The Effects of Exogenous Application of Abscisic Acid and α,α'-Dipyridyl on Cold Acclimation and Physical Characteristics of *Pisum sativum* 'Alaska' Seedlings

by

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(ABSTRACT)

Cold acclimation entails changes in membrane composition, osmotic adjustment, alterations in the cell wall-plasma membrane interface, sugar deposition, and changes in cell wall proteins. There is evidence that a rigid cell wall may be necessary for cold acclimation. Difficulties arise in studying plant material acclimated by exposure to low temperatures, because extraneous changes in the plant material occur that are unrelated to the development of cold tolerance. In order to determine whether cell wall changes are necessary for acclimation, peas were acclimated at warm temperature (26°C) by the application of exogenous ABA, desiccation, light exposure, and an experimental cryoprotectant (GLK 8908). Electrolyte leakage, elastic and plastic bend angles, and stem elongation were used to evaluate freezing injury, cell wall rigidity, and growth, respectively. The role of extensin, a structural hydroxyproline-rich glycoprotein suspected of being involved in cell changes during acclimation, was examined using the hydroxylation inhibitor α,α'-dipyridyl.

Exogenous ABA application and drought stress decreased freezing injury by approximately a 10% -6°C compared to controls. In one experiment light was found to be more effective than ABA at acclimating peas at warm temperatures. Foliar application of GLK 8908 decreased freezing injury (30% at -6°C). Stem bendability was not correlated with freezing resistance. ABA treated peas grown in the dark had reduced growth rates and increased stem rigidity, but exhibited greater injury at -6°C than untreated dark grown peas. Extensin content was not related to cold hardiness. Although acclimation of 'Alaska' peas did occur at warm temperatures with various treatments the reductions in freezing injury were minor when compared to plants acclimated by exposure to low temperatures.

GLK 8908 was also evaluated for its effects on 'Alaska' pea survivability and yield. Peas treated with GLK 8908 (1 and 10% aqueous) and subjected to a -6.7°C freeze were found to have increased survival without significant changes in days to first flower, leaf surface area/plant, and yield/plant.

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DEDICATION

I have worked on this thesis almost continuously for the last two years, although I have received valuable help from faculty and friends, the most unwavering constant support (moral and financial) has come from my parents, Milton and Shirley Tignor. In his lifetime my father has been a sergeant in the Marine Corps, cable technician, avid gardener, and computer enthusiast. My mother worked as a nurse until my parents started a family, at which time she gave up her career to raise her children, she is currently employed as a nurse at an elementary school. Together they not only raised three of their own children (myself and my two sisters Robin and Lisa), but unselfishly opened their home as foster parents. Over a period of ten years they nurtured fifteen newborns and toddlers as volunteers for the Children's Home Society of Richmond, VA. My parents, by example, have instilled a sense of love and pride that I will always carry with me. This thesis is dedicated to "*Mom and Dad*".

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Chapter 1: Literature Review

Cold Acclimation

The major limiting factor in plant distribution worldwide, water availability aside, is temperature (Lyons et al., 1979; Parker, 1963; Sakai and Larcher, 1987). Low rather than high temperatures tend to limit plants geographically (Parker, 1963). In non-acclimated crops, or in plant species that do not cold acclimate, chilling or freezing temperatures can cause severe injury or death (Levitt, 1980). Therefore, study leading to the understanding of cold acclimation has the potential to improve crop productivity in marginal climates.

There are two types of plants with respect to cold tolerance, acclimating and non-acclimating. Alberdi and Corcuera (1991) described cold acclimation as "the non-heritable modification of structures and functions as a response to cold which minimizes damage and improves the fitness of a plant". This may occur during acclimation by the induction of either tolerance or avoidance mechanisms (Levitt, 1980). When acclimated, plants may tolerate extracellular freezing, but intracellular freezing is always lethal since it causes irreversible membrane damage (Burke et al., 1976; Levitt, 1980). A plant exposed to freezing temperatures can either avoid intracellular freezing by the exclusion of nucleators (supercooling) (Burke et al.,

1976; George and Burke, 1984) or by tolerating extreme dehydration by the removal of all free water from the cytosol (non-supercooling) (George and Burke, 1984; Levitt, 1980). Along with these changes in freezing behavior during cold acclimation, changes also occur in membrane composition (Steponkus, 1990; Yoshida and Uemura, 1989) and cell wall proteins (Weiser et al., 1990). Although many changes during cold acclimation have been characterized individually, integration into a cohesive theory has yet to be accomplished (Alberdi and Corcuera, 1991; Guy, 1990; Steponkus, 1984).

Cold acclimation occurs naturally in hardy plants by a gradual exposure to low temperatures and in many cases a decreased photoperiod. The necessary time of exposure and specific photoperiod varies with species. There are other means by which plants may be induced to cold acclimate at non-hardening temperatures. These include, drought stress, high light levels, high phosphorus and low nitrogen fertilizer regimes (Salisbury and Ross, 1992), and the exogenous application of abscisic acid at room temperature (Chen et al., 1983; Keith and Mckersie, 1986; Lee et al., 1991, 1992; Orr et al., 1986; Reaney and Gusta, 1987; and Tanino et al., 1990).

Abscisic Acid

Abscisic acid (ABA) is a 15 carbon sesquiterpenoid (Fig. 1.1) synthesized in chloroplasts and other plastids via the mevalonic acid pathway or by the breakdown of

cartenoids (Goodwin and Mercer, 1983). ABA is a plant growth regulator that appears to be universal among vascular plants (Salisbury and Ross, 1992) and was first characterized by Addicott et al. (1964).

In 1967(a,b) Irving and Lanphear suggested that an "inhibitor" or "dormin", now known as ABA, might have been responsible for hardiness in *Acer negundo* exposed to short days. Similar rises in endogenous ABA were seen in the autumn with *Prunus cerasus* (Mielke and Dennis, 1978). Differences in endogenous ABA levels were reported to occur in different winter wheat cultivars; the more hardy wheat cultivars had more endogenous ABA (Wightman, 1979).

In addition to the correlation between cold hardiness and the rise in endogenous ABA, cross reactivity among stresses also indicates a relationship between ABA and freezing tolerance. For example, tobacco has been found to have increased endogenous ABA levels and increased freezing tolerance when exposed to drought, salinity, and nitrogen deficiency stresses (Boussiba et al., 1975). Imposing drought stress on cabbage (Rosa, 1921) and dogwood (Chen and Li, 1977) also increased freezing tolerance. Therefore, it seems logical that ABA applied exogenously to a plant might increase freezing resistance.

Exogenous ABA is only effective on plants which have the inherent ability to cold acclimate (Chen and Gusta, 1983; Mohapatra, 1988). For example, *Solanum commersonii,* which can cold acclimate with exposure to low temperatures, also acclimates with exogenous ABA, while *Solanum tuberosum* (a non-hardy species) does

not acclimate with exogenous ABA application (Chen et al., 1983).

Cold acclimation by the application of exogenous abscisic acid at room temperature has been more successful with cell cultures, than whole plants. Rye (Churchill, personal communication), alfalfa (Mohapatra et al., 1988), and winter wheat (Lalk and Dörffling, 1985) seedlings have been shown to cold acclimate after the application of exogenous ABA. Plants that have been shown to undergo inducible cold hardiness by the application of exogenous ABA to cell cultures include: *Lotus corniculatus* (Keith and Mckersie, 1986), *Brassica napus* (Orr et al., 1986), *Bromus inermis* Leyss (Reaney and Gusta, 1987; Tanino et al., 1990), *Medicago sativa* L. (Reaney and Gusta, 1987), and *Solanum commersonii* (Chen et al., 1983). Cell cultures are believed to be more responsive to cold acclimation by exogenous ABA, because the cells are grown in a sterile media and continuously exposed to ABA (Reaney et al., 1989). Although ABA has been shown to cold acclimate various economically important crops, it is cost prohibitive for use on field crops. However, experimental cryoprotectants have been developed for use on field crops including GLK 8908 (Great Lakes Chemical Corporation and QO Chemicals).

It has been proposed that ABA has two possible modes of action (Li et al., 1989) membrane alterations (Zeiger, 1983) and alterations in gene expression (Mozer, 1980). As first suggested by Weiser (1970), the development of cold hardiness by low temperatures is the result of changes in gene expression. Since the application of ABA has been shown to induce cold hardiness, it is plausible that it also regulates

gene expression (Lee, 1992; Skriver and Mundy, 1990; Robertson et al., 1987).

As mentioned previously, there are many changes that take place during cold acclimation that may be regulated by ABA. While considering the cell wall, Bartolo et al. (1987) found that suspension cultured cells were more freezing tolerant than their respective protoplasts. This indicates that the cell wall may have and important role in freezing tolerance. It has been suggested that cell wall polymers may reduce freezing damage by altering the shapes of ice crystals that form or that the cell wall may restrict the growth of ice (Burke et al., 1976). How the cell wall is important is still under investigation.

Singh and Johnson-Flanagan (1987) found an increased number of cell wall membrane adhesion sites during acclimation of *Brassica napus* cell. Wallner et al. (1986) found deposition of callose in pear cell walls during acclimation which typically occurs at the wall membrane interface. Perhaps the most well founded rationale for the cell wall involvement in freezing tolerance is as a physical barrier to the dehydrative forces acting on the protoplasm of cells when extracellular ice is present. Anderson et al. (1983) and Hansen and Beck (1988) have suggested that the cell wall may be a barrier that stops dehydration and collapse during freezing. More specifically, in non-ideal equilibrium freezing the cell wall allows a negative turgor pressure to develop (Anderson et al., 1983, Hansen and Beck, 1988). This is different from species that show ideal equilibrium freezing when water moves out of the cell along a potential gradient as extracellular ice forms (Hansen and Beck, 1988;

Rajashekar and Burke, 1982). Much of the injury that occurs during freezing has been attributed to dehydration (Levitt, 1980). One change in the cell wall that occurs during cold acclimation, is the increase in deposition to the cell wall of the hydroxyproline-rich glycoprotein extensin. Weiser (1990) showed that extensin mRNA increased with levels of cold acclimation in *Pisum sativum* 'Alaska'. The increase in mRNA would seem to agree with previous work that showed an alteration in gene expression during cold acclimation (Lee, 1992; Mozer, 1980).

Extensin

In plant cells the plasmalemma is covered with a comparatively tough multi-component layer known collectively as the cell wall which includes, polysaccharides, lignins, proteins, water, and incrusting substances (Goodwin and Mercer, 1983). Proteins found in the cell wall can be broken down into two categories, enzymes and structural proteins. Extensins, first characterized by Lamport and Northcote (1960), are the major class of hydroxyproline-rich structural glycoproteins (Wilson and Fry, 1986).

The backbone of extensin (Fig. 1.2) is made almost entirely of hydroxyproline with intermittent serine, lysine, and tyrosine residues and side chains of arabinose and galactose (Lamport et al., 1973; Wilson and Fry, 1986). The precursor of extensin, consisting of proline, is synthesized in the endoplasmic reticulum (ER) (Fig. 1.3).

Proline residues are then hydroxylated in the ER lumen. The resulting hydroxyproline is glycosylated in the golgi apparatus with arabinose and galactose (Wilson and Fry, 1986). After glycosylation extensin is packaged in golgi vesicles and secreted to the cell wall where isodityrosine crosslinks are formed between molecules, building a network of extensin that exists as a mesh among, but separate from the cellulose matrix (Fig. 1.4)

Incorporation of extensin into the cell wall was found to correlate with light exposure and tissue age (Sadava et al., 1973a). Peas grown in the light had twice as much hydroxyproline as peas grown in the dark. Lower internodes (mature tissue) had more extensin in the cell wall (Sadava et al., 1973a), indicating that extensin deposition increases with plant age.

Sadava et al. (1973b) used α,α'-dipyridyl to alter the form of extensin being secreted to the cell walls of peas. Alpha, alpha dipyridyl inhibits hydroxylation of extensin, because it chelates the iron necessary for the hydroxylation reaction (Fig 1.3). Since hydroxylation step of extensin synthesis is blocked, glycosylation can not occur. The resulting protein synthesized is coiled and not structurally rigid like its native counterpart (Fig. 1.5) (Stafstrom and Staehelin, 1986; van Holst and Varner, 1984). Smith (1981a, 1981b) arrived at similar conclusions with carrot sections. He synthesized an artificial extensin backbone with radiolabeled proline which would have the same sequence as extensin treated with α,α'-dipyridyl. Smith created an antiserum to this protein which recognized most of the radiolabeled proline in the cell

wall. Thus, Smith confirmed that using α,α'-dipyridyl resulted in an extensin molecule which lacks the structural integrity of normally synthesized extensin. One drawback to using this inhibitor is that since the chelating action is not specific, it could cause an increase in tissue elongation unrelated to the disruption of extensin synthesis (Lang, 1976).

The function of extensin is not fully understood, but appears to be manifold. Extensin is generally accepted to be a structural protein (Wilson and Fry, 1986). It has a high level of periodicity (Lamport and Catt, 1981) which typifies structural non-enzymatic proteins (Wilson and Fry, 1986). Extensin increases greatly in plant cell walls during the transition from rapidly growing to mature non-elongating tissue (Cleland and Karlnes, 1967; Wilson and Fry, 1986).Extensin has also been shown to play a role in plant defense against pathogens. For example, hydroxyproline-rich glycoprotein levels increase ten-fold in plants inoculated with the fungus *Colletotrichum logenarium* (Esquerré-Tugayé and Lamport, 1979). In addition, inhibiting extensin synthesis also increased the pathogen levels in the plants (Esquerré-Tugayé et al., 1979).

Although extensin has been shown to be involved in cell wall structural enhancement, cessation of growth, and defense, only correlative evidence exists to relate extensin deposition with freezing tolerance (Weiser, 1990).

Research

As mentioned earlier, many changes take place during cold acclimation of plants, but which of these changes are essential for an increase in freezing tolerance is not clear (Alberdi and Corcuera, 1991; Guy, 1990; Steponkus, 1984). This study was performed to further our understanding of how the cell wall, and specifically extensin, may be involved in freezing tolerance. Experiments were completed to characterize the effects of exogenous application of abscisic acid on acclimation and physical characteristics of warm grown 'Alaska' pea seedlings. Second, work was also completed using α,α'-dipyridyl to examine the role extensin plays in cold hardiness.

Abscisic acid was applied exogenously to pea seedlings grown at non-hardening temperatures. This resulted in a small, but significant degree of acclimation as reported in Chapter 2. Since these plants were not exposed to cold temperature for acclimation, the physical changes induced were not merely a side-effect of exposure to cold. Several physical characteristics of peas acclimated with ABA were examined in Chapter 3. Stem strength and stem elongation were used as indicators for increased cell wall rigidity. The role of extensin in the acclimation of 'Alaska' pea seedlings was examined in Chapter 4 using the extensin hydroxylation inhibitor α,α'-dipyridyl. Treatment with this inhibitor resulted in an increase in stem elongation which may have resulted from the loss of the structural function of extensin. In the final chapter, GLK 8908, an experimental cryoprotectant designed

for application to field crops, was evaluated for its effectiveness in increasing the survival and final yield of pea seedlings subjected to an early freezing stress.

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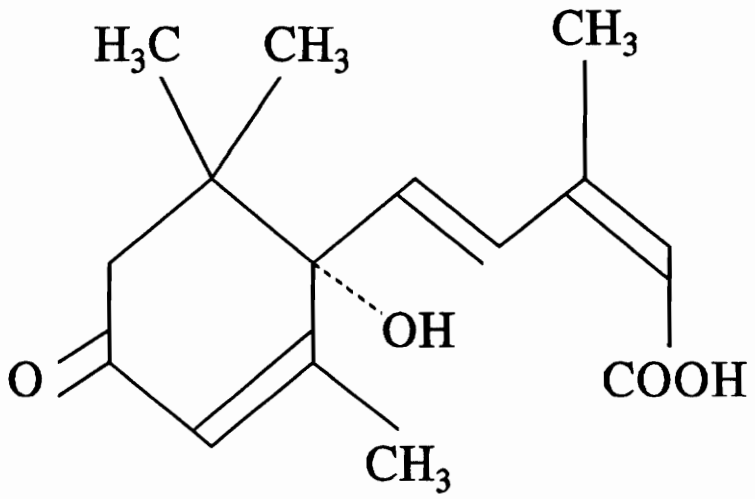

(+) Abscisic Acid

mol. wt.= 264.3 g/mol

Figure 1.1. Structural diagram of abscisic acid.

