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The Manganese Toxicity of Cotton[1]

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ABSTRACT

Cotton plants (*Gossypium hirsutum*. Linn. var. Sankar 4) were grown at normal and toxic levels of substrate manganese, and the altered metabolism of manganese toxic plants was studied. The tissues of plants exposed to toxic levels of manganese had higher activities of peroxidase and polyphenol oxidase, and the activities of catalase, ascorbic acid oxidase, glutathione oxidase and cytochrome c oxidase were lowered. In addition, the high manganese tissues had lower contents of ATP and glutathione but higher amounts of ascorbic acid. The respiration of the partially expanded leaves and the growing tips of toxic plants were depressed when compared to that of the normal tissues. The metabolic changes of manganese toxicity of cotton are placed in the following order: accumulation of manganese in the leaf tissue; a rise in respiration; stimulation of polyphenol oxidase; the appearance of initial toxicity symptoms; the evolution of ethylene and stimulation of peroxidase; the presence of severe toxicity symptoms; the depression of terminal oxidases and respiration; abscission of the growing tip and proliferation of the stem tissue. The early stimulation of polyphenol oxidase may be used to detect potential manganese toxicity.

McHargue (16) first showed Mn to be an essential plant nutrient. The element is involved in many oxidation-reduction reactions of plants and is reported to be a cofactor of polyphenol oxidase and IAA oxidase systems (10, 13, 17, 32). Plant disorders due to high concentrations of Mn are of practical significance and are known to occur in soils that contain extremely high amounts of Mn (22) and in acid soils of moderate Mn content. Neal and Lovett (19) were the first to identify the crinkle leaf disease of cotton as caused by Mn toxicity. Symptoms of the toxicity include puckering and distortion of the leaves which also developed partial mottled chlorosis. Necrotic lesions appeared along and between leaf veins and fasciation of branches usually developed. Plant disorders caused by high amounts of Mn have also been reported in tobacco, barley, potatoes, and palms (2, 3, 5, 29).

Recent studies indicate that the Mn toxicity of plants affects their auxin metabolism. Joham and Amin (12), Morgan *et al.* (18), and Taylor *et al.* (30) have investigated the response of cotton to high levels of Mn. They found that relatively high concentrations of Mn were required to produce toxicity and that IAA oxidase was stimulated and its inhibitor was lowered at toxic levels of Mn in plant tissues. Morgan *et al.* (18) have proposed that Mn toxicity symptoms in cotton are expressions of auxin deficiency caused by IAA oxidase activity increased by abnormal tissue levels of Mn. The investigation reported here further characterizes the metabolism of Mn toxic cotton plants and catalogues the sequence of metabolic changes detected during toxicity development.

MATERIALS AND METHODS

Cotton plants (*Gossypium hirsutum*, Linn. var. Sankar 4) were grown in washed builder's sand placed in earthen pots. After the seedlings emerged, each pot received 1 liter of a modified Hoagland's solution (pH 6.0) daily. At 30 days, the plants were at the four leaf stage, and the Mn levels of the nutrient solutions were altered to establish normal (1 mg/l) and toxic (100 mg/l) treatments. When the plants of the toxic treatment had developed severe Mn toxicity symptoms, which included proliferation of the stem tissue, the first partly expanded leaves of both sets of plants were harvested, and the activities of catalase, peroxidase, polyphenol oxidase, glutathione oxidase, ascorbic acid oxidase, and Cyt *c* oxidase were determined. ATP, glutathione, and ascorbic acid contents of the tissue were also measured. In another experiment, Mn levels of 1, 9, 27, and 81 mg/l were used, and the respiration of growing tips and the first partly expanded leaf were determined after severe toxicity symptoms were observed in cotton plants treated with 81 mg/l Mn.

