Distribution of Indoleacetic Acid Oxidase and Inhibitors in Light-Grown Cotton [1, 2, 3]

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Extracts of green plant tissues exhibiting IAA-oxidase activity characteristically contain inhibitors of the enzyme (4, 11). The high concentration of such inhibitors prompted the discoverers of IAA-oxidase to suggest that the system does not function in green tissue (16). Later, Galston and Dalberg (6) found that in etiolated tissue the in vitro IAA-oxidase activity increases with age or IAA treatment. Similar studies of this enzyme in green tissue have been handicapped by the presence of the inhibitory substances. As will be pointed out in the discussion, the limiting effect of this handicap on other work has clearly left the adaptation and distribution of IAA-oxidase in green plants an open question. The recent finding that riboflavin and light will reduce or eliminate the effect of native inhibitors (11) has provided a method to survey the potential IAA destroying capacity of various green tissues and facilitated the present study.

Cotton contains an IAA-oxidase-inhibitor system (11). This paper presents evidence for an increase, with age, of IAA-oxidase activity in light-grown cotton tissues. The evidence is compatible with the in vivo function of the enzyme proposed by Galston and Dalberg (6).

Methods and Materials

The procedures used in this study have been described previously (8, 11); therefore, only a general description with emphasis upon the modifications is given here. In preliminary tests, both enzyme and inhibitor were observed to vary widely with plant age, variety, and seasonal growing conditions. For this reason, no effort was made to determine absolute enzyme and inhibitor values. That young leaves exhibited a higher activity than older leaves was considered an adequate demonstration of the distribution pattern of IAA-oxidase or inhibitor activity for any particular stage of development. Thus, activity from 1 tissue will be described as high or low relative to the same activity in younger or older tissue from the same plant. For convenience, basipetal gradients of enzyme and inhibitors will be referred to as increases or decreases with age, but there is no inference that aging (the passage of time) is the sole factor producing the gradients.

Plant Culture. Commercial varieties of upland cotton, *Gossypium hirsutum* L. (a few exceptions are noted), were used for the study and grown in a greenhouse as described previously (11).

Extraction and Purification. Each sample contained equivalent leaves or other tissue from several uniform plants. Separation of plant parts into age groups was done on the basis of location relative to the apex for primary leaves and on the basis of size (weight) for flower buds (squares), flowers and fruit (bolls). Fresh tissue was weighed, washed, and homogenized immediately with water in a blender or mortar in a 2° cold room. The brei was squeezed through cheese cloth and centrifuged (11) to produce a liquid hereafter termed extract.

One portion of the extract was assayed for IAA-oxidase activity immediately (usually diluted 10 or 20 times). A second aliquot was boiled to inactivate the endogenous enzyme, returned to volume, centrifuged, and stored at 0 to 5°. The heat-stable inhibitor (11) was assayed within 5 days. Preliminary tests had shown that inhibitor activity remained constant in the cold room for up to 23 days.

A third portion of the extract was dialyzed as previously described (11) and then assayed for IAA-oxidase activity on an equal volume or tissue basis. In addition, the nitrogen content of some of the dialyzed extracts were determined using a Coleman nitrogen analyzer. These extracts were diluted with water to allow assay at equal nitrogen concentrations within the linear limits of the assay system. The total IAA-oxidase activity was then calculated from the dilution factor.

In all cases the relative results from assays of crude extracts on a volume basis or dialyzed extracts on a volume or protein basis were the same; therefore, only 1 type of data will be presented for each experiment.

Inhibitor Extraction. To confirm the distribution pattern indicated by inhibitor activity in the extracts prepared primarily for enzyme assays, a second ex-

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traction procedure was used. Inhibitor was extracted by boiling washed, intact leaves with 5 ml of water per gram of fresh tissue for 20 minutes (5). The liquid was decanted, combined with 2 rinses of the residue, brought to the original volume, and assayed for IAA-oxidase inhibitor activity and total phenol content on a fresh weight basis.

