Dose Responses for *Colletotrichum lindemuthianum* Elicitor-mediated Enzyme Induction in French Bean Cell Suspension Cultures

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Abstract. The induction of L-phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) and flavanone synthase in French bean cell suspension cultures in response to heat-released elicitor from cell walls of the phytopathogenic fungus *Colletotrichum lindemuthianum* is highly dependent upon elicitor concentration. The elicitor dose-response curve for PAL induction shows two maxima at around 17.5 and 50 μg elicitor carbohydrate per ml culture, whereas the flavanone synthase response shows one maximum at around 100 μg ml^{-1}. The PAL response is independent of the elicitor concentration present during the lag phase of enzyme induction; if the initial elicitor concentration is increased after 2 h by addition of extra elicitor, or decreased by dilution of the cultures, the dose response curves obtained reflect the concentration of elicitor present at the time of harvest. PAL induction is not prevented by addition of methyl sugar derivatives to the cultures; α-methyl-D-glucoside, itself a weak elicitor of PAL activity, elicits a multiphasic PAL response when increasing concentrations are added in the presence of *Colletotrichum* elicitor. Eight fractions with different monosaccharide compositions, obtained from the crude elicitor by gel-filtration, each elicit different dose-responses for PAL induction; the response to unfractionated elicitor is not the sum of the responses to the isolated fractions. There is no correlation between the ability of the fractions to induce PAL in the cultures and their ability to act as elicitors of isoflavonoid phytoalexin accumulation in bean hypocotyls.

Key words: Cell suspension culture – *Colletotrichum* – Elicitor – Flavanone synthase – *Phaseolus* – Phenylalanine ammonia-lyase – Phytoalexin.

Introduction

The accumulation of phytoalexins (host-synthesised, low molecular weight antimicrobial compounds) in plant cells in response to phytopathogenic fungi and bacteria is believed to be induced by fungal metabolites termed elicitors. Such molecules have been isolated from fungal culture filtrates and mycelial walls and appear to be either polysaccharides (Ayers et al. 1976; Anderson-Prouty and Albersheim 1975) or glycoproteins (Daniels and Hadwiger 1976; Stekoll and West 1978; Dow and Callow 1979a; De Wit and Roseboom 1980).

Treatment of French bean cell suspension cultures with a crude elicitor from cell walls of *Colletotrichum lindemuthianum*, the causal agent of anthracnose disease, results in the accumulation of the isoflavonoid-derived phytoalexin phaseollin, preceded by a rapid, transient increase in the activity of L-phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), this change resulting from increased de novo synthesis of the enzyme (Dixon and Lamb 1979). The extent of PAL induction in the cultures is highly dependent on elicitor concentration, and the complex dose-response curve observed results from differential effects of elicitor concentrations on PAL synthesis and removal (Lawton et al., 1980).

Elicitors may exert their effects by binding to sites in the host cell plasma membrane, and elicitor dose-response curves may possibly reflect binding phenomena (Albersheim and Valent 1978). Indirect evidence for cell surface binding comes from studies in which low molecular weight sugar derivatives have been shown to inhibit elicitor-mediated responses (Ayers et al. 1976; Marcan et al. 1979). In addition, the high molecular weight glucan elicitor from *Phytophthora infestans* can cause agglutination of potato protoplasts (Peters et al. 1978; Doke and Tomiyama 1980), and low molecular weight glucans from compatible

races of *P. infestans* inhibit elicitor activity by a process which may involve competition for membrane receptor sites (Doke et al. 1979). Radiolabelled glycopeptides from culture filtrates of *Cladosporium fulvum* have been shown to bind directly to isolated tomato leaf mesophyll cells (Dow and Callow 1979b). Dose-response profiles for elicitor activity vary from log-linear (hypersensitivity of potato protoplasts to *P. infestans* elicitor; Doke and Tomiyama 1980), linear saturating at higher concentrations (glyceollin accumulation in soybean hypocotyls in response to *Phytophthora megasperma* var. sojae (PMS) elicitor; Albersheim and Valent 1978), hyperbolic (PAL induction in soybean cell suspensions in response to PMS elicitor; Ebel et al. 1976) to more complex relationships (phaseollin accumulation or PAL induction in French bean cell suspension cultures in response to *C. lindemuthianum* elicitor; Dixon and Lamb 1979; Lawton et al., 1980).

In view of the value of suspension cultured material for elicitor binding studies, we have now investigated further the induction of PAL and flavanone synthase (the first enzyme unique to 5-hydroxyflavonoid/isoflavonoid biosynthesis) in response to varying elicitor concentrations in the French bean cell cultures. We conclude that the elicitor-mediated induction of PAL in the cultures may involve a complex interaction between various fractions present in the crude elicitor preparation, but that such phenomena may not be directly related to the induction of isoflavonoid phytoalexin accumulation in the whole plant.

Materials and Methods

Cell suspension cultures of dwarf French bean variety Canadian Wonder were initiated and maintained by regular subculture at 14-day intervals in a modified Schenk and Hildebrandt medium as previously described (Dixon and Fuller 1976). Unless otherwise stated, cultures were grown at 25° C in a Gallenkamp Model INR-401 orbital incubator at 110 rev min^{-1} and illuminated by a single 30 W fluorescent tube approximately 0.5 m above the cultures. All cultures used in the following experiments were in exponential growth phase (6–7 days after subculture).

