# THE ROLE OF DAILY CHANGES IN LEAF WATER CONTENT IN CHILLING RESISTANCE

BY

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TABLE OF CONTENTS

# LIST OF FIGURES

# CHAPTER I

# INTRODUCTION

## Factors That Control Leaf Relative Water Content

The water content of leaf tissue is determined by the balance of water uptake from the soil and water loss into the atmosphere by transpiration [Korshonova, 1982]. Thus, leaf relative water content (RWC) is strongly affected by changes of stomatal aperture and root water uptake. Daily changes in RWC, because of daily changes in leaf stomatal movement and root water uptake, occur under non stress conditions[Ehrler et al., 1965].

The opening of stomata is mediated by an increase in the turgor pressure of the guard cells, caused by an increase in the $K^+$ concentration [Sweeney, 1979]. Turgor pressure in the guard cells of closed stomata begins to increase after midnight and reaches its maximum level at a time when the sun would normally rise [Raschke, 1979]. Young at al. [1981] observed a significant positive correlation between turgor potential and total water potential. They measured the diurnal variations in the stomatal resistance with well-

watered peach seedlings. The measurement started at the beginning of the day with average stomatal resistance of 75 sec/cm; the resistance continued to decrease until it reached the low point of about 10 sec/cm, and remained low for about 4 to 5 h around midday. Then the resistance started to increase and reach a high level of 130 sec/cm at the beginning of the dark period.

The diurnal stomatal movement can be regulated by internal and external factors. Stomatal opening and closure can be affected by many external factors such as light[Evans and Allaway, 1972), and temperature [Stalfelt, 1962]. In addition, stomatal behavior is under the influence of certain endogenous rhythms [Brun, 1962; Stalfelt, 1963, 1965]. Stalfelt [1963] showed diurnal periodicity of stomatal opening and closure even during constant darkness. The patterns observed were the same as that of Raschke[1979] and Young [1981]. The stomatal movement might be endogenously associated with a circadian clock [Edmunds, 1988; Sweeney, 1987].

Root water uptake shows daily fluctuations. Such fluctuations are demonstrated by changes in the rate of exudation of decapitated sunflower [Vaadia, 1960]. The highest and lowest rate of exudation and root pressure are found at about noon and midnight, respectively. These highest and lowest exudation rates are inversely related to

root resistance. Similar patterns, i.e. low root resistance around noon and high root resistance around midnight were observed in about 30 other root systems [Barrs and Klepper, 1968], including cotton [Parsons and Kramer, 1974].

Studies [Plaut et al., 1975] show that RWC of irrigated plants decreases at the beginning (95%) of the light period and reaches a low point at middle of the day (80%). RWC remains low around 80% for the remainder of the day. Stomatal apertures also started to increase from the beginning of the light period (4μ) and reaches the largest (10μ) at the middle of the day. Then, the aperture decreases gradually throughout the remaining light period. But Plaut et al. point out that there is no correspondence between diurnal changes of shoot water potential and that of stomatal aperture. Stomatal aperture increases from 2μ at 6AM to a maximum of 10μ at 9AM. Then stomates start to close in the afternoon in the well irrigated condition. On the other hand, shoot water potential decreases from -6 MPa at 6AM to -13 MPa at 9AM, and remains low for the rest of the day.

## Effects of Chilling on Plants

Tropical and subtropical plants that are killed or injured by temperatures above the freezing point of their

tissues are defined as chilling sensitive plants. Plants that are able to survive without injury, or to grow near freezing point are defined as chilling resistant plants, although their optimal growth temperature might be much higher [Graham and Patterson, 1982]. Chilling injured plants usually show morphological symptoms such as water-soaked leaves or soft spots on fruits due to a loss of membrane semi-permeability and leakage of electrolytes into the intercellular space. Symptoms often depend on the environmental conditions. Plants chilled at low RH often wilt due to decreased water absorption insufficient to match transpirational water loss. Plants chilled at high RH do not experience the same high transpirational demand and do not wilt [Graham and Patterson, 1982].

Stomatal opening and subsequent leaf dehydration are major factors in the development of chilling injury. Under chilling temperature, stomata of chilling sensitive plants remain open despite severe wilting of the leaves. In contrast, stomata of chilling resistant plants close rapidly upon chilling exposure, and subsequently reopen to a new reduced aperture. When chilling sensitive plants are subjected to low, non-damaging temperature before chilling, they respond similarly to chilling resistant plants when exposed to lower temperatures: the stomatal aperture is

reduced while a positive leaf turgor is maintained [Eamus et al., 1983].

Many species of tropical and subtropical plants show daily changes in chilling resistance. For instance, cotton seedlings grown under light-dark-cycles of 12:12h are most chilling sensitive at the middle of the day and attain maximal chilling resistance at the middle of the night [Rikin, 1991,1992; McMillan and Rikin, 1990]. At present it is not clear whether there is any relationship between the changes in RWC before chilling and the changes in chilling resistance.

## Plant Adaptation to Chilling

Many plants under environmental stresses undergo an acclimation process that involves metabolic changes such as synthesis of new structural proteins, enzymes and lipids [Levitt, 1980].

**Lipids**. A change in the composition of membrane lipids is induced in many plants when they are subjected to non-damaging low temperature. An increase in chilling resistance by low temperature in many cases coincides with an increase in unsaturation of lipids[Somerville and Browse, 1991]. Usually, fatty acid desaturation increases during cold acclimation [Kodama et al., 1995]. Evidence that

chilling tolerance is affected by the level of unsaturated membrane lipids has been found by using transgenic tobacco plants with cDNA for glycerol-3-phosphate acyltransferase from the chilling sensitive plant squash. Phosphatidyglycerol has significantly lower level(about three times) of unsaturation in the thylakoid membrane of transformed plants. These plants are more sensitive to photoinhibition and slower to recover from photoinhibition at low temperature [Moon et al., 1995]. Rikin et al. [1993] observed daily fluctuations in the amount of the unsaturated fatty acids linoleic (18:2) and linolenic (18:3). The amount of 18:2 and 18:3 is higher in the middle of the dark period, which is the most chilling resistant point, than in the middle of the day, which is the most chilling sensitive point. They also demonstrated that the amount of 18:2 and 18:3 fatty acids is higher in acclimated seedlings when compared with non-acclimated seedlings in their chilling sensitive phase.

**Gene Expression and Protein Synthesis.** Under many environmental stresses the synthesis of certain proteins can substantially increase [Levitt, 1980]. Some proteins can be induced by both drought and low temperature, raising the possibility that similar proteins are important for resistance to both chilling and drought [Guy et al., 1992].

These includes CAP85 in spinach[Neven et al., 1993], COR series (COR6.6, COR15, COR47, and COR160) in *A. thaliana* [Thomashow, 1990,1993; Thomashow et al., 1992], and dehydrins [Close et al., 1989]. All these proteins have some common characteristics. They all show high hydrophilicity, boiling solubility and the presence of repeating motifs.

The CAP85 that is associated with freezing tolerance in leaf tissues of spinach is also synthesized to high levels under water deficit[Neven et al., 1993]. The sequence of the CAP85 protein has a similarity to the Group 2 LEA (Late Embryogenesis Abundant protein). The sequence showed a typical Group 2-like 11 residue lysine rich repeat: LDKIKDKLPGQ contained within a large 22 amino acids sequence that is repeated 11 times. CAP85 protein differs from the Group 2 LEAs in not having a serine cluster (Neven et al., 1993).

All four cor genes isolated from *Arabidopsis* are induced by drought and low temperature. The transcript levels for each of the cor genes in *A. thaliana* increases dramatically in response to water stress as well as cold acclimation. The cor6.6 encoded protein (COR6.6) is composed of 66 amino acids and is rich in alanine, lysine and glycine residues. It lacks tryptophan, histidine proline, tyrosine and cysteine. The polypeptide is

hydrophilic and basic[Gilmour et al., 1992]. The COR15 is composed of 140 amino acids, and lacks cysteine, methionine, glutamine, arginine, histidine, tryptophan, and proline. It is hydrophilic and acidic. COR47 seems to be related to Group 2 LEA proteins. The COR160 is rich in glutamic acid and lacks cysteine. It is hydrophilic and acidic[Thomashow, 1993].

