells. TTC and FDA reflect cytoplasmic dehydrogenase (Steponkus, 1968) and membrane-bound epimerase activity (Widholm, 1972), respectively. Thus, both cytoplasmic and membrane associated components appear to be injured during cell exposure to pressure stress. Microscopic examination and counting of isolated cells indicated that pressure injury did not involve cell rupture (Table 1). The extent of ABA-induced pressure tolerance in this study coincides with the upper limit of pressure tolerance observed in plant species (Vidaver, 1972).

Viability was not affected by immediate compression/decompression (Fig. 1), but was dependent on the length of exposure to high pressure(s). In Euglena, Gross (1965) observed that tolerance to augmented pressures was dependent on the magnitude and duration of pressure exposure. In addition to membrane phase transitions, hydrosatic pressure has also been observed to disrupt chemical equilibria, pH (Kinne, 1972) and to cause molecular volume reduction (Morita, 1967). These effects result in numerous metabolic aberrations that have both functional

and structural consequences (Morita, 1967, Vidaver, 1972, Marquis, 1976).

The stress resistance of bromegrass cells increases to both freezing and hydrostatic pressure with duration of ABA treatment (Fig. 2). This implies that the mechanisms of tolerance to both stresses may be similar. Studies of plant cell resistance to hydrostatic pressure, elicited by ABA, may prove useful in elucidating mechanisms of ABA-induced tolerance to freezing stress.

ABA, which is normally found in frost hardy plants, has also been recently found to occur in fungi (Okamoto et al., 1988; Dahiya et al., 1988), algae (Boyer and Dougherty, 1988), and mammalian brain cells (Chen et al., 1988). The functional role of ABA in these organisms is not understood.

Marine organisms that migrate vertically are particulary subject to variations in both pressure and temperature. Under these conditions, the requirement of a continual response to changing environmental conditions would favour the presence of a hormone such as ABA. However, it is not known yet whether ABA occurs, or plays a role in the stress adaptation of such organisms.

These results re-emphasize the possibility that ABA may play a central role in the adaptational responses of terrestrial plants and marine organisms to a variety of environmental stresses.

Table 6.1 Cell viability after different hydrostatic pressure treatments in bromegrass cells with and without 7.5 x 10^{-5} M ABA. TTC = 2,3,5-triphenyltetrazolium chloride, FDA = fluorescein diacetate. "+/-" indicate viability greater or less than 50% of viability at 1 atm.

PRESSURE (ATM)	VIABILITY TESTS					
	TTC		FDA		CELL WALL INTACTNESS	
	C	ABA	C	ABA	C	ABA
1	+	+	+	+	+	+
250	+	+	+	+	+	+
500	-	+	-	+	+	+
750	-	+	-	+	+	+
1000	-	-	-	+	+	+

