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Changes in Sugars, Starch, RNA, Protein, and Lipid-soluble Phosphate in Leaves of Cotton Plants at Low Temperatures[1]

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ABSTRACT

Cotton plants (*Gossypium hirsutum* L., cv 'Westburn') were severely injured when chilled at 5 C without prior hardening. Exposure to hardening temperatures of 15 C day and 10 C night prevented subsequent injury at 5 C. Sugars and starch increased during exposure to hardening temperatures, but RNA, protein, and lipid-soluble phosphate decreased. Except for increases in sugars, these changes in cotton during exposure to hardening temperatures are opposite changes which have been reported in plants that can be hardened to withstand subzero temperatures.

Additional index words: **Carbohydrates, Chilling injury, Cold hardening, Leaves, Phospholipids.**

MANY workers have investigated the hardening of frost-hardy plants against subzero temperatures, but relatively little attention has been given to hardening of thermophilic plants against injury caused by chilling. Young cotton plants can be very sensitive to chilling. Severity of injury, however, is strongly influenced by previous treatment. Exposure of young cotton plants to 2 or more days at about 15 C causes them to become much more resistant to subsequent chilling at 5 C (9 and Fig. 1). Hardened plants showed little injury even at 0 C, but were killed at —5 C (Guinn, unpublished). Presumably, changes occur during preconditioning at 15 C which decrease susceptibility to chilling injury. Tests were conducted in an attempt to determine the nature of these changes.

A large number of components have been correlated with hardening of frost-hardy plants (21). Sugars almost always increase during exposure to hardening temperatures; these increases are often accompanied by a decrease in starch (17, 22, 23, 27). Starch may be detrimental (25, 26), whereas sugars apparently increase hardiness in at least some plants (12, 21, 22, 23, 27). Heber (12) reported that sucrose protected membranes against damage by freezing. However, high sugar concentrations do not always insure cold hardiness (17, 23). Many workers (12, 14, 18, 21, 26, 27) have shown a correlation between amount of protein (usually water-soluble protein) and frost hardiness. In some cases, increases in protein were shown to be accompanied or preceded by increases in RNA (14, 18, 21, 26). Hardening may also involve a change in the amount (7, 26) or nature (7, 15, 16, 26) of lipids. Siminovitch et al. (26) reported convincing evidence that lipid phosphorus is closely correlated with cold hardiness in living bark of the black locust. They suggested that hardening included conversion of starch to sugar and the synthesis of RNA, protein, and phospholipids.

It is possible that some of the changes which increase frost-hardiness may also increase resistance to injury caused by chilling. Membranes appear to be the primary site of freezing injury (12, 21, 23, 26) and may also be involved in chilling injury (5, 9, 19, 20, 28). Because cotton can be hardened against chilling injury and because decreases in starch and increases in sugars, protein, RNA, and lipid-soluble phosphate have been shown to correlate with development of hardiness in frost-hardy plants, I investigated the effects of hardening temperatures on concentrations of these compounds in leaves of young cotton plants. I did not attempt to measure soluble protein because J. T. Cothren[3] was investigating the effects of temperature on soluble protein in cotton.

Fig. 1. Young cotton plants after chilling at 5 C. The plants on the right were hardened for 2 days at 15 C day and night with 14 hr at 3,500 ft-c per day prior to chilling. The plants on the left were subjected to the chilling treatment without previous hardening.

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[3] Cothren, J. T. 1971. Some effects of low temperatures on soluble protein, protein-sulfhydryl content, and electrophoretic protein patterns in young cotton plants. Ph.D. Thesis, Oklahoma State University, Stillwater.

MATERIALS AND METHODS

Plant Culture. Cotton seedlings (*Gossypium hirsutum* L., cv 'Westburn') were cultured in nutrient solutions as reported previously (10). Two tests were conducted to determine the effects of hardening and chilling temperatures on sugars, starch, RNA, protein, and lipid-soluble phosphate.

