

Changes in Indoleacetic Acid Oxidase Isoenzymes in Tobacco Tissues after Treatment with 2,4-Dichlorophenoxyacetic Acid¹

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ABSTRACT

2,4-Dichlorophenoxyacetic acid had a multiple effect on the development of indoleacetic acid oxidase isoenzymes in tobacco callus tissues (*Nicotiana tabacum*, cv. White Gold) cultured *in vitro*, and the development of these isoenzymes was differentially associated with growth promotion or inhibition depending on the concentration of 2,4-dichlorophenoxyacetic acid. At low concentrations (0.1 to 1 μM) it promoted the development of a fast migrating isoenzyme A_3 accompanied by stimulation of a tumor-type growth. At high concentrations (10 to 100 μM), it inhibited the development of the fast migrating isoenzymes but promoted a sharp rise in others with slower electrophoretic mobilities, which was accompanied by growth inhibition. The implications are that 2,4-dichlorophenoxyacetic acid might alter the level of endogenous auxins through its dual effects on the oxidase isoenzyme system.

MATERIALS AND METHODS

Tobacco callus tissue (*Nicotiana tabacum*, cv. White Gold) cultured *in vitro* on Linsmaier-Skoog medium (11) was used as the plant material. The level of kinetin was 0.2 μM , but the level of 2,4-D varied from 0.1 to 100 μM . Five pieces of tissue were grown in each 125-ml Erlenmeyer flask and five replicate flasks were used for each treatment. Each experiment was repeated two to four times. The cultures were grown for 20 to 25 days at 28 C under weak light (20 ft-c) on 16-hr day and 8-hr night cycles.

Enzyme Preparation. The procedures for enzyme extraction, dialysis, and subsequent separation by polyacrylamide gel electrophoresis were essentially the same as used previously (8). Because of the high enzyme activity in A_3 and A_5 fractions of certain treatments, these samples were further diluted before electrophoresis so that the aliquot used per gel was equivalent to 0.5 mg dry weight of tissue. After electrophoresis, each gel was sliced into 1-mm sections, and each section was extracted with 2 ml of phosphate buffer (0.03 M, pH 6) on a shaker at 4 C overnight.

IAA Oxidase Assay. The extract of each 1-mm gel slice was assayed for IAA oxidase activity by incubation with 0.3 mM IAA, 0.1 mM 2,4-dichlorophenol and 0.1 mM MnCl_2 in a total volume of 2.5 ml in a shaking water bath at 37 C for 30 min. The amount of IAA remaining was estimated with the modified Salkowski reagent (2) at 530 nm on a Unicam SP8000 recording spectrophotometer. The IAA oxidase activity is expressed as μg of IAA destroyed per mg dry weight of tissue at 37 C in 30 min. The amount of protein in the samples did not interfere with the color development because the samples applied to each gel contained only 6 to 20 μg of proteins, of which only a small fraction went into each 1-mm gel slice. The IAA oxidase activity was also determined directly by recording the changes in ultraviolet absorption during the course of the reaction with the same instrument.

Growth Determination. The growth of the tobacco tissue is expressed in terms of increase in soluble proteins, fresh weight and dry weight per callus. The amount of protein in the dialyzed extract after precipitation with 10% (w/v) trichloroacetic acid was estimated by the method of Lowry *et al.* (13).

RESULTS

Effect of Low Concentration of 2,4-D. The tobacco tissue grown with 0.2 μM kinetin but without auxin had four distinct IAA oxidase isoenzymes of slow and moderate electrophoretic mobilities, but there was little or no activity of the fast migrating isoenzyme A_5 . Addition of 1 μM 2,4-D promoted the development of this isoenzyme (Fig. 1). Although 2,4-D at 1 μM also increased the levels of A_1 and A_3 , the increase in A_5 was most significant. To test whether the increased isoenzyme A_5

It has been suggested that auxin-herbicides might act by changing the level of endogenous auxin (18). A lower level of endogenous auxin was found in dicotyledonous plants sprayed with 2,4-D than in the control (4, 12), but it was not known whether this decrease was due to inhibited auxin production or to increased destruction. In separate experiments, an increase in IAA oxidase activity was found in 2,4-D-treated plants (1). With the crude extract used, it was uncertain whether the increased IAA oxidase activity was due to stimulated enzyme synthesis or to changes in the level of inhibitors or activators.

Indoleacetic acid oxidase has a wide occurrence in plants. With a chemically defined tobacco tissue culture system, I found that IAA oxidase was composed of at least two groups of isoenzymes which had different responses to growth hormones and that the development of these isoenzymes was differentially associated with the promotion or inhibition of plant growth (8-10). Using this system as a model, I tested a group of herbicides with two main objectives: (a) to determine the effect of herbicides on the level of IAA oxidase; (b) to further test the IAA oxidase isoenzyme-growth relationships. In this paper I report a multiple effect of 2,4-D on the development of IAA oxidase isoenzymes, which was similar but not identical to that of IAA, and confirm the isoenzyme-growth relationships.

