Effects of the Phosphorus-Manganese-Atrazine Interaction in Soybean Plants

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Possible mechanisms for the mode of action of the atrazine-phosphorus interaction in soybeans were investigated. Use of ring labeled C^{14}-atrazine established that increasing P uptake did not increase C^{14} accumulation by soybean shoots. Early in the study Mn was found to be involved in the interaction. By increasing the Mn content of the nutrient solution, inhibition of photosynthesis by excess P or atrazine treatments could be delayed. Similar subcellular effects were observed in the chloroplasts from Mn deficient, excess P, or atrazine treatments. Individually or in combination these treatments reduced the number of grana in the chloroplasts and disrupted intergranal connections so that the thylakoids no longer remained in stacks. In the advanced stages of injury the thylakoids became swollen. The data suggested that the function of Mn in membrane structure and photosynthesis was inhibited by either P or atrazine or both.

Several workers have observed that phosphorus (P) in the soil may influence the activity of herbicides. Upchurch *et al.* (1963) and Adams (1965) reported that simazine [2-chloro-4,6-bis(ethylamino)-*s*-triazine] was more phytotoxic when the P content of the soil was high. Adams and Espinoza (1969) recently reported a similar interaction between P content of the soil and phytotoxicity of atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine) to soybeans. Based on chemical analysis of the tissue and visual symptoms of atrazine injury, they suggested that P might have increased the sensitivity of soybean plants to atrazine.

The literature reports similar subcellular effects in manganese (Mn) deficient and atrazine injured plants. In Mn deficient algae, Teichler-Zallen (1969) reported a reduced tendency of the discs to form stacks, as evidenced by the appearance of many isolated or singlet discs, and of discs already within stacks to dissociate from each other. Ashton *et al.* (1963) studied the effect of atrazine on the ultrastructure of chloroplasts of kidney bean plants. They concluded that the changes were the result of modification of the various membranes, which could account for the breakdown of intergranal connections, the swelling of compartments, and the dissociation of compartments within the grana.

Although the authors are not aware of literature reports of a direct effect of P on Mn availability, zinc (Zn) and iron (Fe) deficiencies of high P soils have been observed frequently (Brown *et al.*, 1959; Burleson *et al.*, 1961; Stukenholtz *et al.*, 1966; Watanabe *et al.*, 1965).

Boawn and Brown (1968) have pointed out that inducing a growth disorder by increased P had no effect on the Zn concentration in, or the total accumulation by, beans or potatoes. They concluded that P induced Zn deficiency is a manifestation of a physiological imbalance between P and Zn.

Paulsen and Rotimi (1968), using two soybean varieties differing in sensitivity to P nutrition, found that added Zn overcame the effect of P on the tolerant variety but not on the sensitive variety. Their data suggested that excess P may affect two mechanisms. Boawn and Brown (1968) reached similar conclusions studying the behavior between plant species that exhibited distinct differences in susceptibility to Zn deficiency in the field. In addition to Zn metabolism, the metabolism of other nutrient elements, such as Mn, may be affected.

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Mn is essential for photosynthesis in algae and higher plants (Pirson, 1937). It acts as a cofactor in the oxygen-evolving sequence of photosynthesis (Kessler, 1955). Mn has been established as a constituent of higher plant chloroplasts (Possingham and Spencer, 1962). These workers also found a close correlation between the Mn content of chloroplasts and their photochemical activity (as measured by Hill reaction activity). Thus, Mn and atrazine affect the same chemical systems in plants.

As the Mn involved in photochemical reactions seems to be tightly bound to the chloroplast, Possingham and Spencer (1962) proposed that Mn is a constituent of some macromolecular complex, such as a manganoprotein, and may have a structural as well as a functional role at the molecular level. Several workers have observed restoration of Hill reaction activity in Mn-deficient algae by the addition of Mn salts to whole cells. Cheniae and Martin (1966) suggested that the restorative effect of Mn was by binding of the metal to an apoenzyme and not merely an increase in synthesis of cellular material. They proposed that Mn^{2+} was bound to an apoenzyme in a dark thermal reaction. In the complexed form, Mn would be inactive as a catalyst in oxygen evolution. If these mechanisms are operative, both biochemical and physical effects from atrazine, phosphorus, and/or manganese might be expected.