Dehydrins are characterized by a consensus 15 amino acids sequence domain EKKGIMDKIKEKLPG, which is always present at or near the carboxyl terminus, and repeated upstream of the terminus once or more times [Close et al., 1993a]. The dehydrins are generally induced by many environmental factors including desiccation of leaves and embryos, low temperature, abscisic acid (ABA), salinity and darkness [Close et al., 1993b]. Antibodies which are produced against a synthetic peptide containing the consensus sequences are widely used in immunoblots to detect dehydrins in a wide range of plants of about 30 species [Asghar et al., 1993].

The roles of dehydrins are currently being investigated. The amino acids sequence of many higher plant dehydrins contain the lys-rich amino acids consensus sequences at the carboxyl terminus and repeated from 1 to 10 times. The size of dehydrins varies from 10 KD to 150 KD. Dehydrins are intracellular proteins and are

associated with the cytoplasm and nucleus. Several hypotheses have been proposed for the roles of dehydrins. The wide range of sizes of dehydrins is more similar to seed storage proteins than what is typically found in enzymes. Since dehydrins represent 0.5% of soluble embryo protein, they are probably structural proteins rather than enzymatic proteins. The presence of dehydrins in the cytoplasm and their accumulation during dehydration-related stresses similarly to solutes such as proline, betaine, sucrose and glucose, suggest that dehydrins may have an analogous role to these solutes. Other possible roles for dehydrins such as free radical scavenging, ion sequestration or ice inhibition are also suggested [Close et al., 1993b].

# CHAPTER II

# OBJECTIVES

The small changes of leaf RWC that occur naturally under normal environmental conditions may be an important but yet unexplored factor in regulation of plant response to the environment.

This research tries to determine whether small fluctuations (about 10%) in leaf RWC of cotton seedlings are related to the plant response to chilling. Two types of small fluctuations are studied. First, daily fluctuations that occur under normal light:dark cycles under non stressing conditions (optimal temperature and water supply, and constant relative humidity). Second, fluctuations caused by short changes in air relative humidity. The next goal of this research is to find the type of possible mechanism(s) by which small fluctuations in leaf RWC regulate chilling resistance. Two lines of possibilities will be investigated:

1. Does the changes in leaf RWC before chilling

exposure affect chilling resistance ?

2. Is there a relationship between the fluctuations in leaf RWC, dehydrin synthesis and chilling resistance?

So far, studies related to dehydrins are mainly with plants under water stress conditions which decrease RWC substantially. At present it is not known whether dehydrins are induced by small fluctuations of RWC at certain points during the light:dark cycle under non stress condition.

# CHAPTER III

# MATERIAL AND METHODS

## Plant Material and Growth Conditions

Cotton seeds(*Gossypium hirsutum L. cv.* Deltapine 50, obtained from Delta and Pine Land Co., Scott, Miss., USA) were sown in plastic pots (10 cm diameter, 8.5 cm high) filled with a mixture of peat and vermiculite (Terra-lite, Redi-Earth: W.R.Grace & Co., Cambridge, Mass., USA). The pots were placed in growth chambers at 30°C, 65% RH under cycles of 12 h light and 12 h dark. The light source was fluorescent lamps(F48T18-CW-VHO; Sylvania, Danver, Mass., USA). The seedlings were thinned to one per pot and fertilized with 100 ml of 3 g/l 20N-20P-20K soluble fertilizer (Peters; W.R.Grace & Co.) one week after germination and irrigated with water as needed. The seedlings used in all experiments were 18 days old.

## Measurement of Leaf Diffusive Resistance, Transpiration Rate and Temperature

Leaf diffusive resistance, transpiration rate and leaf temperature were measured using LI-1600 Steady State Porometer (LI-COR, Inc.). The sensor head was equalized to the same temperature at which the seedlings were grown prior to the measurement. All measurements were conducted as quickly as possible.

## Chilling Exposure and Evalution of Chilling Resistance

Eighteen-days-old seedlings grown under normal conditions were transferred to low temperature of 5 $^{0}$C and 65% RH for 36 h. At the end of the chilling exposure, seedlings were returned to normal growth conditions at 30$^{o}$C for another 4 days. Then, the chilling resistance of the seedlings was evaluated by measuring the shoot fresh weight above the third leaf and total chlorophyll content of the third leaf. The whole third leaf above the cotyledons was put into a test tube with 10 ml N,N-Dimethyl Formamide for 48 h. At the end of this extraction, absorbance at wavelengths of 647 nm and 664.5 nm were measured, and the total chlorophyll contents in each third leaf were determined according to the following equation [Inskeep and Bloom, 1985]:

$$\text{Total Chl (mg)} = 17.9A_{647} + 8.08A_{664.5}$$

## Measurement of Relative Water Content

Six leaf discs were taken from each third leaf above the cotyledon and immediately weighed to determine the fresh weight (FW). Then, the discs were floated on distilled water in a petri dish and saturated weight (SW) was measured after 6 h. Dry weight (DW) of the discs was measured after they were kept in an oven at 70 $^0$C for 24 h. The leaf RWC was estimated according to the following formula:

$$\frac{FW - DW}{SW - DW} \times 100\% = RWC\ (\%)$$

## Protein Extraction

All samples were frozen immediately after collection and then lyophilized using Freeze Dry System (Model 77510, LABCONCO Co.). 50 mg of lyophilized tissue was extracted in 1.0 ml of 30 mM 3-[N-tris(Hydroxymethyl)methylamino]-2-hydroxypropanesulfonic acid (TES-HCl), pH 7.5, 20 mM NaCl, 1 mM phenylmethylsulfonyfluoride (PMSF), and 20% 2-mercaptoethanol extraction buffer. The extraction was done in a glass-to-glass homogenizer on ice. The homogenate was centrifuged at 15,000 g for 15 min in the cold room (+4 $^0$C). The concentration of protein in the supernatant was determined by a dye-binding analysis method [Bradford, 1976]

with BSA as standard. The extract was mixed with sample buffer (125 mM Tris-HCl, PH6.8, 20% glycerol, 10% sodium dodecyl sulfate (SDS), and 20% 2-mecaptoethanol)at a ratio of 4:1, and boiled in 100 $^{0}$C water bath for 4 min. Each lane was loaded with the same amount of protein unless otherwise indicated.

**Western Blot Analysis**

Proteins were fractionated by discontinuous SDS polyacrylamide gel electrophoresis (PAGE), using 13% (w/v) acrylamide separating gel and 4% stacking gel by mini-protein II dual slab electrophoresis cells (BioRad Laboratories). Then the separated proteins were transferred to a nitrocellulose membrane in 1 h using the mini-blot electrophoretic transfer cell (BioRad Laboratories). The nitrocellulose membrane was blocked by 3% non-fat dry milk in TBST buffer for 40 min., and then incubated with rabbit anti-dehydrin antibodies (1:500 v/v, kindly provided by Dr. Timothy Close), which was produced against a synthetic peptide containing the consensus sequence EKKGIMDKIKEKLPG [Close, 1993a], washed 3 times for 10 min. with TBST buffer, followed by incubating with goat anti-rabbit IgG alkaline phosphatase conjugate (1:10,000) (Sigma). The reaction of alkaline phosphatase was developed with 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-

phosphate (BCIP) in the alkaline phosphatase buffer [Promega, 1991]. Fractionated protein in a duplicated gel was visualized with brilliant coomassie blue.

## Experimental Design

In all experiments, each treatment was conducted with at least 3 seedlings. The results shown are the arithmetic mean + SE. Each experiment was repeated at least three times.

