EFFECTS OF INDOLEACETIC ACID ON LIGNIFICATION IN WHEAT INTERNODES AND *IN VITRO*

By R. W. Parish*

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Summary

The formation of lignin was determined in wheat internodes incubated with ferulic acid, hydrogen peroxide, and various concentrations of indoleacetic acid (IAA). IAA was found to markedly inhibit lignification in the lower two-thirds of the internode. Experiments which were carried out *in vitro* utilized the polymerization of free radicals, formed after eugenol had been oxidized by peroxidase and hydrogen peroxide, as a model system for lignification. The polymerization was inhibited considerably by IAA. A mechanism is postulated to explain the effects of IAA.

I. Introduction

Lignification generally does not occur in young cells (see Parish and Miller 1969). Brown (1961) has suggested that, since the concentration of plant growth regulators is higher in young than mature cells, these may inhibit lignification. Siegel (1955) reported that eugenol polymerization by *Elodea densa* tissue, a reaction claimed to simulate lignification, was inhibited by indoleacetic acid (IAA). Petinov and Urmantsev (1964), after adding various levels of IAA to wheat–couch grass hybrids, concluded that levels of this growth regulator are important in resistance to lodging. They suggested that varieties with a strong lodging tendency have a large content of IAA, while this is small in non-lodging varieties. Further, Stafford (1965) has shown that IAA inhibits the synthesis of lignin in *Phleum* and *Elodea*.

The present paper is divided into two main sections. The first reports experiments conducted *in vivo* to examine the effects of IAA on the incorporation of ferulic acid into lignin by wheat internodes. The results indicate that IAA does inhibit this incorporation. The second section presents experiments carried out *in vitro*, in which eugenol polymerization was used to simulate lignification, and the effects of IAA, gibberellic acid, and kinetin on the system were determined.

II. Materials and Methods

For the *in vivo* experiments, the second internodes of wheat (cv. Olympic) in which the third internode had just completed elongating were divided into thirds. The segments were each placed in a screw-cap phial with the reaction mixture. The control solutions consisted of 50 mM KH_2PO_4 buffer (pH 4·5), 1 mM ferulic acid, and 3 mM H_2O_2 in a final volume of 10 ml. The treatment phials contained in addition 1 μM, 10 μM, 100 μM, or 1 mM IAA. The phials were agitated in a water-bath at 28°C for 2 days. The segments were then removed and dried between

* Botany Department, University of Melbourne, Parkville, Vic. 3052; present address: Institut für Allgemeine Botanik, Eidg. Techn. Hochschule, Universitätsstr. 2, Zürich 8006, Switzerland.

blotting paper. They were cut into smaller sections and homogenized in a Servall Omnimixer for 3 min at 240 V with 6·7 mM phosphate buffer (pH 7·0). The homogenate was centrifuged at 1500 *g* for 10 min and the supernatant liquid removed. This was used for peroxidase and soluble protein assays. The precipitate, after being dried and weighed, was extracted for lignin by the method of Miller and Anderson (1965). Extraction of lignin was performed with cold alcoholic alkali (CAA; 2% NaOH–60% ethanol) at 30°C for 48 hr. The extract was then filtered and the filtrate adjusted to pH 7·2 with 2N HCl. The lignin was then precipitated with concentrated HCl, separated by filtration onto glass–fibre filter paper, and dried to constant weight at 65°C. Lignin content was expressed as percentage of dry weight. Initial values for lignin were obtained from internode segments which had not been incubated. Each treatment had six replicates and the average of these is expressed in the results. The least significant difference between mean values was calculated after analysis of variance.

Peroxidase activity was estimated by the method of Lück (1963), but guaiacol rather than *p*-phenylenediamine was used as the substrate. Protein was estimated by the method of Lowry *et al.* (1951).

In the *in vitro* experiments the reaction mixture contained 0·1 μM horseradish peroxidase and 40 μM eugenol in 0·05M KH_2PO_4 buffer (pH 4·5). Various concentrations of H_2O_2 or plant growth regulators were added in different experiments. The changes in absorbance at 420 mμ were measured after H_2O_2 was added to the sample cuvette. In the experiments with plant growth regulators, 0·4 mM H_2O_2 was added.

III. Effects of Indoleacetic Acid on Lignification in Wheat Internodes

Lignin is a complex polymer formed after spontaneous polymerization of free radicals derived from certain phenylpropanes (Brown 1961). Plant peroxidase is involved in the oxidation of these phenylpropanes to form free radicals (Siegel 1953, 1954, 1955).

