Published July, 1971

Description and CO_2 Metabolism of Aberrant and Normal Chloroplasts in Variegated Cotton, *Gossypium hirsutum* L.[1]

R. J. Kohel and C. R. Benedict[2]

ABSTRACT

Mutant chloroplasts in variegated cotton leaves contained reduced amounts of pigments, were smaller, and lacked any well defined grana system when compared with normal green chloroplasts. Replication of the defective chloroplasts was not altered. Mutant leaves fixed reduced amounts of $^{14}CO_2$ by photosynthesis, and the white leaves had a shift in the distribution of the products of $^{14}CO_2$ fixation. Mutant leaves utilized ^{14}C-2-acetate as effectively as green leaves.

Additional index words: **Chlorophyll and carotenoid concentration, $^{14}CO_2$ fixation, ^{14}C-2-acetate metabolism, Chloroplast growth and replication.**

CHLOROPLAST variegation in cotton occurs frequently as a spontaneous mutation. Individual variegated plants may vary from a single leaf to the entire plant expressing the variegated condition. The frequency of occurrence, and the stage and degree of spontaneous expression is associated with periods of unusually cool temperatures during the various stages of plant growth. Genetic analysis of these variegated plants has demonstrated that the mutant condition results from a permanent genetic change, which is maternally inherited (Kohel, 1967).

We became interested in further study of this variegated condition because it represents nonnuclear genetic variability that involves the development of the chloroplasts. Increased interest is being placed on the study of photosynthetic efficiency of crop plants (San Pietro, Greer, and Army, 1967), and there is a need for more information about the genetic processes controlling the photosynthetic apparatus.

In this paper we present the results of experiments that describe some of the developmental features of chloroplasts in the variegated cotton mutant.

MATERIALS AND METHODS

Variegated and normal green cotton plants, *Gossypium hirsutum* L., were grown in the greenhouse. Variegated plants were progeny from a common source. Leaf tissue for the various experiments was obtained by separating the mottled leaves into normal or mutant portions, or by selecting leaves of a homogeneous phenotype. Only a few leaves of homogeneous mutant expression are produced on the variegated plants.

The chlorophylls and carotenoids were extracted from the leaves by absolute methyl alcohol until the tissue became colorless. The absorption spectrum of this extract was determined with a Beckman[3] DK-2 recording spectrophotometer, and the amount of chlorophyll was determined by the method of MacKinney (1941). The methanol extract was saponified by treatment with 30% methanolic KOH at room temperature for 8 hours in the dark. The carotenoids were transferred to hexane by partition and the absorption of the hexane fraction was determined in the visible range.

CO_2 fixation in detached leaves was measured as previously described (Benedict and Kohel, 1968), with these exceptions: A 500-ml separatory funnel $^{14}CO_2$ fixation chamber was illuminated with 5,000 ft-c of light provided by two 150-watt flood bulbs through two flat, rectangular chromatography jars of water. The leaves were equilibrated 2 min in the light, exposed to $^{14}CO_2$ for 8 min, and then removed from the funnel and plunged into boiling 95% ethyl alcohol. The leaves were simmered for 20 min and extracted 4 times with boiling 95% ethyl alcohol. The extracts were combined and evaporated to dryness in a flash evaporator, in the presence of formic acid. The residue was dissolved in H_2O and ether, and the ether- and H_2O-soluble phases were separated and assayed for radioactivity.

The utilization of ^{14}C-2-acetate by the leaf tissue and the separation of the H_2O soluble radioactive compounds was performed by previously described procedures (Benedict and Kohel, 1968; Rinne, et al., 1965). Sodium ^{14}C-bicarbonate and sodium ^{14}C-2-acetate were purchased from New England Nuclear Corporation[3].

The amount of radioactivity in the aqueous samples was assayed in a Beckman[3] liquid scintillation system. A 0.2-ml aliquot of each radioactive sample was added to 15.0 ml of scintillation fluid containing 5 g of PPO (diphenyloxazole), 100 g of napthalene, 10 ml H_2O, and dioxane to one liter. The scintillation vials were dark adapted for several hours, and the counts assayed with $\pm$ 2% error.