In the first test, seedlings were cultured for 13 days in a growth chamber at 35 C day and 30 C night temperatures. Light was supplied at approximately 3,000 ft-c by a mixture of incandescent and cool-white fluorescent lamps for 14 hr daily. Half of the seedlings were hardened for 2 days at 15 C day and 10 C night. Half of the control and half of the hardened seedlings were then harvested. All of the remaining seedlings were chilled for 2 days at 5 C day and night and then harvested. Plants were illuminated 14 hr daily during hardening and chilling treatments. Light intensity dropped to approximately 2,500 ft-c at 15 C and to 1,800 ft-c at 5 C.

The second test was conducted to determine changes in young cotton leaves with time at hardening temperatures. Seedlings were cultured for 3 weeks in a fiberglass greenhouse during early March. (These seedlings were cultured longer than those in the first test because they developed more slowly in the greenhouse than in the growth chamber.) Temperatures ranged from 25 to 35 C and the natural photoperiod was approximately 12 hr. Light intensities were not recorded. Half of the seedlings were then transferred to a growth chamber to harden at 15 C day and 10 C night with 14 hr of light at 2,500 ft-c per day. Hardened and control plants were harvested 0, 1, 2, 3, and 4 days later.

Immediately upon harvest, leaf blades were detached from petioles and lyophilized: They were then ground to pass a 60-mesh screen and stored over silica gel.

Sugars. Sugars were removed from 100-mg samples by three successive 10-min extractions with 10-ml portions of 70% ethanol at 80 C. Ethanol was removed *in vacuo* and reducing and total sugars were determined as reported previously (11), except that sugars were hydrolyzed in 0.05 *N* HCl instead of 0.5 *N* H_2SO_4. (Sulfate ions interfered with the ferricyanide test at low sugar concentrations.)

Starch. The residue left after extraction of sugars was used for starch determination. Residual lipids were removed with a 2:1 mixture of 95% ethanol and ether heated to boiling. Most of the residual phenolic materials were then removed with a 4:1 mixture of 95% ethanol and 10% aqueous NaCl at 80 C. Extraction with this mixture was continued until the supernatant fraction was colorless. The residue was then extracted once with 10 ml of ice-cold water. Insoluble material was sedimented by centrifugation after each extraction. Starch was solubilized in 8 ml of 1 m*M* citrate-10 m*M* NaCl at pH 7.0 for 30 min at 100 C. The suspension was cooled and 2 ml of alpha amylase (prepared by dissolving 5 mg of Sigma[4] Type II-A amylase in 20 ml of 1 m*M* citrate-1 m*M* $CaCl_2$, pH 7.0) were added to each tube to digest starch during 30 min at 25 C. After centrifugation, portions of the supernatant fractions were diluted and used in the carbohydrate test of Dubois et al. (6).

RNA. Nucleic acids were extracted by a modified Schneider procedure (24). Lipids and pigments were removed from 200-mg samples of dry leaf material with 95% ethanol and a 4:1 mixture of ethanol: 10% NaCl at 70 C (8). Remaining free nucleotides were extracted with ice-cold 0.5 *N* $HClO_4$, and nucleic acids were then hydrolyzed during 30 min at 70 C in 0.5 *N* $HClO_4$. Total nucleic acids were determined by ultraviolet absorbance, DNA was determined by a diphenylamine test (4), and RNA was estimated by difference.

In order to check the validity of the results, two other extraction procedures were also used. Nucleic acids were extracted with hot 10% NaCl (8) and with a mixture of phenol, bentonite, Na lauryl sulfate, EDTA, and tris buffer. Results with $HClO_4$ and NaCl were almost identical; the phenol procedure extracted somewhat smaller amounts of nucleic acids, but the trends were the same. Results given are those obtained with $HClO_4$.

Protein. The pellets, which remained after hydrolysis of RNA and DNA with $HClO_4$, were washed with water and then transferred to 30-ml micro-Kjeldahl flasks and digested in 2-ml portions of H_2SO_4. Selenized boiling stones were used as catalysts. Ammonia was freed by adding NaOH, steam-distilled into boric acid, and titrated with dilute H_2SO_4. I assumed that all the nitrogen found was protein N, because chlorophyll, amino acids, vitamins, nucleosides, nucleotides, RNA, and DNA would have been removed by extraction procedures prior to digestion.

