SOLUBLE PROTEINS IN GERMINATING COTTONSEED, *GOSSYPIUM BARBADENSE*, AS RELATED TO CHILLING TOLERANCE

by

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ABSTRACT

This study took advantage of the technique of polyacrylamide gel electrophoresis to identify soluble protein banding pattern differences between chilling-susceptible and chilling-resistant cotton seedlings of two genotypes of 'Pima S-4' and E-2 (Gossypium barbadense L.).

Cotton seedlings were selected for damage after chilling stress was applied. The results of this study showed that there were no significant soluble protein level differences between susceptible and resistant cotton seedlings from each genotype after germination. However, differences were observed between injured and normal cotton seedlings of each genotype when treated with cold stress at a critical time period. The concentration of soluble protein in chill-treated seedlings was generally higher than untreated cotton seedlings at the same stage of germination. The reverse condition occurred when the radicle portion of seedlings was analyzed.

INTRODUCTION

Cotton (Gossypium) seedlings are very sensitive to low temperature. Seed that germinated and emerge during low soil temperature (5 to 10 C) are oftentimes damaged by chilling stress. A characteristic physical expression of chilling injury is apparent in many genotypes of cotton seedlings. Differences in injury between genotypes are often due to the degree of chilling stress applied. Chilling of cotton seedlings results in poor field stand formation and subsequent low yield. Replanting of a stand is uneconomical for a grower. In order to avoid severe damage and loss, attempts have been made to breed for new cotton varieties which are chilling resistant.

Extensive research by breeders at the cotton research center in Phoenix, Arizona and El Paso, Texas has produced certain varieties of Pima (Gossypium barbadense L.) cotton that are relatively resistant to chilling injury. Physiological and biochemical tests have also been developed to screen for cottonseed which exhibit chilling resistant qualities. However, the mechanisms involved in chilling injury are not well understood.

Proteins are the recognized end product of a complex gene function which involves both DNA and RNA. The activity of many important plant enzymes, some of which are in the soluble protein fraction, have been noted to decrease when temperatures deviate from an optimum to a minimum value. As a result certain metabolic activities critical to

plant growth and development are often reduced. Therefore, the analysis of soluble proteins from cotton seedlings at different developmental stages and grown at several temperature regimes could be an important factor in fostering an understanding of the chilling injury phenomena. By using polyacrylamide gel electrophoresis, it may be possible to compare banding patterns of soluble protein from chill-resistant and chill-sensitive cotton seedlings. Therefore, the objectives of this study are as follows:

1. To determine if there are any differences in soluble protein banding patterns between susceptible and resistant cotton seedlings.
2. If no differences are observed, determine if qualitative and quantitative differences are evident after the application of chilling stress to cotton seedlings.

LITERATURE REVIEW

Soluble Proteins

For over a century proteins have been arbitrarily classified by their variant solubilities in aqueous systems. Soluble protein, globular in nature, consists of an albumin and globulin fraction which are soluble in a weak salt solution (Lehninger, 1972). In general, most enzymes in a dormant seed belong to this group. They usually have a specifical biological significance, such as the activation of respiration, metabolism of proteins, fats and carbohydrates.

Denis (1899) first demonstrated that proteaceous material could be extracted from various seeds with 10% aqueous NaCl solution. Northrop (1930) demonstrated that enzymes were proteins which served as catalysts for biochemical reactions in biological systems. Generally, a sequence of chemical reactions within a cell cannot be completed if specific enzymes are not present or inactivated.

Protein Synthesis in Germinating Seeds

Protein synthesis appears to be one of the earliest processes activated in a germinating seed (Chen, Sarid and Kalchalski, 1968; Marcus, 1969). In wheat, protein synthesis begins immediately following 10 min. of water uptake while DNA synthesis is still undetectable (Marcus, Feeley and Valcani, 1966; Mory, Chen and Sarid, 1972). In cottonseeds, protein synthesis cannot be inhibited during the first three days of germination; even detectable RNA synthesis is inhibited

by actinomycin D (Waters and Dure, 1966; Merrick and Dure, 1972). Protein synthesis in an early germinating seed is, however, not de novo; the synthesis of enzymes is present in the cotyledon which in turn is transcribed during embryogenesis, but inhibited by abscisic acid and reactivated during germination (Ihle and Dure, 1969).

Marcus, Feeley and Valcani (1966) divided protein synthesis in a germinated wheat embryo into two phases. One occurred directly upon imbibition of water, the other was triggered by DNA and RNA replication. In the first phase, there is no cell division actually (Ihle and Dure, 1972), but more polyribosomes and more activated amino acids have been detected (Marcus and Feeley, 1965), as more proteins were succeedingly synthesized. In fact, 25% of the total protein in a mature cottonseed was synthesized at this time as a storage protein (Merrick and Dure, 1972). Walbot, Capdevila and Dure (1974) found polyadeylic acid region (poly-A) or heterogeneous nuclear ribonucleic acid (hn RNA) in germinated cotton seedling which presumably represents a presynthesized messenger ribonucleic acid (m-RNA) that is transported from the nucleus to the cytoplasm for function. These proteins synthesized from stored m-RNA are some storage proteins while some are specific enzymes which are used during the seed germination process (Ihle and Dure, 1972). These storage proteins are broken down to peptides and amino acids in order to translocate into developing embryos for resynthesis (Subramanian, 1974). At phase two, DNA and RNA have been activated by the preliminary work of phase one, i.e., protein synthesis followed by transcription and translation. Activation of

amino acids with ATP, catalyzed by aminoacyl-tRNA synthetase and the concomitant formation of a specific aminoacyl-tRNA has been demonstrated by Vanderhoff, Bohannon and Key (1970). Transfer RNA is primarily involved in the first reaction in the assembly of protein being responsible for the proper translation of the sequence of bases in m-RNA into prescribed order of amino acid in protein in soybean hypocotyl (Vanderhoff, Bohannon and Key, 1970). The binding of the initiating met-tRNA took place on the 40 S ribosomal subunit (Seal, Bevley and Marcus, 1972). During the process of peptide bond formation four soluble factors were found in the wheat embryo. Two of these factors, designated C and D, are initiators similar to F factors in procaryotes, which are required for amino acid polymerization and the formation of the messenger-ribosome initiation complex. The mechanism of peptide chain termination in germinating seedlings has not been investigated; it is, however, well known in other eucaryotes from studies in yeast and rats (Tate and Caskey, 1974) that peptide chain termination occurs on the ribosome and required a soluble protein factor(s) R.F. which recognized the terminator codons.

Chilling Tolerance and Cold Hardiness

As early as 1897, Molisch suggested that low temperature damage in the absence of freezing should be called chilling injury as opposed to frost injury. The former generally occurs at temperatures above zero (5 to 12 C), while the latter is often noted at subzero temperatures (-2 to -8 C; Weiser, 1970). This generalization does not apply

in all cases, however, in that some species may differ in chilling tolerance depending upon their region of origin. For example, the low temperature limit for no chilling damage is 12 C for cotton seedlings as compared to 5 C for cucumbers.

Plant physiologists infer that damage by frost is due to the formation of ice crystals in plant tissues (Levitt, 1956). Asahina (1956) suggested that damage is mainly by desiccation of cells as water is withdrawn while ice is being formed. While the mechanism of chilling injury has not been well understood, several possibilities have been proposed. Molisch (1897) proposed a stress in the balance between physiological processes; Lieberman, Craft, Avdia and Wilcox (1958) speculated that a large increase in the permeability of the plasma membranes takes place, and Lyons and Raison (1970) suggested that phase transations in the physical state of certain cell membranes may be involved.

Generally speaking, cold hardiness is referred to as the resistance of plants to freezing; that is, they are able to survive in extremely low temperatures for longer periods of time (Levitt, 1956). Chilling tolerance, on the other hand, is often associated with plants from tropic and subtropic regions.

Chilling Injury and Protoplasmic Streaming

Lambers (1925) observed that the rate of protoplasmic streaming is closely related to temperature within a definite range. Lewis (1956) found that streaming ceased or nearly ceased after one or two minutes at 10 C in chilling sensitive plants. Seifriz (1943) also

pointed out that low temperature treatment will bring about a cessation of streaming.

