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CONVERSION OF TRYPTOPHAN TO AUXIN BY PHENOLIC ESTERS FROM LEAVES OF DWARF FRENCH BEAN (*PHASEOLUS VULGARIS* L.)

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Abstract—Macerated bean leaves (*Phaseolus vulgaris*) produced more auxin when incubated at pH 7·5 in the presence of toluene for 2 days with tryptophan than without. Boiled or autoclaved leaves did not produce auxin when incubated alone, but did so after adding tryptophan. Conversion of tryptophan to auxin during incubation of macerated leaves was attributed to phenols, later identified as esters of caffeic, ferulic and *p*-coumaric acids. The phenolic esters extracted from bean leaves promoted growth of wheat coleoptile sections only when assayed with tryptophan, and produced auxin only when incubated or hydrolysed with tryptophan. Caffeic acid reacted with tryptophan like the bean phenols but ferulic and *p*-coumaric acids were less effective. Phosphate buffer, pH 7·5, removed phenolic esters, but not auxins, from the surface of bean leaves, and like the extracted bean phenols they produced auxin when incubated with tryptophan.

INTRODUCTION

A FREE auxin, extractable in ethyl acetate, was released from a non-extractable, bound auxin or auxin precursor during the senescence of primary leaves of dwarf French bean (*Phaseolus vulgaris*).[1] Leaves, 15 days old, also produced auxin when macerated at pH 7·5 and then incubated at 37° in the presence of toluene for 2 days, or hydrolysed with normal potassium hydroxide at 100° for 2 hr. The tryptophan content of the leaves also increased during senescence, and when they were incubated, suggesting that tryptophan may be an intermediate in the release of auxin, and we now describe experiments to test this possibility.

Gordon and Paleg[2] described the conversion of tryptophan to auxin by phenols. In mung bean this reaction occurred spontaneously at pH 9·5 or was catalysed by polyphenolases at pH 8·5. We have tested the ability of phenolic esters extracted from dwarf bean leaves, and of some phenolic acids, to convert tryptophan to auxin during bio-assay, incubation and hydrolysis. The phenolic esters in bean leaves were extracted and after hydrolysis the components of the esters were isolated and examined chromatographically.

RESULTS

Conversion of Tryptophan to Auxin in Macerated Bean Leaves

When 1 mg of tryptophan was added to 5 g of macerated leaves, 15 days old, and incubated at 37° and pH 7·5 for 2 days in the presence of toluene, more auxin was produced than from leaves incubated without tryptophan (Table 1). This auxin, whether derived from macerated leaves incubated with or without tryptophan, ran at R_f 0·2–0·5 on chromatograms with ammoniacal *iso*propanol; indolyl-3-acetic acid (IAA) ran at R_f 0·26–0·43.

[1] A. W. WHEELER, *J. Exptl Botany* **19**, 102 (1968).
[2] S. A. GORDON and L. G. PALEG, *Plant Physiol.* **36**, 838 (1961).

TABLE 1. EFFECT OF TRYPTOPHAN ON THE RELEASE OF BOUND AUXIN FROM 5 g BEAN LEAVES, MACERATED THEN INCUBATED AT 37° FOR 2 DAYS

		IAA μg released from 5 g leaves	
		Without tryptophan	With 1 mg tryptophan
A—Treatment of macerated leaves before incubation with toluene	Unsterilized	0·76	2·63
	Boiled	0·08	0·39
	Autoclaved	0·06	0·27
	S.E.	±0·04	
B—Preservatives used during incubation	None	0	—
	Toluene	0·80	1·95
	Hibitane	1·30	1·95
	Chloramphenicol	0·80	2·05
	Streptomycin	0·06	2·10
	Penicillin	2·45	1·75
	S.E.	±0·05	
C—Inhibitors used during incubation with toluene	None	0·21	0·69
	Cysteine	0·12	0·12
	Ascorbic acid	0·20	0·16
	S.E.	±0·05	

When bean leaves were boiled before maceration, or autoclaved after maceration, auxin was not produced during incubation with toluene (Table 1A) but when 1 mg of tryptophan was added to these sterile macerated leaves, then incubated, auxin was released. The amount of auxin produced when macerated leaves were incubated increased with increase in the amount of tryptophan added, but not proportionally. Tryptophan autoclaved alone in phosphate buffer, pH 7·5, did not release auxin when incubated for 2 days.