The present investigation studied the physiological effect of this interaction with an attempt to provide some information concerning the mechanism.

MATERIALS AND METHODS

Chippewa 64 variety soybean seeds, weighing 160 to 200 mg, were germinated in the dark on deionized water-saturated paper. After 5 days, five seedlings were transferred to a polyethylene container holding 2 l. of nutrient solution. One-half strength Hoagland minus-P nutrient solution (Hoagland and Arnon, 1950) was used with designated amounts of P and Mn added in the forms of NaH_2PO_4 and $MnCl_2$. Sequestrene 330 Fe at a rate of 2.5 ml of stock solution (40 g per l.) per l. of nutrient solution was used in place of ferric tartrate. The solution was adjusted with NaOH to pH 5.5 and aerated continuously throughout the growth period. The nutrient solution was replaced every 5 days.

The plants were grown in a growth chamber with a 16-hr day and 21° C day and 15.5° C night temperatures. The light intensity was approximately 2000 ft-candles.

Mineral Analysis of Soybean Tissue. The plants were grown in nutrient solutions containing two levels of Mn (0

Table I. Effect of Atrazine on the Growth and Mineral Composition of Soybean Shoots Grown in Nutrient Solutions Containing Different Levels of Mn and P

Mn added ppm	P added ppm	Atrazine added ppm	Dry matter per plant g	P %	K %	Ca %	Fe ppm	Mg %	Zn ppm	Mn ppm	B ppm
0	0	0	0.44	0.20	4.50	1.01	99	0.38	38	5.2	44
		0.2	0.31	0.28	5.28	1.07	122	0.47	49	8.2	56
		0.4	0.28	0.26	4.46	1.08	134	0.45	49	6.1	55
	30	0	0.48	1.32	4.46	1.10	110	0.39	37	4.7	49
		0.2	0.32	1.60	5.64	1.23	113	0.46	45	5.9	56
		0.4	0.33	1.42	5.78	1.09	126	0.43	42	5.7	51
	300	0	0.40	2.13	5.23	1.05	80	0.40	35	4.9	47
		0.2	0.32	2.38	5.60	1.10	118	0.42	36	6.5	53
		0.4	0.28	2.64	5.55	1.08	131	0.42	39	6.7	58
0.5	0	0	0.44	0.08	2.66	0.94	96	0.35	27	43.1	36
		0.2	0.36	0.12	3.63	1.11	107	0.43	40	42.4	46
		0.4	0.37	0.16	4.38	1.19	122	0.46	39	51.1	46
	30	0	0.62	1.16	3.69	1.05	130	0.34	24	42.4	45
		0.2	0.54	1.58	4.84	1.31	135	0.45	31	51.5	50
		0.4	0.44	1.54	4.94	1.33	126	0.46	34	50.5	50
	300	0	0.58	1.49	4.16	0.95	98	0.35	26	31.2	36
		0.2	0.43	1.99	5.17	1.14	139	0.46	36	41.8	55
		0.4	0.41	2.24	5.28	1.17	116	0.44	42	40.3	49

and 0.5 ppm), three levels of P (0, 30, and 300 ppm) and combinations of Mn and P (Mn 0.5 ppm + P 30 ppm, and Mn 0.5 ppm + P 300 ppm). When 17 days old, the soybean seedlings were transferred to nutrient solution containing three levels of atrazine (0, 0.2, and 0.4 ppm). After 4 days the seedlings were harvested and oven-dried at 60° C. Dry weights of seedlings were recorded and the dry material was ground and ashed at 550° C overnight. A Jarrell-Ash Model 66-000 emission spectrograph was used to analyze the ash for P, K, Ca, Fe, Mg, Zn, Cu, Mo, Mn, and B content. These data were submitted to analyses of variance to determine the main and interaction effects of treatments on dry matter produced, the concentration of each element in the dry plant material, and total accumulation of each element. No significant effects on Cu and Mo were observed and these data are not reported.