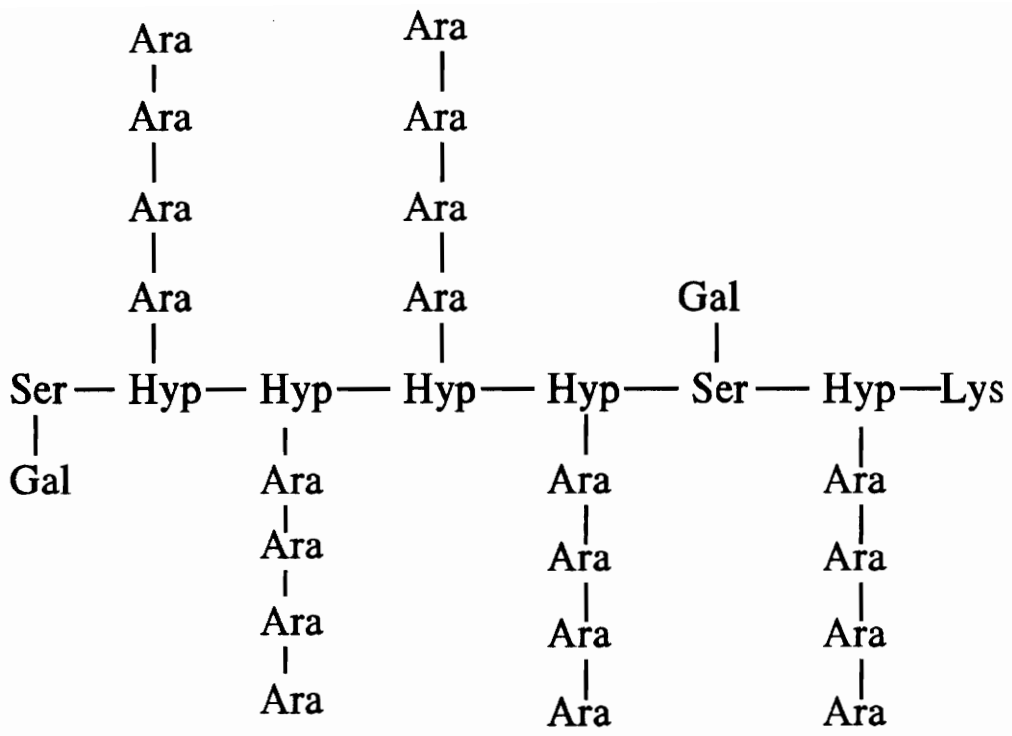

Figure 1.2. Structure of extensin from cultured tomato cell walls (Lamport et al., 1973).

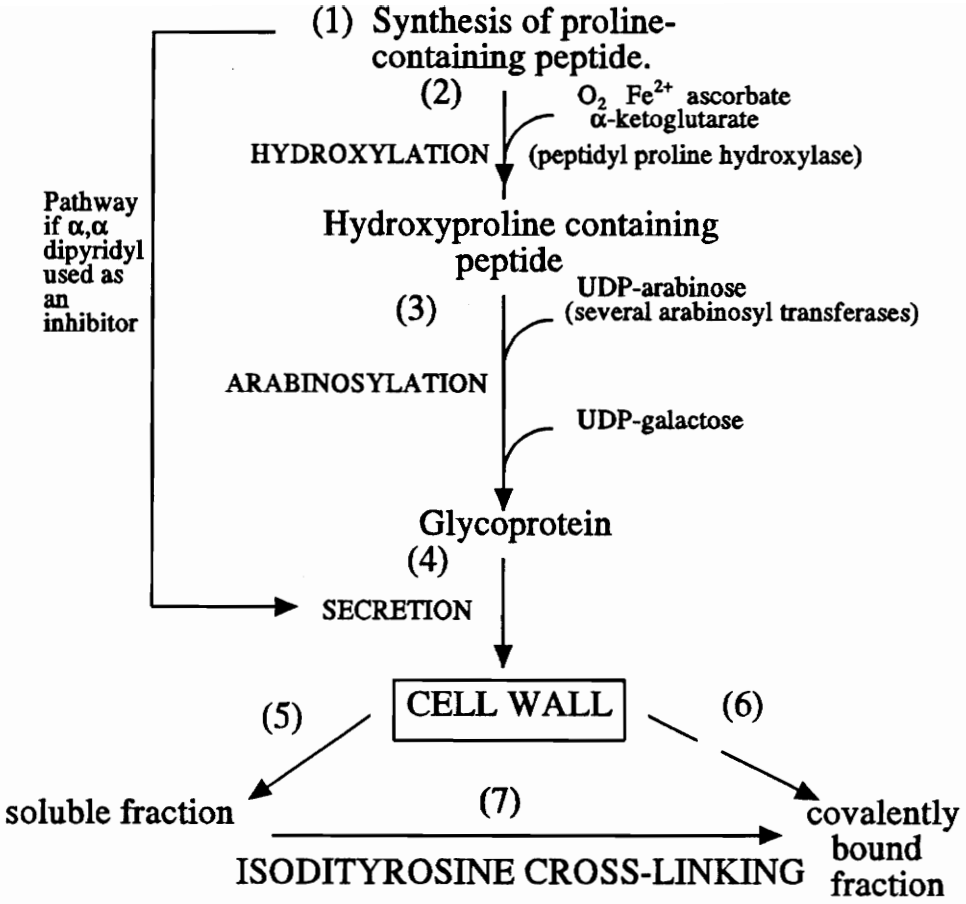

Figure 1.3. Biosynthesis of Extensin as adapted from Wilson and Fry, 1986.

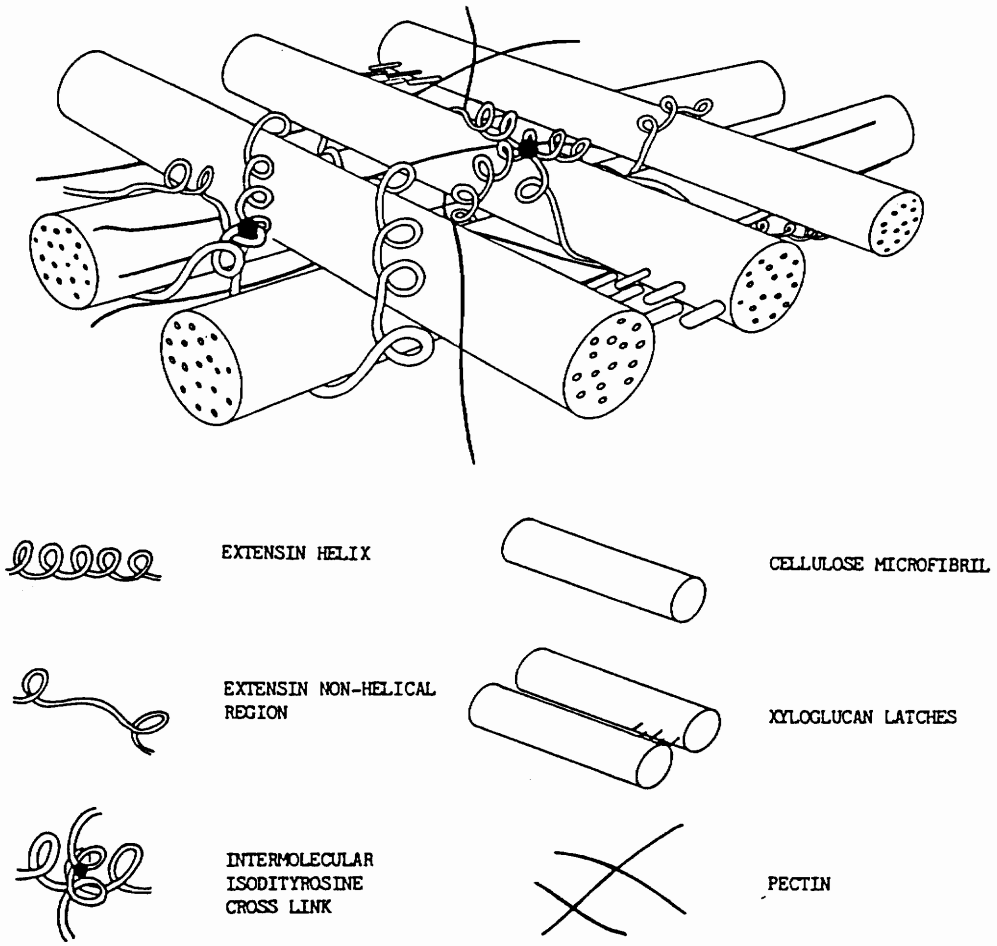

Figure 1.4. Diagram of extensin after incorporation into the cellulose matrix of a plant cell wall (Wilson and Fry, 1986).

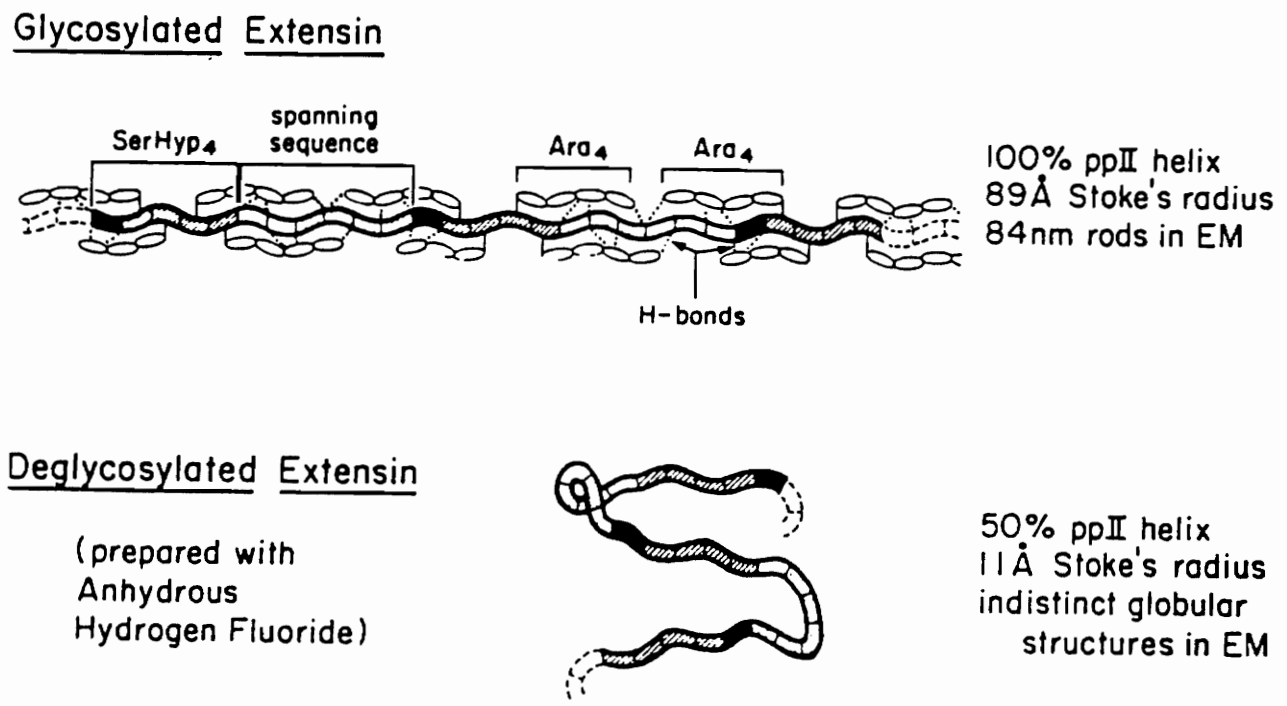

Figure 1.5. Depiction of the levels of polyproline II conformation in extensin as affected by the amount of glycosylation with arabinose (Stafstrom and Staehelin, 1986).

Chapter Two: Changes in Freezing Tolerance of Warm Grown Pea Seedlings Resulting From Treatment With Exogenous Abscisic Acid

Abstract

Many changes occur in plant material exposed to low temperatures, not all of which are related to cold acclimation. Therefore, the study of cold acclimation at warm temperatures is an important research tool. Acclimation of *Pisum sativum* 'Alaska' pea seedlings treated with either exogenous abscisic acid (ABA) or drought stress at non-hardening temperatures (26°C) was characterized by measuring electrolyte leakage from epicotyls frozen to: -3, -6, or -9°C. Exogenous ABA application was found to reduce freezing injury at -6°C at concentrations of 1 X 10^{-5} M to 50 X 10^{-5} M. However, the freezing tolerance of ABA treated peas did not approach the levels observed in peas acclimated with low temperatures. Drought stress acclimated peas to the same level as exogenous ABA (10^{-4}M) at 26°C. Light exposure (1 min /24 hours) was in some cases more effective than exogenous ABA in acclimating peas at warm temperatures.

Introduction

In addition to exposure to low temperatures, conditions such as drought stress, high light levels, high phosphorus, nitrogen deficiency (Salisbury and Ross, 1992), and exogenous application of abscisic acid (ABA) (Chen et al., 1983; Keith and McKersie, 1986; Lee et al., 1991, 1992; Orr et al., 1980; Reaney and Gusta, 1987; and Tanino et al., 1990) increases cold tolerance. Physiological changes in cold acclimation at low temperatures may include changes in membrane composition (Steponkus, 1984), exclusion of nucleators (Burke et al; 1976), osmotic adjustment (Levitt, 1980), and protein changes in the cell wall (Weiser, 1990). Although many physiological changes correlate with cold acclimation, it is unclear which of these changes are important for increased freezing tolerance, and furthermore, how these changes are integrated to result in the tolerant plant (Alberdi and Corcuera, 1991; Guy, 1990; Steponkus, 1984). To eliminate the extraneous effects of low temperature exposure not related to cold hardiness, we applied ABA to warm grown *Pisum sativum* 'Alaska' pea seedlings.

Whole plants of rye (Churchill, personal communication), winter wheat (Lalk and Dörffling, 1985), and alfalfa seedlings (Mohapatra et al., 1988) have all been acclimated by the application of exogenous ABA. However, exogenous ABA application has been a more successful method of cold acclimation at warm temperatures with the cell cultures of many plant species. Bromegrass suspension

cultures treated with ABA have a reduction in lethal temperature for 50% percent of the cells of 23°C (LT_{50}) in 5 days (Lee et al., 1991). Reaney et al. (1989) suggested that cell culture systems may acclimate more readily than whole plants because the cells are grown on a sterile media, (which may keep pathogens from diverting energy from acclimation processes) or because the cells are continuously exposed to ABA.

For whole plants, ABA was applied either by using a root drench (Mohapatra et al., 1988) or a foliar spray (Lalk and Dörffling, 1985), at either 10^{-4} M (Lalk and Dörffling, 1985) or at a range of concentrations from 0 to 10^{-4} M (Mohapatra et al., 1988). Light (Gusta and Fowler, 1979; Steffen and Palta, 1986) and drought (Boussiba et al., 1975; Chen and Li, 1977; Rosa, 1921) have both been shown to be involved in cold acclimation as determined by increased freezing tolerance. We hypothesized that the exogenous application of ABA, light, or desiccation to warm grown 'Alaska' pea seedlings will increase cold acclimation.

ABA, light, drought, and low temperature acclimating treatments were applied to peas several days or weeks prior to exposing them to a freezing stress. Significant differences in freezing tolerance were identified depending on the method by which the abscisic acid was applied. However, the difference was small and not on the order of the acclimation seen with ABA used in cell cultures of bromegrass and potato. Light and desiccation also played a major role in the limited cold acclimation of pea seedlings grown at warm temperatures.

Materials and Methods

Plant Material

Peas (*Pisum sativum* 'Alaska') were planted between two sheets of germination paper along one edge (Anchor Paper Co.), rolled into a cylinder, and held in place by a rubber band. Fourteen of these rolls were placed on end (with the pea seeds in the top 1 cm of the roll) in a one gallon glass jar with a reservoir of 350 ml distilled water. Pea seeds were treated with a 1% aqueous solution (w/v) of fungicide (Thiram). Peas were then placed in a dark germinator (Percival) and held at 26°C for approximately 3 days, or until a 90% germination was reached; after which treatments were initiated. Peas placed in the germinator were exposed to short periods of light for watering. In some experiments, pea seedlings were germinated and grown in a sealed incubator.

Method of ABA Application

Unless otherwise indicated, ABA treatments were applied as a root solution. This was accomplished by removing the distilled water from the 1 gallon jars in which the peas were grown and replacing it with an aqueous solution of 10^{-4} M cis/trans abscisic acid (Sigma) for 4 days. In some experiments, a spray treatment was evaluated and ABA was applied at the same concentration via atomizer to the pea epicotyls.

Freezing Tolerance

Epicotyl sections (1.5 cm) were excised from beneath the "hook" of pea seedlings and placed in test tubes. Six replicates of 4 epicotyl sections were each placed in test tubes for each stress temperature and 100 μl distilled water (conductivity $<$ 5 μmhos) added. Test tubes were placed in racks which were immersed in a methanol bath (2 cm above the bottom of the tubes) and equilibrated at -1°C for 30 minutes. Unfrozen controls were removed and incubated at 1°C, while the remaining samples in the methanol bath were nucleated using a fine mist of ice crystals. After nucleation, the tubes were corked and programmed cooling initiated.

A cooling rate of 5°C was produced using a Neslab MTP-5 programmer in conjunction with the Neslab Exatrol temperature controller. The samples were held for 30 minutes at each stress temperature. The frozen tubes were incubated at 1°C to thaw slowly overnight (18 hours).

After thawing, 2 ml of distilled H_2O were added to each of the sample tubes, (enough water to cover the tissue sections) which were incubated for 2 hours and vortexed. Conductivity of the solution surrounding the tissue samples was measured using an CDM 83 conductivity meter (Radiometer/Copenhagen). Glass marbles were placed on top of the tubes to prevent evaporation. The tubes were then immersed 2 cm in a Precision 185 water bath at 100°C for 25 minutes, to simulate 100% membrane damage. Following boiling, the tubes were cooled to room temperature and conductivity measured again.