The sequence of enzymatic changes during the development Mn toxicity was studied in two experiments. In the first, the treatments (1 mg/l and 200 mg/l) were started when the plants were 4 weeks old. The polyphenol oxidase activity was assayed on the 5th, 9th, and 15th days of the treatment. The peroxidase activity was determined on the 7th, 10th, and 19th days, and the ascorbic acid oxidase activity of the tissue was assayed on the 8th, 11th, and 20th days. In the second experiment, the substrate Mn levels of the normal treatments were unchanged, but the Mn content of the toxic nutrient solution was reduced to 100 mg/l. The peroxidase was determined on the 2nd, 5th, 13th, and 19th days of the treatment, and the catalase activity was assayed on the 5th, 13th, and 19th days of the treatment.

All the experiments were conducted in a randomized plot design involving a minimum of five replicates. Thirty such experiments were conducted during the course of this investigation; the results were statistically analyzed by the sum of the least squares method. When only two treatments were used in the experiment, *F* tests were used to judge the significance of the data. In experiments in which more than two treatments were involved, multiple range testing techniques were employed, and the significance at 0.05 level was noted by subscript letters.

ATP. Two g of the fresh cotton leaf tissue were boiled for 20 min in distilled water; after filtration and cooling, the final volume was adjusted to make a 20% extract. ATP content of

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the extract was determined by the firefly luminescence assay method (27).

Ascorbic Acid. A 0.2% extract of the tissue was prepared in 5% metaphosphoric acid. The anthocyanin pigments of the extract were adsorbed on activated charcoal, and the extract was filtered. Ascorbic acid was estimated by the titrametric method of Roe (23) using 0.00025% 2,6-dichloroindophenol solution containing sodium bicarbonate.

Glutathione. Two g of the fresh leaf tissue were homogenized in a chilled mortar and pestle with 3 ml of 1% metaphosphoric acid. The residue was centrifuged in the cold, and the supernatant was applied to a 10-cm starch column in a 2-cm glass tube. Glass-distilled water was used as a partitioning solvent and flowed at a rate of 0.5 ml/min. The eluates were collected and estimated for glutathione by the nitroprusside test of Grunert and Phillips (11). Total amount of glutathione was obtained by combining the values obtained in different aliquots.

Enzyme Assays. All steps in the preparation of cell-free extracts for enzyme assays were carried out at 4 C.

Catalase and Peroxidase. The cell-free extract preparation for catalase and peroxidase assays were the same and have been described elsewhere (14). The catalase assay was carried out at 15 C according to the method of Euler and Josephson (6). One unit of catalase is that amount of the enzyme which oxidizes 1 μmole of hydrogen peroxide/min at 15 C.

The Worthington procedure was used for the peroxidase assay (34). One unit of the peroxidase is that amount of enzyme which oxidizes 1 μmole of the dye/min at 25 C.

Ascorbic Acid Oxidase. The plant tissue was ground in a mortar and pestle in 0.15 M citrate buffer (pH 5.7) to make a 20% extract. The homogenate was centrifuged at 5000*g* for 15 min, and the supernatant was used for the enzyme assay. The standard reaction mixture containing 1 mg of ascorbic acid, 300 μM citrate phosphate buffer (pH 5.7), and an appropriate aliquot of enzyme solution in a total volume of 25 ml was incubated at 30 C. At various time intervals, aliquots were removed and assayed for residual ascorbic acid by the method of Roe (23). One unit of ascorbic acid oxidase is that amount of enzyme which oxidizes 1 mg of ascorbic acid/min at 30 C.

Glutathione Oxidase. The enzyme extract used was the same as that for ascorbic acid oxidase. GSH (50 μM), 300 μM citrate phosphate buffer (pH 5.7), and an appropriate aliquot of the enzyme solution in a final volume of 10 ml was incubated at 30 C. Aliquots were withdrawn at periodic intervals and GSH content was estimated by the nitroprusside test of Grunert and Phillips (11). One unit of GSH oxidase is that amount of enzyme which oxidizes 1 μmole of GSH/min.

