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Phenolic Components of the Primary Cell Wall and Their Possible Rôle in the Hormonal Regulation of Growth

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Abstract. The insoluble cell wall polymers of cultured spinach cells contained esterified ferulic acid at 2–5 mg g^{-1} dry weight. Gibberellic acid (GA_3, 10^{-11}–10^{-6} M) promoted the expansion of these cells and simultaneoulsy suppressed peroxidase secretion, reduced the activity of cellular phenylalanine ammonia-lyase and favoured the accumulation of wall-esterified ferulate and of extracellular soluble phenolic aglycones. When growth was prevented with 0·7 M sorbitol, GA_3 still evoked the phenolic and peroxidase effects. It is suggested that peroxidase restricts growth by rigidifying the cell wall in two ways: (*a*) covalently by catalysing the conversion of feruloyl side-chains into diferuloyl cross-links and (*b*) non-covalently by catalysing the conversion of soluble phenolics into hydrophobic quinones (or polymers). GA_3 is hypothesised to prevent this rigidification by inhibiting peroxidase secretion.

Key words: Cell growth regulation – Ferulic acid – Gibberellin – Hydrophobicity – Peroxidase – *Spinacia.*

Introduction

Polysaccharide esters of ferulic acid are abundant in monocotyledons (Fausch et al., 1963; Levand and Heinicke, 1968; Fulcher et al., 1972; Hartley, 1973; Whitmore, 1974; Harris and Hartley, 1976). These esters are not restricted to lignified cells but also occur in the cell walls of many growing tissues (Harris and Hartley, 1976). They are interesting for several reasons:

(*a*) Feruloyl side-groups endow certain soluble polysaccharides with the property of forming gels upon oxidation with H_2O_2 plus peroxidase (e.g. *Triticum* endosperm arabinoxylan: Fausch et al., 1963). This property is attributed to the formation of diferuloyl bridges (Fig. 1) between formerly separate polysaccharide molecules (Geissmann and Neukom, 1973; Neukom, 1976). Such "oxidative gelation" of important matrix polysaccharides within the primary cell wall could drastically affect the wall's extensibility and therefore its growth rate (speculations of Markwalder and Neukom, 1976; Hartley and Jones, 1976; Lamport, 1978).

(*b*) Feruloyl groups alter the properties of the walls in which they occur; in particular they protect the polysaccharide from enzymic attack (Hartley and Jones, 1977). This could make them important in restricting the digestion of cell walls by animals, pathogens, leaf-litter microbes or the endogenous enzymes that may loosen the wall during the normal course of growth.

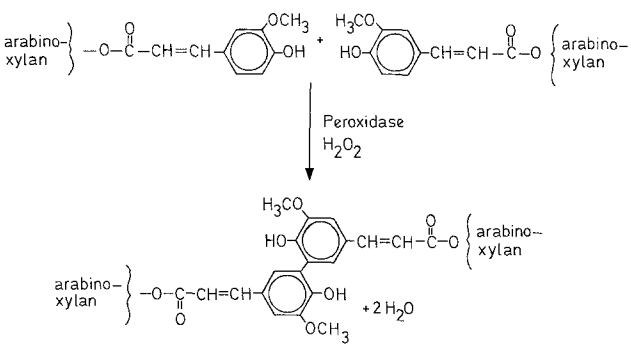

Fig. 1. Proposed mechanism for the oxidative gelation of *Triticum* endosperm feruloyl-pentosans (after Geissmann and Neukom, 1973)

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Abbreviations: A_{280} = absorbance at 280 nm; $a_{1\,cm}^{1\%}$ = absorptivity coefficient; 2,4-D = 2,4-dichlorophenoxyacetic acid; EtOAc = ethyl acetate; GA_3 = gibberellic acid; mol wt = molecular weight; PAL = phenylalanine ammonia-lyase; PCV = packed cell volume; sh = shoulder or inflection; TLC = thin-layer chromatography; UV = ultra-violet; λ = wavelength; IAA = indoleacetic acid

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(*c*) Ferulic acid released from the cell walls by leaf-litter decomposition could play an ecological rôle as a germination inhibitor (Chou and Lin, 1976; Turner and Rice, 1976).

(*d*) Finally, polymer-bound ferulate could seriously interfere in the assay of protein (Lowry et al., 1951) based on Folin and Ciocalteu's phenol reagent.

It therefore seemed worthwhile to explore in more depth the occurrence and functions of wall-bound ferulate esters. No evidence was available for their occurrence in more than trace amounts in dicotyledons (Harris and Hartley, 1976; Hartley and Jones, 1977), suggesting a possible monocotyledon/dicotyledon distinction. Further, despite the suggestion that ferulate esters play a rôle in the regulation of growth, the effects of growth hormones upon ferulate levels had not been studied. This paper reports quantitative analyses of ferulate in the cell walls of a gibberellin-sensitive suspension culture of the dicotyledon *Spinacia* (Fry, 1978).

Besides binding phenolic substances in the cell wall, plant tissue cultures also secrete them into the medium. It is quite possible that such secreted compounds may be substances which in the intact plant are present in the soluble phase of the cell wall matrix; they could affect the properties of the wall by interacting non-covalently with the wall polymers. The metabolism of phenolic compounds is affected by hormones such as cytokinins (Miller, 1978) and gibberellins (Haddon and Northcote, 1976; Heinzmann and Seitz, 1977). I have therefore also studied the effect of gibberellins upon the levels of low mol wt extracellular phenolic compounds in *Spinacia* cultures.

Materials and Methods

Tissue Culture

Stocks of a well-disaggregated, green, auxin-independent suspension culture of *Spinacia oleracea* L., cv. Monstrous Viroflay (line G-10 of Dalton and Street, 1976) were maintained in a salts solution (after Murashige and Skoog (1962) with $FeCl_3$ replaced by equimolar Fe-EDTA) plus 1.0% sucrose (final *p*H = 4.4; no hormones or vitamins) at 20° C under continuous fluorescent lighting (6×10^{-5} mol photons $m^{-2}\,s^{-1}$) on an orbital shaker (diameter of orbit = 2 cm; frequency = 120 min^{-1}) in wide-mouthed 250 ml conical flasks fitted with a glass centre well containing a rolled filter paper disk (7 cm) and 5 ml of 9% mercury perchlorate (Dalton and Street, 1976) as ethylene absorbent. The flasks were sealed hermetically with ethanol-sterilised polythene sheeting (24 g m^{-2}) and the suspension was sub-cultured every two weeks (14 ml into 50 ml of fresh medium). Under these conditions the cells routinely contained chlorophylls at 0.2–0.5 mg g^{-1} dry weight.

