The Relationship of the Peroxidative Indoleacetic Acid Oxidase System to in Vivo Ethylene Synthesis in Cotton

ABSTRACT

Since peroxidase and manganese have been implicated in both auxin destruction and ethylene production, the effect of auxins and high tissue levels of manganese on the peroxidative indoleacetic acid oxidase system and the internal level of ethylene was determined in cotton (Gossypium hirsutum L. cv. Watson GL-7). The highest level of manganese tested produced manganese toxicity symptoms, including necrotic lesions, accompanied by an increase in internal ethylene levels at about 15 days after treatment initiation. Statistically significant increases in indoleacetic acid oxidase and peroxidase activity were first observed 2 days later and were paralleled by tissue manganese levels above 7.4 milligrams per gram dry weight and internal ethylene levels of 0.77 microliters per liter air. Eight hours after application of 2, 4-dichlorophenoxyacetic acid or indoleacetic acid, the internal levels of ethylene were increased to above 6.6 microliters per liter air in cotton plants, and levels of this magnitude were maintained for a 72-hour period of observation. Modification of peroxidase and indoleacetic acid oxidase activity in auxin-treated plants definitely occurred well after the elevation of internal ethylene levels. While ethylene levels and indoleacetic acid oxidase activity were increased by both experimental approaches, the earlier appearance of increased ethylene indicates that the peroxidative indoleacetic acid oxidase system in cotton is not involved in ethylene synthesis or that this enzyme is not the rate-limiting factor when ethylene synthesis is increased. Ethylene, as well as auxin destruction, may be involved in some of the long term plant responses to toxic levels of manganese. The findings also suggest that auxin-induced ethylene may play a role in the elevation of peroxidase and indoleacetic acid oxidase activity eventually seen in extracts of plants treated with auxins. The data support the assumption that the enzymatic portion of the indoleacetic acid oxidase system in cotton is a peroxidase.

In 1934, Thimann (36) reported the inactivation of native auxins by homogenates of plant tissue. Over the years since Thimann's observation, sufficient evidence has been accumulated to define the major substance responsible for auxin activity as indole-3-acetic acid and to attribute the destruction of IAA within the plant, primarily, to a peroxidase-based enzyme system generally known as IAA-oxidase (11, 15, 20, 28, 34, 37). By virtue of its role in limiting the supply of IAA within the plant, the IAA-oxidase system has been recognized as a plant growth regulatory mechanism.

The potential role of the IAA-oxidase system as a plant growth regulatory mechanism assumed new dimensions with Yang's (39) discovery that an enzyme system strikingly similar to the peroxidative IAA-oxidase system is capable of synthesizing the plant hormone ethylene. If this ethylene-synthesizing system functions in vivo, the growth regulatory activities of the peroxidative IAA-oxidase system may encompass those areas in which ethylene is involved as well as the growth phenomena associated with IAA. Additional regulatory role for peroxidases is supported by their association with in vitro ethylene synthesis in several studies (22, 23, 25).

The micronutrient manganese has been implicated as a cofactor in both the IAA-destroying system (15, 20, 21) and the ethylene-synthesizing system (1, 39). High levels of substrate manganese resulting in high levels of tissue manganese reportedly increase both the peroxidase activity and the IAA-oxidase activity in cotton (31, 35). The evidence also suggests that higher than normal levels of tissue manganese may affect flower differentiation in cotton (3, 18). Both ethylene and peroxidase have been implicated as factors affecting tissue differentiation in certain other plant species (6, 9, 17, 32).

After our study was completed, Kang et al. (19) published evidence that peroxidase is not the rate-limiting enzyme in auxin-induced ethylene synthesis in subapical sections of etiolated pea seedlings. Our own studies were undertaken realizing that, even if the peroxidase-based enzyme system in not involved in in vitro ethylene synthesis, additional information is needed for a fuller understanding of the system's activity.

We report here observations of the changes of the activity of the peroxidative IAA-oxidase system and ethylene levels in cotton after treatments which are known to promote peroxidase activity and ethylene synthesis. For the intact, vegetative cotton plant these treatments are supraoptimal levels of substrate manganese (31, 35) and supraoptimal levels of auxin (27, 30).

MATERIALS AND METHODS

Plant Culture. Glandless cottonseed (Gossypium hirsutum L. cv. Watson GL-7) were planted in washed builder's sand in 20.3-cm pots, placed in a controlled environment room (2500 ft-c, 16-hr photoperiod; relative humidity, 35 – 5%; temperature, 29 ± 1.0 C day, 24 ± 1.0 C night). Plants were irrigated with a complete nutrient solution (18) made with distilled

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water adjusted to pH 6.0. Pots were thinned to leave 8 to 12 plants per pot approximately 2 weeks after planting.  

