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Chilling Injury and Changes in Adenosine Triphosphate of Cotton Seedlings[1]

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Abstract. Young *Gossypium hirsutum* L. seedlings chilled at 5°showed a continual decrease in ATP concentration with time of chilling. Chilled plants returned to optimum conditions were able to restore the initial ATP concentration when chilled only 1 day, but not when chilled 2 days. The decrease in ATP with chilling was prevented by hardening the seedlings at 15° for 2 days (14-hr-day-length) immediately before chilling. The ATP level of hardened plants was higher than of unhardened plants. When hardened plants were chilled at 5°, the ATP level increased in the leaves but decreased in the roots.

A mechanism of chilling injury is discussed in relation to the decrease in ATP concentration at low temperature.

Many tropical and subtropical plants are injured or killed by low non-freezing temperatures (chilling injury). Seedling cotton is one of the most chilling sensitive of the major crop plants. The detrimental effects of chilling have been recognized for years, but the specific causes of chilling injury have not been extensively studied. Most of the research on low temperature has been concerned with frost or freezing injury. For example, Heber (1) and Heber and Santarius (2) showed that oxidative- and photophosphorylation were adversely affected by freezing. Two reports suggest that oxidative phosphorylation may also be inhibited by chilling in chilling-susceptible plants. Lieberman *et al.* (6) found that oxidative and phosphorylative activities at 25° of mitochondria from sweet potato roots were decreased when the roots were stored at 7.5° but not when they were stored at 15°. Lyons *et al.* (7) studied the nature of mitochondrial membranes from plants which differed in chilling sensitivity. They reported that mitochondria obtained from chilling-resistant species had a higher content of unsaturated fatty acids and were more flexible (as measured by their greater ability to swell) than mitochondria obtained from chilling-susceptible plants. They suggested that the metabolic injury in chilling-susceptible tissues may be due to an inability of the relatively inflexible mitochondria to function at low temperatures. Although ATP is required in nearly every synthetic process of plant metabolism, low temperature inhibition of phosphorylation would be important only if it resulted in a depletion of ATP to the point of disrupting metabolism (through an imbalance between phosphorylation rate and ATP utilization rate). A determination of the effects of chilling on ATP concentration in a chilling-susceptible plant should give an indication of whether or not chilling slows phosphorylation more than it slows ATP utilization. This study reports changes in the ATP contents of leaves and roots of cold-hardened and non-hardened cotton seedlings in response to chilling at 5°.

Materials and Methods

Methods of Culture. Acid delinted cotton (*Gossypium hirsutum* L., cv. Parrott) seeds were germinated in moist vermiculite under greenhouse conditions for 5 to 6 days. Vigorous seedlings were selected and transferred to 12-liter polyethylene buckets containing a continuously aerated, modified Hoagland's solution (3). The seedlings were grown under near optimum conditions for 2 weeks in a greenhouse or growth chamber. When the growth chamber was used a 14-hr light period of approximately 3000 ft-c was provided by fluorescent lights supplemented by incandescent lamps. Temperatures were maintained at 30° day and 20° night.

Methods of Treatment and Harvest. Chilling treatments varying from 6 hr to 4 days were imposed by placing the buckets of plants in a growth chamber

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maintained at 5° day and night. In experiments where the cotton seedlings were hardened against chilling injury, the plants were placed in a growth chamber at 15° for 2 days before the 5° chilling regime was imposed. In both cases the light intensity was 3000 ft-c and the light period was 14-hr per day. Cool temperatures and illumination are necessary for hardening. (R. Muckel, personal communication).

Each treatment was replicated 4 times, with 6 plants per replicate. The roots and leaves of each replicate were harvested and placed in a deep-freeze within 2 min of harvest. All harvests were made at the same time of day. The frozen tissue was lyophilized, ground to pass a 60-mesh screen, and the powdered tissue stored at −26° over $CaCl_2$ until it was assayed.

Extraction of ATP. A number of methods for extracting ATP were compared, including 0.5 N perchloric acid, 50 % aqueous ethanol, 0.2 N formic acid in 20 % ethanol, 5 % (w/v) trichloroacetic acid, and hot water. Of these, hot water extracted the highest amount of ATP, gave the most reproducible results, and did not require the purification of ATP on activated carbon. In this method, 5 ml of boiling deionized water was quickly added to 25 mg of the dried, ground tissue and placed in a boiling water bath for 3 min. The extract was rapidly cooled in an ice bath, then centrifuged at 0° to remove the insoluble residue. The supernatant fraction was used directly for assay.

