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Structure and Function of Tomato Leaf Chloroplasts During Ammonium Toxicity[1]

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Summary. Ammonium toxicity resulted in morphological modifications of tomato leaf chloroplasts. The chloroplasts, which are normally flattened around the protoplast periphery, became ellipsoidally rounded and dispersed through the protoplasm. The first apparent effect of plastid degradation was development of many vesicles from the fretwork. Later the grana lamellae swelled, and some disappeared. Eventually, distinct grana could not be detected.

Ammonium accumulation, chlorophyll loss, and photosynthetic decrease occurred simultaneously. Initial changes in these processes preceded the detection of modifications of fine structure; however, each continued with further breakdown of the chloroplasts.

In many higher plants prolonged application of ammonium as a source of nitrogen leads to serious physiological and morphological disorders resulting in chlorosis, restricted growth, and in some cases death (4, 5, 10, 29, 30). With extended ammonium nutrition major alterations in protein metabolism occur. Workers have noted (4, 34) that in plants treated with ammonium, the synthesis of insoluble nitrogen compounds within the leaves levelled off and eventually showed a small decrease; subsequently, soluble nitrogen increased. Barker et al. (4) showed that the high concentrations of the soluble nitrogen compounds accumulating under ammonium nutrition were largely derived from endogenous sources.

An increase in concentration of free ammonium may disrupt various aspects of plant metabolism. Ammonium ions uncouple photophosphorylation (2). In concentrations of 0.6 mM, ammonium ions inhibit ATP formation by 50% (17). This results in a reduction of CO_2 fixation within the chloroplast (26). Although in low concentrations the ammonium ion enhances electron transport in photosynthesis by uncoupling photophosphorylation, at concentrations of 6 mM and greater it inhibits the reduction of ferricyanide in the Hill reaction (11). Similar experiments have also revealed that it inhibits the reduction of NADP (28).

Despite intensive investigations on the physiological responses of plants to ammonium nutrition, very little has been done to relate such responses to morphological changes. The symptoms of ammonium toxicity suggest that morphological changes might occur in the chloroplasts. Chlorosis of plants treated with ammonium has been reported to occur in conjunction with an imbalance between the soluble and insoluble nitrogen compounds (4). Chlorophyll synthesis has been shown to be dependent on protein synthesis (16). Within the leaves of higher plants, the majority of proteins are chloroplastic (19, 35) and exist in a state of turnover. The proteins of chloroplasts are found in the stroma and in the complex system of lamellar membranes of lipoprotein (31). The organization of the lamellar network of the chloroplast is dependent on the cellular environment of the leaf (7, 14, 20, 24), particularly the nutrient composition. It was felt that an ultrastructural examination of the chloroplasts of plants treated with ammonium would provide an indication of the morphological aspects of ammonium toxicity and permit further correlation between morphological features and physiological symptoms of ammonium toxicity.

Materials and Methods

Tomato plants (*Lycopersicon esculentum* Mill., cv. Heinz 1350) were chosen for this study because they develop ammonium toxicity symptoms rapidly and sequentially. Seeds were sown in flats of soil, and the seedlings were grown for 5 weeks in a greenhouse. The young plants were then transplanted into 8-inch plastic pots containing a 1:1 mixture of coarse and fine silica sand and were treated with Hoagland's No. 1 nutrient solution (12) for 15 days. At the end of this period, the plants were randomly divided into 2 groups to be treated with NH_4^+ or NO_3^-. The root medium of the group to be treated with ammonium was washed with the ammonium nutrient solution, which was supplied as a modified Hoagland's

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solution with all the nitrogen as $(NH_4)_2SO_4$. The other group was retained on the Hoagland No. 1 solution and was termed the nitrate or control group. Solutions were applied twice daily at 250 ml per culture. The minor element solutions of Hoagland and Arnon (12) were used to supply micronutrients. Experiments were conducted in April, June, and July, 1966, in which ammonium was applied for 28, 20, and 12 days respectively.