**Manganese Treatments.** This phase of the study included three replications of three treatment levels and four harvest dates. At 10 days after planting, manganese treatments were initiated. Manganese was applied in a nutrient solution (16) modified with manganese chloride to give levels of 0.5, 1.35, and 324 mg Mn\(^{2+}\) per liter. In each treatment level, pH was adjusted to 6.0 with dilute (0.5% NH\(_4\)) ammonium hydroxide solution. Beginning on the 8th day of treatment, the substrate manganese level in the highest treatment was progressively increased to 624 mg liter\(^{-1}\) by the 11th day to attain the desired development of manganese toxicity symptoms during the experiment.

The first plant harvest was made on the 11th day after treatments were initiated. Three additional harvests were made every other day. Tissue above a point just below the attachment of the petiole of the third youngest, expanded true leaf was harvested (Fig. 1). Each replication of a treatment included two pots of cotton plants. The plants of each pot were harvested and divided equally, as to number and condition, into two samples (Fig. 1). One sample was weighed and extracted immediately to determine internal ethylene content. The other sample was weighed, washed, and placed in a deep freeze for later enzyme extraction. The sample weights, in most cases, ranged from 10 to 20 g.

**Auxin Treatments.** This experiment consisted of three treatments (a control, IAA treatment, and 2,4-D treatment) and three harvest dates replicated three times. At 35 days after planting, cotton plants previously designated for treatment were removed from the growth room and sprayed without runoff with either a 9.25 mm solution of IAA prepared in 10% aqueous ethanol (30) or a 1.36 mm (acid eq) aqueous solution of the sodium salt of 2,4-D (27). One percent Tween 20 was used as a wetting agent in both solutions, and the soil surface was covered during spraying. After drying, the plants were returned to the growth room. Based on evidence from an earlier study (30), it was not necessary to spray control plants with the carrier solvent.

Harvesting was accomplished at 8, 25, and 72 hr after treatment in a manner similar to that described for the manganese-treated plants.

**Ethylene Assays.** Internal ethylene concentration was determined by gas chromatography with the vacuum extraction method of Beyer and Morgan (4). The details of the chromatographic procedure of ethylene analysis and the verification of ethylene identity were as previously reported (26).

**Enzyme Extraction.** All extraction procedures were carried out in a cold room or ice bath at 0 to 5 C. Frozen tissue was ground by hand with mortar and pestle and some purified quartz sand in 3 ml of 0.1 M K\(_2\)HPO\(_4\), containing 2 mM EDTA tetrasodium salt per g plant tissue (23, 28). The brei was squeezed through four layers of cheesecloth and centrifuged at 30,000g at 0 C for 20 min, and the supernatant was brought to a volume of 4 ml per g tissue with buffer. This resulted in a crude extract with a pH of approximately 7.2.

The crude extract was dialyzed in the cold against five changes of glass-distilled water for 72 hr to remove the endogenous inhibitors. After dialysis the extract was centrifuged at 30,000g at 0 C for 10 min and the supernatant was diluted 1 to 10 with glass-distilled H\(_2\)O for use in IAA-oxidase and peroxidase assays.

**IAA-Oxidase Assay.** The IAA-oxidase activity of the dialyzed extracts was determined after dialysis with the use of a manometric technique (28) modified by deletion of riboflavin from the reaction medium. Flasks containing 1 ml of diluted enzyme extract were assayed in light by placing a 200-w incandescent bulb directly above the Warburg assembly. Duplicate flasks were assayed, and reagent blanks containing boiled extract were used to adjust for any \(\text{O}_2\) uptake due to light, reagents, or nonenzymatic substances in the extract.

**Peroxidase Assay.** Total peroxidase activity was measured by following the change in absorbance at 460 nm due to the oxidation of dianisidine in the presence of H\(_2\)O\(_2\) (38). The units of activity reported from duplicate assays of samples correspond to the same quantity of enzyme used in the IAA-oxidase assays. One unit of peroxidase activity is that amount of enzyme decomposing 1 \(\mu\)mole of H\(_2\)O\(_2\) per min at 25 C.

**Tissue Manganese Assays.** After determination of internal ethylene levels, these samples were thoroughly washed in distilled water, rinsed in glass-distilled water, pressed between paper towels, dried in a forced draft oven, and ground in a Wiley mill. Approximately 1-g samples of the ground material were ashed at 550 C, and extracts were prepared (12). The manganese content of these extracts was determined by atomic absorption spectrophotometry.