Percent injury was calculated using the following equation developed by Martineau et al., (1979).

$$\%Injury = [\ 1\ -\ \frac{(1-T_1/T_2)}{(1-C_1/C_2)}\ ] \times 100$$

This equation includes factors to account for damage before (T_1) and after boiling (T_2). The damage caused during tissue harvest and sample preparation are factored out using electrolyte leakage values from the unfrozen controls (C_1 before boiling, C_2 after boiling).

Light Treatment

Light treatments were applied in an enclosed incubator with a cool white 20 watt fluorescent tube (Phillips F20T12/CW) at the time intervals indicated. Examination of plant materials in this experiment took place under green safe light to insure that no phytochrome response could occur.

Alternative Acclimating Treatments

Peas were acclimated with cold temperatures by exposing them to 3°C for up to three weeks. Some peas were acclimated at 26°C for four days by exposing the epicotyls to dry circulating air.

Results and Discussion

The freezing injury at -6°C was reduced when peas were pretreated with ABA. Epicotyl sections from seven day old seedlings germinated and grown in the dark at 26°C had an increase in freezing tolerance when treated with 10^{-4} M ABA by applied as a root solution, spray treatment via atomizer, or a combination of both (Fig. 2.1). When ABA was applied as either a foliar spray or root solution, injury was reduced by about 4% after four days. When both treatment methods of ABA were used simultaneously, the peas had about a 9% reduction in injury after 4 days compared to plants without ABA. Although the ABA treatments used resulted in statistically significant increases in freezing tolerance of the pea seedlings, these increases are not as great as those achieved after 3 weeks of low temperature cold acclimation at 3°C (Fig. 2.3). Furthermore, ABA applied in the root solution resulted in less reduction in %injury as compared to the spray and root solution together, the root solution could be applied once initially and the germinator could remain unopened until the harvest of the epicotyl sections. The most effective concentration of ABA for acclimation has been found to vary with species and method of ABA delivery to the plant tissue (Chen et al., 1983; Keith and McKersie, 1986; Lalk and Dörffling, 1985; Lee et al., 1991, 1992; Mohapatra et al., 1988; Orr et al., 1980; Reaney and Gusta, 1987; Tanino et al; 1990). Therefore, concentrations were tested from 1 to 50 X 10^{-5} M of abscisic acid (Fig. 2.2). All the concentrations of

ABA tested enhanced freezing tolerance. However, there was no significant difference in freezing tolerance among the different concentrations of ABA. Again, as determined in the previous experiment, the changes %injury were small, on the order of 10%.

To compare freezing resistance resulting from ABA treatment to levels obtained with other acclimatizing treatments, peas were acclimated at low temperatures or by desiccation. Acclimation at low temperature requires more time and therefore the plant material was twice as old as the warm grown treatments. However, all peas were approximately the same height and had the same number of internodes, and thus were at a similar physiological age. The cold treated peas had the lowest injury at -6°C, with 40% injury, while the controls had approximately 80% injury (Fig. 2.3). There was no significant difference in freezing tolerance between the desiccated and ABA treated peas which only had a 10% reduction in injury as compared to control peas grown at warm temperatures. This again is a small gain in freezing tolerance.

Exposure to light is another treatment that has been shown to induce acclimation (Gusta and Fowler, 1979; Steffen and Palta, 1986). Controlled light experiments were performed in closed incubators, and treatments applied under green safe light. While there was no significant acclimation by ABA treated seedlings grown in either light or dark (Fig. 2.4), peas exposed to only 1 min of light per day were significantly more resistant to freezing injury. Exogenous application of ABA

may not have acclimated these peas because the sealed incubator may have had a different humidity, different CO_2 content, or ethylene build up not present in the open growth chamber. However, these parameters were not measured.

To summarize, ABA does appear to be involved in the development of cold hardiness as suggested by the significant differences in freezing tolerance as compared to control peas (Fig. 2.1, 2.2, and 2.3) except when the peas were grown in the sealed incubator (Fig. 2.4). However, these gains in freezing tolerance are small when compared to plant material acclimated with low temperatures. The increased freezing tolerance of ABA treated pea agrees with results obtained in other species. Mohapatra et al. (1988) found that exogenous ABA applied in a root drench at 10^{-4} M to alfalfa had the same survival rate when exposed to -10°C (50%) as alfalfa acclimated for one week at 4°C. Lalk and Dörffling (1985) found that applying 10^{-4} M ABA via foliar spray at 24 hours before a freezing stress further acclimated hardened winter wheat cultivars and increased freezing resistance 15% at -15°C. The lethal temperature for 50% of the cells (LT_{50}) of rye seedlings treated with 10^{-4} M ABA was reduced from -3 to -10°C after 3 days (Churchill, personal communication). The ABA effect we observed was also minor when compared to the increases in cold hardiness of cell suspension cultures of other plants. In bromegrass suspension cultures 50% of the cells (LT_{50}) dropped from -7 to -30°C in 5 days at 23°C (Lee et al., 1991), and in potato cell suspension cultures the LT_{50} fell from -5 to -11.5°C in 2 days (Lee et al., 1992).

To compare the changes in freezing injury that occur with exogenous application of ABA in 'Alaska' pea to other systems, a desiccation treatment was used. Drought stress, imposed by exposing the seedlings to circulating air, acclimated peas to the same level as peas treated with exogenous ABA (Fig 2.3). This is not unexpected since drought stress has previously been reported to cause an increase in endogenous ABA and cold hardiness in tobacco (Boussiba et al., 1975). Drought stress has also been reported to cause an increase in freezing resistance in dogwood (Chen and Li, 1977), and cabbage (Rosa, 1921).

Light exposure has been shown to be necessary for the cold acclimation of many woody and non-woody plants (Gusta and Fowler, 1979; Li et al., 1987; Steffen and Palta, 1986). Similarly, light exposure is also important for the acclimation of 'Alaska' pea seedlings at warm temperatures (Fig. 2.4). However, this light requirement is not universal; cell cultures of bromegrass treated with exogenous ABA (7.5×10^{-5} M in the medium) have been shown to acclimate to the same level with or without light (Reaney et al., 1989).

In conclusion, although application of exogenous ABA and desiccation partially acclimate 'Alaska' peas grown at warm temperatures, the level of acclimation is small compared to peas acclimated by exposure to low temperatures. Thus, the physiological changes in these peas can only represent some of the changes that take place during cold acclimation.

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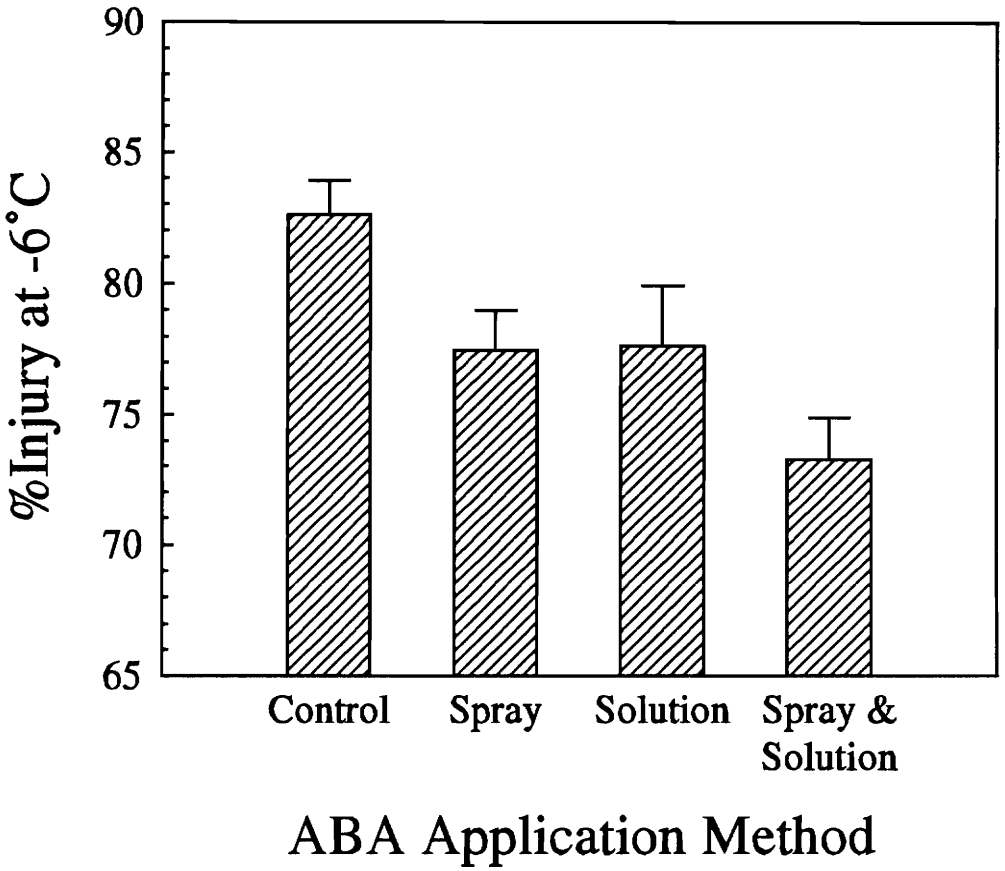

Figure 2.1. Percent injury at -6°C for 7 day old warm grown pea epicotyl sections. ABA was applied at a conc. of 10^{-4} M in all cases. ABA was applied either via atomizer (spray), in a root solution, or a combined treatment using both methods. The control and solution treatments were sprayed with distilled water when ABA was sprayed on to other peas. Bars represent SE, n=6.

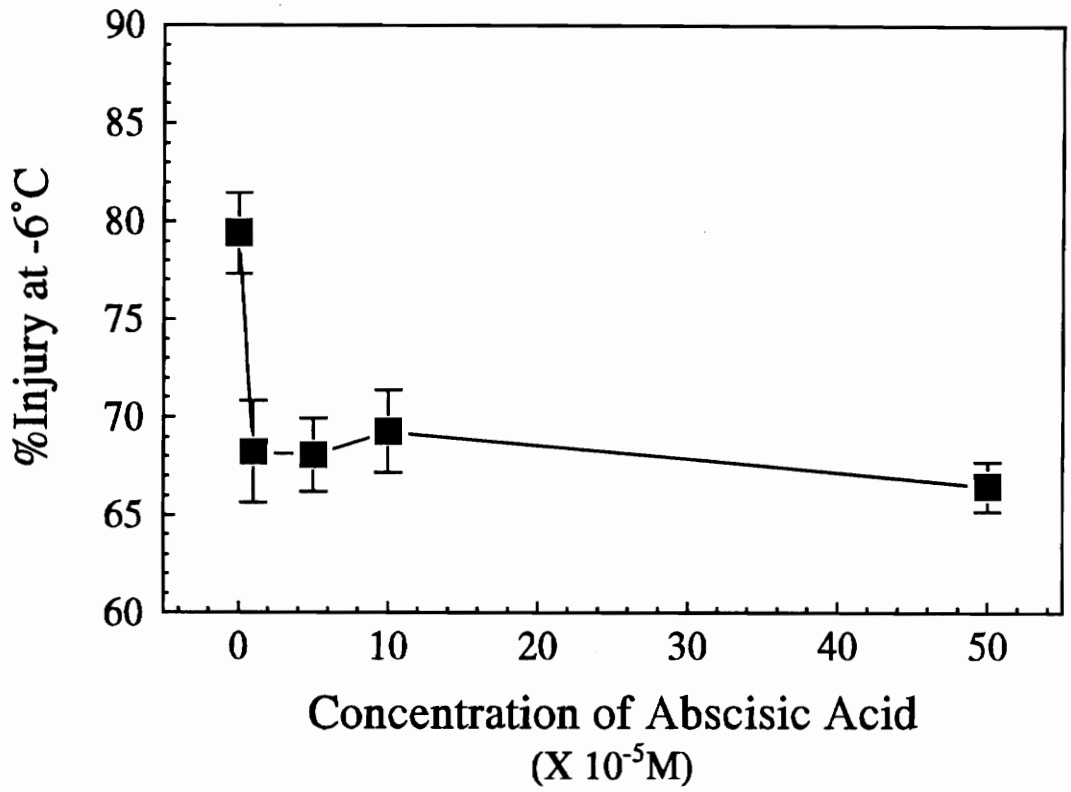

Figure 2.2. Percent injury at -6°C for 7 day old warm grown pea epicotyl sections. ABA was applied at 1, 5, 10, and 50 X 10^{-5} M in a root solution. Bars represent SE, n=6.

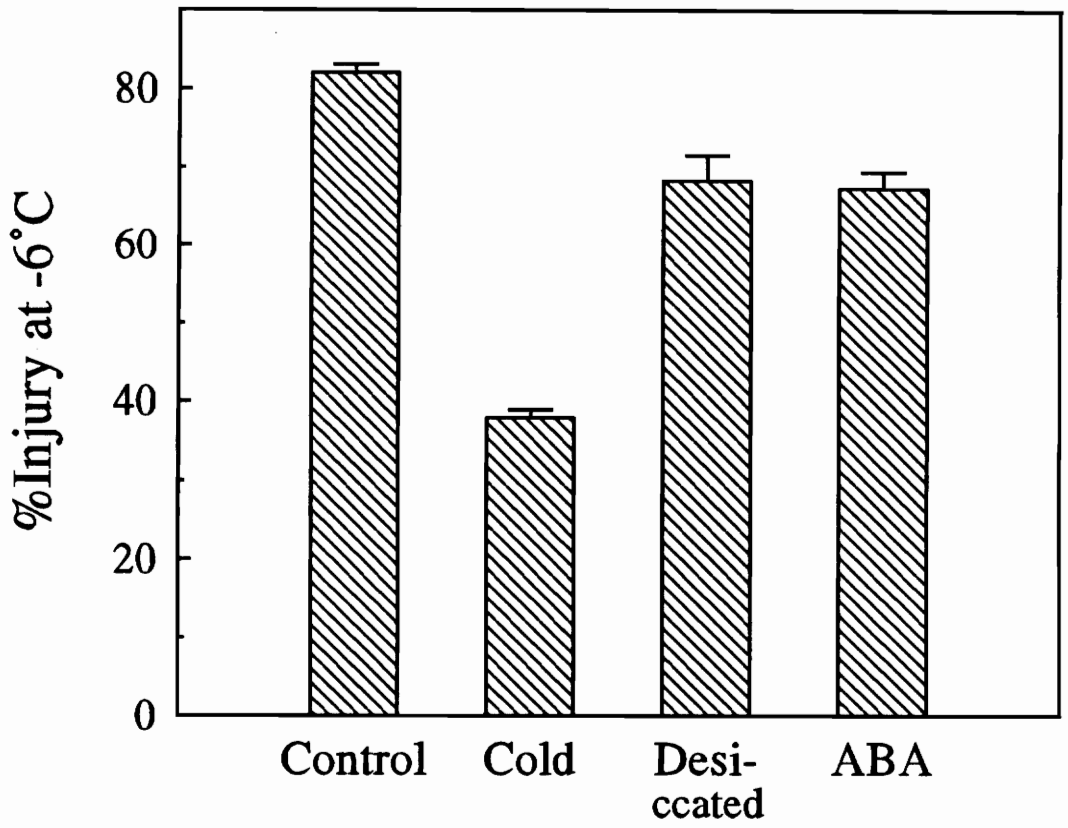

Figure 2.3. A comparison of several cold acclimating treatments. Cold peas were grown at 1°C for 2 weeks. The desiccated peas were desiccated for 1 week. ABA was applied at 10^{-4} M in a root solution for 1 week. Bars represent SE, n=6.

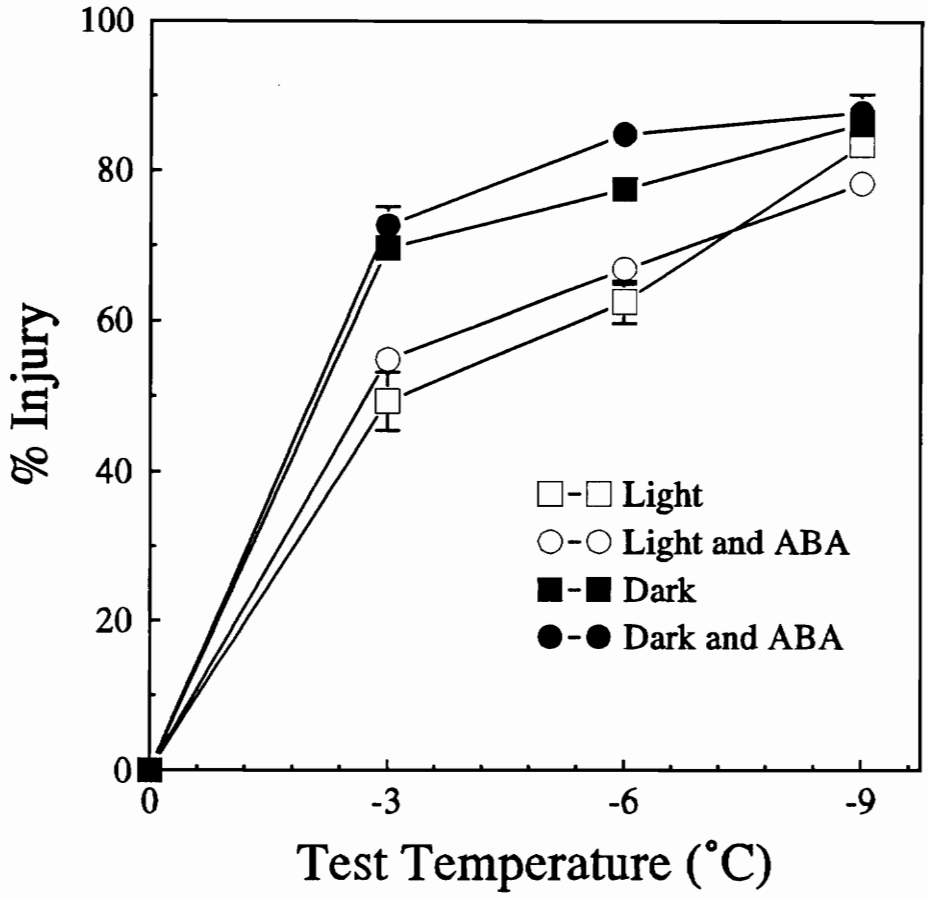

Figure 2.4. Percent injury of light and dark grown peas treated with ABA. Light was applied with florescent lamps for 1 minute / 24 hours in a sealed incubator. ABA was applied in a root solution at 10^{-4} M. Bars represent SE, n=6.