Polyphenol Oxidase. The tissue was homogenized in a mortar and pestle in 0.01 M sodium acetate buffer (pH 5.6) to yield 20% extract. The homogenate was centrifuged at 5000*g*, and the supernatant was used for enzyme assay, according to method of Ponting and Joslyn (20). The reaction system contained 800 μM sodium acetate buffer (pH 5.6); 250 μM catechol, and the enzyme extract in a final volume of 10 ml. The assay was conducted at 30 C, and the change in absorbance was observed every 2 min for a period of 15 min. The unit of polyphenol oxidase was that amount of enzyme which produced a change of one unit of absorbance at 420 nm at 30 C.

Cytochrome *c* Oxidase. The cotton leaf mitochondria were isolated by the method of Matsumoto *et al.* (15). The components of the grinding medium were essentially similar to those of Srivastava and Sarkissian (26) and included 0.5 M sucrose; 50 mM potassium orthophosphate; 4 mM cysteine; 2 mM magnesium chloride; 10 mM potassium chloride; and 50 mM EDTA. The homogenate was centrifuged at 2,000*g* for 10 min, and the supernatant was recentrifuged at 15,000*g* for 15 min. The pellet from the second centrifugation was washed with the grinding medium and recentrifuged at 15,000*g*. The final mitochondrial pellet was suspended in 2 ml of 0.25 M sucrose in 0.05 M tris-HCl buffer (pH 7.5). Mitochondrial suspension (0.1 ml) was added to a mixture consisting of 27 μM phosphate buffer (pH 7.4) and 30 μM Cyt *c*. The reduced Cyt *c* was obtained by the method of Cooperstein and Lazarow (4). The assay was carried out at 30 C, and the change in absorbance at 550 nm was recorded for a period of 5 min. The unit of Cyt *c* oxidase is that amount of the enzyme which produces a change of one unit of absorbance/min at 550 nm at 30 C.

Respiration. The manometric techniques described by Umbreit *et al.* (31) were used to determine the respiration of plant tissues in a Warburg apparatus. Respiration studies were conducted on freshly excised tissue. The leaves were washed with distilled water and a No. 3 cork borer was used to cut 6-mm leaf disks. The surface moisture from the disks was absorbed; the disks were weighed immediately and transferred to Warburg flasks containing distilled water. A filter paper wick soaked in 0.2 ml of 20% KOH was placed in the center well of the vessel to absorb CO_2. The system was equilibrated at 25 ± 0.5 C for 30 min, and the respiration of plant tissues was measured for the next 2 hr. Tissue from each replicate of the experiment was examined, and the averages of the data were reported as μmoles of O_2 consumed/hr·g fresh weight of the tissue.

RESULTS

Two weeks after the initiation of treatment, dark necrotic spots appeared in the first partly expanded leaves of cotton plants of the toxic treatments. They were the initial symptoms of Mn toxicity. Later, pronounced marginal cupping and chlorosis were noticeable. As the disease developed, the leaves became small, puckered, mottled, and leathery and eventually abscised. In the next stage, the apical meristem was affected, and the growing tip was abscised. Proliferation of the stem and initiation of secondary meristem could be observed and a general reduction in plant growth was apparent. The results were similar to those obtained by Neal and Lovett (19) and Joham and Amin (12).

The activities of catalase, peroxidase, and polyphenol oxidase of the leaves from normal and manganese toxic cotton plants are presented in Table I. The toxicity resulted in a 4-fold reduction of the catalase activity, and increases in the activities of peroxidase and polyphenol oxidase were 10-fold and 4-fold, respectively. The activities of the leaf oxidases of ascorbic acid, Cyt *c*, and GSH (Table II) were greatly decreased

Table I. *Activities of Peroxidase, Catalase, and Polyphenol Oxidase of Leaves from 80-day-old Normal and Mn Toxic Cotton Plants*

First partially expanded leaves were used.

