Effect of Manganese Toxicity on the Indoleacetic Acid Oxidase System of Cotton[1, 2]

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Summary. The effect of substrate manganese on tissue manganese levels and activity of the indoleacetic acid (IAA)-oxidase system of cotton (*Gossypium hirsutum*, L.) was investigated. A sand culture technique was used with 1, 3, 9, 27 and 81 mg manganese ($MnSO_4$) per liter nutrient solution applied in various experiments.

The following relationships held for both long-term (126 days) and short-term (12-14 days) exposures to manganese treatment: A) There was a direct relationship between substrate and tissue manganese. B) Only the 81 mg/liter Mn plants exhibited severe manganese toxicity symptoms. C) At the toxic level of manganese an increased IAA-oxidase activity and decreased IAA-oxidase inhibitor activity was observed. There was a direct relationship between degree of enzyme response and severity of visible symptoms. D) With the manganese toxicity plants, but none of the other treatments, extracts of the young leaves contained as much IAA-oxidase activity as extracts of much older leaves. E) Crude extracts from the plants grown with 81 mg manganese per liter solution, in contrast to those of other treatments, destroyed IAA without addition of $MnCl_2$ to the assay medium.

A hypothesis is advanced stating that manganese toxicity symptoms in cotton are expressions of auxin deficiency caused by IAA-oxidase activity increased by the abnormal tissue levels of manganese.

Cotton was observed to exhibit unusual growth responses to toxic levels of manganese during an investigation of absorption and distribution of the element (6). Severe stunting, death of the apex, proliferation of tissue at the cotyledonary node, formation of branches with short internodes and malformed leaves were noted.

There is evidence relating manganese as a stimulator or cofactor for many IAA-oxidase systems, including the one from light-grown cotton (see 4, also 7, 8, 11, 12, 13, 14, 15). The element has been included in hypothetical mechanisms for IAA-oxidase (7, 15) and has been related to in vitro IAA-oxidase inhibitor destruction (2). A relationship between IAA-oxidase and manganese nutrition of plants would indicate an in vivo role for the enzyme, and such a role is compatible with other indirect evidence from cotton (10).

In view of our previous findings with the IAA-oxidase system of cotton, the known relationship of manganese with IAA-oxidase and the appearance of the toxicity in cotton, we suspected that the high level of manganese was accelerating enzymatic destruction of auxin. This hypothesis is supported by the data reported here.

Methods and Materials

Plant Culture. Cotton, *Gossypium hirsutum* L. variety Deltapine TPSA, was grown in a greenhouse from seed planted in 2 gallon crocks filled with washed sand. The plants were watered daily with Hoagland's solution, containing a base level of 1 mg manganese per liter. Manganese (as $MnSO_4$) was added to the Hoagland's solution to provide the treatments in table I at the times indicated. At the end of the period of nutrient treatment (table I), plant parts were harvested, pooled according to leaf age and treatment, and assayed for IAA-oxidase activity and manganese content.

Enzyme Extraction and Assay. As previously described (10, 11), tissue samples were weighed, washed, homogenized, filtered and centrifuged. The supernatant fraction (extract) was divided into 3 portions: the first was immediately assayed manometrically for IAA-oxidase activity (11), the second was dialyzed for 24 to 48 hours then assayed and the third was boiled and assayed for relative IAA-oxidase inhibitor activity (10, 11). Unless excep-

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[2] A preliminary report of this work appeared in Plant Physiol. 38: xvii.

Table I. *Details of Experiments*

Expt no.	Treatment manganese, mg/liter	Period of Mn treatment, days from planting	Replications	Plants/replication
I	1, 3, 9, 27, 81	0–126	4	2
II	1, 27, 81	14–28 (First harvest)	7	6
		14–42 (Second harvest)		
III	1, 27, 81	21–34	7	2
IV	1, 27, 81	21–33	12	4

tions are specifically noted, all IAA-oxidase assays included manganese at 3 μmoles per flask in the medium and were conducted in the light with added riboflavin to inactivate inhibitors (11, 12). In cotton heat-stable, endogenous inhibitors delay O_2 uptake, and this lag is removed without major effect on the reaction kinetics by riboflavin in the light (10, 11). Although riboflavin often causes a slight rate suppression, this effect is not dependent upon the presence or absence of inhibitors (fig 7, ref 11) and all data here were corrected for blank O_2 uptake in the absence of enzyme. In this (see fig 3) and previous work (6, 10), at least part of the assays with low inhibitor levels were repeated without added riboflavin and the results agreed with those obtained with riboflavin.