Gibberellins, when used, were sterilised by filtration. In most experiments involving GAs, slightly different culture conditions were used: inocula were transferred into 150 ml of medium in plain 250 ml conical flasks which were then sealed (non-hermetically) with sterile aluminium foil; incubation conditions were otherwise as for the stock cultures. The modified conditions gave more reproducible growth rates and a more pronounced response to GA_3 (Fry, 1978), but they were not used for the stocks because the cells became rather aggregated (and tuned a paler green) during prolonged culture in foil-sealed flasks.

Analysis

Polymer-esterified ferulic acid. "Cell walls" were obtained as the residue after extraction of other cellular constituents by the following sequence: (*a*) water, 100° C, 30 min; (*b*) 3% aqueous sodium dodecyl sulphate, 120° C (in an autoclave), 30 min; (*c*) repeat (*b*); (*d*) 60% aqueous ethanol, 85° C, 30 min; (*e*) acetone, 56° C, 30 min; (*f*) diethyl ether, 34° C, 30 min. The wall residue was dried and stored at −20° C. It was rehydrated in 0.1 M NaOH (about 0.1 ml mg^{-1} dry weight) in the presence of a trace of 2-ethylhexan-1-ol and incubated overnight (20° C, in vacuo) to saponify esters. The solution was then adjusted to *p*H 2.5 with HCl and partitioned once against an equal volume of EtOAc. The A_{320} of the organic phase was taken as a measure of ferulate concentration.

Qualitative phenolic analysis was by UV-spectroscopy after separation by TLC on Si-gel and by low-voltage paper electrophoresis and chromatography on Whatman No. 1 paper; solvents are given in Table 1. Phenolic compounds were located by UV-fluorescence ($\pm NH_3$) and staining with Folin and Ciocalteu's phenol reagent ($\pm NH_3$) or iodine in CCl_4.

Peroxidase (EC 1.11.1.7) was assayed by a modification of the Worthington (1972) method. An aliquot (50 µl) of enzyme solution was mixed with 500 µl of a substrate solution containing H_2O_2 (0.8 mM), *o*-dianisidine dihydrochloride (0.8 mM) and acetate buffer (100 mM, *p*H 5.0). The A_{460} was recorded continuously for 0.5–5.0 min at 25° C. The enzyme solution contained 1 "unit" ml^{-1} if the coloured product accumulated at 1 A_{460}-unit min^{-1}.

Total haemoprotein was estimated from A_{402} after clarification by centrifugation (27,000 *g*, 20° C, 20 min). The $a_{1\,cm}^{1\%}$ of haemoprotein at 402 nm was taken as 20 (= approximate value for pure horseradish peroxidase).

Amino acids were analysed with a 'Technicon' automated ion-exchange apparatus, using a lithium buffer system (Horner, 1977).

Phenylalanine ammonia-lyase (EC 4.3.1.5) was assayed in *Spinacia* cell acetone-powder. A sample (1.95–2.05 mg dry weight) of the powder was incubated at 25° C for 2 h with 60 µl of a solution containing 6.7 mM L-[*U*-^{14}C]-phenylalanine (150 kBq ml^{-1}), 10 mM mercaptoethanol, 0.05% Triton X-100 and 100 mM Tris-HCl, *p*H 8.8. The [^{14}C]trans-cinnamate produced was purified by TLC on Si-gel in toluene:ethyl formate:formic acid (7:2:1) and assayed by scintillation-counting.

Microscopy

Samples of cultured cells and hand-sections of whole plant material were mounted in water or 5% NH_3 and examined by UV-fluorescence microscopy (Harris and Hartley, 1976). NH_3-enhanced UV-fluorescence was taken as evidence of phenolic material. Lignin was located with phloroglucinol-HCl (Johansen, 1940).

Results

Preliminary Microscopical Survey

UV-fluorescence microscopy confirmed the presence of phenolic material in the cell walls of *Lolium* (a monocotyledon) internode cortex (a non-lignified tissue) (Harris and Hartley, 1976). Non-lignified, UV-

fluorescent cell walls were also present in the pith and cortex of young petioles and internodes of *Sagina procumbens* (Caryophyllaceae) and *Spinacia oleracea* (Chenopodiaceae), but not of *Rumex obtusifolius* (Polygonaceae), *Senecio vulgaris* (Compositae) or *Phaseolus vulgaris* (Leguminosae). In *Sagina* and *Spinacia* cortex and pith cell walls the fluorescence was intensified by NH_3, suggesting a phenolic compound. The xylem vessel elements stained strongly for lignin, the phloem fibres weakly and the cortex and pith not at all.

In order to obtain large numbers of cells with purely primary walls, tissue cultures were studied. In exponentially growing suspension cultures of *Spinacia* essentially all the cell walls showed NH_3-enhanced UV-fluorescence; none stained for lignin. Extraction of the soluble components of the cells did not diminish the intensity of cell wall fluorescence, suggesting a covalently bound phenolic component.

Identification of Esterified Ferulic Acid

After a cell wall macromolecule preparation from the suspension culture had been saponified, the residue no longer fluoresced and the acid EtOAc-extractable material had a UV-absorption spectrum very similar to that of ferulic acid (λ_{max} of extract = 232^{sh}, 295^{sh} and 318 nm; λ_{max} of pure ferulic acid = 234, 295^{sh} and 321 nm; solvent = 80% ethanol). Upon chromatography and electrophoresis under seven sets of conditions (Table 1), the only compounds detected in the extract ran exactly with trans- and cis- ferulic acids and stained and fluoresced identically with them. The fluorescent material in the cell walls is concluded to be bound ferulic acid. The slight difference in the λ_{max} values of the extract and pure ferulic acid could have been due to a difference in the cis:trans ratio. This ferulic acid made up 0.2–0.5% of the dry weight of the wall macromolecule preparation.