Root Absorption of ^{14}C-Atrazine. In a factorial experiment, similar to that above, soybeans were grown in nutrient solutions containing four levels of Mn (0, 0.25, 0.50, and 1.00 ppm) and three levels of P (0, 30, and 300 ppm), and combinations of Mn and P. After 2 weeks the soybean seedlings were transferred to nutrient solutions containing 0.2 ppm of ring-labeled ^{14}C-atrazine. Atrazine was added to the nutrient solutions from a stock solution containing 20 ppm of atrazine with a specific activity of 1.5×10^{-7} μCi per ml. At the end of 24 hr the seedlings were removed, the roots were rinsed three times in deionized water, and they were blotted dry with filter paper. The plants were oven dried as before. Shoots and roots were separated and dry weights were recorded. ^{14}C-Activity was determined by the combustion method of Buyske *et al.* (1963) using a Beckman liquid scintillation spectrometer.

Photosynthetic Rates. Plants were grown for 3 weeks in the nutrient solution using treatments similar to those in the ^{14}C-atrazine absorption study. In one experiment, photosynthetic measurements were made using 26 to 28 day-old plants. The apparatus used was described by Dregor (1967). In a second experiment the change in photosynthetic rate with time after the application of atrazine was observed. The latter experiments were performed on the apparatus described by Imbamba (1969). Measurements were made 4, 8, 12, 24, 36, and 48 hr after the application of atrazine. The plants had been treated with atrazine so that they would not enter the dark cycle in the growth chamber before the 12-hr measurements were made.

Electron Microscopy. Small strips of leaf tissue were excised, infiltrated with 6% glutaraldehyde in 0.1 *M* phosphate buffer and 0.2 *M* sucrose solution, and fixed at room temperature for 3 to 4 hr in 2% aqueous osmium tetroxide solution. The tissue was then dehydrated in an acetone series and embedded with Epon. Sections were cut with a glass knife on LKB ultratome. They were mounted on uncoated copper grids and stained for 5 to 10 min with lead citrate. The grids were viewed on a Philips EM 300 electron microscope.

RESULTS

When high concentrations of P were supplied to the soybean plants, growth stunting occurred. Chlorosis in varying degrees was observed on the leaves. The primary leaves were first to show a peculiar mottled type of chlorosis with islets of light green tissue scattered between dark green leaf veins (Figure 1). A striking resemblance between P toxicity symptoms and Mn deficiency symptoms was observed. Atrazine in the nutrient solution at a concentration of 0.2 ppm or more caused a rapid desiccation and eventual abscission of the leaves. However, atrazine at a concentration of 0.05 ppm or less caused chlorosis similar to that induced by P toxicity or Mn deficiency. Symptoms appeared gradually, requiring 7 to 10 days to develop.

Table I shows the effect of atrazine, P, and Mn on the dry matter production and mineral composition of soybean shoots. Analyses of variance were performed on both the concentration and total accumulation of the mineral elements. Only the concentrations are given in Table I. (Total accumulations may be obtained by multiplying the concentration by the weight of dry matter.) Effects are listed in Table II as significant only when both the concentration and total accumulation of a mineral element in soybean shoots were significantly affected by a given treatment or interaction [discussed by Adams and Espinoza (1969)]. Maximal dry weight accumulation was obtained with 0.5 ppm Mn and 30 ppm P in the absence of atrazine. Hereafter, 30 ppm P is referred to as

Table II. Summary of Significant Effects Due to Mn, P and Atrazine Treatments on the Yield of Dry Matter and Accumulation of Certain Elements in Soybean Shoots as Shown by F Ratios in Analysis of Variance