To check the effects of any contaminating bacteria, various preservatives were added to the macerated leaves before incubation. The amount of auxin produced from 1 mg of tryptophan incubated with 5 g of macerated leaves was the same when toluene (8 drops), hibitane (chlorhexidine diacetate, 2·5 mg), chloramphenicol (500 μg), streptomycin (480 μg) or penicillin (25 i.u.) were also added to 25 ml of macerate (Table 1B). When incubated without tryptophan, streptomycin decreased and penicillin increased the production of auxin from leaves. Auxin was not detected when trytophan was incubated alone with any of these preservatives; nor when 5 g of macerated leaves were incubated alone without any preservative.

Cysteine and ascorbic acid inhibit the catalytic effect of polyphenolase on the conversion of tryptophan to auxin by phenols[2] and these oxidation inhibitors were added to macerated leaves before incubation with toluene. Less auxin was produced when 1 mg of tryptophan and 5 g of macerated bean leaves were incubated with 10^{-2} M cysteine or ascorbic acid (Table 1C) but they affected the production of auxin less in the absence of tryptophan.

Hydrolysing 5 g of macerated leaves in N KOH at 100° for 1 hr with 1 mg of tryptophan yielded 0·60 μg auxin whereas 5 g of similar leaves hydrolysed alone yielded only 0·48 μg auxin. Both these auxins ran at R_f 0·2–0·5. Auxin was not detected when 1 mg of tryptophan was hydrolysed alone in similar conditions.

Conversion of Tryptophan to Auxin by Phenols Extracted from Bean Leaves

Zones R_f 0·08–0·20 on chromatograms of the acidic fraction of ethyl acetate extracts of leaves, 15 days old, slowly became violet with Salkowski reagent and gave a positive test for phenols with diazotized sulphanilic acid. When segments R_f 0–0·2 of these chromatograms were assayed in 10 ml of N/100 K_2CO_3 with 2 mg of tryptophan, the wheat coleoptile sections grew more than with the tryptophan or the bean phenols alone. This suggested that the bean phenols converted tryptophan to auxin (Table 2A). The bean phenols produced less auxin

TABLE 2. CONVERSION OF TRYPTOPHAN TO AUXIN BY PHENOLS

	Phenol	IAA μg produced after treatment: Without tryptophan	IAA μg produced after treatment: With 2 mg tryptophan
A—Bio-assay in 10 ml N/100 K_2CO_3 for 19 hr at 25°	None	0	0·18
	Bean phenols	0·25	1·85
	Caffeic acid	0·03	1·70
	Ferulic acid	0	0·71
	p-Coumaric acid	0·05	0·58
	o-Coumaric acid	0	0·19
	p-Hydroxybenzoic acid	0·09	0·20
	Vanillic acid	0·06	0·13
	Chorogenic acid	0	1·65
	Catechol	0·01	3·75
	S.E.	±0·06	
B—Hydrolysis in 10 ml N/10 K_2CO_3 for 1 hr at 100°	None	0	0·05
	Bean phenols	0	0·60
	Caffeic acid	0·03	0·66
	Ferulic acid	0	0·14
	p-Coumaric acid	0·06	0
	S.E.	±0·03	
C—Incubation in 10 ml N/10 $KHCO_3$ for 24 hr at 37° with toluene	None	0	0·47
	Bean phenols	0·01	2·35
	Caffeic acid	0	2·85
	Ferulic acid	0·07	0·60
	p-Coumaric acid	0·05	0·65
	S.E.	±0·04	

After treatments B and C the auxins were extracted, separated on chromatograms, and bio-assayed.

from tryptophan when assayed in less alkaline conditions. Eight phenols, each at 10^{-4} M, did not affect growth of coleoptile sections in 10 ml of N/100 K_2CO_3, but when catechol and its derivatives, caffeic and chlorogenic acids, were assayed with 2 mg of tryptophan the coleoptile sections became much longer than in tryptophan alone. Ferulic and *p*-coumaric acids had smaller synergistic effects with tryptophan and the other phenolic acids were ineffective. The increased growth of the coleoptile sections was attributed to auxin derived from tryptophan by the phenols, and the amounts are expressed as IAA in Table 2A.