As previously discussed (6, 10), measurement of absolute enzyme and inhibitor levels is impractical, but for a given tissue the present assay does show which, if any, treatment increases or decreases enzyme activity with respect to a control. For each assay all flasks contained enzyme from equal fresh weights of tissue (27 mg/flask for 1 to 10 dilutions and 13.5 mg/flask for 1 to 20 dilutions). Except in experiment I, comparisons were made between the same morphological tissues, using weight to estimate tissue volume, before treatments produced great differences in size. In other studies with cotton IAA-oxidase (6, 10), assays on a protein basis and converted to total enzyme activity per g fresh weight produced the same relative results as assays made on the basis of fresh weights. The magnitude of the differences of IAA-oxidase and inhibitor activity between plants grown with 81 mg/liter Mn and all lower levels (Results section) led us to conclude that in this study, comparisons on a fresh weight basis were adequate.

Manganese Assay. Manganese content of leaf tissue was determined by the potassium periodate method (1).

Individual Experiments. In experiment I, samples of young and old leaf blades were taken from all plants. In the 1, 3, 9, and 27 mg/liter Mn plants, the youngest leaf over 51 mm in diameter near the top of the main stem and the oldest primary leaf from each plant were harvested. In the 81 mg/liter Mn plants, the young and old leaves were the youngest and oldest leaves over 25 mm in diameter from near the tip and base respectively of the largest lateral branches. Thus, the young and old leaves from the 81 mg/liter Mn plants were nearer in age than young and old leaves in other treatments.

In experiment II, 2 sets of samples of leaf blades were collected. One set, based on leaf position, was taken from plants with 6 leaves. In these plants leaf number 1, 2 and 3 were the youngest unfolded, next to youngest and third youngest leaves respectively. The second set of samples, based on stage of leaf expansion, was collected from plants with either 5 or 7 leaves. In this set, leaf number 1 was the youngest leaf over 51 mm in diameter. Leaves number 2 and 3 were collected from the next 2 nodes down the plant.

Plants remaining after the first harvest were exposed to an additional 2 weeks of nutrient treatment. The 81 mg/liter Mn plants with dead apices were discarded, and the remaining plants were matched with equivalent plants from the 1 and 27 mg/liter Mn treatments. The first, second and third opened leaf blades below the apex were harvested.

In experiment III, the first sample included the youngest leaf 3 mm to 9 mm in diameter and all tissue above it. The second sample was the next leaf blade down the plant, and the third sample was leaf blade 3.

In experiment IV, the apex was harvested to include all tissue above the point of attachment of the youngest leaf 51 mm or more in diameter.

Results

Long Term Nutrient Treatments.

Experiment I. Visible Responses and Manganese Assays. After 126 days of nutrient treatment in experiment I only those plants receiving 81 mg/liter Mn were markedly affected by treatment. Within 2 weeks after emergence visible symptoms of manganese toxicity were evident on the 81 mg/liter Mn plants. Maximum development of the main stem apex of those plants was achieved with only partial expansion of the first true leaf. Dark brown necrotic spots appeared scattered over the surface of the true leaf, and by the time the plants were 27 days old the main stem apex was dead. Following this, a bud in the axil of each cotyledon began to elongate and each produced a very short branch before dying. The tissue at the cotyledonary node began to swell, and several additional axillary buds made limited growth. Eventually buds were produced at the cotyledonary node which grew to form branches with short internodes and malformed leaves.

Total dry weight of 126-day-old plants and leaf manganese content are given in table II. The 81 mg/liter treatment restricted growth severely. Substrate and leaf manganese content were directly related.