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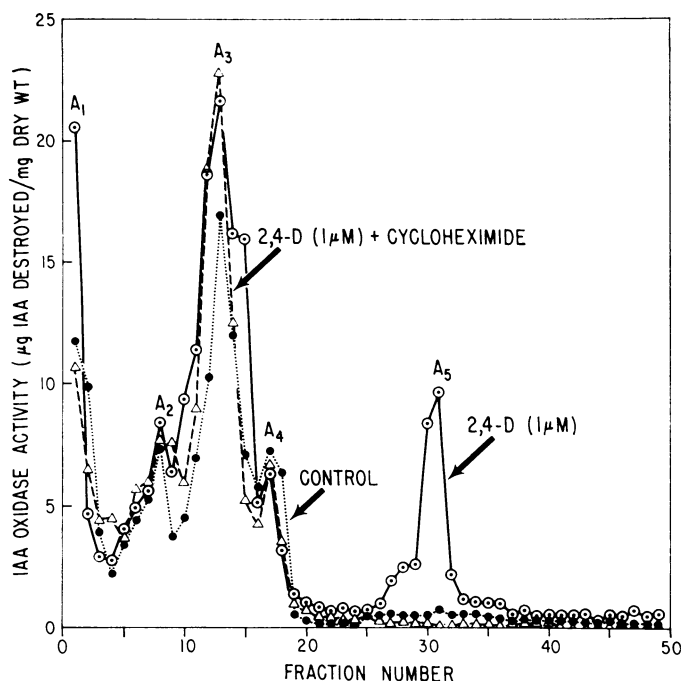


FIG. 1. Promotion of the fast migrating IAA oxidase isoenzyme A_5 by a low concentration of 2,4-D in tobacco callus tissues. The media contained $0.2 \mu\text{M}$ kinetin. The concentrations of 2,4-D and cycloheximide were $1 \mu\text{M}$ and 1 mg/l , respectively. The growth period was 25 days.

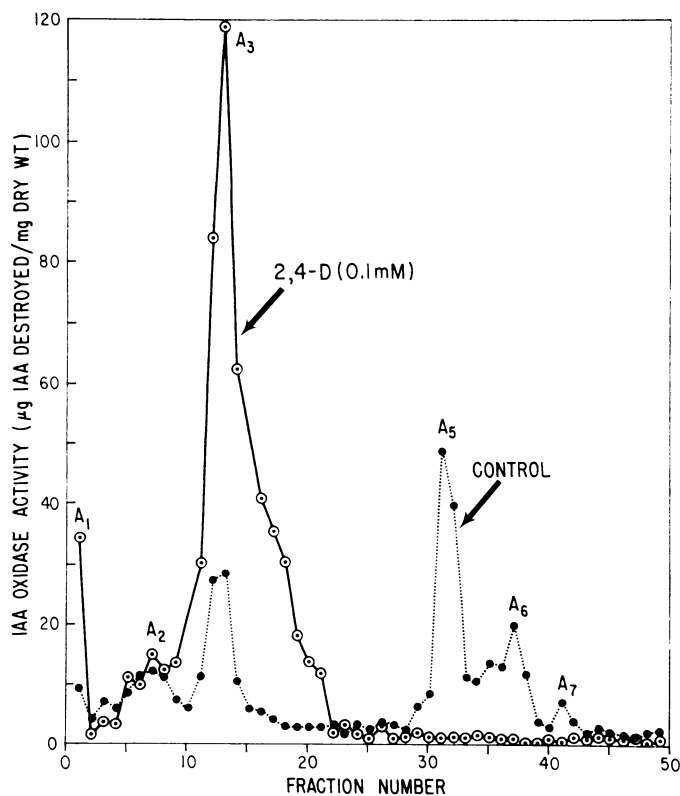


FIG. 2. Inhibition of the fast migrating IAA oxidase isoenzymes and promotion of the slow migrating isoenzymes by a high concentration of 2,4-D in tobacco callus tissues. The media for both 2,4-D treatment and control contained $2 \mu\text{M}$ GA, $10 \mu\text{M}$ IAA, and $0.2 \mu\text{M}$ kinetin. The concentration of 2,4-D was $100 \mu\text{M}$. The growth period was 24 days.

required protein synthesis, cycloheximide (1 mg/l) was added to the medium in the presence of $1 \mu\text{M}$ 2,4-D. As a result, the development of isoenzyme A_5 was inhibited (Fig. 1). A similar result was observed with actinomycin D (5 mg/l), suggesting a requirement for RNA synthesis.