Assay of ATP. A firefly luciferin-luciferase system similar to that of Strehler and Totter (8) was used to measure ATP. The enzyme was prepared by grinding 130 mg of dried lanterns with 10 ml of ice-cold 0.1 M sodium arsenate buffer at pH 7.4 in a hand homogenizer. After washing the homogenizer with an additional 2 ml of buffer, the homogenate was centrifuged 10 min at 27,000*g*. The supernatant fraction was retained and mixed with 100 mg of $MgSO_4$. This mixture was used as the luciferin-luciferase preparation.

A Beckman DU-2[3] spectrophotometer with a recording adapter was used to measure light produced in the reaction. The reaction vessels were 4-ml cuvettes raised about 1 cm in the cuvette chamber so that maximum light detection was possible. The reaction mixture consisted of 0.6 ml of 0.05 M glycine buffer, pH 7.4, 0.2 ml of the luciferin-luciferase preparation and 0.2 ml of the sample or standard containing 0.1 to 1.5 μg of ATP. The sample was added last by forcible injection with an air-tight syringe through a small hole in a piece of plastic tape applied to the lid of the chamber. Maximum light intensity was recorded and related to ATP concentration by the inclusion of standards in each day's assays. Responses were linear within the concentration range of ATP used. The standard error was determined for each set of replications.

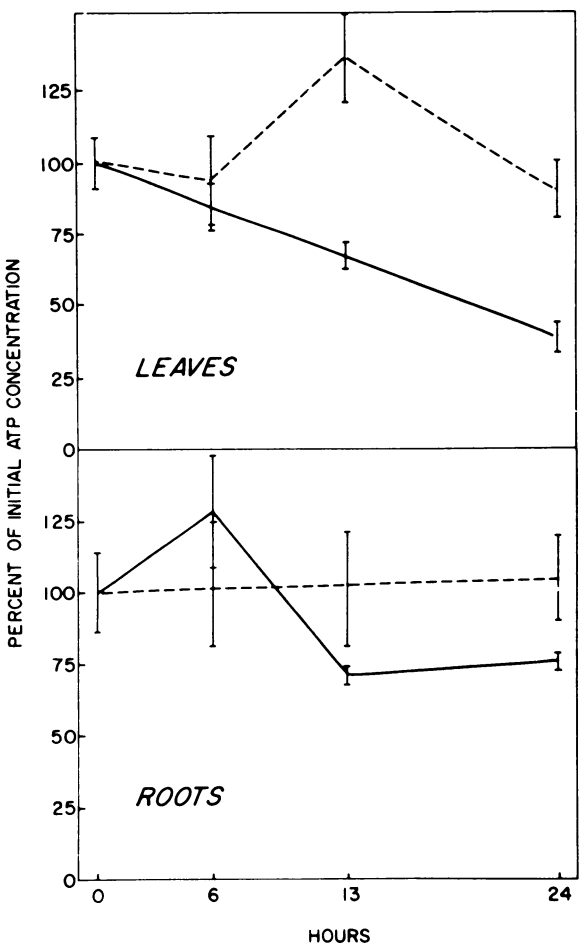
Results and Discussion

When 2-week-old cotton seedlings were subjected to chilling treatment at 5°, the ATP level began to decrease in a relatively short time. Fig. 1 shows the effects of chilling the entire plant for 6, 13, and 24 hr at 5° compared to control plants maintained at 30° day and 20° night. In the leaves the continual decrease in ATP concentration over the 24 hr chilling period is apparent. No decrease occurred in the control plants. The increase in ATP in the leaves of the control plants at 13 hr was probably due to the 20° temperature of the dark period, since in a separate experiment ATP was shown to increase at cool temperatures above 10°. In the root tissue there was an initial increase in ATP level followed by a decrease. The liquid nutrient solution buffered the roots against sudden temperature changes such that the root temperature had decreased to only 10° by 6 hr. By 13 hr the temperature was 5°. Some hardening may have occurred during the cooling period (*cf.* below).