Variable	Dry matter per plant	P	K	Ca	Fe	Mg	Zn	Cu	Mo	Mn	B
Mn	*a*	n.s.	*a*	*b*	n.s.	n.s.	*b*	n.s.	n.s.	*a*	*a*
P	*a*	*a*	*a*	*b*	n.s.	n.s.	*a*	n.s.	n.s.	*a*	*a*
Atrazine	*a*	n.s.	*a*	*b*	*a*	*a*	n.s.	n.s.	n.s.	*b*	n.s.
Mn × P	*a*	*b*	*a*	n.s.	n.s.	n.s.	*a*	n.s.	n.s.	*a*	n.s.
Mn × Atra	n.s.[c]	n.s.	*a*	n.s.	n.s.	n.s.	*a*	n.s.	n.s.	n.s.	n.s.
P × Atra	*a*	n.s.	*a*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*b*	*b*
Mn × P × Atra	*a*	n.s.	*a*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

[a] Significant at 1% level. [b] Significant at 5% level. [c] n.s. = not significant.

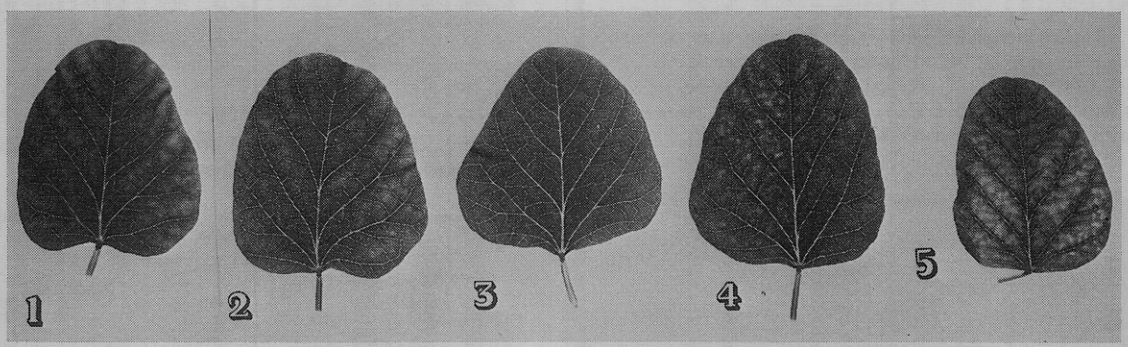

Figure 1. Composite of primary leaves from various treatments producing chlorosis: (1) Mn-deficient treatment (0.06 ppm); (2) excess-P treatment (300 ppm); (3) control; (4) 30 ppm P and 0.05 ppm atrazine; and (5) 300 ppm P and 0.05 ppm atrazine

adequate-P and 300 ppm P is referred to as excess-P. The addition of adequate-P increased the dry matter production of soybean shoots but excess-P depressed growth. Atrazine also decreased dry matter production. The analyses of variance indicated that both two-way and three-way interactions were involved. The total production of dry matter and accumulation of K were the only variables affected by the three-way interaction (P-Mn-atrazine).

The application of P increased the shoot concentration of P and K. The Mn and Zn concentrations were depressed. The effect of P on the accumulation of Ca and B was nonlinear. Atrazine increased the concentration of K, Ca, Mg, and Mn in the soybean shoots. The interaction between P and atrazine reduced the Mn concentration and caused greater accumulation of K and B in soybean shoots.

Root Absorption of ^{14}C-Atrazine. The absorption of ^{14}C-atrazine by soybean plants grown in nutrient solutions containing different levels of Mn and P is given in Table III. The ^{14}C-activity per mg of shoot dry matter was greatest when the plants were grown in solutions containing adequate Mn (0.5 ppm) and P. These data indicated that greater herbicide uptake did not account for the increased atrazine sensitivity of the soybean plants when large amounts of P were present. However, the uptake and translocation of ^{14}C decreased by 10% when the plants were given a double amount of Mn (1.0 ppm) in the nutrient solution. This factor should be considered when examining data presented later in this discussion.

Photosynthetic Rates. The treatments receiving adequate-P exhibited greater photosynthetic activity than treatments either deficient in P or containing excess-P (Table IV). Similar effects were obtained with manganese. Treatments receiving adequate-Mn exhibited greater photosynthetic activity than those either deficient in Mn or containing excess Mn.