Photosynthesis and respiration measurements were determined on a Gilson Differential Respirometer. Small leaves were selected from the second largest branch from the tops of plants and were placed in 15 ml Gilson reaction vessels containing 3 ml of 0.2 M tris solution adjusted to pH 7.2 with 0.1 N HCl. A constant atmosphere of 1% CO_2 in air was obtained by placing 0.6 ml of Pardee buffer in the center well of the reaction vessel (cf 27). Oxygen evolved during photosynthesis was measured at 10-minute intervals over a 60-minute period. During this interval the reaction vessels were shaken in a water bath at 25° and illuminated from below with a light intensity of 1000 ft-c. Dark respiration, in terms of O_2 consumption, was measured at 10-minute intervals over a 30-minute period after photosynthesis.

Soluble nitrogen constituents were extracted by homogenizing 5 g or 10 g of leaf tissue in 70% (v/v) ethanol, filtering by suction, and washing several times with 70% ethanol. Ammonium and amide fractions of the ethanol extract were determined by a modified Kjeldahl method (3). The chlorophyll content of the leaves was determined by the method of Arnon (1) using a wavelength of 652 mμ.

Leaf sections for the ultrastructural examinations were selected from the middle of the leaf samples, cut 1 mm² and fixed in unbuffered 2% (w/v) $KMnO_4$ for 20 minutes at room temperature. The Luft (18) procedure for dehydration and embedding was followed. The sections were embedded in a 1:1 mixture of Epon A to Epon B, polymerized at 60° for 24 hours and sectioned with a diamond knife on a Porter-Blum microtome (Servall MT-1). The sections were collected on 200-mesh formvar-coated copper grids and examined with a Carl Zeiss EM-9 electron microscope.

Results

Photosynthetic Oxygen Evolution. Ammonium nutrition restricted the rate of net photosynthesis (table I). Two days after initiation of the treatment with ammonium, net photosynthetic O_2 evolution rates were nearly 30% less than those of the nitrate controls and continued to be lower throughout the experiment. Values near the compensation point were reached after 17 and 19 days of treatment. As seen below, the decreases in photosynthesis were not accompanied by any large increases in respiration.

Table I. *The Effects of Ammonium and Nitrate Nutrition on the Apparent Oxygen Evolution by Tomato Leaves*

Within columns, $LSD_{.05}$ = 0.72 for July series and 0.30 for June series and $LSD_{.01}$ = 0.97 for July series and 0.40 for June series. All values are means of 5 samples.

July series treatment	Days after initiation of ammonium treatment				
	2	5	8	10	12
	μl O_2 10 min^{-1} g^{-1} fr wt				
NH_4^+	106a*	112a	134a	75a	109a
NO_3^-	141a	132a	209ab	259b	263b
June series treatment	**Days after initiation of ammonium treatment**				
	13	15	17	19	
	μl O_2 10 min^{-1} g^{-1} fr wt				
NH_4^+	86b	63b	—8a	15a	
NO_3^-	163ab	189b	173ab	149a	

* Within rows, means not followed by the same letter are significantly different at the 5% probability level.

Respiratory Oxygen Consumption. Initially the leaves from the plants treated with ammonium showed a respiratory rate equal to that of the control plants. After 8 days of treatment, the O_2 consumption rate of the leaves from plants treated with ammonium increased 26% over the respiratory rate of the control plants (table II). The rate of O_2 consumption then declined with further treatment until it levelled off after 13 days of treatment.

Chlorophyll Content. Rapid and consistent losses of chlorophyll were shown by the series ran in June and July (table III). Agreement is found between the first losses of chlorophyll, accumulation of ammonium and amides, and decline in photosynthetic rate.

Table II. *The Effects of Ammonium Nutrition on the Apparent Oxygen Consumption by Tomato Leaves*

Within columns, $LSD_{.05}$ = 0.19 for July series and 0.21 for June series and $LSD_{.01}$ = 0.25 for July series and 0.28 for June series. All values are means of 5 samples.