Colletotrichum lindemuthianum (Commonwealth Mycological Institute, isolate IMI 112166) was maintained on a semi-solid glucose-neopeptone medium (Mathur et al. 1949) in a continuous black light incubator. Conidia were removed from the cultures by vigorous shaking with sterile distilled water, and transferred to 500 ml batches of the Mathur medium (modified by the addition of 15 g glucose l^{-1}) in 2 l conical flasks. These were incubated on an orbital shaker at 25° C and 120 rev min^{-1} for 14 days. Mycelium was harvested on 4 layers of muslin, and cell walls prepared as described elsewhere (Anderson-Prouty and Albersheim 1975). Walls were autoclaved (100 ml H$_2$O per g walls) at 120° C for 30 min to release elicitor, the preparation then being filtered through sintered glass, clarified by centrifugation, dialysed for 24 h against three 10 l changes of distilled H$_2$O and concentrated 5-fold under reduced pressure. This preparation is referred to as the crude elicitor. Elicitor (from 5 g of walls) for fractionation studies was lyophilized and re-dissolved in 5 ml of distilled H$_2$O prior to column chromatography.

Enzyme induction was measured in 20 ml batches of suspension (5 ml packed cell volume) incubated for 7 h with 1.5 ml elicitor solution under normal culture conditions. Controls (minus elicitor) were treated with 1.5 ml sterile distilled H$_2$O. Each individual experiment was performed with cells from the same culture batch. Cell samples for PAL assay were harvested by suction filtration on porous polythene filters, transferred to small stoppered vials and stored at −70° C until required. Extraction and assay of the enzyme were as described elsewhere (Dixon et al., 1980). Flavanone synthase activity, in extracts from freshly harvested cells, was determined by a radiochromatographic method using [2-^{14}C]malonyl coenzyme A (Radiochemical Centre, Amersham, U.K.) and p-coumaroyl coenzyme A as substrates, as previously described (Dixon and Bendall 1978b). p-Coumaroyl coenzyme A was sythesised from p-coumaric acid via the N-hydroxysuccinimide ester (Stöckigt and Zenk 1975) and purified from unreacted coenzyme A by chromatography on Sephadex G-10 (Lindl et al. 1973). Protein was measured by a modification of the method of Lowry et al. (Legett-Bailey 1962) and total carbohydrate by the α-napthol/sulphuric acid method (Fuller and Northcote 1956).

For determination of monosaccharide composition, crude and fractionated elicitor preparations were adjusted to a concentration of 100 µg glucose equivalents in 100 µl of distilled H$_2$O in small capped vials. Trifluoroacetic acid (2 M, 0.5 ml) was added, and the samples hydrolysed at 100° C for 6 h. Samples were then reduced to dryness over KOH in a vacuum dessicator for 24 h, taken up in 50 µl H$_2$O, and 4 µl aliquots applied to Merck No. 5748 silica gel 69 thin-layer sheets. The 10 cm sheets were developed three times in ethyl acetate:pyridine:acetic acid:H$_2$O (6:3:1:1, v/v), and the separated monosaccharides quantitatively determined by direct densitometric scanning of the plates (Menzies et al. 1978). Lactose was included in all samples as internal standard.

Phytoalexin induction by crude and fractionated elicitor preparations was determined by application of the elicitor to the wounded surfaces of excised, etiolated hypocotyls from 7-day old seedlings of French bean variety 'The Prince'. For each treatment, elicitor was applied to five 2 cm long hypocotyl sections as described elsewhere (Anderson-Prouty and Albersheim 1975). After 48 h incubation in the dark at 25° C, each batch of hypocotyl sections was extracted in a pestle and mortar with 20 ml ethanol. Samples were worked up for TLC analysis of isoflavonoids as previously described (Dixon and Bendall 1978a), except that diethyl ether was used in place of petroleum ether for partitioning against the aqueous phase. Chromatograms (Machery-Nagel silica gel G/UV$_{254}$) were developed in toluene:ethyl formate:formic acid (7:2:1 v/v). Isoflavonoids were eluted from chromatograms in 1 ml ethanol. Phaseollin (R$_f$ 0.60) and kievitone (R$_f$ 0.27) were quantitatively determined from their reported extinction coefficients at λ_{max} of 280 and 293 nm respectively (Bailey and Burden 1973). Kievitone was further characterised by the 21 nm bathochromic shift observed in the UV spectrum following addition of ethanolic AlCl$_3$ (Bailey and Burden 1973). Although traces of phaseollidin, phaseollinisoflavan and coumestrol were consistently observed on chromatograms from elicitor-treated material, the other major compound present was a 5-OH-isoflavone (R$_f$ 0.32; λ_{max} (EtOH) 259, 280 (sh); λ_{max} (EtOH/AlCl$_3$) 270, 280 (sh)). It was eluted from chromatograms as above, and its concentration determined in arbitrary units from its absorption at 259 nm.