# CHAPTER IV

# RESULTS

## Daily Changes in Diffusive Resistance, Transpiration, Relative Water Content and Temperature of Cotton Leaves

In the first experiment, several daily fluctuations that are related to the seedling water balance were characterized. Seedlings were grown at 30 $^{0}$C for 18 light : dark cycles of 12 h light and 12 h dark from sowing. Then, the measurements were taken throughout the 19th light : dark cycle at 6-h intervals (Fig. 1).

The diffusive resistance started to decline from the beginning of the light period. It reached its lowest level at middle of the light period, and remained low until the beginning of the dark period. Then it increased and remained high throughout the dark period (Fig. 1A). As expected, the transpiration rate showed essentially an opposite pattern to that of diffusive resistance (Fig. 1A).

The relative water content started to decline from the beginning of the light period, reaching the lowest level at the end of the light period. Afterwards, it increased

throughout the dark period. The difference between the highest value of RWC at the beginning of the light period and the lowest value at the end of the light period was about 10 % (Fig. 1B).

Leaf temperature showed a negative correlation with the transpiration rate. It was the lowest at the middle of the light period and the highest at the beginning of the light period corresponding to the highest and lowest transpiration rates (Fig. 1C).

## Daily Changes in the Resistance of Cotton Seedlings to Chilling

Chilling resistance as determined by shoot fresh weight and leaf chlorophyll after chilling exposure showed daily fluctuations (Fig. 2). The seedlings were the least chilling resistant at the middle of the light period. The resistance increased toward the end of the light period and reached its highest level at the middle of the dark period. Then it declined toward the end of the dark period (Fig. 2).

## The Effect of Relative Humidity on Leaf Relative Water Content and Chilling Resistance

In the first two experiments daily changes in RWC were correlated with chilling resistance (Fig. 1, 2). In the following experiments (Fig. 3-6), I examined how changes in

atmospheric RH affect leaf RWC and whether such possible changes affect chilling resistance.

Seedlings grown under the standard conditions of lower RH of about 65 % showed the same pattern of daily changes in RWC as described previously (Fig. 1B). Transfers of seedlings to 100 % for 6 h at any time during the light : dark cycle resulted in an increase in RWC. The RWC increased 5 to 10 % depending on the time in the daily cycle. The exposure to 100 % RH almost eliminated the daily fluctuations of RWC of the seedlings. All seedlings under RH of 100 % reached RWC of about 95 % (Fig. 3).

Transfers of seedlings to 100 % RH 6 h before chilling exposure changed their chilling resistance depending on the time in the light : dark cycle (Fig. 4). Treatment of 100 % RH for 6 h before chilling decreased chilling resistance only when chilling started at 12 h of the daily cycle. Such treatment had a very small or no apparent effect when chilling started at 6 h, 18 h, and 24 h of the daily cycle (Fig. 4). A possible reason for the absence of a response to 100 % RH at these points is the specific length of chilling exposure used in this experiment i.e. 36 h. For instance, at 6 h the seedlings were already very chilling sensitive, and any further decrease in their resistance could not be achieved. On the other hand, at 18 h, the

seedlings were very resistant, and a chilling exposure for 36 h is not enough to inflict a substantial damage.

To clarify whether the lack of response to 100 % RH at certain points during the light : dark cycle is due to the previous chilling conditions, I exposed seedlings at 6 h and 18 h to various lengths of chilling after pre-exposure to 100 % RH (Fig. 5). When seedlings were exposed to chilling starting at 6 h (the middle of the light period i.e. the most sensitive point), there was a big difference between the resistance of seedlings grown under the low RH (65 %) and the seedlings transferred to high RH (100 %) when measurement were taken after 12 h of chilling exposure. After such chilling exposure the seedlings transferred to high RH were more injured than the seedlings grown under low RH. After a longer chilling exposure seedlings of the two treatments showed the same degree of high injury. When seedlings were exposed to chilling starting at 18 h (the middle of the dark period, i.e. the most resistant point), there was no big difference between the two treatments until the exposure reached 84 h. At this time the seedlings transferred to high RH were more injured than the seedlings grown under low RH (Fig. 5).

The next experiment was conducted to examine whether interference with the normal daily fluctuations in RWC would affect chilling resistance of cotton seedlings. The

experimental setting is described in Fig. 6A. Seedlings not transferred to high RH before chilling showed the usual low chilling resistance at 6 h (treatment #1) and high chilling resistance at 12 h (treatment #2) (Fig. 6B). Transfers of seedlings to 100 % RH for 6 h and 12 h followed by 12 h and 6 h at 65 % RH (treatment #3, #4) before chilling starting at 18 h did not change the level of chilling resistance as compared to treatment #2 (Fig. 6B). Only transfers to 100 % RH started at 0 h and continued up to the beginning of the chilling exposure (treatment #5) resulted in reduced chilling resistance (Fig. 6B).

**Immunological Detection of Dehydrins in Cotton**

In the first experiment I tried to find what types and quantities of dehydrins are synthesized in cotton plants as compared to other plants. First I extracted proteins from embryos of cotton seedlings after treatment with abscisic acid and tried to detect dehydrins immunologically (Fig. 7). Such a treatment resulted in a synthesis of one dehydrin with a molecular weight (15,000) slightly lower than the major dehydrins in corn embryos treated with abscisic acid. Dehydrin from cotton seeds treated with abscisic acid was used in subsequent experiments as a positive control.

Proteins extracted from cotton leaves showed no specific immunological response to antibody against dehydrins (Fig. 8

and data not shown). Such negative results were obtained under many extraction conditions and all amounts of proteins loaded on the gels. Extraction of proteins from cotton leaves is known to be very difficult probably because of the extremely high levels of polyphenols. In order to find out whether the lack of dehydrin detection in leaf extracts is because of factors that are related to the extraction I checked the effect of leaf extract of dehydrins in cotton seed embryos. Such experiments showed that leaf extract did not affect dehydrin detection in cotton seed embryo (Fig. 8). These results indicated that dehydrins were not detected in cotton leaves because they actually were not synthesized there or their levels were very low, below the sensitivity threshold of the technique.

## Immunological Detection of Dehydrins in Cotton Leaves at Various Times During the Light : Dark Cycle

Many processes in cotton leaves show daily and circadian changes [McMillan and Rikin, 1990; Rikin et al., 1985]. To check whether dehydrin synthesis was time-dependent, dehydrin synthesis was checked throughout the light : dark cycle. No dehydrin was detected at any time during the light : dark cycle (Fig. 9).

**Effect of Drought on Dehydrin Synthesis in Cotton Leaves**

Leaf dehydration caused by withholding watering did not cause dehydrin synthesis in both young and old leaves (Fig. 10)