July series treatment	Days after initiation of ammonium treatment				
	2	5	8	10	12
	μl O_2 10 min^{-1} g^{-1} fr wt				
NH_4^+	70a*	64a	123c	103bc	83ab
NO_3^-	73a	70a	97b	99b	92b
June series treatment	**Days after initiation of ammonium treatment**				
	13	15	17	19	
	μl O_2 10 min^{-1} g^{-1} fr wt				
NH_4^+	59a	62a	70a	55a	
NO_3^-	106b	88a	89a	100b	

* Within rows, means not followed by the same letter are significantly different at the 5% probability level.

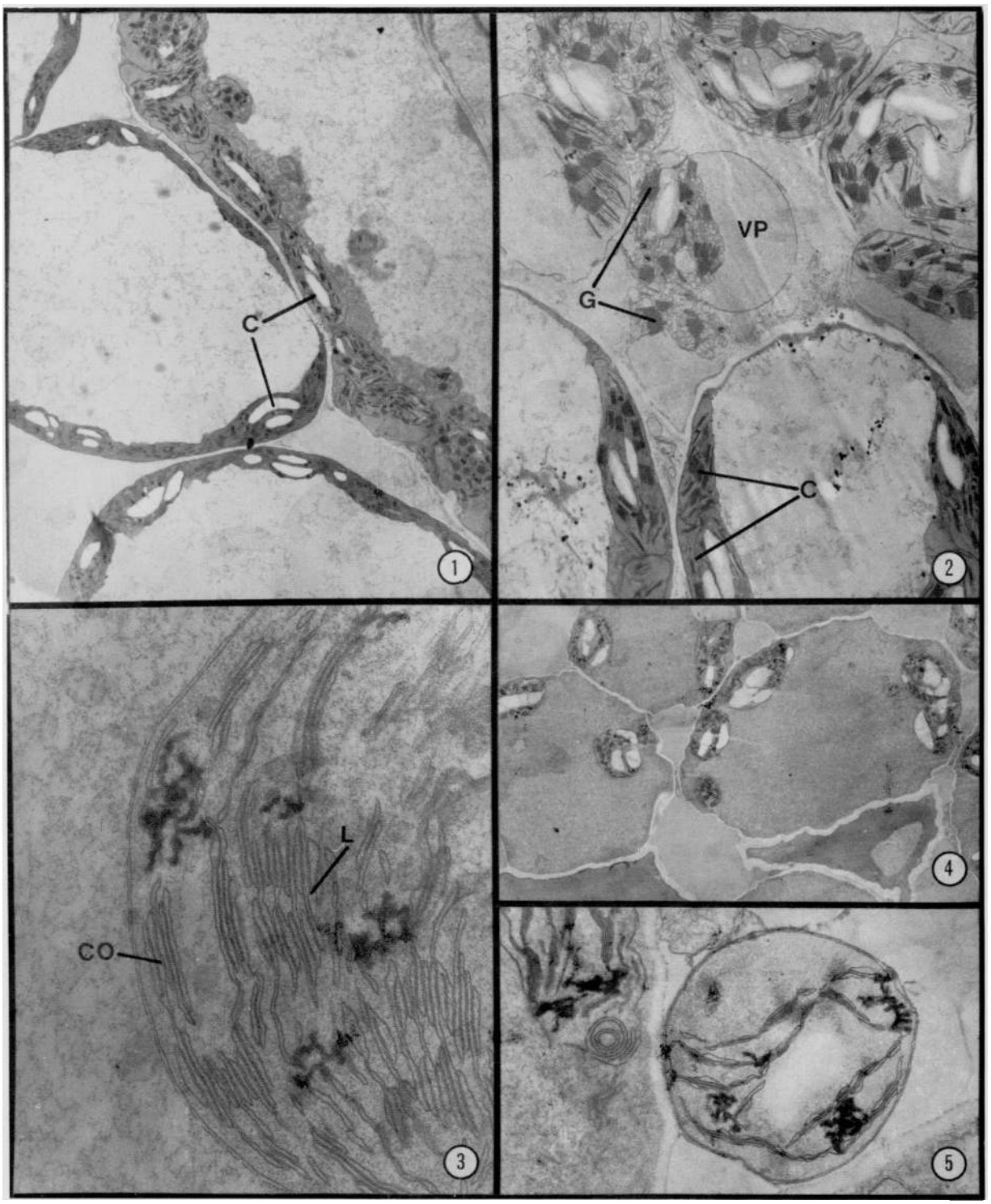

FIG. 1. Chloroplasts (C) from plants 3 days on ammonium treatment. Plants appear similar in position and structure to control plastids, × 5,546.