Hydrolysing the bean phenols in 10 ml of N/10 K_2CO_3 at 100° for 1 hr yielded auxin only when tryptophan was added; caffeic acid, 10^{-3} M, reacted similarly with tryptophan but

ferulic and *p*-coumaric acids were ineffective (Table 2B). Incubating the bean phenols, or caffeic acid, 10^{-3} M, in 10 ml of N/10 $KHCO_3$ at 37° for 1 day with toluene also yielded auxin only when tryptophan was added, and the yield was more than after hydrolysis (Table 2C). Incubating ferulic or *p*-coumaric acids with tryptophan also yielded auxin, but in this experiment some auxin was produced when tryptophan was incubated alone.

Phenols separated on chromatograms from the remaining ethyl acetate fractions after partitioning with 5 per cent $NaHCO_3$, or in the aqueous fractions, did not affect elongation of wheat coleoptile sections, even in presence of tryptophan.

Identification of Phenolic Esters from Bean Leaves

The R_f values and reactions of the three phenolic esters (A, B and C) extracted from bean leaves, shown in Table 3, differed from those of phenols commonly occurring in plants. Alkaline hydrolysis yielded caffeic, ferulic and *p*-coumaric acids from A, B and C, respectively, and a single aliphatic acid, common to all three esters. The phenolic acids were identified by their R_f values, the appearance of the spots under u.v. radiation and their subsequent reactions with the ferricyanide reagent and diazotized *p*-nitroaniline/Na_2CO_3, which were identical with the properties of the corresponding marker acids. Confirmation of these results was given from the identity of each u.v. absorption spectrum with the authentic phenolic acid.

TABLE 3. R_f VALUES AND REACTIONS OF THE THREE PHENOLIC ESTERS EXTRACTED FROM BEAN LEAVES

Reactions		Phenolic ester A	B	C
R_f	1st way solvent*	0·65	0·73	0·78
	2nd way solvent†	0·88	0·92	0·95
Colour under u.v. radiation	Alone	Blue	Blue-violet	None
	with NH_3	Bright green-white	Green	Violet
Reaction with ferricyanide reagent		Strong	Weak	Very weak
Reaction with diazotized *p*-nitroaniline	Alone	Yellow	Faint yellow	None
	with Na_2CO_3	Yellow-brown	Pink	None

* 6% Acetic acid.
† *Sec* Butanol: acetic acid: H_2O, 14:1:5.

TABLE 4. R_f VALUES OF BEAN ACID, *meso*-TARTARIC ACID, AND TARTARIC ACID ON THIN LAYER CHROMATOGRAMS

Acid	Solvent* (a)	(b)	(c)	(d)
Meso-tartaric	0·19	0·64	0·51	0·33
Tartaric	0·24	0·58	0·52	0·38
Dwarf bean	0·19	0·65	0·73	0·23

* (a) *n*-Butyl formate: formic acid: H_2O; 10:4:1; (b) Ethyl formate: formic acid: H_2O; 12:5:3; (c) Ethanol: H_2O: NH_3; 35:13:2; (d) *n*-Butanol: acetic acid: H_2O; 6:1:2.

The R_f values for thin-layer chromatograms of the aliphatic component of the three phenolic esters was unlike most of those of the common organic acids of plants, although in solvents (a) and (b) the values were similar to those of *meso*-tartaric acid (Table 4).

Conversion of Tryptophan to Auxin by Bean Leaf Washings

Primary leaves were shaken gently in 0·5 M phosphate buffer, pH 7·5, for 1 min. This solution yielded only 0·08 μg auxin when incubated at 37° alone, but yielded 2·40 μg auxin when incubated with 1 mg tryptophan for 2 days. When the solution was boiled for 2 min, or autoclaved at 15 lb/in.^{2} for 15 min, then incubated with 1 mg of tryptophan, 1·15 and 2·40 μg auxin were produced respectively. Autoclaving, then incubating 1 mg of tryptophan alone in similar conditions, yielded only 0·11 μg auxin. Ethyl acetate extracts of the solutions obtained by washing leaves also contained a substance that ran at R_f 0–0·05 on chromatograms and gave a typical phenolic reaction with diazotized sulphanilic acid. Thus, the auxin was probably derived from the tryptophan by phenolic esters washed from the surface of the leaves.