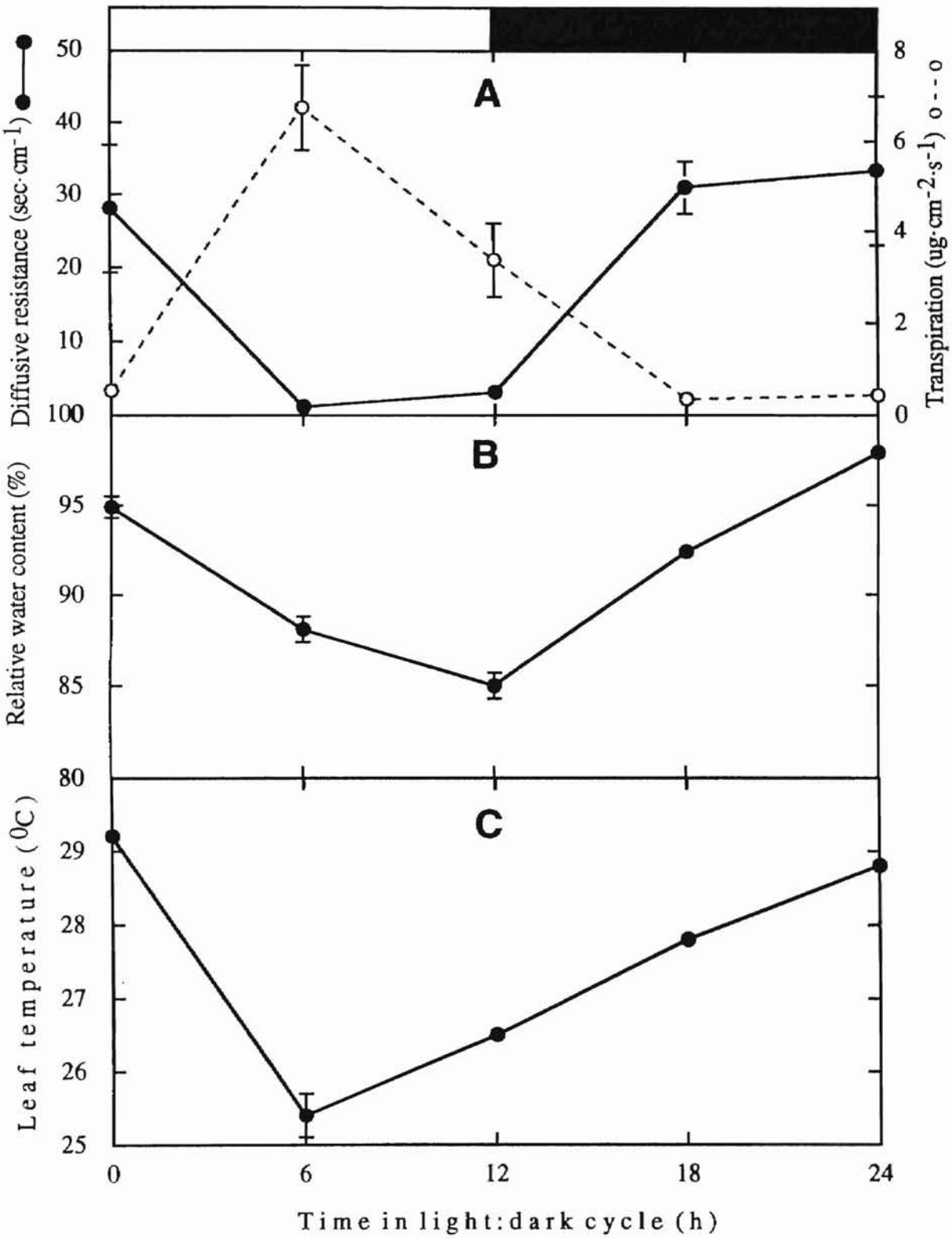


Figure 1. Daily changes in diffusive resistance, transpiration, relative water content and temperature of cotton leaves. Seedlings were grown at 30 $^{0}$C for 18 light:dark cycles of 12h L:12h D from sowing. Then, during the 19th cycle at 6-h intervals measurements were taken.

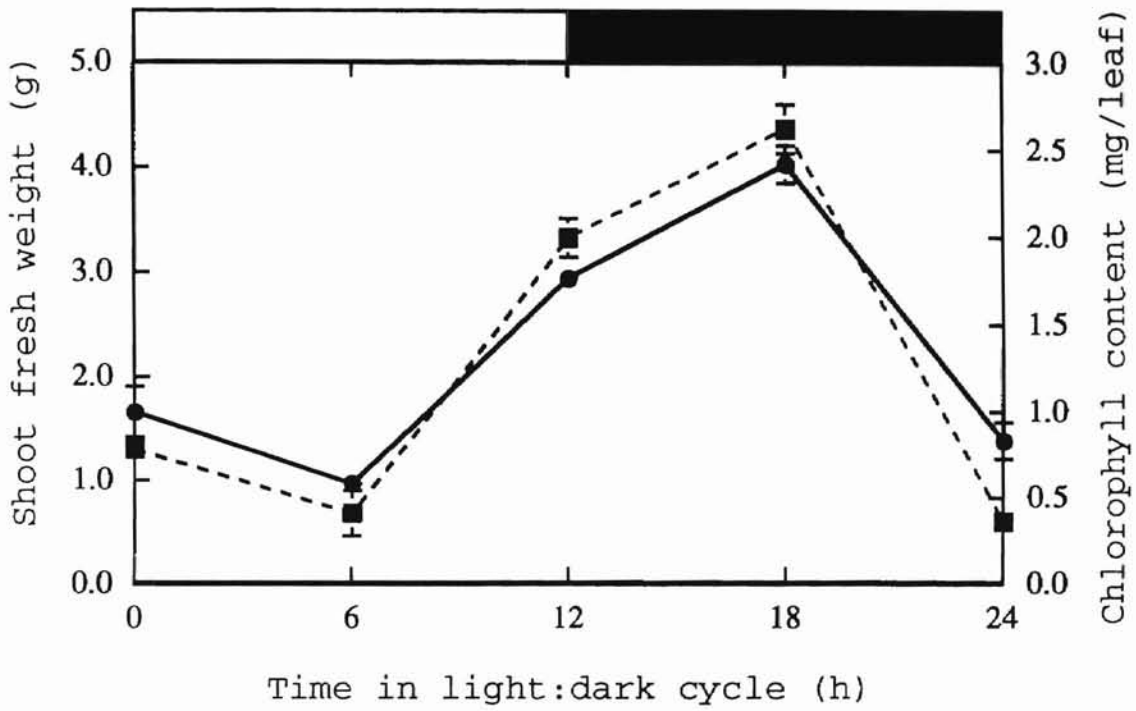



Figure 2. Daily changes in the resistance of cotton seedlings to chilling. Seedlings were grown at 30 $^0$C for 18 light:dark cycles of 12h L: 12h D from sowing. Then, during the 19th cycle at 6-h intervals seedlings were exposed to chilling of 5 $^0$C for 36h. After chilling, the seedlings were returned to 30 $^0$C for 4d and at the end of this period chilling resistance was evaluated by determination of shoot fresh weight and leaf chlorophyll. ( ● ) Shoot fresh weight and ( ■ ) chlorophyll content.