Mn Concn	Peroxidase	Catalase	Polyphenol Oxidase
mg/l	*units/g fresh wt*		
1	2.98	3.30	0.05
100	26.73	0.82	0.20
F value obtained	22.20	22.50	51.10
F value required			
0.05	7.71	10.13	7.71
0.01	21.20	34.12	21.20

by the toxicity. The decrease was more pronounced in ascorbic acid and Cyt *c* oxidases.

Data on the respiration of young leaf and growing tips of cotton plants as influenced by substrate Mn are shown in Table III. Cotton leaf disks from plants subjected to high substrate levels of Mn (27 and 81 mg/l) had depressed rates of respiration when compared to the respiration of normal tissues (1 mg/l). The leaves of plants subjected to 9 mg/l manganese treatment had the highest respiration rate. When compared with the respiration of growing tips of the normal plants, that of the growing tips of 81 mg/l manganese plants was significantly decreased; the respiration of the growing tips subjected to 27 mg/l Mn treatment was not changed significantly. As compared to the normal plants the leaf tissue of the Mn toxic cotton plants had significantly smaller amounts of ATP and GSH, but the ascorbic acid content was higher in the toxic tissue (Table IV).

The sequence of metabolic changes during the development of Mn toxicity was examined in two experiments. The plants were supplied with solutions containing 1 mg/l, 100 mg/l, and 200 mg/l of Mn. The results of the 200 mg/l Mn experiment are presented in Table V. The polyphenol oxidase activity was stimulated on the 5th day after the treatment initiation. This increase of the polyphenol oxidase activity preceded the appearance of the first toxicity symptoms. The toxic symptoms first appeared on the 7th day in the form of a few black spots. The activity of polyphenol oxidase increased further with the development of the toxicity symptoms. The stimulation of the peroxidase was detected only after the toxicity symptoms were general and moderate, and the activity was found to rise sharply with further development of toxicity. The ascorbic acid oxidase was not affected during the course of the experiment. The toxic symptoms of the 100 mg/l experiment were much milder than those obtained in the 200 mg/l experiment. The peroxidase activity of the toxic tissue (100 mg/l) showed a tendency to increase only after the appearance of the toxicity symptoms (Table VI). The catalase activity of the toxic leaves was not affected at that time.

Table II. *Activities of Oxidases of Ascorbic Acid, Cyt c and Glutathione of Leaves from 80-day-old Normal and Mn Toxic Cotton Plants*

First partially expanded leaves were used.

Mn Concn	Ascorbic Acid Oxidase	Cyt *c* Oxidase	Glutathione Oxidase
mg/l	*units/g fresh wt*		
1	0.071	0.016	0.093
150	0.017	0.004	0.048
F value obtained	13.27	35.03	9.07
F value required			
0.05	7.71	7.71	7.71
0.01	21.20	21.20	21.20

Table III. *Respiration of Young Leaf and Growing Tips of 80-day-old Cotton Plants as Influenced by Substrate Manganese*

First partially expanded leaf was used. Figures followed by the same letter are not significantly different at 0.05 level.

Mn Concn	Respiration		
	Leaf disks	Growing tips	
mg/l	*μmole O_2/hr·g fresh wt at 25 C*		
1	16.2c	21.0b	12.5a
9	18.1cd		
27	13.6b		14.5ab
81	10.8a	17.9a	

Table IV. *ATP, Glutathione, and Ascorbic Acid Content of Cotton Leaves as Influenced by Mn Nutrition*

Uppermost partially expanded leaves were used. The plants were 80 days old.