Results

The dose-response curve for PAL activity induced in the cultures by varying concentrations of the crude *Colletotrichum* elicitor showed two maxima at around
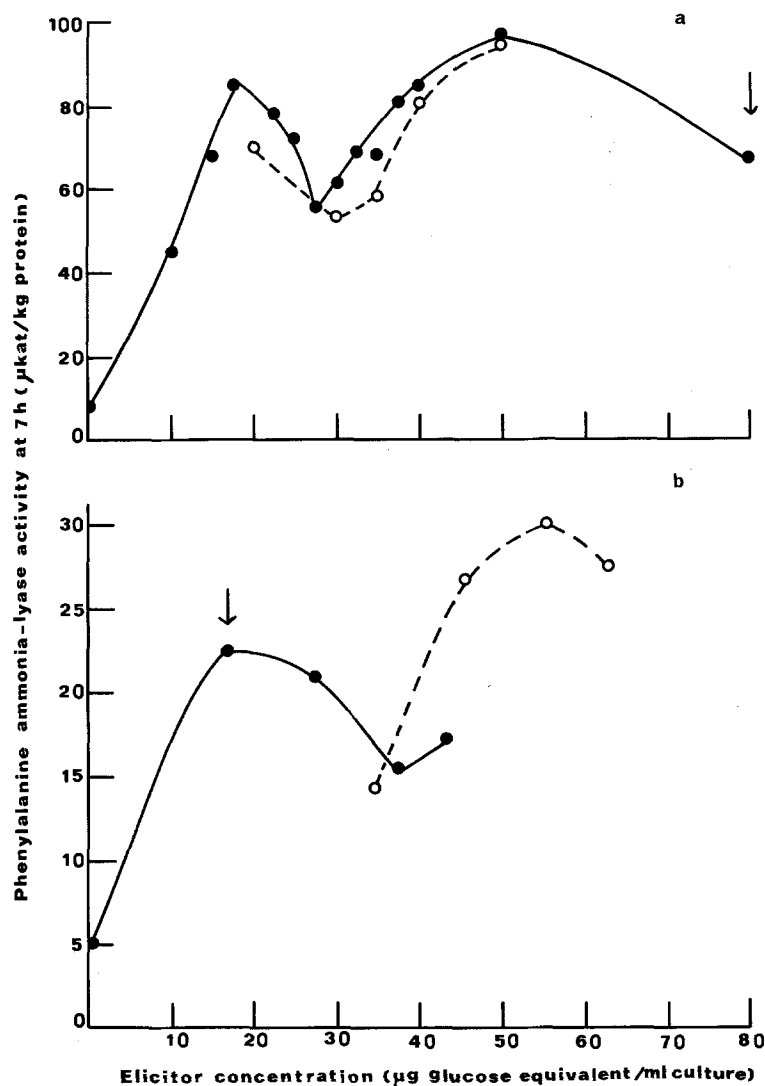
Fig. 1a, b. Dose response curves for the effects of crude *Colletotrichum* elicitor concentration on the induction of PAL in French bean cell suspension cultures. **a** Elicitor was added at zero time at the concentrations shown (●——●), or 80 μg ml⁻¹ elicitor (arrow) was added at zero time and the cultures diluted with conditioned medium 2 h later to give the final concentrations shown (o——o). **b** Elicitor was added at zero time at the concentrations shown (●——●), or 17.5 μg ml⁻¹ elicitor (arrow) was added at zero time and extra elicitor added 2 h later to give the final concentrations shown (o——o)

17.5 and 50 μg glucose equivalents ml⁻¹ (Fig. 1a, filled-in circles). This response has been consistently observed in at least ten independent experiments; in all cases the activity induced by 27.5–40 μg carbohydrate per ml culture was less than that induced by 17.5 and 50 μg ml⁻¹. The absolute activity values of the maxima and minima vary with different elicitor preparations and different culture batches; in the first report of the phenomenon, the activity at 37 μg ml⁻¹ was even lower than that obtained in controls minus elicitor (Lawton et al, 1980). Similar dose response curves for PAL induction have been observed with crude elicitor preparations from races γ and δ of *C. lindemuthianum* (D.L. Murphy, unpublished observations).