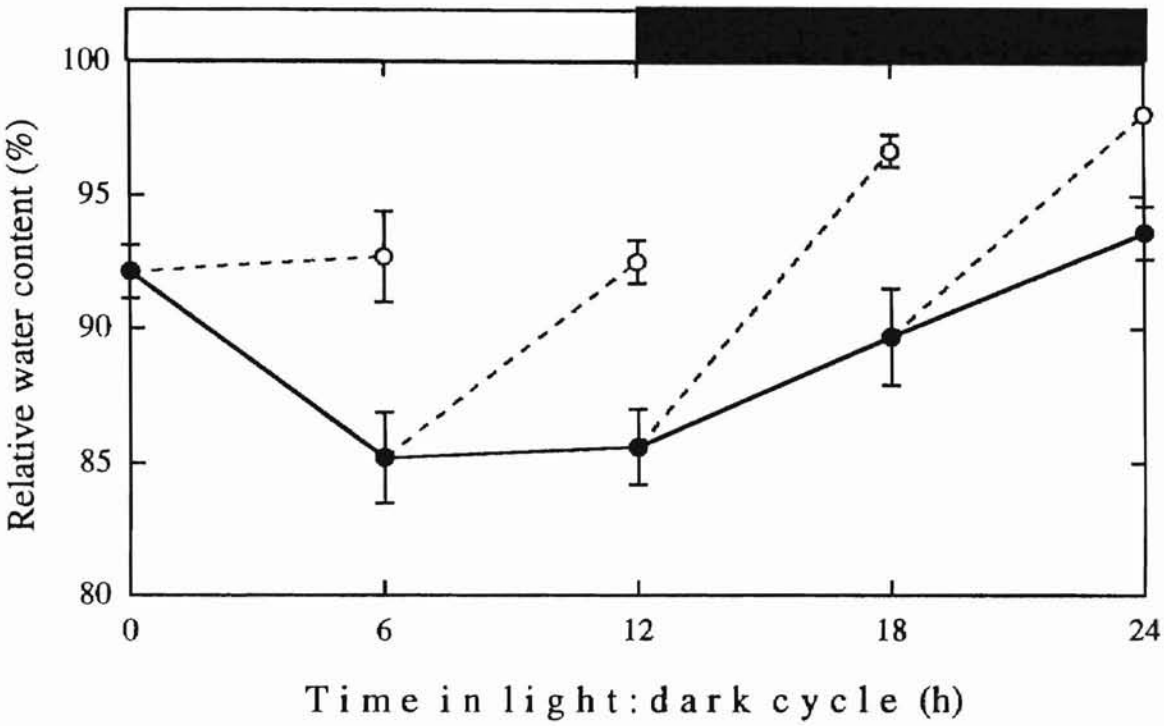



Figure 3. The effect of high relative humidity on leaf relative water content of cotton seedlings. Seedlings were grown at 30 $^{o}$C and 65 % RH for 18 light:dark cycles of 12h L:12h D from sowing. Then, during the 19th cycle at 6-h intervals seedlings were transfered to 100 % RH for 6h. At the end of this period leaf relative water content was determined. ( ● ) Seedlings under 65 % RH and ( O ) under 100 % RH

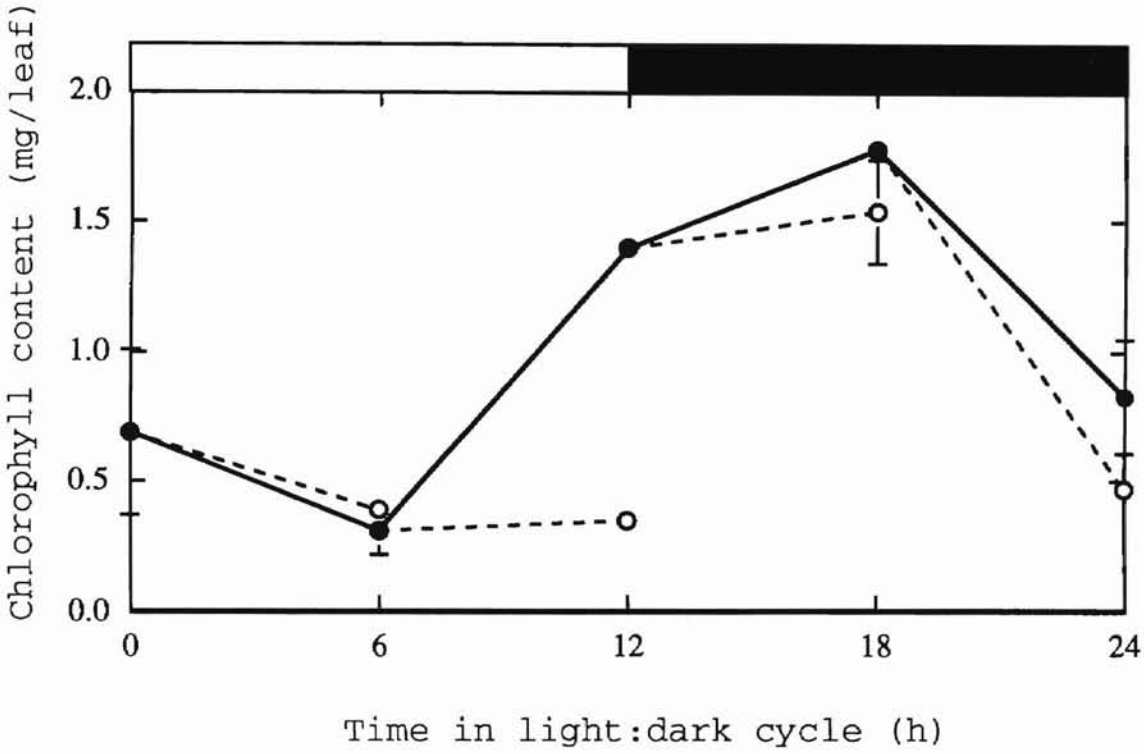



Figure 4. Effect of high relative humidity before chilling on chilling resistance of cotton seedlings. Seedlings were grown at 30 $^{0}$C and 65% RH for 18 light:dark cycles of 12h L: 12h D from sowing. Then, during the 19th cycle at 6-h intervals seedlings were transferred to 100% RH for 6h. At the end of this period the seedlings were exposed to chilling of 5 $^{0}$C for 36 h at 65% RH. After chilling, the seedlings were returned to 30 $^{0}$C for 4d and at the end of this period chilling resistance was evaluated by determination of leaf chlorophyll. ( ● ) Seedlings under 65% RH and (O ) under 100% RH for 6h before chilling.

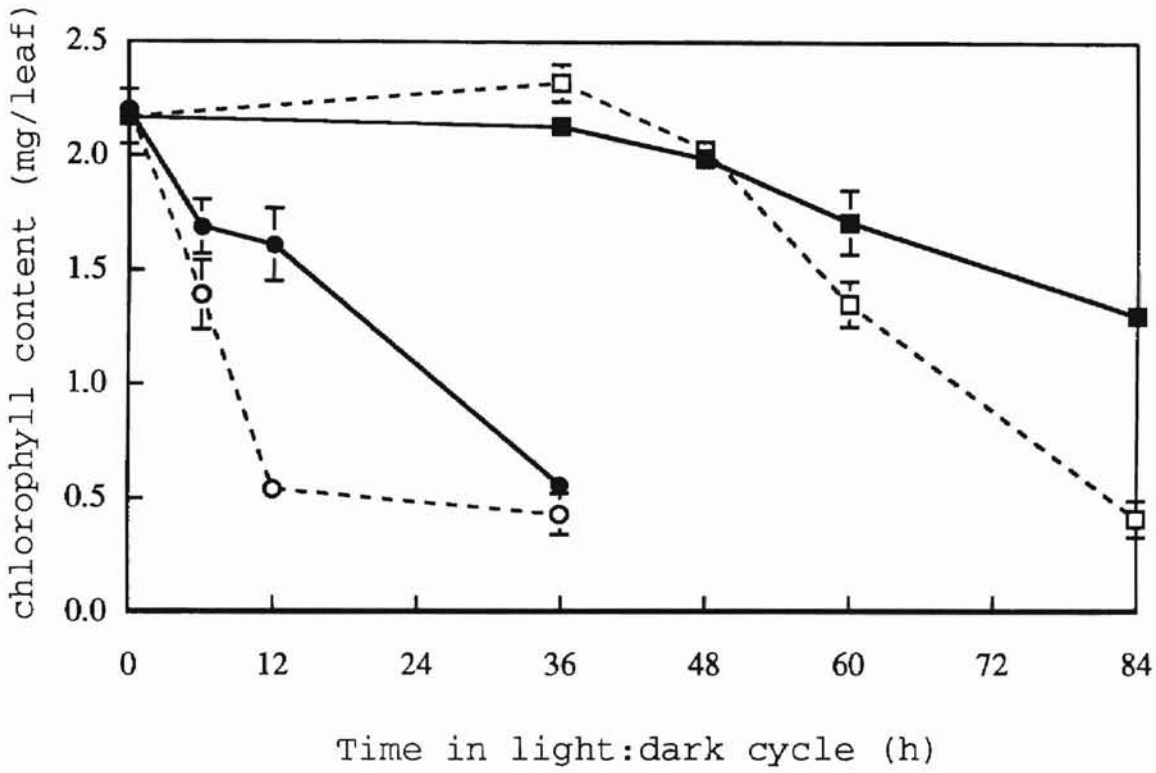
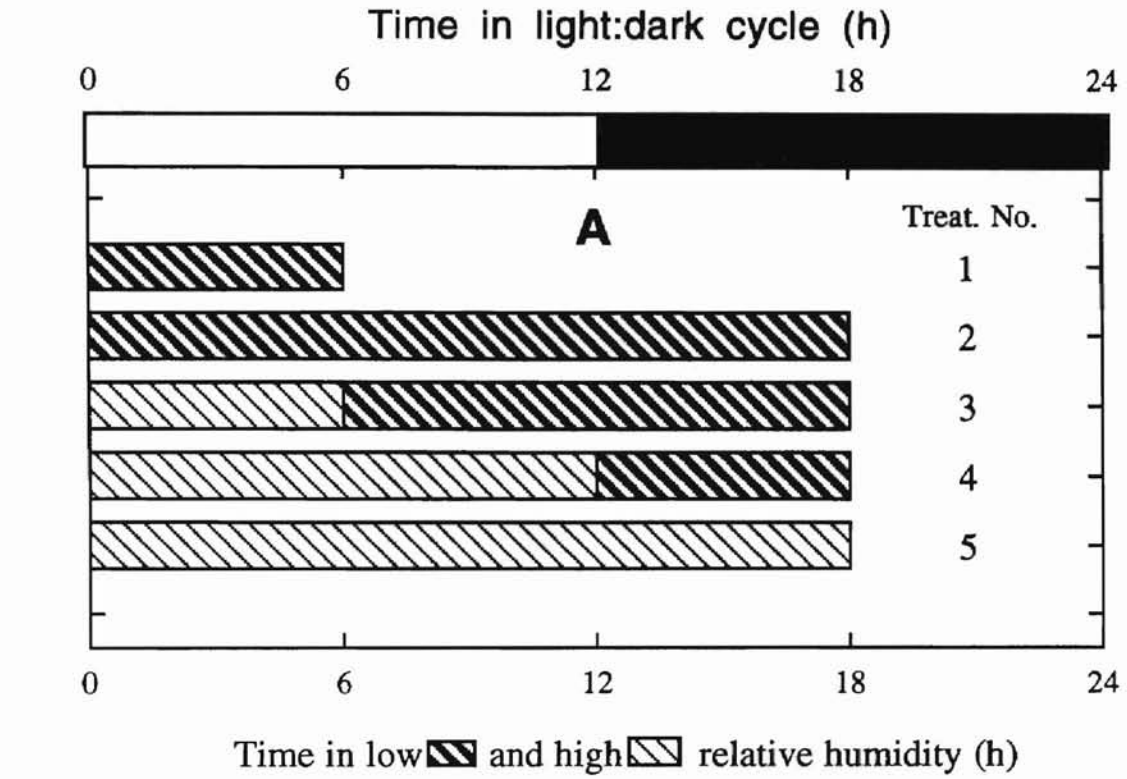



