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Influence of Substrate and Tissue Manganese on the IAA-Oxidase System in Cotton[1, 2]

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Abstract. **Tissue manganese was found to influence the indoleacetic acid (IAA) system of cotton over a wide range of concentrations. The cofactor and inhibitor activities of the IAA-oxidase system were affected as the concentration of manganese in the tissue was varied. Maximum inhibitor activity was found in leaf extracts from the plants grown in 0.5 mg/l manganese (Hoagland's level). The inhibitor activity decreased in the leaf extracts of plants grown at concentrations of manganese either higher or lower than 0.5 mg/l. Abnormally high IAA-oxidase activity was found in the leaves of plants grown in deficient levels of manganese ($<$0.0005, 0.005 mg/l) and the extracts from plants in the $<$0.0005 mg/l Mn treatment showed IAA-oxidase cofactor activity.**

Manganese has been reported to be either required or stimulatory for the activity of the indoleacetic acid (IAA) oxidase system from pea (13), bean (5, 13), pineapple (2), lupine (12), wheat (14), Omphalia (10), and cotton (8). Furuya and Galston (1) found that preincubation of tissue homogenate with manganese overcame the effects of the inhibitor of IAA-oxidase. Morgan, Joham, and Amin (7) found that leaf extracts from plants grown with toxic levels of manganese (81 mg/l of nutrient solution in sand culture) exhibited a higher capacity for the destruction of IAA than leaf extracts from plants grown with usual levels of manganese (1 mg/l). Analysis showed lower inhibitor activity in leaf extracts from plants displaying symptoms of manganese toxicity. These studies (1, 7), involving addition of manganese either to substrate (nutrient solution) or to *in vitro* enzyme systems, indicate that the function of manganese in the oxidation of IAA may not be limited to its role as a cofactor for the enzyme. The effect of low levels of substrate manganese and the resulting reduction in tissue manganese on the IAA-oxidase system has not been reported.

This paper deals with the relationship between the concentration of manganese in cotton leaf tissue and IAA-oxidase activity. Tissue manganese was varied by growing cotton plants at substrate manganese concentrations ranging from deficient to toxic levels of the element. The effects of tissue manganese on the IAA-oxidase system were tested with assays of enzyme, inhibitor, and cofactor activity.

Methods and Materials

Hydroponics Techniques. Cotton plants (*Gossypium hirsutum* L.), variety Deltapine 15, were grown in a greenhouse in water culture using Hoagland's solution. Levels of substrate manganese (as $MnCl_2$) were $<$0.0005, 0.005, 0.5 and 5.0 mg/l of solution. These treatments were shown to represent respectively, deficiency, border line deficiency, standard Hoagland's, and border line toxicity levels of manganese for cotton (D. M. Taylor, 1965, The Manganese Nutrition of Cotton. Ph.D. Dissertation, Texas A & M University, College Station).

The plants were grown in 6 liter plastic containers which were placed inside a metal can to eliminate light. The nutrient solutions were adjusted daily to pH 6.0 and were aerated with filtered air which passed through a glass tube into the solution. The culture solutions were changed weekly to assure a constant supply of nutrients. Transpired water was replaced with deionized water as required.

Purification of Chemicals. The low levels of nutrient manganese used in this study required that all nutrient salts be purified and that double deionized water be used. Trace amounts of manganese impurities were removed from all the macronutrients, except phosphate, using a hydrogen sulfide co-precipitation technique. A near saturated solution of each salt was prepared to which 2 grams of

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zinc chloride and 1 gram of ammonium chloride were added. The stock solutions were made alkaline with concentrated ammonium hydroxide and heated to 80°. Hydrogen sulfide was bubbled through each solution for 1 hour. During this stage, additional concentrated ammonium hydroxide was added as needed to keep the solution alkaline. The solutions were then cooled and allowed to stand overnight. Subsequently, each solution was centrifuged and the supernatant liquid boiled to drive off the unreacted hydrogen sulfide and ammonia. The colloidal sulfur was removed by filtering the solutions through Whatman No. 42 paper. Each solution was then analyzed for the element in question and made to volume.

Since phosphate salts of zinc and manganese are insoluble, the trace amount of manganese occurring in the potassium bi-phosphate stock solution was removed by adding 100 mg of zinc chloride per liter of solution. The resulting co-precipitates of zinc and manganese phosphate were removed by centrifugation.

The micronutrients, except iron, were purified by recrystallization. Iron was purified by a modified isopropyl ether extraction technique outlined by Piper (9). Ferric chloride was dissolved in concentrated hydrochloric acid and extracted 3 times with isopropyl ethyl. The iron was removed from the ether fraction by adding water and heating to drive off the ether. The solution was then analyzed for iron and made to volume. Concentrated nitric acid was added to convert the iron to the ferric form.