Chapter Three: Physical Characteristics of 'Alaska' Peas Cold Acclimated by Treatment With Exogenous Abscisic Acid, Desiccation, Light Exposure, and Cold Temperature

Abstract

Exogenous application of abscisic acid (ABA) increased the freezing resistance of pea epicotyls (*Pisum sativum* 'Alaska') at warm temperatures (26°C) (chapter 2). We hypothesized that this increase in resistance was due to a rigid cell wall that allowed the cytoplasm to avoid dehydration and collapse during freezing. To test this hypothesis, stem strength (elastic and plastic bendability) and stem elongation were recorded for plants at various degrees of freezing tolerance induced by exogenous application of ABA, light exposure, drought, and cold temperature. Application of exogenous ABA (root solution) was found to reduce stem elongation by 35% and decrease stem bendability by 25% (increased elastic stem strength). In controlled light experiments, ABA was found to cause a 55% decrease in stem elongation and 65% increase in stem strength (elastic) regardless of light exposure. Reduced stem elongation and increased stem rigidity correlated with increased freezing tolerance (chapter 2) with exception of the light exposure experiments. Peas grown in the dark

treated with exogenous ABA had increased stem rigidity and decreased stem elongation, but exhibited significantly greater injury than light exposed pea seedlings (Fig. 2.5). Therefore, increased cell wall rigidity (as determined by stem bendability) is not necessary for increased freezing tolerance.

Introduction

Freezing temperatures can injure plants in several ways. Intracellular freezing is always lethal, while extracellular freezing in many species is often only injurious to non-acclimated plants (Levitt, 1980). There are alterations that take place in the plasmalemma (Steponkus, 1990), protein synthesis (Guy, 1990), osmotic potential (Levitt, 1980), and the cell wall (Weiser et al., 1990) during cold acclimation.

The role of the cell wall in freezing tolerance has not been extensively investigated, but some evidence suggests it may have an important function in preventing freezing injury. Bartolo et al. (1987) found that cultured plant cells were more hardy than their respective protoplasts. Singh and Johnson-Flanagan (1987) found that the number of cell wall membrane adhesions increased with cold acclimation and Wallner, et al., 1986 found that callose deposition to the cell wall increased with cold hardiness. It has been suggested that during "non-ideal equilibrium freezing" the cell wall may act as the barrier to desiccation, therefore preventing cytoplasmic collapse and allowing negative pressure potentials to develop

within the cells (Anderson et al., 1983; Hansen and Beck, 1988).

We measured stem bendability and stem elongation in peas which were acclimated with exogenous ABA, light, drought, and growth at low temperatures. Stem elongation was measured for the pea seedlings since a rigidifying cell wall would not likely be elongating and in previous studies reduced stem elongation was correlated with increased freezing resistance (Irving and Lanphear, 1967).

In general, peas with stiffer stems were more cold hardy. However, in the experiments which tested the effects of light exposure on stem elongation and stem rigidity, ABA applied to dark grown epicotyls resulted in increased stem elongation and stem rigidity without increases in cold tolerance (data chapter 2). Therefore, the data refutes our hypothesis that the exogenous application of ABA might improve cold hardiness by increasing cell wall rigidity and reducing stem elongation.

Materials and Methods

Plant Material

The pea seeds (*Pisum sativum* 'Alaska') were planted between two sheets of germination paper (Anchor Paper Co.) then rolled into a cylinder and held in place by a rubber band. Fourteen of these rolls were then placed in a one gallon glass jar with a reservoir of 350 ml distilled water. Pea seeds were treated with a fungicide (1% (w/v) Thiram). The jars were then placed in a dark germinator at 26°C (Percival

Co., Boone, Iowa) and held for approximately 3 days, or until a 90% germination level was reached. At this stage treatments were initiated. Peas propagated for the light sensitive experiments were grown in a sealed incubator (no air exchange) and were exposed only to green light aside from the timed fluorescent light exposure.

Method of ABA Application

Unless otherwise stated ABA treatments were applied as an aqueous solution of 10^{-4} M cis/trans abscisic acid (Sigma). Root solutions were applied by exchanging distilled water with an ABA solution when the seedlings reached 90% germination. Spray application of 10^{-4} M ABA (via atomizer) was accomplished by misting epicotyls until runoff of the ABA solution occurred.

Stem Elongation

Stem elongation was recorded initially when the seedlings were approximately 3 days old and measured from the "hook" (Fig. 3.1) to the point were the epicotyl met the seed coat. Final measurement of stem elongation was recorded at the final harvest in conjunction with the measurement of freezing tolerance.

Stem Strength Evaluation

Stem strength was evaluated in terms of elastic and plastic bend angles from horizontal (Fig. 3.2). A 5 cm section including the "hook" was removed from the

epicotyl and placed into the sleeve as shown. A 3.32 g weight was placed on the tip of the hook for 30 seconds. The angle from horizontal was recorded as the elastic bending angle, after which the weight was removed and the epicotyl section was allowed to return to horizontal for 30 seconds. This angle was recorded as the plastic bending angle. Method adapted from Yoda and Ashida (1960).

Light Treatment

Light treatments were applied in an enclosed incubator with a cool white 20 watt fluorescent bulb (Phillips F20T12/CW) 1 min per day every 24 hours. Examination of plant materials in this experiment took place under green safe light.

Results and Discussion

When ABA was applied using a root solution, foliar spray, or a combination of both the spray and solution treatments, the peas had significantly less stem elongation than the controls (Fig. 3.3.A). There was no difference in stem elongation whether ABA was applied as a spray or solution. The greatest reduction in stem elongation was observed where spray and solution were used together, these plants had a 50% reduction in stem elongation compared to untreated peas, and also had the largest decrease in stem elastic and plastic bend angles (Fig. 3.3.B). A decrease in stem elastic and plastic bend angle reflects an increase in stem strength. By this

measure all the ABA treatments showed increases in stem strength compared to controls. The solution treatment resulted in more rigid peas than foliar spray based on the elastic and plastic bend angles measured. Increased stem strength and reduced stem elongation correlated well with increases in freezing resistance (Fig. 2.1), but no causative connection can be made. In further experiments, ABA was administered in a root solution treatment because the plant material required less handling since the ABA root solution was applied once and the germinator sealed until final harvest.

A range of ABA concentrations was tested using the root solution method of ABA application (Fig. 3.4). Abscisic acid at 50 X 10^{-5} M produced the greatest reduction in final plant height. Concentrations of 5 and 10 X 10^{-5} M produced approximately a 25% reduction in plant height. Abscisic acid applied at 10^{-5} M had no significant reduction in final plant height compared to controls. Interestingly, this reduction in plant height with the varying concentrations did not correlate with changes in freezing tolerance (Fig. 2.2, 3.4). That is, while all of the ABA concentrations tested reduced freezing injury to a similar level, these same incremental increases in ABA continued to reduce stem elongation. For example, 10^{-5} M ABA increased freezing tolerance significantly, but had no affect on growth rate. This suggests that ABA may signal multiple changes in the plant tissues. Cold hardiness and reduced growth may not be mutually dependent as has been suggested by Levitt (1980) and Weiser (1970).

Other treatments applied to dark grown peas resulted in changes in stem

properties and freezing tolerance. Cold treated peas that were at 3°C for two weeks had 75% less stem elongation than controls over the treatment period, (Fig 3.5) even though that period was twice as long for the cold acclimated plants. ABA treated peas had a 50% reduction in stem elongation, while the desiccated peas showed only a small reduction in stem elongation. Acclimation by cold (3°C for 2 weeks) resulted in the greatest reduction in % injury at -6°C. Abscisic acid and desiccated peas, although acclimated less, were significantly more tolerant than control peas (Fig. 2.3). Increased freezing tolerance of desiccated peas agrees with past research that showed similar cross reactivity of stresses in tobacco, cabbage and dogwood (Boussiba et al., 1975; Rosa, 1921; Chen and Li, 1977).

Light also had an effect on freezing tolerance. Peas germinated in a dark incubator (closed air flow) and given only one daily pulse of fluorescent light had 20% less growth than plants grown entirely in the dark (Fig. 3.6). Kigel and Cosgrove (1991) also found that red and blue light reduced elongation stem elongation in peas. Light-flashed peas treated with ABA had the greatest reduction in stem elongation (66% as compared to dark grown peas); followed by ABA treated dark grown peas (50% reduction). There was no difference in injury between light grown peas and light grown peas treated with ABA, however, the dark grown peas exhibited significantly more injury (Fig 2.4). This suggests that light may be more important in the acclimation of peas at non-hardening temperatures than exogenous ABA. Stem bend angles for light grown pea epicotyls were significantly greater (indicates less

rigid stem) than peas grown in the dark. This is contrary to the findings of Kigel and Cosgrove (1991) who found that exposure to light decreased the plasticity of cell walls (Fig 3.7). Dark grown peas treated with ABA had increased stem rigidity and decreased stem elongation but were no hardier (Fig. 2.4) than their dark grown (no ABA) counterparts which had the greatest stem elongation and half the stem rigidity (Fig. 3.7). This data is contrary to the argument that stiffer stems result in increased cold hardiness.

In conclusion, the data reported here refutes our original hypothesis that the application of exogenous ABA results in increased cell wall rigidity which helps protect the cytoplasm from dehydration injury during extracellular freezing. Peas treated with exogenous ABA showed increases in freezing resistance and stem rigidity in some experiments, but this result was not consistent. In fact, when ABA treated peas were grown in total darkness the opposite trend was observed. Highly reduced stem elongation and increased stem rigidity were associated with low freezing resistance. This suggests that the development of a rigid cell wall may not be involved in non-ideal equilibrium freezing (as described by Anderson et al., 1983; Hansen and Beck, 1988) for peas. The data also suggests a phytochrome mediated response may be involved in the induction of cold acclimation. The data presented by Kigel and Cosgrove (1991) also suggests that there is a plastic component of cell wall extensibility which may not be detected using stem elasticity and plasticity bending measurements.

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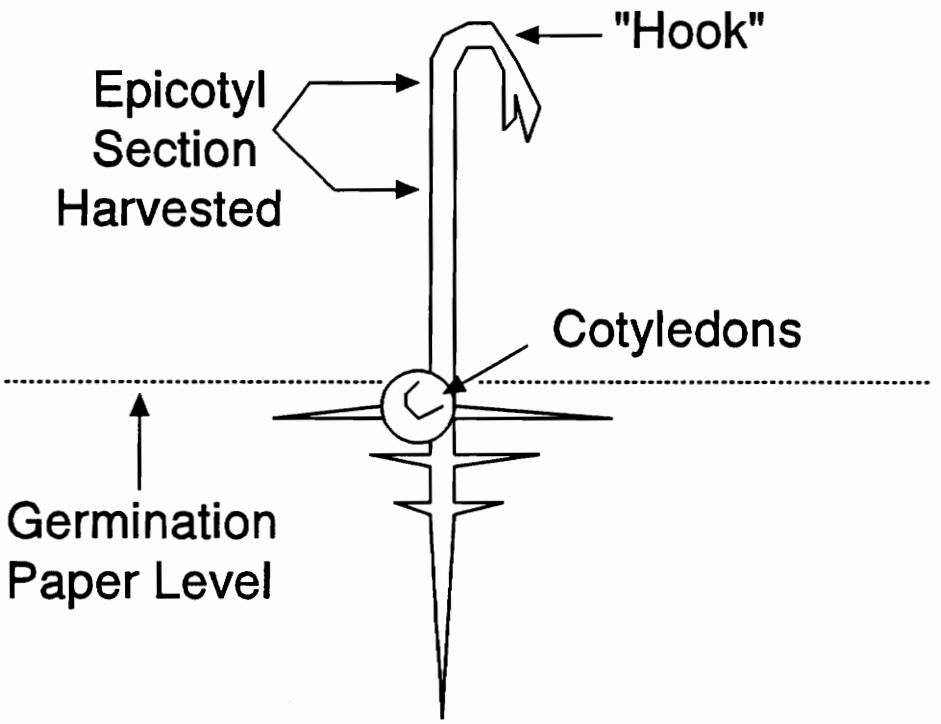

Figure 3.1. Diagram of *Pisum sativum* 'Alaska' pea epicotyl.

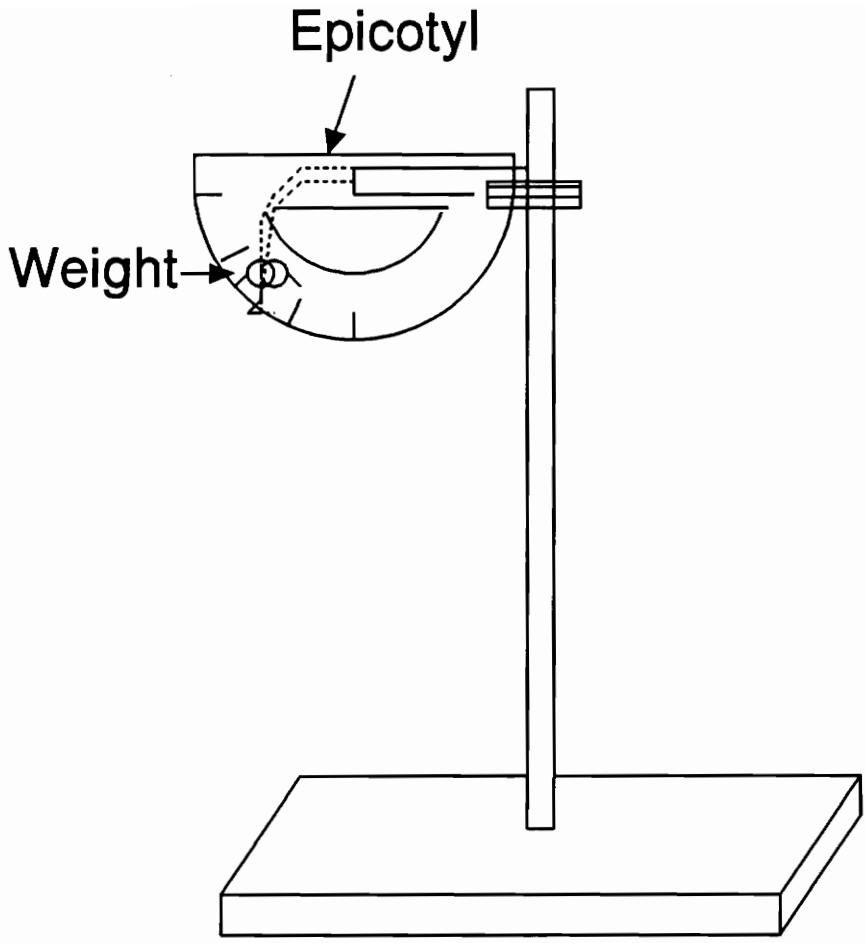

Figure 3.2. Schematic of pea stem bendability testing device. The epicotyl sections were 5 cm in length with 3 cm between the fulcrum and the "hook". The weight applied to the peas for elastic and plastic measurement had a mass of 3.32g.

