IAA Oxidase Inhibitors From Normal and Mutant Maize Plants

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Abstract. Extracts of maize (Zea mays L.) plants contain substances which, in vitro, inhibit an indoleacetic acid (IAA) oxidase enzyme from maize. The extracts can be freed of inhibitors by dialysis or by passage through columns of polyvinylpyrrolidone powder. Inhibitor-free extracts contain an IAA oxidase enzyme which requires a phenolic co-factor and is stimulated by Mn2++.

IAA oxidase inhibitor and total phenol levels were compared for normal maize and for the maize mutant Knotted (Kn). In plants up to 18 days old the level of heat stable, water soluble IAA oxidase inhibitors increases with increasing dosage of the Kn allele. Increased inhibitor content is accompanied, but not paralleled, by increased content of total phenols. Although several inhibitors are present in crude extracts, most inhibition can be attributed to 1 compound. This compound is not destroyed by horseradish peroxidase in the absence of IAA. At pH 3.5 it is not extracted into ether, but it is rendered ether-extractable by incubation in 2 N KOH for 5 hr at room temperature. This compound is tentatively identified as an ester of ferulic acid and some unknown moiety.

A basic goal of biological research is the elucidation of mechanisms which enable information encoded in DNA to determine the morphology of an organism. One approach to this problem is through the use of single-gene mutations altering morphology (13,14, 15,17,20). The altered morphology of affected organisms results from a limited number of biochemical events. Biochemical comparison of normal and mutant plants should reveal points of difference and thus provide information concerning the molecular basis for morphogenesis. However, a serious impediment is inherent in this approach. Most morphological mutants provide no clue concerning the underlying biochemical lesions. Consequently, the utility of such mutants in the study of morphogenesis is limited by the lack of any clear indication of where to begin biochemical studies. The Knotted mutant of maize is unusual in that it provides an obvious biochemical difference, anthocyanin production, associated with the morphological abnormality.

The Knotted mutant develops hollow, finger-like outgrowths (knots) on the leaf blade (4,7,16). The Kn allele is dominant, but shows dosage effects (7,16). Kn/Kn plants are shorter and more severely knotted than Kn/+ plants. Knotted plants genetically capable of forming anthocyanin accumulate pigment in the knots, but nowhere else in the blade. Anthocyanin per se has no causal relation to knot formation. Kn/Kn and Kn/+ plants genetically unable to produce anthocyanin do produce knots. Anthocyanin formation, in a sense, a bioassay for some condition uniquely associated with knotted tissue in the leaf blade. Furthermore, this condition must obtain in knotted tissue regardless of the plant's ability to produce anthocyanin.

Anthocyanin formation is triggered by a wide variety of conditions (3). Consequently, its presence in knots may provide no useful clue concerning biochemical lesions associated with knot development. On the other hand, 2 observations suggest that anthocyanin may provide a useful clue. First, knots result from abnormal cell division and elongation (7). Second, the anthocyanins are biochemically related to substituted phenols, compounds known to affect growth (21,22,23,24). A disturbance in the metabolism of phenolic compounds might result in both abnormal growth and anthocyanin production. Since growth-regulating activity of substituted phenols results at least in part from their effect on IAA oxidase (23), IAA oxidase inhibitor levels were compared for normal and mutant plants.

Materials and Methods

Knotted seed of Zea mays L. obtained from the maize genetics cooperative was grown in the field, where appropriate crosses were made to produce seed known to be +/-, +/-/Kn, or Kn/Kn, all in a W23/L31F background. With the exception of 1 group maintained in a controlled climate chamber, plants were grown in a greenhouse in ordinary soil and with water as necessary. A 16 hr photo-

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period was maintained by a combination of natural and artificial lighting. Temperature was usually within the range of 24° to 30°, but on occasion reached 38°. Because of these large fluctuations in temperature comparisons were made only among plants of the same age grown simultaneously, thereby assuring that all 3 genotypes were exposed to identical conditions. All data reported here are derived from plants genetically unable to produce anthocyanin. Only a limited number of samples could be examined because the plants were segregating 3 colored to 1 green. Use of anthocyanin-producing plants could cause complications at the biochemical level as a result of the close relation between anthocyanins and phenolic acids.