Chemical Methods and Sampling. Manganese was determined by the phosphate-potassium periodate method (3). Oven-dried samples were ashed at 550° in a muffle furnace. The ash was then digested using concentrated nitric and 70 % perchloric acid. The residue was dissolved in nitric acid and phosphoric acid (85 % v/v) was added. The solutions were warmed on a hot plate and the color was developed by adding 30 mg of potassium periodate. After cooling, the transmittance was read at 530 mμ with a Spectronic 20 colorimeter. The concentration of manganese in each sample was determined by comparison to a standard curve.

Leaf samples were harvested at 12, 37, 72, and 135 days after the nutrient treatments were started. Each sample was then divided into 2 fractions which were assayed for manganese and IAA-oxidase activity. Since results at all harvest dates were similar, only the data from the 37 day-old plants are presented.

For the 37 day-old plants, the upper leaf samples included the 3 youngest main stem leaf blades greater than 2.5 cm in diameter; while the lower leaf samples included the oldest 3 main stem leaf blades. Due to limited growth (0.67 g dry wt per plant) of the plants in the <0.0005 mg/l Mn treatment, all leaf blades greater than 2.5 cm in diameter were included in 1 sample for assay of IAA-oxidase activity.

The leaf sample fractions used for IAA-oxidase determinations were weighed immediately after harvest, washed with deionized water, blotted dry and stored at minus 20° until assayed. All the steps in the preparation and purification of the leaf extracts were carried out in a 2° cold room. The samples were homogenized with a glass mortar with 3 ml of deionized water per gram of fresh tissue. The brei was squeezed through 4 layers of cheese cloth and centrifuged at 22,000 $\times$ g for 20 minutes at 0°.

The resulting supernatant liquid (crude extract) was divided into 3 parts. One portion of each crude extract was diluted 10 times with water and assayed for IAA-oxidase activity without further treatment. A second portion was dialyzed in the cold in 10 liters of water for 72 hours to remove the endogenous inhibitor. After dialysis the extract was centrifuged as before and assayed for IAA-oxidase activity. The third portion was boiled for 10 minutes to inactivate the enzymes and precipitate the protein. It was centrifuged, returned to volume and endogenous inhibitor activity was determined in the resulting supernatant.

IAA-oxidase was assayed by a manometric system (6,8). Each flask contained 30 μM of IAA in the side arm, 0.2 ml of 20 % KOH in the center well with a filter paper wick and 1 ml of 0.4 M Na_2HPO_4-NaH_2PO_4 buffer (pH 5.8), 3 μM of 2,4-dichlorophenol, 3 μM of $MnCl_2$, 0.0319 μM of riboflavin, 1 ml of leaf extract, and water in the main compartment to give a total volume of 3.3 ml. The assay was conducted at 25° in room light after being equilibrated for 15 minutes. Duplicate flasks of each extract were assayed. Each flask contained extract from 27 mg fresh weight of tissue. Omission of IAA, 2,4-dichlorophenol, manganese or enzyme was previously shown to eliminate oxygen uptake in the assay (6, 8). Other mono hydroxy phenols will substitute for 2,4-dichlorophenol (11).

The assay for cofactor activity was identical to the IAA-oxidase assay except that 2,4-dichlorophenol was omitted from the reaction flask. Under this condition oxygen uptake by the crude leaf extracts indicated presence of both IAA-oxidase and a native phenolic cofactor. Appropriate blanks, containing boiled extract, were run to determine oxygen uptake due to the other constituents.

In order to determine the amount of endogenous inhibitor, the boiled extracts were assayed with a standard acetone purified, cotton-leaf enzyme preparation (6). The IAA-oxidase assay was used. Relative inhibitor activity was measured by the length of the lag period which was induced by 0.3 ml of the boiled extract. A control flask, which contained water instead of the boiled extract, was included with each assay.

The inhibitor(s) in cotton is heat stable, dialyzable and its activity follows total phenol level

(6, 8). Increasing the amount of inhibitor in the form of boiled, cotton-leaf extract or phenolic materials such as gossypol, kaempferol, scopoletin, pyrogallol, ferulic acid, gallic acid, catechol, quercitrin, caffeic acid or chlorogenic acid increased the lag in oxygen uptake in the assay in a proportional manner (8, 11). The lag-induction by either native inhibitor or phenolic materials is reduced by conducting the assay in the light with added riboflavin (8).

