Differential responses of bean (Phaseolus vulgaris L.)
to elicitor fractions from Colletotrichum lindemuthianum

by

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Contents

Abstract

Polysaccharide elicitor preparations from culture filtrate and cell walls of Colletotrichum lindemuthianum had broadly similar monosaccharide compositions. Treatment with culture filtrate or cell wall elicitors had no effect on the electrolyte leakage from bean leaf slices or mesophyll cells and resulted in similarly reduced viability of suspension cultured bean cells. Both elicitor preparations had similar effects on the induction of extractable activities, synthesis and mRNA activities of phenylalanine ammonia-lyase, chalcone synthase and chalcone isomerase, on the induction of synthesis and mRNA activities of chalcone synthase multiple subunit isoforms in bean cell suspension cultures and on the patterns of active phenylalanine ammonia-lyase and chalcone synthase multiple forms separated by chromatofocussing. Both qualitative and quantitative differences were observed in the effects of the two elicitor preparations on the accumulation of 5-deoxy and 5-hydroxy isoflavonoids, deposition of wall-bound phenolics and on the levels of free and esterified hydroxycinnamic acids. The two elicitor preparations induced prolyl hydroxylase activity although only the cell wall elicitor induced accumulation of hydroxyproline in the cell walls of suspension cultured bean cells. In addition, although the overall patterns of polysomal mRNA activities in bean cell cultures following treatment with cell wall or culture filtrate elicitors were broadly similar, distinct differences were observed in the effects of the two elicitor preparations on the induction/reduction of the activities of mRNAs encoding a number of polypeptides as shown by two-dimensonal gel electrophoresis.

An unaltered monosaccharide composition and ability to induce phenylpropanoid biosynthetic pathway enzymes were associated with all fractions obtained after chromatography of culture filtrate elicitor on the basis of size and charge. Fractions obtained after affinity chromatography on Concanavalin A-Sepharose had different monosaccharide compositions but exhibited similar effects on the viability of cultured cells and on the induction of synthesis of phenylalanine ammonia-lyase, chalcone synthase and chalcone isomerase as did the crude culture filtrate elicitor. However, although the three Concanavalin A-Sepharose-

purified fractions induced chalcone synthase activity to higher levels than the crude culture filtrate elicitor preparation, little or no induction of phenylalanine ammonia-lyase and chalcone isomerase activities was observed following treatment with the Concanavalin A-unbound and α-methyl mannoside-eluted fractions. In addition, the Concanavalin A-Sepharose-purified fractions induced the activities of mRNAs encoding phenylalanine ammonia-lyase and the most basic chalcone synthase subunit isoform to higher levels than the culture filtrate elicitor. The three Concanavalin A-Sepharose-purified fractions had broadly similar effects on the overall patterns of protein synthesis in vitro although their effects were clearly different from those obtained with crude elicitor preparations. Possible physiological importance of putative multielicitor components with non-identical biological activities and their value in elucidating biochemical control mechanisms underlying regulation and co-ordination of host gene expression are discussed.

ACKNOWLEDGEMENTS

List of Abbreviations

AMPS	-	Ammonium persulphate
BSA	-	Bovine serum albumin
CHI	-	Chalcone isomerase
CHS	-	Chalcone synthase
DNAase	-	Deoxyribonuclease
DTT	-	Dithiothreitol
EDTA	-	Ethylenediaminetetra-acetic acid (disodium salt)
HEPES	-	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
IEF	-	Isoelectric focussing
MES	-	2-[N-Morpholino]ethanesulphonic acid
NP-40	-	Nonidet P-40
PAGE	-	Polyacrylamide gel electrophoresis
PAL	-	Phenylalanine ammonia-lyase
PCPA	-	p-Chlorophenoxy acetic acid
PMSF	-	Phenylmethylsulphonyl fluoride
PPO	-	2,5-Diphenyloxazone
PVP	-	Polyvinylpyrrolidone
RNAase	-	Ribonuclease
SDS	-	Sodium dodecyl sulphate
TCA	-	Trichloroacetic acid
TEMED	-	N,N,N',N'-tetramethylethylenediamine
Tris	-	Tris(hydroxymethyl)methylamine
TTC	-	2,3,5-Triphenyltetrazolium chloride

CHAPTER 1

INTRODUCTION

Contents

INTRODUCTION

1.1   Physiological and biochemical events associated with the expression
        of resistance of plants to disease

Plants are continually exposed to a wide array of pathogens, yet most
plants are resistant to most pathogens, the resistance usually
amounting to total immunity.   The cellular events occurring in
resistant and susceptible plants are briefly outlined and the
mechanisms of resistance to infections discussed in the following
sections.