In these experiments a lignin precursor (ferulic acid) and hydrogen peroxide were supplied to segments from wheat internodes in the presence of various concentrations of IAA. The amounts of lignin formed and changes in the specific activity of peroxidase were then determined.

The segments of internodes incubated with ferulic acid and H_2O_2 showed a brown coloration which was more marked in some areas than others. No consistent pattern of coloration was observed, but sometimes brown rings were present circling the segment. This coloration was reduced in the presence of IAA.

IAA generally inhibited lignification in wheat internode segments (Table 1), the only exception being top segments treated with 10 μM IAA. The only statistically significant inhibition in these segments occurred with 1 μM IAA. However, all concentrations of IAA significantly inhibited lignification in middle and base segments. The lowest (1 μM) and the highest (1 mM) concentrations were the least effective inhibitors in these two segments, but large differences did not exist between the IAA treatments.

The changes in the specific activity of peroxidase in the top segments of internodes after incubation are shown in the following tabulation, where peroxidase activity is expressed as units per milligram of soluble protein:

	Control	Incubated with:			
		1 μM IAA	10 μM IAA	100 μM IAA	1 mM IAA
$10^3 \times$ specific activity of peroxidase:	1·75	4·54	4·14	4·12	4·93

The peroxidase activity prior to incubation was $1 \cdot 1 \times 10^{-3}$ units/mg soluble protein. IAA, at all concentrations, considerably stimulated peroxidase activity in the internodes. Increased peroxidase activity and synthesis has also been observed in tobacco leaf disks treated with IAA (Parish, unpublished results).

TABLE 1

EFFECTS OF INDOLEACETIC ACID ON CAA–LIGNIN FORMATION IN SECOND INTERNODES OF WHEAT

Treatment	CAA-lignin Content (%) of:		
	Top Segment	Middle Segment	Base Segment
Initial*	42	22	37
Control†	100	100	100
Incubation with:			
1 μM IAA	70	39	71
10 μM IAA	118	34	60
100 μM IAA	94	27	71
1 mM IAA	93	50	77
Least significant difference ($P = 0 \cdot 05$)	18	22	14

* Before incubation. † Incubated with ferulic acid and H_2O_2 only.

IV. Effects of Plant Growth Regulators on Ferulic Acid Oxidation and Eugenol Polymerization *in vitro*

The fact that peroxidase is involved in both IAA oxidation (Ray 1958) and lignification (Brown 1961) suggested that it might be concerned in the IAA control of lignification. If lignin precursors, peroxidase, and H_2O_2 are all present, IAA may be acting in two ways:

(1) The growth regulator may compete successfully against lignin precursors for peroxidase; or

(2) IAA may inhibit polymerization of the free radicals formed by oxidation of phenylpropanes.

The first experiment in this section indicates that IAA does not prevent oxidation of phenylpropane by peroxidase. Eugenol and ferulic acid will inhibit the oxidation of IAA by peroxidase and oxygen, but lose their inhibitory effects when themselves oxidized (by peroxidase and H_2O_2). Oxidized ferulic acid and eugenol actually stimulate IAA oxidation (Parish 1969), and the relevance of this will be discussed later.

The effects of IAA on the non-enzymatic polymerization of free radicals formed by oxidation of phenylpropane was studied using eugenol. When H_2O_2 and horseradish peroxidase are added to a eugenol solution, the solution becomes increasingly cloudy as initial oxidation and polymerization take place. As further polymerization occurs and larger particles form to eventually settle out, the cloudiness decreases. These increases and decreases in cloudiness can be estimated by measuring changes in

apparent absorbance at 420 mμ (although it should be noted that this is a measurement of light scattering and not true absorbance), and the effects of various concentrations of IAA on the reaction determined.

How closely eugenol polymerization resembles lignification is difficult to ascertain. Siegel (1955) found that *Elodea densa* could convert eugenol to lignin, but Stafford (1960) suggests that only "aged" eugenol is convertible. Nevertheless, eugenol does closely resemble known lignin precursors in chemical structure, and, like these precursors, does polymerize after oxidation by peroxidase and H_2O_2 (Bland 1961). Bland (1961) has studied in detail the resemblances and differences between eugenol lignin and natural lignin. The two do differ in certain respects and he urges caution when drawing conclusions from experiments with eugenol lignin. So while it is stressed that extrapolation from the results obtained *in vitro* to the plant may be dangerous, the present data both support the results obtained *in vivo* and suggest a mechanism of lignification control.