The linkage between ferulate and the macromolecule(s) was probably an ester formed through the carboxyl group of the ferulate. Evidence for this is that in the saponification treatment the white macromolecule preparation immediately turned yellow on the addition of NaOH (this yellowing was reversible with HCl) but after a few hours in NaOH the yellow colour faded; this behaviour is typical of the phenolate anions of glycosyl-ferulate esters, which have a higher λ_{max} than, and are hydrolysed to, the corresponding aglycone (Hanson and Zucker, 1963).

Methods of Hydrolysis

Various treatments of hydrolysis were tested on the ferulate esters (Table 2). None was as effective as saponification. Also, a desalted preparation of 'Onozuka SS' cellulase failed to release ethanol-soluble ferulate under conditions worked out for the isolation of protoplasts from the *Spinacia* culture (cf. Hartley, 1973).

Table 1. Chromatography and electrophoresis of ferulic acid. PC = paper chromatography, TLC = thin-layer chromatography, PE = paper electrophoresis at 15 V cm^{-1}. "Mobility" values for PC and TLC are R_f; values for PE are mobility relative to the main spot given by bromophenol blue

Method	Solvent	Mobility[a]
PC	Acetic acid, conc. HCl, H_2O (10:3:30)	0.60, 0.78
PC	Propan-2-ol, 25% NH_3, H_2O (8:1:1)	0.57
PC	0.3 M ammonium acetate and 3 mM Na_2 EDTA in 63% ethanol	0.69
TLC	EtOAc, benzene (9:11)	0.10, 0.18
PE	40 mM Acetate (Na^+) buffer, *p*H 5.0	0.44, 0.80
PE	50 mM Phosphate (Na^+) buffer, *p*H 7.0	0.61, 0.95
PE	0.4% Ethanolamine (*p*H 9.8)	1.43

[a] The presence of two spots probably indicates resolution of the cis and trans isomers

Table 2. Methods for the hydrolysis of *Spinacia* cell walls. A cell wall macromolecule preparation was hydrolysed under the conditions indicated (the NaOH treatment was in vacuo). The hydrolysate was then adjusted to *p*H 2.5 with HCl or NaOH and partitioned once against EtOAc. Ferulic acid was assayed in the EtOAc extract

Method of hydrolysis				Ferulic acid yield (mg g^{-1} dry weight)
Agent	Conc. (M)	Temp. (°C)	Time (h)	
H_2O	–	100	3.0	< 0.1
NaCl	0.5	100	3.0	< 0.1
NaCl	2.0	100	3.0	< 0.1
HCl	0.1	70	0.5	0.1
HCl	0.5	70	0.5	0.3
HCl	2.0	70	0.5	1.1
HCl	0.5	100	1.0	1.3
HCl	2.0	100	1.0	1.5
HCl	0.5	100	3.0	0.8
HCl	2.0	100	3.0	1.3
NaOH	0.1	20	15.0	2.4
NaOH	1.0	20	15.0	2.5

Soluble Phenolic Compounds

Spinacia cultures secreted into their medium a soluble material of λ_{max} = 280 nm. This material partitioned readily into EtOAc from both acidic and neutral aqueous solutions. It was resolved by TLC (in EtOAc: benzene, 9:11) into five main UV-fluorescent phenolic compounds designated X_1–X_5 (Table 3). Compounds X_3 and X_4 were interconvertible by UV light, suggesting a pair of geometrical isomers. Electro-

Table 3. Properties of five phenolic compounds secreted into the medium by suspension cultures of *Spinacia*. R_f is after development by TLC in EtOAc:benzene (9:11). The λ_{max} values were measured in 80% ethanol; sh = shoulder. Colours were observed under neutral (n) and alkaline (alk) conditions, by daylight. Fluor. = UV-fluorescence as observed on chromatograms with (alk) or without (n) exposure to NH_3 fumes. Colour code: bg, blue-green; br, brown; dv, dull violet; v, violet; wh, white; y, yellow. F = Reaction with Folin and Ciocalteu's phenol reagent, without NH_3. I = Retaining stain after treatment with iodine in CCl_4. Geom. isom. = interconvertible upon exposure to UV lamp

Cmpd, R_f	Absorption maxima (nm)	Colour		Fluor.		F	I	Geom. isom.
		n	alk	n	alk			
X_1, 0.00	275, 230^{sh}	br	y	v	bg	+	+	?
X_2, 0.04	317^{sh}, 279, 230^{sh}	wh	y	v	bg	+	+	−
X_3, 0.16	318, 289, 230^{sh}	wh	y	v	bg	+	+	+
X_4, 0.20	311^{sh}, 282, 230^{sh}	wh	y	v	bg	+	+	
X_5, 0.45	314^{sh}, 305^{sh}, 265	br	br	dv	dv	+	+	−

phoresis showed X_1–X_5 to be uncharged. None of the five compounds was affected in R_f by standard hydrolytic methods (1.0 M HCl at 100° C for 2 h, or 1.0 M NaOH at 25° C for 16 h), suggesting that they were aglycones.

Effect of Gibberellins

GA_3 at 10^{-11}–10^{-6} M promoted cell expansion in the *Spinacia* culture; cell division was little affected (Fig. 2; and Fry, 1978). In the presence of GA_3 a high level of macromolecule-esterified ferulic acid accumulated compared with the total dry mass of macromolecules (Fig. 2). Chromatography revealed no evidence for other macromolecule-esterified phenolic compounds than ferulate, in any treatment.

In view of the continuity of the cell wall matrix and culture medium in suspension cultures of *Spinacia*, one might predict an effect of GA_3-treatment upon the soluble phenolic compounds of the spent medium in parallel with the effect seen on wall-bound ferulate. This did turn out to be the case (Fig. 2) and several further experiments were performed on soluble secreted phenolics.

Seven different gibberellins evoked the response (Table 4), the order of effectiveness being $GA_3 > GA_1 > GA_7$[1] $> GA_2 > GA_4 > GA_{13} > GA_9$. The effect was not evoked by 2,4-D nor by kinetin; 2,4-D antagonised the effect of simultaneously applied GA_3 (Table 5).