Even though the real mechanism of streaming has not been delineated, respiration and growth require effective cellular streaming. Research has shown that protoplasmic streaming requires energy. Stewart and Guinn (1971) observed that cotton seedlings chilled at 5 C showed a continual decrease in ATP concentration with time of chilling.

Chilling Injury and Membrane Phenomena

Recently it has been shown that low temperatures induce phase changes in cell membranes from sensitive species (Luzzati and Husson, 1962). The cell membrane changes from a flexible liquid-crystalline to a solid-gel structure at 10 to 12 C.

Evidence has shown that chilling susceptible plants and animals have a higher concentration of saturated fatty acids associated with their lipids than resistant counterparts (Lyons and Asmundson, 1965; Richardson and Tappel, 1962). Membranes with a higher degree of unsaturated fatty acids are more flexible in comparison to the saturated analogues. Lyons and Asmundson (1965) showed that a change of as little as 5% in the amount of unsaturated fatty acids in artificial mixtures of fatty acids would change the temperature of solidification of the lipid mixtures by 15 C.

The mechanism postulated by Lyons (1973) suggested that the membrane liquid will solidify at a critical temperature and that the resulting change in membrane state would bring about a contraction which causes cracks or channels. This would lead to increase

permeability and cessation of protoplasmic streaming. These facts are able to explain somewhat the phenomenon of "discontinuity" in an Arrhenius temperature plot (Raison, 1973; Kumamoto, Raison and Lyons, 1971).

Chilling Injury and Protein Metabolism

Many workers have observed that protein synthesis and enzyme activity are often correlated with chilling damage. Guinn (1971) showed that chilling decreased RNA, protein and lipid phosphate in cotton seedlings. Stewart and Guinn (1969) indicated a decline in ATP and other nucleotides as well. Mohapatra, Smith, Fites and Noggle (1970) showed that the primary effect of chilling injury on cotton seedlings is a depression of isocitratase activity as well as retardation of lipid utilization. Quimio and Noggle (1971) showed that two hours of chilling of whole cotton seedlings at the initial phase of germination showed alteration in the isozymic patterns of the total protein, glutamic glucose-6-P, lactic and malic dehydrogenases. On the other hand, Pierre and Lascombers (1971) noted that the low temperature did not result in the disappearance of any of the initial bands of the protein banding patterns of Bryophyllus diagramontianum. Quantitative changes in these patterns, however, were observed. Thormason (1970) pointed out that the basic protein variation is an indication of physiological difference in barley seedling. Furthermore, evidence from Hedera helix showed protein from cold acclimated tissue exhibited a higher sugar binding capacity than protein from non-acclimated tissue (Steponkus, 1971).

Since Arrhenius plots for enzyme activity showed a discontinuity or break at 0 C, an important consideration is that the phase change in the reaction components may be determined by the conformational changes in enzyme proteins which are mediated through a phase change in the membrane lipids (Kumamoto, Raison and Lyons, 1971). Evidence from animal studies, on the other hand, showed that the change in discontinuity of Arrhenius plot probably corresponds more with the change in activation energy of enzymes than the phase transition of membranes (Raison, Lyons and Thomson, 1970; Raison and Lyons, 1971).

The association of ATPase with membranes suggests that configurational changes in this enzyme are associated with chilling injury and death. Although the available data established membrane lipid as the locus of temperature sensitivity, it does not provide any direct evidence to indicate whether all membrane lipids undergo phase transitions or only the membranes associated with enzymes (Raison, 1973). Therefore, chilling damage as related to enzyme activity, conformation and kinetics are still not well understood.

Chilling Injury and Genetics

Some research has been undertaken to determine if genetic variation exists in a seed moisture chilling relationship. Christiansen (1972) suggested that variation in resistance to hydration chilling injury is a heritable factor. Ivanov and Valchev (1971) studied cold resistance and downy mildew resistance in interspecific hybrids of vines. They found F_1 progenies from crosses Vitis amurennis with

varieties of Vitis vinifera cultivars have a high degree of cold resistance.

Significant differences have been shown for the unsaturated/saturated fatty acid ratio between susceptible and resistant cotton seedlings (Bartkowski, 1974). Ponelut (1972) postulated that a single gene in the opaque-2 locus was found to control 10 to 15 percent of the linoleic acid in the germ oil of maize. Thus, the unsaturated/saturated fatty acid ratio may be under explicit genetic control.

MATERIALS AND METHODS

Genotypes Compared

Two different genotypes of Gossypium barbadense L. were used for the analysis of soluble proteins in germinating cotton seedlings. These are E-2, an experimental line developed by USDA researchers, and 'Pima S-4,' a released cultivar (supplied through the courtesy of C. V. Feaster and E. L. Turcotte, Research Agronomists, ARS, USDA, Phoenix, Arizona). They were collected from replicated yield trials at Safford, Arizona in 1972. The elevation of Safford is at 890 m and the average temperature in spring, summer, and autumn in 1972 were 17.98 C, 29.24 C, and 17.77 C, respectively (Water Bureau Climatological Data, Arizona 76). Seeds were acid delinted (Bartkowski, 1974) and selected for undamaged seed coat.

Sterilization

Fifty seeds of each genotype were sterilized in a 4:1 ratio of water:chlorox and stirred about two minutes. They were placed into a strainer and washed with tap water.

Conditions of Germination

The sterilized cottonseed were arrayed into two lines on a wet germination paper, rolled and put into a 1,000 ml beaker filled with 500 ml of water. These beakers were covered with a glass jar to insure constant moisture. The seeds were incubated in a growth chamber at

34 C for 24 hours, 36 hours, and 48 hours. Each experiment was replicated at least twice.

Chilling stress was applied to cotton seedlings in a similar manner. Seeds were incubated in the growth chamber at 34 C for 24, 30, 33, 35, and 42 hours. They were then placed in a cold, dark chamber at 4 C. Seedlings were chilled for 24 hours and then returned to the growth chamber set at 34 C for another 24, 18, 15, 13 and 6 hours of incubation respectively.

Extraction of Soluble Proteins

Seedlings were harvested and measured for radicle length. Seedlings with intermediate radicle length were used for extraction purposes. The protein extraction was divided into three parts for analysis. First, the entire seedling extraction; second, radicle extraction; and third, cotyledon extraction. Seed coats were removed from the seedlings. The seedlings were cooled at 4 C about 10 minutes. All the extractions were carried out at 4 C. Weighed seedlings were ground in a mortar and pestle with Tris-glycine buffer (pH 8.3, 0.01 M). The ratio of extracting solution to plant material was 2:1 (v/w) for the entire seedling and cotyledon and 1:2 (v/w) for radicles.

The crude extracts were partially purified by centrifugation for 10 minutes at 4300 g in a Sorvall refrigerated centrifuge at 4 C. The supernatant fraction (pH 6.4) was adjusted to pH 8.3 with concentrated Tris-buffer solution. 2-mercaptoethanol was added to 0.001 M before recentrifugation for 60 minutes at 79,100 g (36,000 rpm), on a 50 sw rotor in a refrigerated Beckman Model-L centrifuge. The middle

layer of the supernatant fraction was then drawn off with a pasture pipette and subjected to electrophoresis. Protein content in the supernatant was measured by the method of Lowry et al. (1951). The amount of soluble proteins used in each gel column are 230-153 µg for the entire seedling, 132-110 µg for the cotyledon, and 280-210 µg for the radicles. The standard curve of soluble protein concentration is based on bovine serum albumin.

Enzymes Extracted from Seedlings after Cold Stress

Seedlings were harvested after the 72 hours germination with 24 hours chilling stress given between 35 to 59 hours time period. The harvested seedlings were divided into three groups: the chill tolerant; morphologically by changed or damaged chill sensitive seedlings; and controls (non-chill treated). The determination of a chilling tolerant seedling was made by comparison to a control one; a chilling sensitive seedling is dependent on whether the seedling is injured and transparent in the middle part of the radicle, or injured at the apical tip. Undamaged and damaged seedlings were selected for maximum length and analyzed separately as the entire seedlings, cotyledons and radicles.