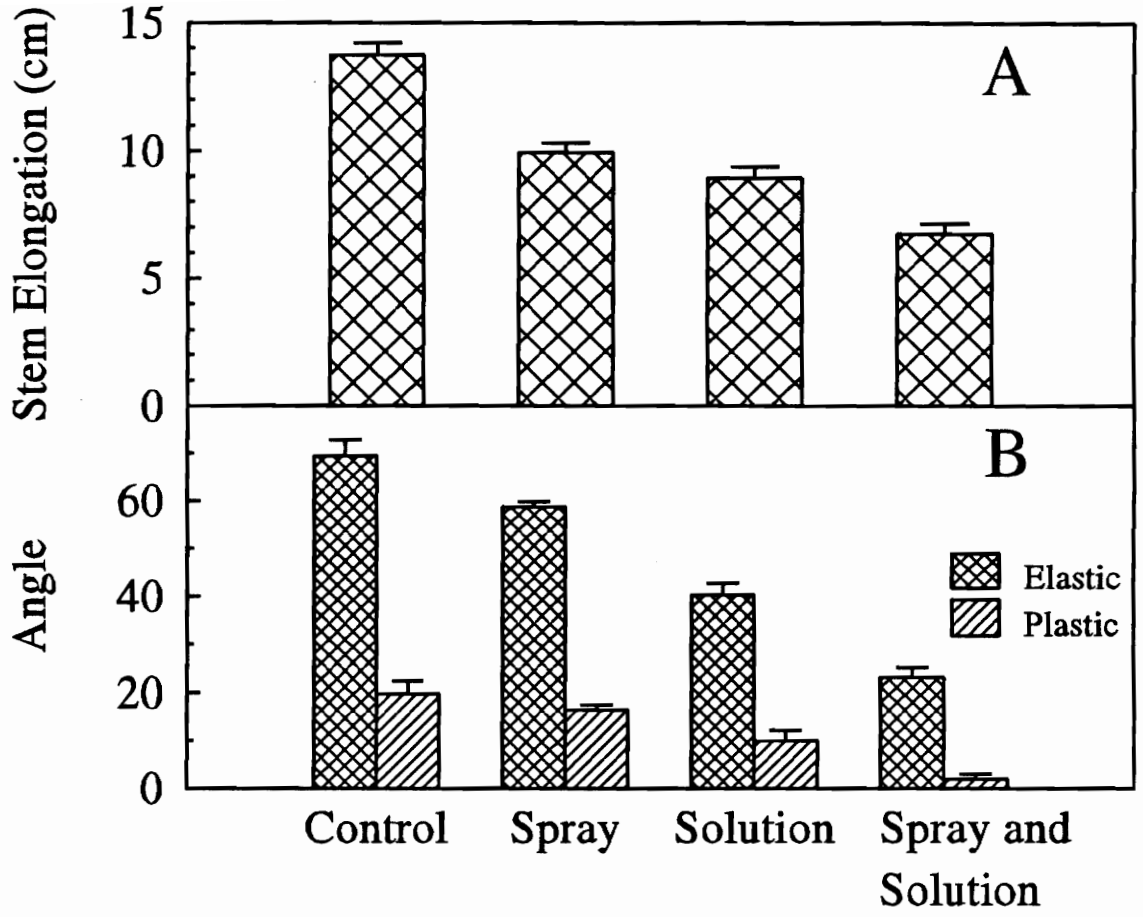

Figure 3.3. Stem elasticity, plasticity, and stem elongation measurements for different methods of ABA application. ABA was applied at 10^{-4} M in a root solution, spray via atomizer, or a combination of both treatments. The peas were dark grown at 25°C. Bars represent SE, n=6.

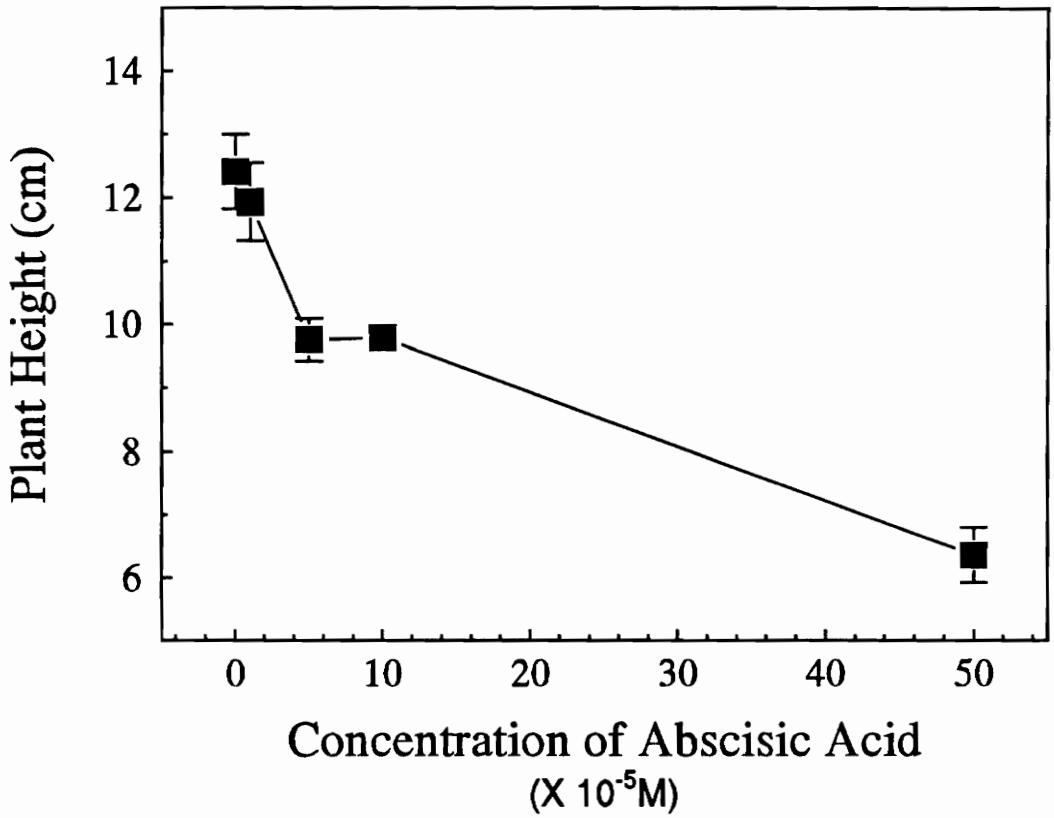

Figure 3.4. Final plant height of dark grown peas (at 26°C) treated with a root solution containing either 1, 5, 10, or 50 X 10^{-5} M ABA. Plant height was measured from the "hook" to the base of the cotyledon. Bars represent SE, n=10.

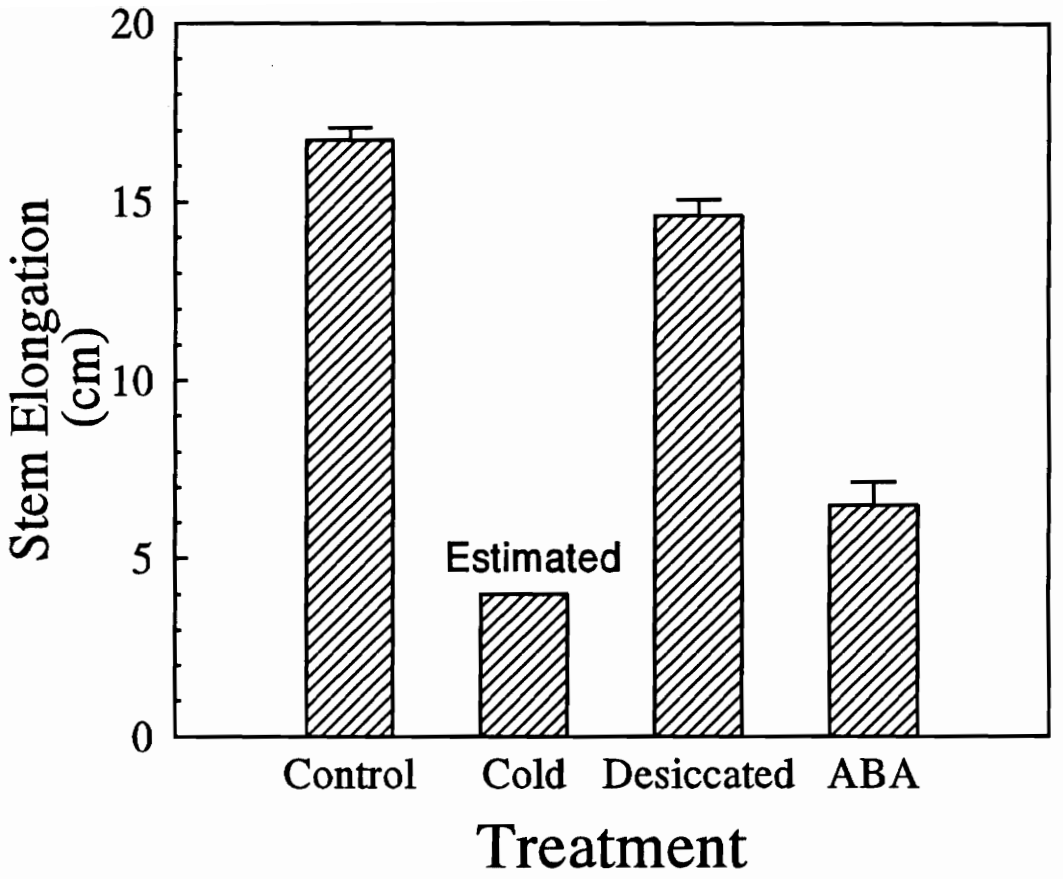

Figure 3.5. Stem elongation of pea seedlings treated under several different acclimating conditions. Cold treated peas were stored in an incubator for two weeks at 3°C. Desiccated peas, though well watered, were exposed to dry circulating air for 1 week. ABA was applied for 1 week at 10^{-4} M in a root solution. Bars represent SE, n=10. Cold height was estimated since the plant material was twice as old and cannot be statistically compared to the others.

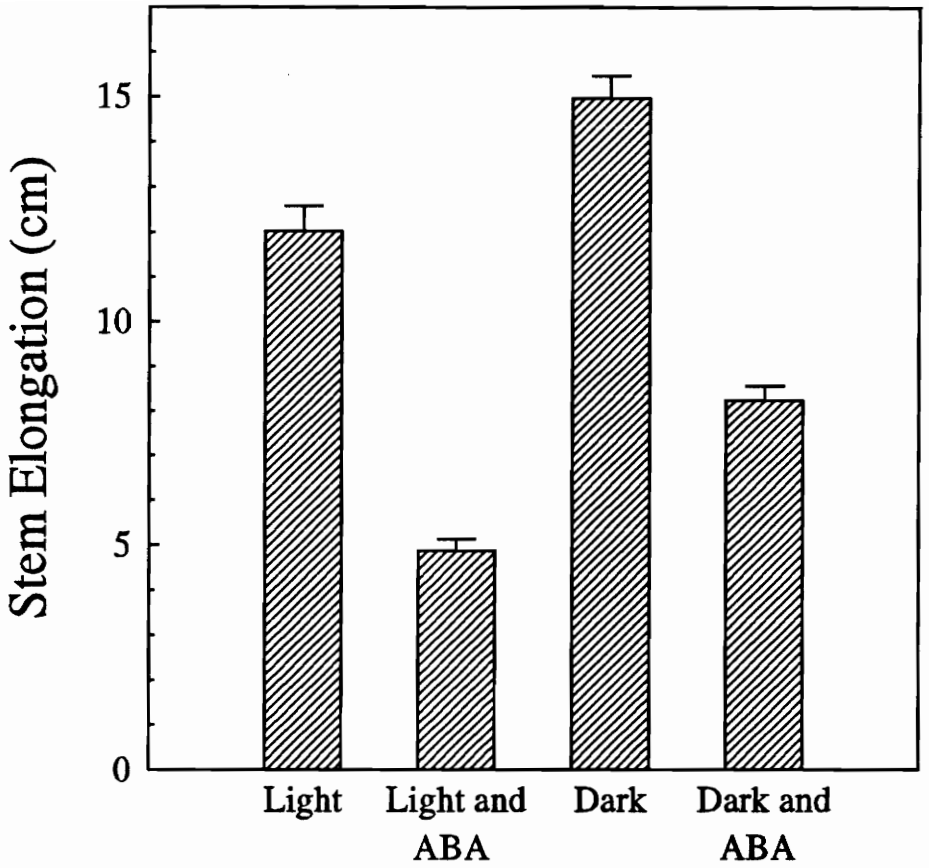

Figure 3.6. Stem elongation of peas treated with 10^{-4} M ABA in a root solution. The peas were placed in dark incubators and half were flashed with 1 min of fluorescent light every 24 hours and the second group remained in total darkness. Stem elongation was measured from the "hook" to the base of the cotyledons. Bars represent SE, n = 10.

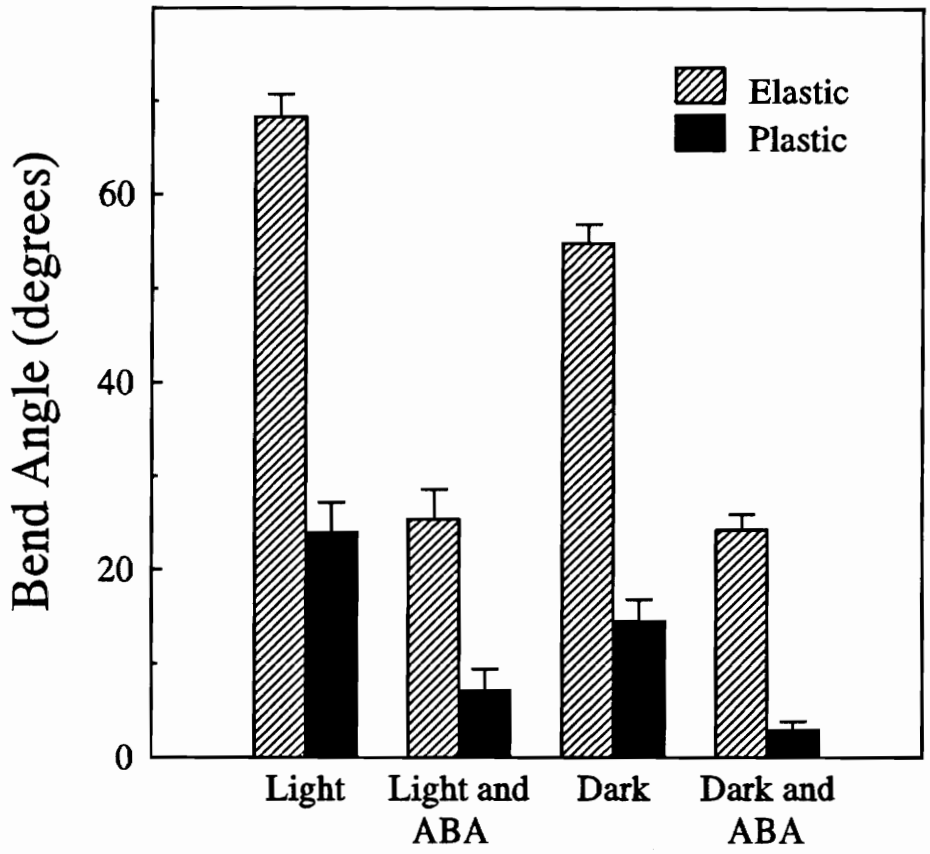

Figure 3.7. Stem elasticity and plasticity of peas treated with 10^{-4} M ABA in a root solution. The peas were placed in dark incubators and half were flashed with 1 min of florescent light every 24 hours and the second group remained in total darkness. The stem sections were 5 cm long with 3 cm between the fulcrum and the "hook". The weight used had a mass of 3.32g. Bars represent SE, n = 5.

Chapter Four: Freezing Tolerance of 'Alaska' Pea Seedlings was Unaffected by the Application of an Extensin Hydroxylation Inhibitor

Abstract

Exogenous abscisic acid has been shown to partially cold acclimate pea seedlings (chapter 2) at warm temperatures. This increase in freezing tolerance was correlated with reductions in stem elongation and increases in stem strength (chapter 3). We hypothesized that the exogenous application of ABA increased cell wall strength and thus decreased dehydration injury caused during extracellular freezing due to increased extensin deposition to the cell wall. Extensin is a hydroxyproline-rich glycoprotein. Weiser et al., (1990) found that extensin mRNA levels increases with increasing cold tolerance in pea epicotyls. Alpha, alpha'-dipyridyl is an extensin hydroxylation inhibitor that was used in the following experiments to ascertain if extensin deposition to the cell wall had a role in cold acclimation in peas grown at warm temperatures (26°C). Alpha, alpha'-dipyridyl was found to significantly increase the stem elongation of both control and ABA treated peas, while increasing stem rigidity of control peas and decreasing stem rigidity of peas treated with exogenous ABA at 10^{-4} M. No significant difference was found in freezing injury among any treatment, regardless of ABA application. Therefore, extensin deposition

was found to have no role in cold hardiness at warm temperatures.

Introduction

In chapters 2 and 3 application of exogenous abscisic acid (ABA) to *Pisum sativum* 'Alaska' pea seedlings was found to increase the plants resistance to freezing injury, reduce stem elongation, and increase stem strength except in experiments with controlled light exposure. Freezing temperatures injure plants in a variety of ways. Intracellular freezing is always lethal, while extracellular freezing in many species is often only injurious to non-acclimated plants (Levitt, 1980). One theory is that during cold acclimation the cell wall is strengthened so that the cell can resist dehydration injury caused by extracellular ice formation. The molecular events that comprise this increase in cell wall strength aren't presently understood. During the cessation of growth the cell wall is thought to be strengthened by the covalent binding of certain proteins among the cellulose microfibrils of the primary cell wall (Wilson and Fry, 1986). This may be related to non-ideal equilibrium freezing, in which the cell wall is suspected in being the physical barrier (by way of decreased elastic modulus) to allow the cell to develop a negative turgor and stop dehydration injury (Anderson et al., 1983, Hansen and Beck, 1988). There is evidence to show that extensin, a hydroxyproline-rich glycoprotein, rigidifies cell walls (Wilson and Fry, 1986) and may be involved in the acquisition of cold hardiness in *Pisum sativum* 'Alaska'

(Weiser et al., 1990).