De novo synthesis of PAL in the cell cultures may be measured within 2 h following addition of *Colletotrichum* elicitor (Dixon and Lamb 1979). However the extent of the induced response is not finally determined by this time; treatment of cultures with elicitor at a concentration of 80 μg ml⁻¹ for 2 h, followed by dilution of the cultures with conditioned medium to give various lower final elicitor concentrations resulted in a dose response curve of similar shape to that obtained if the final elicitor concentrations had been present throughout the 7 h induction period (Fig. 1a) The dose-response curve for cultures treated with 17 μg elicitor carbohydrate per ml, followed by addition at 2 h of extra elicitor to give various higher final concentrations (Fig. 1b) likewise in-
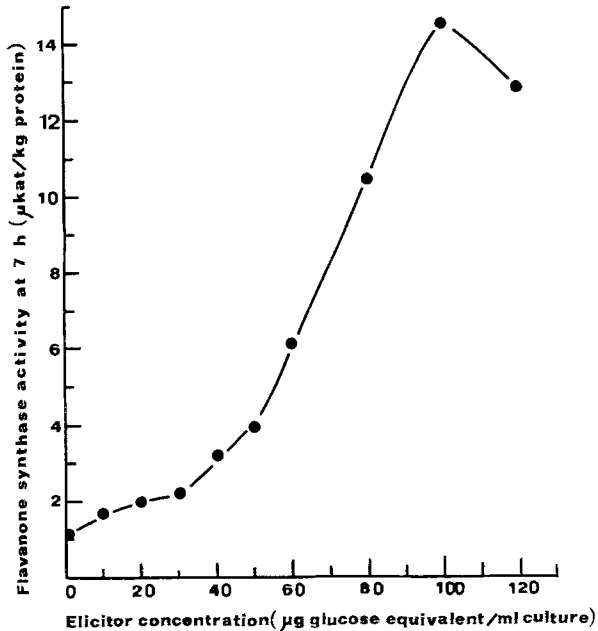
Fig. 2. Dose-response curve for the effects of crude *Colletotrichum* elicitor concentration on the induction of flavanone synthase in French bean cell suspension cultures
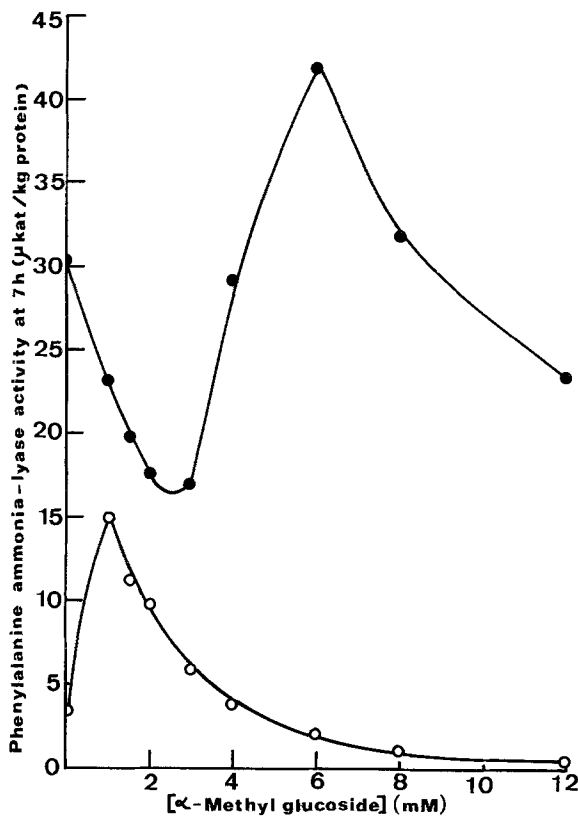

Fig. 3. PAL induction in French bean cell suspension cultures in response to varying concentrations of α-methyl D-glucoside alone (o——o) or in the presence of 20 μg ml⁻¹ crude *Colletotrichum* elicitor (●——●)

dicates the reversibility of the elicitor-mediated response. The lower enzyme activities observed in Fig. 1b are the result of using cultures grown under a lower level of illumination than those used in all other experiments.

The dose-response curve for elicitor induction of flavanone synthase showed only one maximum at around 100 μg carbohydrate per ml culture (Fig. 2). There was little induction of the enzyme over the range of elicitor concentrations which induced the first peak in PAL activity.

In soybean cotyledons, methyl α-D-mannoside inhibits phytoalexin accumulation in response to the elicitor from cell walls of *Phytophthora megasperma* var. sojae (Ayers et al. 1976), whilst in potato tuber discs *Phytophthora infestans* elicitor activity is inhibited by methyl β-D-glucoside and laminaribiose (Marcan et al. 1979). These low molecular weight sugar derivatives may possibly act as "haptens", competing with elicitor for binding sites at the host cell surface (Ayers et al. 1976; Marcan et al. 1979). If the complex elicitor dose-responses observed in the present work are due to the presence in the elicitor preparation of more than one active component, it may be possible, by the use of such compounds, to inhibit selectively only a part of the elicitor dose-response curve. Methyl α- and β-D-glucosides, methyl α- and β-D-galactosides and methyl α-D-mannoside were therefore tested at a range of concentrations for their effects on elicitor-mediated PAL induction in the French bean cultures. Of these derivatives, only methyl α-D-glucoside had significant effects on the PAL response. This compound acted as a weak elicitor of PAL activity when supplied to the cultures in the absence of elicitor (Fig. 3), although its effectiveness declined exponentially with concentrations above 1.0 mM. In contrast, when applied at the same time as crude elicitor (20 μg ml⁻¹), increasing concentrations of methyl α-D-glucoside first inhibited and then stimulated extractable PAL activity.