1.1.1   The infection process

In many cases, pathogens arrive at the host surface as a propagule
such as a spore which must germinate before entry and colonisation
of the host is achieved. Generally, the initial infection process is
similar in both compatible (susceptible) and incompatible (resistant)
interactions.   For   example,   in   the   interaction   between   the
hemibiotrophic anthracnose fungus Colletotrichum lindemuthianum and
bean, fungal conidia germinate to form a swelling, the appressorium.
From the underside of the appressorium an infection peg is developed
which   penetrates   the   cuticle   and   outer   epidermal   cell   wall.
Differences between susceptible and resistant tissues become apparent
following plant cell wall penetration. In compatible interactions the
infection peg within the lumen of the epidermal cell enlarges to form
an intracellular infection vesicle from which intracellular primary
hyphae are developed.   The primary hyphae, surrounded by a host-
produced extrahaustorial matrix (O'Connell et al, 1986) separating the
hyphal wall from the invaginated host plasmalemma, colonise further
host cells by penetrating plant cell walls.   After a period of
biotrophy lasting less than 24 h infected cells degenerate and die
and lesions appear on the plant surface. After an extensive primary
mycelium is established, the mode of parasitism becomes destructive

with secondary hyphae growing within cell walls. Death of host cells and wall dissolution then occurs in advance of secondary hyphae and spreading lesions are formed (O'Connell et al, 1985). In an incompatible interaction however, infection vesicles are not formed and the fungus is restricted to initially infected single hypersensitive epidermal cells, with brown flecks appearing on the surface of infected tissue.

1.1.2 Mechanisms of resistance

Generally, both static and dynamic means of resistance are adopted by host tissues. Static protection is conferred on plants by virtue of preformed structural barriers such as waxy cuticles and suberised epidermal cells. In addition, a number of constitutive substances which possess antimicrobial activity have been reported in several plants. These include alkaloids, phenolic compounds and glucose esters (Sequira, 1980). The classic example of preformed antimicrobial substances are the phenolics catechol and protocatechuic acid governing resistance in coloured onion scales against Colletotrichum circinans (Walker & Stahman, 1955). Dynamic resistance is a term which refers to the sum of active post-infectional metabolic processes that result in inhibition or killing of the pathogen and expression of resistance. The mechanisms of recognition leading to expression of such resistance are the subject of much pathophysiolgical research and will be referred to later in Section 1.3.

1.1.2.1 The hypersensitive response (HR) and the role of phytoalexins

The hypersensitive response is an active defence system of higher plants typified by necrosis of host cells at the site of infection and a concomitant reduction in colonisation rate by the pathogen (Tomiyama et al, 1979). At the microscopic level the HR is characterised by cytoplasmic aggregation, rapid loss of cytoplasmic streaming and cell death (Doke & Furuichi, 1982; Davis & Currier,

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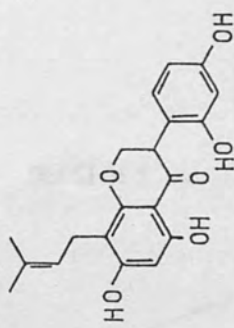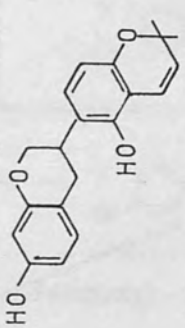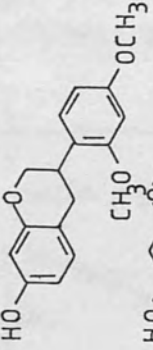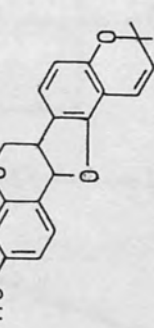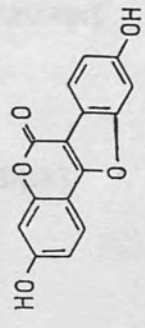
1986). Measurement of death of plant cells has relied on observing their stainability with vital dyes, plasmolysing ability and granulation of cytoplasm (Doke, 1982; O'Connell et al, 1985). Complications to the assessment of the role of HR in disease resistance however, may arise as a result of the reported varying time sequences between plant cell necrosis and inhibition of fungal growth for different cultivars and races and of different levels of resistance of different tissues from a single plant (Bell, 1981).