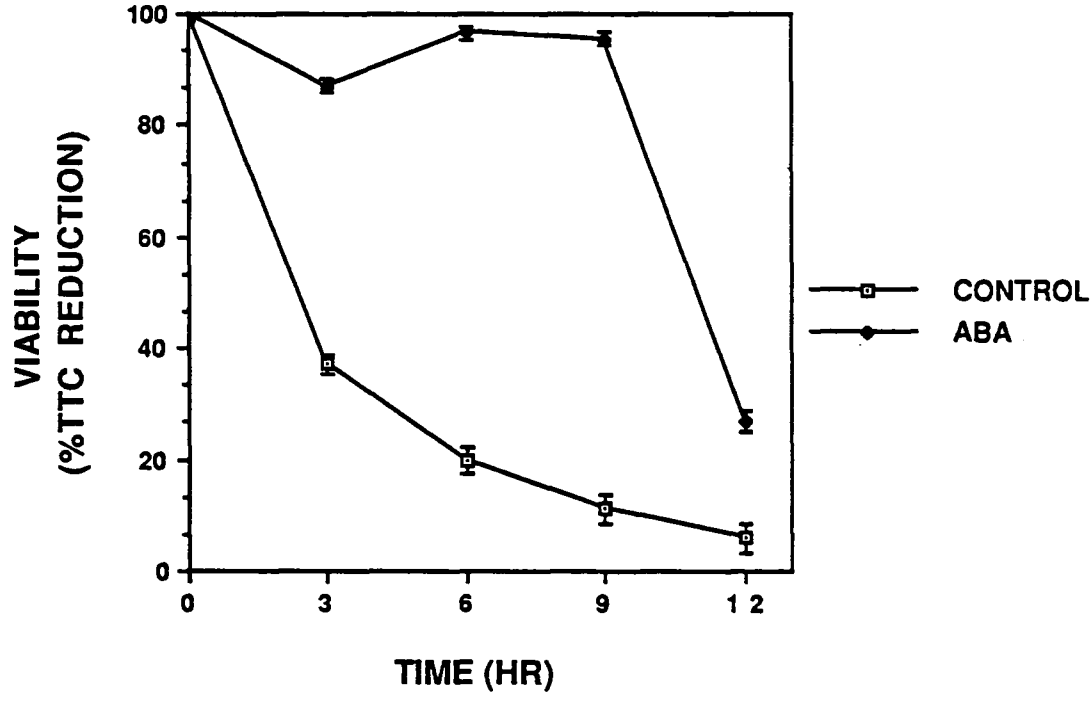

Figure 6.1 Tolerance of 7 day old bromegrass cells at 1000 atm pressure with and without 7.5 x 10^{-5}M ABA over a 12 hour period. Viability is expressed as a percentage of cell viability at 1 atm for each time period.

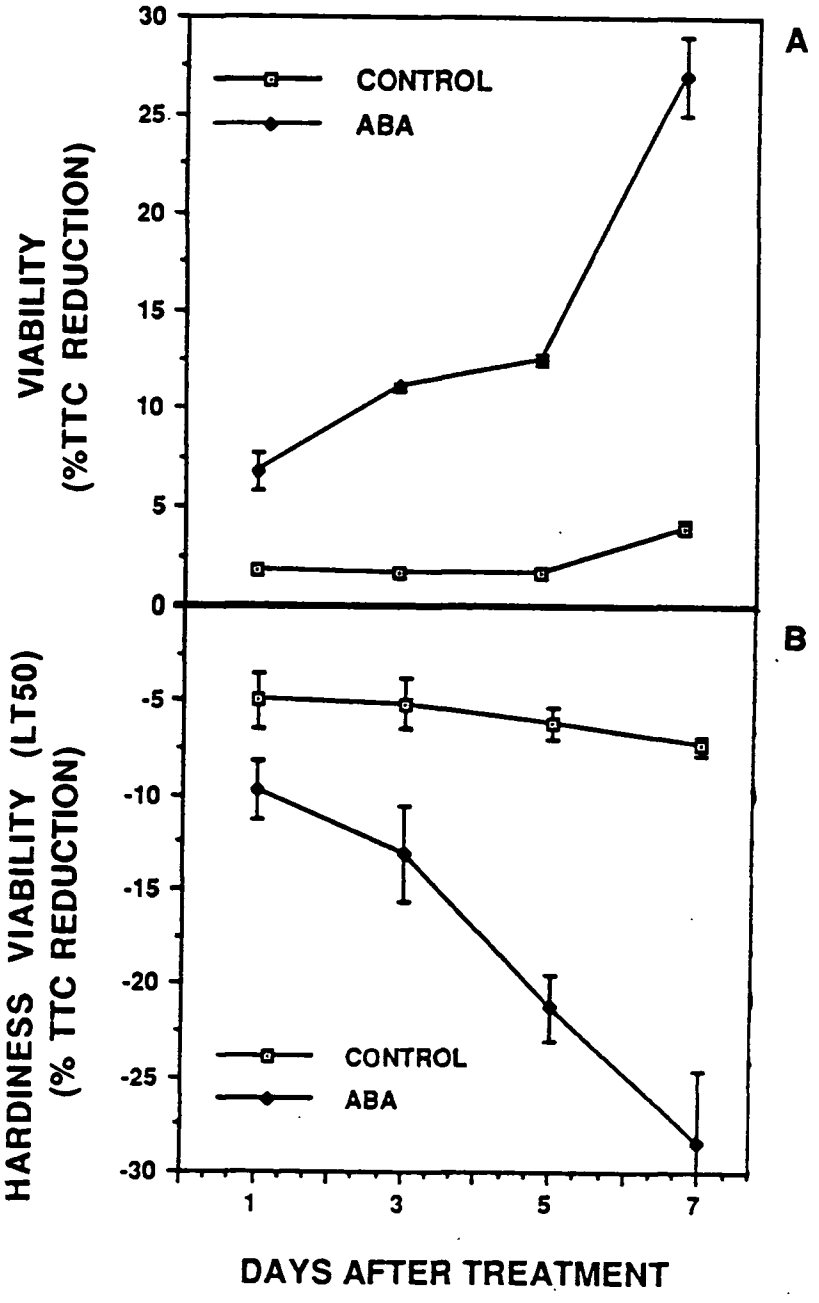

Figure 6.2 A. Hydrostatic pressure tolerance of ABA treated (7.5 X 10^{-5}M) and control bromegrass cells after 12 hours of exposure to 1000 atm pressure on days 1, 3, 5 and 7 days of treatment. Viability is expressed as a percentage of the viability of cells held at 1 atm for 12 hours. B. Freezing tolerance of bromegrass cells during 1, 3, 5 and 7 days with and without 7.5 x 10^{-5}M ABA treatment. Frost hardiness is expressed as the LT_{50} (temperature at which 50% decrease in TTC reduction was observed).