About 10 selected hulled seedlings and cotyledons were weighed, seed coat(s) were then removed from seedlings and cotyledons, and placed in the refrigerator for about 10 minutes before extraction. The ratio of extracting solution to seedlings and cotyledons was 2:1 (v/w). Twenty-five to 30 selected radicles were also weighed and

placed in the refrigerator for about 10 minutes before extraction. The ratio of extracting solution to radicles was 1:2 (v/w). Weighed seedlings, cotyledons and radicles were ground with Tris-glycine buffer (pH 8.3, 0.01 M), and centrifuged at 4300 g in a Sorvall refrigerated centrifuge for 10 minutes. The procedure of protein extraction then followed exactly the same as noted for the controls.

Polyacrylamide Gel Electrophoresis

A Canalco Model 1200 electrophoretic apparatus and a Beckman Model Rd-2 Duostat power source were used. The method of preparation of the gel and the electrophoretic analysis was similar to that of Davis (1964), but with three specific modifications. These were (1) the pH value of the running buffer solution used was 9.5 and 0.05% 2-mercaptoethanol was added, (2) the voltage started at one milliampere (mA) per gel tube as it went through the stacking gel and increased to two mA per sample when the bromophenol mark moved down the acrylamide gel, and (3) the gel tube was 7.5 x 12 cm.

The gels were stained 15 minutes with Buffalo Black NBR (one gm of NBR added to 100 ml of 7% acetic acid) immediately after the run. The staining of the gels was followed by soaking with 7% acetic acid with frequent changes, for one or two days in order to remove excess stain from the gels. The results were photographed for permanent records.

Gel Scanning Chromatography

The destained gels were scanned at 560 nm using a Gilford spectrophotometer Model 240 equipped with an automatic absorbance recorder. The ratio switch position is 0.1, while the ratio of absorption is 1.5. The graph has been condensed into one half of its origin.

RESULTS

A conclusive determination of whether soluble protein extracts exhibit any noticeable or significant differences in electrophoretic banding patterns between chill resistant and susceptible varieties of cotton depends to a large extent upon the elimination of several variables which might confuse the final results. The first of these is the electrophoretic assay itself. Even though acrylamide gel electrophoresis has been used quite extensively and with a good degree of success on soluble protein extracts from animal tissues and microbial organisms, the same degree of resolution is often lacking in similar investigations on higher plants due to the occurrence of less discrete bands against a more diffuse background. One of the main reasons for this anomaly can be traced to the various heterogeneous isozymes often found in many diploid plants as opposed to a lower haploid type of plant (Steward and Barber, 1964). Another reason is that a given extraction method, successful for one type of plant, viz., tobacco, must be modified or altered to suit another class of higher plant. The method of extraction developed for this present investigation was a combination and modification of several previously developed methods for cotton (Van Loon and Van Kammen, 1968; Cherry, Katterman and Endrizzi, 1970). It was noted that when these methods were used individually and without modification only a few distinct bands were produced. Resolution of the bands was also increased when the running

was adjusted to a pH of 9.5 along with a voltage of 8.5 volts per gel tube.

Another significant source of error could be that of environmental influence on seed development. It is conceivable that seed lots from different areas of Arizona exhibit differences in protein banding patterns just as found for a wild uncultivated species of cotton (Cherry and Katterman, 1971). Figure 1 indicates a slight but noticeable difference in banding patterns between Pima S-4 seed lots from the Safford and Wenden-Salome areas of the state. Note a band in the 7 cm region of the depicted protein band pattern from the Wenden-Salome area that is not present in the Safford figure. Both of these seed growing areas represent two extreme types of environment with respect to altitude and temperature differential during the growing season. Therefore, comparisons of chilling treatment between varieties was limited to one region of seed cultivation and production, namely that of Safford, for this investigation.

Developmental stages of seedling growth can also be related to significant changes in quantitative amounts as well as with changes in the soluble protein banding pattern (Bhatia and Nilson, 1969). This fact has a direct bearing on the present work in that the average length of radicle elongation for the chill resistant variety E-2 is slightly shorter at a given time period of germination than that of the chill susceptible S-4 variety (Table 1). It was imperative therefore to compare the protein banding patterns of these two varieties at selected time periods of germination to determine if the difference in

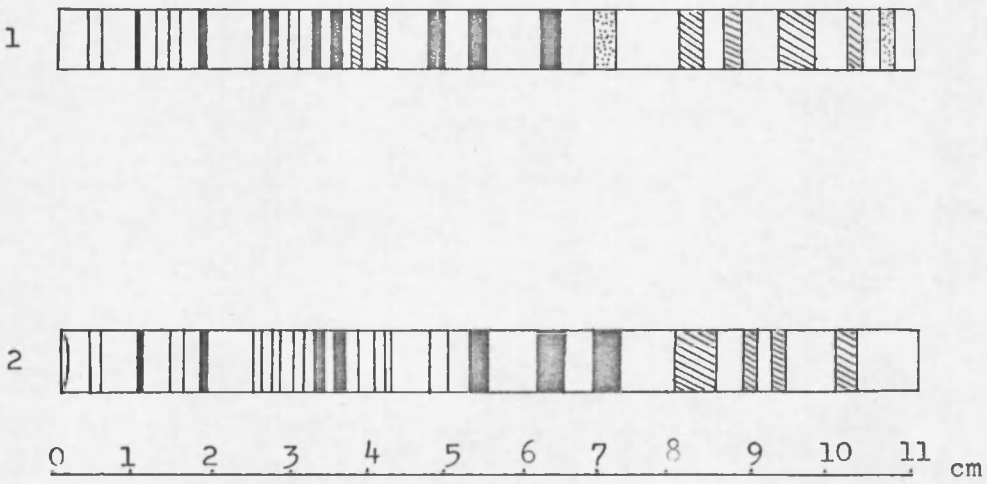

Figure 1. Zymograms of soluble protein electrophoretic patterns from 'Pima S-4' of 36 hours germination of different seed lots between Safford (1) and Wenden-Salome area (2).

Table 1. Data from each gel column.

Gel Column	Number of Seedling		Total Weight		Average Length	
	S-4	E-2	S-4	E-2	S-4	E-2
I.	10	12	2.4 gm.	2.7 gm.	12.2 mm.	9.6 mm.
II.	15	13	4.2	3.7	41.9	35.0
III.	15	12	5.5	4.2	89.4	64.6
a.	16	25	7.5	6.1	40.1	21.4
b.	17	15	4.8	3.9	25.5	21.2
c.	25	32	2.9	1.9	54.3	22.6
d.	21	32	1.9	2.1	48.7	22.5
e.	8	32	2.4	4.1	36.1	22.6
f.	8	32	2.3	4.1	30.1	22.5

Note: Columns I, II, III represent materials from 24, 36, and 48 hour germinated S-4 and E-2. Columns a, b, c, d, e, f represent materials from each normal and damaged chilled seedlings on Figures 6 and 8.

the rate of radicle growths of these varieties would produce different gel patterns at a given time of seed germination. Figure 2 shows that the soluble protein bands are the same for both varieties at all time periods except those of E-2 at 48 hours of germination. The latter variety has one more band at the 2.4 cm mark than the 48 hour gel of S-4 (Figures 2 and 3). Note that the protein bands decrease in number and intensity for both the total seedling and excised radicles (Figures 2 and 4) as the germination period progresses. Previous work showed that the protein bands of the dormant seed were even more numerous and greater in intensity than that shown for the 24 hours germination period in Figure 2 (Cherry, Katterman and Endrizzi, 1970). Although the foregoing results indicate that protein banding pattern changes are associated with seed germination, the radicle length differences between the two varieties as noted in Table 1 were not enough to elicit protein banding patterns that uniquely differ from one variety to the other at a given time of seedling germination. Thus, a direct comparison of chill treatment between both varieties with respect to soluble protein banding patterns at a given time of seedling development is entirely feasible.