There now exists a large body of evidence, much of it indirect, to suggest that induction of phytoalexins may be a major feature of the HR, and thus a determinant of resistance in many plant-pathogen interactions. The concept of phytoalexins derives from the work of Müller and Börger (Müller & Börger, 1941) but it was not until the discovery of the presence of the isoflavonoid pisatin in infected pea tissue (Cruickshank & Perrin, 1963) that the validity of the concept was established. A simple 'working redefinition' of phytoalexins states that "phytoalexins are low molecular weight, antimicrobial compounds that are both synthesised by and accumulated in plant cells after exposure to microorganisms" (Paxton, 1981). Phytoalexin accumulation is a wide-spread phenomenon during expression of localised resistance in plant-pathogen interactions. They have been isolated from plants of many families and different plant species were found to produce different groups of closely related phytoalexins. Also the same phytoalexin may be found in a variety of different, but closely related species (Table 1.1).

The main biosynthetic origins of phytoalexins are acetate-polymalonate, shikimate-polymalonate and terpenoid and by far the most studied class is that of the isoflavonoids of the Leguminosae. Isoflavonoid phytoalexins are derived from the amino acid L-phenylalanine via the central phenylpropanoid pathway which is common to the synthesis of phenolics such as hydroxycinnamic acids, lignin and flavonoid pigments (Figure 1.1). In addition to fungal infection, a wide range of chemicals including salts of heavy metals (Hargreaves, 1979), surfactants (Hargreaves, 1981) and U.V. light

Table 1.1

Structures and sources of selected isoflavonoid phytoalexins of the Leguminosae

Chemical class	Trivial name	Structure	Species
Isoflavanone	Klevitone		Phaseolus vulgaris Vigna unguiculata Vigna sinensis
Isoflavan	Phaseollinisoflavan		Phaseolus vulgaris
Isoflavan	Sativan		Medicago sativa
Pterocarpan	Phaseollin		Phaseolus vulgaris Vigna sinensis
Coumestan	Coumestrol		Phaseolus vulgaris Medicago sativa

Shikimate pathway   Proteins

$CH_2CH\,NH_2COOH$
L-phenylalanine

$CH=CH-COOH$
t-cinnamic acid

$CH=CH-COOH$
HO
4-coumaric acid

R $CH=CH-COOH$ ⟶ COUMARINS
HO

DEPSIDES ⟵   R $CH=CH-COSCoA$ ⟶ LIGNINS
HO

3×malonyl CoA

XANTHONES   CHALCONE   BENZOIC
HYDROXYSTILBENES   ACIDS

FLAVANONE

ISOFLAVONES   FLAVANONOL   FLAVONES

PTEROCARPANS

FLAVONOLS   ANTHO-
                                    CYANIDINS

$R = -OH\,, -OCH_3$
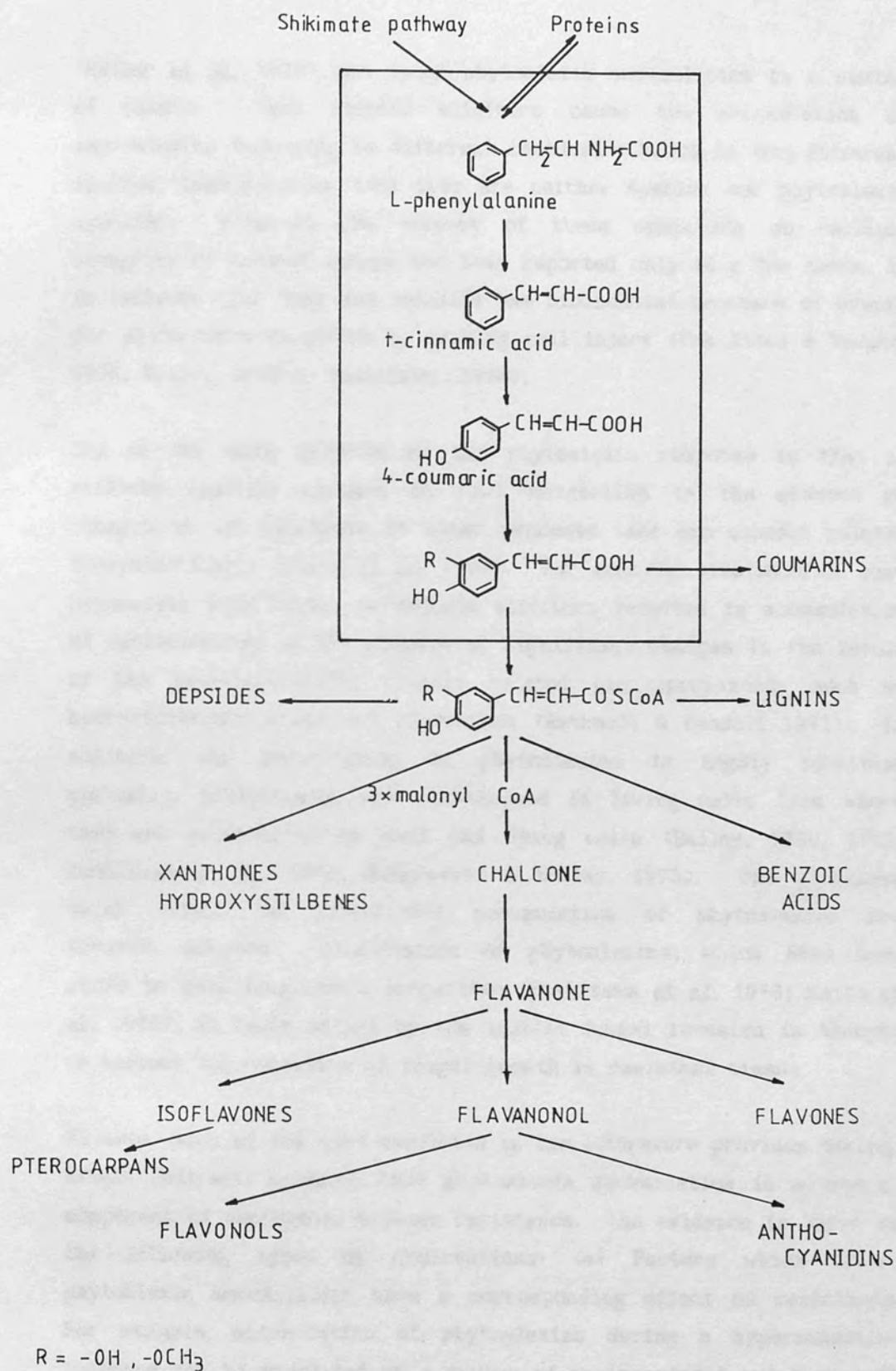
Figure 1·1 — The pathway of phenolic biosynthesis