**Statistical Procedures.** The data reported were subjected to analysis of variance and correlation analysis as indicated (33). Duncan’s Multiple Range Test was used to test significance of differences among means (24).

**RESULTS**

**Manganese Study.** During the course of this experiment, manganese toxicity symptoms became apparent in the plants and developed progressively as tissue manganese concentration increased. Initial symptoms included chlorosis of leaves followed by cupping of leaf margins. Development of moderate symptoms was expressed as small necrotic spots which subsequently developed into the large necrotic spots, completely desiccated young leaves, and, in some cases, death of the apical tissue characteristic of severe manganese toxicity in cotton. At the highest level of manganese, initial symptoms were observed at the 13 day harvest, and moderate to severe symptoms were observed at 15 days after treatment initiation. The proportion of plants in the highest manganese treatment exhibiting severe symptoms increased at the 17 day harvest.
Table I. The Effect of Tissue Manganese Level on the Peroxidase and IAA-Oxidase Activities of Dialyzed Cotton Tissue Extracts and the Relationship of These Enzymes to Internal Ethylene Concentration

<table>
<thead>
<tr>
<th>Mn* Level</th>
<th>Peroxidase Activity</th>
<th>Internal Ethylene Concentration</th>
<th>IAA-Oxidase Activity, μ/liter</th>
<th>Oxidation, ml/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>Tissue</td>
<td>units/min-mg</td>
<td>μ/liter</td>
<td></td>
</tr>
<tr>
<td>mg/liter</td>
<td>g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13 day harvest</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>46</td>
<td>0.781 a</td>
<td>0.193 a</td>
<td>1.29 a</td>
</tr>
<tr>
<td>135</td>
<td>1387</td>
<td>0.688 a</td>
<td>0.123 a</td>
<td>1.14 a</td>
</tr>
<tr>
<td>342+</td>
<td>5322</td>
<td>0.672 a</td>
<td>0.183 a</td>
<td>1.18 a</td>
</tr>
<tr>
<td>15 day harvest</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>32</td>
<td>0.598 a</td>
<td>0.126 a</td>
<td>1.43 a</td>
</tr>
<tr>
<td>135</td>
<td>1751</td>
<td>0.579 a</td>
<td>0.152 a</td>
<td>1.02 b</td>
</tr>
<tr>
<td>324+</td>
<td>7238</td>
<td>0.694 a</td>
<td>0.347 b</td>
<td>1.47 a</td>
</tr>
<tr>
<td>17 day harvest</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>40</td>
<td>0.688 a</td>
<td>0.216 a</td>
<td>1.49 a</td>
</tr>
<tr>
<td>135</td>
<td>2110</td>
<td>0.573 a</td>
<td>0.228 a</td>
<td>1.23 a</td>
</tr>
<tr>
<td>324+</td>
<td>7460</td>
<td>0.881 b</td>
<td>0.766 b</td>
<td>2.32 b</td>
</tr>
</tbody>
</table>

1 Any two means followed by the same letter are not significantly different at the 0.05 level of probability as determined by the Duncan’s Multiple Range Test.
2 r: Correlation coefficient.
3 Significant at the 0.05 level.
4 Significant at the 0.01 level.

The relationships between peroxidase activity, IAA-oxidase activity, and ethylene content in cotton plants treated with concentrations of substrate manganese ranging up to supranormal levels are summarized in Table I. Neither peroxidase nor IAA-oxidase activity increased significantly due to manganese treatment until toxicity symptoms were observed. This occurred only at extremely high levels of tissue manganese at 17 days after initiation of treatment. A trend was evident in the intermediate levels of tissue manganese which suggest a reduction of IAA-oxidase activity within a range of 1.2 to 2.0 mg manganese per g dry weight of plant tissue.

Peroxidase activity was positively correlated with IAA-oxidase activity throughout the observation period. This was determined by the linear relationship obtained when one was plotted against the other and by the highly significant correlation coefficient (r value) of +0.648.

Internal ethylene concentration was also correlated with development of manganese toxicity symptoms. The increase in internal ethylene levels was only two to four times that of the controls, but a significant change was apparent as early as the 15 day harvest in the high substrate manganese treatment (Table I). This change in ethylene synthesis occurred at a threshold tissue manganese level of approximately 7.2 mg manganese per g dry weight. At this time severe manganese toxicity symptoms were becoming apparent but were not extensive. Internal ethylene concentration increased in a manner proportional to the development of manganese toxicity lesions. These results suggest a direct relationship between degree of toxicity symptom expression and synthesis of ethylene.