RESULTS

Variegated plants, as the name implies, produced normal and mutant tissue. Leaves that developed from the mutant tissue were either mottled (green with yellow or white sectors, Fig. 1) or entirely yellow or white. Genetic analysis of variegated cotton mutants has shown that they are maternally inherited. The frequency of mutant expression increases with the degree of mutant expression in the maternal germinal tissue, and the degree of expression increases with cool temperatures (Kohel, 1967). Similar results have been reported in *Nicotiana* (von Wettstein and Ericksson, 1964), *Dianthus* and *Euphorbia* (Stewart, 1965), and in *Hosta* (Woods and du Bury, 1946).

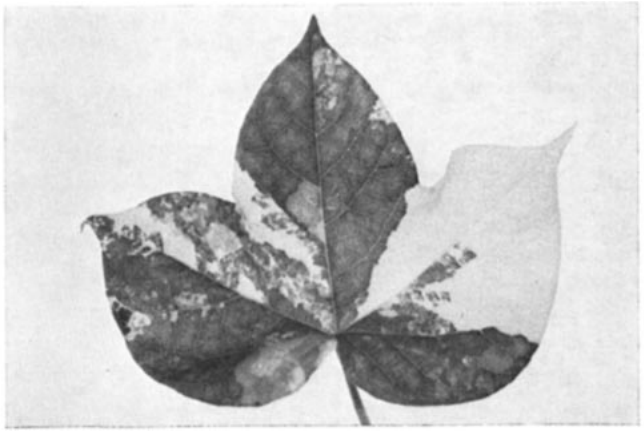

Fig. 1. Variegated cotton leaf with green and white sectors. The gossypol glands appear as prominant dark dots in the white tissue.

[1] Cooperative investigations of the Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture and Texas Agricultural Experiment Station. This work was supported in part by the Cotton Producers Institute and the National Cotton Council of America. Received September 19, 1970.

[2] Research Geneticist, Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture and Professor of Plant Physiology, Plant Sciences Department, Texas A & M University, respectively, College Station, Texas 77843.

[3] Mention of corporate or brand names does not imply USDA approval to the exclusion of products of other firms.

Table 1. Pigment concentration per gram fresh weight of normal and mutant tissue.

Leaf tissue	Chlorophyll$_{a+b}$ mg	Chlorophyll$_{a+b}$ %	Chlorophyll$_a$ / Chlorophyll$_b$	Carotenoids* µg	Carotenoids* %
Green	1.16	100	8.64	476	100
Yellow	.52	45	5.48	212	44
White	.06	5	1.17	22	5

* Carotenoids calculated from a $E^{1\%}_{1\ cm}$ β carotene of 2,500.

Table 2. Chlorophyll analysis of green, yellow, and white tissue from five variegated plants, from a genetic standard, Texas Marker 1, and spinach.

Leaf tissue	Chlorophyll$_{a+b}$ mg/g fresh weight	Chlorophyll$_a$ / Chlorophyll$_b$
Variegated - Green	1.46 ± .48	12.74 ± 7.84
Yellow	0.19 ± .21	4.00 ± 3.14
White	0.03 ± .02	0.62 ± 0.43
Texas Marker 1	1.35 ± .73	4.59 ± 2.41
Spinach	1.21 ± .53	4.84 ± 0.94

White leaf tissue contained small amounts of chlorophyll and carotenoids (Table 1). The yellow leaf tissue contained not quite one-half the pigments of green tissue. The yellow tissue bleached to white. Similar bleaching was observed in variegated tobacco (von Wettstein and Ericksson, 1964; Schmid, 1967). The rate and time at which it bleached was influenced by environmental conditions. High light intensities apparently increased bleaching. It appeared that the white leaves had bleached during their early developmental period prior to expansion, whereas, other leaves were fully expanded before bleaching occurred. In these leaves chlorophyll and carotenoids were lost at the same rate. The ratio of chlorophyll a to b indicated a differential loss of these two chlorophylls in the bleaching process. The loss of chlorophyll and the associated change in chlorophyll a to b ratios was highly correlated in the mutant tissues ($r = .85$, $df = 15$). The chlorophyll a to b ratios of the variegated samples were higher than normally expected (Table 2). However, the range of mean a/b determinations in normal green cotton varied from 4 to 8. The variability of a/b ratios between sample periods was probably due to variation in light intensities as has been demonstrated experimentally in *Chlorella* (Reger and Krauss, 1970). The presence of pigments in the mutant tissue establishes the presence of a functional enzyme system for the production of these pigments, but the loss of these pigments by bleaching indicated that the pigment-lamellar complex was unstable.