Levels of extensin deposition have been examined along the pea stem from base to the apex using the internodes as markers for different growth and tissue maturation levels. (Sadava et al., 1973 and Fennoy and Jones, 1991). Researchers found that extensin content is highest in the base of the pea where active growth has ceased and the levels of extensin decrease along the pea epicotyl towards the apex (Sadava et al, 1973). Exposure to temperatures low enough to acclimate peas also results in the slowing of stem elongation and growth. Therefore, it is conceivable that increased freezing resistance could be linked to extensin deposition in the cell wall. We hypothesized that decreased stem elongation and increased stem strength, and increased freezing resistance were related to increased extensin deposition. Extensin deposition to the cell wall, however, is only one change of many that take place during the development of cold hardiness. Other changes include alteration in membrane composition (Steponkus, 1984), exclusion of nucleators (Burke et al., 1976), and osmotic adjustment (Levitt, 1980).

The extensin hydroxylation inhibitor, α,α'-dipyridyl, was applied to 'Alaska' pea seedlings to test our hypothesis. Alpha, alpha'-dipyridyl is an iron chelator which prevents the hydroxylation of extensin and thus prevents the glycosylation of the extensin backbone (Smith, 1981; Wilson and Fry, 1986). As a result, extensin deposition to the cell wall may take place, but the protein exists in a weak coil instead of its normal elongated rigid state (Stafstrom and Staehelin, 1986; van Holst and

Varner, 1984).

Application of α,α'-dipyridyl to both control and ABA treated peas resulted in significant increases in stem elongation from 48 to 72 hours, but even with the markedly different rates of stem elongation there was no difference in freezing injury among the treatments. Alpha, alpha'-dipyridyl was also able to partially counteract the increase in stem rigidity that occurs with exogenous ABA treatment. This data shows that increased stem rigidity and decreased stem elongation via increased extensin deposition does not result in increased freezing tolerance. We conclude that although extensin deposition may occur during cold acclimation, it is not essential for the increased freezing tolerance observed.

Materials and Methods

Plant Material

Pea seeds (*Pisum sativum* 'Alaska') were planted between two sheets of germination paper (Anchor Paper Co.) then rolled into a cylinder and held in place by a rubber band. Fourteen of these rolls were then placed on end (with the peas at the top) in a one gallon glass jar with a reservoir of 350 ml distilled water. Pea seeds were treated with a fungicide (1% (w/v) Thiram). The jars were then placed in a dark germinator at 26°C (Percival Co., Boone, Iowa) for approximately 3 days, or

until a 90% germination. At this stage treatments were initiated. Peas propagated for the light sensitive experiments were grown in a sealed incubator (no air exchange) and were exposed only to green light aside from the timed fluorescent light exposure some of the plants received.

Method of ABA and α,α'-Dipyridyl Application

Unless otherwise stated, ABA was administered in a root solution treatment at concentration of 10^{-4} M. This was accomplished by removing the distilled water and replacing it with the ABA solution when the seedlings reached 90% germination. Alpha, alpha'-dipyridyl (10^{-3} M, Sigma Chemical) was applied once to the epicotyls, via atomizer, four days before harvest.

Stem Elongation

Stem elongation was recorded initially when the seedlings were approximately 3 days old and measured from the "hook" (Fig. 3.1) to the point were epicotyl met the seed coat. Final measurement of stem elongation was always recorded the same day as freezing tolerance was assessed.

Stem Strength Evaluation

Stem strength was evaluated in the terms of elastic and plastic bend angles

from horizontal (fig. 3.2). A 5 cm section from the "hook" was removed from the epicotyl and placed into the sleeve as shown. A 3.32 g weight was placed on the tip of the hook for 30 seconds. The angle from horizontal was recorded as the elastic bending angle, after which the weight was removed and the epicotyl section was allowed to return to horizontal for 30 seconds. This angle was recorded as the plastic bending angle. Method adapted from Yoda and Ashida (1960).

Freezing Tolerance

Freezing tolerance was assessed by electrolyte leakage determination as described in detail in chapter 2. Pea epicotyl sections harvested below the "hook" were placed in distilled water, nucleated with ice, subjected to a specific freezing test temperature, and thawed slowly at a constant temperature overnight. Electrolyte leakage from the stressed peas was then measured before and after boiling (boiling simulated 100% injury). Electrolyte leakage values from unfrozen controls were then compared to the values from frozen epicotyls. Injury percent was calculated from the standard equation listed in chapter 2 (Martineau et al., 1979).

Results and Discussion

Pea epicotyls treated with the extensin inhibitor α,α'-dipyridyl showed significantly greater stem elongation than controls did at 48 and 72 hours after

treatment, while ABA treated peas had a reduction in stem elongation (Fig. 4.1). Application of the extensin inhibitor also significantly increased stem elongation in ABA treated pea seedlings. This increase in stem elongation suggests that extensin has been deposited to the cell wall, but in a coiled form (Stafstrom and Staehelin, 1986) that is not involved in the cessation of growth. However, by its nature of α,α'-dipyridyl as an iron chelator, could also illicit an increase in stem elongation unrelated to its affect on extensin deposition (Lang, 1976).

Abscisic acid treatment reduced stem flexibility (increased "stiffness") as measured by both elastic and plastic bend angles (Fig. 4.2). This increase in stem stiffness caused by exogenous ABA was partially blocked by the α,α'-dipyridyl.

Despite the growth reduction and increases in stem stiffness caused by ABA, the freezing tolerance was not changed (Fig. 4.3). Furthermore, application of the extensin inhibitor sped stem elongation in the cold (data not shown), but had no effect on cold acclimation as characterized by % injury. Thus, our hypothesis that the application of exogenous ABA increases cold acclimation by increased extensin deposition was not supported by this evidence.

Although researchers agree that hydroxyproline-rich glycoproteins increase in maturing tissue regions where growth cessation occurs (Cleland and Karlnes, 1967; Sadava et al., 1973), there is debate about the timing of extensin deposition (Klis, 1976; Van Holst et al., 1980). If extensin deposition occurs after the cessation of growth it would not be responsible for decreases seen in stem elongation.

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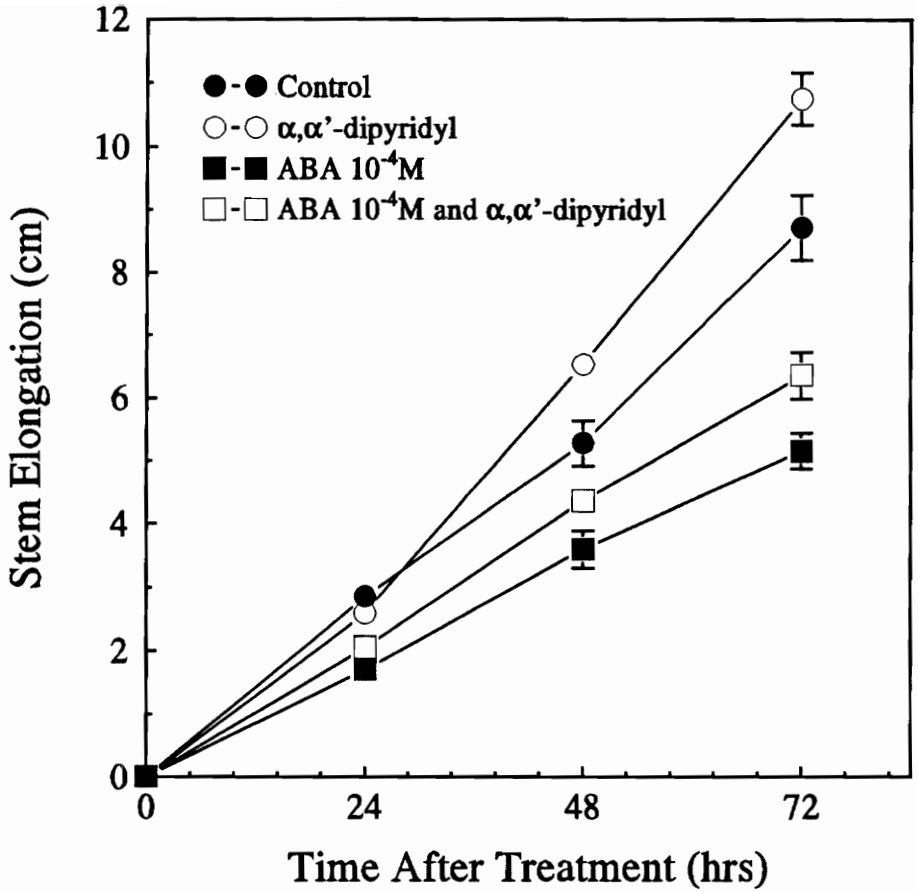

Figure 4.1. Stem elongation over time of control and ABA peas treated with the extensin hydroxylation inhibitor α,α'-dipyridyl. ABA was applied at 10^{-4} M in a root solution and the inhibitor was applied at time zero with an atomizer at a concentration of 10^{-3} M. Bars represent SE, n = 15.

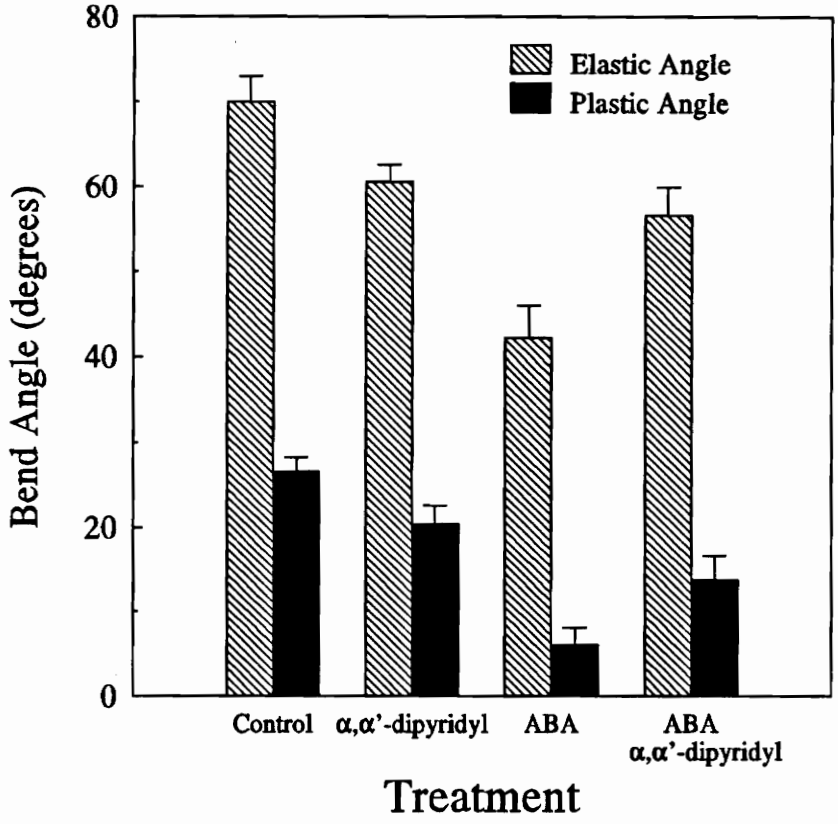

Figure 4.2. Stem elasticity and plasticity of control and ABA and α,α'-dipyridyl treated peas. ABA was applied at 10^{-4} M in a root solution and α,α'-dipyridyl was applied at time zero with an atomizer at a concentration of 10^{-3} M. The stem sections were 5 cm long and the weight applied had a mass of 3.32g. Bars represent SE, n = 5.

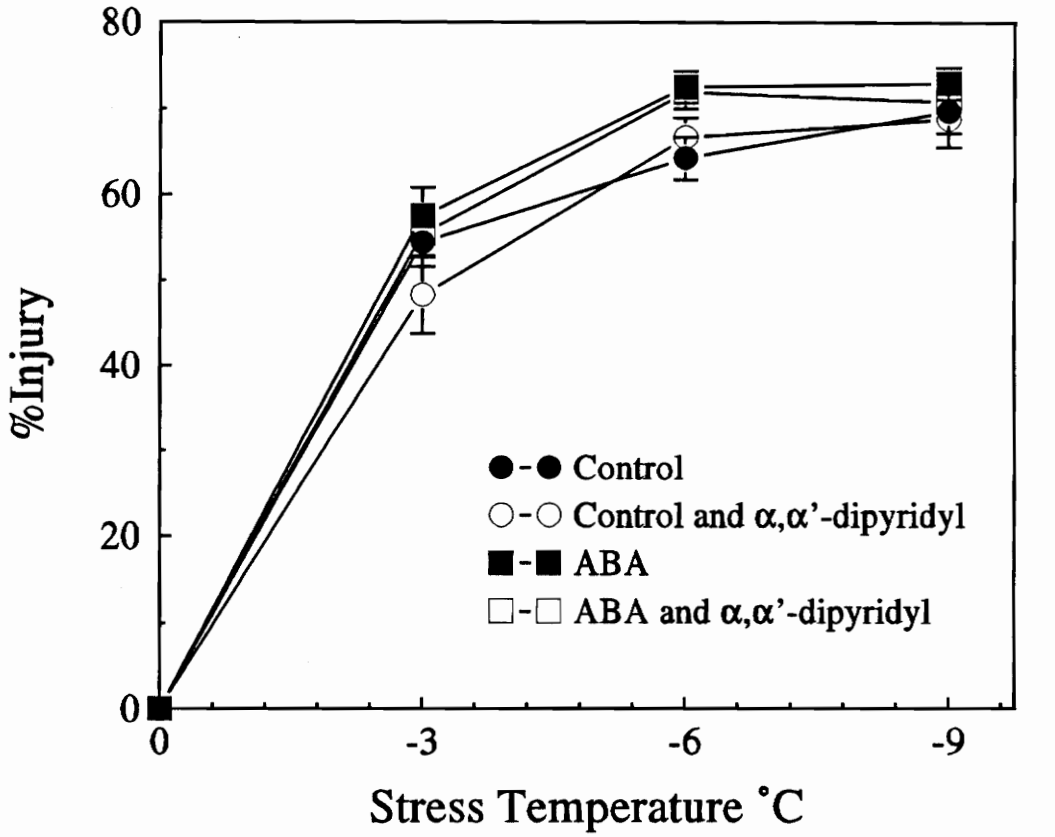

Figure 4.3. Percent injury of 'Alaska' pea seedlings treated with ABA and or α,α'-dipyridyl. ABA was applied at 10^{-4} M in a root solution and α,α'-dipyridyl was applied at time zero, via atomizer, at 10^{-3} M. Bars represent SE, n=6.

Chapter Five: Acclimation of Warm Grown 'Alaska' Pea Seedlings Using The Experimental Cryoprotectant GLK 8908

Abstract

Application of exogenous ABA to *Pisum sativum* 'Alaska' seedlings grown at warm temperatures has been shown to increase freezing resistance, decrease stem elongation, and increase stem rigidity (Chapter 2). However, the cost of ABA would be prohibitive for use in a field application. An experimental cryoprotectant, GLK 8908, was examined for its ability to acclimate peas at warm grown temperatures. Non-hardening temperatures were used to alleviate the extraneous effects caused in plant tissue with exposure to low temperatures unrelated to cold hardiness. Warm grown pea seedlings treated with GLK 8908 were more freezing resistant than controls, but as with ABA (Chapter 2) the acclimation was minimal when compared to peas acclimated by low temperature exposure. GLK 8908 also decreased stem elongation and slightly increased stem rigidity as compared to controls. GLK 8908 had a significantly greater reduction in freezing injury than desiccated or ABA treated peas. In a companion study to examine the effects of GLK 8908 on yield, peas treated and grown in the greenhouse were found to have increased survival without

significant changes in days to flowering, leaf surface area, and yield/plant as compared to control seedlings.

Introduction

Many physiological changes occur with the induction of cold hardiness by exposure to low temperatures. These include changes in membrane composition (Steponkus, 1984), exclusion of nucleators (Burke et al., 1976), osmotic adjustment (Levitt, 1980), and protein changes in the cell wall (Weiser, 1990). Guy (1990) and Steponkus (1984) stated that although there was a large amount of accumulated data on changes during cold acclimation, there has been little integration of this information into an overall theory of cold acclimation.

Further integration of this knowledge could be accomplished in an experimental system in which cold acclimation of plants could be induced at warm temperatures. This would be useful because it would eliminate the extraneous changes in plant tissue, caused by exposure to low temperatures, that are unrelated to cold acclimation. The application of exogenous ABA at warm temperatures has been successful in acclimating cell cultures (Chen et al., 1983; Keith and McKersie, 1986; Lee et al., 1991, 1992; Orr et al., 1980; Reaney and Gusta, 1987; Tanino et al; 1990) and whole plants (chapter 2; Churchill, personal communication; Lalk and Dörffling, 1985; Mohapatra et al., 1988). Other chemical cryoprotectants have also

been shown to provide plants with protection from freezing injury. These include: DMSO (Ketchie and Murren, 1976), n-D-decenylsuccinic acid (Kuiper, 1964), and mefluidide (Li et al., 1989). The experimental cryoprotectant GLK 8908 (Great Lakes Chemical Corporation and QO Chemicals) was evaluated for its ability to cold acclimate *Pisum sativum* 'Alaska' pea seedlings at warm grown temperatures. GLK 8908 had previously been shown to improve the yields of beans, tomatoes, and green peppers under chilling conditions for 3 to 4 days (Cryoban Data Sheet).