Experiment I. Enzyme and Inhibitor Assays. In 126-day-old tissues, crude extracts (1–10 dilution)

Table II. *Total Dry Weight and Leaf Manganese Content of 126-Day-Old Cotton Plants as Influenced by Substrate Manganese (Expt I)*

Treatment mg Mn/liter	Dry wt g/plant	Leaf Mn μg/g tissue
1	86.9 a b*	84.1 a*
3	94.3 a	176.3 a b
9	77.1 b	516.1 b
27	77.4 b	2314.6 c
81	8.8 c	6208.3 d

* Indicates the Duncans Multiple Range test significance at the 0.05 level.

of both young and old leaves from the 81 mg/liter treatment had a much greater capacity for oxidation of IAA than tissue of either age from any of the other treatments. A second assay (fig 1) at increased dilution revealed that: A) extracts from the 81 mg/liter Mn plants still exhibited the highest IAA-oxidase activity, B) extracts of all other old leaves displayed significant IAA-oxidase activity, C) all young leaves except those from 81 mg/liter Mn plants exhibited the normal, low IAA-oxidase activity of such tissue (10).

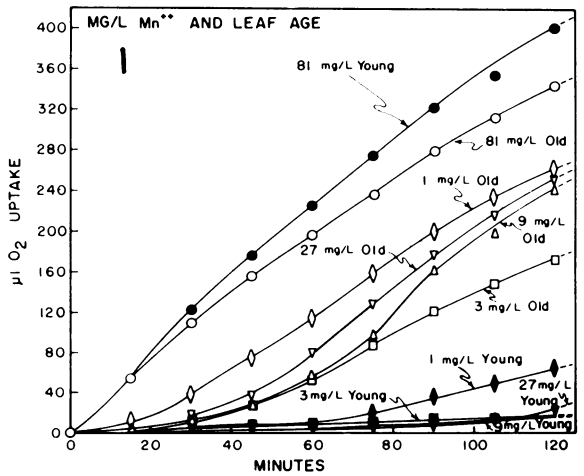

FIG. 1. IAA-oxidase activity of crude extracts of cotton grown with different levels of substrate Mn. 1 to 20 dilution of enzyme, experiment I.

When manganese was withheld from the assay system, extracts from the 81 mg/liter Mn plants showed IAA-oxidase activity (fig 2), but extracts from plants of all other treatments were inactive (compare fig 1 and fig 2 and see 11). These differences were due to tissue manganese since: A) all dialyzed extracts assayed without manganese had very little IAA-oxidase activity in contrast to other assays with manganese (fig 3, 4), B) dialyzed extracts of the 81 mg/liter Mn leaves exhibited high IAA-oxidase activity when assayed with boiled, crude extracts of the 81 mg/liter Mn leaves substituted for manganese in the assay medium.

Following dialysis extracts were assayed in the dark without added riboflavin due to the low inhibitor content (fig 3). Extracts from plants grown with 81 mg/liter manganese again exhibited the highest capacity for destruction of IAA and unusually high activity in the young leaves. These results (fig 3) were confirmed by an assay in the light with riboflavin.

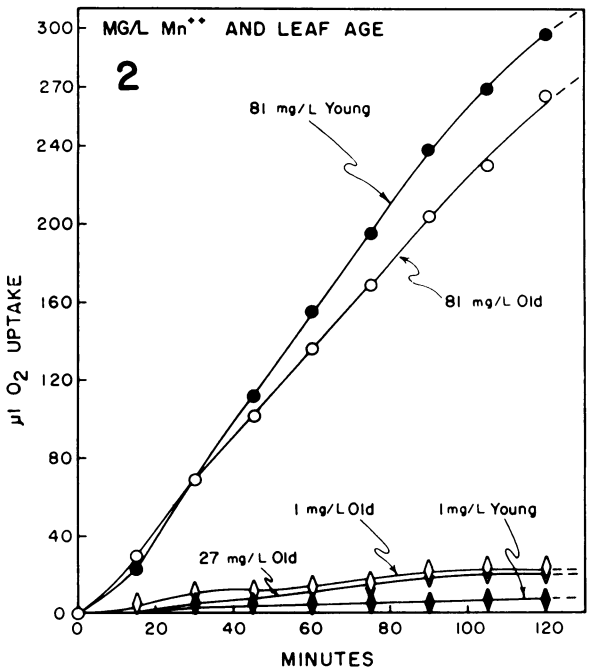

FIG. 2. IAA-oxidase activity of crude extracts of cotton grown with different levels of substrate Mn. 1 to 20 dilution of enzyme assayed without $MnCl_2$, experiment I.