With the clarification of the variables enumerated above, the next step was then to determine which time period after the onset of germination was the most favorable for the expression of morphological damage due to chilling. It was learned previously that such a susceptible period for the cultivated hirsutum short staple cotton was between 30 and 40 hours of germination with the most critical time

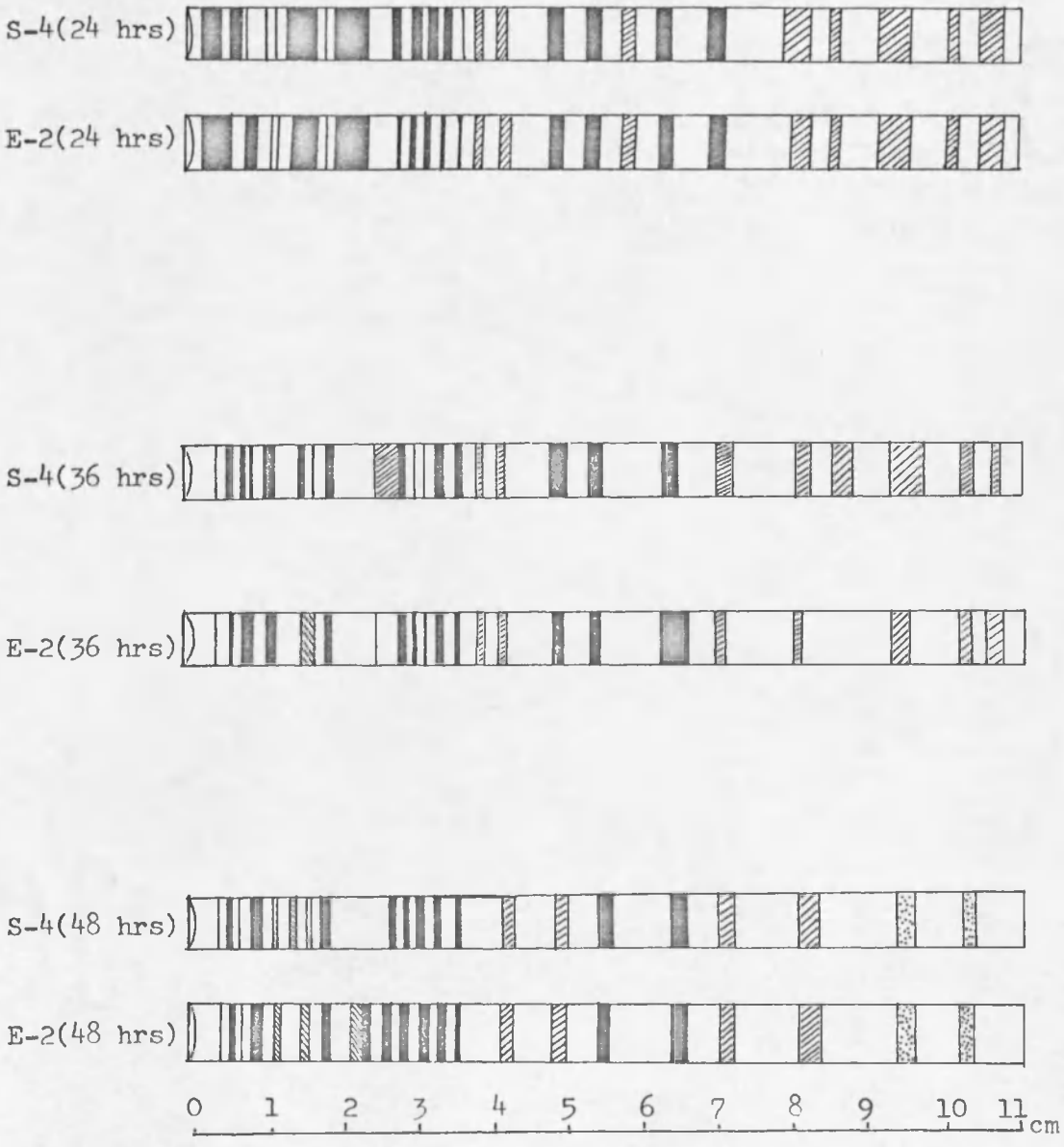

Figure 2. Zymograms of soluble protein from 24, 36 and 48 hours germination at 34 C.

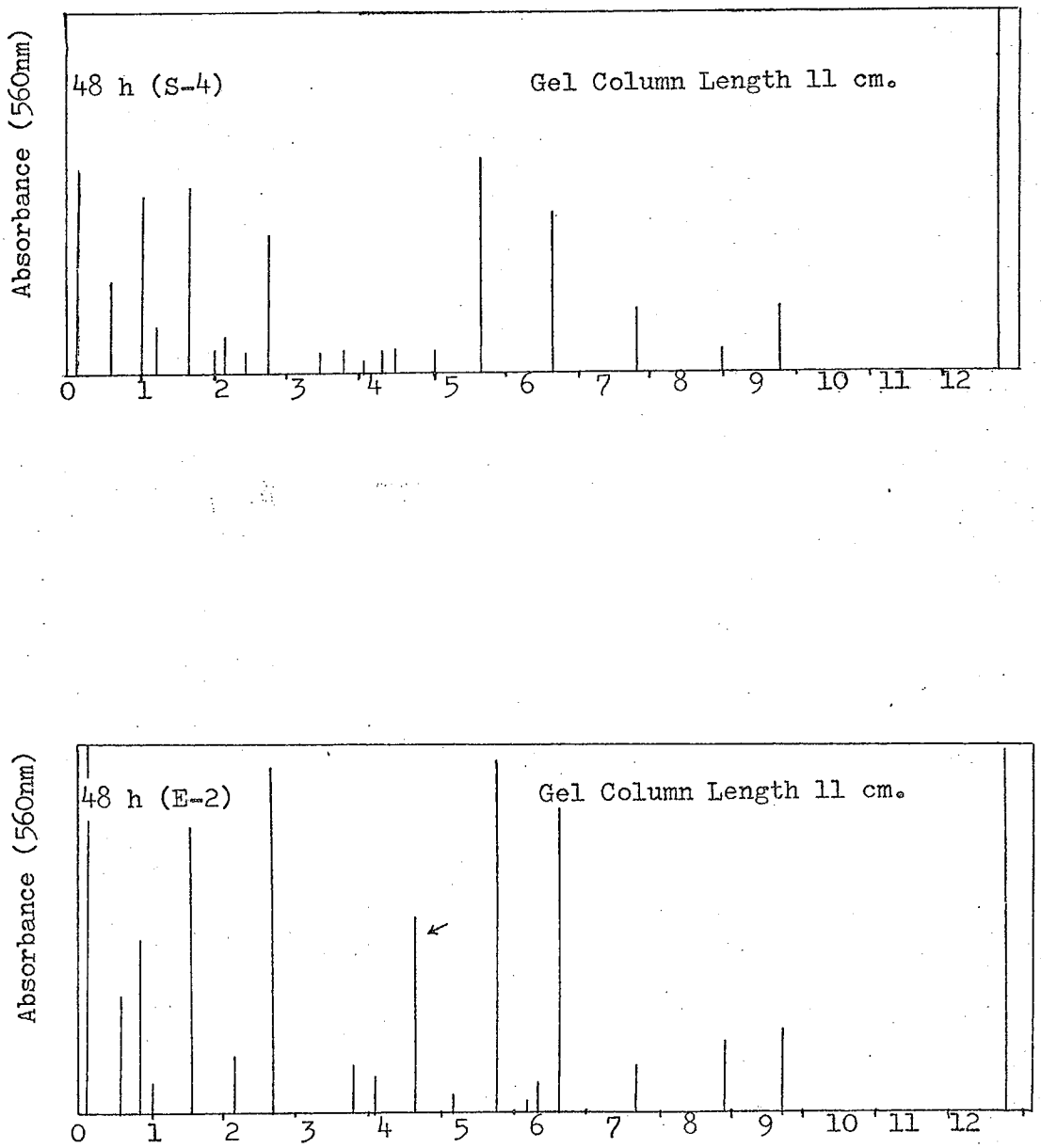

Figure 3. Chromatography of soluble proteins from 48 hours germination at 34 C. (The arrow means one more band in E-2 than the 48 hour gel of S-4.)