FIG. 2. Chloroplasts from 10-day ammonium treated plants. Note the occurrence of vesicle-filled plastids (VP) among the normal plastids (C). Grana (G) still appear structurally intact. Appearance of grana-fret components within the cytoplasm shows evidence of plastid disruption, × 8,002.

FIG. 3. Details of a chloroplast from a 10-day ammonium treated plant. Compartments (CO) are greatly swollen and loculi (L) can be seen opening into frets, × 33,480.

FIG. 4. Plastids from a 17-day ammonium treated plant. The chloroplasts are rounded in contour and appear widely spaced from each other, × 6,255.

FIG. 5. Chloroplasts from a plant 20 days on ammonium treatment showing reduction in granal number and swollen compartment number, × 17,800.

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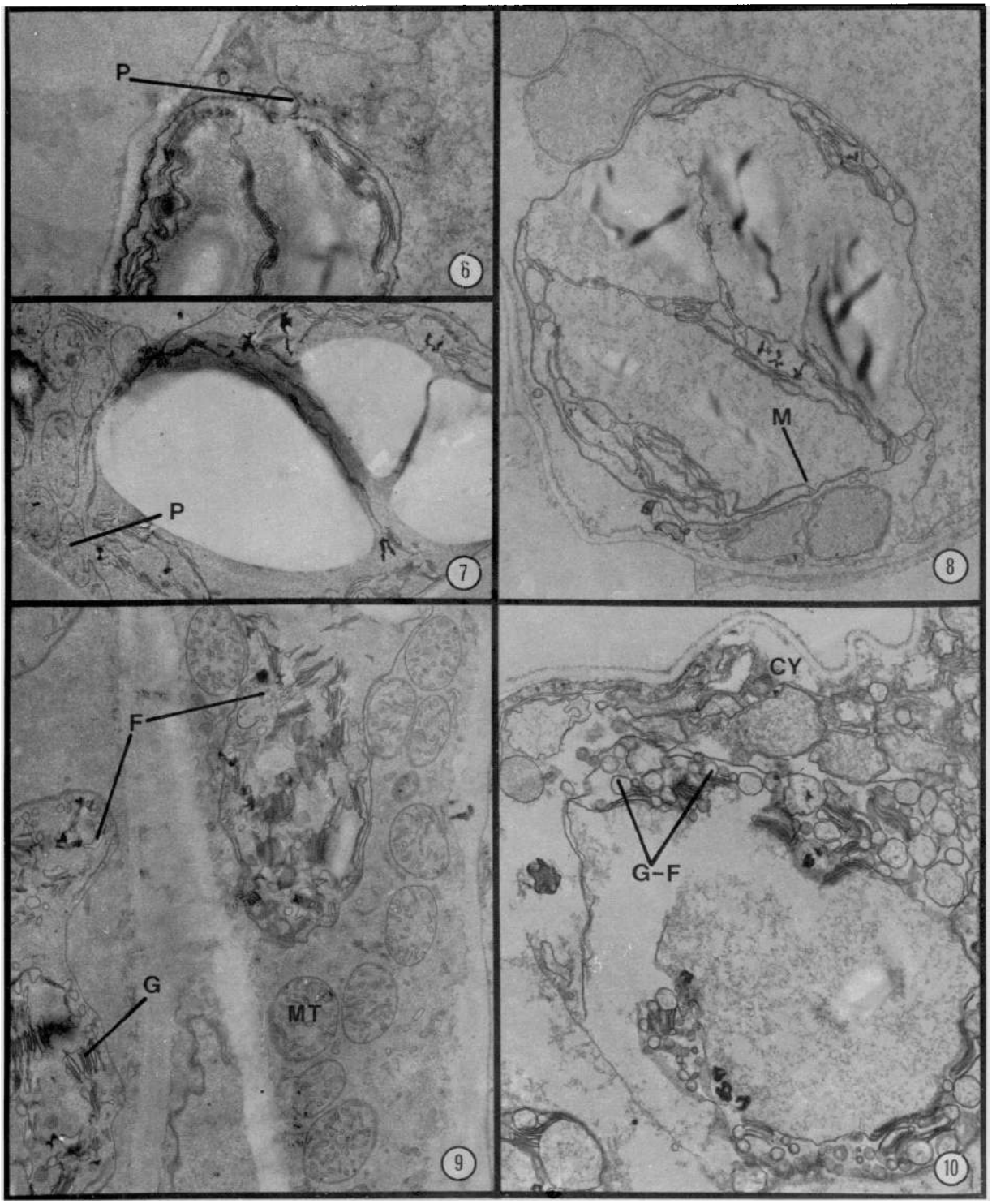