The effect of GA_3 upon the accumulation of phenolics in the medium was most pronounced during the period of rapid growth (Table 6); it was thus

[1] Calculated by difference, $(GA_4+GA_7) - (GA_4$ alone)

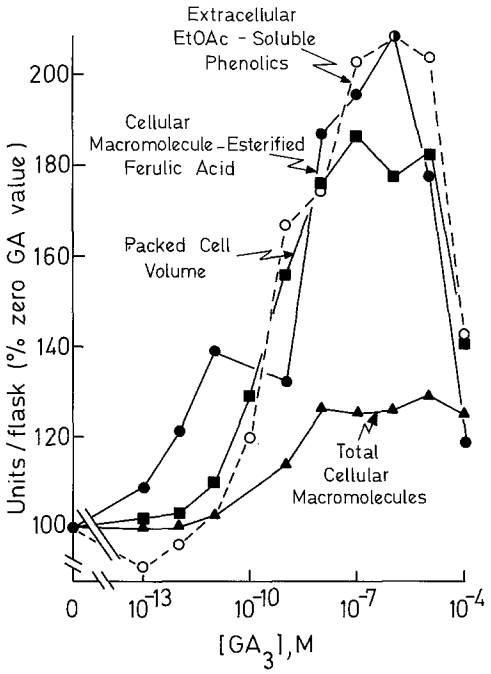

Fig. 2. Effect of GA_3 on suspension cultures of *Spinacia*. The cultures were inoculated at 4 µl PCV ml^{-1} into various concentrations of GA_3 in culture medium and harvested for analysis after 7 days' growth. For determination of total cellular macromolecules the living cells were briefly washed in water, then killed and exhaustively extracted in 70% ethanol, dried and weighed. For determination of extra-cellular phenolics, culture medium was adjusted to *p*H 2.5 with HCl and partitioned once against an equal volume of EtOAc; phenolics were assayed as A_{280}. PCV was measured after centrifugation at 1,500 *g* for 5 min. Esterified ferulate was assayed after saponification

Table 4. The effect of several gibberellins on the accumulation of soluble phenolic compounds in the culture medium of *Spinacia*. Two replicate cultures were used for each treatment; the means are shown. PCV on day 0 was 8 µl ml^{-1}. Values entered in the Table were measured after 14 days' growth. Phenolic concentration was measured as A_{280} of the spent medium after clarification by high-speed centrifugation. A_{280} on day 0 was *ca.* 0.3

GA	Conc.	Growth as PCV		Phenolics level	
	(nM)	(µl ml^{-1})	(Δ control)	(A_{280})	(Δ control)
None	−	47	0	0.76	0
GA_1	10	58	+28%	0.98	+48%
	1000	73	+67%	1.10	+74%
GA_2	10	49	+ 5%	0.79	+ 7%
	1000	65	+46%	1.01	+54%
GA_3	10	54	+18%	1.13	+80%
	1000	59	+31%	1.13	+80%
GA_4	10	47	0	0.73	− 7%
	1000	58	+28%	0.98	+48%
GA_{4+7}	10	58	+28%	0.91	+33%
	1000	57	+26%	1.05	+63%
GA_9	10	44	− 8%	0.80	+ 9%
	1000	45	− 5%	0.83	+15%
GA_{13}	10	47	0	0.67	−20%
	1000	52	+13%	0.95	+41%

Table 5. Hormonal interactions in the effect of GA_3 upon phenolic accumulation in the culture medium of *Spinacia*. The 2,4-D was used at 5.0 µM, the kinetin at 2.3 µM and the GA_3 at 0.1 µM. The cultures were harvested on day 8. Phenolics were assayed as in Table 4. A_{280} due to hormones was negligible

Hormones supplied	Phenolics level (A_{280}) on day 8
None	0.38
GA_3	0.84
2,4-D	0.36
GA_3 + 2,4-D	0.41
Kinetin	0.46
Kinetin + GA_3	0.73
Kinetin + 2,4-D	0.40
Kinetin + GA_3 + 2,4-D	0.43

Table 6. Time-course for the effect of GA_3 upon growth and the accumulation in the medium of soluble phenolic compounds. GA_3, where present, was at 10^{-7} M. Each value is the mean from two replicate cultures. Phenolic compounds were assayed as in Table 4

Time (days)	PCV (µl ml^{-1})		Phenolics (A_{280})	
	$-GA_3$	$+GA_3$	$-GA_3$	$+GA_3$
0	12	–	0.33	–
5	21	25	0.67	1.02
10	26	34	0.73	1.38
15	32	42	1.01	1.25
20	44	42	0.93	1.33

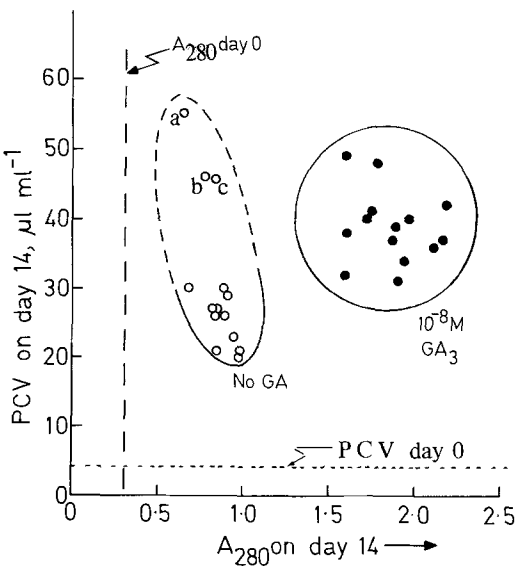

Fig. 3. The relationship between growth (PCV) and the accumulation of soluble phenolic compounds in the culture medium (measured as A_{280}). Fourteen replicate *Spinacia* cultures were incubated with GA_3 (10 nM) and fourteen without; conditions were as for the *stock* cultures. The inoculum density was 3.5 µl PCV ml^{-1} and the cultures were harvested on day 14

Table 7. The effect of sorbitol (0.7 M) and gibberellic acid (10^{-7} M) upon growth and the accumulation in the medium of soluble phenolic compounds and peroxidase activity. PCV on day 0 was 11 µl ml^{-1}, A_{280} was 0.32 and peroxidase activity was negligible (the cells had been washed in sterile water prior to inoculation). Each value is the mean from two replicate cultures after 9 days' growth

Treatment	PCV (µl ml^{-1})	Phenolics (A_{280})	Peroxidase (units ml^{-1})
$-GA_3$ − sorbitol	55	0.68	1.00
$+GA_3$ − sorbitol	92	0.89	0.49
$-GA_3$ + sorbitol	12	0.47	0.47
$+GA_3$ + sorbitol	13	0.71	0.32

not a symptom of senescence. Furthermore, GA_3 did not influence the viability of the *Spinacia* cultures as assessed by the fluorescein diacetate test (Widholm, 1972).