IAA-Oxidase Assay. Assays were conducted in the light with 1×10^{-5} M riboflavin in the flask using the method previously described (11). This procedure was found to reduce or eliminate the lag in O_2 uptake caused by the native inhibitor (11). In some cases riboflavin reduced the rate of the reaction slightly (from 0 to 10 % depending on the enzyme concentration and light intensity), but if riboflavin was not added, the length of the lag often prevented a useful assay (11), especially with nondialyzed samples. The rate of the reaction in the presence of riboflavin was not effected significantly by the presence or absence of inhibitors (fig 7, ref 11). When tissue inhibitor levels were low (winter-grown tissue), some dialyzed extracts were diluted and assayed successfully in the dark without riboflavin. In these tests the relative results with or without riboflavin were the same. All assays were corrected by subtracting blanks determined under similar conditions and with all of the reagents except for the test enzyme.

Inhibitor Assay. Boiled extracts were assayed for relative inhibitor activity by measuring the lag induced when they were added to a standard cotton-leaf IAA-oxidase purified by acetone precipitation (7, 11). The same batch of enzyme was used for all assays of a given experiment, but not for all experiments. Therefore, the assay only gives relative inhibitor activity, and the data are not comparable from 1 experiment to another. Inhibitor activity, which varied from 1 experiment to the next, was either increased or decreased to give a lag that was measurable but not excessive. When inhibitor levels were high, extracts were diluted and assayed in the light with riboflavin. With very low inhibitor concentrations, samples were not diluted and assays were conducted in the dark without riboflavin.

Phenol Assay. The total phenol concentration of some of the boiled extracts was determined by the method of Swain and Hillis (15) and reported as μmoles of resorcinol based on a standard curve prepared with that material.

Results

Enzyme Occurrence. As one aspect of the present study a survey was completed in which IAA-oxidase activity was found in the following leaf extracts: *Gossypium stocksii* Mast., *G. harknessii* Brandg., *G. thurberi* Tod., *G. longicalyx* Hutch. et Lee, *G. sturdii* F. Muell., *G. armourianum* Kearney, *G. gossypioides* (Ulb.) Standley, *G. raimondii* Ulb., *G. arboreum* L., *G. barbadense* L., *G. klotzschianum* Anderss., *G. robinsonii* F. Muell., *G. nandewarense* Derera, and *G. klotzschianum* var. davidsonii (Kell.) Hutch., Silow et Steph. IAA-oxidase and inhibitor activity was previously demonstrated in 11 other species of cotton (11) which together with the present findings illustrate that the capacity to destroy IAA exists in extracts of leaves of all known species of Gossypium.

Table I. *Distribution of IAA-Oxidase Activity in Variety Deltapine TPSA Cotton Seedlings*

Plants 2 weeks old with 2 true leaves each were used. Data are averages of duplicate samples.

Tissue	Specific activity μl O_2 per min per μg N	Total activity μl O_2 per min per g tissue
Apical bud and leaf blades	0.17	124
Cotyledons (blades)	0.47	230
Stems and petioles	0.43	26

Enzyme Distribution. Representative data on in vitro IAA-oxidase activity are presented for cotton plants of several ages.

In seedlings, IAA-oxidase activity was greater in the cotyledons than in the younger true leaves (table I). The older cotyledons contained more activity on both a protein and tissue basis.

A pattern of enzyme activity similar to that observed in seedlings was also found in 37 day-old vegetative plants, (6 true leaves) in that IAA-oxidase activity increased from young to old leaves, and from the upper to the lower stem (fig 1).