Although the decrease in ATP was observed after relatively short periods of chilling, the initial concentration was restored if chilling was not continued. The original ATP level of both roots and leaves was regained within 1 day under greenhouse conditions after 24 hr of chilling at 5° (Fig. 2). When the plants were chilled for 2 days, however, necrosis developed; and the leaf ATP concentration remained low when the plants were returned to the greenhouse for 2 days. The slight recovery of ATP was probably due to the growing points which seemed best able to survive chilling conditions. Prolonged chilling caused expanded leaves to become flaccid and necrotic, but growing points still appeared normal. Roots chilled for 2 days showed an erratic ATP response (as indicated by the standard error) when returned to warm conditions.

Fig. 1 and 2 show results with plants grown under near optimum conditions before chilling conditions were imposed. Seedlings of Parrott cotton can be hardened against chilling injury by subjecting them to a 15° temperature with 14-hr-day length for 2 or more days immediately before the chilling treatment. When the seedlings were hardened before the chilling treatment was imposed, the ATP concentration did not decrease in the leaves (Fig. 3). On the contrary, it increased, possibly because ATP utilizing reactions were slowed more than were ATP generating reactions. Similarly, the level of reduced nicotinamide adenine dinucleotide phosphate increased when pea plants were hardened (4). When the hardened cotton plants were returned to the

[3] Mention of a trademark name or a 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.



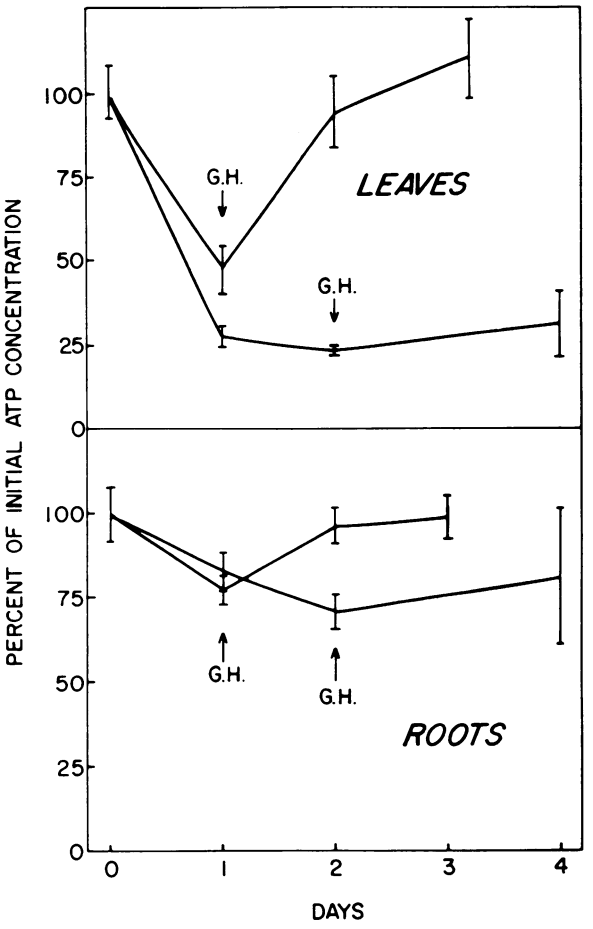


FIG. 1. (left) Comparison of ATP levels in chilled and control cotton seedling leaves (upper) and roots (lower). (———) chilled at 5°; (-----) control with 30° day, 20° night.

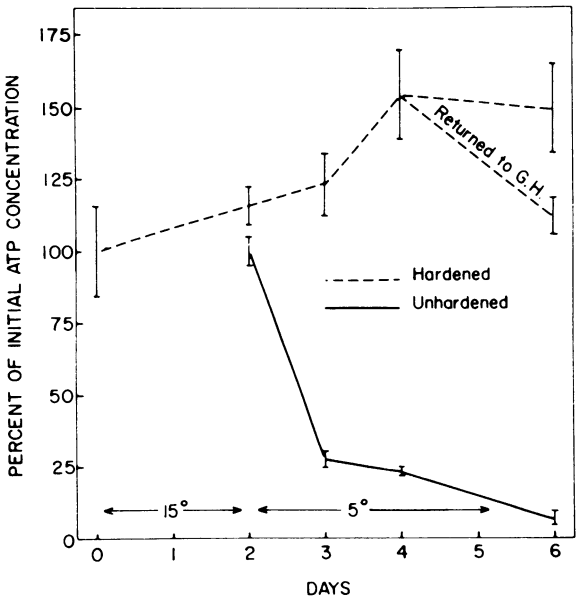
FIG. 2. (right) Effect of chilling on subsequent ATP levels in cotton seedling leaves (upper) and roots (lower). Plants were chilled for 1 or 2 days at 5° in the growth chamber then returned to the greenhouse (G.H.) for 2 days.

greenhouse after 2 days of chilling, the ATP level returned to normal. No visible damage could be detected in hardened plants after chilling.