**Preparation of IAA Oxidase.** Leaves of 11 day old +/+ plants were homogenized in cold 0.1 M sodium phosphate buffer, pH 6.0 (5 ml per g fresh wt of tissue) in an ice-jacketed Virtis 45 homogenizer. The homogenate was filtered through 4 layers of cheesecloth, then centrifuged 20 min in the cold at 16,000g. Portions (10 ml) of the supernatant fluid were either dialyzed for 3 days at 4° against 5 changes of deionized water, or filtered through 2 successive 1.0 × 5.0 cm columns of polyvinylpyrroldione powder (Polyclar AT) (11). Two 5 cm columns were used rather than one 10-cm column in order to improve flow rate. The filtering operation, which requires about 1 hr with vacuum assistance, was carried out at 4°. The extracts subjected to dialysis or Polyclar AT filtration were used as a source of IAA oxidase. Protein measurements were made on trichloroacetic acid precipitates by the method of Lowry et al. (12).

**IAA Oxidase Inhibitors.** Leaves were homogenized in cold 0.1 M sodium phosphate buffer, pH 6.0 (5 ml per g fresh wt of tissue) in an ice-jacketed Virtis 45 homogenizer. The homogenate was filtered through 4 layers of cheesecloth, then centrifuged 20 min in the cold at 16,000g. Portions of the supernatant fluid (10 ml) were heated for 10 min in boiling water. The extracts were cleared by centrifugation at 16,000g and the clear, yellow supernatant fluid used as a source of IAA oxidase inhibitor.

**Assay for IAA Oxidase and IAA Oxidase Inhibitors.** The reaction mixture contained 0.5 ml IAA oxidase enzyme preparation, 0.2 mM IAA, 0.1 M sodium phosphate buffer, pH 6.0, and, except as indicated, 0.1 mM dichlorophenol^a^ (DCP) and 0.1 mM MnCl₂, in a final volume of 10 ml. The same reaction mixture was used to measure IAA oxidase inhibitors, except that A) horseradish peroxidase, (11 μg Sigma type 2, RZ 14) was substituted for IAA oxidase from maize; B) 2 ml of a 1 to 10 dilution of inhibitor preparation were added; C) the final volume was 20 ml. The destruction of IAA was checked at 10 min intervals by adding 1.0 ml portions of the reaction mixture to 2.0 ml of the Gordon and Weber (8) modification of the Salkowski reagent. The tubes were allowed to stand in the dark for approximately 1 hr before recording the percent transmittance at 525 nm. For most experiments the percent transmittance data were plotted without modification, although for a few experiments these data were converted to μg of IAA destroyed.

**Phenol Estimation.** Total phenol content of the IAA oxidase inhibitor preparations was estimated by mixing 0.1 ml of undiluted inhibitor preparation with 5 ml of the Lowry reagent (12). The tubes were allowed to stand for 45 min at room temperature, then 0.5 ml of the Folin phenol reagent (12) was added. The tubes were allowed to stand for another 15 min, then were diluted with deionized water to 10 ml total volume. The percent transmittance of 750 nm was recorded and converted, by means of a standard curve, to μg equivalents of p-coumaric acid. Phenol estimation on chromatograms was accomplished by shaking the sections in 5 ml of the Lowry reagent at 30° for 1 hr, then adding the Folin phenol reagent and proceeding as described above. Since most of the protein was precipitated from the inhibitor extracts by heating, the phenol results should represent only non-protein phenols.