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7.0 Abscisic Acid Induced Frost Hardiness in a Newly Initiated and a Twenty Year Old Bromegrass Cell Culture

7.1 ABSTRACT

A higher mutation frequency is often present in cell cultures. The response of cells subcultured for numerous generations, therefore, can be associated with either the inherent traits of the plant species or with mutations selected in culture. This study demonstrates that the abscisic acid-induced frost hardiness response is not simply the result of a mutation originating in culture. A new cell suspension culture was established from the same cultivar as a bromegrass (Bromus inermis Leyss cv. Manchar) cell culture propagated for twenty years and known to increase frost hardiness in response to abscisic acid treatment. This new cell culture also significantly increased frost hardiness in response to abscisic acid treatment. Similar to that previously reported in the older cell line, response to abscisic acid was concentration-dependent and the same abscisic acid concentration (7.5×10^{-5}M) induced optimum hardiness in the new culture. Under the optimal abscisic acid concentration, the new cell line expressed a higher rate of acclimation than did the twenty year old culture. By 5 days after treatment, the new cell line survived -33C temperatures while the established culture hardened to only -23C. In addition, the new cell cultures were able to regenerate into green plants, while regeneration was not possible using the old cultures. This study indicates that the abscisic acid-induced freezing tolerance in the twenty year old culture is not a unique mutant, but represents the characteristics of bromegrass. Since green plants can be regenerated and abscisic acid appears to elicit

a greater cold hardiness response in the new cell line, the newly initiated cell culture will represent a more useful system with which to examine mechanisms of abscisic acid-induced cold hardiness.

7.2 INTRODUCTION

The bromegrass cell culture initiated in 1969 by Schenk and Hildebrandt (5) has proven to be a useful system to study abscisic acid (ABA)-induced frost acclimation (1, 2, 8, 9). The ABA-induced frost hardiness system was established on cultures of at least 12 years age (1). Since the frequency of mutation appears to increase with the number of cell divisions (6, 7), cells maintained in culture by continuous sub-culturing for decades may be the product of unintentional mutational selection. As a result, the possibility exists that the ABA induced hardiness response reflects a mutant cell line selected in culture over time rather than the inherent plant cell response from which the culture was generated.

The practical use of plant cell culture in the elucidation of frost hardiness at the molecular level requires regeneration of intact plants from the culture. A cell culture that is both responsive to ABA treatments and that regenerates green plants will provide a valuable system to examine mechanisms and methods of frost hardiness improvement in plants.

This study was conducted to: 1) compare a culture maintained for twenty years and of known ABA-induced frost hardiness levels with ABA response of a new bromegrass cell line, generated from the same seed source, and 2) compare the regeneration of green plants between new and old cell cultures.

7.3 MATERIALS AND METHODS

Culture Source, Growing Conditions and Regeneration: The old culture, generated from cv. Manchar hypocotyls in 1969 by Shenk and Hildebrandt (5) was obtained from Dr. L.V. Gusta, Department of Crop Science and Ecology, University of Saskachewan, Canada. Suspension cultures were maintained in the same media as the newly initiated cultures.

To initiate the new culture, bromegrass (Bromus inermis Leyss. cv. Manchar) seeds were obtained from Pickseed West, Inc., Tangent, Oregon. Calli were induced by placing surface sterilized seeds onto Murashige and Skooge medium (1962) containing 2mg/l 2,4-D and solidified with agar (8 g/l). Two month old calli were transformed into modified Erickson's liquid media supplemented with .5 mg/l 2,4-D (1) to establish new suspension cultures. Cells were grown in 250 ml. erlenmeyer flasks with 50 ml media and maintained in the dark (23C) on a 110 rpm reciprocating shaker.

To test the plant regeneration potential, cells were plated onto the surface of plant growth regulator free Erickson's medium solidified with agar (8 g/l). Plant regeneration was evaluated two months after plating.

Hormone Treatment: Abscisic Acid (ABA) (Calbiochem) was serially diluted from 1 x 10^{-4} M ABA dissolved in several drops of 1N NaOH to concentrations of 7.5 x 10^{-5}, 5 x 10^{-5}, 1 x 10^{-5}, 1 x 10^{-6}, 1 x 10^{-7}, 1 x 10^{-8}, and 0 M ABA. After addition of ABA to the

media, the media was adjusted to pH 5.8 with NaOH, and autoclaved at 121 C for 15 minutes. A 3.0 ml packed cell volume (PCV) of cell inoculum was placed into the media at 0 time and frost hardiness was monitored after 1, 3, 5 and 7 days of treatment.