(Heller et al, 1979) can cause phytoalexin accumulation in a number of plants. Such abiotic elicitors cause the accumulation of phytoalexins belonging to different chemical classes in many different species, demonstrating that they are neither species nor phytoalexin specific. Although the effects of these compounds on cellular integrity of treated tissue has been reported only in a few cases, it is believed that they may initiate the biochemical sequence of events for phytoalexin induction by causing cell injury (Van Etten & Peupke, 1976; Bailey, 1982 a; Yoshikawa, 1978).

One of the main features of the phytoalexin response is that it reflects specific changes in host metabolism in the absence of changes in the synthesis of other products that are closely related biosynthetically (Dixon et al, 1983). For example, treatment of bean hypocotyls with biotic or abiotic elicitors resulted in accumulation of isoflavonoids in the absence of significant changes in the levels of the biosynthetically closely related phenylpropanoids such as hydroxycinnamic acids and flavonoids (Rathmell & Bendall 1971). In addition, the accumulation of phytoalexins is highly localised spatially; phytoalexins are synthesised in living cells from where they are accumulated in dead and dying cells (Bailey, 1980, 1982; Yoshikawa et al, 1978; Hargreaves & Bailey, 1978). The processes which effect the directional accumulation of phytoalexins are however, unknown. Localisation of phytoalexins, which have been shown to have fungistatic properties (Yoshikawa et al, 1978; Smith et al, 1974), in cells killed by the initial fungal invasion is thought to account for cessation of fungal growth in resistant tissue.

To date, much of the work available in the literature provides strong, albeit indirect, evidence that phytoalexin accumulation is a crucial component of necrogenic disease resistance. The evidence is based on the following types of observations: (a) Factors which affect phytoalexin accumulation have a corresponding effect on resistance. For example, accumulation of phytoalexins during a hypersensitive response can be modulated by a number of environmental and ontogenic variables which can be related to corresponding changes in resistance

