Published September, 1972

Cyanogenesis in Green Tissues of Cotton Plants[1]

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ABSTRACT

A cyanogenic compound was present in extracts of nearly all tissues of cotton plants (*Gossypium hirsutum* L.), with the greatest amounts present in photosynthetic tissues. Measured as HCN evolved from aerated aqueous homogenates, amounts in leaves varied from 27 to 92 μg/g (fresh wt basis). No significant correlation was found with leaf age. Unlike other green tissues, carpel walls contained little cyanide. Cyanogenesis in cotyledons depended upon light. Turnover of the cyanogenic compound was also accelerated in light, with more than 50% of the pool of cyanogenic compound being metabolized each day.

Additional index words: Cyanide, Asparagine, Photosynthesis, *Gossypium hirsutum* L.

CYANOGENESIS in some higher plants has been well known for more than a century. Its physiological roles, however, are unclear. Recently an enzyme has been purified that condenses cysteine and cyanide to form β-cyanoalanine (9). This compound is then further metabolized to asparagine in many species (2, 3, 13). In addition to this pathway, asparagine may be synthesized from aspartic acid (14). Accordingly, roots of cotton (*Gossypium hirsutum* L.) have been shown to synthesize asparagine from both cyanide and aspartic acid (17). This investigation was undertaken to determine whether cotton plants are cyanogenic, and to elucidate the conditions under which cyanide might play an important role in their metabolism.

MATERIALS AND METHODS

Leaves of cotton (*Gossypium hirsutum* L. 'Deltapine 16') were collected from plants grown in a greenhouse and watered with a modified Hoagland's solution. Lamina length was measured to the tip of the middle lobe. Bracts, bolls, and flowers were taken from the same plants. In addition, bolls and leaves were collected from plants of the glandless cultivar 'M-8' grown under the same conditions. Roots were taken from plants that had been grown in aerated liquid nutrient culture, either in a greenhouse or in a growth chamber.

Cotyledons were obtained from seeds germinated in darkness at 29 C in trays containing vermiculite moistened with nutrient solution. Trays containing the etiolated seedlings were transferred to a growth chamber. The chamber maintained a 16-hour daylength with day and night temperatures of 32 and 22 C, respectively. Relative humidity was approximately 30%, and light intensity was 42,000 lux. Dark-treated control plants were also transferred to the growth chamber, but were covered with a light-proof box.

Samples to be analyzed for cyanide were homogenized in distilled water in a Virtis[3] mixer and placed in flasks with 1 mg of β-glucosidase (Sigma Chemical Co.[3]). In early work the homogenates were incubated in closed vessels with sodium picrate paper (prepared by dipping filter paper in a solution of 0.01 *M* picric acid and 0.25 *M* NaOH and drying). The papers were then extracted with water and the color intensity measured in a colorimeter. This procedure was found to be unreliable, and was greatly improved by aerating the homogenates with CO_2-free air for 2 to 3 hours. The HCN produced was carried into the airstream and removed by a trap containing 10 ml of the alkaline picrate reagent described above. The resulting color, which developed fully only after 8 hours at room temperature, was measured at 485 nm. Aldehydes and some methyl ketones are known to interfere with this test (15); passing the airstream through I_2 solution (to form cyanogen iodide from HCN), however, or through 2,4-dinitrophenylhydrazine (to form the hydrazone derivative of aldehydes and ketones) indicated a maximum error of only 2 μg/g fresh wt. in the estimation of cyanide. Standards were run with each set of assays; all points were replicated at least 3 times except when noted otherwise.

Amino acids were determined by homogenizing samples in 80% ethanol and boiling for 5 min. The extracts were cooled, filtered, and partitioned three times with petroleum ether. The aqueous phase was treated with Dowex 50-X8 (H^+ form)[3], eluted from the resin with 2 *M* ammonia, taken to dryness, and redissolved in a small known volume of water. Aliquots were chromatographed in two dimensions on thin-layer plates with *n*-butanol-acetic acid-water (4:1:1 v/v/v) and phenol-water (75:25 w/w). The plates were sprayed with ninhydrin reagent (18) and heated. Amino acid spots of interest were scraped into test tubes and reacted further with 1 ml ninhydrin reagent in a boiling water bath for 20 min. The solutions were cooled, diluted with 5 ml 50% ethanol, filtered, and absorbance measured at 570 nm. Standards were processed similarly.