An alternative approach to investigate the possibility of multicomponent elicitor activity was to fractionate the crude elicitor on Biogel P-150 (Fig. 4). The elution profile for total carbohydrate showed much heterogeneity; the major carbohydrate-containing fraction eluted in the void volume of the column, but a further seven fractions of lower molecular weight were obtained. All fractions were lyophilized prior to further analysis. Fractions A and D contained the most protein, the high 280 nm absorption of fractions G and H being due to phenolic pigments. Each fraction was tested at five concentrations for its ability to induce PAL in the cultures (Fig. 5). Fractions A to H all induced significant levels of the enzyme, but each fraction produced a different dose response
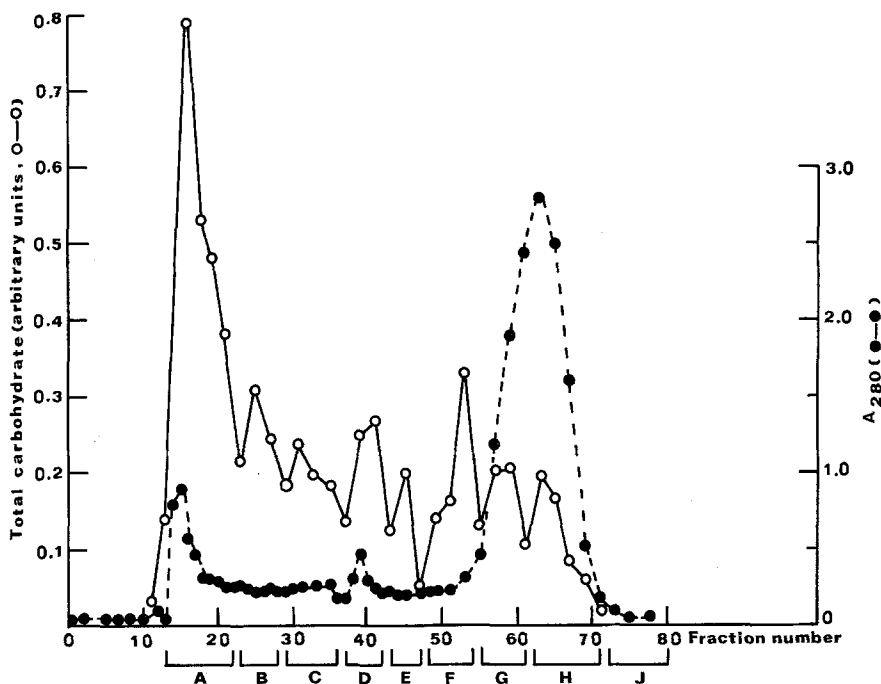
Fig. 4. Fractionation of crude *Colletotrichum* elicitor. A 2 ml sample was applied to a Biogel P-150 column (83·1.5 cm) and eluted with deionized H_2O at a flow rate of 12 ml h^{-1}; 3 ml fraction were collected. Units for total carbohydrate represent the absorbance at 555 nm obtained from a 20 μl aliquot of the fraction following reaction with α-naphthol/sulphuric acid reagent. Fractions corresponding to peaks A to J were pooled as shown, and freeze-dried prior to further analysis
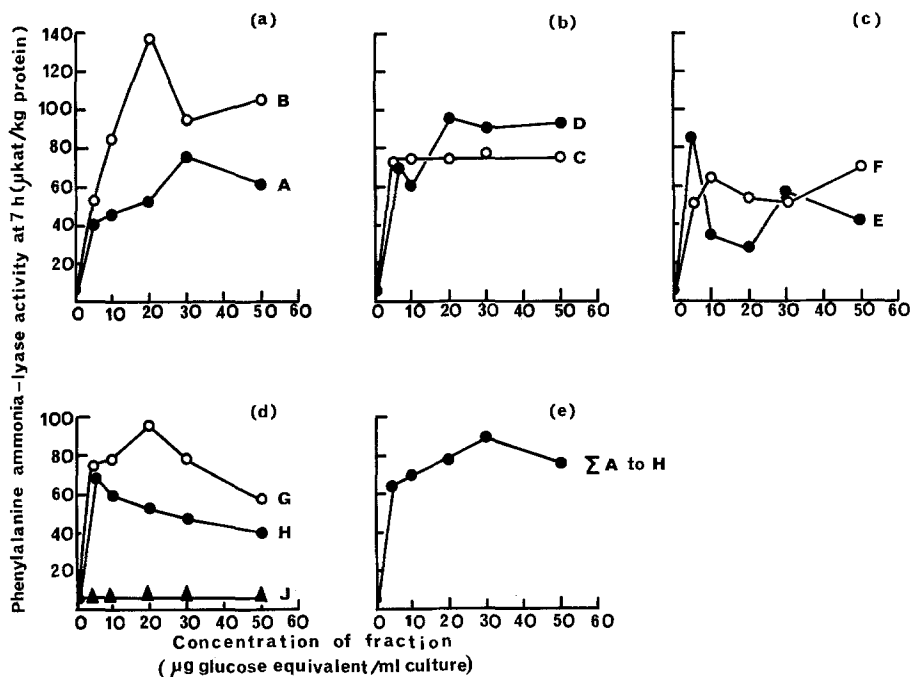

Fig. 5a–e. Dose-response curves for the effects of *Colletotrichum* elicitor fractions A to J as elicitors of PAL activity in French bean cell suspension cultures [graphs (a) to (d)]. Analysis of fractions A to J was performed in a single experiment using cells from the same culture batch. The sum of the curves for the individual fractions, weighted in accordance with the relative proportions of each fraction in the crude elicitor, is shown in graph (e)

curve. Fraction B (20 μg ml^{-1}) gave the highest level of enzyme induction, whereas the response to fraction C was saturated by concentrations above 5μg ml^{-1}. The only fraction to show a double peak in the dose-response was fraction E. The sum of the individual

dose-response curves, when weighted for the relative proportions of each fraction in the crude elicitor, was not equivalent to that obtained for the unfractionated preparation (Fig. 5e).