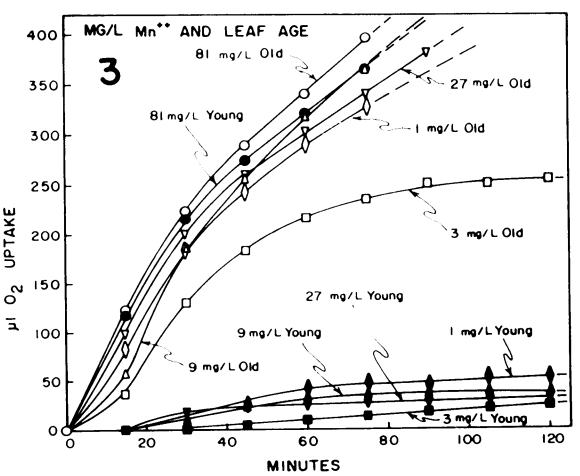

FIG. 3. IAA-oxidase activity of dialyzed extracts of cotton grown with different levels of substrate Mn. 1 to 20 dilution of enzyme, experiment I. Assays in dark without riboflavin.

That inhibitor levels were higher in all other samples than in the 81 mg/liter Mn samples was indicated by: A) their inactivity when assayed as crude 1 to 10 dilutions (text), B) their increased activity with increased dilution (fig 1), C) removal of a lag in O_2 uptake with dialysis (compare fig 1 and 3) and D) direct inhibitor measurements (table III). The major difference in the IAA-oxidase system of old leaves was in inhibitor levels (compare inactivity in first assay, text with fig 3).

The findings from the 126-day-old plants in experiment I prompted the question: Was the acceleration of IAA-oxidase a cause or effect of the toxicity? We, therefore, examined the IAA-oxidase system in the early stages of manganese toxicity (12–14 days).

Table III. *Relative IAA-Oxidase Inhibitor Activity of Boiled, Crude Extracts of Cotton Grown with Different Levels of Substrate Manganese (Expt I)*

Treatment mg Mn/liter	Length of lag period min*	
	Old leaves	Young leaves
1	19	>135
3	...**	>135
9	21	>135
27	10	>135
81	3	10

* Longer lag indicates higher inhibitor level.
** Sample lost.

Short-Term Nutrient Treatments.

Experiment II. After 2 weeks of nutrient treatments in experiment II, the 81 mg/liter Mn plants showed chlorotic and necrotic spots on the older leaves and in addition, puckering and retarded expansion of the youngest leaves. Visible symptoms were restricted to the 3 leaves developing after treatment was imposed, and growth abnormalities clearly increased in severity from older to younger leaves.

Results from IAA-oxidase assays of leaf samples, selected either on the basis of node number or size of the first open leaf were similar, and therefore, averages of the data are presented. In extracts from the youngest open leaf, IAA-oxidase activity was much higher from the 81 mg/liter Mn plants than from the other, visibly normal plants (table IV). Differences between IAA-oxidase activity of 81 mg/liter Mn plants and others declined in leaf 2 and even more in leaf 3. The decrease in enzyme activity with leaf age paralleled the expression of toxicity symptoms in the leaves examined. When these crude extracts were assayed without $MnCl_2$ in the medium, leaf 1 from the 81 mg/liter Mn plants exhibited high IAA-oxidase activity, and the other plants were inactive (table IV). With leaf 2 and leaf 3, the 27 mg/liter plants yielded extracts with some IAA-oxidase activity, but the differences between 81 mg/liter plants and 27 mg/liter plants were greater without added manganese than where manganese was added to the assay medium (table IV).

Table IV. *IAA-Oxidase Activity of Crude Extracts of Cotton Plants as Influenced by Different Levels of Substrate Manganese (Expt II, First Harvest)*

Treatment mg Mn/liter	μl O_2 Uptake/75 min*		
	Leaf 1	Leaf 2	Leaf 3
	With Mn in the assay medium		
1	19	74	94
27	19	52	78
81	128	115	112
	Without Mn in the assay medium		
1	0	0	0
27	0	36	86
81	113	137	132

* 1 to 20 dilution of enzyme extract, data are averages of duplicate samples.

Following dialysis the extracts from apical tissue above leaf 1 had IAA-oxidase activity. The 81 mg/liter Mn plants yielded extracts with higher IAA-oxidase activity than other treatments from the apical bud tissue (fig 4a), leaf 1 (fig 4b) and leaf 2 (fig 4c). As with the crude extracts (table IV), differences in enzyme activity declined in samples successively farther away from the apex where visible damage was slight (fig 4d).