Table III. Absorption of ^{14}C-Atrazine by Soybean Plants Grown in Nutrient Solutions Containing Different Levels of Mn and P to which 0.2 ppm Atrazine Was Added 24 hr prior to Harvest

Mn ppm	Atrazine in Shoot μ/Shoot, rate of P, ppm: 0	30	300
	μmoles/mg dry matter		
0	22.9	24.8	21.8
0.25	23.0	26.2	22.6
0.50	27.2	29.9	27.2
1.00	23.1	26.8	25.9

Averages of triplicates.

Table IV. Photosynthetic Rate of Soybean Plants Grown in Nutrient Solutions Containing Different Levels of Mn and P

Mn	Photosynthetic Rate, rate of P, ppm: 0	30	300
	mg CO_2/dm^2/hr		
0	3.2	6.5	1.8
0.25	8.1	11.4	8.8
0.50	8.8	15.8	9.2
1.00	...	13.4	7.8

Averages of 3 to 6 replicates.

Figure 2 shows the change in relative photosynthetic rates with time after the application of 0.25 ppm of atrazine to soybean plants grown in nutrient solution containing three levels of Mn (0.06, 0.5, and 1.0 ppm) and two levels of phos-

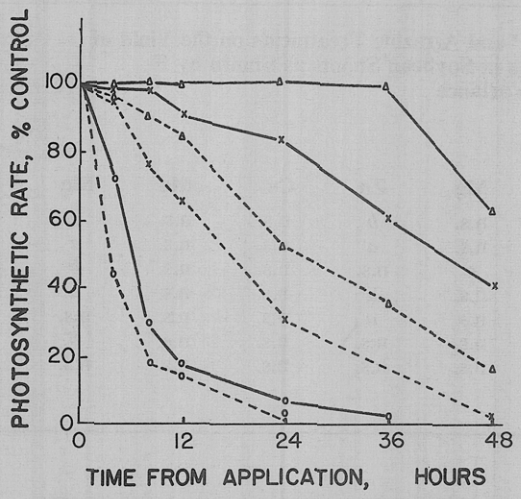

Figure 2. The reduction of photosynthesis with time of soybean plants grown in nutrient solutions containing three levels of Mn (—○—○—, 0.06 ppm; —X—X—, 0.50 ppm; and —△—△—, 1.0 ppm Mn) and two levels of P (———, 30 ppm and ----------, 300 ppm) following the application of 0.25 ppm atrazine

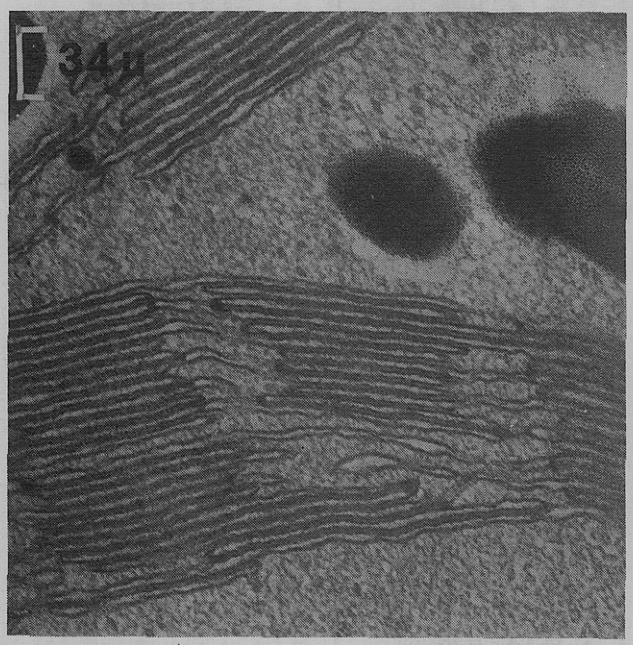

Figure 3. Electron micrograph of a normal chloroplast from the mesophyll cells of soybeans grown in nutrient solution