Phenolic accumulation was not an inevitable consequence of rapid growth, as can be seen from the relationship between A_{280} (due to phenolics) and PCV (growth) shown in Fig. 3. The PCV of replicate cultures was more variable than their A_{280} value. Occasional GA_3-free cultures grew as fast as did GA_3-treated ones; in particular, three GA_3-free cultures (a, b and c in Fig. 3) yielded high PCV values without any increase in A_{280}.

Phenolic accumulation in the medium was not the result of GA_3-promoted growth: when growth was prevented by the addition of sorbitol (0.7 M), GA_3 still promoted phenolic accumulation (Table 7).

Qualitative analysis suggested that the phenolic compounds whose accumulation was favoured by GA_3-treatment were X_1, X_2, X_3 and X_4. In contrast, X_5 was somewhat diminished by GA_3-treatment.

Possible Enzymic Basis

The increased levels of cell wall ferulate and of soluble phenolics seen in the presence of GA_3 could be due to increased synthesis or to decreased degradation (here taken to include oxidative polymerisation). One key enzyme that could increase the rate of phenolic synthesis is phenylalanine ammonia-lyase (PAL), and an enzyme that degrades phenolics is peroxidase (Fig. 1). Some attempt was made to distinguish between these two possibilities.

Measurements of the concentration of free amino acids in the cell sap showed that GA_3-treatment caused an increase in the level of phenylalanine (the substrate of PAL) and of the related amino acid tyrosine (Table 8). These data are compatible with the idea that GA_3 does not increase PAL activity. GA_3-

Table 8. The effect of GA_3 upon the amino acid composition of *Spinacia* cell sap. The cells were cultured for two weeks with or without GA_3 (10^{-8} M). PCV at harvest was 105 µl ml^{-1} ($-GA_3$) or 188 µl ml^{-1} ($+GA_3$). The cells were collected on a filter and killed in hot 80% acetone. The amino acids in the acetone extract were analysed. The concentrations are given as mmol l^{-1} PCV. nd = not determined

Amino acid	Concentration		Amino acid	Concentration	
	$-GA_3$	$+GA_3$		$-GA_3$	$+GA_3$
Glycine	0.25	0.21	Lysine	0.15	0.09
Alanine	8.6	5.2	Arginine	<0.01	<0.01
Valine	0.99	1.9	Histidine	0.08	0.06
Leucine	0.35	1.0	Tryptophan	<0.01	<0.01
Isoleucine	0.34	1.3	Phenylalanine	0.28	0.35
Serine	1.8	1.6	Tyrosine	0.24	0.40
Threonine	0.67	0.81	Proline	1.5	0.73
Aspartate	nd	nd	α-Amino-*n*-		
Asparagine	2.2	2.0	-butyrate	0.02	0.02
Glutamate	6.7	6.0	γ-Amino-*n*-		
Glutamine	10.3	8.1	-butyrate	0.76	1.4
Cysteine	<0.01	<0.01	Unknown	~0.5	~0.7
Methionine	<0.01	<0.01			

Table 9. The effect of GA_3-treatment upon the activity of cellular phenylalanine ammonia-lyase. Four cultures were inoculated (day 0) at 7 µl PCV ml^{-1} with 10^{-8} M GA_3 and four without GA_3

Incubation time (days)	PAL activity (nmol cinnamate h^{-1} mg^{-1} dry wt)	
	$-GA_3$	$+GA_3$
0	0.60	–
1	1.57	2.05
2	1.60	0.55
4	0.84	0.59
7	0.35	0.33

Table 10. The effect of GA_3 upon the secretion of haemoprotein (largely peroxidase) into the culture medium of a suspension culture of *Spinacia*. GA_3 was added, where appropriate, to 50 nM on day 7

Incubation time (days)	Haemoprotein (mg l^{-1})	
	$-GA_3$	$+GA_3$
0	0.0	–
1	6.3	–
3	11.2	–
5	17.2	–
7	25.1	–
7+3 h	20.1	19.8
7+8 h	20.8	20.5
8	21.5	20.5
9	24.4	22.1
10	25.4	23.5
12	29.7	23.8

treatment was actually found to repress PAL activity in *Spinacia* suspensions, after an initial (GA_3-independent) peak (Table 9). [The peak was probably due to dilution shock at inoculation, as reported for *Petroselinum* (Hahlbrock and Schröder, 1975) and *Phaseolus* (Dudley and Northcote, in press)]. There are two reports that GA_3 also represses PAL in solid calli (Haddon and Northcote, 1976; Heinzmann and Seitz, 1977).

The spent medium of *Spinacia* showed peroxidase activity and a small sharp absorption peak at 402 nm. The material absorbing at 402 nm was insoluble in 75% ethanol, it eluted in the void volume on 'Sephadex G-25' dextran gels, it salted out in saturated $(NH_4)_2SO_4$, and its λ_{max} increased from 402 to 427 nm upon reduction with dithionite. It co-eluted exactly with peroxidase activity on 'Sephadex G-150' dextran gels ($K_{av}=0.42$, suggesting mol wt = 40,000 if a globular protein). These properties show the A_{402} material to be mainly peroxidase (Saunders et al., 1964).

Secretion of peroxidase activity into the culture medium was suppressed by GA_3 treatment; this effect was seen whether or not growth was prevented by the simultaneous addition of 0.7 M sorbitol (Table 7). The effect was seen whether peroxidase was assayed enzymically (Table 7) or as haemoprotein (Table 10).