Large chloroplasts with pronounced grana were present in green palisade cells of a variegated cotton leaf; in contrast, mesophyll cells in the white sector contained only small plastids (Fig. 2). No internal structure of these mutant chloroplasts was apparent in the light photomicrograph. The mutant chloroplasts developed to about one-third the size of normal, but the number of aberrant chloroplasts in palisade cells was only slightly reduced when compared with the number of white chloroplasts. The mean number of chloroplasts per palisade cell was 25.54 ± 1.36 (11 cells counted) in green tissue and 21.16 ± .71 (19 cells counted) in the white tissue. The mutation resulting in variegated white leaf sectors did not terminate chloroplast replication, but it did limit chloroplast growth.

Electron micrographs of mutant chloroplasts from a white leaf showed no lamellar-grana system and are a striking contrast to the structure of normal chloroplasts (Fig. 3). Yellow leaf tissue of variegated leaves contains chloroplasts that develop to the primary lamellar stage, but they subsequently degrade (Benedict and Kohel, unpublished). The degradation of the lamellar structure was similar to that reported for tobacco (von Wettstein and Ericksson, 1964; Schmid, 1967) and accounts for the observed bleaching.

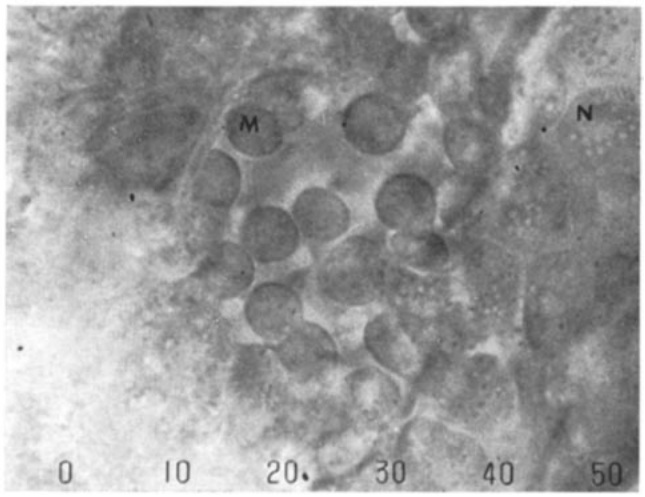

Fig. 2. Photomicrograph of a water-mounted fresh-cut section through a variegated leaf. The palisade cells contained normal (N) and the mesophyll cells contained mutant (M) chloroplasts (1500X).

Table 3. Distribution of radioactivity from photosynthetic $^{14}CO_2$ fixation in normal and mutant leaves.

Fraction	Radioactivity: Green CPM/g fr wt	Green Recovery, %	Yellow CPM/g fr wt	Yellow Recovery, %	White CPM/g fr wt	White Recovery, %
Ether-soluble	559,000		121,000		16,200	
H_2O-soluble	17,012,000	100	2,050,000	100	99,400	100
Basic (amino acids)	3,169,000	19	301,000	15	78,100	79
Neutral (sugars)	12,319,000	72	1,513,000	74	12,300	12
Acidic (organic acids)	1,793,000	11	270,000	13	19,600	20

Photosynthetic $^{14}CO_2$ fixation in yellow leaves was drastically reduced compared to the $^{14}CO_2$ fixation in green leaves (Table 3). The distribution pattern of radioactivity from $^{14}CO_2$ fixation in the yellow leaves was similar to the pattern in green leaves. The photosynthetic rate of yellow leaves in variegated cotton was in agreement with that of yellow-green sectors of variegated tobacco leaves (Schmid, 1967).