FIG. 6. Chloroplast from the cells of an ammonium treated plant (28 days on treatment) with a protrusion (P) extending from the plastid membrane, × 22,240.

FIG. 7. Mitochondrial-like protrusion (P) extending from the plastid membrane of a chloroplast from an ammonium treated plant (21 days on treatment), × 8,757.

FIG. 8. Chloroplast from an ammonium treated plant (28 days on treatment) showing absence of granal organization. The plastid membrane (M) appears continuous with the granal lamellae system, × 15,012.

FIG. 9. Chloroplast from ammonium treated plant (28 days on treatment). The granal components (G) are swollen and the frets (F) are evident as tubules or vesicles. Normal appearing mitochondria (MT) surround the plastid, × 15,012.

FIG. 10. Disrupted chloroplast from a 28-day ammonium treated plant. Plastid membrane is broken and the grana-fret components (G-F) are vesiculated and dispersed throughout the cytoplasm (CY), × 12,788.

Insert

Table III. *The Effects of Ammonium and Nitrate Nutrition on the Chlorophyll Content of Tomato Leaves*

Within columns, $LSD_{.05}$ = 0.40 for June series and 0.48 for July series and $LSD_{.01}$ = 0.53 for June series and 0.64 for July series. All values are means of 5 samples.

June series treatment	Days after initiation of ammonium treatment									
	1	4	6	7	9	11	13	15	17	19
	mg chlorophyll/g fr wt									
NH_4^+	1.93d*	2.01d	1.32c	1.57c	0.81b	0.73b	0.94b	0.74b	0.43a	0.28a
NO_3^-	1.66a	2.52b	2.75b	2.59b	2.66b	2.90b	2.91b	2.59b	2.76b	2.63b

July series treatment	Days after initiation of ammonium treatment				
	2	5	8	10	12
	mg chlorophyll/g fr wt				
NH_4^+	2.37b	2.51b	2.51b	1.47a	1.40a
NO_3^-	2.54a	2.48a	3.54b	3.37b	3.50b

* Within rows, means not followed by the same letter are significantly different at the 5 % probability level.

After 19 days of treatment, nearly 90 % of the chlorophyll was lost from the leaves.

Ammonium and Amide Contents. The patterns of ammonium and amide accumulations are shown in table IV. Only the June series is presented since there was little difference between the 2 series analyzed. Differences in ammonium accumulation between plants treated with ammonium and nitrate occurred 4 days after the initiation of the ammonium treatment. These differences became larger as time progressed. The accumulation of amides was similar.

Chloroplastic Ultrastructure. Selected micrographs from the series of experiments are shown in figures 1 to 10. During the first 10 days of treatment, plastids from leaves of plants receiving ammonium were not detectably different from plastids of plants treated with nitrate (fig 1). However, sections from leaves of plants treated for 10 days with ammonium in the April series showed vesicle-filled plastids interspersed among normal plastids (fig 2). These vesicles developed from the fretwork. The grana appeared to be relatively unaffected by this vesiculation since they maintained their structural integrity. Some of the plastids appeared broken, and lamellae were scattered throughout the cell. The effect on chloroplastic ultrastructure after 10 days of ammonium treatment was similar in the June series. Vesicles were not apparent although the plastids showed structural modifications such as swollen compartments, reduced grana and disrupted frets (fig 3).