(*a*) *Effects on Ferulic Acid Oxidation*

The control assay solution was 40 μM ferulic acid, 0·12 mM H_2O_2, and 0·02 μM horseradish peroxidase in 0·05M KH_2PO_4 buffer (pH 4·5) or 0·05M Tris–HCl buffer (pH 6·8), final volume 2·5 ml. The oxidation of ferulic acid was determined by following the decrease in absorbance at 320 mμ. The absorbance fell from approximately 0·70 to 0·10 (there was slight variation in these values between experiments) in less than 1 min and then remained constant. Various concentrations of plant growth regulators were also added in different experiments but no effect on ferulic acid oxidation was observed.

(*b*) *Effects on Eugenol Polymerization*

(i) *H_2O_2 Concentration*

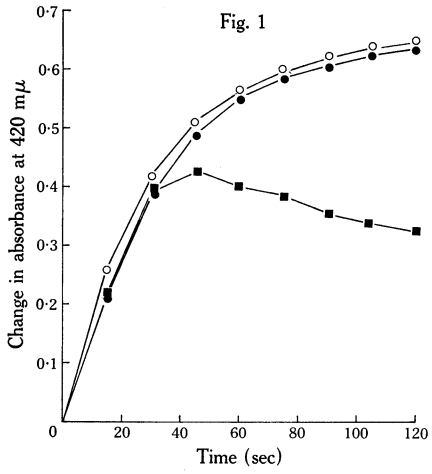
H_2O_2 was necessary for the reaction to proceed, and the amount added considerably modified eugenol polymerization (Fig. 1). At all H_2O_2 concentrations added, the initial rates of increase in absorbance at 420 mμ were similar. However, the lower the concentration the sooner maximum absorbance was reached, and absorbance then began to decrease. The rate of decrease was not significantly affected by H_2O_2 concentration.

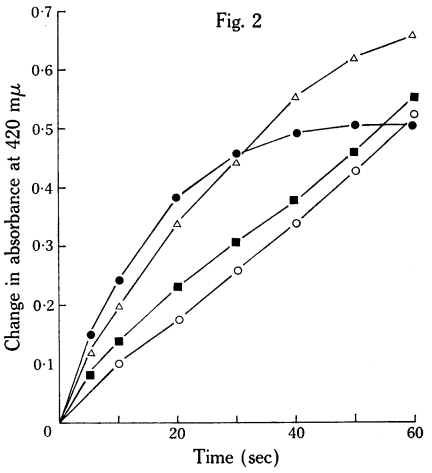
(ii) *Indoleacetic Acid*

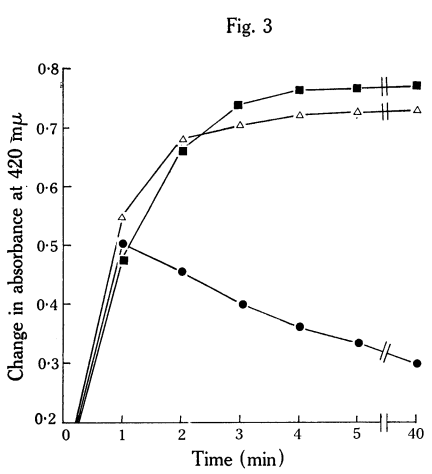
When IAA was present a higher maximum absorbance was reached, and the time taken to reach this absorbance increased with increasing concentrations of IAA. Thus, IAA slowed the initial rate of eugenol polymerization (Fig. 2). The presence of IAA also inhibited the decrease in absorbance at 420 mμ (Fig. 3).

(iii) *Gibberellic Acid and Kinetin*

Gibberellic acid tended to inhibit the increase in absorbance at 420 mμ, while kinetin slightly stimulated this increase (Fig. 4). However, the effects were not marked in comparison to results with IAA.