Effect of High Concentration of 2,4-D. The promotion of the fast migrating IAA oxidase isoenzyme A_5 by 2,4-D was observed only at low concentrations. Increasing the 2,4-D level to $10 \mu\text{M}$ or higher inhibited the development of this isoenzyme. To test this inhibitory action, 2,4-D ($100 \mu\text{M}$) was added to a medium containing $10 \mu\text{M}$ IAA, $0.2 \mu\text{M}$ kinetin and $2 \mu\text{M}$ GA, a condition most favorable for the development of the fast migrating IAA oxidase isoenzymes (9). The results show that 2,4-D completely inhibited the development of A_5 , A_6 , and A_7 (Fig. 2).

In sharp contrast to the inhibition of the fast migrating isoenzymes, 2,4-D at $100 \mu\text{M}$ increased the level of other IAA oxidase isoenzymes of slower electrophoretic mobilities (Fig. 2). The most significant increase was in A_3 and possibly A_1 , which was not resolved from A_3 at the concentration used. The levels of these isoenzymes rose progressively with increasing concentrations of 2,4-D (Table I). With 2,4-D at $100 \mu\text{M}$, the IAA oxidase activity of A_3 was four times as high as that of the control without 2,4-D. Also the increase in A_1 in 2,4-D-treated tissue was more than 3-fold.

The enzymatic oxidation of IAA was also followed by recording the change in ultraviolet absorption every 10 min for 1 hr during the reaction and the spectral changes observed were in accordance with the results obtained by the colorimetric method.

Growth Response. Promotion or inhibition of growth by 2,4-D was dependent on the concentration (Table I). With 2,4-D at $0.1 \mu\text{M}$, the fresh weight of the tissue increased by 10-fold in 25 days, and the dry weight and the amount of soluble proteins increased by 7-fold. Increasing the 2,4-D level to $1 \mu\text{M}$ further increased the fresh weight yield, but the dry weight and the level of soluble proteins per callus remained unchanged. Consequently the protein level per unit fresh weight of tissue decreased. The tissue was soft and friable with an appearance of tumor-type growth. Interestingly, this tissue contained a higher level of IAA oxidase isoenzyme A_5 than that in any other tissue treated with higher or lower levels of 2,4-D (Table I).

Further increase of the 2,4-D level to $10 \mu\text{M}$ inhibited tissue

Table I. Responses of Plant Growth and IAA Oxidase Isoenzymes to Different Concentrations of 2,4-D

All media contained $0.2 \mu\text{M}$ kinetin. The growth period was 25 days.

2,4-D Concn	IAA Oxidase Isoenzymes ¹			Soluble Proteins		Tissue Fresh Wt	Tissue Dry Wt
	A_1	A_3	A_5	mg/ callus	mg/g fresh wt	g callus	mg/ callus
μM	$\mu\text{g IAA destroyed/mg dry wt}$						
0	13.5	36.2	1.7	0.4	2.3	0.17	21
0.1	10.5	25.2	10.0	2.8	1.8	1.56	142
1	18.0	30.2	14.9	2.6	0.6	4.02	148
10	24.5	75.9	2.5	0.8	1.1	0.74	44
100	50.9	262.9	1.5	0.5	3.3	0.15	16

¹ For isoenzyme A_3 , only the reading in the peak region of the isoenzyme profile is shown, because this fraction was not completely resolved. For A_1 and A_5 the enzyme activity represents the total of each isoenzyme fraction, which covered a region of 3 to 5 mm.

growth in terms of increase in soluble proteins and fresh and dry weights per callus. Accompanying these changes were an inhibition of the development of A_5 and a sharp rise in other IAA oxidase isoenzymes, notably A_3 . There was also an increase in soluble proteins per unit fresh weight of tissue. These changes were particularly evident when the concentration of 2,4-D was increased to 50 or 100 μM . Further evidence for the apparent association of growth with the development of IAA oxidase isoenzymes as affected by GA and 2,4-D is presented in Table II. Clearly, a shift in the isoenzyme distribution was accompanied by a drastic change in growth.

DISCUSSION

Evidently 2,4-D had a multiple effect on the development of IAA oxidase isoenzymes, but it differed from IAA, not only in the effective concentration, but also in the response of the isoenzymes. IAA at 10 μM promoted the development of both IAA oxidase isoenzymes A_5 and A_6 (10), but 2,4-D at this concentration was inhibitory. At low concentrations (0.1 to 1 μM), 2,4-D promoted IAA oxidase A_5 , but the level of this isoenzyme promoted by 2,4-D at an optimal concentration was not as high as that promoted by IAA. Furthermore, at these and other concentrations tested, 2,4-D did not promote IAA oxidase A_6 , which was usually present in the tissue grown with 10 μM IAA and 0.2 μM kinetin. At high concentrations (10–100 μM), 2,4-D completely inhibited the development of the fast migrating isoenzymes, but significantly increased those with slow electrophoretic mobilities. The extent of such inhibition and promotion was much greater with 2,4-D than IAA. Thus, when the isoenzyme level is considered, IAA was more effective than 2,4-D for promotion of the fast migrating IAA oxidases; but for promotion of the slow migrating IAA oxidases 2,4-D was more effective than IAA. When the effective concentration of the compound is considered, 2,4-D was more effective than IAA in both cases.