Chlorophyll was extracted by homogenizing samples for 1 min in 80% acetone. The extract was filtered and the precipitate washed. The filtrate and washings were combined and diluted to 100 ml, and absorbancy was measured at 660 nm. A single wash removed all green color from the precipitate.

RESULTS

Analysis of homogenates by incubation with picrate paper failed to detect any cyanide. In fact, homogenates of leaves, even when strongly buffered at acid pH, prevented volatilization of added cyanide (Table 1). This effect, although diminished, was also noticeable with glandless leaf extracts. Previous workers using this method also failed to observe cyanogenesis in cotton (A. Bell, personal communication). Table 1 suggests that gossypol or some related aldehyde might condense in equilibrium with cyanide to form a cyanohydrin. To eliminate this possibility, homogenates were continuously aerated to remove free HCN, thus shifting the equilibrium toward dissociation of cyanohydrins. All studies reported here are based upon this revised analytical method.

Compounds that could be hydrolyzed to form hydrocyanic acid occurred in virtually all tissues studied (Table 2). Only the photosynthetic tissues, leaves, green cotyledons, and bracts, however, contained large

Table 1. Recovery of HCN from leaf extracts to which KCN was added. Measurements were made with sodium picrate paper as described in text. Extracts were of 2 g fresh wt. leaf tissue and contained 0.5 *M* KH_2PO_4.

KCN added, μmoles	HCN recovered, μmoles	
	Glanded	Glandless
0.0	0.0	0.0
0.77	0.05	0.23
1.54	0.18	1.36
2.31	0.15	0.93
3.08	0.59	1.36

[1] Contribution of the Plant Science Research Division, ARS, USDA, and the Arizona Agricultural Experiment Station. Journal Paper No. 1890 of the Arizona Agricultural Experiment Station. Received Feb. 28, 1972.

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[3] 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.

Table 2. Cyanide released by homogenates of various parts of cotton plants. The homogenates were aerated with CO_2-free air for 2 to 3 hours to remove HCN from solution.

Tissue	Cyanide content, μg/g fresh wt.		
	Mean	Range	No. of replicates
Leaves	51	27-92	56
Bracts	17	10-29	5
Green cotyledons	45	39-51	6
Etiolated cotyledons	9	4-14	15
Carpel walls	1	0-3	12
Immature fibers	0	0	3
Immature seeds	7	4-12	3
Flowers	5	4-6	2
Roots	4	2-8	8
Mature seeds*	0.6	0.4-0.8	2

* Hulls removed.

amounts. Carpel walls, which are also green and presumably photosynthetic, released very little cyanide (Table 2).

Cyanide content varied from sample to sample, even when collected from the same plant. In 56 analyses, leaves were found to contain 27 to 92 μg HCN per g fresh wt (1 to 3.4 μmoles/g), with a mean of 51 (Table 2). These leaves, however, were collected without regard to position on the plant. When young, expanding leaves were analyzed, little or no relationship was found between leaf size and cyanide content (Fig. 1). Thus, the differences in cyanide content may have resulted from external factors affecting each leaf individually, rather than from reproducible developmental changes during maturation.