Freezing Test: Cells were harvested, and washed with 200 ml dd$H_2$0 (about 20 volumes) to remove the possible cryoprotective effects of the media residue during freezing. Cell samples of 200 mg or more fresh weight were frozen at a controlled cooling rate in 10x75 mm glass culture tubes. After 30 min. equilibration in a Neslab LT-50 low temperature bath at -1C samples were nucleated with ice crystals. Nucleated cell samples were held overnight at -1C and slowly cooled the next day at a rate of 2C/hr to -10C and at 3C/hr thereafter. Cell sample tubes were removed at a series of test temperatures from -3 to -31C and rewarmed at 4C for 24hrs.

Viability Test: Cell samples (50 mg) were placed into shell vials and cell viability was estimated by a 2,3,5-triphenyltetrazolium chloride (TTC) reduction method modified from Towill and Mazur (10). Sample vials were sealed with a serum stopper and incubated in the dark at 23C for 24 hrs.

TTC was extracted with 3ml of 95% ethanol per vial for three days. After extraction, 300 ul samples of the ethanol extractant were transferred to Eliza plates and absorbance was read at 495 nm. with a Biotek Microplate Autoreader EL 309. In each experiment, three samples were analyzed for each freezing temperature test for each cell line. The experiment was repeated

six times. Viability was expressed as LT_{50}, the temperature at which 50% of the cells were killed, as determined by the TTC test.

7.4 RESULTS AND DISCUSSION

Somaclonal variation arising from mutations in cultures increases with the number of cell division cycles (6, 7). As a result, cell cultured for years may develop abnormal physiological responses.

In this study, ABA significantly increased cold hardiness in both the old and new bromegrass cell culture (Fig. 7.1). An ABA concentration of 7.5×10^{-5} M elicited the optimum cold hardiness response in both cell lines. However, under identical culture and freezing systems the rate of acclimation of the older line was less than that of the new cell culture (Table 7.1).

Both green and albino plants were generated from the new cell culture (Fig. 7.2) whereas only albino plants could be regenerated from the old cell line. In contrast, sixteen years previously, plants were regenerated from the old cell line (11).

This study shows that the cold hardiness response induced in bromegrass cells by ABA is not due to mutations arising during the long subculturing maintenance in cultures. Thus, the ABA-induced increase in freezing tolerance of Bromegrass is an inherent characteristic of this species. It is interesting that ABA elicited a more rapid cold hardiness response in newly established cells than that of the old cell culture of the same plant species. This difference, however, may be the result of genetic differences inherent in the seeds rather than due to the age of the culture. It is also observed that the newly established cell culture, but not the old cell line had the capacity to regenerate into normal green

plants. The apparent "loss" of normal regenerative ability of the old culture suggests that the loss may be due to mutation occurring during prolonged subculture or a result of cell selection pressures eliminating the cells with normal regenerative potentials.

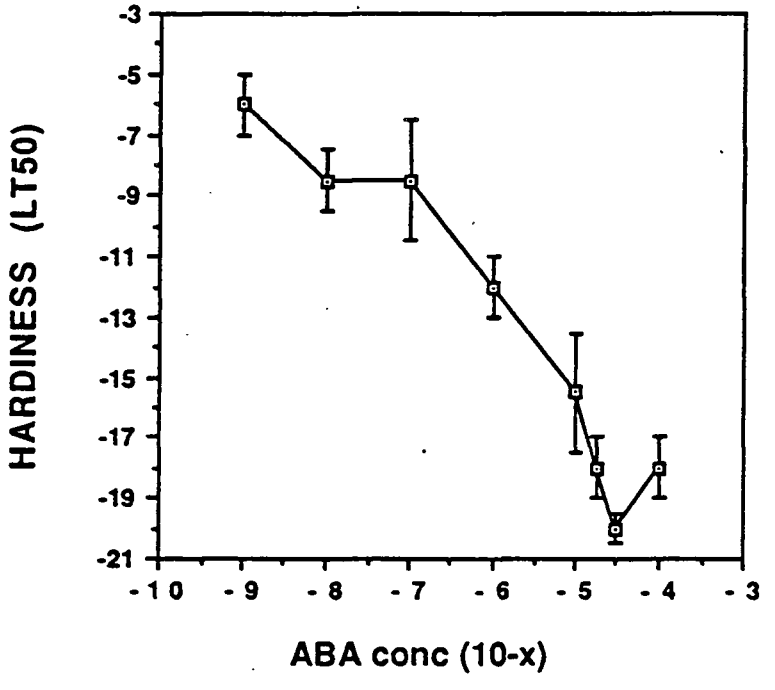

Figure 7.1 New cell culture cold hardiness (LT_{50}) at seven ABA concentrations after 3 days of treatment. Values represent means ±S.D. of three separate experiments with two subsamples for each experiment.