Figure 5. Effect of the duration of chilling exposure on the response of cotton seedlings treated with high relative humidity before chilling. The experimental setting of this experiment is similar to that in figure 4, except that seedlings were transferred to 100% RH for 6h only at the beginning of the light period and the beginning of the dark period. Then , 6 h later at the middle of the light period and the middle of the dark period, the seedlings were exposed to various lengths of chilling up to 84h. (●) Seedlings grown under 65% RH and exposed to chilling starting at the middle of the light period, ( ○) seedlings transferred to 100% RH at the beginning of the light period and exposed to chilling starting at the middle of the light period, (▬) seedlings grown under 65% RH and exposed to chilling starting at the middle of the dark period, (□ ) seedlings transferred to 100% RH at the beginning of the dark period and exposed to chilling starting at the middle of the dark period,


Time in light:dark cycle (h)
0
6
12
18
24
A
Treat. No.
1
2
3
4
5
0
6
12
18
24
Time in low and high relative humidity (h)

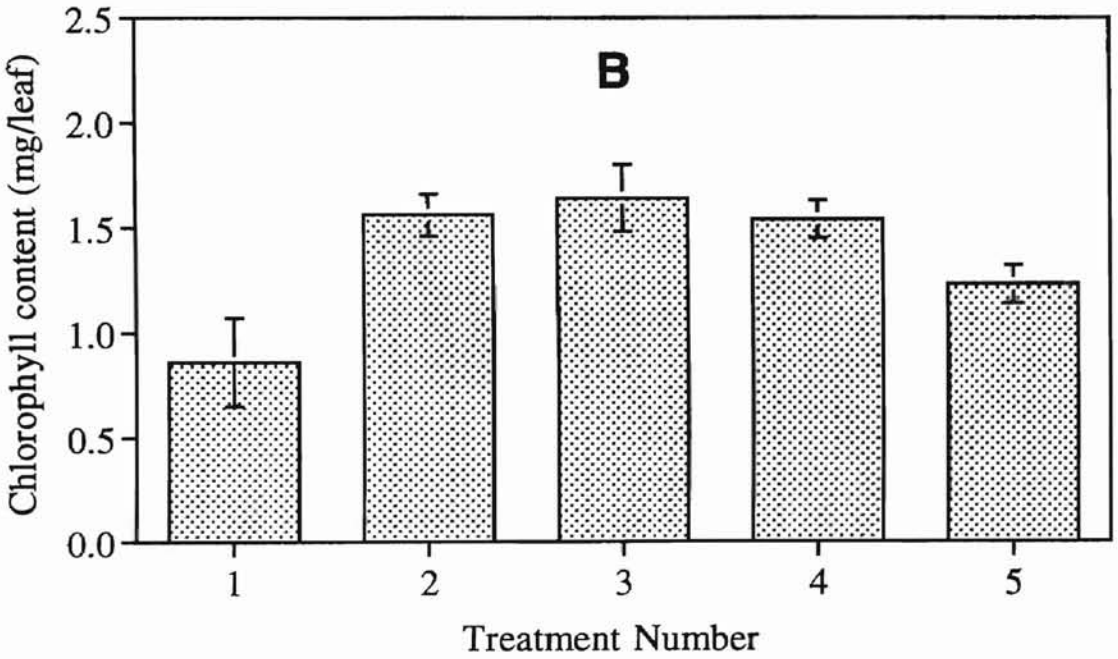

B
Chlorophyll content (mg/leaf)
2.5
2.0
1.5
1.0
0.5
0.0
1
2
3
4
5
Treatment Number

Figure 6. The effect of high relative humidity for various durations at different times during the light : dark cycle on chilling resistance of cotton seedlings. Seedlings were grown at 30 $^{0}$C and 65 % RH for 18 light:dark cycles of 12h L: 12h D fromsowing. Then, during the 19th cycle seedlings were transferred to and from 100 % RH as described in (A). Treatment of 5 were exposed to chilling of 5 $^{0}$C for 60 h at 65 % RH starting at the middle of the dark period. Treatment 1 was exposed to the same chilling conditions starting at the middle of the light period. After chilling, the seedlings were returned to 30 $^{0}$C for 4d and at the end of this period chilling resistance was evaluated by determination of leave chlorophyll (B).


65 % RH


100 % RH

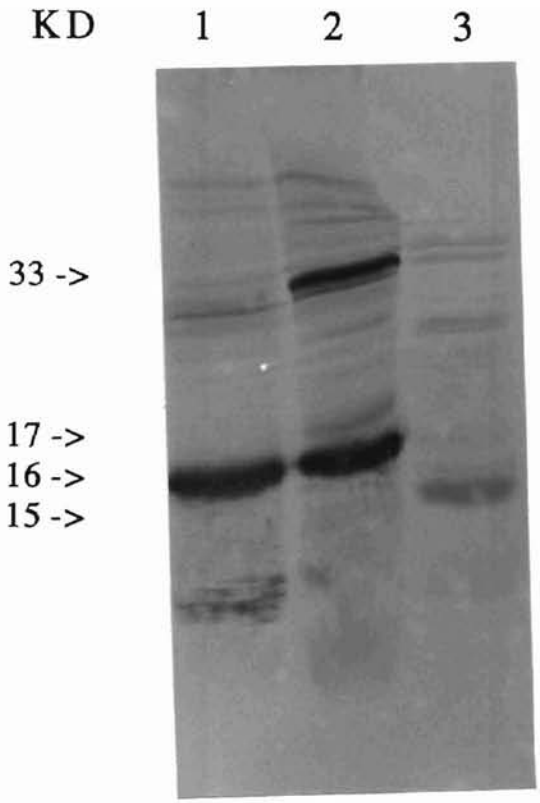



Figure 7. Immunological detection of dehydrins in embryos of maize and cotton. Maize and cotton seeds were incubated in 10-4 M abscisic acid for 48 h. Proteins were extracted from the whole maize embryos and the hypocotyl-root axis of the cotton embryos. Proteins were fractionated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane and detected with antibody raised against the consensus sequence of dehydrin.