**Results**

**IAA Oxidase.** No IAA oxidase activity could be demonstrated in maize extracts prior to dialysis or Polyclar treatment (Fig. 1). After either treatment the extracts showed IAA oxidase activity. DCP and Mn²⁺ were required for full activity (Fig. 1). Polyclar AT removed the inhibitor at least as well as dialysis for 3 days against 2 l of deionized water (changed 5 times) (Fig. 1).

**IAA Oxidase Inhibitors.** Extracts of maize shoots contained a heat-stable, water soluble IAA oxidase inhibitor of low molecular weight. In the presence of HRP, IAA, and cofactors, the inhibitor caused a lag prior to the destruction of IAA (Fig. 2). Destruction of the inhibitor occurred only in the presence of both HRP and IAA. A 40 min incubation of the inhibitor in a reaction mixture lacking only IAA produced no reduction in inhibitor capacity. Subsequent addition of IAA led to IAA destruction after a lag period of approximately 40 min, indicating that the inhibitor had retained full activity (Fig. 2).

The relationship between the duration of the lag period and the inhibitor concentration was elucidated using 8 concentrations (from 0.5 ml per 20 ml total volume to 4.0 ml per 20 ml volume, increasing in steps of 0.5 ml) of the same inhibitor preparation.

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^a^ The abbreviations used are: DCP, dichlorophenol; HRP, horseradish peroxidase; BAW, butanol, acetic acid, water (6:1:2 v/v).
This series bracketed the concentration used in subsequent experiments (2.0 ml per 20 ml total volume).

Measurements of IAA concentration in the reaction mixtures were made at 10 min intervals. Sampling was started at 0 min for the control and for inhibitor concentrations 0.5 ml to 2.0 ml. For inhibitor concentrations 2.5 ml to 4.0 ml sampling was begun only when IAA destruction was complete at the preceding inhibitor concentration. Plotting increase in percent transmittance concentration by visually extending the linear portion of the curves (Fig. 3) to 10% transmittance (no destruction of IAA) The point at which the extrapolated curve intersects the abcissa represents the total lag period. This value, minus the lag of the control, equals the inhibitor-induced lag. Plotting the log_{10} of the inhibitor-induced lag for each inhibitor concentration against the log_{10} of that inhibitor concentration in units yields a straight line (Fig. 4). The definition of an inhibitor unit is arbitrary, 0.5 ml of the extract employed being said to contain 5 units of inhibitor. The inhibitor concentration of any extract can be quantitatively expressed by measuring under standard conditions the inhibitor-induced lag period and, using Fig. 4 as a standard curve, converting this value to standard inhibitor units. The results of applying this measurement technique to inhibitor extracts of normal

![Figure 1](https://via.placeholder.com/150)
**Fig. 1.** Characteristics of IAA oxidase prepared from maize extracts. 1) Enzyme preparation run through 2 small columns of Polyclar AT. Complete reaction mixture contains IAA, Mn^{2+}, DCP, 0.1 M phosphate buffer, pH 6.0. 2) Enzyme preparation dialyzed 3 days, reaction mixture lacking Mn^{2+}. 3) Enzyme preparation dialyzed 3 days, reaction mixture complete. 4) Enzyme preparation dialyzed 3 days, reaction mixture lacking DCP. 5) Enzyme preparation dialyzed 3 days, reaction mixture lacking DCP and Mn^{2+}. 6) Untreated enzyme preparation, reaction mixture complete.

![Figure 2](https://via.placeholder.com/150)
**Fig. 2.** Demonstration that IAA is required for enzyme catalyzed destruction of inhibitor. 1) Control, containing IAA, Mn^{2+}, DCP, HRP, and phosphate buffer, pH 6.0, but lacking inhibitor. 2) Complete reaction mixture plus inhibitor, producing a 40-min lag period. 3) Reaction mixture lacking IAA until 40 min (arrow indicates addition of IAA) but containing inhibitor and HRP from time 0.