Elicitor fractions A to H significantly differed in

Table 1. Monosaccharide composition of the crude *Colletotrichum* elicitor and the fractions obtained from it by chromatography on Biogel P-150. The figures in brackets after the fraction number are the percentage total carbohydrate content of the fraction with respect to the crude elicitor. The compounds x_1, x_2, x_3 and x_4 were unidentified pentose-containing oligosaccharides, showing low mobilities in the TLC system used (see Materials and Methods) and giving a pink colour with p-aminobenzoic acid reagent. Their presence may have resulted from incomplete hydrolysis of the fractions

| Monosaccharide		% Composition									
		Galactose	Glucose	Mannose	Arabinose	Ribose	Rhamnose	x_1	x_2	x_3	x_4
R_G^a		0.87	1.00	1.09	1.22	1.39	1.57	0.17	0.29	0.35	0.72
Fraction:											
Crude elicitor	(100)	15.5	17.0	22.9	25.8	14.8	2.9	0.36	—	—	0.74
A	(42.0)	9.2	17.6	21.3	23.5	14.1	4.4	0.91	3.10	—	—
B	(10.9)	11.0	15.3	21.3	27.3	12.95	4.2	0.60	0.84	0.84	5.75
C	(13.5)	24.1	14.5	40.4	12.2	2.5	6.2	—	—	—	—
D	(7.2)	23.2	11.9	38.8	9.3	10.3	6.5	—	—	—	—
E	(4.7)	13.0	13.5	17.6	29.3	15.6	3.2	0.87	0.92	0.92	5.40
F	(6.5)	14.0	12.7	21.0	29.3	11.0	4.0	—	—	1.9	6.01
G	(8.1	10.9	24.8	20.9	23.2	13.9	4.2	—	—	—	2.0
H	(5.9)	1.9	9.6	13.5	25.0	43.6	3.5	—	—	—	1.8

[a] TLC mobility relative to glucose
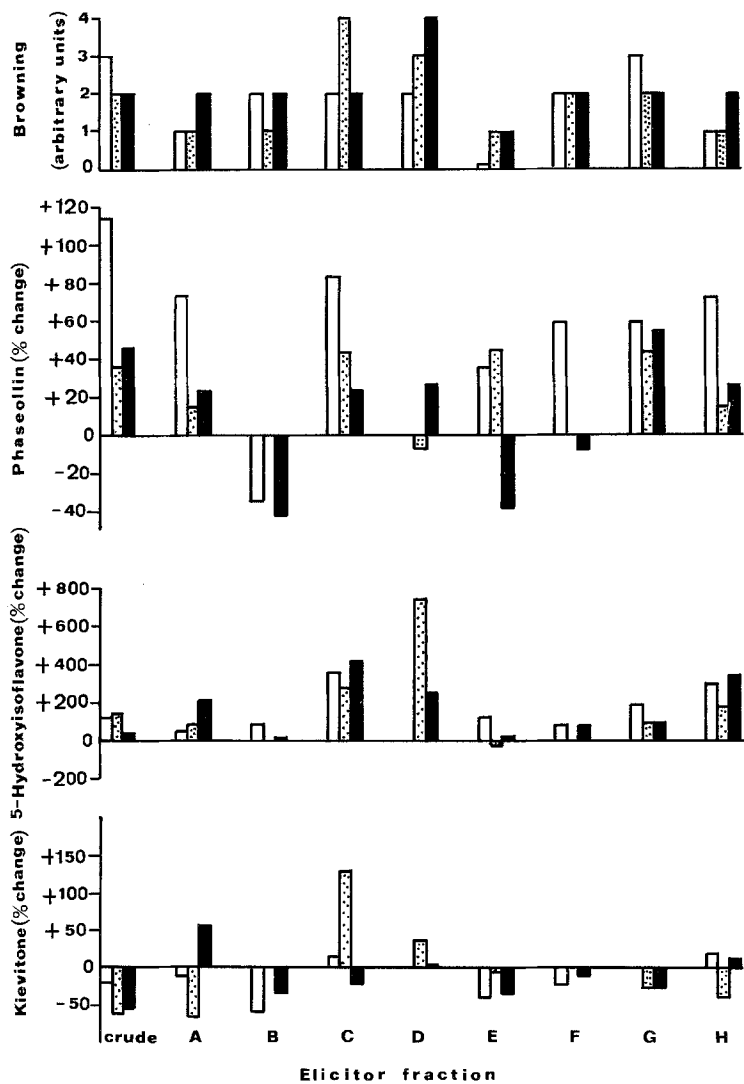
Fig. 6. Response of French bean hypocotyls to crude and fractionated *Colletotrichum* elicitor preparations. Elicitor samples (100 μl) containing 5, 20 or 50 μg glucose equivalents ml^{-1} (open, dotted and filled-in histograms respectively) were applied to the wounded surfaces of four 2 cm-hypocotyl segments, and the hypocotyls incubated in the dark at 25° C for 48 h. Browning was measured on an arbitrary scale of 0–4 (0 = no visible browning; 4 = very dark brown, streaky lesion). Isoflavonoid levels are expressed as the % change compared to control hypocotyls treated with 100 μl sterile distilled H_2O. Control values (per treatment) were: browning, 1.0; phaseollin, 55 μg; 5-hydroxyisoflavone, A_{259} of 0.30; kievitone, 26 μg

monosaccharide composition (Table 1). Fractions A and B appeared similar, with glucose, mannose and arabinose as the major monosaccharides present. Fractions C and D contained mannose as the major monosaccharide, with much lower levels of pentoses. Only fraction G contained glucose as the major monosaccharide. The results are in contrast to a previous report of the carbohydrate composition of the partially purified elicitor from the α race of *Colletotrichum lindemuthianum* (Anderson-Prouty and Albersheim 1975), where only glucose, galactose and mannose were observed following gas chromatography of acetylated elicitor preparations.

