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Altered Development of Ribonuclease Activity and Formation of Polyribosomes in Chilled Cotton Cotyledons[1]

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ABSTRACT

Sensitivity of cotton (*Gossypium hirsutum* L.) seedlings to 6 C chilling was measured by following the development of ribonuclease (RNase) activity in cotyledons during the first week of germination. Increases in RNase activity observed in control tissues were delayed during chilling. Development of RNase activity was most sensitive to chilling during the second day of germination.

Both total and specific activities of RNase preparations were similarly affected by chilling. Alterations of protein synthesis were suggested by comparisons of free polyribosome profiles from control and chilled tissues.

Chilled seedlings partially recovered RNase activity following return to warm temperature. Recovery was slowest in seedlings that were chilled during both the second and third days of germination.

Additional index words: ***Gossypium hirsutum*** **L., Cold tolerance, Germination.**

COTTON (*Gossypium hirsutum* L.) originated as a tropical or subtropical plant (Lewis and Richmond, 1968) and requires high soil temperatures for germination and seedling establishment (Raney and Cooper, 1968). Chilling temperatures during early seedling growth have been found to impair subsequent development of the plant (Christiansen and Thomas, 1969).

When cotton is grown in comparatively temperate areas, such as the mid-South, the high soil temperature requirement and the need to minimize risk of seedling chill injury delay planting until late spring. Late planting in turn delays crop development, renders the crop vulnerable to late-season insect attack, and postpones harvest until late fall, when adverse weather conditions frequently reduce the quality of the crop. The undesirable agronomic aspects of late planting have stimulated the investigation of various features of chill injury to cotton seedlings in recent years.

Exposure of cotton seedlings to chilling temperatures has been shown to affect membrane integrity in cellular organelles (Smith and Fites, 1973; Fites, 1974), in the seedling radicle (Christiansen, 1967, 1968), and in cotyledonary tissues (Guinn, 1971a).

Stewart and Guinn (1969, 1971a) found that chilling 2- to 3-week cotton plants decreased nucleotide levels and increased free nucleosides. They also observed reduced mitochondrial activity in chilled tissues but these reductions were less striking than were those of nucleotide pools (Stewart and Guinn, 1971b). Guinn (1971b) found chilling significantly changed the chemical composition of cotton leaves. Common to each of these experiments was the protective effect of a 2-day hardening period imposed prior to the chill.

Chills early in germination caused radicle malformations (Christiansen, 1967, 1968). At least two periods of sensitivity were identified by Christiansen (1967), one at the inception of imbibition and another after 18 to 30 hours of germination at 31 C. Solute loss from cotton roots into a bathing solution was increased following either a chill, anaerobiosis, or low pH (Christiansen et al., 1970). The effects of chilling or anaerobiosis treatments could be reversed or prevented by the addition of either calcium or magnesium sulfates to the bathing solution.

Short term exposure of germinating cotton seedlings to chilling temperatures resulted in depressed levels of isocitratase activity, retarded lipid consumption, and lowered soluble sugar levels but had no effect upon isocitrate dehydrogenase activity (Mohapatra et al., 1970). These data suggested that one of the earliest effects of chill injury was manifested within the glyoxysomes. Smith and Fites (1973) pursued this implication and found that chilling temperatures altered glyoxysome membrane permeability to succinate, which accumulated both in organelles from chilled seedlings and in chilled isolated organelles. Fites (1974) examined membrane permeability of isolated glyoxysomes and found that chilling reduced permeability to succinate, which accumulated to levels sufficient to inhibit isocitratase activity.

Manifestations of chill injury in cotton are, therefore, widespread. The works of Mohapatra et al. (1970) and Smith and Fites (1973) are specific examples of enzymic lesions resulting from chilling of cotton seedlings. Regulation of nucleic acid and pro-

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tein metabolism have been implicated (Christiansen, 1967) but little effort has been directed in this area of investigation. It was therefore decided to study one aspect of nucleic acid and protein metabolism by examining the development of ribonuclease (RNase) activity during early germination of cotton and the response of this activity to the exposure of seedlings to chilling temperatures.