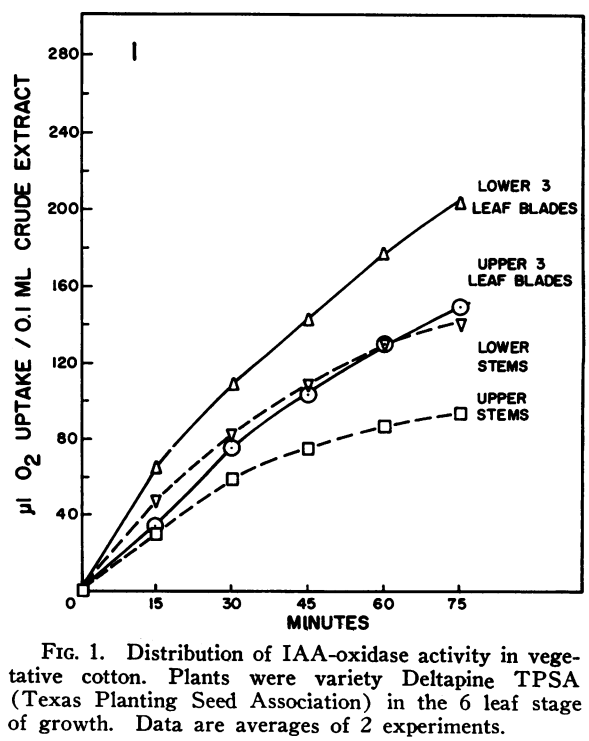

FIG. 1. Distribution of IAA-oxidase activity in vegetative cotton. Plants were variety Deltapine TPSA (Texas Planting Seed Association) in the 6 leaf stage of growth. Data are averages of 2 experiments.

Table II. *Distribution of IAA-Oxidase Activity in Flowering Cotton*

Doubled haploid (M-11) plants 47 days old with 10 to 12 true leaves each were used.

Tissue	Specific activity μl O_2 per min per 0.04 mg protein	Total activity μl O_2 per min per ml extract
Young leaves	2.9	9.4
Intermediate leaves (fully expanded)	2.0	48.1
Old leaves (fully expanded)	2.3	46.2

Total IAA-oxidase activity increased from young to old (fully expanded) leaves in flowering cotton plants (table II), and thus followed the pattern noted with younger cotton. In some experiments there were no significant differences in IAA-oxidase activity between fully expanded leaves differing in age or position on the plant (table II). On other occasions the activity gradient continued stepwise from the apex to the base of the plant. For example, in a test with flowering Deltapine 14 cotton where dialyzed extracts were compared on an equal tissue basis, the intial rate of O_2 uptake was 4.08, 5.07, 6.02, and 7.07 μl per minute for youngest, one half to just fully expanded, intermediate fully expanded, and oldest fully expanded leaves respectively.

IAA-oxidase activity was demonstrated in fruiting forms of cotton. With the flower and square, most of the activity was found in extracts from the peduncle, bracts, and calyx. Little or no enzyme activity was detected in the corolla, pistil, and stamen tissue.

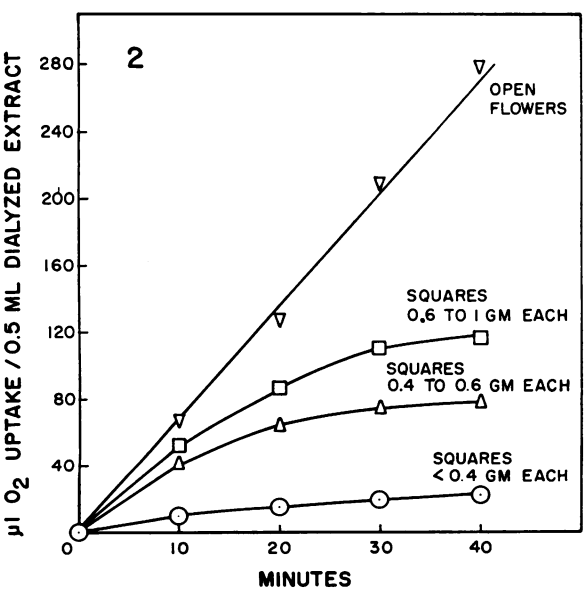

FIG. 2. Distribution of IAA-oxidase activity in cotton fruiting forms of various ages. Plants were variety Deltapine TPSA. The age of the fruiting forms increases progressively from the bottom to the top curve of the figure.

The boll contained significant IAA-oxidase activity in the peduncle, bracts, calyx, and green carpel walls, but no activity could be detected in tissue from the interior of the boll. Only the tissue shown to have IAA-oxidase activity was extracted in subsequent studies with fruiting forms. IAA-oxidase activity increased with age of the square, and the open flower contained much more enzyme activity than the squares (fig 2). An increase in IAA-oxidase activity with age of the boll was also observed in a separate experiment.