Table 7.1 LT_{50} in twenty year old cell lines and new cell cultures with and without 7.5 x 10^{-5} M ABA treatment. Values represent means ±S.D. of at least three separate experiments with two subsamples per experiment.

culture		1	3	5	7
new	control	-5 ± 1	-5 ± 1	-6 ± .5	-7 ± 1
	ABA	-10 ± 1	-20 ± .5	> -33	> -33
old	control	-4 ± .5	-5 ± 1	-4 ± .5	-5 ± 1
	ABA	-10 ± 2	-13 ± 3	-23 ± 2	-28 ± 3

Figure 7.2 Plant regeneration from a newly established suspension culture of Bromus inermis cv. Manchar.

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A.0 Appendices

A.1 Effect of Dormancy-Breaking Agents on Acclimation/Deacclimation of Dogwood

ABSTRACT

Hydrogen cyanamide or hot-water treatment (47C) for 1 hr at the 250°GS (Growth Stage) effectively broke rest in dogwood buds within 10 to 12 days. At this growth stage, control plants grown at 25/18 (day/night) maintained an LT_{50} of -25C throughout the 3-week study period, whereas plants treated with H_2CN_2 or hot water gradually lost hardiness. After 3 weeks at 5/2C (day/night), the control plants hardened to a hardiness (LT_{50}) of -45C, and H_2CN_2-treated plants maintained an LT_{50} of -25C. The results demonstrate that the extent of acclimation and deacclimation of dogwood plants may be influenced by environmental temperatures and rest status.

INTRODUCTION

Hardy woody plant species undergo a seasonal change in cold hardiness in response to the environment (1, 2, 10, 13). The ability of these plants to cold-acclimate and deacclimate depends on the physiological status of the plant (3-5, 7, 8, 12). During the period of increasing rest development (180-270°GS), the temperature range effective for acclimation is very wide; e.g., 0C to 20C. In contrast, as the rest period is broken (270-315°GS), the temeprature range for acclimation gets narrower while the effective range for deacclimation widens (4). Consequently, maintenance of rest and/or low temperatures during mid-winter is essential for maintaining maximum hardiness.

In previous studies concerning the relationship between acclimation/deacclimation and dormancy, the precise developmental stage of the plants was unknown (2, 3, 6, 10, 13). To clarify this relationship, the °GS model, developed by Fuchigami et al. (4), was used in this study. This model defines and numerically expresses the stage of physiological development for dogwood. The annual growth status is expressed with a 360° cycle with the following point events: spring budbreak (0/360°GS), maturity induction point (90°GS), vegetative maturity (180°GS), maximum rest (270°GS), and end of rest (315°GS).

The rest period of dogwood can be overcome at any growth stage by exposing buds to sublethal stresses. Nee (11) found that hydrogen cyanamide, hot water, and freezing temperatures were each effective in breaking rest. However, there is little

information available on the effects of artificially breaking rest (i.e., without natural chilling temperatures) on the acclimation/deacclimation of plants. Exposing plants to chilling temperatures for prolonged periods breaks rest, but also can have a separate influence on acclimation/deacclimation (4). Artifically breaking rest without prolonged exposure to chilling temperatures could provide a systematic approach for studying the relationship of rest and acclimation/deacclimation.

The objective of this study was to determine the effects of breaking rest on acclimation/deacclimation in dogwood plants at warm and cool temperatures.

MATERIAL AND METHODS

Stem cuttings of a clone of red-osier dogwood native to Wayland, Mass. were rooted in February in a greenhouse mistbench, transplanted into a 1 soil: 1 sand: 1 bark mixture (by volume) in 15-cm pots (1 liter) and grown in a lath-house (20% shade) under natural conditions in 1986. Beginning 15 September and at approximately 2 week intervals, five plants were randomly selected, defoliated and placed in a greenhouse at 25/18C (day/night) under a 16-hr photoperiod. The daylength was extended from 0600 to 2200 hr with General Electric Lucalux LU400 lamps, 150 $umol \cdot s^{-1} \cdot m^{-2}$. Degree of rest, expressed as °GS, was determined by observing the number of days to vegetative budbreak (11). Budbreak was recorded at first appearance of green leaves. At the 250°GS, when plants were approaching deepest rest, 72 plants were sampled from the lath-house and hand-defoliated. The entire stem was either painted with a 2.0 M hydrogen cyanamide solution or foil-wrapped and immersed in a 47C water bath for 1 hr. After treatment, plants were placed in either a greenhouse [25/18C (day/night)] under natural short-day (12-hr) conditions of October or in a growth chamber 5/2C (day/night) under a 12-hr photoperiod. Plant hardiness was determined after 0, 1, and 3 weeks of incubation. Time of first budbreak was evaluated as stated previously, (11).