RNase activity is easily extracted from cotton cotyledons (Leffler, 1974). Increased RNase activity has been shown during germination of corn (Ingle and Hageman, 1965) and peas (Barker et al., 1974). Experiments with corn indicate that increased RNase activity might result from protein synthesis (Shannon et al., 1964) while the early increase in peas apparently does not (Barker et al., 1974).

MATERIALS AND METHODS

Two cultivars of *Gossypium hirsutum* L. were compared in this study. One was 'Gregg 35W,' a glandless cotton from the Texas high plains, and the other was a glandless derivative of 'Deltapine Smooth Leaf,' provided by Dr. W. R. Meredith of this laboratory.

Cottonseed were planted in trays of moist vermiculite and placed into a 30 C germinator. At selected times after planting, some trays were transferred into a 6 C germinator. For most experiments, cotyledons were harvested immediately after a 24 hour chill. For recovery experiments, seedlings were chilled for 24 or 48 hours, replaced into the 30 C germinator, and sampled periodically thereafter. All experiments were performed at least twice with each cultivar.

Cotyledons were harvested, rinsed free of vermiculite, blotted dry, weighed, and placed on ice. Parallel samples were taken for dry matter determination.

Triplicate extracts of cotyledons were assayed for RNase activity. Minced tissue (0.5 g) was homogenized in 10 volumes of 25 m*M* Tris-HCl, pH 7.6, 25 m*M* $MgCl_2$, in a chilled Ten Broeck tissue grinder. Homogenates were centrifuged 20 min at 30,000 × *g* and the supernatants were decanted through a layer of Miracloth[3] to remove the lipid layer at the surface. These extracts were used directly as sources of enzyme activity.

The reaction mixture for RNase assays, slightly modified from that of Wilson (1967), was composed of 100 m*M* sodium acetate, pH 5.4, 10 m*M* $MgCl_2$, 4.0 mg RNA (Soluble RNA from *Torula*) in a final volume of 2.5 ml. Cotton RNase activity was enhanced about 5% by addition of $MgCl_2$. Reactions were incubated for 30 min at 37 C and were halted by the addition of 0.5 ml of 25% perchloric acid containing 0.75% uranyl acetate. Stopped reactions were chilled in an ice bath for at least 20 min before pelleting undigested material by centrifugation at 4,000 × *g* for 20 min. Aliquots of the supernatant were diluted tenfold with water and the A_{260} determined. One unit of RNase was taken as that amount of activity which would effect an increase of one A_{260} in the reaction mixture through a 1-hour incubation. Protein measurements were made on the extracts with the method of Lowry et al. (1951).

Free ribosomes were extracted from duplicate 6.0 g samples of cotyledons, frozen to —75 C (Cherry, 1974). Cotyledons were homogenized with a mortar and pestle according to the methods of Davies et al. (1972). Ribosomes were pelleted through 1.75 *M* sucrose (containing 40 m*M* Tris-HCl, pH 8.5, 10 m*M* $MgCl_2$, and 20 m*M* KCl) by centrifugation for 2 hours at 42,000 rpm (195,000 × *g*, r_{max}) in a 42.1 rotor in a Beckman L5-50 ultracentrifuge. The sucrose was decanted and the pellet dissolved in 1 ml of 40 m*M* Tris-HCl, pH 8.5, 10 m*M* $MgCl_2$, 20 m*M* KCl overnight at 3 C. Linear sucrose gradients (12.5 to 37.5%, wt/vol. containing 20 m*M* Tris-HCl, pH 8.5, 10 m*M* $MgCl_2$, and 20 m*M* KCl) were prepared and aliquots (0.5 to 0.8 ml) containing six A_{260} units of the dissolved ribosomal material were gently layered over the top. Gradients were then centrifuged for 2 hours at 27,000 rpm (135,000 × *g* r_{max}) in an SW 27.1 rotor of the ultracentrifuge. Gradients were fractionated with an ISCO Model 183 density gradient fractionator; the A_{254} of the gradient was measured during fractionation with an ISCO Model UA-4 absorbance monitor. Recorder tracings of the A_{254} profiles were made to compare corresponding control and chilled samples.