(Bell, 1981). Also, treatments that induce resistance or susceptibility were frequently found to be associated with induction and reduction of phytoalexin synthesis, respectively e.g. inhibition of glyceollin synthesis and accumulation in soybean by treatment with blasticidin S or actinomycin D resulted in suppressed resistance to Phytophthora species normally pathogenic to soybean (Yoshikawa, 1978). It has been shown however, that if pea pods were inoculated with the pathogen Fusarium solani, incubated for 6 h and then heat shocked, total resistance was observed although only about 10% of the normal level of pisatin was obtained (Hadwiger & Wagoner, 1983). This suggests that phytoalexin induction may not be the only process conferring resistance to F. solani. (b) For many host-pathogen interactions, pathogens are less susceptible (more tolerant) to phytoalexins than non-pathogens. In the Phytophthora megasperma f.sp. glycinea-soybean interaction lesions that developed from mixed virulent and avirulent inoculum had reduced levels of glyceollin, necrosis and browning compared to lesions that developed from avirulent inoculum (Ward, 1983). The survival of the compatible race in incompatible lesions and its ability to escape therefrom after a lag of 24-48 h suggests some mechanism of tolerance of the inhibitory environment within the incompatible lesion. Studies on 59 isolates of Nectria haematococca showed that virulence on pea closely correlated both with tolerance and ability to demethylate the phytoalexin pisatin (Van Etten et al, 1980). Exceptions to this characteristic have however been reported e.g. no correlation was observed between sensitivity to kievitone and pathogenicity to bean for a number of fungi (Smith et al, 1974). (c) Pathogenic fungi can metabolise phytoalexins in a variety of ways involving hydroxylation, methylation and demethylation to less-fungitoxic or non-fungitoxic compounds (Dixon et al, 1983a). For example, in pea cultivars the ability of the pathogen Fusarium solani f.sp. pisi to detoxify pisatin has correlated well with virulence (Fuchs et al, 1980). The ability to metabolise phytoalexins does not however appear to be consistently related to virulence, as shown for example for the Colletotrichum lindemuthianum-bean interaction; both virulent and avirulent races of Colletotrichum convert phaseollin to 6a-hydroxyphaseollin which is

not toxic to the fungus (Dixon et al, 1983). This apparent inconsistency may be due to the fact that bean produces multiple phytoalexins of which kievitone, phaseollidin and phaseollinisoflavan as well as phaseollin are often prominent early in resistance responses (Bailey 1974; Smith et al, 1974). (d) Phytoalexins accumulate rapidly and are closely associated with the expression of necrogenic resistance. In a number of host-pathogen interactions resistance to partially biotrophic organisms is characterised by quicker initiation of phytoalexin accumulation in resistant than in susceptible cultivars, although inhibitory amounts of phytoalexins may accumulate in the susceptible cultivar as necrosis becomes prominent, for example as shown for the P. megasperma-soybean and C. lindemuthianum-bean interactions (Yoshikawa et al, 1978; Bailey & Deverall, 1971; Rahe, 1973; Bell et al, 1984). Thus, in the Phaseolus vulgaris - Colletotrichum interaction it has been proposed that compatibility is associated with the ability of the pathogen to maintain biotrophic growth for an extended period of time as compared with the incompatible interaction where rapid necrosis (hypersensitivity) occurs (Bailey & Deverall, 1971; Bailey et al, 1980). Rapid necrosis of plant cells in an incompatible interaction has been proposed to be the trigger which activates the plant defense responses including phytoalexin accumulation (Bailey, 1982 b; Mansfield 1982). In this respect it is interesting to note that susceptible bean cells were dead 48 h after infection with Colletotrichum lindemuthianum (O'Connell et al, 1985) although there was no detectable accumulation of phytoalexins at this time (Bailey & Deverall, 1971). It is not yet known however, whether death of the initially infected plant cell is a direct consequence of fungal invasion or a result of its synthesising and accumulating phytoalexins above a critical level. Injury to adjoining cells is probably prevented by the plant's detoxification of phytoalexins, a process which has been shown for a number of plants (Dixon et al, 1983).

One important factor in ascertaining the significance of phytoalexins in plant disease resistance is the knowledge of their spatial and

temporal quantitative distribution within plant tissue at or near the site of infection. Recent work on the interaction between soybean and *P. megasperma* f.sp. *glycinea* using radioimmunoassay for quantification of glyceollin and an immunofluorescent stain for localisation of the fungus supports the hypothesis that accumulation of glyceollin is an important early response of soybean roots to infection (Hahn et al, 1985). However, it appears that glyceollin accumulation may not be solely responsible for inhibition of fungal growth in the resistant response as hyphae of the incompatible race were located in tissue containing inhibitory glyceollin concentrations (Hahn et al, 1985). Thus, it appears that the significance of phytoalexins should be sought not only in their antifungal activities or in the timing of their accumulation. Theoretically, some metabolite(s) may enter and regulate microbial cells to become non-aggressive without killing them and without accumulating in detectable amounts at a certain stage of infection.

It is clear from the preceding discussion that there are numerous papers which provide evidence, albeit circumstantial, supporting a primary role for phytoalexin accumulation in disease resistance in a number of host-pathogen interactions. An outstanding exception to this general pattern concerns the infection of pea seedlings by *Aphanomyces euteiches*. *A. euteiches*, sensitive to pisatin *in vitro* , continued to grow in expanding lesions in which pisatin concentration was 8 times the inhibitory concentration *in vitro* (Peupke & Van Etten, 1974). One further question remaining concerns the ubiquity of phytoalexins throughout the plant kingdom. Cucumber and wheat are two examples of plants that do not produce phytoalexins. In these and possibly other plants, processes other than phytoalexin accumulation may contribute to the restriction of microorganisms. Some of these processes are briefly discussed in Section 1.1.2.2 below.