The relationship between peroxidase activity, IAA-oxidase activity, and internal ethylene concentration is not sufficiently distinguishable from the data in Table I to allow conclusions regarding cause and effect. Internal ethylene concentration was correlated with both IAA-oxidase activity and peroxidase activity only at extremely high levels of tissue manganese at 17 days after treatment initiation (correlation coefficients of +0.91 and +0.88, Table I). This was the point at which the initial, significant increase in both IAA-oxidase and peroxidase activities occurred. However, a significant change in ethylene synthesis was detected at 15 days after treatment initiation, a time when there was a nonsignificant trend for an increase in peroxidase activity and no increase in IAA-oxidase activity.

The data suggest that ethylene synthesis may be altered before the change in peroxidative IAA-oxidase activity is expressed.

Auxin Treatments. The time sequence of the effects of foliar applications of IAA and 2,4-D on internal ethylene concentration, peroxidase activity, and IAA-oxidase activity of cotton plants are summarized in Table II. Neither peroxidase activity nor IAA-oxidase activity increased significantly due to auxin at 8 hr after treatment. However, at this time the internal ethylene concentration of cotton tissue was greatly increased by both auxin treatments. At 25 hr after treatment, IAA-oxidase activity increased significantly in response to the 2,4-D treatment, but the trend of increasing peroxidase activity was not statistically significant. By 72 hr after treatment, the extracts of both the IAA- and the 2,4-D-treated plants significantly increased in peroxidase activity and IAA-oxidase activity as compared to the extracts of the untreated plants. There was a highly significant, positive correlation between these enzymatic functions throughout this experiment, as indicated by an r value of +0.948 (Fig. 2).

Increased internal ethylene levels clearly preceded changes in either peroxidase or IAA-oxidase activity (Table II). After the initial dramatic rise in internal ethylene levels, there were some fluctuations associated with the two different auxins, but concentrations above 5 μl ethylene per liter persisted for the 72-hr experimental period. The experiment was designed to give a major alteration of ethylene levels with the two auxins and not to compare them.

Table II. The Effect of Foliar Applications of IAA and 2,4-D on the Peroxidase and IAA-Oxidase Activities of Dialyzed Cotton Tissue Extracts and the Relationship of These Enzymes to Internal Ethylene Concentration

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Peroxidase Activity</th>
<th>Internal Ethylene Concentration</th>
<th>IAA-Oxidase Activity, μ/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units/min-mg</td>
<td>μ/liter</td>
<td></td>
</tr>
<tr>
<td>8 hr after treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.603 a</td>
<td>0.209 a</td>
<td>1.15 a</td>
</tr>
<tr>
<td>IAA</td>
<td>0.605 a</td>
<td>7.961 c</td>
<td>1.08 a</td>
</tr>
<tr>
<td>2,4-D</td>
<td>0.623 a</td>
<td>6.670 b</td>
<td>1.20 a</td>
</tr>
<tr>
<td>r² = +0.107</td>
<td></td>
<td></td>
<td>r = -0.100</td>
</tr>
<tr>
<td>25 hr after treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.725 a</td>
<td>0.231 a</td>
<td>1.28 a</td>
</tr>
<tr>
<td>IAA</td>
<td>0.807 a</td>
<td>9.005 b</td>
<td>1.58 ab</td>
</tr>
<tr>
<td>2,4-D</td>
<td>0.924 a</td>
<td>17.799 c</td>
<td>2.02 b</td>
</tr>
<tr>
<td>r = +0.511</td>
<td></td>
<td></td>
<td>r = +0.642</td>
</tr>
<tr>
<td>72 hr after treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.504 a</td>
<td>0.144 a</td>
<td>0.78 a</td>
</tr>
<tr>
<td>IAA</td>
<td>0.931 b</td>
<td>7.286 c</td>
<td>1.65 b</td>
</tr>
<tr>
<td>2,4-D</td>
<td>1.224 c</td>
<td>5.539 b</td>
<td>2.51 c</td>
</tr>
<tr>
<td>r = +0.779</td>
<td></td>
<td></td>
<td>r = +0.673</td>
</tr>
</tbody>
</table>

1 Any two means followed by the same letter are not significantly different at the 0.05 level as determined by the Duncan’s Multiple Range Test.
2 r: Correlation coefficient.
3 Significant at the 0.05 level.
This investigation was designed to evaluate the effect of varying tissue manganese levels and exogenous auxins on the in vivo synthesis of ethylene in cotton. In both approaches changes in the peroxidative IAA-oxidase system were followed. The results reflect on both the mechanism of ethylene synthesis and the physiological roles of peroxidase in the vegetative cotton plant.