The peroxidase from *Spinacia* spent medium catalysed the destruction of the soluble phenolic compounds in vitro. For instance, when the peroxidase was added to purified compound X_4 (adjusted to $A_{280}=1.0$) in a buffer containing H_2O_2 (0.8 mM), NaCl (100 mM), and acetate (25 mM, *p*H 5.0), a considerable decrease occurred in the shoulder at ~310 nm, and the λ_{max} increased from 278 to 282 nm (the initial λ_{max} was lower than in Table 3 owing to the use of water as solvent in the present experiment), and a substantial turbid precipitate formed which was insoluble in hot water, 80% ethanol or EtOAc. The spectral changes were also catalysed by horseradish peroxidase, in which case they took about 1 h to complete despite the use of a high concentration of peroxidase (33 µg haemoprotein ml^{-1}, equivalent to about 80 peroxidase units ml^{-1} by the *o*-dianisidine assay) and H_2O_2. This suggests that peroxidases have a low catalytic centre activity for the destruction of compound X_4.

Discussion

The Diphenyl Bridge Hypothesis

GA_3 is a plant hormone that promotes cell expansion, probably via effects on the physical properties of the cell wall (Stuart and Jones, 1977). Little is known

of its mode of action. One hypothesis is that hormones promote growth by stimulating the secretion into the cell wall of "wall-loosening factors" (Cleland, 1977); alternatively, GA_3 could promote growth by suppressing the secretion of factors that rigidify the cell wall. One such rigidifying factor could be peroxidase, the enzyme that catalyses the oxidative gelation of feruloyl polysaccharides (Fig. 1). This idea is supported by the observations that genetically dwarf plants possess higher peroxidase levels than do tall varieties (Kamerbeek, 1956; Galston and Davies, 1969; Palmieri et al., 1978) and that GA_3 reduces peroxidase levels whilst reversing genetic dwarfism or promoting cell expansion in non-dwarf genotypes (McCune and Galston, 1959; Halevy, 1963; Ram et al., 1976); however, for the idea to be convincing the peroxidases involved must be compartmentalised in the cell wall. There is evidence that the peroxidase activity of the spent medium closely parallels that of the cell wall matrix in suspension cultures (King, 1976). I therefore conclude from my data that GA_3 reduces the peroxidase activity of the cell walls. Since this effect of GA_3 was seen whether peroxidase was assayed enzymically or as haemoprotein, it is concluded to be an effect on enzyme secretion rather than on enzyme activation. (It is unlikely that the haem would be added to the apoenzyme extracellularly.)

Many workers have ascribed the negative correlation between peroxidase content and growth rate to the peroxidase-catalysed oxidation of indole auxins (van Overbeek, 1935; Skytt Andersen and Muir, 1969). However, that this hypothesis is the complete answer seems unlikely because added auxins to not usually mimic the action of GA_3 (Cleland, 1969; this work). The widespread occurrence of wall-bound ferulic acid suggests an alternative hypothesis: peroxidase-catalysed diferuloyl bridge formation (Fig. 1). *Spinacia* cell walls contain less peroxidase and more (monomeric) feruloyl residues in the presence of GA_3 than in its absence. Thus I wish to suggest that the growth of GA_3-free cultures might be limited by a high degree of matrix polysaccharide diferuloyl cross-linking and that GA_3 may stimulate growth by suppressing the peroxidatic formation of these cross-links.

Does the primary cell wall contain enough feruloyl residues for this hypothesis to be feasible? It can be calculated from the data of Neukom (1976) that the formation of about one diferuloyl bridge per 3,000 sugar residues sufficed for the oxidative gelation of arabinoxylans. If it is assumed that the particular polysaccharide capable of gelling makes up 10% of the cell wall polymers, then a ferulate level of about 0.01% of the dry weight of the polymers should allow gelation. The values obtained for the *Spinacia* cells were 20–50 fold in excess of this, and similar values have been obtained for *Triticum* (Fausch et al., 1963) and *Lolium* (Hartley, 1973). Thus the oxidation of ferulate at the high levels that occur in *Triticum*, *Lolium* and *Spinacia*, and perhaps also at the lower levels detected in other dicotyledons (Hartley and Jones, 1977), could have profound effects upon the physical properties of the primary cell wall matrix and thus on the rate of cell expansion.

Esterified diferulic acid was not detected in the present work. Nevertheless, its precursor (esterified ferulic acid) was present, as was the appropriate enzyme (peroxidase), and it has been claimed that peroxidase can itself generate H_2O_2 from O_2 (Chance, 1952; Saunders et al., 1964) given a reducing agent, a phenolic co-factor and Mn^{2+}. The reducing agent can be indolyl-acetic acid (Engelsma, 1964), glutathione (Stonier and Yang, 1973) or NADH (Zmrhal and Macháčková, 1978); IAA is widespread in plants, suspension cultures can secrete large amounts of reduced glutathione (Bergmann and Rennenberg, 1978) and a system has been proposed for the generation of cell wall NADH (Gross et al., 1977). Furthermore, ferulic acid itself is weakly effective as phenolic co-factor (Zmrhal and Macháčková, 1978) and Mn^{2+} was present in the medium at 10^{-4} M (Murashige and Skoog, 1962). It is thus quite conceivable that diferuloyl bridges would have formed. Diferuloyl residues are present in vivo on polysaccharides of *Triticum* (Markwalder and Neukom, 1976) and *Lolium* (Hartley and Jones, 1976). The failure to detect diferulic acid in the present work could have been due to the presence of a large excess of ferulic acid; or the hypothetical oxidation product could have been a higher polymer than diferulate, perhaps akin to "acid lignin" (Stafford, 1964), which is insoluble in EtOAc and would therefore not have been detected.

Peroxidase is an abundant enzyme in plants and it might be argued that its activity is unlikely to be a limiting factor. However, since peroxidase and ferulate co-existed, it seems that the peroxidatic reaction was indeed the limiting factor. The substrate of peroxidase is H_2O_2 and this is also a substrate for catalase (which was also present in the medium: Fry, unpublished). In the cell wall, therefore, there is a possible competition for H_2O_2 between these two enzymes, especially if the H_2O_2 concentration in the cell wall is well below the K_m of peroxidase. Under these conditions, the peroxidase concentration might limit the rate of oxidation of monomeric ferulate esters to diferulate esters. In this way GA_3-treatment, which reduces the amount of peroxidase in the cell wall, might suppress the gelation of polysaccharides and hence increase the growth rate.