![Figure 3](https://via.placeholder.com/150)
**Fig. 3.** Relationship between inhibitor concentration and duration of the lag period. All reactions conducted with a complete reaction mixture (see Materials and Methods). 1) Control, no inhibitor. 2) 0.5 ml inhibitor. 3) 1.0 ml inhibitor. 4) 1.5 ml inhibitor. 5) 2.0 ml inhibitor. 6) 2.5 ml inhibitor. 7) 3.0 ml inhibitor. 8) 3.5 inhibitor. 9) 4.0 ml inhibitor. Curve 7 has been extrapolated to 10% transmittance (no destruction of IAA).

![Figure 4](https://via.placeholder.com/150)
**Fig. 4.** Relationship between inhibitor concentration in arbitrary units and duration of the lag period. One unit of inhibitor equals that amount of inhibitor present in 0.1 ml of the extract employed in Fig. 7.
and Knotted plants are shown in Fig. 5. Knotted plants consistently had higher levels of IAA oxidase inhibitors than did normal plants of the same age. The increased inhibitor levels in Knotted plants were demonstrable prior to morphological expression of the Kn allele. Furthermore, from day 11 through at least day 18 the concentration of inhibitor increased with increasing dosage of the Kn allele. In order to confirm this result seeds of each genotype were germinated and grown for 14 days in a controlled climate chamber at 30° and with a 16 hr photoperiod. Plants of each genotype were divided into 2 groups, each group being homogenized and assayed independently. The results confirm that increasing dosage of the Kn allele correlates with increasing inhibitor concentrations (Table I). This relationship holds regardless of the basis for comparison: per g fresh weight; per mg protein; or per whole shoot. When inhibitor concentrations were compared for normal and Knotted plants producing anthocyanin, the inhibitor level of normal plants sometimes exceeded that of Knotted plants of the same age (unpublished data).

**Total Phenol.** Portions of the extracts used for inhibitor measurements were assayed for total phenols. The results, expressed as μg equivalents of p-coumaric acid, revealed that Knotted plants consistently had higher levels of total phenols than did normal plants (Fig. 6). The phenol concentration did not parallel the inhibitor concentration, however. At 14 days the phenol concentration of Kn/Kn plants was higher than at 11 days (Fig. 6), but the inhibitor concentration was lower than at 11 days (Fig. 5). Comparison of the position of phenols with the position of inhibitors on paper chromatograms of inhibitor extracts revealed a close correspondence (Fig. 7).

**Nature of Inhibitor.** Adjusting an inhibitor extract to pH 3.5 and partitioning with ether did not remove the inhibitor from the aqueous phase. Treating an inhibitor preparation with 2 N KOH for 5 hr at room temperature, followed by neutralization with HClO₄, removal of KClO₄, and readjustment by evaporation to the original volume, caused only a negligible loss of inhibitor activity (unpub-

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**Fig. 5.** Comparison of inhibitor content (units per g fresh wt) in Kn/Kn (1), Kn/+ (2), and +/+ (3) plants at various ages.

**Fig. 6.** Comparison of total phenol content (mg equivalents of p-coumaric acid per g fresh wt) in Kn/Kn (1), Kn/+ (2), and +/+ (3) plants at various ages.

**Fig. 7.** Comparison of location of inhibitors (top) and total phenols (bottom) on paper chromatograms of inhibitor extract. Horizontal lines represent controls (segments of chromatogram from above the spotting line). Whatmann No. 3MM paper developed in 15% acetic acid.

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**Table 1. Inhibitor Content of 14-day-old Plants Grown Under a Constant Temperature of 30° and a Photoperiod of 16 Hours**

<table>
<thead>
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<th>Sample</th>
<th>Units of Inhibition per:</th>
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<tbody>
<tr>
<td></td>
<td>g Fresh wt</td>
<td>mg Protein</td>
</tr>
<tr>
<td>Kn/Kn</td>
<td>329</td>
<td>8.9</td>
</tr>
<tr>
<td>Kn/Kn</td>
<td>327</td>
<td>7.7</td>
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<tr>
<td>Kn/+</td>
<td>177</td>
<td>5.3</td>
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<tr>
<td>Kn/+</td>
<td>155</td>
<td>5.8</td>
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<tr>
<td>+/+</td>
<td>109</td>
<td>3.7</td>
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<tr>
<td>+/+</td>
<td>107</td>
<td>3.7</td>
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lished data). However, if the preparation was then adjusted to pH 3.5 and partitioned with ether, little inhibitory activity remained in the aqueous phase (Fig. 8).