The mode of action of these cryoprotectants is not well understood. The cell wall has been hypothesized to play an important role in cold hardiness. Bartolo et al., (1987) found that suspension cultured cells were more hardy than their respective protoplasts. Other researchers have found increases in cell wall membrane adhesion sites (Singh and Johnson-Flanagan, 1987) and the deposition of callose (Wallner et al., 1986). Anderson et al. (1983) and Hansen and Beck (1988) suggested that the cell wall could be a barrier that stops dehydration and cytoplasmic collapse during the formation of extracellular ice. This may result in non-ideal equilibrium freezing and the development of negative turgor in the cytoplasm (Anderson et al., 1983; Hansen and Beck, 1988). We hypothesized that GLK 8908 may improve freezing resistance in warm grown pea seedlings by increasing cell wall rigidity which can protect the cell from dehydration damage during extracellular injury. Stem elongation and elastic and plastic bending angle were used as indicators for stem rigidity and freezing injury was evaluated using electrolyte leakage.

In 1987 the total crop value for processed peas was 98 million (USDA, 1988) and between the years of 1962 and 1985 21.6% of peas were lost to cold damage (Rieger, 1989). For these reasons GLK 8908 was evaluated for its effects on final yield of peas.

Exogenous applications of GLK 8908 were found to decrease stem elongation and slightly reduce stem rigidity, while reducing freezing injury as compared to untreated peas. In a greenhouse study GLK 8908 was found to increase survival after a -6.7°C freeze without significantly altering days to flower, leaf surface area, and yield per plant as compared to control seedlings.

Materials and Methods

Plant Material

Pea seeds (*Pisum sativum* 'Alaska') were planted between two sheets of germination paper (Anchor Paper Co.) then rolled into a cylinder and held in place by a rubber band. Fourteen of these rolls were then placed on end in a one gallon glass jar with a reservoir of 350 ml distilled water. Pea seeds were treated with a fungicide (1% (w/v) Thiram). The jars were then placed in a dark germinator at 26°C (Percival Co., Boone, Iowa) for approximately 3 days, or until a 90% germination. At this stage treatments were initiated. Peas for use in the greenhouse study were planted in plug trays of soilless media (Sunshine Mix LC3) and exposed to light 9

hours (21,520 lumens/m^2) per day for three days prior to the freezing stress to simulate field conditions. Each treatment in the greenhouse consisted of replications of one seedling each.

GLK 8908

GLK 8908, an anti-chilling and anti-frost agent was provided by Great Lakes Chemical Corporation and QO Chemicals. In all laboratory experiments GLK 8908 was applied via foliar spray at the recommended 1% solution (v/v) to pea seedlings. GLK 8908 was applied to the pea epicotyls one time only, every 24 hours, or every 36 hours with an atomizer depending on the experiment. The chemical nature of GLK 8908 has not been released.

Other Acclimating Treatments

Some peas were acclimated by growth at 3°C and some were acclimated by exposure of the epicotyls to dry circulating air in a dark incubator for one week. Peas were also acclimated using exogenous abscisic acid applied at 10^{-4} M applied using a root solution.

Stem Elongation

Stem elongation was recorded initially (prior to treatment) when the seedlings were approximately 3 days old and measured from the "hook" (Fig. 3.1) to the point

were epicotyl met the seed coat. Final measurement of stem elongation was always recorded the same day as freezing tolerance was assessed.

Whole Plant Freeze Stress and Survival

Peas were placed in a germinator and cooled from 26°C to -6.7°C at 8°C per hour. Peas were returned to 26° at the same rate. Thermocouples were used to record temperatures during the cooling process. Twenty-four hours after the freezing stress peas were evaluated for survival. Peas with a flaccid water soaked appearance were considered non-viable.

Freezing Tolerance

Freezing tolerance was assessed by electrolyte leakage determination as described in detail in chapter 2. Pea epicotyl sections were harvested below the "hook" and were placed in distilled water, nucleated with ice, subjected to a specific freezing test temperature, and thawed at a constant temperature overnight. Electrolyte leakage was then measured for previously frozen epicotyl sections before and after boiling (boiling simulated 100% injury). Electrolyte leakage values for unfrozen controls were then compared to the values from frozen epicotyls and % injury calculated with the equation developed by Martineau et al. (see Chapter 2).

Stem Strength Evaluation

Stem strength was evaluated in the terms of elastic and plastic bend angles from horizontal (Fig. 4.2). A 5 cm section from the "hook" was removed from the epicotyl and placed into the sleeve as shown. A 3.32 g weight was then placed on the tip of the hook for 30 seconds. The angle from horizontal was then recorded as the elastic bending angle, after which the weight was removed and the epicotyl section was allowed to return to horizontal for 30 seconds. This angle was recorded as the plastic bending angle (method adapted from Yoda and Ashida (1960)).

Determining % Moisture Content

At harvest fresh weight (FW) was recorded for shoots and pods. The plant material was then placed in a drying oven for 1 week at 96°C at which time dry weights (DW) were recorded. Moisture content was calculated with the following equation: moisture content = (((FW-DW)/FW)*100). Final yields are presented on a fresh weight basis.

Measuring Leaf Surface Area

Immediately following harvest leaf surface area was determined by using LI-3050A surface area meter. All the leaves from each plant were measured.

Results and Discussion

Multiple applications of GLK 8908 reduced plant height significantly as compared with control and desiccated peas. Applying GLK 8908 every 24 hours for three days reduced plant height by one third (Fig. 5.1.A) and freezing injury was reduced by 10% (Fig. 5.1.B). Desiccated peas also showed a small decrease in injury at -6°C, but there was no difference between a single GLK 8908 treatment and the control peas.

The ability of GLK 8908 to induce acclimation was also compared to acclimation by exogenous ABA and exposure to cold temperatures. Pea seedlings treated with a root solution of 10^{-4} M ABA had the greatest reduction in stem elongation (33%). Stem elongation was reduced by a small amount with GLK 8908 treatment (Fig. 5.2.A). Acclimation by long term exposure to low temperatures resulted in the greatest level of reduction in stem elongation, but these peas could not be statistically compared because they were twice as old as seedlings in other treatments. In order to further test our hypothesis that decreased stem elongation and increased cell wall rigidity are important for increased freezing tolerance, stem rigidity was measured (Fig. 5.2.B). Acclimation by exposure to low temperatures resulted in the most rigid epicotyls (as measured by elastic and plastic bend angles). ABA treated peas had a significantly more rigid stem sections that GLK 8908 and untreated peas (Fig. 5.2.C) however, GLK 8908 treated peas had less injury at -6°C

than ABA or control pea seedlings. The greatest reduction in freezing injury was observed in peas acclimated with cold temperatures. In summary, treatment with GLK 8908 resulted in greater increases in freezing resistance than ABA treated peas without the reductions in stem elongation and increases in stem rigidity that occurred with ABA treatment. Thus, the data presented here does not support the hypothesis that GLK 8908 increases freezing resistance by increasing cell wall rigidity.

When compared to other cold acclimating treatments, GLK 8908 at a single, double, or triple application reduced freezing injury to pea seedlings significantly more than either the desiccated or ABA treated peas (Fig. 5.3). In fact, GLK 8908 applied three times caused a 30% reduction in %injury at -6°C. Only peas acclimated with low temperatures were more freezing tolerant.

Pretreatment with exogenous GLK 8908 (single foliar application) was evaluated for its effect on final yield of peas exposed to a -6.7°C freeze. Days to first flower, moisture content at harvest, and leaf surface area were also evaluated. The pretreatment with GLK 8908 at 1 and 10% solutions had an approximately 50% increase in survival, after a -6.7°C freeze, while GLK 8908 applied at 50 and 100% solutions only increased survival slightly (Fig. 5.4).

Although GLK 8908 applied at 1 and 10% solutions resulted in increased survival no significant differences in days to first flower (Fig. 5.5), moisture content of pods (Fig. 5.6.A), and yield/plant (Fig. 5.8) resulted. GLK concentrations of 50 and 100% resulted in only a small increase in survival and significant delays in

flowering (Fig. 5.5) and decreased yield/plant (Fig. 5.8) as compared to controls. Concentrations of 50 and 100% did not significantly differ from control peas with respect to pod moisture content (Fig. 5.6.A) and leaf surface area (Fig. 5.7).

Interestingly, GLK 8908 applied at any of the concentrations significantly increased the moisture content of pea shoots 27 days from treatment. This suggests that effects of GLK 8908 were either still prevalent or that GLK 8908 resulted in less damage during freezing and left the pea seedlings in a more vigorous state.

In conclusion, the results presented here show that GLK 8908 improves pea cold hardiness but not by increases in cell wall rigidity which protect the cytoplasm from dehydrative injury during extracellular freezing as was hypothesized. Based on the concentrations tested, 1 and 10% (v/v, aqueous) foliar applications gave maximum freezing protection and yield in plants that were exposed to a freeze stress early in their development and then grown to maturity in the greenhouse. GLK 8908 reduced freezing injury second only to growth at cold temperature. In this respect it performed better than desiccation or ABA treatment and shows promise for use in protection from freezing damage to crops on a large scale.

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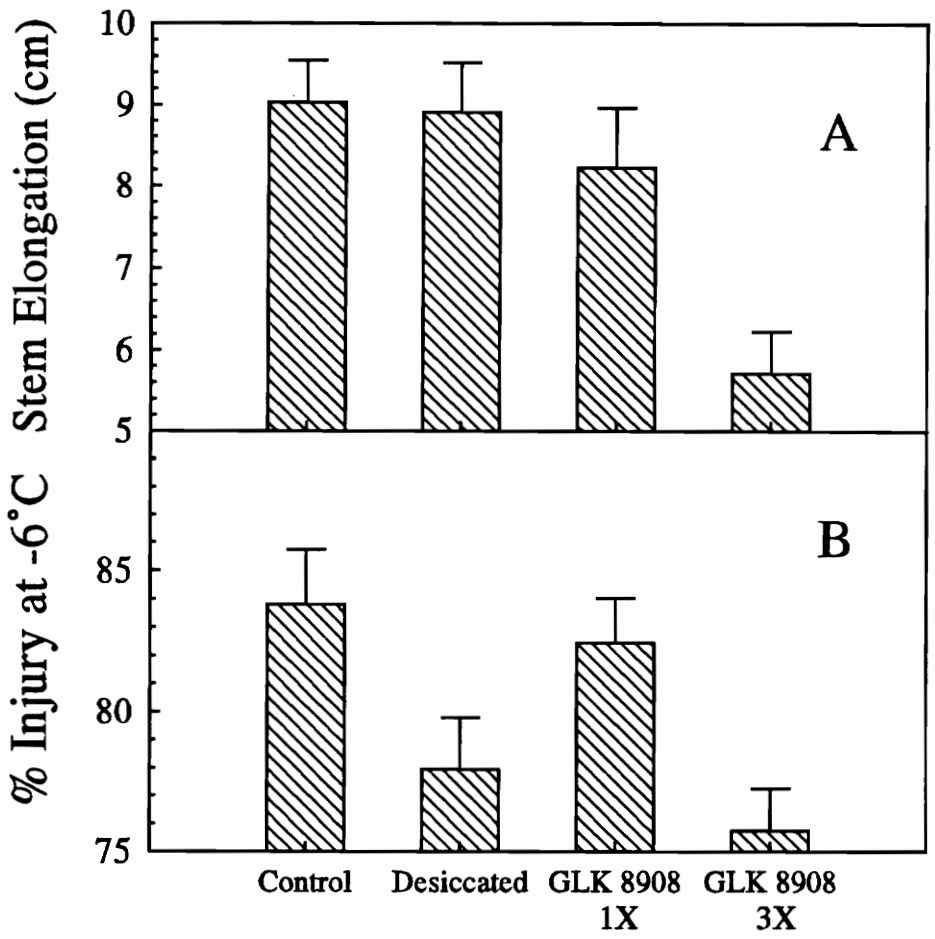


Figure 5.1. A comparison of stem elongation (A) and % injury at -6°C (B) of 'Alaska' peas treated with GLK 8908 as compared to control and desiccated peas. GLK 8908 was applied in a 1% solution to the peas either once or three times. Injury was estimated by electrolyte leakage. The desiccated peas were kept well watered, but the epicotyls were exposed to dry circulating air for 1 week. Bars represent SE, $n_A = 10$ and $n_B = 8$.

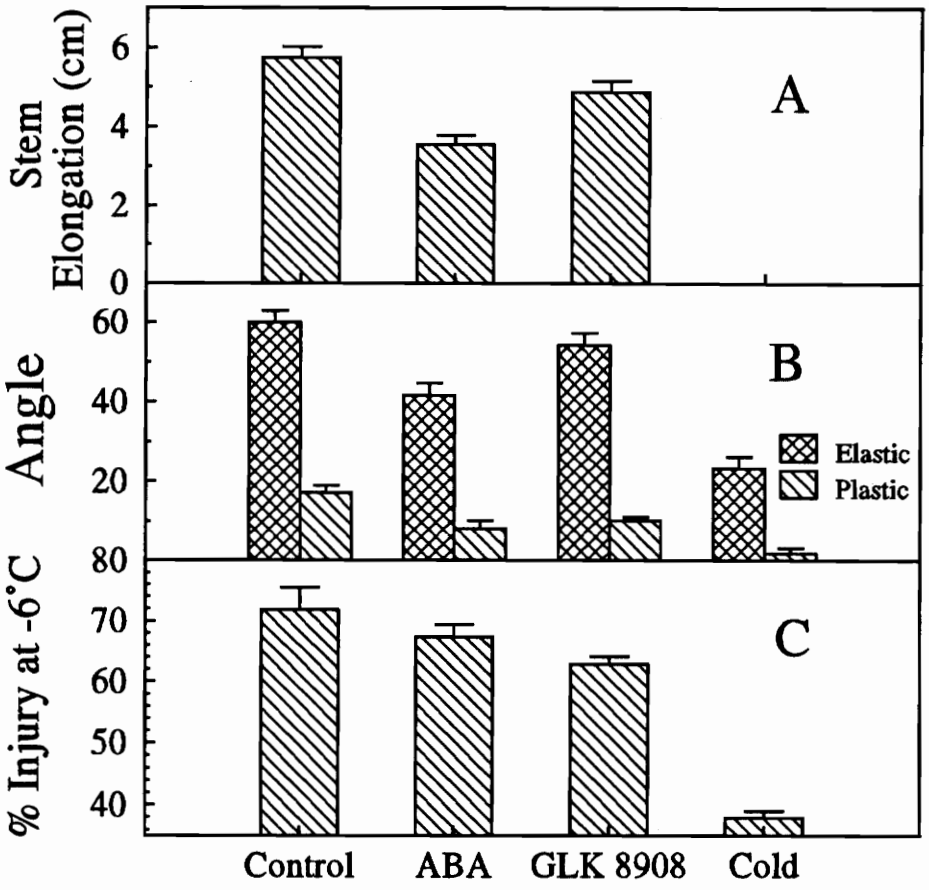


Figure 5.2. A comparison of stem elongation (A), stem strength (B), and % injury at -6°C (C) of peas treated with GLK 8908, ABA, or cold temperatures. GLK was sprayed on in a 1% solution to the peas every 48 hrs and ABA (10^{-4}M) was applied using a root drench. Cold treated peas were grown for 3 weeks at 3°C and so stem elongation could not be analyzed with the other treatments. Bars represent SE, $n_A = 10$, $n_B = 5$, $n_C = 8$.

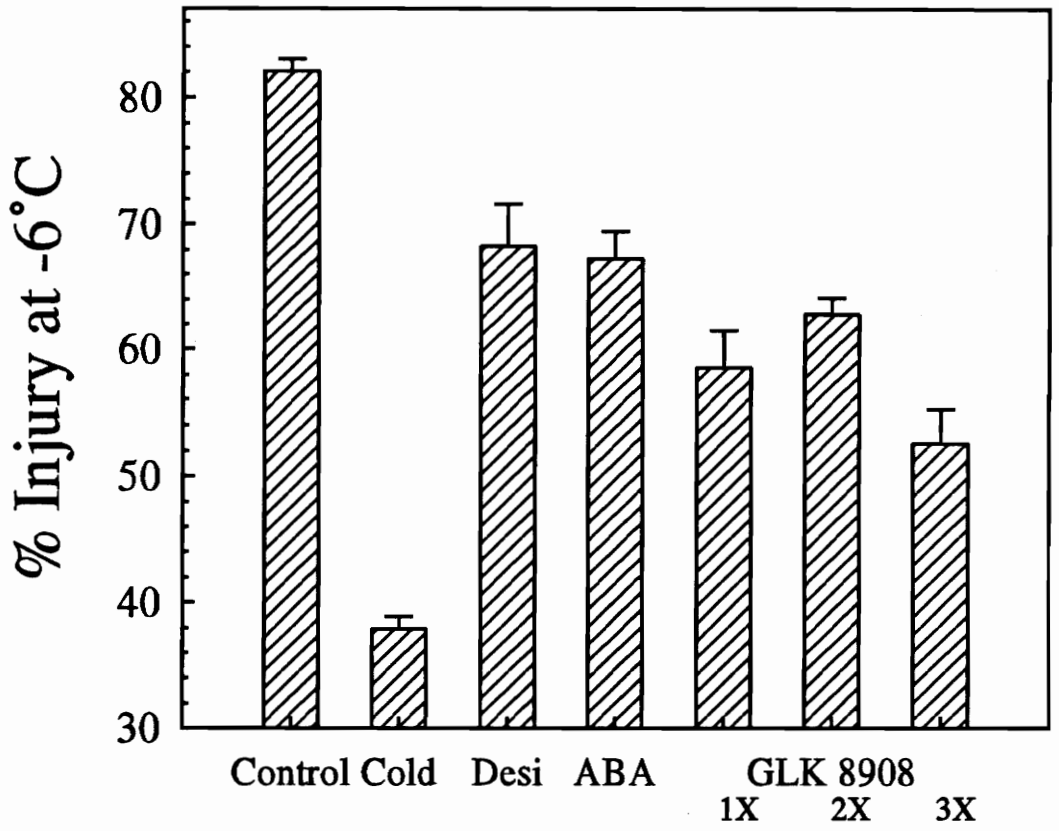


Figure 5.3. Freezing injury of 'Alaska' peas treated with GLK 8908, desiccation, ABA, or cold temperatures. GLK 8908 was sprayed on the epicotyls in a 1% solution either once, every 48 hours, or every 24 hours. Desiccated peas remained well watered, but were subjected to circulating dry air for 1 week. ABA was applied at a concentration of 10^{-4} M in the root solution and cold treated peas were grown for 3 weeks at 3°C. Bars represent SE, n = 8.