If esterified diferulate does play a rôle in rigidifying the cell wall, then extracellular esterases could be very important in the control of growth.

A peroxidatic reaction related to the formation of diferuloyl bridges is the cross-linking of proteins by means of dityrosine bridges. This reaction could restrict cell expansion by creating a network of the cell wall protein extensin (Lamport, 1978). The reaction has a *p*H optimum of 9.5 (Aeschbach et al., 1976) and is thus another one where, in a typical cell wall (*p*H 4–6), the reaction would be very slow so that peroxidase concentration could be the limiting factor in vivo.

The Hydrophobicity Hypothesis

GA_3-treatment promoted the accumulation in the medium of a group of low mol wt phenolic aglycones. These were not identified but from their UV-absorption spectra seem likely to have been phenylpropanoids; they were uncharged. As with the esters (see above), their accumulation could a priori have been due to increased synthesis and secretion or to decreased degradation. The decrease in extracellular peroxidase activity upon GA_3-treatment suggests decreased oxidative degradation; the decrease in PAL (probably a rate-limiting enzyme in phenolic synthesis) supports this suggestion.

The growth of GA_3-free cultures may have been limited by the presence in the cell walls of oxidation products of the soluble phenolics. These hypothetical oxidation products would probably have been quinonoid (Young and Steelink, 1973) or polymeric (Siegel, 1955), and therefore hydrophobic; in this case they could have lowered the effective concentration of cell wall water, considered to be an important structural component (Northcote, 1972). Exclusion of water would rigidify the wall by strengthening the hydrogen-bonds between adjacent matrix polymers, and could also restrict the access of the wall glycanases (or transglycosylases) that may be important in loosening the wall by means of enzymic excision of polysaccharides. Upon the addition of GA_3, peroxidase secretion was suppressed and thus the quinone/polymer formation might have been slowed down, resulting in a less hydrophobic wall that was more susceptible to loosening by water and glycanases.

Support for this hypothesis comes from the observation that GA_3 decreased (*sic*) the concentration of X_5, which, from its chromatographic properties, seems to have been the most hydrophobic of the five soluble extracellular aromatic compounds detected.

The low catalytic centre activity of peroxidase for compound X_4 supports the view that peroxidase concentration was the limiting factor in phenolic oxidation within the cell walls.

The GA_3-induced increase in the concentration of free phenolics was typically about $+10$ mg l^{-1} medium (based on the measured value $a^{1\%}_{1\,cm}=80$ for purified compound X_4 at $\lambda=280$ nm), *i.e.* about 1% of the total cell dry weight per unit volume of culture. This quantity seems enough for their hypothetical oxidation products to have a significant effect on the hydrophobicity of the cell walls, especially if adsorbed at a specific wall site.

It might be argued that since compounds X_1–X_5 are unusual substances, perhaps unique to the Chenopodiaceae, their possible rôle in growth regulation would be taxonomically restricted and therefore of no great importance. However, a culture of *Rosa* responded to GA_3 with promoted growth and greatly increased accumulation in the medium of a phenolic compound different from X_1–X_5 and tentatively identified as a low mol wt glycosyl ester of *p*-coumaric acid (Fry, 1978). Thus it could be that "secondary" products are widely involved in the regulation of cell expansion, the precise substance depending on the plant species tested.

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References

Aeschbach, R., Amadò, R., Neukom, H.: Formation of dityrosine cross-links in proteins by oxidation of tyrosine residues. Biochim. Biophys. Acta **439**, 292–301 (1976)

Bergmann, L., Rennenberg, H.: Efflux und Produktion von Glutathion in Suspensionskulturen von *Nicotiana tabacum*. Z. Pflanzenphysiol. **88**, 175–185 (1978)

Chance, B.: Oxidase and peroxidase reactions in the presence of dihydroxymaleic acid. J. Biol. Chem. **197**, 577–589 (1952)

Chou, C., Lin, H.: Autointoxication mechanism of *Oriza sativa*: I. Phytotoxic effects of decomposing rice residues in soil. J. Chem. Ecol. **2**, 353–367 (1976)

Cleland, R.E.: Gibberellins. In: Physiology of Plant Growth and Development, pp. 49–81, Wilkins, M.B., ed. London: McGraw-Hill 1969

Cleland, R.E.: The control of cell enlargement. In: Integration of Activity in the Higher Plant, pp. 101–115, Symposium XXXI, Society for Experimental Biology. Cambridge University Press 1977

Dalton, C.C., Street, H.E.: The role of the gas phase in the greening and growth of illuminated cell suspension cultures of spinach (*Spinacia oleracea* L.). In Vitro **12**, 485–494 (1976)

Dudley, R.K., Northcote, D.H.: Regulation of induction of phenylalanine ammonia lyase in suspension cultures of *Phaseolus vulgaris*. Planta (in press)

Engelsma, G.: Destruction of hydroxycinnamic acids coupled to oxidation of indolyl-3-acetic acid by peroxidase as a possible