A causal relationship between the IAA oxidase isoenzymes and the promotion of tumor-type growth or the inhibition of normal growth has not been shown. A key question is whether or not the different isoenzymes can catalyze the oxidation of IAA through different pathways, thus forming intermediates and products with different growth regulating activities. This question is difficult to resolve since the course of enzymatic oxidation of IAA is highly dependent on the concentration of all reaction components and the pH of the medium (5) and since certain intermediates formed in the reaction are unstable during isolation (15). From the ultraviolet absorption spectra obtained from one simple *in vitro* test system, there appeared to be no substantial differences in the products formed in the reactions catalyzed by IAA oxidase isoenzymes A_3 and A_5 . The changes were similar to those reported by Ray (14) with enzymes of *Omphalia flavida* and by Hinman and Lang (5) with horseradish peroxidase.

From the pioneer work of Skoog and Miller (16), it is known that an auxin-cytokinin balance controls the pattern of plant growth. It has been suggested that a disturbance in this natural hormone balance is responsible for 2,4-D-induced abnormal growth (7, 17). In tobacco tissues the cytokinins do play a significant regulatory role in the development of the fast migrating IAA oxidases (8, 9). Thus, in this respect, it is likely that the cytokinins are involved in 2,4-D action.

The results from the experiments with cycloheximide and actinomycin D suggest that protein and RNA syntheses were required for the development of the fast migrating IAA oxidase A_5 in response to a low concentration of 2,4-D. This agrees with previous results which showed a requirement for both RNA and protein syntheses for the increase of fast migrating

Table II. Comparison of Growth of Tobacco Callus Under Conditions Favorable for Development of IAA Oxidase Isoenzymes of High or Low Electrophoretic Mobilities

All media contained 10 μM IAA and 2 μM GA. The growth period was 24 days.

Treatment		IAA Oxidase Activity ¹		Soluble Proteins		Tissue Fresh Wt	Tissue Dry Wt
Kinetin	2,4-D	Slow migrating	Fast migrating	mg/g fresh wt	mg/g dry wt	g/callus	mg/callus
μM		$\mu\text{g IAA destroyed/mg dry wt 30-min}$					
5.0	0	140	1	2.2	41.1	1.74	91
0.2	0	180	189	0.3	8.1	5.64	206
0.2	100	675	3	3.5	33.5	0.16	17

¹ The activity of slow migrating isoenzymes represents fractions 1 to 20, and the activity of fast migrating isoenzymes represents fractions 29 to 42.

IAA oxidase isoenzymes in response to cytokinin, GA, and IAA (8–10). Promotion of RNA and protein syntheses by 2,4-D has been well established (3, 6, 7, 19).

Protein synthesis might also be involved in the increase of the slow migrating IAA oxidases in response to high concentrations of 2,4-D. This is suggested by the partial inhibition of the activities of IAA oxidases A_1 and A_3 in the presence of cycloheximide (partly shown in Fig. 1) and by the increase in the incorporation of ³H-leucine in 2,4-D-treated tissues into a fraction which coincided with IAA oxidase isoenzyme A_3 (unpublished). However, in contrast to the complete inhibition of the fast migrating isoenzymes, cycloheximide had only a limited effect on the level of the slow migrating IAA oxidases. Perhaps this was due to a high stability of these isoenzymes pre-existing in the tissue at the onset of the experiment and a low level of protease because of the inhibitory action of cycloheximide. It is also possible that these isoenzymes were continuously formed despite the presence of cycloheximide at the 1 mg/l level.

The herbicidal action of 2,4-D has been related primarily to its hormonal effect on RNA and protein syntheses, followed by abnormal cellular proliferation and vascular disorganization, rather than to its direct interference with intermediary metabolism, respiration, or photosynthesis (3, 6). From the present work, the multiple effect of 2,4-D on IAA oxidase not only provides supporting evidence at a specific enzyme level but also suggests a role of 2,4-D in the regulation of endogenous auxin. Since growth promotion and inhibition were observed, it can be assumed that the basic biochemical functions of 2,4-D were operative. The dual effects on IAA oxidase isoenzymes might contribute to the herbicidal action.

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