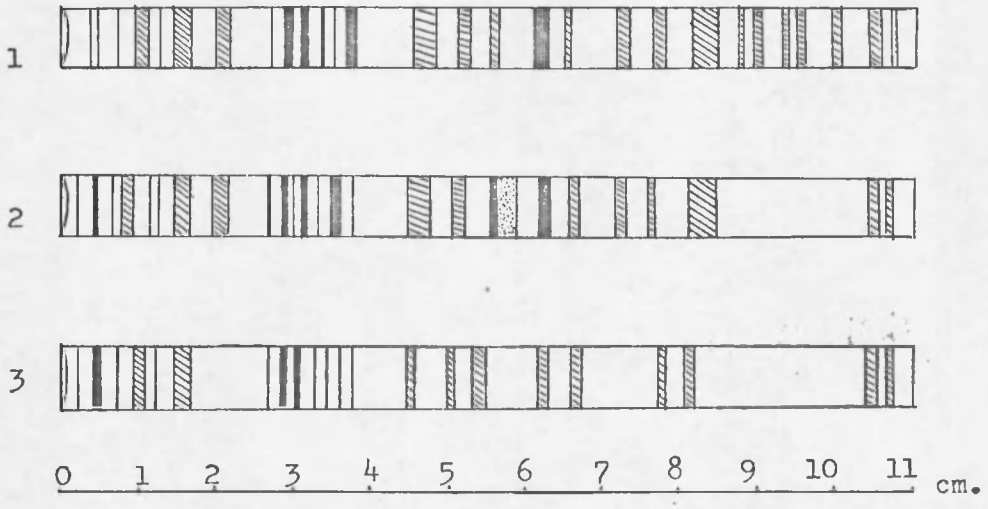

Figure 4. Zymograms of soluble proteins of 'Pima S-4' radicles from various hours of germination at 34 C.

1. 24 hours germination.
2. 36 hours germination.
3. 48 hours germination.

period of seedling damage at 36 hours (Christiansen, 1967). Using these results as a guide for long staple barbadense cotton, seeds were germinated at 24, 30, 33, 35 and 42 hours. At the termination of each germination period, the developing seedlings were subjected to a 24 hour period of chilling at 5 C as described in the methods section. At the end of the stress period the seedlings were placed once more at incubation temperature until a total time, including the chill stress period, of 72 hours had elapsed. The results of Table 2 show that the seedlings exhibiting the most injury were those exposed to a chill stress after 30 or 35 hours of germination at the normal incubation temperature. The same level of chilling injury was observed for both the S-4 and E-2 varieties. The type of injury on the extended radicles can be seen in Figure 5. These results are in essential agreement with those observed for short staple cotton.

In order to avoid the effect of developmental stages on protein content and banding patterns within a single batch of germinating seedlings, it was necessary to select only those seedlings whose radicle lengths, damaged and undamaged, were both uniform and represented the majority of the seedling radicles during a particular period of germination. As a result, the percentage of chill damaged seedlings in Table 2 for the critical time periods of both varieties was lower than if all of the germinating seedlings of varying lengths were included. The results of this table also indicate that both varieties exhibited the same amount of chilling damage. Previous work (Clay and Katterman, 1975) revealed that a shorter exposure time (about 5 hours) and a

Table 2. Percent of damaged seedlings at 72 hours germination with 24 hours chilling stress after various hours germination time period.

Time of Treatment (hour)	% of Total Damaged Seedling		% of Mature Damaged Seedling	
	S-4	E-2	S-4	E-2
24	28.57	27.45	9.5	15.68
30	36.95	20.33	21.73	6.77
33	28.0	14.03	10.54	6.77
35	25.0	40.62	14.28	20.31
42	0	0	0	0
24	21.77	13.51	8.06	5.6
30	25.49	31.48	15.68	16.66
33	22.22	23.07	9.87	16.92
35	26.53	30.50	22.44	20.33
42	0	0	0	0

Figure 5. Damaged seedlings from 'Pima S-4' of the 72 hours germination which included 24 hours cold treatment. (Arrows indicate the injured portion of damaged seedlings.)

milder chilling temperature (10 to 15 C) were necessary to bring out observable differences in seedling damage between the chill resistant versus the chill susceptible varieties. In the present investigation, it was necessary to use a more severe regime of chilling time and temperature to obtain enough damaged seedlings for soluble protein extraction under the restrictions of uniform seedling lengths mentioned previously. Apparently the resistance to chilling damage is a quantitative rather than a qualitative genetic effect and reaches a limit with respect to severity of applied chill stress.

In addition to the scaling effect of seedling damage within respect to lowered temperature and time of treatment, it can be seen in Figure 2 that the banding patterns of the 36 hour period for both varieties are essentially identical. Since the 36 hour period represents the optimum time for a maximum amount of radicle damage, it is most likely that any qualitative differences noted in the soluble protein banding patterns between the susceptible and chill-resistant varieties under a more permissive stress regime would also be noted between the damaged seedlings of a seed lot from either variety under the more severe stress application of this present investigation. Thus Pima S-4 seeds were germinated at 34 C for 36 hours, then chilled at 4 C for 24 hours and finally germinated for 12 hours at 34 C before the soluble proteins were extracted and analyzed quantitatively as well as qualitatively for both damaged and undamaged seedlings.

The quantitative levels of total soluble proteins between damaged and whole seedlings from a stress-treated seed sample were

nearly identical. Upon comparison with control seedlings of approximately the same radicle length however, it was found that the stressed seedlings contained significantly more soluble protein than that of the controls (Table 3). Analysis of the radicles from both the control and treated seedlings revealed that there was more soluble protein in the radicles of the control or non-chill-treated seedlings. Apparently, the transport of soluble proteins from the cotyledons to the growing radicle in chilled-treated seedlings is impaired to some extent at a particular time period of germination.

The protein banding patterns, as shown by acrylamide gel electrophoresis, were compared for both undamaged and damaged chilled seedlings. Figures 6, 7, 8 and 9 show that these banding patterns for both types of chilled seedlings are nearly identical. Subsequent comparison of banding patterns were alike. The possibility that small differences such as the quantity of each individual could be present in both types of seedlings (Figures 7 and 9) was then investigated since DNA snythesis has been shown to reach a maximum in the radicle during the 36 hour time period (Katterman and Clay, 1975). Again, as seen in Figures 6 and 8, no differences could be noted between the radicles of the damaged and undamaged chilled seedlings. Thus, the principle effect of chilling temperatures on the soluble protein fraction of germination cotton seedlings appears to reside in the total amount rather than class of protein available to the developing radicle during a certain stage of growth in respect to chilling damage.

Table 3. Comparison of protein concentration from cold-treated to untreated longest S-4 seedlings and radicles.

	With Chilled (µg/ml)	Without Chilled (µg/ml)
Radicles	87.5 ± 1.5	95.63 ± 1.5
Seedlings	370.0 ± 2.5	305.00 ± 2.5

Optical Density (O.D.): 600 mu.
Standard Curve: by using bovine serum albumin.
Ratio: 1:2 (W/V).
Average length of chilled longest seedlings and radicles are 74.54 mm and 69.54 mm.
Average length of untreated longest seedlings and radicles are 101.70 mm and 95.77 mm.