Lipid-soluble Phosphate. The method of Bligh and Dyer (3) was scaled down and used for extracting lipid-soluble phosphate from 200-mg portions of dry leaf tissue. The tissue was shaken in stoppered centrifuge tubes with 19-ml portions of a 5:10:4 mixture of chloroform: methanol: water. After centrifugation and transfer of the supernatant fluid to clean tubes, the pellets were shaken with 5-ml portions of chloroform which was then separated from the residue by filtration and added to the first extract. Five-ml portions of water were added to cause phase separation. After thorough mixing, the extracts were centrifuged, and 3-ml portions of the lower phase were transferred to 30-ml micro-Kjeldahl flasks. Chloroform was driven off by gentle heating, and the residues were then digested in 1-ml portions of concentrated H_2SO_4. After all frothing ceased, 2 drops of 30% H_2O_2 were added, and heating was continued for 30 min to complete the digestion and drive off any residual H_2O_2. (A separate test with reagent phosphate indicated that no phosphate was lost during the digestion procedure used.) Phosphate was measured by Bartlett's modification (2) of the Fiske-Subbarow test.

[4] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U. S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

RESULTS AND DISCUSSION

Of the components investigated, only sugars showed the same trends during exposure of cotton plants to low temperature as have been reported for frost-hardy plants (Tables 1 and 2). Sugars increased at hardening and, to a lesser extent, at chilling temperatures. Most of the sugars in leaves were reducing sugars; the predominant sugars in cotton leaves are glucose and fructose (11). Whether the increase in sugars was a cause of hardening against chilling or was merely coincidental cannot be ascertained from the data.

Table 1. Concentrations of reducing sugars, total sugars, starch, RNA, protein, and lipid-soluble phosphate in cotton leaves as influenced by hardening for 2 days at 15 C day and 10 C night and by chilling for 2 days at 5 C day and night*.

Treatment	Reducing sugars	Total sugars	Starch	RNA	Protein	Lipid-soluble phosphate
	/g dry weight					μmoles/g dry wt.
Control	22.6±1.3	31.4±1.3	15.2±1.4	12.6±0.4	198±6.1	24.5±2.3
Hardened	75.3±2.3	83.4±4.0	68.4±1.8	11.4±0.3	167±3.9	18.3±1.6
Chilled	38.4±1.8	47.7±3.0	18.8±1.0	11.8±0.3	195±2.2	22.4±1.3
Hardened and chilled	68.3±3.6	72.5±4.8	73.2±7.5	9.0±0.4	148±9.1	18.8±3.8

* The data are averages of 4 replications. Standard errors of the means are shown.

Table 2. Time course study of changes in reducing sugars, total sugars, starch, RNA, protein, and lipid-soluble phosphate in cotton leaves as influenced by hardening temperatures of 15 C day and 10 C night*.

Treatment	Days of treatment 0	1	2	3	4
	Reducing sugars, mg/g dry weight				
Control	17.8 ± 3.0	19.2 ± 1.6	23.4 ± 1.2	27.0 ± 2.1	--
Hardened	--	58.0 ± 3.9	66.8 ± 5.0	79.2 ± 3.6	82.8 ± 5.8
	Total sugars, mg/g dry weight				
Control	31.8 ± 2.0	24.0 ± 1.8	27.0 ± 2.4	42.5 ± 4.1	--
Hardened	--	63.5 ± 7.9	73.5 ± 3.9	90.5 ± 12.8	93.8 ± 11.1
	Starch, mg/g dry weight				
Control	31.4 ± 1.1	13.7 ± 1.1	12.9 ± 0.5	35.8 ± 2.2	--
Hardened	--	89 ± 3.9	107 ± 11.5	116 ± 16.2	160 ± 4.8
	RNA, mg/g dry weight				
Control	22.6 ± 0.56	22.8 ± 0.46	22.1 ± 0.61	22.2 ± 0.58	--
Hardened	--	18.9 ± 0.53	16.3 ± 0.35	15.8 ± 0.48	11.2 ± 0.37
	Protein, mg/g dry weight				
Control	209 ± 5.0	217 ± 4.8	214 ± 5.1	220 ± 3.5	--
Hardened	--	188 ± 4.4	177 ± 5.8	171 ± 4.7	138 ± 3.8
	Lipid-soluble phosphate, μmoles/g dry weight				
Control	33.8 ± 1.8	29.0 ± 3.5	28.4 ± 2.9	36.6 ± 1.9	--
Hardened	--	23.0 ± 1.0	23.2 ± 1.7	28.9 ± 1.7	17.9 ± 0.7

* Data are averages of 4 replications. Standard errors of the means are shown.