The distribution of cyanide (Table 2) suggested an influence of light. This influence was studied in young cotyledons, as uniformity of samples was easier to obtain than with leaves. In seedlings germinated in the greenhouse, cyanide began to increase in cotyledons about 4 days after imbibition, or about 1 day after emergence and subsequent chlorophyll formation (Fig. 2). This increase continued for 4 days, and then cyanide levels began to decrease. In dark-grown seedlings cyanide remained low throughout the period of measurement. This course of development of the cyanogenic compound suggested that it could have been a photosynthetic product. In addition, the decline in cyanide after 8 days suggested that seedlings were capable of degrading the cyanogenic compound. To test the latter possibility, 4-day-old dark-germinated seedlings were transferred to the growth chamber (16-hour daylength), then replaced in continuous darkness at various times. Again, cyanide increased after a 1-day lag, and the control plants that remained in continuous darkness contained little cyanide (Fig. 3). The behavior of seedlings that were exposed to light and subsequently replaced in continuous darkness depended upon the number of days of light. Seedlings transferred after 1 day (5 days old) gained a small amount of cyanide in the dark, whereas seedlings transferred after 2 and 3 days (6 and 7 days old) lost cyanide at the net rates of 2 and 6 μg per pair cotyledons per day, respectively (Fig. 3). Thus, even though cyanide began to increase 1 day after transfer to the growth chamber, loss of cyanide in darkened plants did not occur until after 2 days. The greater rate of loss of cyanide with increased exposure to light suggests that degradation as well as synthesis was stimulated by light. The increase in rate of degradation, however, may have resulted simply from greater availability of substrate. Degradation was initiated 1 day later than synthesis, and thus one might expect a net rise, then

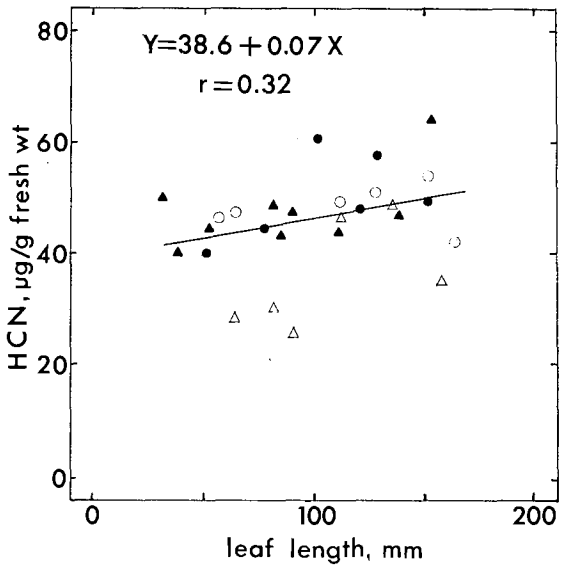

Fig. 1. Linear regression of cyanide content of leaves plotted against lamina length. Different symbols denote separate experiments. Points were not replicated.

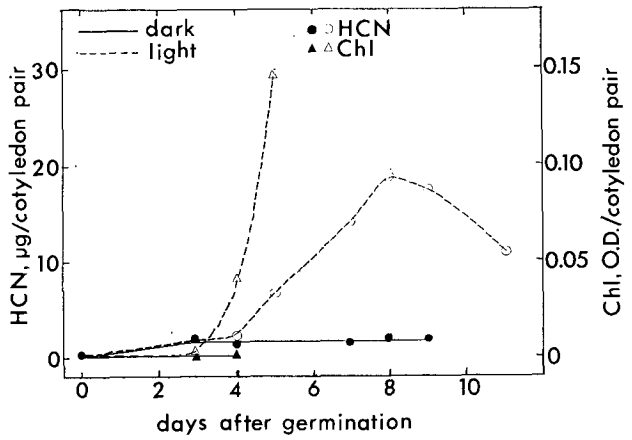

Fig. 2. Cyanide and chlorophyll (chl) contents of cotyledons of seedlings germinated and grown either in light or in darkness.

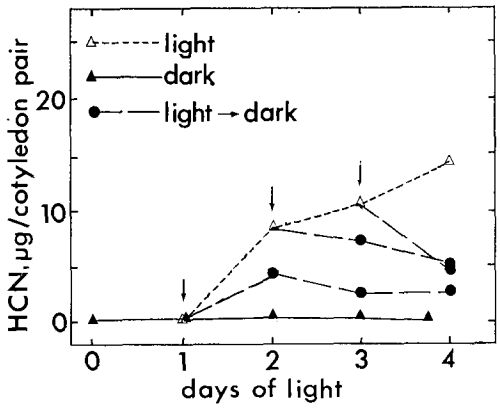

Fig. 3. Cyanide content of cotyledons of 4-day-old dark-grown seedlings transferred to light, then replaced in continuous darkness. Light treatment was in a growth chamber with 16-hour daylength, as described in text. Arrows indicate times of transfer to continuous darkness.