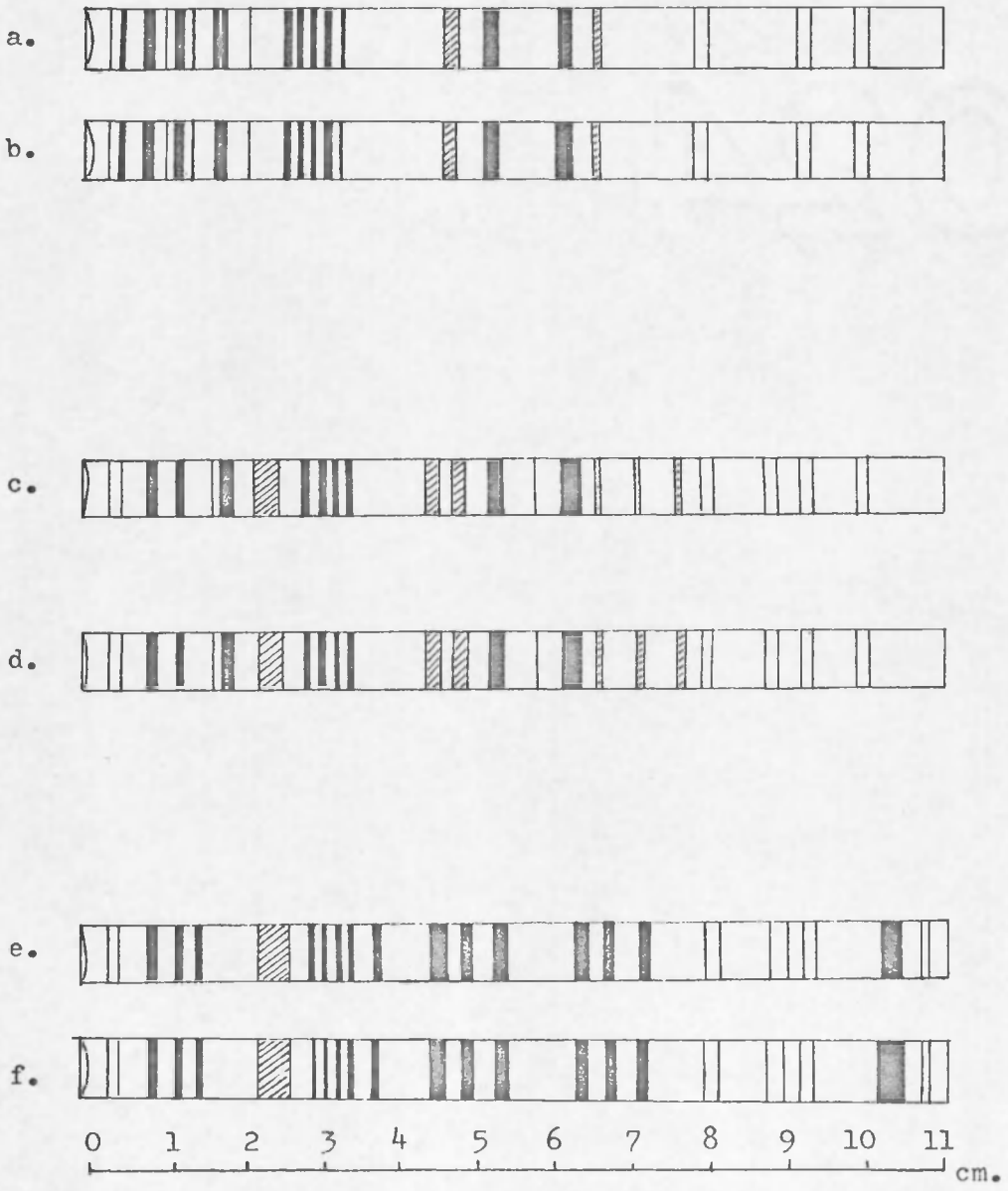

Figure 6. Zymograms of soluble proteins from 'Pima S-4' of the 72 hours germination with cold treatment.

a. normal seedlings
b. chill damaged seedlings
c. normal radicles
d. chill damaged radicles
e. normal cotyledons
f. chill damaged cotyledons

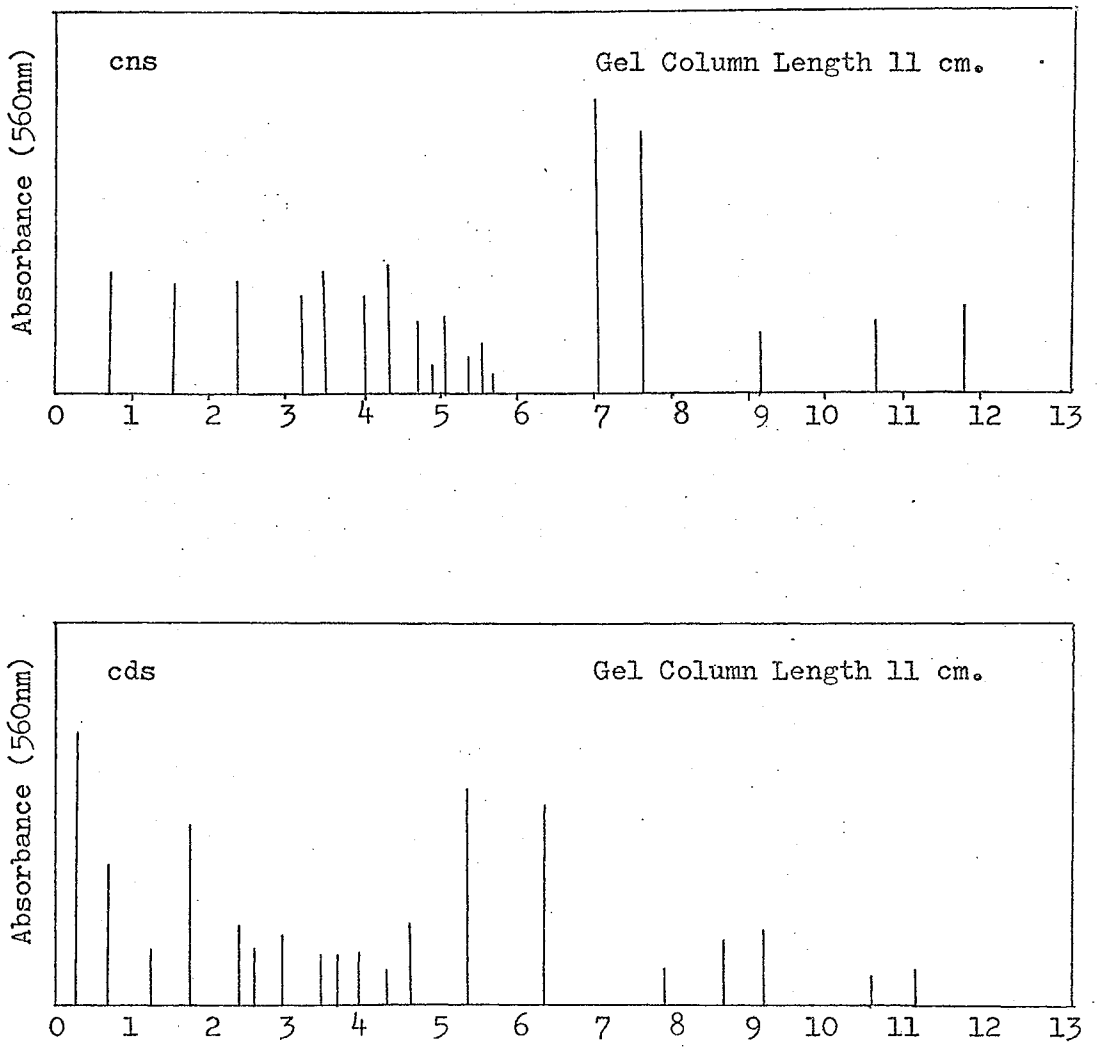

Figure 7. Chromatography of soluble proteins from chilled injured and normal seedlings of 'Pima S-4'.

cns means chill normal seedlings.
cds means chill damaged seedlings.