Inhibitor Distribution. IAA-oxidase activity increased with age of a given tissue. The reverse pattern, however, was observed with respect to the inhibitor. In leaves from seedling, vegetative, and flowering plants the highest inhibitor activity occurred in the apex and decreased basipetally (table III). When the inhibitor was extracted with boiling water, a similar decline in activity was noted from the apex to the base of the plant (table IV). Total phenol

Table III. *Distribution of IAA-Oxidase Inhibitor in the Cotton Plant*

Age and tissue	Length of lag period Minutes*
Seedlings**	
Apical bud and leaf blades	> 195
Cotyledons	18
Stems and petioles	4
Vegetative plants***	
Upper 3 leaf blades	74
Lower 3 leaf blades	35
Cotyledons	29
Flowering plants†	
Young leaves	166
Intermediate leaves (fully expanded)	51
Old leaves (fully expanded)	18

* Longer lag indicates higher inhibitor activity.
** Data from same plants as table I.
*** Data from same plants as figure 1.
† Data from same plants as table II.

Table IV. *Distribution of IAA-Oxidase Inhibitor Activity in Flowering Cotton*

Extraction of inhibitor from the variety Deltapine TPSA plants was with boiling water.

Leaf number Apex to base*	Length of lag period, Minutes	μmole phenol/ml as Resorcinol
1	> 135	11.0
2	> 135	5.5
3	76	3.0
4	42	2.2
5	29	1.9
6	37	2.1
7	29	1.8
8	27	2.0

* Sample designated leaf 1 included the apical bud and 1 leaf 12.7 mm in diameter from 6 plants. Leaf 2 was between 12.5 and 50 mm in diameter.

content followed inhibitor activity (table IV). As noted with enzyme activity, on some occasions there was (table III) and at other times there was not (table IV) a significant difference in inhibitor activity between fully expanded leaves which differed with respect to age or verticle position on the stem. The present data indicate that total capacity to destroy IAA may frequently increase with age of fully expanded leaves due either to increasing enzyme or decreasing inhibitor; however, the relationship of the IAA-oxidase system to leaf senescence will be the subject of further detailed investigations.

IAA-oxidase inhibitor activity decreased with increasing age of squares, and open flowers contained much less activity than squares 3 to 5 days younger. The same decline of inhibitor activity was demonstrated with different age groups of bolls. The bolls and squares were taken from different plants. The comparisons of both enzyme and inhibitor from fruiting forms of different ages illustrate a general trend, but are not considered detailed enough to define conditions during critical developmental stages of the square associated with high rates of shedding.

Field-Grown Cotton. The distribution patterns noted for in vitro IAA-oxidase and inhibitor activity from greenhouse plants were verified with leaf tissue from field-grown cotton in the flowering stage.

Glandless Cotton. Pigment glands, present on cotton leaves, stems and fruiting forms, contain gossypol, which has IAA-oxidase inhibitor activity (11). The glands could contain other inhibitors of the enzyme. Therefore, the observed distribution pattern of the inhibitor could be related to the content, structural integrity or other condition of the glands and not be related to the cytoplasmic IAA-oxidase system. To investigate this possibility, the distribution of the IAA-oxidase inhibitor was determined using glandless cotton (Acala 4–42–77, B.C. 2, F_6 and Acala 4–42–77, B.C. 4, F_4) and the boiling water extraction technique. The inhibitor activity of both glandless lines was found to follow the same pattern as that obtained with the gland containing cotton.

Other Species. An obvious question is whether the IAA-oxidase distribution pattern observed here is unique to Gossypium. In tests in our laboratory, a similar gradient of enzyme and inhibitor was noted in leaves of peach seedlings (*Prunus persica* Batsch) and rhubarb (*Rheum rhaponticum* L.). IAA-oxidase activity was found to increase significantly from leaflets in the apical bud to the older leaflets of the Alaska pea (*Pisum sativum* L.). For the latter test greenhouse-grown, 14 day-old seedlings were used which had 4 leaves and a terminal bud. In an additional test with 7 day-old pea seedlings, a significant stepwise increase in IAA-oxidase activity with age was noted in leaflets. Assays of the latter group of extracts were conducted in the dark without riboflavin.