DISCUSSION

Macerated bean leaves produced tryptophan in addition to auxin when they were incubated,[1] but they produced more auxin when incubated with added tryptophan, confirming that tryptophan was an intermediate in auxin production. The auxin was probably IAA but this was not confirmed with other chromatographic solvents. The bound auxin, or auxin precursor, described earlier,[1] was probably a protein or polypeptide that was degraded by endogenous proteases or epiphytic bacteria to amino acids, including tryptophan, during leaf senescence or when macerated leaves were incubated. Bean phenols then converted the tryptophan in alkaline conditions to auxin. The yield of auxin when macerated leaves were incubated with tryptophan was less than 1 per cent of the theoretical yield and the percentage yield decreased when more tryptophan was added.

Leaves sterilized by heat did not produce auxin when incubated without added tryptophan presumably because heat inactivated the proteases or killed the epiphytic bacteria that converted protein to tryptophan. However, tryptophan yielded more auxin when incubated with unsterilized than with sterilized macerated leaves, because the bean polyphenolases were more effective than the spontaneous reactions in yielding auxin from tryptophan and phenols.[2]

The preservatives used during incubation of macerated leaves did not affect the conversion of added tryptophan to auxin, whereas Libbert *et al.*[3] reported that chloramphenicol and streptomycin prevented bacterial conversion of tryptophan to auxin. Conversely, cysteine and ascorbic acid inhibited only the conversion of added tryptophan to auxin, probably by blocking the polyphenolase reaction, as reported by Gordon and Paleg.[2]

Macerated leaves hydrolysed in boiling alkaline, presumably sterile, conditions produced less auxin than incubated leaves. Adding tryptophan increased the yield of auxin only slightly, showing that inadequate tryptophan limited production of auxin less when the leaves were hydrolysed than when they were incubated.

The three phenolic esters from leaf extracts had R_f values different from those of phenols common in plants. When these phenolic esters were hydrolysed with alkali or acid, caffeic, ferulic and *p*-coumaric acids were liberated, also a single aliphatic acid, common to all three esters. Approximate measurements from the u.v. absorption spectra showed that the relative amounts of the esters were in the order caffeoyl > feruloyl > *p*-coumaryl; there was about

[3] E. Libbert, S. Wichner, U. Schiewer, H. Risch and W. Kaiser, *Planta* **68**, 327 (1966).

ten times more caffeoyl ester than ferulyl ester. The appearance of the esters under u.v. radiation, and their behaviour with diagnostic chromatographic reagents, was identical with that of the corresponding phenolic acids; also, the u.v. absorption spectrum of each ester resembled that of its parent phenolic acid, but with small differences in the wavelength of maximum absorption. These results indicate that, in each ester, the carboxyl group of the phenolic acid is linked to a hydroxyl group of the aliphatic acid, leaving free the phenolic function of each ester.

The aliphatic components of the phenolic esters could not be identified unambiguously by chromatographic examination of an aqueous extract of the hydrolysed esters. The R_f values of the acid on paper, and on thin layers of cellulose, developed with two of the four chromatographic solvents employed, are similar to those of *meso*-tartaric acid. Phenolic esters of the tartaric acids are rare, however. A monocaffeoyl[4] and a dicaffeoyl tartaric acid[5] from chicory leaves (*Chicorium intybus*) have been reported and a dicaffeoyl *meso*-tartaric acid has been synthesized.[5]

Caffeic acid, and the phenolic esters extracted from leaves, assayed with tryptophan elongated wheat coleoptile sections, presumably because auxin was derived from tryptophan by the phenolic acids. The amounts of auxin are probably underestimated, because conversion of tryptophan to auxin probably continued after the coleoptile sections had stopped elongating. The small elongating effect of tryptophan alone could be attributed to auxin derived from the tryptophan by phenols in the coleoptile sections. The auxin derived from tryptophan by hydrolysis or incubation with the bean phenols, or with caffeic acid, had a similar R_f to IAA but its identity was not confirmed with other solvents. Caffeic acid promoted growth of oat mesocotyl sections in presence of tryptophan,[6] and chlorogenic acid had a similar synergistic effect but *p*-coumaric acid inhibited their growth.

Phosphate buffer solutions used to wash the surface of bean leaves removed phenols and probably any epiphytic bacteria, but as the production of auxin proceeded at the same rate when sterile or unsterile solutions were incubated with tryptophan, the bacteria evidently had little effect. These phenols occurred on the surface of the leaves but the leaf cells were easily ruptured so releasing chloroplasts into the solution. The conversion of tryptophan to auxin, attributed by Libbert and Wichner[7] to epiphytic bacteria on pea stem sections, could easily be caused by phenols on the pea stems or from ruptured cells.