Results

The content of manganese in the upper and lower leaf fractions at each level of substrate manganese used in this study is given in table I. The manganese content of the leaf fractions varied from 5.6 µg/g dry weight of leaf tissue from the upper leaves of plants grown with 0.005 mg/l manganese to 1282.7 µg/g in the lower leaves of plants grown with 5.0 mg/l manganese. Presumably, tissue manganese in the plants grown in <0.0005 mg/l manganese was lower than the level observed with the 0.005 mg/l treatment. In all treatments, the concentration of manganese was higher in the lower than in the upper leaf fractions.

Table I. *The Influence of Substrate Manganese on the Manganese Concentration in Leaf Tissue of 37 Day-old Cotton Plants*

Manganese in nutrient solution	Manganese Leaf sample Upper	Lower
mg/l	*mg/gm dry wt*	
<0.0005	...[1]	...
0.005	5.6f[2]	11.5e
0.5	168.7c	251.2c
5.0	746.5b	1282.7a

1 The concentration of manganese in this tissue was not determined due to lack of plant growth at this nutrient concentration.

2 Each datum is the mean of 4 replications. Means followed by the same letter are not significantly different at the 0.05 level of probability.

The effect of different levels of nutrient manganese on the IAA-oxidase activity of crude extracts of upper and lower leaves is shown in figure 1. At all nutrient manganese levels, IAA-oxidase activity was found to be highest in extracts obtained from the lower leaves. The leaf extracts from the upper leaves exhibited a lag before oxygen uptake occurred, while no lag period was evident in any of the lower leaf extracts. Leaf extracts from plants grown with <0.0005 mg/l manganese had the highest IAA-oxidase activity, and there was no lag period.

Although differences in enzyme activities of the lower leaf extracts from the different manganese treatments were small, the order of activity from highest to lowest was the same as in the upper leaf extracts. IAA-oxidase activity in the leaf extracts from plants grown with <0.0005 and 0.005 mg/l manganese (deficient levels) was highest followed in order by leaf extracts from the 5.0 and then the 0.5 mg/l manganese treatments.

After the leaf extracts were dialyzed to remove endogenous inhibitors, the relative IAA-oxidase activities for different leaf extracts were again measured (fig 2). In spite of the fact that the sample from the plants in the <0.0005 mg/l manganese treatment contained upper leaves, which normally have low IAA-oxidase activity (6), this extract demonstrated the highest activity. Among the other 3 manganese levels, the upper leaf extracts from the plants in the 0.005 mg/l Mn treatment were higher in IAA destroying capacity than the lower leaf extracts from the plants grown with 0.5 and 5.0 mg/l Mn. This high activity in the upper leaves from plants in the 0.005 mg/l Mn

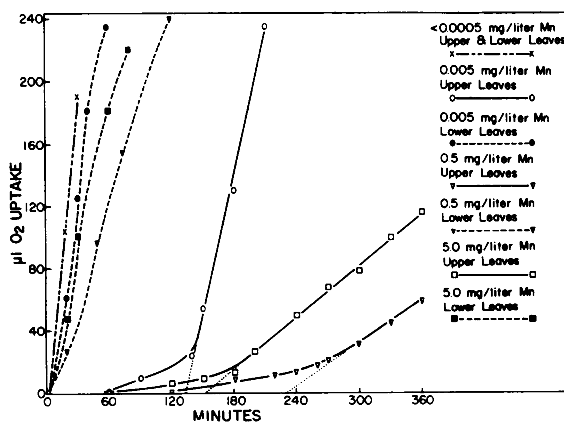

FIG. 1. IAA-oxidase activity of crude (non-dialyzed) extracts from leaves of 37 day-old cotton plants grown at different substrate levels of manganese.

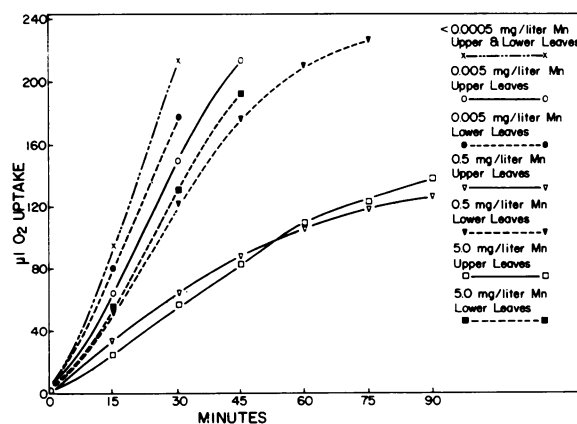

FIG. 2. IAA-oxidase activity of dialyzed extracts from leaves of 37 day-old cotton plants grown at different substrate levels of manganese.