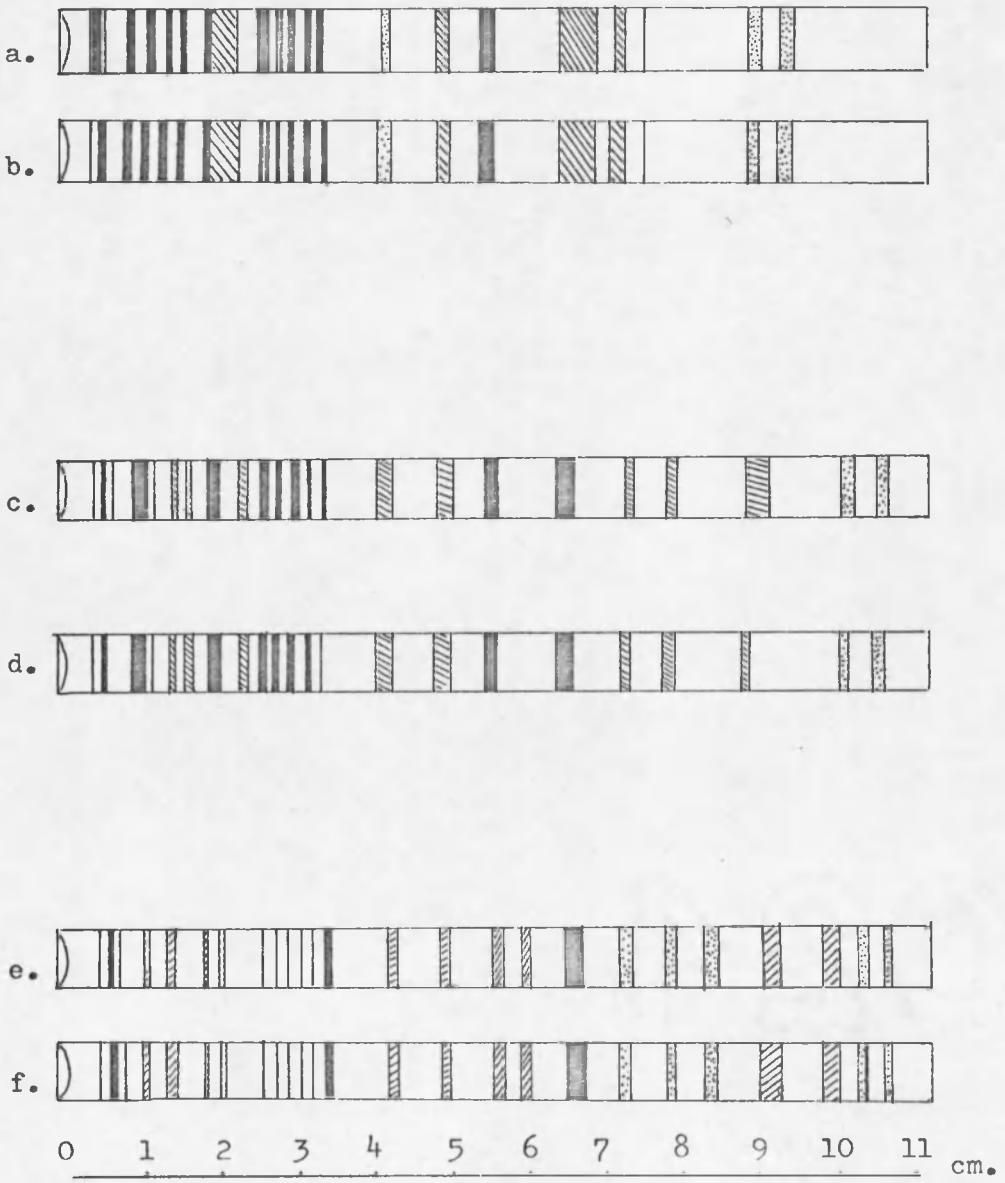

Figure 8. Zymograms of soluble proteins from E-2 of the 72 hours germination with cold treatment.

a. normal seedlings
b. chill damaged seedlings
c. normal radicles
d. chill damaged radicles
e. normal cotyledons
f. chill damaged cotyledons

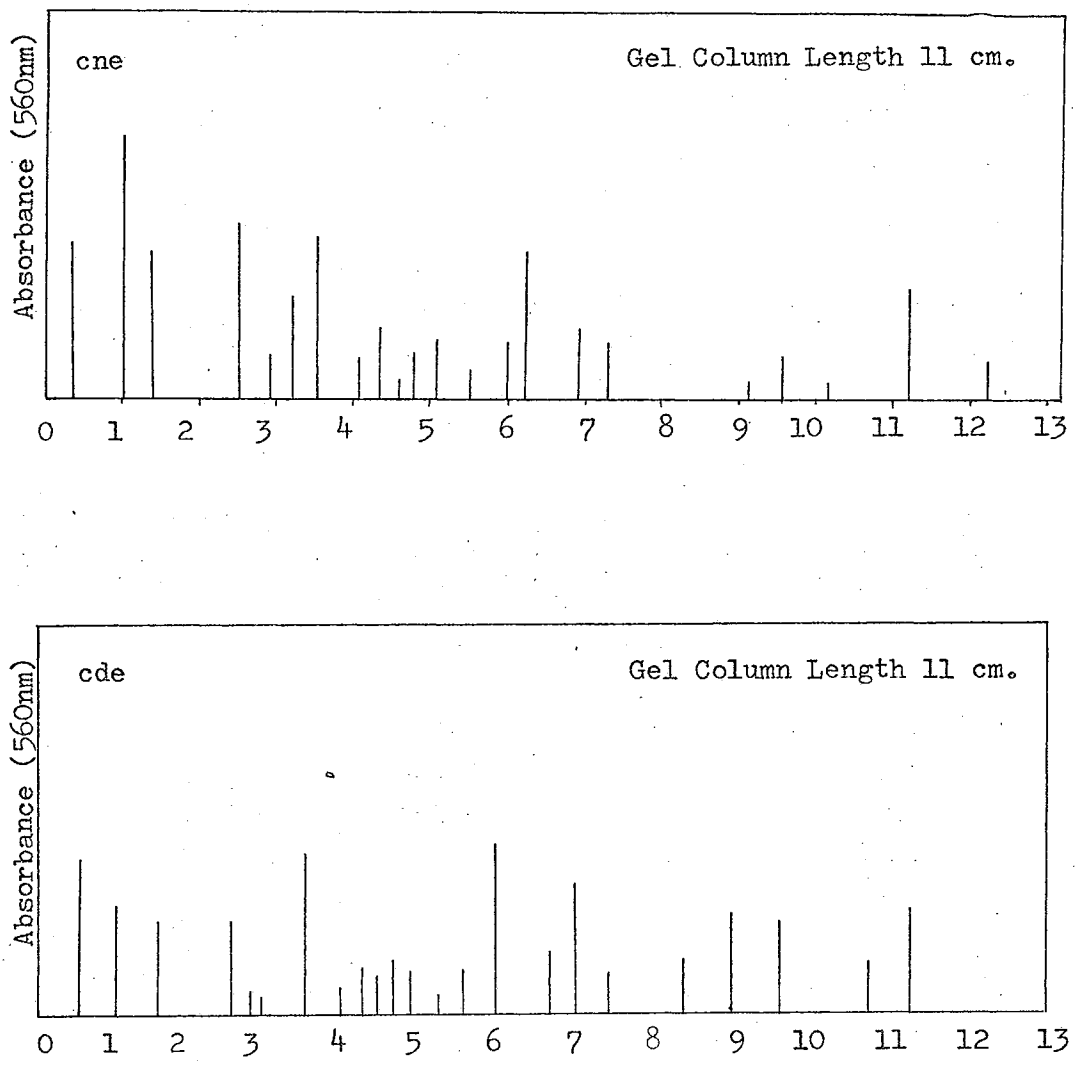

Figure 9. Chromatography of soluble proteins from chilled injured and normal seedlings of E-2.

cne means chill normal E-2
cde means chill damaged E-2

DISCUSSION

The exact reason for the observed relationship between the degree of seedling injury and time period of stress application is as yet unclear. Of several possible explanations, the following appears to be the most plausible as to the most direct cause. It can be noted from Figure 5 that the area of injury for most of the seedlings appears to be localized. When the temperature drops to 4 C for 24 hours there may be temperature induced phase changes in the cellular membranes that result in a solid gel instead of the normal flexible liquid-crystalline structure (Kumamoto, Raison and Lyons, 1971). Earlier investigations on DNA replication in the radicle tip demonstrated that the latter was membrane bound during the germination time period between 36 to 42 hours (Clay and Katterman, 1975). It is most likely that the observed injury by means of chilling may be due to an irreversible physical change in the membrane attached to the replicating DNA which in turn would alter the normal process of cellular division in the chilled meristimatic tip. In spite of the irreversible damage there are probably some of these meristimatic cells which survive the effects of the temperature induced phase change of the nuclear membrane as with the radicle cells in the undamaged chill-treated seedlings. The process of cell division and growth of the cells is then continued upon a return to normal germinating temperature.