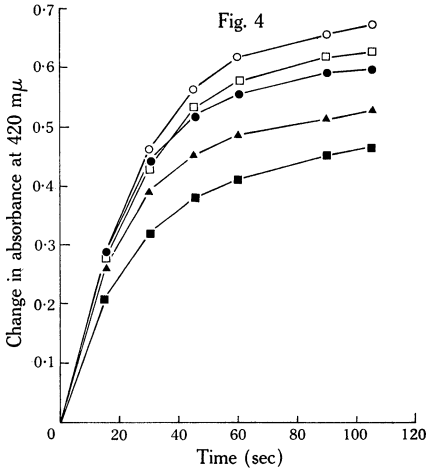


Fig. 1.—Effects of H_2O_2 on the initial polymerization of eugenol (40 μM) in the presence of 0·1 μM horseradish peroxidase in 0·05M KH_2PO_4 buffer (pH 4·5). Concentrations of H_2O_2 were 0·24 mM (■), 0·48 mM (●), and 0·72 mM (○).

Fig. 2.—Effects of IAA on the initial polymerization of eugenol (40 μM) in the presence of 0·1 μM horseradish peroxidase and 0·4 mM H_2O_2 in 0·05M KH_2PO_4 buffer (pH 4·5). Concentrations of IAA were 0·29 mM (△), 0·57 mM (■), 0·86 mM (○), and nil (●).

Fig. 3.—Effects of IAA on polymerization of eugenol (40 μM) treated as in Figure 2. Concentrations of IAA were 0·29 mM (△), 0·57 mM (■), and nil (●).

Fig. 4.—Effects of kinetin and gibberellic acid on the initial polymerization of eugenol (40 μM) treated as in Figure 2. ● Control: ▲ 1·2 mM gibberellic acid; ■ 4 mM gibberellic acid; □ 6·2 μM kinetin; ○ 25 μM kinetin.

V. General Discussion

The *in vivo* experiments with wheat internodes demonstrated that the incorporation of ferulic acid into lignin is inhibited by IAA. Experiments carried out *in vitro* showed that IAA considerably inhibited the polymerization of free radicals formed after oxidation of eugenol by peroxidase and H_2O_2.

A mechanism to explain the effects of IAA *in vitro* may be derived from two facts:

(1) Once oxidized, eugenol forms free radicals which then polymerize.

(2) During the oxidation of IAA by peroxidase two complexes (denoted compounds I and II) involving IAA and the enzyme are formed. The formation of compound II from compound I is a one-electron reduction, the electron being supplied by the enzyme or any added reducing agent (Fox, Purves, and Nakadi 1965).

The stimulation of IAA oxidation by 2,4-dichlorophenol is a result of electron provision by the latter compound. The free radicals, formed from eugenol oxidized by peroxidase and H_2O_2, stimulate IAA oxidation (Parish 1969), presumably also by acting as a reducing agent. Therefore, while unoxidized eugenol actually inhibits IAA oxidation, eugenol free radicals are apparently able to supply electrons. Once having lost electrons, however, the eugenol free radicals are unable to polymerize. In this way the effects of IAA on eugenol polymerization may be explained. If eugenol polymerization provides a model system of lignification (see introduction to Section IV), a similar mechanism may function in the inhibition of lignification by IAA. Gibberellic acid and kinetin may inhibit lignification but do not markedly affect eugenol polymerization *in vitro*.

If, as this work suggests, IAA inhibits lignification, the higher levels of this hormone in young tissue could account for the absence of lignin. Lignin levels may also be controlled by the activity of enzymes involved in the synthesis of lignin precursors. Higuchi (1966) found that synthesis of phenylalanine deaminase and tyrase occurs just prior to lignification in bamboo shoots. IAA may act by controlling the synthesis of these and other enzymes as well as via the mechanism suggested above. Synthesis of peroxidase increases in senescing leaf tissue (Parish 1968), but activity is present in young tissue. IAA does not act by limiting synthesis of peroxidase, and synthesis is in fact stimulated by the growth regulator. The synthesis of enzymes which produce or destroy H_2O_2, for example glycollic acid oxidase and catalase, would also be important in controlling lignification. Catalase activity does fall in senescing leaf tissue (Parish 1968).

Higuchi *et al.* (1955) discovered that the levels of reduced ascorbic acid and glutathione decreased with increasing distance from the apex of bamboo shoots. They also showed that these reducing substances inhibited the condensation of coniferyl alcohol, by peroxidase and H_2O_2, to form lignin. The levels of reduced ascorbic acid and glutathione may, therefore, provide another means of controlling lignification. In turn auxins, by controlling these levels, may indirectly affect lignification. However, the effects of IAA on ascorbic acid levels are uncertain as experimental evidence is conflicting (Butt 1959; Mertz 1964; Purohit and Nanda 1966; Schopfer 1966).

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