Direct stimulation of in vivo ethylene synthesis by supraoptimal levels of tissue manganese was not indicated by this study. A correlation was noted, however, between internal ethylene concentration and the development of manganese toxicity lesions. That is, at the threshold level of tissue manganese at which severe manganese toxicity lesions began to appear in cotton, an increased internal ethylene concentration was simultaneously observed. These results have an interesting parallel in the work reported by Morgan et al. (31), which demonstrated a stimulation of IAA-oxidase activity at a threshold level of tissue manganese corresponding to the development of toxicity symptoms in cotton. Moreover, these investigators reported a direct relationship between degree of enzyme response and severity of visible symptoms. A similar relationship was observed in the present study with respect to both ethylene synthesis and IAA-oxidase activity. Correlations were also noted between IAA-oxidase activity and peroxidase activity and between peroxidase activity and internal ethylene concentration at tissue manganese levels causing severe toxicity symptoms. Since the initial significant increase in ethylene concentration observed in this study preceded that of both IAA-oxidase activity and peroxidase activity, there is no basis to conclude that there is a causal relationship.

The stimulation of both IAA-oxidase activity and peroxidase activity in cotton tissue by exogenous ethylene fumigation has been reported by others (8, 13, 29). However, none of these studies used ethylene levels as low as those which occurred in the high manganese plants in the present study. Thus, the evidence indicates that peroxidase activity did not increase before ethylene increases, and the change in ethylene levels was too small to assume without reservation that the ethylene produced the changes in peroxidase activity.

The highest level of ethylene occurring in manganese-treated plants (0.77 μl/liter, Table I) would be near the threshold necessary to inhibit auxin transport and hasten abscission of cotton cotyledons (5). Over a period of weeks this concentration of ethylene applied exogenously to cotton plants would inhibit growth by shortening the internodes and cause some leaf abscission (14). Thus, some of the long term effects of manganese toxicity observed after weeks of treatment (18, 31) may be due to both reduced auxin levels as a result of peroxidase activity and increased ethylene levels.

Both exogenously applied IAA and 2,4-D greatly enhanced ethylene synthesis in cotton, as has been reported by others (13, 27, 30). Peroxidase activity and IAA-oxidase activity were altered significantly by both these auxins at 72 hr after treatment, but no correlation was evident between either of these enzymatic activities and ethylene synthesis in the plants harvested at 8 or 25 hr after auxin application. The failure of both IAA-oxidase and peroxidase to increase due to auxin at 8 hr after treatment while ethylene concentration increased by over 30-fold in both treatments, plus the significant increase in peroxidase and IAA-oxidase activity in response to auxin application after 72 hr, implies that the change in the activity of enzyme(s) assayed may be the result of and not the cause of the accelerated ethylene production. Auxins, as well as ethylene, have been observed to increase peroxidase activity (9, 10, 17). Many responses to supraoptimal levels of auxins are now known to be actually a response to ethylene whose synthesis was modified by the exogenous auxins (2, 6, 7, 13, 27, 30, 32). The present stimulation of first internal ethylene levels and subsequently peroxidative IAA-oxidase activity may be another case of auxin acting through modified ethylene synthesis.

The stimulation of peroxidative IAA-oxidase activity only after a definite surge in ethylene synthesis observed in this study supports the conclusion that the processes are independent or that ethylene mediates this change in enzymatic activity. These results, in conjunction with those of the manganese study, strongly indicate that the level of peroxidase, IAA-oxidase, or both is not the rate-limiting factor in the in vivo ethylene-synthesizing system of cotton. If increased ethylene synthesis in cotton is dependent upon induction of a protein neither IAA-oxidase nor peroxidase appears to be that protein.

The very consistent, highly significant correlation between peroxidase activity and IAA-oxidase activity throughout these studies supports the assumption that the IAA-oxidase system in cotton is peroxidase based and, generally, measurement of one activity indicates the other (13, 28, 29). Increases in the quantity of peroxidases and the isozyme complement are characteristics of the normal aging process (9), and it may be useful to view the effects of manganese and auxins on the peroxidative IAA-oxidase system from this perspective.

FIG. 2. Relationship of peroxidase activity and IAA-oxidase activity of dialyzed cotton tissue extracts as influenced by foliar applications of IAA and 2,4-D. The correlation coefficient (r) of +0.948 is significant at the 0.01 level of probability.
PEROXIDASE AND ETHYLENE SYNTHESIS

LITERATURE CITED