1.1.2.2. Other post-infectional processes

It has been shown that for a number of host-pathogen interactions, host cell walls are modified during infection by lignification (Hammerschmidt, 1984; Hammerschmidt et al, 1984) and accumulation of hydroxyproline-rich proteins that are glycosylated with arabinose and galactose (Esquerré-Tugayé et al, 1979; Hammerschmidt et al, 1984). Some of the possible roles of lignification in plant resistance to fungal attack have been listed by Ride (1978); in addition to increasing mechanical resistance of plant cell walls to the compressive forces during fungal penetration, lignin may also protect the wall against fungal hydrolases and reduce diffusion of fungal enzymes and toxins. Similarly, activation of phenolic metabolism for lignification would generate small M_r phenols which might disrupt fungal membranes or inhibit enzymes and which might reduce the plasticity of the fungus for growth by polymerising within fungal hyphae. The mechanism of action of the hydroxyproline-rich glycoproteins (HRGPs) is not known although they may function as lectins. Proteinase inhibitors, reported to be induced from zero basal levels following infection (Fielding, 1981) may also play a role in disease resistance in some plant-pathogen interactions.

1.2 Fungal elicitors of resitance-related responses

The first published report of an elicitor was of a polypeptide, called monilicolin A, present in mycelial extracts of Monilinia fructicola, which induced phaseollin accumulation in non-host bean endocarp tissue. Although the role of an elicitor molecule from a non-pathogen in vivo is difficult to assess, the term 'elicitor' has since come into general usage and it depicts molecules of microbial origin which induce a host response believed to be characteristic of active defence. Because of their potential role in activation of disease resistance responses, elicitors have been the subject of numerous studies (Section 1.2.1). To date, much of the work on elicitors has concentrated on their purification and characterisation coupled with a bioassay to monitor the presence of elicitor-active molecules. Generally, the bioassay involves introduction of the elicitor preparation to plant tissue cultures or to wounded surfaces of hypocotyls or cotyledons followed by qualitative or quantitative measurement of a selected host resistance response after a suitable incubation period. Typical responses induced by elicitor treatment include a) phytoalexin accumulation or parameters related to it such as optical density of wound droplets (e.g. Albersheim & Valent, 1978), b) induction of phytoalexin biosynthetic enzymes (Dixon et al, 1981; Whitehead et al, 1982), c) accumulation of lignin (Pearce & Ride, 1982; Robertsen, 1986), d) tissue browning associated with cellular necrosis (Marcan et al, 1979), e) leakage of electrolytes from isolated cells (Dow & Callow, 1979 a), f) production of ethylene (Toppan & Esquerré-Tugayé, 1984) and g) accumulation of HRGPs (Roby et al, 1985). Some elicitors of one or more of the above responses have also been shown to induce systemic accumulation of proteinase inhibitors in tomato (Walker-Simmons et al, 1984).

It is important to note that many of the elicitor preparations whose effects have been described to date are impure and that their involvement in intact plant-parasite interactions is difficult to ascertain. A recent report using immunogold labelling techniques showed the presence of antigens known to be present in culture fluids

of the pathogen Colletotrichum lindemuthianum within the matrix layer
separating the fungal cell wall and host plasmalemma (O'Connell et
al, 1986), although recognition of the fungal elicitor, which may be
required for host cell survival, is much more difficult to
demonstrate.

1.2.1   Nature of fungal elicitors

1.2.1.1 Polysaccharides

(i)   Phytophthora megasperma f.sp. glycinea

The most widely investigated elicitors are those obtained from
Phytophthora megasperma f.sp. glycinea, the causal agent of stem and
root rot in soybean. Preparations from cell walls and culture fluids
from this fungal pathogen contain components which induce glyceollin
accumulation in soybean tissue (Ayers et al, 1976 a, b).   Elicitor
activity in preparations from culture fluids of this fungus, partially
purified by ion-exchange chromatography appeared to be associated
with polysaccharides containing predominantly 3-linked glucan (Ayers
et al, 1976 a).   Material solublilised from P. megasperma cell walls
by autoclaving, in addition to inducing phytoalexin accumulation in
soybean, was found to be non-host specific resulting in induction of
a number of phytoalexins in bean cotyledons, rishitin in potato tuber
tissue (Cline et al, 1978) and phenylalanine ammonia-lyase (PAL)
activity in cell suspension cultures of sycamore and parsley (Ebel et
al, 1976).