[3] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

RESULTS

Extraction buffers (Wilson and Shannon, 1963; Scrubb et al., 1972; Arad et al., 1973; Wilson, 1973) were evaluated and modified to determine which would provide maximum RNase activity in a homogenate of cotton cotyledons. The buffer finally adopted (25 m*M* Tris-HCl, pH 7.6, 25 m*M* $MgCl_2$) was an adaptation of one used by Wilson and Shannon (1963), in which the $MgCl_2$ replaced 0.5 *M* KCl. This buffer provided more soluble RNase activity than did the other extraction media, with or without polyvinylpyrrolidone or Triton incorporated.

Extractable RNase activity increased (expressed on a gdw^{-1} base) between the second and seventh day of germination (Table 1). Although the values differed slightly between the two cultivars, the pattern was the same. Lower levels of RNase activity were extracted from seedlings of each genotype if they had been subjected to a chill immediately before harvest. The magnitude of the reduction of enzyme levels decreased as the seedlings aged. Generally the enzyme level in chilled tissue at any given day resembled the level in control tissue on the previous day more closely than it did the level in control tissue of the same chronological age.

The possibility that an inhibitor was affecting RNase activity of chilled tissue extracts was examined with a mixing experiment. Extracts of both control and chilled tissues were assayed for RNase activity separately and in combination. The presence of an inhibitor would be indicated if the observed RNase activity in the combined assays was below the midpoint of the separate assays. This experiment showed that an inhibitor was unlikely: RNase activities of the combined assays were 99.4, 99.7, 101.5, and 102.4% of the expected value for four separate analyses.

Specific activities of these same preparations revealed a similar pattern (Table 2). In control tissues, the specific RNase activity of preparations increased rapidly between the second and fifth days of germination. In tissues that had been chilled the day prior to harvest, the specific activity of the preparations was significantly lower than that of control tissues at days 2, 3, and 4. This was principally the result of the restricted increase in RNase specific activity

Table 1. Cotyledonary RNase activity during early germination.

Seedling age at harvest	Ribonuclease activity			
	Deltapine		Gregg	
	Control*	Chilled†	Control*	Chilled†
days	units, gdw^{-1}			
2	1,132 ± 75‡	475 ± 47	967 ± 45	388 ± 18
3	1,813 ± 45	1,039 ± 42	1,440 ± 65	737 ± 53
4	2,830 ± 63	1,904 ± 63	2,528 ± 121	1,799 ± 71
5	3,480 ± 81	3,224 ± 82	2,907 ± 103	2,620 ± 100
6	4,051 ± 84		3,132 ± 122	3,356 ± 98
7	4,526 ± 73		3,817 ± 83	

* Control seedlings germinated at 30 C. † Chilled seedlings germinated at 30 C until 24 hours before harvest when they were transferred to a 6 C chamber. ‡ Data are presented as means ± standard errors of the means.

during the chill, rather than a decrease during the chilling period.

The dry weight of cotyledons decreased during germination as reserves were mobilized and exported to the axes (Table 3). If seedlings had been chilled prior to harvest, the dry weight of these cotyledons was always greater than the dry weight of control cotyledons. In most cases, differences were significant. Increased enzyme activity (on a gdw^{-1} basis) between fifth and seventh days reflected continued decreases in cotyledon dry weight, as neither specific activity nor enzyme activity per cotyledon (data not presented) changed appreciably during this stage.

Experiments summarized in Tables 1, 2, and 3 indicated that the most severe perturbations (as measured by enzyme and dry weight parameters) were effected by chilling at the earliest stages of germination. It was therefore decided to chill seedlings on the second day, on the third day, and on both the second and the third days of germination, and determine the effects of these chills on subsequent seedling growth at 30 C (recovery). RNase activity from these tissues is presented in Table 4. The severest of the chill treatments was the 48 hour chill covering days 2 and 3; the mildest was the 24 hour chill on day 3. Following replacement into the 30 C germinator all tissues recovered enzyme activity; the rate of recovery reflected the severity of the damage originally caused by the treatments. By the seventh day, seedlings which had been chilled only on day 3 had recovered RNase activity equivalent to that of control seedlings. In both treatments which had been subjected to a chill on day 2, however, enzyme levels were still below control levels.