Micrographs from plants under ammonium treatment for longer than 10 days revealed further structural alterations. The plastids no longer appeared abutted to each other but were widely spaced and dispersed within the cell (fig 4). Some compartments of grana were lost, and others had expanded loculi (fig 5). After 20 days of ammonium nutrition, marked differences in chloroplastic organization were evident in all specimens observed. The chloroplasts appeared to have a rounded contour as opposed to the rectangular or spindle shape of the plastids of control plants. The chloroplastic membrane sometimes formed small protrusions (figs 6, 7) and in a few cases appeared broken. Some plastids illustrated a continuity between the chloroplast membrane and the granal lamellar system (fig 8). Although the

Table IV. *The Effects of Ammonium and Nitrate Nutrition on the Concentration of Ammonium and Amide Nitrogen in Tomato Leaves*

Within columns, $LSD_{.05}$ = 0.11 for ammonium and 0.07 for amide nitrogen and $LSD_{.01}$ = 0.14 for ammonium and 0.09 for amide nitrogen. All values are means of 3 samples.

June series treatment	Ammonium-nitrogen Days after initiation of ammonium treatment									
	1	4	6	7	9	11	13	15	17	19
	mg nitrogen/g fr wt									
NH_4^+	0.09a*	0.13ab	0.21abcd	0.20abcd	0.33d	0.17abc	0.33d	0.23bcd	0.26bcd	0.27cd
NO_3^-	0.08d	0.05bc	0.03a	0.06c	0.04ab	0.06c	0.06c	0.03a	0.05bc	0.03a

June series treatment	Amide-nitrogen Days after initiation of ammonium treatment									
	1	4	6	7	9	11	13	15	17	19
	mg nitrogen/g fr wt									
NH_4^+	0.11a	0.20abc	0.19abc	0.15ab	0.24bc	0.19abc	0.16ab	0.28c	0.24bc	0.18abc
NO_3^-	0.08b	0.12c	0.07b	0.08b	0.07b	0.07b	0.08b	0.06a	0.06a	0.09b

* Within rows, means not followed by the same letter are significantly different at the 5 % probability level.

plastids contained grana, they were commonly distorted due to swollen compartments (fig 9). In contrast to the control plants, the plastids of the plants treated with ammonium were marked by an absence of large grana. Only 1% of the injured plastids observed contained grana with greater than 20 compartments, the average number per granum being approximately 5. In more extreme cases, the grana completely lost their orientation within the plastid and appeared as a dispersed lamellar system (fig 8) or as various sized vesicles (fig 10). The fretwork showed a reduction and was typically swollen into vesicles and tubules (fig 9).

Discussion

It is evident that ammonium nutrition altered the physiological mechanisms of the tomato leaf and disrupted its morphological organization. The sequential study of the effects of ammonium nutrition permits an evaluation between the physiological disorders and the morphological alterations as well as a correlation of both of these factors with the functional capacity of the leaf.

One of the characteristic signs of ammonium toxicity is the yellowing of the leaves (5, 10, 15, 30). The results of the chlorophyll determinations in this investigation indicate that the chlorophyll content of the leaves was reduced very early after the initiation of ammonium treatment and continued to decline with increasing time on ammonium nutrition. The rapid chlorophyll loss might be explained by an effect of ammonium toxicity on the biosynthesis of chlorophyll (6, 9, 16, 32, 33).