Leaf 1 from the 81 mg/liter manganese plants had substantially less inhibitor activity than similar tissue from other treatments (table V). Significant differences in tissue manganese between 27 and 81 mg/liter Mn plants are most noticeable in the youngest tissue (table VI) and this was where large differences in enzyme (fig 4a, 4b) and inhibitor (table V) activity also occurred.

Experiment II, Second Harvest. The second harvest of experiment II was made 2 weeks after the first. The apical meristems of the most severely affected plants were similar in appearance to those of the previous harvest. Symptoms declined with successively older leaves, but in all cases, the symptoms were more severe than those exhibited by leaves 2 and 3 of the first harvest. Results of IAA-oxidase assays were quite similar to those observed at the previous harvest (table IV, fig 4) and, therefore,

Table V. *Relative IAA-Oxidase Inhibitor Activity of Boiled, Crude Extracts of Cotton Grown with Different Levels of Substrate Manganese (Expt II, First Harvest)*

Treatment mg Mn/liter	Length of lag period Min*		
	Leaf 1**	Leaf 2	Leaf 3
1	53	31	29
27	55	35	24
81	30	30	28

* Longer lag indicates higher inhibitor level.
** See text for leaf description.

are not reported in detail. The greatest increase in IAA-oxidase activity with 81 mg/liter Mn was in leaf 1, but in keeping with the additional period of treatment and the more severe symptoms noted, all differences were larger than in the first harvest. As before (table V), inhibitor activity was also lower in leaf 1 of the 81 mg/liter Mn plants.

Table VI. *Tissue Manganese Content as Influenced by Substrate Manganese (Expt II, First Harvest)*

Treatment mg Mn/liter	Tissue Mn µg/g tissue			
	Apical tissue*	Leaf 1	Leaf 2	Leaf 3
1	61	20	53	9
27	57	...**	2713	3259
81	1071	2026	9747	8168

* See text for sample description.
** Sample lost.

After 3 weeks of nutrient treatment in experiment II, young leaves, which showed necrotic spots, and retarded expansion, began to abscise from the 81 mg/liter Mn plants (fig 5). The abscising leaves had unfolded, were green and turgid, but not fully expanded. Abscission continued for about 1 week and then ceased. No fully expanded leaves abscised from the 81 mg/liter Mn plants, and no leaves abscised from plants in the 1 and 27 mg/liter treatments. This abscission response of cotton to toxic levels of manganese was subsequently repeated.

Experiments III and IV. Results of experiments III and IV agreed with those of experiment II at the first harvest. Plants grown on 81 mg/liter Mn