Mn Concn	ATP	Glutathione	Ascorbic Acid
mg/l	*mg/g fresh wt*		
Normal (1.0)	0.076	0.10	3.05
Toxic (150.0)	0.025	0.03	3.51
F value obtained	46.20	56.60	10.95
F value required			
0.05	7.71	7.71	7.71
0.01	21.20	21.20	21.20

Table V. *Influence of Development of Mn Toxicity of Cotton Plants*

Results show the activities of polyphenol oxidase, peroxidase, and ascorbic acid oxidase of the partly expanded first leaf (200 mg/l) Mn experiment. The plants were 30 days old at the start of the treatments.

Days of Treatment	Polyphenol Oxidase		Peroxidase		Ascorbic Acid Oxidase	
	N[1]	T	N	T	N	T
	units/g fresh wt					
0						
5	0.117	0.170				
7			2.06	2.69		
8					0.083	0.071
9	0.185	0.622				
10			5.75	13.78		
11					0.099	0.100
18	0.378	1.034				
19			0.65	15.94		
20					0.109	0.102

[1] N: normal; T: toxic.

Table VI. *Activities of Peroxidase and Catalase of Cotton Leaves as Influenced by Development of Manganese Toxicity*

First partially expanded leaf was used. The plants were 30 days old at the start of the treatments. The level of Mn was 100 mg/l.

Days of Treatment	Peroxidase		Catalase	
	Normal	Toxic	Normal	Toxic
	units/g fresh wt			
0				
2	13.93	12.12		
5	5.45	5.15	1.98	1.98
13	2.73	3.33	1.32	1.32
19	2.73	3.33	1.32	0.99

DISCUSSION

Manganese accumulates in growing regions of the cotton plants, and the symptoms of Mn toxicity first appear in young leaves. Later, the loss of apical dominance results in extensive

proliferation of the tissue in the stunted plants (12). In these experiments the partly expanded leaf was used most often. The technique allowed collection of sufficient number of samples to analyze the results statistically. The growing tips were not suitable for the purpose because of their small size. The nutrient solutions of the extreme toxic treatments were varied from 81 to 200 mg/l in different experiments. Two factors determined the choice of the substrate Mn levels: the need to control the rate of toxicity development and the variation in light intensity. The lower light levels prevalent in the monsoon season (July to October) necessitated the use of stronger Mn concentrations.

Oxidative Enzymes in Mn Toxicity. The Mn toxic tissues of the cotton plants are characterized by increased polyphenol oxidase and peroxidase activities (Table I), and by decreased activities of catalase, GSH oxidase, ascorbic acid oxidase, and Cyt *c* oxidase (Tables I and II). Mn is known to be a cofactor of polyphenol oxidase, IAA oxidase, and peroxidase. Morgan *et al.* (18) and Taylor *et al.* (30) have shown high IAA oxidase activities in Mn toxic cotton leaves; they did not measure the peroxidase activity. However, a number of workers have advanced arguments in favor of the view that IAA oxidase is a peroxidase (7–9, 13, 33). The higher activities of the polyphenol oxidase and the peroxidase in the Mn toxic tissues of this experiment and increased IAA oxidase activity detected by Morgan *et al.* (18) form a pattern. Mn is a cofactor of all the enzymes stimulated during Mn toxicity.

Abutalybov *et al.* (1) and other Russian workers reported a stimulation of peroxidase and polyphenol oxidase in cotton plants sprayed with high levels of Mn. Our results agree with their findings, except that they also reported increased ascorbic acid oxidase and catalase activities and we find that these activities are suppressed during Mn toxicity. The experimental techniques of the Russian workers were different from those employed here. They sprayed high levels of Mn on field-grown cotton plants, whereas our cotton plants were grown in the greenhouse and supplied with high levels of substrate Mn using sand culture techniques. In the Russian studies, there is no mention of the sampling time in relation to toxicity development, but there is a hint that the samples may have been collected shortly after spraying the plants. Their results could have been due to a salt shock rather than the physiological effects of Mn toxicity. The tissues of this study were sampled well after the development of Mn toxic symptoms and reflect conditions prevailing in severe Mn toxicity.