Hardiness determination consisted of sampling 10-cm stem sections from the mid-sections of the plant. Stem sections were pooled and four stems randomly sampled per treatment per freezing

temperature. The sections were wrapped in foil with moist cheesecloth and placed on an aluminum plate in a programmable freezer. Samples were held overnight at -4C, and then frozen at a cooling rate of 5C/hr to -45C. Freezing was induced by initiating ice formation in the moistened cheesecloth at -4C. Samples were withdrawn from the freezer at -10, -17, -24, -31, -38, and -45C and thawed slowly at 4C overnight. Hardiness was evaluated at thaw based on percent conductivity (6) and reported as LT_{50}.

RESULTS AND DISCUSSION

Hydrogen cyanamide and hot-water treatment resulted in budbreak after 10 to 12 days in the warm-temperature greenhouse, while buds of the control plants remained dormant beyond 90 days (Fig. A.1). Plants incubated at low temperatures (5/2C) did not break bud, but rather remained in a quiescent state throughout the experiment. Conductivity reading of nonfrozen control plants and those treated were similar and thus the treatments were considered to be non-injurious at the time of sampling.

Control plants grown at 25/18C (day/night) maintained an LT_{50} of about -25C throughout the experimental period, whereas, in those plants grown at 5/2C, the LT_{50} decreased to -45C in 3 weeks (Fig. A.1). Plants given either rest-breaking treatment lost hardiness following 3 weeks of incubation at 25/18C. Under 5/2C incubation, H_2CN_2-treated plants maintained initial hardiness levels, but were unable to express acclimation to the extent of the controls. The hot water-treated plants, however, deacclimated under 5/2C incuation. These results support the findings of Fuchigami et al. (4) that acclimation and deacclimation are influenced by the stage of rest. At the 250°GS, they found that plants continued to acclimate between 5C and 20C, whereas after rest was broken by chilling (after 315°GS), plants deacclimated at all temperatures tested. The extent of and the temperature requirement for deacclimation was dependent on the growth stage.

That the rest-broken plants did not acclimate at 5/2C (day/night) and lost hardiness at 25/18C may indicate that factors

present in the dormant buds are essential for optimal hardiness attainment and maintenance. The basis for the differential plant response to each dormancy-breaking treatment under 5/2C (day/night) incubation is unclear. However, since the possibility also exists that the imposed treatments may directly interfere with the acclimation process, the hot-water treatment may have more severely stressed the plant than the H_2CN_2 dormancy-breaking treatment. Nee (11) has shown that sublethal stress, such as freezing, can break dormancy. It is conceivable that dormancy may be broken under natural freezing conditions, resulting in premature deacclimation. Thus, premature dormancy breaking may have significant practical implications on acclimation and deacclimation under natural conditions.