In white leaves, the photosynthetic $^{14}CO_2$ fixation was only 0.5% that of green leaves. There was no measurable dark CO_2 fixation. Most of the radioactivity in the white leaves was found in the amino acids and not in the sugars. The low rate of CO_2 fixation and accumulation of radioactivity in basic compounds accompanied the loss of photosynthetic pigments. The decreased amount of incorporation of ^{14}C into the ether-soluble fraction may have been a reflection of the lesser amount of photosynthetic pigments. The alteration of CO_2 metabolism was directly related to the presence of aberrant plastids.

The metabolism of ^{14}C-acetate into H_2O soluble compounds was similar in green, yellow, and white leaf tissues (Table 4). All leaf tissues absorbed an equal amount of radioactive acetate from the bathing solution. The low incorporation of radioactivity in the ether-soluble compounds in the yellow and white

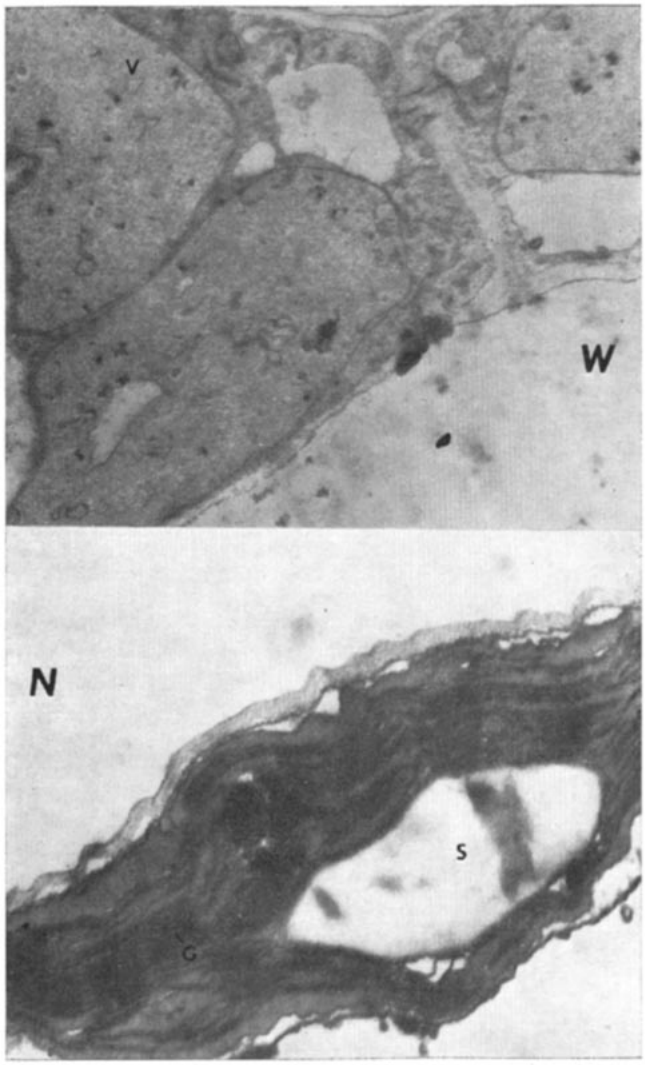

Fig. 3. Electron micrographs of mutant chloroplasts (14,500X) from a white leaf (W) and normal chloroplasts (10,000X) from a green leaf (N). The only visible structural organization in the mutant chloroplasts is the presence of vesicles (V). The normal chloropalst contain well defined grana (G) and starch (S). Sections were fixed with OsO_4 and stained with methanolin uranyl acetate and elad citrate.

tissues again may have been a reflection of the low content of pigments. Apparently the machinery responsible for acetate utilization into H_2O soluble compounds was not altered in the leaf cells containing aberrant plastids.

DISCUSSION

Examination of the mutant tissue of variegated cotton by pigment analysis, light and electron microscopy, and metabolic studies has shown that this tissue contained aberrant chloroplasts. The presence of chlorophyll and carotenoid pigments demonstrated the presence of the enzymes necessary for their production, and the chloroplasts were capable of reproduction, although their growth was arrested. Variegated cotton most certainly differs from variegated iojap maize in which the mutant chloroplast lacked ribosomes (Shumway and Weier, 1967) or variegated *Arabidopsis,* which showed extensive nondiscriminant

Table 4. Distribution of radioactivity from ^{14}C-2-acetate metabolism in normal and mutant leaf tissues.