Respiration and Mn Toxicity. Mn deficiency increases the respiration of potato and barley tissues (24, 29). Subba Rao and Lal (29) found that the respiration of Mn toxic barley plants was depressed. In the experiments reported here, the respiration of the Mn toxic cotton leaves was depressed. Thus the effects of Mn deficiency and those of Mn toxicity on plant respiration are opposite. In several other instances, the effect of a nutrient deficiency on the plant respiration are opposite to those of toxicity (25), but this may be an oversimplified point of view. Any dying tissue is likely to have severely reduced respiration, and the results of initial nutritional disorder may be opposite to those observed when the disorder is well advanced. In the present instance, plants subjected to moderate concentrations of Mn (9 mg/l leaf tissue and 27 mg/l growing tips) show a slight increase in the respiration rate, whereas the plants subjected to more severe treatments have depressed respiration. The rise in the respiration of mildly Mn toxic cotton tissue (Table III) may be due to higher activity of polyphenol oxidase which is stimulated very early during Mn toxicity and which can serve as one of the terminal oxidases. The other terminal oxidases of respiration do not appear to be affected at this stage. It is also possible that the ATP levels of toxic tissues may have dropped and thus the ATP/ADP ratio which normally controls respiration would be lowered resulting in increased respiration of the slightly affected cotton plants. In the experiments reported here, the data on ATP concentration were collected at a much later stage and does not help in judging the trend of events.

The possibility of ethylene being responsible for the early rise in the respiration of Mn toxic tissue was considered. Fowler and Morgan (7) state that in their studies ethylene was evolved after the appearance of Mn toxic symptoms. We have detected the rise in respiration of the cotton tissues only in the milder treatments, and at that time the symptoms of Mn toxicity were absent. Therefore the ethylene effect does not appear to be involved in this phenomenon. Respiration is also known to increase when plant tissues are injured or infected. It is possible that the leaves of the 9 mg/l Mn plants were injured mildly and therefore the tissue respiration was increased.

Sequence of Mn Toxicity Development. Results of the two experiments of the sequence studies indicate that the polyphenol oxidase is accelerated well before the initial toxicity symptoms appear. In the 200 mg/l experiment, the initial Mn toxic symptoms appeared 7 days after the treatment but the rise in polyphenol oxidase activity could be detected as early as 5 days after treatment initiation (Table V). At this time, the peroxidase activity had not started to rise. Under certain conditions, it may be possible to use the early stimulation of polyphenol oxidase activity to detect a potential Mn toxicity. In fact, this may be one of the relatively rare times that a nutritional disorder could be detected enzymatically even before the appearance of the initial toxicity symptoms. The peroxidase shows high activity along with the appearance of the toxicity smyptoms. It is clear that the activation of peroxidase and the depression of Cyt *c*, ascorbic acid, and catalase may be a secondary effect of Mn toxicity. Fowler and Morgan (7) state that the stimulation of peroxidase and the production of ethylene may be a secondary phenomena during Mn toxicity. Thus the primary effect may well be the stimulation of polyphenol oxidase. It may be noted that a phenol is an inhibitor of the IAA oxidase system (21, 28). Hence the early stimulation of the polyphenol oxidase during Mn toxicity of cotton can remove the phenolic inhibitor and consequently produce high IAA oxidase activity.

The sequence of events during the development of toxicity by Mn is placed in the following order: (*a*) accumulation of Mn in the young leaf tissue; (*b*) the rise in respiration of Mn toxic tissue; (*c*) the increased polyphenol oxidase activity; (*d*) the appearance of the initial Mn toxic symptoms in the young leaves; (*e*) the evolution of ethylene and the stimulation of the peroxidase; (*f*) the presence of extremely severe symptoms; (*g*) the depression of the respiratory terminal oxidases and the depression of respiration; (*h*) abscision of the growing tips; (*i*) the proliferation of the tissues.

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