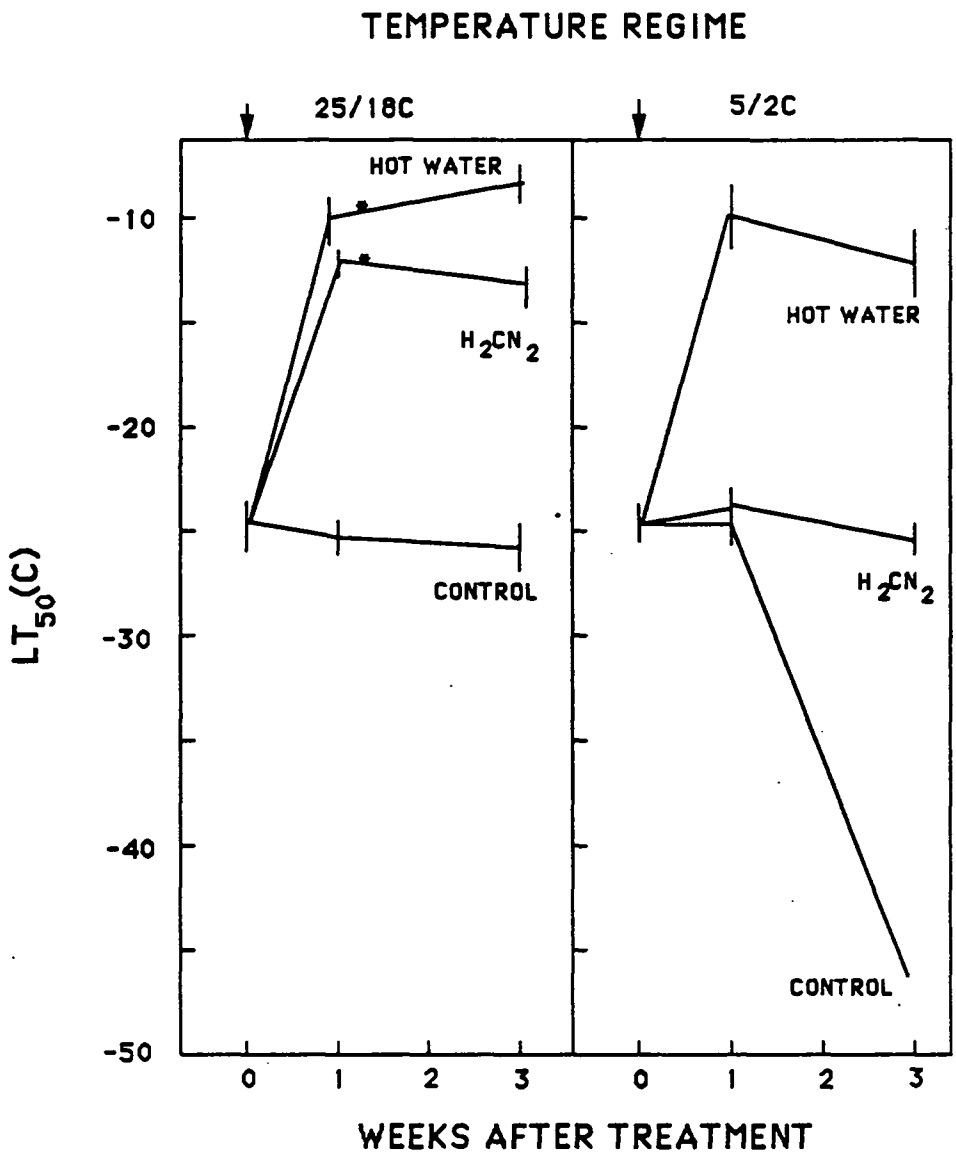

Figure A.1 Hardiness of dogwood plants (LT_{50}) held at deacclimating [25/18C (day/night)] and acclimating (5/2C) regimes for 3 weeks following treatments at the 250°GS (Growth Stage). Budbreak (*) occurred 10 1 days after treatment with H_2CN_2 (2 M) or hot water (47C) at 25/18C. Buds of control plants remained dormant after 90 days. Buds of plants held at 5/2C remained quiescent during the experiment. Vertical bars represent means ±SD of four replicate samples.

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A.2 Major conclusions from Dogwood, Cell Culture, Rhododendron Plant Systems

September, 1984 - February, 1987

1. Dogwood

i. Pre-freezing the stem segments down to non-lethal temperatures is effective in elevating hardiness levels only after the 180°GS. Pre-freezing, although effective in inducing ethylene levels before 180°GS, did not acclimate the stem segments above controls. The °GS tested were: 160, 180, 180-270.

ii. Stem segments sampled between 160-180°GS were unable to withstand prolonged ice formation in the tissues. Samples were nucleated in test tubes and held at -4°C for 0, 3, 5, and 7 days. Controls were held at -4°C without nucleation. Nucleation was identified by the presence of ice in the 200ul dH_2O at the bottom of the tube. Control samples survived through 7 days while treatments were killed at 3 days but were alive at 0 days (held frozen for 1 hour). This implies that injury was due to desiccation stress.

iii. At the growth stage that pre-freezing is effective (see i.), incubation of samples at 4°C after the first thaw and before the second freeze is more effective to enhance hardiness levels than a 23°C incubation.

iv. ABA appears to be induced by pre-freezing followed by a 4°C incubation temperature. This result however, needs to be verified again. Samples form (i) were used.

v. Desiccation-related stresses are effective to induce further acclimation (freezing, desiccation) while non-desiccation related stresses (hot water, H_2CN_2) are non-inductive.

vi. ABA enhancement of hardiness was effective only around 180°GS. Vacuum infiltration of samples before and after this time did not increase hardiness and samples that were incubated together with both ABA and 4°C lost hardiness at the 270°GS. The enhancement of hardiness was only 3°C. I believe that poor infiltration of samples was partially the reason for this result. Samples were incubated for 3 days after treatment.

vii. Once dormancy is broken artificially with both H_2CN_2 and hot water, the plant is unable to re-acclimate. Under hot water treatment, the plant loses hardiness under 4°C conditions. Since non-frozen treated tissues did not leak out significantly different amounts than non-frozen controls, the treatments were assumed to be non-injurious themselves for the duration of the experiment (hardiness tested after 1, 2, and 3 weeks after initial treatment). The H_2CN_2 treatments eventually died after 3 months. The hot water treatments were still viable. In both cases, shoots had stunted growth.

viii. Pre-freezing does not acclimate the tissue to heat stress. Plants were sampled between 180-270°GS.

ix. There is no linear relation between the degree of pre-freezing stress and the degree of re-acclimation of the stem segments. Pre-freezing must be held at 4°C for maximum effectiveness. Pre-freezing temperatures were: -10, -15, -20°C at >180°GS.