1. Maize, line A-632, 39 μg total proteins loaded.
2. Maize, line 17-3, 22 μg total proteins loaded
3. Cotton, 27 μg total proteins loaded.

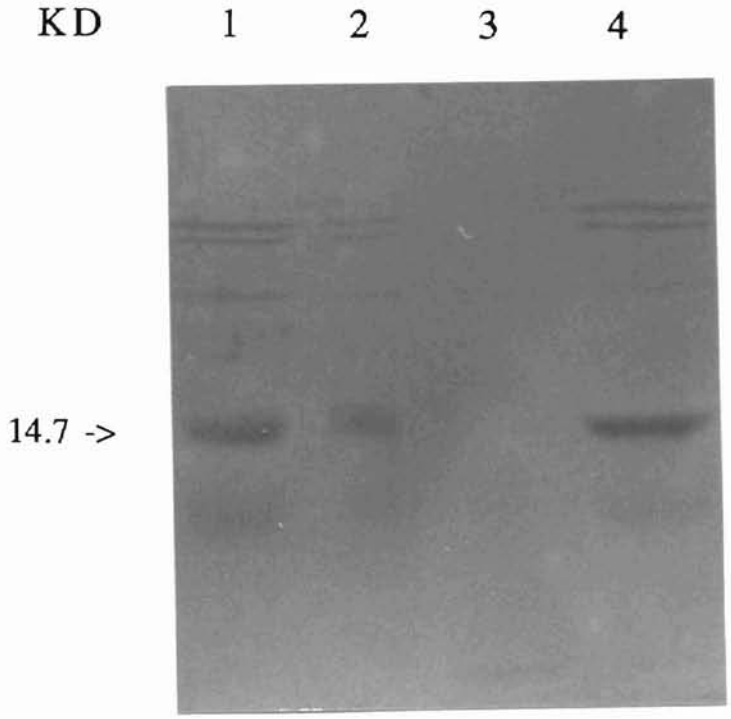
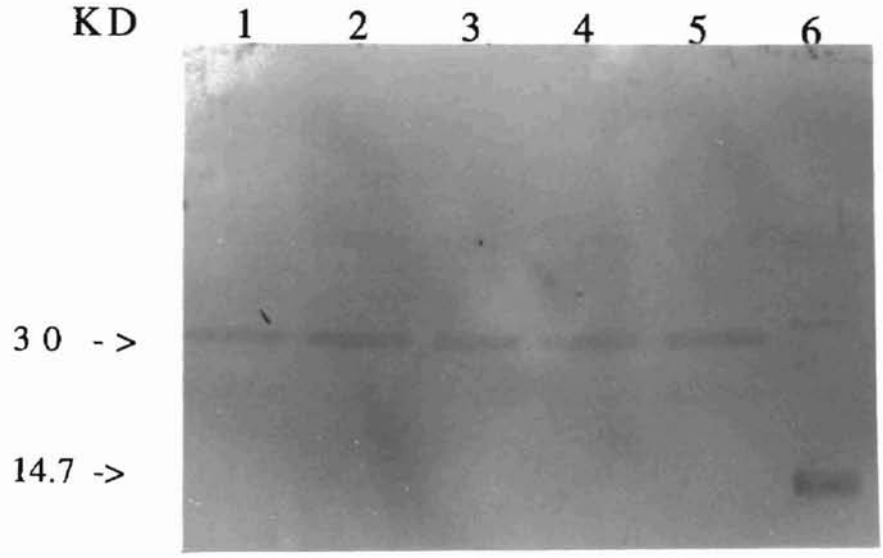
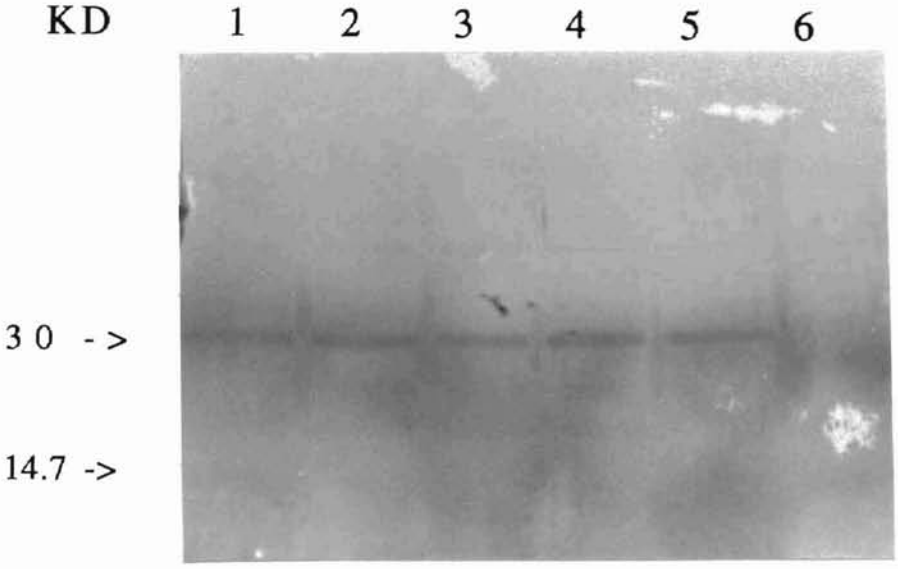



Figure 8. Effect of leaf protein extract from cotton on detection of dehydrins from cotton embryos. Proteins were extracted either from embryos of seeds incubated in 10-4 M abscisic acid for 48 h or from leaf material. In another treatment proteins were extracted from a mixture of embryos and leaf material. Proteins were fractionated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane and detected with antibody raised against the consensus sequence of dehydrin.

1. Embryos, 22 µg total proteins loaded.
2. Embryos, 5.8 µg total proteins loaded.
3. Leaf, 5.8 µg total proteins loaded.
4. Embryos and leaf, 24 µg total proteins loaded.




A




B

Figure 9. Immunological detection of dehydrins in cotton leaves at various times during the light:dark cycle. Cotton seedlings were grown under light:dark cycle of 12 h light and 12 h dark. Leaves were collected and protein extracted at 6 h intervals. Proteins were fractionated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane and detected with antibody raised against the consensus sequence of dehydrin (A). Another membrane with the same treatments was detected with pre-immune serum (B). Equal amounts of total protein (11.5 μg) were loaded in each lane. The light period started at o h and the dark period started at 12 h.