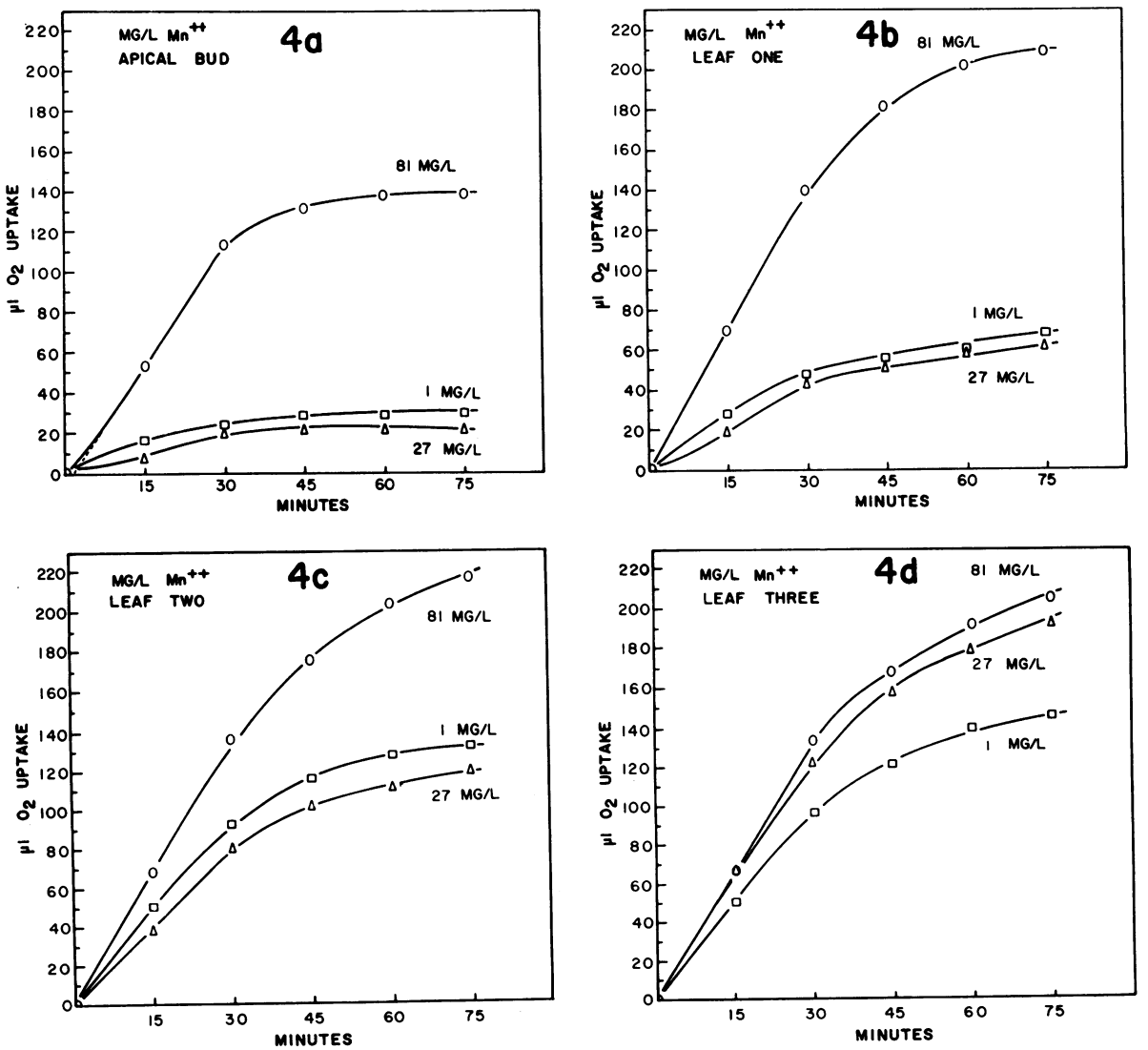

FIG. 4. IAA-oxidase activity of dialyzed extracts of cotton grown with different levels of substrate Mn. Data from apical bud (a), youngest (b), second youngest (c), and third youngest (d) unfolded leaves. 1 to 10 dilution of enzyme, experiment II, first harvest. Average of replicate samples.

FIG. 5. Abscission of cotton leaves in experiment II associated with response to 81 mg/liter nutrient Mn. Shown is leaf scar from which a young leaf has just abscised and the apical bud above the scar.

showed toxicity symptoms in leaves 1 and 2 and produced extracts with unusually high IAA-oxidase activity. Plants grown on nontoxic levels of manganese had lower total IAA-oxidase activity and higher IAA-oxidase inhibitor activity. Assays of crude extracts without added manganese produced results similar to those noted before (table IV).

A second result of experiment IV (fig 6) was to illustrate that extracts from manganese toxicity plants were not high in IAA-oxidase activity simply due to an abundant supply of a cofactor (manganese)

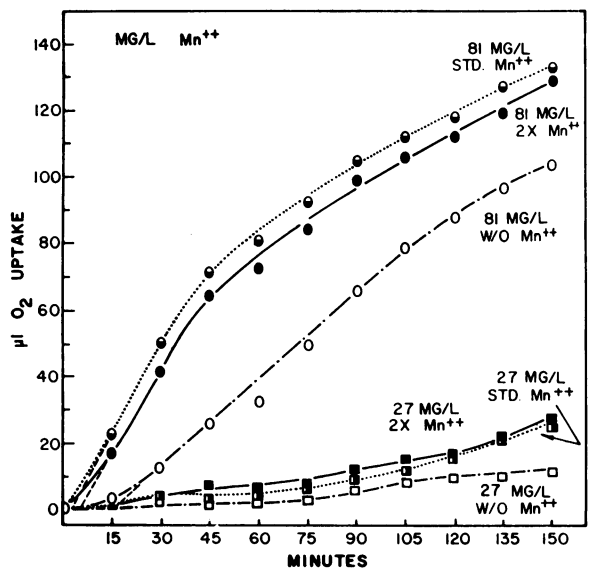

FIG. 6. IAA-oxidase activity of crude extracts of cotton grown with different levels of substrate Mn in experiment IV. 1 to 20 dilution of enzyme assayed in either the standard assay system (std. Mn, 3 μM Mn/flask), without manganese (w/o Mn) or double the standard amount of manganese (2 × Mn, 6 μM Mn/flask). Results from 1 mg/liter plants closely parallel those from 27 mg/liter plants.