Free polyribosomes were prepared from control and chilled cotyledons of both cultivars the second, third, fourth and fifth day of germination. Chills were imposed for the last 24 hour before harvest. Results from the two cultivars were similar. Profiles obtained from the Deltapine cultivar are presented to illustrate the effect of chill conditions imposed upon seedlings at different stages of development (Fig. 1 A-D). Low levels of polyribosomal material were obtained from cotyledons at the second day of germination (Fig. 1A). By the third day of germination, however, dissimilarities between the control and chilled polysome profiles were evident (Fig. 1B). A marked increase in polyribosomal material occurred during the third day of germination in control seedlings; whereas, the third-day chilled profile was quite similar to the second-day profiles. Gradual conversion of control tissue polyribosomal material toward the lower molecular weight region of the profile during the fourth and fifth days of germination was arrested by the imposition of a chill during either of these days (Figs. 1 C and D). On each of these days, the high molecular weight regions of the chilled tissue profiles made up a greater proportion of the total profile than did the corresponding areas of the control tissue profiles.

DISCUSSION

Cotton cotyledon RNase activity is soluble, has a pH optimum of 5.4, and is resistant to gossypol toxicity (Leffler, 1974). More recently, this activity has been found to be slightly enhanced by either $MgCl_2$ or KCl and significantly depressed by either $MnCl_2$ or $ZnCl_2$; there is no DNase activity associated with it (Leffler, unpublished results).

Extractable RNase activity of cotton cotyledons increased in both cultivars during the first week of germination. The increase during the first 4 days may

Table 2. Specific activity of RNase extracts during early germination.

Seedling age at harvest	Specific activity of RNase			
	Deltapine		Gregg	
	Control*	Chilled†	Control*	Chilled†
days	units, mg Protein^{-1}			
2	10.06 ± 0.54‡	4.17 ± 0.32	8.72 ± 0.35	3.27 ± 0.18
3	13.15 ± 0.31	7.79 ± 0.27	12.13 ± 0.31	6.73 ± 0.37
4	15.60 ± 0.30	13.38 ± 0.66	16.11 ± 0.66	12.74 ± 0.57
5	16.85 ± 0.51	18.11 ± 0.87	16.51 ± 0.68	16.74 ± 1.17
6	16.08 ± 0.56		17.44 ± 0.49	20.77 ± 0.75
7	19.43 ± 0.11		18.17 ± 0.44	

* Seedlings were germinated at 30 C. † Seedlings were transferred from 30 C to 6 C 24 hours before harvest. ‡ Data are presented as means ± standard errors of the means.

Table 3. Cotyledonary dry weight during early germination.

Seedling age at harvest	Cotyledonary dry wt			
	Deltapine		Gregg	
	Control*	Chilled†	Control*	Chilled†
days	mg, Cotyledon pair^{-1}			
2	46.6 ± 1.4‡	47.8 ± 0.6	61.6 ± 0.7	62.2 ± 1.7
3	39.8 ± 0.7	46.2 ± 1.0	57.4 ± 0.9	61.7 ± 1.0
4	38.1 ± 0.9	42.1 ± 1.1	51.5 ± 0.6	57.1 ± 1.0
5	35.5 ± 1.3	37.4 ± 0.7	50.5 ± 0.6	55.8 ± 0.8
6	29.7 ± 0.8		44.5 ± 0.8	52.7 ± 1.2
7	27.7 ± 1.6		42.4 ± 1.6	

* Seedlings were germinated at 30 C. † Seedlings were transferred from 30 C to 6 C 24 hours before harvest. ‡ Data are presented as means ± standard errors of the means.

Table 4. Cotyledonary RNase during recovery from early-germination chill.