EXPERIMENTAL

Extraction and Assay of Bean Leaves

Plants of dwarf French bean (*Phaseolus vulgaris*, var. Canadian Wonder), were grown at 25° in an artificially-lit cabinet described previously.[8] Auxins were extracted from macerated primary leaves at pH 3 with ethyl acetate by the usual methods.[8] Auxins in the fraction of the ethyl acetate extracts containing acidic substances were separated on chromatograms of Whatman No. 3 MM paper with *iso*propanol/water/concentrated ammonia solution (9:1:1 v/v) and detected and assayed by their elongation of coleoptile sections of wheat, var. Atle.[9]

Phenolic Esters of Bean Leaves

The phenols of zone R_f 0·08–0·20, from several chromatograms run with ammoniacal *iso*propanol were eluted with water-saturated ethyl acetate. The concentrated eluates were washed with water and the ethyl

[4] M. L. SCARPATI and A. D'AMICO, *Ric. Sci. Roma* **30**, 1746 (1960).
[5] M. L. SCARPATI and G. ORIENTE, *Tetrahedron* **4**, 43 (1958).
[6] J. H. M. HENDERSON and J. P. NITSCH, *Nature* **195**, 780 (1962).
[7] E. LIBBERT and S. WICHNER, *Naturwissenschaften* **50**, 451 (1963).
[8] A. W. WHEELER, *J. Exptl Botany* **11**, 217 (1960).
[9] A. W. WHEELER, *J. Exptl Botany* **12**, 217 (1961).

acetate layer separated and dried (Na_2SO_4). The solution was concentrated and chromatographed 2-dimensionally on Whatman No. 2 paper in 6 per cent acetic acid followed by *sec.*-butanol/acetic acid/water (14:1:5 v/v). The dried chromatograms were examined under u.v. before and after exposure to NH_3 and others were dipped in either the ferricyanide reagent (5 ml 3 per cent $FeCl_3$ diluted to 50 ml with water, with the addition of 50 ml 0·3 per cent $K_3Fe(CN)_6$) or diazotized *p*-nitroaniline, followed by 10 per cent Na_2CO_3 (Table 3).

Isolation of Individual Esters

A mixture of the phenolic esters (84 mg) isolated from the ethyl acetate extract of the leaves was chromatographed in 6 per cent acetic acid. The band corresponding to the caffeoyl ester (A in Table 3) was relatively pure but the ferulyl and *p*-coumaric esters (B and C) overlapped. The esters were eluted with 50 per cent aqueous ethanol and again developed in 6 per cent acetic acid and were then substantially pure. The u.v. absorption spectra of the final eluates, suitably diluted were determined using a suitable blank. The λ_{max} and absorptivities were: caffeoyl ester, 327 nm (2·40); ferulyl ester, 315 nm (0·22); *p*-coumaryl ester, 305 nm (0·08).

Hydrolysis of Individual Esters

The esters were hydrolysed (10 per cent aq. NaOH) for 1½ hr at room temperature. The hydrolysates were acidified (2 N HCl) diluted with water and the phenolic acids extracted with ethyl acetate. The phenolic acids were identified chromatographically using the upper phase of toluene/acetic acid/water (4:1:5 v/v)[10].

Non-Phenolic Components of the Hydrolysate

The extracted aqueous layer from each hydrolysate was concentrated, Na^+ was removed with Amberlite CG120 (H^+ form), and HCl removed by evaporating the de-ionized solution to dryness. The residue was chromatographed on Whatman No. 1 paper and on TLC (250 μ) of cellulose powder (MN 300; Machery, Nagel and Co.) using the solvent systems (a) *n*-butyl formate/formic acid/water, (10:4:1 v/v)[11] (b) ethyl formate/formic acid/water (12:5:3 v/v)[12] (c) ethanol/water/ammonia (35:13:2 v/v)[12] and (d) *n*-butanol/acetic acid/water (6:1:2 v/v) (Table 4). Tests with the sodium metaperiodate–sodium nitroprusside– piperazine reagent[13] showed both quinic and shikimic acids were absent.

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[11] H. A. W. Blundstone, *Nature* **197**, 377 (1963).
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