Ion-exhange and Concanavalin A-Sepharose chromatography of the cell
wall elicitor preparation yielded four distinct fractions with
varying monosaccharide compositions and degree of association with
protein (Ayers et al, 1976 b,c). The most active fraction, fraction I,
was composed predominantly of a branched 3- and 3,6-linked glucan
polymer (Ayers et al, 1976 c) similar in structure to the
extracellular elicitor in the culture filtrate (Ayers et al, 1976 a).
It was concluded that all of the fractions were active because of

their glucan component and that autolytic digestion of the cell wall in later stages of growth was probably responsible for the presence of elicitor components in the culture-filtrate (Ayers et al, 1976 a,b). However, the inhibition of elicitor activity by α-methyl mannoside but not other α-methyl glycosides suggested a possible role for α-linked terminal mannose residues in the elicitor activity of the P. megasperma glucan (Ayers et al, 1976 c).

A complex mixture of elicitor-active and elicitor-inactive oligoglucosides has recently been shown to exist in partial acid hydrolysates of P. megasperma cell walls (Sharp et al, 1984). Only one out of 8 heptaglucosides purified from this mixture was elicitor-active, the nature and activity of this smallest elicitor-active glucoside being confirmed by chemical synthesis (Ossowski et al, 1984). However, it is not clear if the elicitor-active heptaglucoside is the major, or only, molecular determinant involved in induction of plant resistance responses or if other molecules with related or unrelated molecular configurations may have been discarded during purification. The latter point is of particular relevance in view of the isolation from P. megasperma cell walls of a number of elicitor-active molecules (Table 1.2); for example, mycolaminarin (a β-1,3-glucan storage polysaccharide from Phytophthora species) (Ayers et al, 1976 a; Keen et al, 1983), cell surface glycoproteins obtained by NaOH hydrolysis (Keen & Legrand, 1980), or pronase treatment (Keen et al, 1983), of P. megasperma mycelial cell walls and cell wall glucomannans released by soybean β-1,3-endoglucanase treatment of mycelial cell walls (Keen et al, 1983) were all shown to induce phytoalexin accumulation in soybean tissue (Table 1.2). Indeed, the glucomannan fraction was 10 times more active in inducing phytoalexin accumulation than the cell wall β-glucan fraction (Keen et al, 1983).

(ii)   Colletotrichum lindemuthianum

Elicitor-active molecules have been isolated, but only partially purified, from culture fluids and extracts of autoclaved cell walls of

Table 1.2

Nature of fungal elicitors

Source	Bioassay		Chemical nature of elicitor preparation	Reference
	Plant tissue	Plant response		
Phytophthora megasperma cell walls				
a) Autoclaved	Soybean hypocotyls	E_{285} of wound droplet	Heterogenous branched β-1,3-glucan	Ayers et al 1976 a,b; Albersheim & Valent 1978
b) NaOH hydrolysed	Soybean hypocotyls	Glyceollin in wound droplet	Race-specific glycoproteins	Keen & Legrand 1980
c) Pronase treated	Soybean cotyledons	E_{285} of wound droplet	Glycopeptides	Keen et al 1983
d) Soybean β-1,3-endo-glucanase treated	Soybean cotyledons	E_{285} of wound droplet	Race-specific glucomannans, M_r 40 000	Keen et al 1983
Colletotrichum lindemuthianum culture filtrate; autoclaved cell walls	Bean cotyledons and hypocotyls	Browning, phaseollin in wound droplet	Polysaccharides, predominantly 3- and 4- linked glucosyl residues, M_r between 1-5x10^6	Anderson-Prouty & Albersheim 1975
Fusarium solani f.sp. **pisi** & **Fusarium solani** f.sp. **phaseoli**	Pea hypocotyls	Pisatin in wound droplet	Chitosan, a β-1,4-glucos-amine polymer	Hadwiger & Beckman 1980
Cladosporium fulvum				
a) Culture fluids and auto-calved cell walls	Tomato leaves and fruit	Necrosis, rishitin accumulation, electrolyte leakage	Heterogenous peptidogalacto-mannans	De Wit & Roseboom 1980; De Wit & Kodde 1981
b) Intercellular fluids of infected host	Tomato leaves	Necrosis	Race-specific polypeptide, M_r 5000	De Wit et al 1985

Table 1.2 (cont'd)