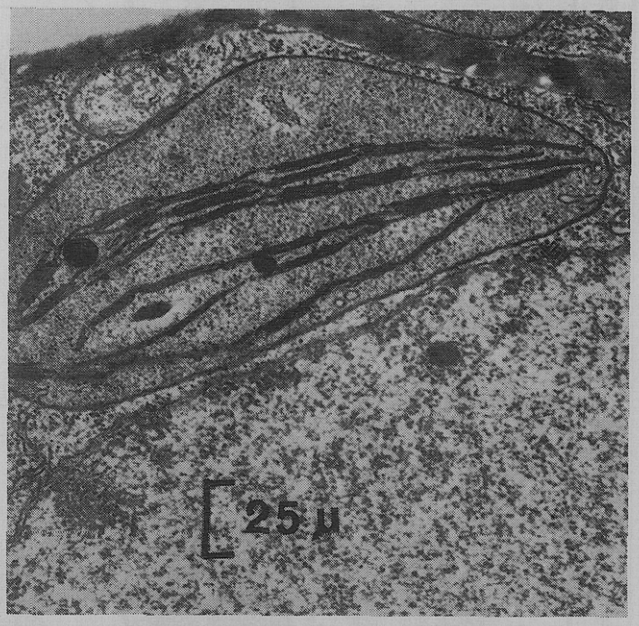

Figure 4. Electron micrograph from the mesophyll cells of a Mn-deficient soybean plant. Note absence of thylakoids

Figure 5. Chloroplasts from soybean grown in 300 ppm phosphorus nutrient solution. Note dissociation of thylakoids from stacks

phorus (30 and 300 ppm). In each case the control used as a comparison contained the respective amounts of Mn and P, but no atrazine.

The rate of photosynthesis was reduced to less than 10% of the control in Mn-deficient plants 24 hr after atrazine treatment. No photosynthesis occurred when Mn was totally absent from the nutrient solution. A rate of 0.06 ppm Mn was arbitrarily chosen as the deficient system so that some photosynthesis would occur initially. Excess-P treatments of Mn-deficient plants seemed to cause more suppression of photosynthesis than the 30 ppm P treatment. However, inhibition was so acute in the Mn-deficient system that these differences were not great. Mn applied at rates of 0.5 and 1.0 ppm to soybean plants grown in either 30 or 300 ppm P significantly (analysis of variance, 5% level) reduced the inhibition of photosynthesis produced by atrazine in the Mn-deficient systems. With 1.0 ppm Mn and 30 ppm P, plants had practically the same photosynthetic capacity 36 hr after atrazine application as the controls. Forty-eight hours after the application these plants still retained about 60% of their capacity to assimilate carbon dioxide. With the 0.5 ppm Mn treatments, photosynthetic activity started to decline 8 hr after the application of atrazine and kept on decreasing to about 40% of the controls after 48 hr. The same relationship was found between plants grown in 0.5 ppm Mn and 300 ppm P, or 1.0 ppm Mn and 300 ppm P, except that photosynthesis in the excess-P treatments apparently was much more affected by atrazine than in the adequate-P treatments. Mn appeared to exert some protection against atrazine injury to the excess-P treated plants.

Electron Microscopy. The basic ultrastructure of normal soybean chloroplasts is shown in Figure 3. In Mn deficient chloroplasts there was an increase in stroma relative to the amount of lamellar structure (Figure 4). The number of