Untreated inhibitor preparations contained a compound with a blue fluorescence (becoming more intense upon exposure to ammonia vapors) on thin layer chromatograms irradiated at 254 nm. This compound was present in the aqueous phase of preparations previously partitioned with ether, but was absent from the aqueous phase of KOH-treated extracts partitioned with ether.

Paper chromatograms (Whatman No. 3 MM paper) of untreated inhibitor extracts developed in BAW had, when irradiated at 254 nm, a blue fluorescing compound with an RF of 0.16. Chromatograms were cut into sections (2 cm × 1 inch) and each section was tested for IAA oxidase inhibitor activity. Although some inhibitory activity was associated with several sections, the section corresponding to RF 0.13 to 0.19 (bearing the blue fluorescing compound) carried the major inhibitor (Fig. 9).

Purification of the major inhibitor was accomplished by streaking 7 ml of concentrated inhibitor extract across 3 sheets of Whatman No. 3 MM paper and developing in BAW. The band containing the blue fluorescing compound was cut out and the compound eluted by rotating the chromatogram pieces in 250 ml of 20% ethanol in a rotary evaporator without vacuum. The filtered, concentrated eluate (about 3 ml) was applied to 1 sheet of Whatman No. 3 MM paper and developed in 15% acetic acid. The blue fluorescing compound (traveling near the solvent front) was again eluted and concentrated as before. A small amount of gummy brown precipitate formed upon the addition of several volumes of absolute ethanol to the concentrated eluate (about 2 ml). An aqueous solution of the precipitate had IAA oxidase inhibitor activity and yielded a single (blue fluorescing) compound upon chromatography on Whatman No. 1 paper developed in BAW, although there was some streaking. Incubation of a portion of the precipitate with 2 N NaOH for 5 hr at room temperature resulted in disappearance of the original blue fluorescing compound and the appearance of a new blue fluorescing compound traveling near the solvent front in BAW and having the same RF as an authentic sample of ferulic acid chromatographed on the same paper. Upon spraying the chromatogram with diazotized p-nitroaniline solution (2), both ferulic acid and the hydrolysis product gave a pink color.

The absorption spectrum of the unhydrolyzed inhibitor (eluted from a chromatogram developed in BAW) resembled that of ferulic acid (Fig. 10). The unhydrolyzed inhibitor underwent a bathochromic shift of 47 nm upon the addition of 2 drops of 2 N NaOH, greater than the alkaline shift for free ferulic acid (30 nm), but less than those reported for glucose esters of hydroxycinnamic acids (50-60 nm) (18). Esterified ferulic acid has been identified as a modifier of IAA oxidase activity in pineapple (19).

**Discussion**

Increased levels of IAA oxidase inhibitors would be of little significance to development unless the plants do in fact contain an IAA oxidase enzyme.
The enzyme found in maize is similar to IAA oxidase enzymes from other sources in that it requires a phenolic cofactor and is stimulated by Mn$^{2+}$ (6). Furthermore, the enzyme is inhibited by dialyzable, heat-stable, compounds present in extracts of maize shoots.

Experiments to measure the IAA oxidase inhibitor content of shoot extracts were performed with HRP rather than IAA oxidase prepared from maize plants. This substitution permitted accurate control of the amount of enzyme present and avoided problems which could accompany the use of maize enzyme preparations, e.g., different degrees of inhibitor removal in different preparations, possible qualitative differences in enzyme among preparations. It is recognized, however, that HRP may not be an exact substitute for maize IAA oxidase (19). Consequently, conclusions reached on the basis of results with HRP may not apply in toto to the IAA oxidase from maize.