The lower curve in Fig. 3 shows the effect of prolonged chilling on unhardened plants. In this particular experiment the ATP concentration of the foliage after 4 days at 5° was only about 6 % of the initial concentration. These plants were severely wilted and showed the necrotic areas typical of chilling injury.

In unhardened root tissue the ATP level continued to decrease with time of chilling (Fig. 4), and was less than 25 % of the original value after 4 days of chilling. Additional experiments in which ATP was purified by ion exchange chromatography indicated that this is a conservative estimate of the ATP decline. If the plants were hardened for 2 days, the concentration of ATP in the roots increased, but upon chilling of the hardened plants at 5° the ATP level in the roots began to decrease. The actual decrease over the 4-day chilling period was approximately the same as for the unhardened plants, although the relative change was less. Apparently, the protective action of hardening in roots was not as great as in the tops. Perhaps this partially explains why even hardened cotton cannot tolerate extended periods of chilling. Two days of chilling did not seem to injure hardened roots, since the ATP level returned to normal when the plants were returned to greenhouse conditions.

Considering the decrease in ATP with chilling at 5°, oxidative and photophosphorylation must be more sensitive to low temperature inhibition than systems that use ATP. However, the observation that the ATP concentration increased at the hardening temperature suggests that this relationship is not simple or direct. Our data supports the suggestion of Lyon *et al.* (7), that mitochondria from chilling-sensitive plants become less functional at low temperature. Membrane inflexibility at low temperatures could explain the decrease in ATP with chilling, since synthesis of ATP is associated with swelling



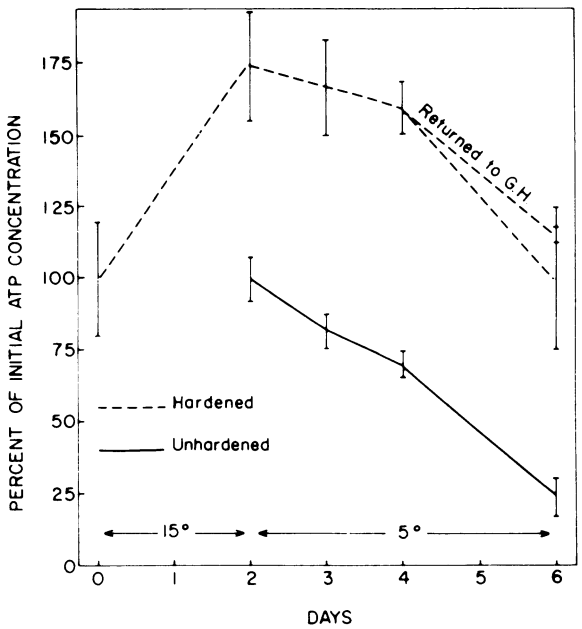


FIG. 3. (left) Effect of chilling on the ATP content of hardened leaves compared to unhardened leaves.
FIG. 4. (right) Effect of chilling on the ATP content of roots from hardened seedlings compared to unhardened seedlings.

and contraction of the mitochondria (5). Inflexibility does not necessarily mean inactivation; therefore, when plants are returned to warm conditions after short periods of chilling, the mitochondria may again be functional and resupply the ATP lost during chilling. Should chilling be continued, hydrolytic processes not associated with membranes would probably continue slowly (9) and consume the phosphorylated compounds present. Without a resynthesis of ATP a general decrease in phosphorylated compounds would result. Preliminary results with activity of isolated mitochondria and with anion-exchange column chromatography of nucleotides support these assumptions. Below a certain point of available energy, the tissue could no longer maintain the metabolic integrity of the cytoplasm necessary for survival. Structural damage could not be repaired and general disorganization of cellular structure and metabolic processes would occur with depletion of the usable cellular energy. Injury of this nature would vary with the length of time at low temperature as suggested in Fig. 2.

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