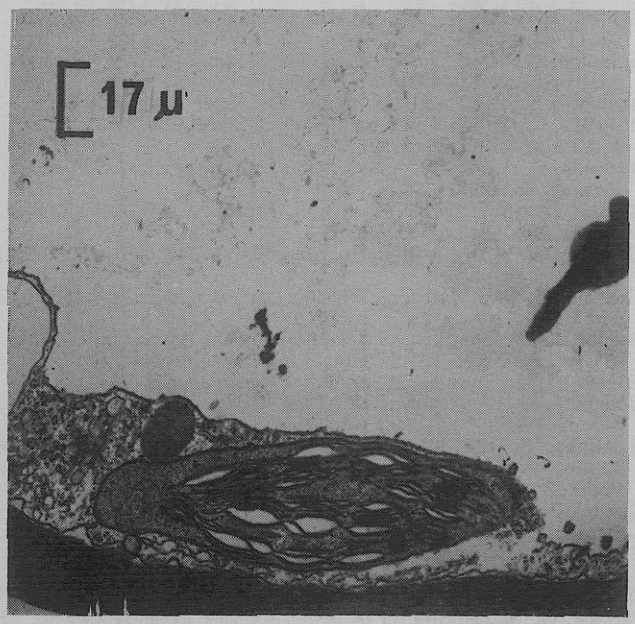

Figure 6. Chloroplast from soybean exposed for 1 hr to nutrient solution containing 5 ppm atrazine. Note swelling of thylakoids

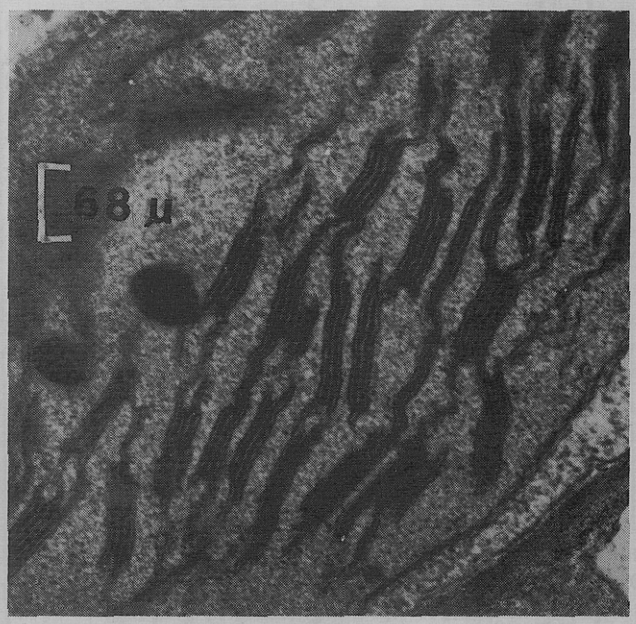

Figure 7. Chloroplast from soybean grown for 3 weeks in nutrient solution containing 0.05 ppm atrazine. Note dissociation of thylakoids

grana per chloroplast was decreased and all grana had fewer compartments than normal chloroplasts. Very few intergranal connections were formed. At a more advanced stage of Mn deficiency, the thylakoids became swollen. The structure of excess-P treated chloroplasts (Figure 5) was similar to that of Mn-deficient ones. The lamellae in the chloroplasts were not well stacked into grana. There was little connective structure between grana. At a more advanced stage the swelling of compartments appeared.

When soybean plants were exposed for a short time (1 hr) to a high concentration of atrazine (5 ppm), two kinds of structural changes were observed in addition to the breakdown of fretwork. In some chloroplasts the first sign of atrazine injury was the swelling of the compartments within the grana. Usually it started with the end compartments (Figure 6). In other chloroplasts the first sign was the dissociation of thylakoids (Figure 7). As the atrazine injury became more severe, both responses took place. There were no detectable external symptoms of atrazine injury at the time the leaves were removed from the plants. After treatment with 0.05 ppm atrazine for 3 weeks, the chloroplasts were more rounded in shape with very little lamellar structure, leaving the chloroplast filled primarily with stroma (Figure 8). The thylakoids became swollen and intergranal connections poorly developed. The effect of atrazine at 0.05 ppm on the ultrastructure of chloroplasts of excess-P treated soybean plants was similar to that just described.