Discussion

The series of assays used here gives a measure of IAA-oxidase and inhibitor activities independent of one another and therefore reflects the true in vitro activity of both enzyme and inhibitor. The design of these assays should allow confidence in interpretation of the data which is not warranted when enzyme preparations containing native inhibitors are assayed with no provision made to inactivate or remove completely the inhibitors. In such cases, the absence of enzyme activity can either be due to low enzyme or high inhibitor or both. To date, the use of dialysis to remove inhibitors appears to have been most successfully applied to tissues with low inhibitor content such as stems.

Data are presented here which illustrate that in vitro IAA-oxidase activity increases from the apex to the base of the cotton plant at several stages of its development and that the inhibitor or inhibitors of this enzyme decrease in activity from the apex to the base of the plant. Similar results for both substances were obtained when small flower buds or fruits were compared to larger flower buds or fruits from the same plants. The findings with leaves, buds, and fruits also illustrate a relative increase in IAA-oxidase activity and a relative decrease in inhibitor activity with age. For example, the apical leaf of a young cotton plant has lower IAA-oxidase activity than the older leaves or cotyledons below it, but with the passing of time, the same leaf would have higher enzyme activity than the younger leaves above it.

The inverse correlation of IAA-oxidase and inhibitor activity in the green cotton plant has several implications to the physiological significance of both the enzyme and inhibitor system in light-grown plants. Previously, Galston and Dalberg (6) found that with inhibitor free, etiolated pea tissue in vitro IAA-oxidase activity increases with age or incubation with IAA. However, the adaptive formation of the enzyme in green plants remained in doubt due to the high concentration of inhibitors in the light-grown pea (7, 16). Presumably, the high concentration of inhibitors would completely inactivate the IAA-oxidase and thereby prevent both functioning and adaptation of the enzyme. Indeed, IAA-oxidase activity appeared to decline rather than increase with age in the light-grown pea stem, and no enzyme activity was detected in leaves (5). The absence of activity in leaves was suggested to either be due to low enzyme, high inhibitor, or both. Enzyme activity and distribution pattern in the pea stem varied with the size of the aliquot assayed. The variation was suggested to be due to the presence of inhibitors in the preparations assayed.

In the case of the inhibitor, a gradient of activity in leaves and stems which declined from apex to base was clearly demonstrated in peas grown under artificial light (5) which agrees with the present findings in cotton. Neither of these studies are in conflict with the observation by Sagi and Garay (13) that IAA-oxidase inhibitor (phenol) content of leaves of *Lupinus albus* increases with age. In the present studies and those of Galston (5), the decrease in inhibitor activity with age refers only to comparisons

between leaves on a given plant and not between plants of different ages. It is possible that the absolute concentration of inhibitor could increase in a given leaf with age while the concentration relative to that of other leaves could decline in the same leaf. In such a case the relative change could be of physiological significance.

Auxin concentrations are generally believed to decline from the apex to the base of the plant (3, 12) and this distribution pattern could be partially explained by its inverse correlation with the pattern of IAA-oxidase activity demonstrated here in cotton. The decline of auxin activity with aging of leaves (14) could also be due in part to the relative increase in IAA-oxidase with age or basipetally. In cotton, a similar decline in detectable auxin was noted just prior to leaf fall (1, 2). On the basis of our tests, an increasing basipetal gradient of IAA-oxidase appears to occur in species other than Gossypium; therefore, such gradients could have general physiological significance.

Several correlations are apparent from the present findings. First, activity of the enzyme which destroys auxin in vitro is inversely correlated with the expected pattern of auxin activity. Secondly, the inhibitor, which normally blocks activity of the enzyme and thereby would protect auxin, is present in a pattern directly related to expected auxin levels and inversely related to IAA-oxidase levels. These observations suggest that IAA-oxidase, regulated by an inhibitor system, may function in the control of auxin-mediated processes in cotton. Such a hypothesis is supported by other types of indirect evidence in cotton including: the universal occurrence of the enzyme in Gossypium (11, present paper), the correlation between ethylene-induced stimulation of both abscission and IAA-oxidase activity (8, 10) and the correlation between symptoms of manganese toxicity and abnormally high IAA-oxidase activity in young leaves of cotton (9). In the latter study, the leaves showing toxic symptoms contained sufficient manganese to oxidize IAA in vitro without the addition of $MnCl_2$ which is normally required (11).