2. Cell Cultures

i. In carrot cell cultures, ABA and low temperatures appear to acclimate the cells in different ways. Growth curves under low temperature are essentially horizontal while ABA profiles are similar to controls (true for both DW and PCV). Internal ABA levels do not increase under low temperature acclimation (monitored over a 4 day period) even though hardiness under 4°C increased during this time. According to the gradient bar experiment, when ABA is incubated together with low temperature, hardiness is reduced below that of low temperature controls. ABA is more effective at high temperatures while control hardiness decreased. The two curves crossover around the 10°C acclimation temperature.

ii. According to TEM studies, under both ABA and low temperature acclimation, the plasma membrane developed infoldings while non-acclimated cells did not.

iii. A 1mM solution of H_2CN_2 in culture media elevated hardiness levels by 24 hrs but had no effect on hardiness at 1 hour. This is according to TTC test.

iv. In alfalfa cell suspension cultures, ABA plus 2,4-D was more effective than ABA alone in acclimation. ABA plus kinetin decreased acclimation, ABA plus 2,4-D plus kinetin also decreased hardiness.

v. Wounding the cells by pipetting or squashing elevated hardiness levels in carrot cell suspension cultures where according to TTC, 485nm absorbance readings were double that of the controls (no wounding) at -16°C.

vi. In carrot cell suspension culture, controls also acclimated over the growth curve as did ABA and low temperature treatments.

vii. Isolated nuclei of control and ABA treated carrot cell suspension under flow cytometry indicate that ABA may be shifting the cells into the "S" or DNA synthesis phase. These results are very preliminary.

viii. Trehalose (1%) did not enhance hardiness in carrot cell suspension.

ix. ABA (10^{-5}M) does not appear to induce ethylene biosynthesis in carrot suspension. The capacity of the cells to generate ethylene did not increase.

3. Rhododendron

i. After a 4 day, 24°C pre-treatment followed by a 1 week 0°C incubation, a 10% trehalose treatment was the only one (out of desiccation, 10^{-5} and 10^{-9} M ABA, 10% proline, spermidine) that elevated hardiness levels. Desiccation increased levels of hardiness above controls, ABA, and proline but failed to enhance hardiness above trehalose treatment, particularly after 1 week at 0°C.

ii. A -5°C pre-freezing treatment with subsequent 0°C incubation significantly increased hardiness above controls.

A.3 Final thoughts...

At all levels of organization, cold acclimation is, in many ways, the management of water. Results from this thesis suggest the importance of water-solute/dry matter interaction in the development of cellular freezing resistance. However, advancements in understanding the mechanisms of acclimation can be realized only if complemented by a clear understanding of the mechanism of injury. Unfortunately, the mechanism of injury has become increasingly isolated in its definition. It now appears to refer to the specific lesion after freezing stress. The mechanism of injury actually revolves about the central question of whether extracellular freeze-dehydration injury occurs at the limit of tolerance or avoidance strategies. The lesion is only a reflection of the loss of these strategies. Although extracellular freeze dehydration tolerance has become a fundamental paradigm, it is largely based upon whole plant model systems. Inferences of cellular mechanisms of resistance/injury from whole plant data may not reflect actual strategies because of the inherent complexities of the various tissues. In fact, results from a limited number of plant cell systems suggest an extracellular freeze-dehydration avoidance strategy. If true, it lends more support for such theories as "vital water" (see Weiser, 1970).

In the interest of space (and time), other musings are presented in question form.

Acclimation usually occurs in a consistently defined and ordered series of biochemical and physiological changes. I've sometimes wondered why a certain change would always precede another. Could the axiom, "ontogeny recapitulates phylogeny" (the

developmental stages of an organism's life reflects it's evolutionary development) be applicable? i.e. Could the seasonal acclimational alterations observed be a reflection of the evolutionary adaptation of plants to stressful environments?

Is ABA really involved in natural plant acclimation? The answer to this question would be facilitated with the use of specific ABA inhibitors during cold acclimation.

There is still some question as to whether dormancy and cold acclimation are interdependent. As we move latitudinally north and south from the equator, both photoperiod and temperature are simultaneously changing. To study the interaction between dormancy and hardiness in nature, one of these parameters must be held constant. Thus, to determine the influence of photoperiod, can an ecological study be performed where hardiness and strategies of adaptation of high altitude plants at the equator are compared with high altitude plants in progressively temperate regions?

Are the exocytotic to endocytotic extrusions (see Steponkus, 1984) observed as protoplasts become less hardy be simply an indication of changing lipid composition from the outer to inner bilayer rather than a form of injury?

In cell cultures, is ABA acting to increase hardiness at the cell or at the clump level? If ABA changes the cell wall composition, will the propagation of ice through the intercellular spaces be altered? Thus, are we merely observing the effect of the alteration of ice (Olien's hypothesis) in these spaces? The cell culture system might be a nice system to test Olien's hypothesis.

If the cell wall/middle lamella is changing, could ABA be predicted to increase resistance to fungal pathogens?

In future, the plasmamembrane-cell wall interaction may acquire greater significance in freeze acclimation/injury. However, this and other possible lesions should be viewed in the larger context of the mechanism of injury and the role of water and its management.