mechanism for adaptive changes in indolyl-3-acetic acid oxidase activity. Nature **202**, 88–89 (1964)
Fausch, H., Kündig, W., Neukom, H.: Ferulic acid as a component of a glycoprotein from wheat flour. Nature **199**, 287 (1963)
Fry, S.C.: Metabolic corollaries of gibberellin-stimulated growth. PhD Thesis, University of Leicester 1978
Fulcher, R.G., O'Brien, T.P., Lee, J.W.: Studies on the aleurone layer. I. Conventional and fluorescence microscopy of the cell wall with emphasis on phenol-carbohydrate complexes in wheat. Aust. J. Biol. Sci. **25**, 23–34 (1972)
Galston, A.W., Davies, P.J.: Hormonal regulation in higher plants. Science **163**, 1288–1296 (1969)
Geissmann, T., Neukom, H.: On the composition of the water-soluble wheat flour pentosans and their oxidative gelation. Lebensm. Wiss. Technol. **6**, 59–62 (1973)
Gross, G.G., Janse, C., Elstner, E.F.: Involvement of malate, monophenols and the superoxide radical in hydrogen peroxide formation by isolated cell walls from horseradish (*Armoracia lapathifolia* Gilib.). Planta **136**, 271–276 (1977)
Haddon, L., Northcote, D.H.: The influence of gibberellic acid and abscisic acid on cell and tissue differentiation of bean callus. J. Cell Sci. **20**, 47–55 (1976)
Hahlbrock, K., Schröder, J.: Specific effects on enzyme activities upon dilution of *Petroselinum hortense* cell cultures into water. Arch. Biochem. Biophys. **171**, 500–506 (1975)
Halevy, A.H.: Interaction of growth-retarding compounds and gibberellin on indoleacetic acid oxidase and peroxidase of cucumber seedlings. Plant Physiol. **38**, 731–737 (1963)
Hanson, K.R., Zucker, M.: The biosynthesis of chlorogenic acid and related conjugates of the hydroxycinnamic acids. J. Biol. Chem. **238**, 1105–1115 (1963)
Harris, P.J., Hartley, R.D.: Detection of bound ferulic acid in cell walls of the Gramineae by ultraviolet fluorescence microscopy. Nature **259**, 508–510 (1976)
Hartley, R.D.: Carbohydrate esters of ferulic acid as components of cell-walls of *Lolium multiflorum*. Phytochemistry **12**, 661–665 (1973)
Hartley, R.C., Jones, E.C.: Diferulic acid as a component of cell walls of *Lolium multiflorum*. Phytochemistry **15**, 1157–1160 (1976)
Hartley, R.D., Jones, E.C.: Phenolic components and degradability of cell walls of grass and legume species. Phytochemistry **16**, 1531–1534 (1977)
Heinzmann, U., Seitz, U.: Synthesis of phenylalanine ammonia-lyase in anthocyanin-containing and anthocyanin-free callus cells of *Daucus carota* L. Planta **135**, 63–67 (1977)
Horner, M.: PhD Thesis, University of Leicester 1977
Johansen, D.A.J.: Plant Microtechnique. London: McGraw-Hill 1940
Kamerbeek, G.A.: Peroxydase content of dwarf types and giant types of plants. Acta Bot. Neerl. **5**, 257–263 (1956)
King, P.J.: Studies on the growth in culture of plant cells. XX. Utilization of 2,4-dichlorophenoxyacetic acid by steady state cell cultures of *Acer pseudoplatanus* L. J. Exp. Bot. **27**, 1053–1072 (1976)
Lamport, D.T.A.: Cell wall carbohydrates in relation to structure and function. In: Frontiers of Plant Tissue Culture 1978, pp. 235–244, Thorpe, T.A., ed. Calgary: International Association for Plant Tissue Culture 1978
Levand, O., Heinicke, R.M.: Ferulic acid as a component of a complex carbohydrate polymer of bromelain. Phytochemistry **7**, 1659–1662 (1968)
Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**, 265–275 (1951)
Markwalder, H.U., Neukom, H.: Diferulic acid as a possible cross-link in hemicelluloses from wheat germ. Phytochemistry **15**, 836–837 (1976)
McCune, D.C., Galston, A.W.: Inverse effects of gibberellin on peroxidase activity and growth in dwarf strains of peas and corn. Plant Physiol. **34**, 416–418 (1959)
Miller, C.O.: Cytokinin modification of metabolism of *p*-coumaric acid by a cell suspension culture of soybean (*Glycine max* (L.) Merrill). Planta **140**, 193–199 (1978)
Murashige, T., Skoog, F.: A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. **15**, 473–497 (1962)
Neukom, H.: Chemistry and properties of the non-starchy polysaccharides (NSP) of wheat fluor. Lebensm. Wiss. Technol. **9**, 143–148 (1976)
Northcote, D.H.: Chemistry of the plant cell wall. Ann. Rev. Plant Physiol. **23**, 113–132 (1972)
van Overbeek, J.: The growth hormone and the dwarf type of growth in corn. Proc. Natl. Acad. Sci. USA. **21**, 292–299 (1935)
Palmieri, S., Odoardi, M., Soressi, G.P., Salamini, F.: Indoleacetic acid oxidase activity in two high-peroxidase tomato mutants. Physiol. Plant. **42**, 85–90 (1978)
Ram, C., Balasimha, D., Tewari, M.N.: Effect of gibberellic acid and auxins on growth, sulphydryl content and peroxidase activity in *Phaseolus radiatus* L. seedlings. Plant Biochem. J. **3**, 128–133 (1976)
Saunders, B.C., Holmes-Siedle, M.A., Stark, B.P.: Peroxidase: the properties and uses of a versatile enzyme and of some related catalysts. London: Butterworths 1964
Siegel, S.M.: The biochemistry of lignin formation. Physiol. Plant. **8**, 20–32 (1955)
Skytt Andersen, A., Muir, R.M.: Gibberellin induced changes in diffusible auxins from Savoy cabbage. Physiol. Plant. **22**, 354–363 (1969)
Stafford, H.A.: Comparison of lignin-like products found naturally or induced in tissues of Phleum, Elodea and Coleus and in a paper peroxidase system. Plant Physiol. **39**, 350–360 (1964)
Stonier, T., Yang, H.: Studies on auxin protectors. XI. Inhibition of peroxidase-catalysed oxidation of glutathione by auxin protectors and *o*-dihydroxyphenols. Plant Physiol. **51**, 391–395 (1973)
Stuart, D.A., Jones, R.L.: Roles of extensibility and turgor in gibberellin- and dark-stimulated growth. Plant Physiol. **59**, 61–68 (1977)
Turner, J.A., Rice, E.L.: Microbial decomposition of ferulic acid in soil. J. Chem. Ecol. **1**, 41–58 (1976)
Whitmore, F.W.: Phenolic acids in wheat coleoptile cell walls. Plant Physiol. **53**, 728–731 (1974)
Widholm, J.M.: The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Technol. **47**, 189–194 (1972)
Worthington Biochemical Corp., N.J., USA. In: Worthington Enzyme Manual 1972
Young, M., Steelink, C.: Peroxidase catalyzed oxidation of naturally occurring phenols and hardwood lignins. Phytochemistry **12**, 2851–2861 (1973)
Zmrhal, Z., Macháčková, I.: Isolation and characterization of wheat peroxidase isozyme B1. Phytochemistry **17**, 1517–1520 (1978)

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