The proposed physiological role of IAA-oxidase in cotton suggested from indirect evidence presented and reviewed here supports the earlier proposal by Galston and Dalberg (6) that IAA-oxidase may function in aging, lateral bud inhibition, interactions of auxins, and exogenous rhythms involving auxin. On the other hand, the patterns reported here may also be viewed as gradients of peroxidase activity and phenol content. Thus the possibility exists that the significance of the present findings involves functions in addition to auxin destruction.

Summary

The distribution of an indoleacetic acid-oxidase-inhibitor system was studied in cotton (*Gossypium hirsutum* L.) grown in a greenhouse. In vitro activity of the enzyme increased in leaves and stems from apex to base of plants in several stages of development. Inversely, inhibitor activity was highest in the apex and declined basipetally. The activity patterns of both substances were also demonstrated with respect to age in fruiting forms. The observations on distribution of enzyme and inhibitor in leaves of greenhouse-grown plants were verified using plants grown in the field. The findings are compatible with a hypothesis that indoleacetic acid-oxidase functions in vivo in cotton to help regulate auxin levels and that the activity of the enzyme is controlled by an inhibitor system.

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Photosynthesis in Climatic Races of Mimulus
II. Effect of Time and CO_2 Concentration on Rate [1]

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Field observations, transplant experiments, and genetic studies have long been used in attempts to elucidate the mechanism of natural selection in the evolution of climatic races of plants. In the preceding paper (5) the possible contribution of physiological measurements to an understanding of the problem was discussed. It was found that the photosynthetic rates of 6 climatic races of *Mimulus cardinalis* respond differently to changes in temperature and light intensity. The differences in response appear to be related to the natural conditions prevailing in the diverse native habitats.

We proceeded then to test the ability of the same races to maintain a high rate of photosynthesis under constant favorable conditions during a 12-hour period. We are not aware of any other work in which the photosynthetic rate has been measured over an extended time at the temperature and light intensity that produce maximum photosynthesis. Such measurements not only have intrinsic physiological interest, but they also offer another means to test for differences of response by climatic races.

Continuing the search for physiological responses that may characterize climatic races of *Mimulus*, we measured the effect of CO_2 concentration on their photosynthetic rates for short and for extended times.

Materials and Methods

The experimental plants were duplicate clone members of the same climatic races of *Mimulus cardinalis* Dougl. that were used in the earlier work. The place names and elevations of the native habitats of the races are: Race 1, Los Trancos, 45 m; 2, Jacksonville, 240 m; 3, Priest Grade, 400 m; 4, Baja California, 550 m; 5, Yosemite, 1220 m; 6, San Antonio Peak, 2220 m (5, table I).

The apparatus and method for measuring photosynthesis were the same as previously described. In prolonged experiments the desired light intensity and temperature were kept constant while the photosynthetic rate was determined each 5 minutes. At first the rate increased with each measurement. The 12-hour period began with the first of 3 or more identical rate measurements.

Unless otherwise noted, all rates reported here are at the temperature for maximum photosynthesis and the saturating light intensity found previously for each clone. Separate experiments showed that within the experimental error the light intensity required to saturate photosynthesis in *Mimulus* and the temperature causing the maximum rate do not differ materially at 0.150 and 0.0425 % CO_2. This may or may not be true for other plants. Both the independence (8) and dependence (2) of these values on CO_2 concentration are reported for crop plants.

In rate measurements over a range of CO_2 concentrations the procedure differed from that used at one concentration. After setting the highest CO_2 level to be used, the leaf chamber was closed and photosynthesis was allowed to proceed until CO_2 was reduced to the compensation point, usually 60 to 90 minutes. The continuous recorder trace permits com-

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