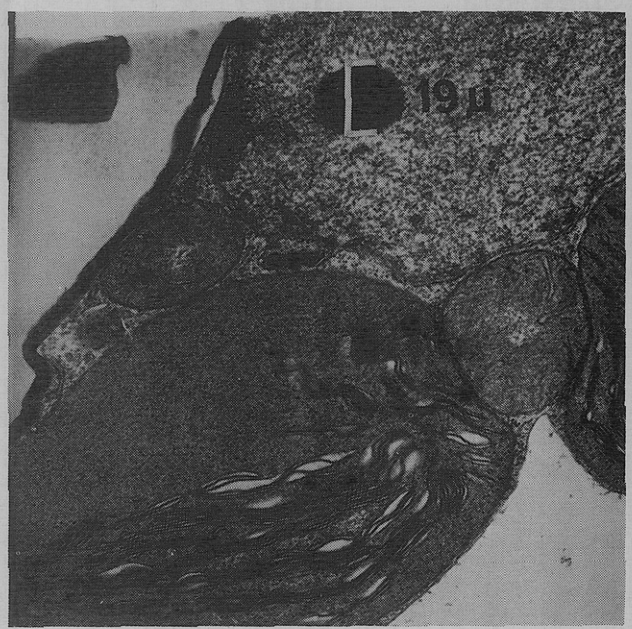

Figure 8. Chloroplast from soybean grown for 3 weeks in nutrient solution containing 0.05 ppm atrazine. Note multiplicity of symptoms

DISCUSSION

Although the excess-P treated plants in this study showed apparent Mn-deficiency symptoms, the content of Mn in the shoots was well above the critical concentration of 20 ppm or less suggested by Labanuska (1966). This indicated that chemical analysis of plant tissue to diagnose or identify certain nutrition problems may not always be satisfactory. The response of plants to one nutrient may depend on the level of other nutrients. A high P to Mn ratio may render the micronutrient less available to plant functions. Such an imbalance would affect metabolism directly and make the deficiency symptoms more apparent. Mn could not be assumed to be the only element affected by P. An effect of P on other microelements may have occurred. The addition of either Mn or P decreased the uptake of Zn. In addition, both Mn-P and Mn-atrazine interactions significantly altered the accumulation of Zn. Thus, the possibility of a very complex four-way interaction (Mn-Zn-P-atrazine) affecting the results cannot be excluded.

Brown *et al.* (1959) suggested that there was an internal inactivation of Fe by P in soybean plants. Olsen (1935), Franco and Loomis (1947), and Biddulph (1948) proposed that Fe was precipitated as $FePO_4$ in the leaf tissue. Excess-P may merely precipitate certain cations and make them unavailable. This would explain the appearance of Mn-

deficiency symptoms in P-treated plants, even though Mn contents were above the critical concentrations. Mn-deficient and excess-P treated plants produced structural changes in chloroplasts similar to those reported by Possingham *et al.* (1964) and Teichler-Zallen (1969). When a very low concentration of atrazine was given to the plants for an extended time, they seemed able to adapt gradually to the adverse situation and chloroplasts became more spherical in form. There was very little lamellar structure and fretwork. A small surface area and reduced lamellar structure become necessary if certain factors involved in membrane formation and/or maintenance are limited by atrazine. We propose that atrazine may exert its herbicidal action by making Mn unavailable for photosynthesis. A similar hypothesis has been published. Cheniae and Martin (1968) stated that the site affected by Mn deficiency is identical to the site of DCMU [3-(3,4-dichlorophenyl-1,1-dimethylurea)] poisoning. DCMU, a substituted urea herbicide, is believed to have the same mode of action as atrazine, a triazine herbicide (Van Overbeek, 1964).

Light is needed for the maintenance of chloroplast structure, as plants kept in darkness for a period of time show disorganization (Ashton *et al.*, 1963). Since atrazine by itself is not effective in the absence of light there is a possibility that atrazine might exert its herbicidal action by preventing the photooxidation of Mn which is essential for the formation and maintenance of the membrane structure of thylakoids and/or of preventing Mn from performing its functional role in the oxygen evolution of photosynthesis. Excess P application could further enhance the effect of atrazine by precipitating Mn in the tissue.

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Received for review July 22, 1970. Accepted December 21, 1970. This research was supported in part by a grant from Geigy Agriculture Chemicals, Division of Geigy Chemical Corp.