Table 3. Amino acid content of cotyledons of seedlings grown in the light. Control: 8-day-old seedlings. Dark: seedlings grown in light for 8 days and put in darkness for 1 day.

Treatment	Amino acid content, μmoles/g fresh wt.		
	Asparagine	Aspartic acid	Glutamic acid
Control	17	5	14
Dark	24	<1	12

a fall, in cyanide. This sequence of events agrees with that shown in Fig. 2.

Several experiments failed to show the release of free hydrocyanic acid in the dark. This failure indicates that all of the liberated cyanide must have been reassimilated. Assuming that the major product of the pathway is asparagine, cyanide turnover at rates demonstrated here would produce about 1 μmole asparagine per g fresh wt per day, equivalent to more than 50% of the total cyanide in the cotyledons. To determine the relative importance of this source of asparagine, 8-day-old greenhouse-grown seedlings were placed in the dark for 24 hours, and the free amino acids of the cotyledons were extracted and analyzed. Asparagine increased by about 40% in the dark, from 17 to 24 μmoles per g (Table 3). At the same time aspartic acid decreased by 4 μmoles per g. Part of this net decrease in aspartic acid may have resulted from conversion to asparagine. The estimated turnover of cyanide could account for about 15% of the increase in asparagine. The rest of the increase in asparagine must have resulted from hydrolysis of protein in darkness (12). In contrast, the level of glutamic acid changed less markedly in darkness, decreasing by only about 14% (Table 3).

DISCUSSION

Asparagine increased greatly when green seedlings were placed in darkness (Table 3). This response has been observed in other species as well (16). Differences in the behavior of glutamic and aspartic acids and asparagine indicate that the asparagine may be in a relatively inactive pool, and may accumulate as an end product. Similar conclusions were reached by Ting and Zschoche (17) for cotton roots. The most obvious use for asparagine in such a system would be the storage and translocation of N. Thus, the cyanogenic pathway of cotton plants may serve, in effect, as a means of mobilizing excess N in leaves. The distribution of asparaginase activity might help resolve this question.

Cyanogenesis has been associated with physiological fitness for warm climates (5), and also with defense against herbivores (10, 11). The factors that regulate the production and utilization of cyanide thus could be of considerable economic importance. It is believed that light and moisture are the most important factors affecting cyanide content (6). Nevertheless, environmental control of cyanogenesis has not been extensively studied, except for the influence of N (7, 8). Genetic variation in cyanogenesis is controlled by two loci affecting production and hydrolysis of the cyanogenic compound (1). The present work has demonstrated that these two systems are both affected by light and that they develop sequentially, since in greening cotyledons, the ability to degrade the cyanogenic compound lags behind the synthesis of the compound itself (Fig. 3).

In cotton plants, the dependence of cyanogenesis upon light is almost absolute (Fig. 2, 3). It is possible that synthesis of the cyanogenic compound occurs only during photosynthesis, as production of most known cyanogenic compounds requires amino acid skeletons, glucose, and UTP (4). All of these constituents might be expected to increase in green tissues during photosynthesis. The increase in degradation of the cyanogenic compound during greening, however, (Fig. 3) is more puzzling. Three possible explanations are a general increase in hydrolytic activity during aging of the cotyledons, a greater availability of cyanogenic compound for hydrolysis by preexisting enzymes, or synthesis of a specific hydrolytic enzyme. The first two are unlikely, as one might expect specific regulation of cyanide production so as to keep the pool of free cyanide small. Daday (5) has proposed cyanide autotoxicity as a mechanism of death under some circumstances; however, that this does not normally occur indicates the existence of some regulatory mechanism limiting the production of cyanide to the capacity of a tissue to assimilate it.

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