In addition to the postulated primary cause of injury discussed above, there may be several interacting factors which, if also irreversibly affected by chilling temperatures (viz., as mitochondrial or glyoxysome membranes), will tend to enhance and be an integral part of the overall injury process. If it is true that the energy producing mechanism is linked with conformational changes of mitochondrial membranes (Hackenbrock, 1968), then it is conceivable that the production of ATP will be reduced by the relatively inflexible mitochondria at chilled temperature. Furthermore, gloxylate cycle will also be inhibited by chilled temperature which altered the gloxysomal membrane permeability as noted by a depressed isocitratase activity within the glyoxysomes (Fites, 1974).

If, on the other hand, some of these secondary factors are undamaged by the chilling temperatures, or are able to adequately recover from chilling injury, they may tend to minimize or even protect the radicle cell against the initiating cause of damage by providing the necessary protective or repair materials needed for a resumption of cellular growth and division. For example, the ATP content of cotton seedlings returns back to a normal level after 24 hours of chilling, especially at the growing point of the cotton seedling radicle where the meristimatic cells start to divide again (Stewart and Guinn, 1969). In corn seedlings, the reversibility of O_2 uptake, ion leakage and leaf symptoms return to a normal condition after a transfer of these seedlings from 36 hours chilling at 0.3 C to 21 C (Creencia and Bramlage, 1971) and it was concluded that as long as mitochondria can quickly

and fully regain their capacity to generate ATP, chilling injury may be comparatively minimized. The energy requirements at the time of chilling tend to come from a type of carbohydrate metabolism that is geared to a lower ATP utilization than that normally observed. Ellen (1974) found that tulip bulbs exposed to a low temperature will increase the amylase activity. Shiomi and Hori (1973) demonstrated that at a low temperature, glycolysis became dominant over the pentose phosphate cycle which in turn represented a less efficient process of carbohydrate metabolism with respect to ATP utilization. Energy derived from the glycolytic cycle, though limited, could sustain the cells' growth needs on a minimal basis even though the tricarboxylic acid cycle and glyoxylate cycle have been shut down.

Results from this present investigation have shown that the soluble protein banding patterns between susceptible and resistant seedlings of the same genotype are similar. It is possible that the observed damage of chilling stress may only affect the membrane structure without any attendant or significant physiological injury during the 24 hours of chilling stress, even though the activity of these soluble enzymes will be changed more or less to adjust for the low unfavorable temperature (Nobuyuki and Hori, 1973). In other words, although conformational isomers or denatured proteins were not detected by a change in band position during gel chromatography, a change in the intensity of absorption of some of these bands noted during a gel scan of these samples (Figures 7 and 9), however, may be a

reflection of a slight change in the activity of these proteins induced by the low temperature.

Isozymes, based on Shaw's (1969) classification, have been divided into two major categories: (a) those that are distinctly different in molecular structure and which are presumably produced from different genetic sites, and (b) those which result from secondary alterations in the structure of a single polypeptide. Since damaged and undamaged cotton seedlings studied in this investigation are from one genotype, there is, theoretically, no question about the first category. The second category, conformational isomers, could be induced by cold temperature (Quimio and Noggle, 1971; De Jong and Olson, 1972). Quimio and Noggle observed a slight alteration in the isozymic patterns of the glutamic, glucose-6-P, lactic and malic dehydrogenase enzyme after two hours of chilling at the initial phase of cotton seed germination. Such an observation which differs from the results of the present experiment could probably be explained on the difference between the time of chill stress application, as well as between the times chosen for protein banding pattern analysis. It can be recalled that there is a gradual change or decrease in protein banding patterns from the dormant seed stage up until the 36 hour period of DNA replication in the radicle tips. At this time, the banding patterns in the seedlings appear to remain constant until the 48 hour period at which time the experiment was terminated. Therefore, the slight alteration of soluble protein banding patterns observed by Quimio and Noggle, after chill stress treatment may be a result of a temporary arrest of

protein degredation by the low temperature used by these workers during the onset of germination. Our results, on the other hand, are derived from a period of relative stability with respect to degredation of soluble proteins. The inhibition of conserved protein degredation by low temperatures has also been shown by other workers to be a process that accompanies prolonged germination under chilling conditions (Teraoka, 1973).

Ihle and Dure (1972) demonstrated that enzyme activity first appeared after 24 hours of germination and continued to increase until the fourth day of germination. Thus, if temperature is to affect the physiological processes of the germinating seed, such a disturbance should appear during 24 to 48 hours of germination and be detected by altered soluble protein electrophoretic banding patterns. However, low temperature treatments did not result in the disappearance of any of the essential soluble proteins as detected in this study, as well as in a similar Bryophyllus d. Berger (Pierre and Lascombers, 1971). Quantative changes, on the other hand, were noted for both our and the latter studies. This seems to indicate that resistance to chilling damage is a quantitative rather than a qualitative effect.

The results of Table 3 showed that chilled tolerant seedlings have greater protein content than those without cold treatment. Radicles, however, exhibited a decline in protein content under the chilling stress. Whether this difference is due to an increase in protein synthesis or an impairment of protein transport from cotyledons to radicles, is still unclear. However, several recent

investigations could be cited as plausible explanations. Free proline accumulated to a high concentration, after an initial cold exposure of two days for barley and declined rapidly after a 24 hour exposure to normal temperatures (Chu, Spinall and Palag, 1974). These facts seem to indicate that protein synthesis in barley has been inhibited by low temperatures and indirectly argues against the increase of protein in germinating cotton under chill stress as being attributable to an increased protein synthesis per se.

The inhibition of protein translocation within a plant exposed to chilling temperature has been studied by Geiger (1969). His results indicated that low temperatures tended to inhibit the transport of protein from source to sink by slowing down the necessary metabolic processes involved in transport as well as causing physical alteration of the membrane components in the transport pathway. Even though the existence of a transport system in a 36 hour germinated cotton seedling has not been well established, a slowing down of metabolic processes and altered membrane components are still worthy of consideration as a possible explanation for the inhibition of protein transport. The translocation process in a young germinating seedling is not separated from the necessary metabolic processes and membrane permeability that control the mechanism of cytoplasmic streaming, mass flow and their corresponding rates of velocity. If any of these phenomena ceased to function, translocation of growth substances from source to sink could decline drastically or even be inhibited. This observation has been demonstrated by Das, Hildebrandt and Riker (1966). They reported that

cytoplasmic streaming ceased during a cooling period in tobacco cells in the range of 5 to -7 C. Geiger and Sovonick (1970) reported that the temporary inhibition of translocation velocity and mass transfer rate by cooling indirectly increased the accumulation of metabolites in the source. Thus, if the quantity of soluble protein increase in a chilled seedling is due to the inhibition of protein translocation as well as to the utilization to growth substance for cell division, the phenomenon of increased protein content in chilled cotyledons along with a decreased protein content in the radicles is explainable.

In conclusion, it can be stated somewhat explicitly that the soluble protein fraction as defined in this investigation does not appear to be directly affected per se by chilling temperatures known to produce obvious symptoms of injury in the germinating and developing cotton seedling. This soluble fraction, however, is indirectly affected with regard to transport from cotyledons to the growing radicle tip of the germinating seedling. Whether or not the interference with such a transport of perhaps vital proteins and enzymes needed for cell division and growth in the developing radicle, has a direct bearing on the initiation of chilling damage in the radicle is not as yet known, but should form the basis for an area of future research.

SUMMARY AND CONCLUSIONS

E-2 and 'Pima S-4' are different cotton genotypes. E-2 has more tolerance under chilling stress temperatures than 'Pima S-4'. However, damages do exist in both genotypes. The reason for this is probably due to the irreversible physical changes in the membrane as well as its composition of each individual rather than that of the whole population. Factors which might affect individual seedlings are their unsaturated fatty acid content and a critical time period sensitive to chilling for rapid cell synthesis of DNA and RNA.

The comparison between a susceptible and resistant cotton seedling for soluble protein banding patterns level is almost the same quantitatively and qualitatively. The difference may be in the accumulation of toxin, conformational changes in membrane bonded proteins or membrane rupture which are not included in this experiment. However, quantative increases in soluble protein after the application of chilling stress to cotton seedlings are evident in this experiment.

Though the slight differences exist between seedlings of genotype E-2 and 'Pima S-4' in soluble protein levels at 48 hours germination, further study is still needed to understand the superiority of E-2 to 'Pima S-4' in chilling tolerance.

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