Perhaps the sugars retained water and protected the tissue from dehydration. Dehydration and changes in membrane permeability appear to be common to both freezing injury (12, 21, 26) and chilling injury (5, 9). Freezing causes dehydration as water is converted to ice; chilling causes dehydration in cotton because evaporation from leaves and cotyledons exceeds water uptake at low root temperatures (1). According to Heber (12), desiccation and resulting concentration of electrolytes are major factors in freezing injury. Sugars apparently protect proteins against dehydration and the denaturing effects of salts (12). Cothren[3] found that hardening prevented the marked decreases in soluble protein which occured in nonhardened cotton plants when chilled at 5 C. Muckel (personal communication) was unable to harden cotton plants in the dark; he obtained greatest hardening under long, cool photoperiods — conditions which cause sugars to accumulate.

In many plants, starch is converted to sugar at low temperatures and this is thought to be a mechanism for hardening in some frost-hardy plants (17, 22, 23, 25, 26, 27). Siminovitch and Briggs (25) and Siminovitch et al. (26) thought decreases in starch were more important than increases in sugars during hardening of black locust. At least some thermophilic plants apparently differ from frost-hardy plants in that they accumulate, rather than deplete, starch at low temperatures (13 and Tables 1 and 2). Starch accumulated to a very high level in cotton leaves during 4 days at hardening temperatures (Table 2).

Hardening temperatures caused apparent decreases, rather than increases, in RNA, protein, and lipid-soluble phosphate (Tables 1 and 2). Cothren[3] found slight decreases in the soluble protein content of cotton cotyledons during hardening. Thus, the increases in RNA, protein, and phospholipids reported in frost-hardy plants (14, 18, 21, 26, 27) apparently do not occur in cotton. Actual decreases in these components were probably less than the measured concentration decreases because of the diluting effect of accumulated sugars and starch. However, it is evident that no increases in RNA, protein, or lipid-soluble phosphate occurred in cotton leaves (Tables 1 and 2). Therefore, an explanation for hardening in cotton is to be sought elsewhere unless the observed increases in sugars are adequate to provide the limited amount of hardening attainable in this thermophilic plant.

A possibility not investigated in this study is that the nature of membrane lipids changes during hardening. Lyons et al. (20) investigated the nature of mitochondrial membrane lipids in several species of plants. They found that the greatest capacity for swelling, and the greatest degree of unsaturation of the membrane fatty acids, were shown by mitochondria from chilling-resistant species, and the lowest values for each of these criteria were shown by chilling-sensitive species. They did not, however, investigate possible changes in membrane lipids at hardening temperatures. They suggested that the metabolic injury caused in chilling-sensitive tissues may be due to inability of the relatively inflexible mitochondria to function at low temperatures. This conclusion was supported by the results of Lyons and Raison (19) who found that the rate of decrease in oxidative activity of mitochondria with decreasing temperature changed in chilling-sensitive plants at about 10 C, presumably because of a phase change in mitochondrial membrane lipids. Stewart and Guinn (28) found that ATP decreased rapidly in leaves of unhardened cotton plants at 5 C, but increased slightly when hardened leaves were kept at 5 C. The nature of lipids in frost-hardy plants has been shown to change during hardening (7, 15, 16). Gerloff et al. (7) found that fatty acids increased in alfalfa roots during hardening. There was a preferential accumulation of polyunsaturated fatty acids, mainly 18:2 and 18:3. Ketchie (15) found an increase in the ratio of unsaturated to saturated fatty acids in peach tree bark during cold hardening. Similar changes may occur in thermophilic plants and contribute to hardiness against chilling. This possibility should be investigated.

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