which was present in limiting quantities when other tissues were assayed. Doubling the amount of $MnCl_2$ added to the assay medium did not increase the activity of any extract above that observed with the standard assay medium (fig 6). An in vivo effect of manganese was also suggested by the persistence of differences in enzyme activity after dialysis and calculations which indicated that the crude extracts themselves added much less manganese than the standard level in the assay medium. An in vivo effect of tissue manganese on the IAA-oxidase system (fig 1, 3, 4, 6 and table IV) should be distinguished from the capacity of extracts of the 81 mg/liter Mn plants to supply the cofactor requirement of manganese. This latter ability was only observed when IAA-oxidase assays were conducted without added manganese (fig 2, table IV).

Discussion

Results of both long-term (126 days) and relatively short-term (12–14 days) experiments reveal a direct correlation between a high (toxic) level of nutrient manganese and both tissue manganese and IAA-oxidase activity. There was an inverse correlation between high manganese and IAA-oxidase inhibitor activity. The degree of enzyme stimulation was directly related to the severity of symptoms. These symptoms can be interpreted to reflect an auxin shortage. The observed stimulation of IAA-oxidase activity in young leaves and buds with the 81 mg/liter Mn treatment may be viewed as: A) an acceleration of the normal increase in the enzyme with aging of leaves (10), B) a suggestion that the usual distribution pattern of the enzyme (10) is important to normal development. This was the first demonstration of IAA-oxidase activity in a cotton-leaf extract without the addition of manganese to the assay system. Straus and Gerding (14) have suggested, based on their work with tissue cultures of *Ephedra*, that the ability of an enzyme preparation to destroy IAA without added cofactors (manganese and 2,4-dichlorophenol) is an argument in favor of in vivo activity of the enzyme.

Based on the informaton above, the following hypothesis is advanced. Above a threshold concentration, tissue manganese allows or causes an increase in activity of the IAA-oxidase system which reduces the supply of auxin. The auxin deficiency produces abnormal growth as reflected by shortened internodes, restricted leaf expansion, abscission of leaves, proliferation of tissues, loss of apical dominance and death of apical buds. The hypothesis would suggest no significant modification of auxin destruction below a threshold concentration of manganese and would allow other effects and interactions of the element at toxic concentrations.

The most apparent explanation for our results is that the high level of tissue manganese catalyzed destruction of IAA-oxidase inhibitor(s), thus allow-

ing the enzyme present to function more actively. This mechanism is supported by the finding of Furuya and Galston (2) that in vitro preincubation of pea homogenates with manganese lowered inhibitor activity and thus increased IAA-oxidase activity. An inhibitor-mediated mechanism is also in agreement with the negative correlation between inhibitor concentration and IAA-oxidase activity in light-grown cotton and the suggestion that this negative correlation indicates a causative relationship (10). Greater IAA-oxidase activity, in response to decreased inhibitor, could also allow adaptive formation of more enzyme as shown by Galston and Dalberg (3) in response to added IAA. Although such a feedback mechanism would explain the apparent increase in enzyme amount (see later), it would require adaptive synthesis in response to an IAA-breakdown product or a decrease in inhibitor content.

Regardless of the mechanism(s) involved, in vivo manganese appeared to affect the absolute amount of both enzyme and inhibitor and not simply the net amount of inhibitor-free enzyme. The normal, relatively low activity of IAA-oxidase from apical tissues is not changed by processes to remove or inactivate inhibitors (i.e. dialysis, acetone precipitation, assay with riboflavin; 10, 11). Preincubation of dialyzed pea homogenates with manganese produced no activation of enzyme (2); whereas, in our studies the young leaves from the 81 mg/liter Mn plants had high IAA-oxidase activity even after dialysis (fig 3, 4a, 4b).

Our results indicate a case of in vivo induction of the IAA-oxidase system in the intact, green plant with a component of the enzyme system. This suggests that the enzyme can function in vivo to regulate auxin levels and processes affected by auxin.

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