The fractionation of the predominantly glucan elicitor from the α race of *C. lindemuthianum* relied upon the bioassay of fractions by measurement of the induction of hypersensitive browning and phytoalexin accumulation in isolated bean hypcotyls and cotyledons (Anderson-Prouty and Albersheim 1975). Three concentrations of each of the present fractions were therefore applied to wounded French bean hypocotyls, and the extent of cellular browning and the levels of three isoflavonoid derivatives (the phytoalexins phaseollin and kievitone and a partially characterised 5-hydroxyisoflavone) were compared to controls treated with sterile distilled H_2O (Fig. 6). There was no constant direct correlation between cellular browning and tissue levels of all three isoflavonoids, but the crude elicitor and fractions A, C and D were the most consistent in inducing positive changes in all four parameters. Fraction B, although inducing slight cellular browning, inhibited phaseollin levels to below those found in wounded-only controls.

Discussion

The dose-response curve for the *Colletotrichum* elicitor-mediated induction of PAL in French bean cell suspension cultures is unusual in that it consists of two maxima separated by a relatively small increment in elicitor concentration. In soybean cell suspension cultures the dose response curves for PAL induction by two partially purified β-1-3-glucan elicitor fractions from *Phytophtora megasperma* var. sojae have single maxima at around 1 and 8 μg hexose equivalents ml^{-1} respectively (Ebel et al. 1976).

There are several possible explanations for the declines in elicitor-mediated responses observed at supra-optimal elicitor concentrations. Firstly, bell-shaped dose response curves of this are typically obtained for responses mediated by binding of lectins to animal cell surfaces (Novogrodsky and Ashwell 1977). If the functional units of fungal elicitors are purely carbohydrate in nature, as has been suggested for the *Colletotrichum lindemuthianum* and *Phytophthora megasperma* elicitors (Anderson 1978; Albers-

heim and Valent 1978), then the lectin component may be a part of the putative receptor for elicitor binding at the host cell surface. Secondly, β-1-3-glucans assume a helical conformation in solution and self-associate at high concentrations (Preston 1979); dose-response curves may therfore reflect elicitor-elicitor as well as elicitor-cell interactions. Another factor, which has received little attention, is the possible toxicity of elicitors at high concentrations. The dose response curves observed in the present work may result from one or more of the above phenomena, although the lowered response observed at intermediate elicitor concentrations would seem to imply the presence in the crude preparation of either two functionally distinct classes of elicitor or a further factor(s) with an inhibitory effect on elicitor-mediated PAL induction.

The elicitor dose-response curve for flavanone synthase induction closely resembles the shape of the curve for *Colletotrichum* elicitor-mediated induction of phaseollin accumulation in the French bean cultures (Dixon and Lamb 1979), although the flavanone synthase assayed in the present work is not believed to be directly involved in the synthesis of 5-deoxyisoflavonoid derivatives such as phaseollin (Dixon and Bendall 1978b). It is not yet clear whether the same or different elicitor components are responsible for the induction of PAL and flavanone synthase; the association of flavanone synthase induction with those elicitor concentrations which potentiate the second peak of the PAL dose-response curve may be coincidental.

Density labelling studies with ^2H from ^2H_2O have demonstrated that PAL induction in response to crude *Colletotrichum* elicitor involves rapid de novo synthesis of the enzyme, and that at intermediate and supra-optimal elicitor concentrations the rate constant for enzyme synthesis is lower than at the two observed optimum elicitor concentrations (Dixon and Lamb 1979; Lawton et al., 1980). Changes in PAL activity therefore reflect changes in PAL synthesis, and the present results suggest that elicitor induction of enzyme synthesis may be reversible. The dose-response curves obtained following dilution of cultures or addition of extra elicitor 2 h after initial treatment (Figs. 1a and 1b) would not be obtained if the overall rates of PAL synthesis during the 7 h induction period were fully determined during the period up to the appearance of newly synthesised active enzyme. Furthermore, if the elicitor dose-response curves reflect elicitor binding phenomena, elicitor must remain reversibly bound to the cells in an active state throughout the early stages of enzyme induction.