The results confirm that an increase in IAA oxidase inhibitors is associated with the mutant allele. Most of this inhibition can be attributed to a compound tentatively identified as an ester of ferulic acid. An increase in total phenols accompanies the increase in IAA oxidase in mutant plants. The phenol content does not exactly parallel the inhibitor content, particularly for days 11 and 14, suggesting that the Kn allele causes an increase in several phenols, only a few of which have IAA oxidase inhibitor activity. In older plants, days 18 and 22, the phenol content does reflect the inhibitor content quite closely. As the plants age qualitative changes may occur in the phenolic compounds synthesized. New phenolic compounds with inhibitor activity may be synthesized in older plants, accounting for the close parallel between total phenols and inhibitor activity. Alternatively, phenols lacking IAA oxidase inhibitor activity may decrease to an insignificant fraction of the total phenol content of older plants. Between days 18 and 22 Kn/+ plants surpass Kn/Kn plants in both phenol and inhibitor content. These results are based upon 1 sampling of each genotype. It is possible that the plants comprising either genotype were atypical in some respect. A more likely explanation relates to differences in maturity between Kn/Kn and Kn/+ plants. Early development of Kn/Kn and Kn/+ plants proceeds at about equal rates. As the plants age, Kn/Kn plants become retarded in development and stature. It then becomes impossible to obtain comparable tissue samples from plants the same age but of different genotypes. The differences between Kn/Kn and Kn/+ plants at 22 days may reflect different proportions of mature to immature tissue comprising the samples. Regardless of the age of the samples, the mutant plants have higher levels of phenols and IAA oxidase inhibitors than the normal plants. The most pertinent data are not the amounts of inhibitor per g fresh weight, per mg protein, or per plant; they are the units of IAA oxidase inhibitor per unit of IAA oxidase. Such data are not available at this writing.

The potency of the inhibitor is not significantly reduced by hydrolysis of the ester linkage. This suggests a lack of specificity between enzyme and inhibitor. Such a lack of specificity is consistent with the suggestion that IAA oxidase inhibitors act by interference with the formation of free radicles (5). The inability of HRP to inactivate the inhibitor except in the presence of IAA suggests that the inhibitor does not function as an alternate substrate for HRP.

From these results it is impossible to determine whether or not the increase in IAA oxidase inhibitors has any causal relation to knotting. The facts that A) inhibitor level corresponds to dosage of the Kn allele (at least in plants up to 18 days old); B) mutant plants have more inhibitor than normal plants; C) the difference in inhibitor content pre-
ceeds morphological expression of the Kn allele; all suggest that a causal relationship may exist between knotting and IAA oxidase inhibitor content. Of course, it is possible that the compounds causing IAA oxidase inhibition in vitro may produce knots by a mechanism entirely unrelated to IAA oxidase in vivo.

The observation that among anthocyanin-producing plants +/+ genotypes may exceed mutant plants in inhibitor concentration certainly suggests that there may be no causal relation between knot formation and inhibitor concentration. However, anthocyanins and related flavonols having IAA oxidase inhibitor activity presumably are restricted to the vacuole and therefore would have little effect on IAA oxidase in vivo. Until more is known concerning the nature of IAA oxidase inhibitors in anthocyanin-producing maize and the intracellular compartmentalization of IAA oxidase and its inhibitors, the results with anthocyanin-producing plants cannot be taken as proof that knotting is not a consequence of increased IAA oxidase inhibitor levels. Another important consideration is that HRP may be affected by a wider range of inhibitors than is the maize IAA oxidase enzyme. The possible significance of the increased inhibitor levels associated with the Kn allele remains uncertain. Regardless of whether IAA oxidase inhibitors are involved in knot production, the increased level of these compounds in mutant plants is in itself a phenomenon worth further investigation.

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Literature Cited