1. Leaf 0 h.
2. Leaf 6 h.
3. Leaf 12 h.
4. Leaf 18 h.
5. Leaf 24 h.
6. Embryos.

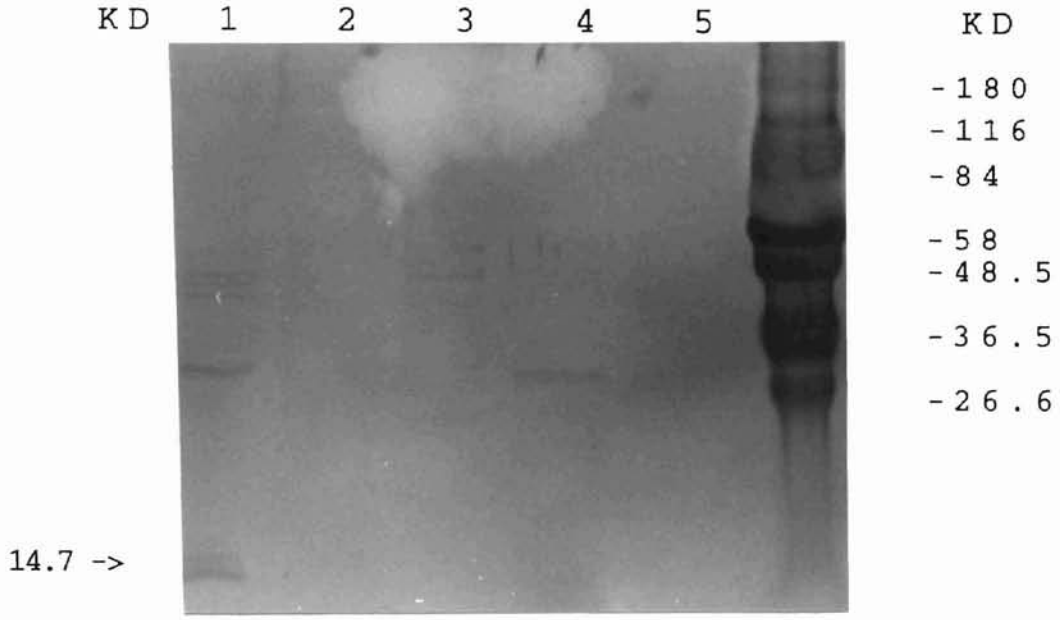



Figure 10. Immunological detection of dehydrins in leaves of cotton seedlings exposed to drought. Seedlings were exposed to drought by withholding watering for 7 days. Proteins were extracted and then fractionated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane and detected with antibody raised against the consensus sequence of dehydrin. Equal amounts of total proteins (7.4 μg) were loaded in each lane.

1. Embryos.
2. Leaf, non-stressed, 6th leaf above the cotyledons.
3. Leaf, stressed, 6th leaf above the cotyledons.
4. Leaf, non-stressed 3rd leaf above the cotyledons.
5. Leaf, stressed, 3rd leaf above the cotyledons.

# CHAPTER V

# DISCUSSION

## Role of Leaf Relative Water Content in Daily Changes of Chilling Resistance of Cotton Seedlings

Resistance to low temperature in plants can be regulated endogenously by a circadian clock [Couderchet and Koukkari, 1987; McMillan and Rikin, 1990] and externally by factors such as temperature and relative humidity [Levitt, 1980]. Cotton plants show daily changes in chilling resistance that are regulated by a circadian clock [McMillan and Rikin, 1990]. Cotton plants also show daily changes in transpiration that control daily changes in leaf RWC and leaf temperature. Therefore, an interesting question is whether these changes in leaf RWC affect or even regulate the daily changes in chilling resistance.

A major symptom of chilling injury is desiccation. The desiccation rate may be affected by the stomatal aperture at the beginning of chilling exposure. However, in cotton the stomatal aperture at the beginning of chilling exposure does not correspond to the degree of chilling resistance. This

lack of correlation excludes the possibility that the daily changes in stomatal aperture directly affect the daily changes in chilling resistance.

Leaf RWC changes during the light:dark cycle. Throughout all the light:dark cycle a period of low RWC precedes a chilling resistant period by 6 h and a period of high RWC precedes a chilling sensitive period by 6 h. Thus, the seedlings are chilling resistant at 12 h and 18 h and the RWC is low at 6 h and 12 h. On the other hand, the seedlings are chilling sensitive at 0 h (=24 h) and 6 h and RWC is high at 18 h and 0 h (=24 h) (Fig. 1 and Fig. 2). The daily decline in RWC may trigger gene expression, the products of which participate in development of chilling resistance. Many genes are turned on by both dehydration and acclimating low temperature [Thomashow, 1993; Close et al., 1993b]. The proteins encoded by these genes may have a role in increasing chilling resistance.

An increase of leaf RWC by high atmospheric relative humidity resulted in a decrease in chilling resistance at any time throughout the light:dark cycle. Despite this overall decrease in chilling resistance the basic daily pattern of chilling resistance is maintained. For instance, the lowest chilling resistance under high relative humidity at the middle of the night (the usual time of highest resistance) is still much higher than the chilling

resistance under low relative humidity at the middle of the day (the usual time of lowest resistance). This type of change indicate that RWC is not the only factor determining the level of chilling resistance and that the sensitivity affected by changes in RWC is superimposed on resistance regulated by other factors.

## No Role for Dehydrins in the Regulation of Chilling Resistance in Cotton Seedlings

The antibody raised against the consensus sequence of dehydrin [Close et al., 1993a] recognizes one major specific protein in the embryo of cotton. This protein is probably a dehydrin because it is detected specifically by this antibody and not by pre-immune serum. Also, it has a molecular weight of 15,000, very close to the molecular weight of the major dehydrin in the embryo of corn.

This antibody does not recognize any protein in mature cotton leaves of well-watered plants at any time during the light:dark cycle. Also, no immunological detection of dehydrin is observed in leaves of cotton plants subjected to drought, low temperature and application of abscisic acid. This lack of immunological detection of dehydrin indicates that dehydrins are not synthesized in leaves of cotton plants. Since dehydrins are not synthesized in cotton

leaves, my hypothesis relating dehydrin synthesis and accumulation to chilling resistance is not valid.

In general, current evidence is consistent with ubiquitous presence of dehydrins in all photosynthetic organisms [Close, 1996]. Therefore, an interesting question is why dehydrins are not detected immunologically in cotton leaves.

One possibility is that although dehydrins are synthesized in all plants, they are much more abundant in developing seeds especially at certain developmental phases. Also, in the mature vegetative parts of plants, dehydrins are relatively more abundant in stems than in leaves [Godoy et al., 1994]. When dehydrins are found in mature leaves of many species such as barley, wheat, maize and rice, their levels are low in well-hydrated leaves but they increase tremendously in dehydrated leaves [Close et al., 1993c]. Dehydrins are not detected in either hydrated, dehydrated or cold-exposed leaves of cotton. Therefore, it is most likely that dehydrins are not synthesized in cotton leaves. Since regulation of many genes by drought, low temperature and abscisic acid is common to most plants [Close et al., 1993c] the lack of synthesis of dehydrins in cotton leaf does not necessarily reflect a general non response of genes to these factors. Probably, proteins synthesized in cotton leaves as a response to drought, cold temperature and abscisic acid

are not recognized by this specific antibody against dehydrins.

Dehydrins are characterized by the conserved 15 amino acid sequence EKKGIMDKIKEKLPG, which is repeated from one to many times at the carboxyl terminus. They are also referred to as the Group2 LEA D-11 family of proteins[Close et al., 1993b]. These proteins usually accumulate under water deficit, low temperatures and exogenous application of ABA [Galau and Close, 1992]. Although all the D-11 proteins studied so far contain the consensus sequence in the carboxyl terminus, cotton D-11 is probably an exception [Galau and Close, 1992]. In cotton leaves, it is possible that in well watered seedlings and in seedlings subjected to water deficit, D-11 either remains unchanged or increases in response to the water stress but cannot be detected by the antibody raised against the 15 consensus sequence. This also explains why I cannot detect dehydrins under other conditions such as low temperatures and ABA application.

Since regulation of many genes by drought, low temperature and abscisic acid is common to many plants [Close et al., 1993c] the lack of synthesis of dehydrins in cotton leaf does not necessarily reflect a general non response of genes to these factors. Probably, proteins synthesized in cotton leaves as a response to drought, cold

temperature and abscisic acid are not recognized by this specific antibody against dehydrins.

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