	Radioactivity					
	Green		Yellow		White	
Fraction	CPM/g fr wt	Recovery, %	CPM/g fr wt	Recovery, %	CPM/g fr wt	Recovery, %
Ether-soluble	305,000		223,000		124,000	
H_2O-soluble	1,121,000	100	1,165,000	100	1,401,000	100
Basic (aaino acids)	244,000	27	200,000	24	169,000	18
Neutral (sugars)	38,000	4	35,000	4	48,000	5
Acidic (organic acids)	616,000	69	603,000	72	730,000	77

RNA degradation (Rédei, 1967). Either condition would terminate all enzyme synthesis and would lead to a complete disappearance of chloroplasts from the leaf cells because of the inability of these organelles to develop the enzyme machinery for replication.

There were similarities between the arrested growth of chloroplasts in mutant cotton tissue and chloroplasts in 5-fluorodeoxyuridine (FUDR)-treated tobacco leaves. Chloroplast growth was arrested when greening tobacco leaves were treated with FUDR, but replication and differentiation were not altered (Boasson and Laetsch, 1969).

Chloroplast growth and differentiation involves plastid enlargement, development of a pigment-lamellar system, production of a protein synthesizing system, synthesis of enzyme and structural proteins, and the development of a plastid replication system. Perhaps chloroplast enlargement and a stable pigment system (but not differentiation) is ultimately linked to the development of a lamellar system in the plastid. Errors in the aggregation of lamellar discs has resulted in reduced pigment concentration (von Wettstein, 1960). A mutation leading to a defective lamellar-grana system would have the net effect of reducing plastid enlargement and pigment concentration but not replication or differentiation.

LITERATURE CITED

Benedict, C. R., and R. J. Kohel. 1968. The characteristics of a virescent cotton mutant. Plant Physiol. 43:1611-1616.

Boasson, R., and W. M. Laetsch. 1969. Chloroplast replication and growth in tobacco. Science 166:749-751.

Kohel, R. J. 1967. Variegated mutants in cotton, *Gossypium hirsutum* L. Crop Sci. 7:490-492.

MacKinney, G. 1941. Absorption of light by chlorophyll solutions. J. Biol. Chem. 140:315-322.

Rédei, G. P. 1967. Biochemical aspects of a genetically determined variegation in *Arabidopsis*. Genetics 56:431-443.

Reger, Bonnie J., and R. W. Krauss. 1970. The photosynthetic response to a shift in the chlorophyll a to chlorophyll b ratio of *Chlorella*. Plant Physiol. 46:568-575.

Rinne, R. W., R. W. Buckman, and C. R. Benedict. 1965. Acetate and bicarbonate metabolism in photosynthetic bacteria. Plant Physiol. 40:1066-1073.

San Pietro, A., F. A. Greer, and T. J. Army. (ed) 1967. Harvesting the sun. Academic Press. New York. 342 p.

Schmid, G. H. 1967. Photosynthetic capacity and lamellar structure in various chlorophyll-deficient plants. J. Microscopie 6:485-498.

Shumway, L. K., and T. E. Weier. 1967. The chloroplast structure of iojap maize. Am. J. Bot. 54:773-780.

Stewart, Robert N. 1965. The origin and transmission of a series of plastogene mutations in *Dianthus* and *Euphorbia*. Genet. 52:925-947.

von Wettstein, D. 1960. Multiple allelism in induced chlorophyll mutants. II. Error in the aggregation of the lamellar discs in the chloroplasts. Hereditas 46:700-708.

———, and G. Ericksson. 1964. The genetics of chloroplasts. *In* Genetics Today. Proc. XI Intern. Congr. Genet. p. 591-612.

Woods, M. W., and H. G. du Bury. 1946. Seasonal changes in biological equilibria involving two chondriosomal systems in variegated *Hosta*. Phytopathology 36:472-478.