Table II. *IAA-Oxidase Inhibitor Activity of Extracts of Leaves of 37 Day-old Cotton Plants Grown at Different Substrate Levels of Manganese*

Manganese in nutrient solution	Leaf description	Inhibitor activity[1] Length of lag period
mg/l		*min*
<0.0005	Upper and lower	0
0.005	Upper	72
	Lower	0
0.5	Upper	140
	Lower	0
5.0	Upper	93
	Lower	0

[1] Inhibitor activity of boiled, crude extracts tested, longer lag indicates more inhibitor activity.

treatment was also in contrast to a much lower activity in upper leaves from plants grown in 0.5 and 5.0 mg/l manganese. The enzyme activity was of the same order of magnitude in the dialyzed extracts for the upper leaves from both the 0.5 and 5.0 mg/l substrate manganese treatments. The same relationship existed for the old leaves of these 2 treatments.

The relative inhibitor activity of the boiled leaf extracts is presented in table II. There was no measurable lag period produced by the extracts from the lower leaves from any of the manganese substrate levels used. The greatest inhibitor activity (lag period) was found in the upper leaf extracts from plants in the 0.5 mg/l manganese treatment. The lowest inhibitor activity in the upper leaf extracts was from plants grown with 0.005 mg/l of manganese with the 5.0 mg/l Mn treatment being intermediate. Although the boiled leaf extracts from plants in the <0.0005 mg/l manganese treatment contained upper leaves, no inhibitor activity could be demonstrated.

The crude leaf extracts were assayed for IAA-oxidase activity without the addition of manganese to the assay system. The crude extracts from the lower leaves of the plants grown in the 5.0 and 0.5 mg/l manganese solutions gave considerable enzyme activity (160 and 60 μl O_2 uptake in 60 mins). All extracts from plants in the 0.005 and <0.0005 mg/l Mn treatment showed slight activity (<20 μl O_2 uptake in 60 mins) and all other samples were inactive.

Cofactor activity was evaluated in the crude leaf extracts which showed low inhibitor activity. Extract from plants in the <0.0005 mg/l Mn treatment showed significant cofactor activity. After blank correction, the cofactor assay from this treatment showed 65.3 μl O_2 uptake in 150 minutes while other low inhibitor samples (0.005, 0.5 and 5.0 mg/l Mn) did not support oxygen uptake.

Discussion

This study confirms that toxic levels of substrate manganese (7), here represented by the 5 mg/l treatment, modify the IAA-oxidase system of cotton as measured by *in vitro* assays (fig 1, table II). Furthermore, these data are the first evidence that deficient levels of manganese produce plants with an abnormal IAA-oxidase system when compared to normal manganese nutrition.

A major effect of manganese nutrition on the IAA-oxidase system in cotton appears to be on the cofactor and inhibitor activity of the enzyme system. Maximum inhibitor activity was present in the upper leaf extracts when the concentration of tissue manganese was 168.7 μg per gm dry weight (0.5 mg/l Mn treatment) table I. The inhibitor activity was reduced when the concentration of leaf manganese was increased or decreased from the level present in the plants grown in 5.0 mg/l manganese. Also, there was significant cofactor and no inhibitor activity in extracts from plants grown in the <0.0005 mg/l Mn treatment, but tissue manganese was not determined due to insufficient leaf material. These results indicate that the biosynthesis or at least the activity of the native cofactor and inhibitor of the IAA-oxidase system was influenced by the concentration of manganese in the leaf tissue. This and previous work (7) shows that both unusually high or low levels of manganese modify IAA-oxidase inhibitor activity.

A second effect of manganese nutrition on IAA-oxidase activity was noted (table II). With extracts dialyzed to remove native inhibitors, IAA-oxidase activity of young leaves of plants in the 0.005 mg/l manganese treatment was similar to that of old leaves of plants grown in 0.5 mg/l manganese (Hoagland's level). This represents an increase of activity in the young leaves since they generally have lower IAA-oxidase activity than old leaves from the same plant (see 0.5 mg/l manganese treatment, fig 2, and ref 6). Although not assayed separately, the same abnormally high IAA-oxidase activity must have been present in young leaves of the plant in the <0.0005 mg/l manganese treatment since a combined sample of young and old leaves produced the highest IAA-oxidase activity per gram fresh weight in this study.

The role of manganese as a cofactor in many IAA-oxidase systems has been established by earlier work (2, 4, 5, 10, 12, 13, 14) and is supported by the present results. Nutrient manganese, therefore, probably has a dual affect on the IAA-oxidase system: A) it affects the level or activity of the cofactor(s) and inhibitor(s) of the system, B) it acts as a cofactor of the enzyme. The results of this study are compatible with the hypothesis that IAA-oxidase functions *in vivo* in light-grown cotton plants (6, 7, 11).

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