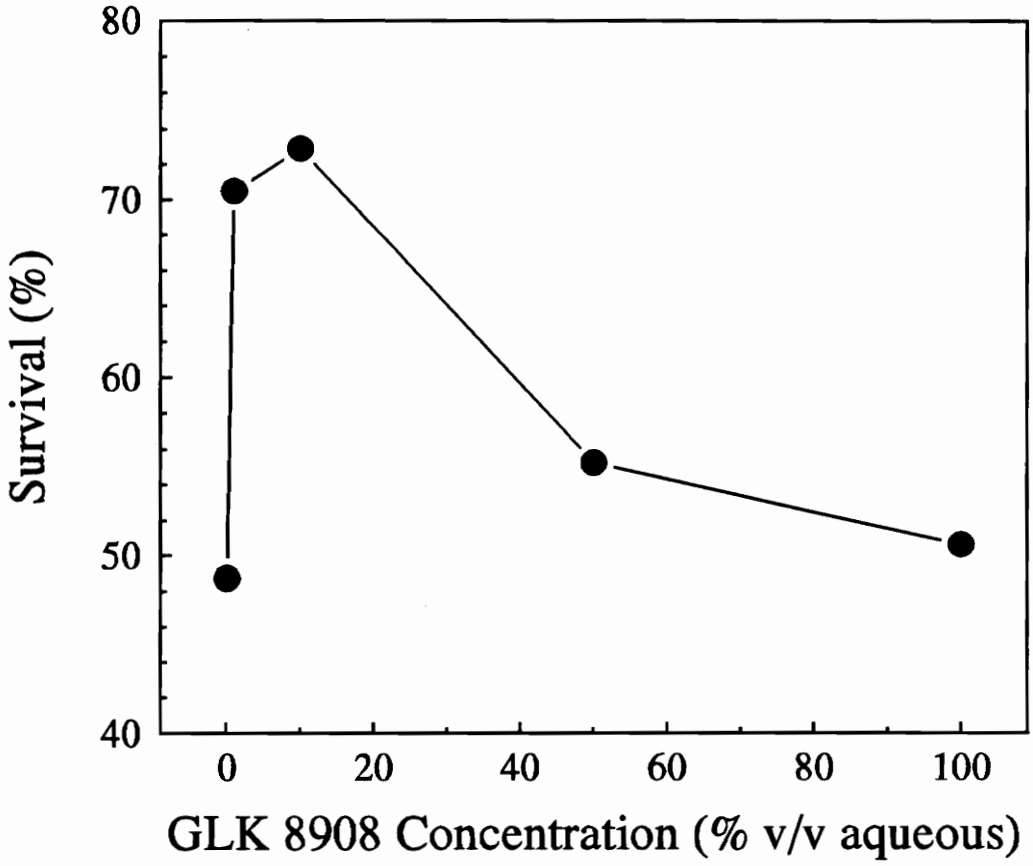


Figure 5.4. Survival of peas treated with varying aqueous concentrations of GLK 8908 after a freeze test. GLK 8908 solutions were applied via atomizer 24 hours before freezing. The peas were then cooled to -6.7°C and mortality of the pea seedlings was recorded after 24 hours. Each treatment contained 80 seedlings.

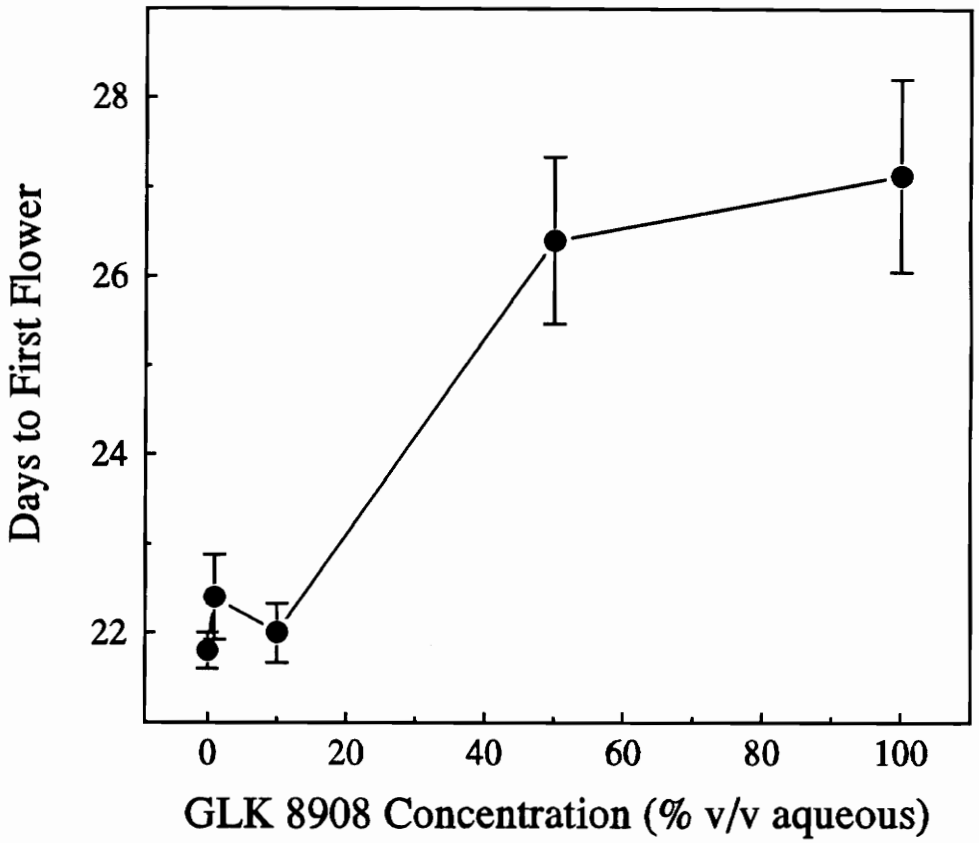


Figure 5.5. Days to first flower of 'Alaska' peas treated with either 0, 1, 10, 50, or 100% GLK 8908 aqueous solutions. Peas were germinated at 26° C and GLK 8908 was applied via atomizer one time 24 hours before the peas were exposed to -6.7° C. The seedlings were then transplanted to 4" pots and placed in a greenhouse on 6/17/92. Days to first flower was recorded when the first bloom had fully expanded.

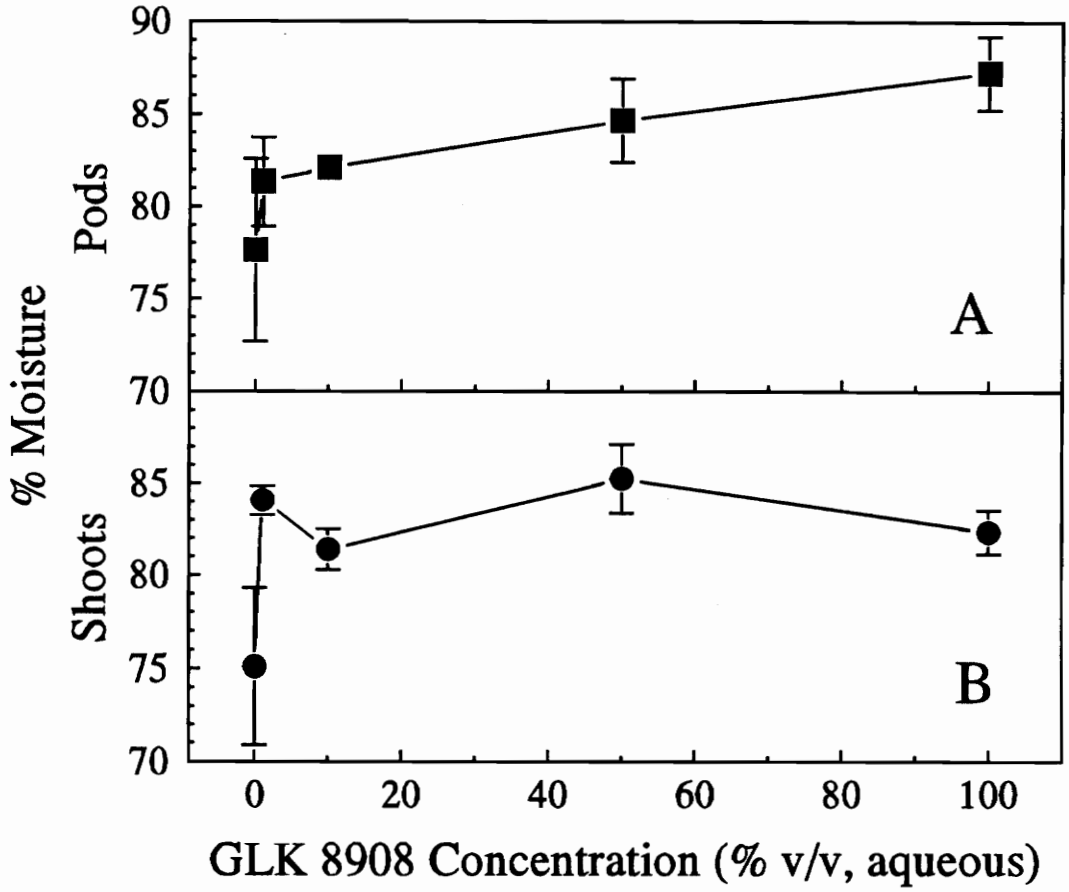


Figure 5.6. Moisture content for the pods (A) and shoots (B) of 'Alaska' peas treated with either 0, 1, 10, 50, or 100% GLK 8908 solutions. Peas were germinated at 26° C and GLK 8908 was applied via atomizer one time 24 hours before the peas were exposed to -6.7° C. The seedlings were then transplanted to 4" pots in a soilless media and placed in a greenhouse on 6/17/92. Peas were harvested on 7/7/92 and fresh weight (FW) recorded. The peas were then placed in an oven for drying for 1 week at 96° C and dry weights (DW) recorded. Moisture content = ((FW - DW)/FW)*100.

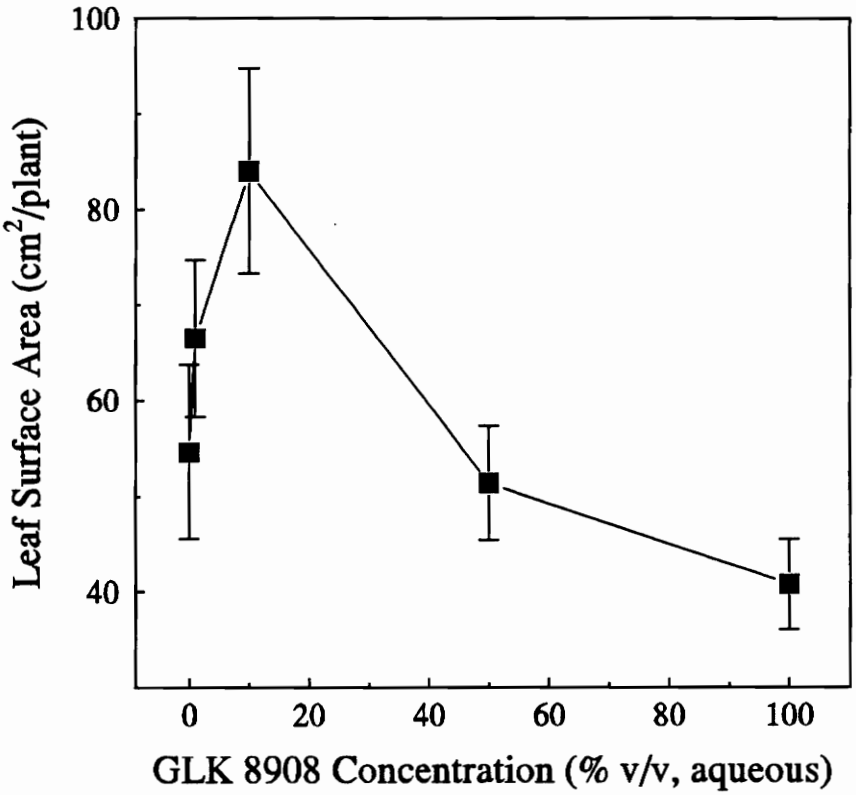


Figure 5.7. Leaf surface area (cm^2/plant) of peas treated with either 0, 1, 10, 50, or 100% GLK 8908 solutions. Peas were germinated at 26° C and GLK 8908 was applied via atomizer one time 24 hours before the peas were exposed to -6.7° C. The seedlings were then transplanted to 4" pots in a soilless media and placed in a greenhouse on 6/17/92. Peas were harvested on 7/7/92 and leaf surface area recorded on a LI-3050A surface area meter.

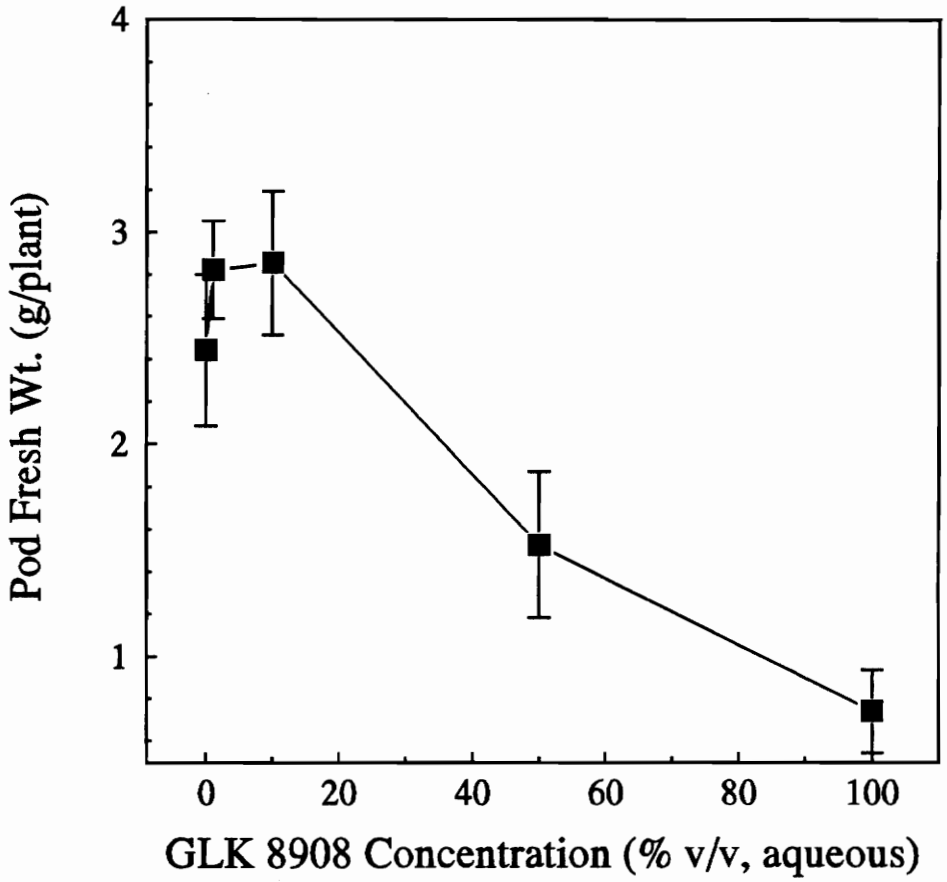
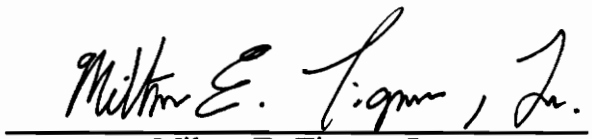


Figure 5.8. Pod yield of pea plants treated with 0, 1, 10, 50, or 100% GLK 8908 solutions. Peas were germinated at 26° C and GLK 8908 was applied via atomizer one time 24 hours before the peas were exposed to -6.7° C. The seedlings were then transplanted to 4" pots in a soilless media and placed in a greenhouse on 6/17/92. Fresh weights for the pods were recorded 7/7/92.

Vita

Milton E. Tignor, Jr. was born to Milton and Shirley Tignor on April 2, 1968, in Richmond, Virginia. His father almost immediately gave him the nickname "Buddy." He was raised by his parents on his maternal grandfather's tobacco farm in Chesterfield County, VA, attended Manchester High School and graduated in June 1986. He earned a Bachelor's of Science in Horticulture in May 1990 and Master's of Science in Horticulture specializing in cold acclimation in September of 1992, both from Virginia Polytechnic Institute and State University, Blacksburg, VA.



Milton E. Tignor, Jr.