The inability of α-methyl sugar derivatives to inhibit all or part of the elicitor dose-response curve may reflect the need for a more complex saccharide structure to compete successfully with elicitor for host

cell recognition sites. High concentrations (>40 mM) of β-methyl-D-glucoside are required to inhibit browning of potato tuber discs in response to mycelial sonicates of *Phytophthora infestans* (Marcan et al. 1979). Similarly, concentrations of water soluble glucans from compatible races of *P. infestans* in the order of 10–100 mg ml^{-1} are required to suppress the hypersensitive response of potato protoplasts to *P. infestans* hyphal wall components (Doke and Tomiyama 1980). In the present work, α-methyl-D-glucoside concentrations above 4 mM inhibit extractable PAL activity in untreated bean cultures (Fig. 3), although stimulation of PAL levels is observed at α-methyl glucoside concentrations of between 4 and 8 mM in the presence of elicitor (20 µg ml^{-1}). There is clearly some functional interaction between α-methyl glucoside and elicitor, but in the present system this may be more complex than simple competition for elicitor binding sites, as has been proposed to explain the effects of methyl sugar derivatives on elicitor activity in other systems (Ayers et al. 1976; Marcan et al. 1979).

The monosaccharide composition of the crude elicitor from the CMI-isolate of *Colletotrichum lindemuthianum* here reported is compatible with the analysis previously reported for crude preparations from the α, β and δ races of the fungus, although significant differences in monosaccharide composition occur between different races (Anderson 1978). Fractionation of the crude elicitor on the basis of molecular size yielded 8 fractions which, on the basis of monosaccharide composition, could be divided into 4 groups; fractions A, B, E and F contained arabinose and mannose as the major monosaccharides, whereas fractions C and D contained approximately 40% mannose, with galactose as the next most abundant sugar and relatively low levels of pentoses. Fraction G was the fraction richest in glucose, while fraction H showed a preponderance of ribose. This fractionation pattern is different from that observed for the α, β and δ races of the fungus, where the highest molecular weight fraction eluted from a Biogel A-5 m column was predominantly glucan in nature and accounted for the major part of the total elicitor activity of the crude preparations (Anderson 1978). The presence of a relatively high proportion of pentose sugars in the hydrolysed crude elicitor agrees with previously published results (Anderson 1978). This pentose material is not the result of cytoplasmic contamination, as a similar amount could be extracted by reautoclaving the wall residue in fresh H_2O for a further 3 h.

In the present work, all 8 fractions obtained by gel-filtration showed elicitor activity as measured by their ability to induce PAL in the bean suspension cultures. The different dose-response curves for PAL induction exhibited by each fraction, and the fact that the sum of these curves does not show the charac-

teristic double maximum observed in the response to the crude elicitor, suggests that heat-treatment of *Colletotrichum* cell walls releases a variety of polysaccharides or glycoproteins differing in molecular size, composition and biological activity which do not act independently when present in a crude mixture. Fractionation studies alone may not therefore resolve the mechanisms underlying the dose-response curves for enzyme induction.

The complexity of the elicitor-mediated response suggested by the above results is based solely upon studies of PAL induction in cell suspension cultures. As the increase in PAL activity generally precedes the accumulation of isoflavonoid phytoalexins in cell suspension cultures (Ebel et al. 1976; Dixon and Bendall 1978b; Dixon and Lamb 1979), such changes in enzyme levels may be a useful marker for elicitor activity. However, the most important criterion for the assessment of elicitor activity is the induced accumulation of phytoalexins in tissues of the whole plant. Assays of *Colletotrichum* elicitor activity in previous work have relied mainly upon the ability of fractions to elicit cellular necrosis when applied to French bean hypocotyls or cotyledons (Anderson-Prouty and Albersheim 1975; Anderson 1978; Theodorou and Smith 1979). Little data has been presented on the actual tissue concentrations of phytoalexins accumulating in response to elicitor preparations, presumably because this involves lengthy extraction procedures. In addition to PAL induction, all the elicitor fractions obtained by gel-filtration in the present work induced cellular necrosis when applied to the wounded surfaces of excised bean hypotocyls, although they did not all induce increased accumulation of phaseollin. Fraction B suppressed phaseollin levels below those observed in wounded-only controls, in spite of the fact that this fraction induced the highest PAL increases in the suspension cultures. Fraction B was also the least effective in inducing 5-hydroxyisoflavone accumulation, and was one of several fractions (including the crude extract) which appeared to suppress kievitone accumulation. Suppressors of hypersensitivity and phytoalexin induction have now been shown in culture fluids from several plant pathogens (Gnanamanickan and Patil 1977; Oku et al. 1977, Doke and Tomiyama 1980), although there have been no reports to date of their occurrence in the cell walls of the pathogen.

A full explanation of the complex dose-response phenomena associated with elicitor-mediated enzyme induction in the cell suspension cultures must await a more systematic study of the interactions between the various elicitor components. The present studies suggest, hower, that such phenomena may not be directly related to the induction of phytoalexin accumulation in the intact plant. Furthermore, attention

is drawn to the possibility that elicitor fractions may have different biological activities, and it may therefore be unwise to rely on a single parameter for the assessment of elicitor activity.

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Note Added in Proof

Separation of monosaccharides by TLC as described under Materials and Methods but using Schleicher and Schüll F1500 silica gel TLC plates in place of Merck No. 5748 silica gel 69 plates indicated the presence of xylose in the hydrolysates of elicitor preparations. This sugar co-chromatographed with ribose on the Merck TLC plates. Values for ribose in the present paper are therefore to be read as values for ribose plus xylose.