Seedling age at harvest	RNase activity							
	Deltapine				Gregg			
	Control*	Chilled day 2†	Chilled day 3†	Chilled days 2+3†	Control*	Chilled day 2†	Chilled day 3†	Chilled days 2+3†
days	units, gdw^{-1}							
2‡	1,132 ± 75§	475 ± 47	1,132 ± 75	475 ± 47	967 ± 45	388 ± 18	967 ± 45	388 ± 18
3	1,888 ± 28	735 ± 53	938 ± 74	489 ± 74	1,218 ± 64	731 ± 73	884 ± 80	269 ± 14
4	2,750 ± 101	1,823 ± 87	1,989 ± 77	881 ± 68	2,520 ± 38	1,330 ± 92	1,826 ± 27	913 ± 146
5	3,477 ± 129	2,654 ± 59	2,848 ± 106	1,817 ± 179	2,813 ± 64	2,474 ± 21	2,963 ± 73	1,770 ± 121
6	3,911 ± 98	2,881 ± 267	3,119 ± 110	2,548 ± 179	3,239 ± 182	2,616 ± 51	2,760 ± 160	2,411 ± 129
7	4,527 ± 73	3,887 ± 147	4,367 ± 319	3,568 ± 120	3,817 ± 83	3,222 ± 26	3,627 ± 273	3,167 ± 92

* Exposed to 30 C throughout germination. † Exposed to 30 C except for indicated period of chill at 6 C. ‡ Data taken from Table 1 for illustrative purposes. § Data are presented as means ± standard errors of the means.

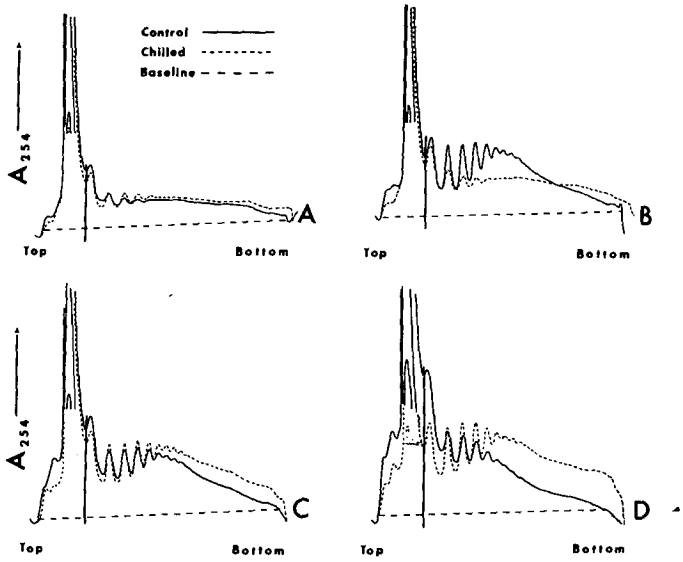

Fig. 1. Profiles of polyribosomes extracted from Deltapine cotton cotyledons on the 2nd (A), 3rd (B), 4th (C), and 5th (D) days of germination. Control seedlings were exposed to 30 C throughout germination; chilled seedlings were transferred from 30 C to 6 C 24 hours before harvest. The solid vertical line through each profile identifies division of monoribosome area (toward top of gradient) from polyribosome area (toward bottom of gradient).

have resulted from new synthesis, as suggested by the increase in specific enzyme activity in the preparations over this period.

Specific activity of an enzyme preparation can be interpreted as a measure of the homogeniety of the preparation with regard to enzymes possessing that particular activity (Dixon and Webb, 1964). Differences among preparations in the proportion of enzymes having a specified activity relative to other protein will therefore appear as changes in the specific enzyme activities of these preparations. In the current experiments, the specific RNase activity of the cotyledon preparations doubled between the second and seventh days of germination. These data are consistent with the concept of preferential development of RNase activity relative to total soluble protein during this phase of germination. Similarly increased specific RNase activity in crude extracts of corn tissue has been shown to be related to protein synthesis (Shannon et al., 1964; Ingle and Hageman, 1965).

Cotyledonary RNase activity of cotton seedlings did not increase while seedlings were exposed to a chill, either on a total or a specific base. Specific activity of preparations from seedlings chilled during any of the first 4 days of germination was always lower than that of preparations from corresponding control seedlings. These data lend additional support to the association between protein synthesis and increases in RNase activity at this early stage of development. Specific activities of preparations from seedlings chilled during the fifth day were not significantly different from those from control seedlings. The specific activity of RNase from Gregg seedlings chilled the sixth day of germination was slightly greater than that of corresponding control seedlings. While the reason for this is not known, it may indicate the bulk of protein synthesis was arrested during this time, while RNase activity was stable.