The initial loss of chlorophyll from leaves of plants treated with ammonium appeared to exert little effect on the chloroplastic ultrastructure although it may have done so after further loss. In any case, a severe disruption of the morphological placement and internal organization of the chloroplasts occurred under ammonium treatment. The chloroplasts from the leaves of plants treated with ammonium typically showed a lack of orientation in relation to the cell (fig 4). In contrast, the chloroplasts of the control cells were closely appressed to the cell wall and tightly abutted to each other. The chloroplasts in leaves injured with ammonium were often round in section and frequently had small protrusions extending from the plastid membrane (figs 6, 7). Possingham et al. (24) reported similar protrusions on the chloroplasts of manganese-deficient spinach and attributed the protrusions to the reduction of the frets. The granal component of the chloroplast was modified by ammonium nutrition. The number of compartments per granum was greatly reduced and in certain cases the grana were absent (fig 8). In more extreme instances, breakdown of the chloroplast was observable with vesiculation of both granal and fret components and disruption of the plastid membrane (fig 10). Plastid breakdown could perhaps result in a large increase in soluble nitrogen compounds such as was observed with *Phaseolus vulgaris* (4).

Photosynthesis was restricted by ammonium toxicity. The concentration of ammonium in the leaves was sufficiently high to uncouple photophosphorylation (2, 17, 26) and to inhibit NADP reduction (28). Reduced photophosphorylation may have lowered the energy available for protein synthesis and restricted net protein synthesis. Plastid breakdown may have occurred as a result of the lower protein synthesis. Uncoupling of electron transport in photosynthesis may also have resulted in disruption of the substructural organization of the plastid (13, 21, 22). It has been determined that electron transfer reactions in cyclic and noncyclic photophosphorylation are responsible for mechanochemical changes in the chloroplastic membranes (22). Measurements of the scattering of light by isolated, intact chloroplasts showed that conditions of electron transport and phosphorylation such as induced by light, or addition of ATP caused a contraction of the plastid (21). Under these conditions the plastids appeared as long spindles or crescents (13). When phosphorylation was interrupted by an uncoupler, e.g., ammonium, or by removal of the light source, the chloroplast swelled and became ellipsoidal (13, 22). Uncoupling of phosphorylation from the electron transport system led to swelling and disruption of the grana-fret network (14). Structural changes of these chloroplasts resembled those of the chloroplasts injured by ammonium.

Another possible mechanism of ultrastructural breakdown of the chloroplast under ammonium nutrition is the disruption of membrane structure through constant loss of chlorophyll. The chlorophyll molecules are a component part of both the grana and the fretwork of the chloroplast. The molecules are uniformly distributed throughout the lamellar structure of the chloroplasts, but chlorophyll may be more evident in the grana regions because of their higher number of chlorophyll-containing lamellae (23). In view of the fact that the chlorophyll molecules are intimately linked with the membranous component of the grana-fret network, under constant chlorophyll loss the chloroplastic ultrastructure might be disrupted. Much of the loss of chlorophyll may be associated with loss of granal lamellae.

A third possibility for the structural modifications of chloroplasts injured by ammonium is derangement of protein metabolism. Work with pole beans established that ammonium nutrition caused a reduction in synthesis of insoluble nitrogen compounds in leaves (4). Use of ^{15}N also revealed that ammonium treatment caused a large increase in the soluble amino acids at the expense of endogenous sources (4). Since chloroplasts contain as much as 70% of the leaf protein (35) and chloroplastic protein undergoes a very rapid turnover rate (25), it appears that altered protein metabolism might exert its main effect on the chloroplast. The high turnover rate of chloroplastic proteins when coupled with decreased protein synthesis would easily manifest an alteration

in structure and function on the chloroplasts of treated plants. Continued degradation without net synthesis would lead to intensive structural breakdown of the plastids and cessation of their physiological processes.

Respiration may be similarly affected by altered protein metabolism. When nitrate is used as a source of nitrogen it may partially replace O_2 as the terminal acceptor of electrons during respiration. Ammonium, on the other hand, will not accept electrons with the result that O_2 would be the major acceptor. This possibly accounts for the greater degree of O_2 consumption in the leaves after 8 days of ammonium nutrition. Later as treatment with ammonium continues, the altered protein metabolism described above may affect the enzymes of the respiratory cycle resulting in a decreased respiration rate.

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