The balance between initiation and release during protein synthesis is reflected in the appearance of the polyribosome profile (Travis et al., 1970; Cherry, 1973, 1974). A large increase in polyribosomes took place during the third day in control seedlings but did not in chilled seedlings. This was the most dramatic indication of arrested protein synthesis caused by chilling. The level of the polyribosomal material declined in control seedlings from the fourth and fifth days of germination; however, decreases in the high molecular weight regions of the profiles were not apparent if seedlings were chilled prior to harvest.

Davies and Larkins (1974) found polyribosome profile quality to be quite sensitive to nuclease activity and the patterns observed here were reminiscent of the nuclease degradation they described. The isolation procedure employed in these experiments minimizes nuclease effects through the combined effects of high pH and high ionic strength (Davies et al., 1972). The basic extraction medium (pH 8.5) greatly depresses cotton RNase activity, which has an optimum pH of 5.4 (Leffler, 1974). Furthermore assays of polyribosome pellets from each of the treatments surveyed have detected negligible nuclease activity (unpublished results). It is possible, however, that some slight nuclease activity, too little to detect by other methods, may have been present in the older control preparations (Davies and Larkins, 1974). Whether these profile shifts reflect some normally occurring processing of the polyribosome complement or the appearance of some nuclease activity associated with this fraction, the control-chilled comparisons attest to disturbances of protein synthesis caused by tissue chilling.

Development of RNase activity was most severely disrupted by chilling on the second day of germination. Development of polyribosomal material was most markedly affected by chilling on the third day however. If increased RNase activity during germination resulted from protein synthesis, these results appear to be in conflict.

The level of polyribosomal material in a preparation provides a static estimate of the dynamic process of protein synthesis. Rates of protein synthesis may be broadly estimated from changes in polyribosome profiles with time or from large differences in the polyribosome profiles of different tissues at one time. Small differences in rates of protein synthesis may be detected in enzyme activity measurements but not in polyribosomal profile analyses, especially when the level of polyribosomal material is low. While this may explain the apparent conflict in results above, verification of this interpretation will require additional investigation.

Seedlings eventually recovered RNase activity if they were chilled only during the third day of germination. Both treatments employing a chill during the second day of growth had more severe effects upon the recovery potential of the seedlings however. None of these seedlings had recovered RNase activity comparable to control tissues by the end of the experimental period. These data agree with the growth results of Christiansen (1963, 1964), Christiansen and Thomas (1969) and Thomas and Christiansen (1971), who obsereved long-term deleterious effects of chilling

early in germination of cotton. Furthermore these results provide additional evidence of the sensitivity of cotton to chilling at the 18 to 30 hour stage of germination (Christiansen, 1967).

Mohapatra et al. (1970) and Smith and Fites (1973) reported that chilling reduced activity of the organelle-associated enzyme, isocitratase. Results presented here extend those findings to another enzyme activity and suggest that chilling may also affect cytoplasmic protein synthesis. Although at least partial recovery of enzyme activity occurred upon replacement of seedlings into warm temperatures, it is likely that altered chronology of metabolic regulation has had effects which are in themselves deleterious. Unaltered chronology during early germination of corn has previously shown to be important (Leffler et al., 1970).

Cotton seedlings are therefore extremely sensitive to chilling temperatures and are noticeably affected at several levels of organization. Disruption of enzyme activities and protein synthesis (Mohapatra et al., 1970; Smith and Fites, 1973; and reported here) would combine to effectively starve the developing seedling of reserves needed for continued growth. Additionally if mitochondrial activity were disrupted during early germination chills as it is during chills later in development (Stewart and Guinn, 1971b), the seedlings would be starved for energy as well as for carbon skeletons.

These observations indicate that successful protection of cotton seedlings from chill injury might lie with a genetic improvement program. Problems associated with this approach are many. It would, however, seem more efficient to investigate cotton cultivars for potential tolerance to chilling, determine heritability of the trait(s), and transfer them to otherwise-adapted cultivars, than to hope to select any one physiological attribute for manipulation to remedy the problem. Although both cultivars examined in this study were found to respond similarly to chilling, other strains of *Gossypium* may possess more desirable attributes (Muramoto, 1975).

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