Source	Bioassay		Chemical nature of elicitor preparation	Reference
	Plant tissue	Plant response		
Colletotrichum lagenarium autoclaved mycelia	Melon petioles	Ethylene synthesis	Galactose-, mannose-rich glycoproteins	Toppan & Esquerré-Tugayé 1984
Phytophthora infestans				
a) Autoclaved cell walls	Potato	Agglutination of protoplasts; cellular necrosis of tuber discs	β-1,3-glucans	Peters et al 1978; Marcan et al 1979
b) Mycelial extracts	Potato tuber discs	Necrosis and rishitin accumulation	Lipids, arachidonic and eicosapentaenoic acids	Preisig & Kuć 1985; Creamer & Bostock 1986
Monilinia fructicola cell walls	Bean	Phaseollin accumulation	Polypeptide, M_r 8 000	Cruickshank & Perrin 1968
Rhizopus stolonifer culture filtrate	Castor bean seedlings	Casbene synthesis	Polygalacturonase, a glycoprotein of M_r 32,000. Carbohydrate moeity composed of 92% 1,2-linked mannosyl residues and 8% glucosamine	Lee & West 1981
Erwina carotovora culture filtrate	Soybean cotyledons	Pterocarpan synthesis	Endopolygalacturonic acid lyase	Davis et al 1984

Colletotrichum lindemuthianum, the causal agent of anthracnose in bean (Anderson-Prouty & Albersheim, 1975; Anderson, 1978; Theodorou & Smith, 1979; Whitehead et al, 1982). The active fractions in culture filtrate elicitor preparations were reported to be high M_r heterogenous polysaccharides containing glucose units in both β-1,3 and β-1,4 linkage together with much smaller amounts of other neutral hexoses, mostly galactose and mannose (Anderson-Prouty & Albersheim, 1975; Anderson 1978). Later reports suggested a possible attachment of elicitor-active polysaccarides to protein and the existence of differences in monosaccharide composition and relative potency of elicitor preparations from different races of the fungus (Anderson, 1980). Elicitor-active components from autoclaved Colletotrichum cell walls were also reported to be heterogenous polysaccharide-containing molecules (Whitehead et al, 1982).

(iii) Phytophthora infestans

Initial reports using a number of bioassay parameters suggested that glucans from the cell wall of Phytophthora infestans, the causative agent of late blight in potatoes, were reponsible for elicitation of resistance responses in potato tubers. Fractions rich in glucan from P. infestans cell walls were shown to agglutinate potato protoplasts (Peters et al, 1978). Later work showed that extracts of P. infestans mycelial homogenates were non-host specific (Doke et al, 1979) and non-race-specific inducers of necrosis and suggested the possible requirement for β-1,3-linked glucan residues for elicitor activity (Marcan et al, 1979). However, the subsequent use of a somewhat different extraction and purification procedures to prepare two fractions from P. infestans mycelia suggested that most of the elicitor activity resides in components other than cell wall glucans (Kuratz & Zacharius, 1981) (see Section 1.2.1.4), and that glucans from P. infestans enhance elicitor activity of lipid fractions from the same fungus (Maniara et al, 1984) but possess no elicitor activity on their own (Kurantz & Zacharius, 1981; Maniara et al, 1984). A recent report indicates that culture fluids of race 4 of P. infestans contains a higher M_r elicitor of both browning and

- 19 -

sesquiterpenoid phytoalexin accumulation in potato tubers (Keenan et al, 1985). Elicitor activity was not reduced by heat and pronase treatments although periodate oxidation resulted in loss of activity suggesting that carbohydrate may be required for elicitation. The culture filtrate elicitor did not interact synergistically with arachidonic acid (see Section 1.2.1.4)

(iv)   Fusarium solani

Recent work has implicated the cell wall polysaccharide chitosan, present in conidia as a dormancy factor, as an elicitor (Hadwiger & Beckman, 1980). Chitosan, a β-1,4-linked polymer of D-glucosamine is a constituent of the cell walls of Fusarium solani and a number of classes of fungi. A role for chitosan in disease resistance was proposed on the basis of its potency as an elicitor of pisatin accumulation in interactions between pea and F. solani (Hadwiger & Beckman, 1980). In addition, chitosan has been shown to induce lignification and the synthesis of proteinase inhibitor in wheat and tomato, respectively (Pearce & Ride, 1982; Walker-Simmons & Ryan, 1986).

1.2.1.2 Glycoproteins

(i)   Cladosporium fulvum

The best characterised fungal polypeptide elicitors have been isolated from culture filtrates of Cladosporium fulvum, the tomato leaf mould pathogen. From the culture fluids, a family of glycopeptides of disperse M_r, charge and sugar composition, containing phosphate in the form of diester, were isolated (Dow & Callow, 1979 a). These were shown to induce electrolyte leakage in leaf mesophyll cells of tomato (Dow & Callow, 1979 b) and the accumulation of rishitin, pisatin and glyceollin in tomato, pea and soybean tissues respectively (De Wit & Roseboom, 1980). Chemical composition of the extracellular peptidogalactomannan elicitor was shown to be dependent on the composition of the growth medium and

age of culutre (De Wit & Kodde, 1981), an important observation in relation to reports of different chemical compositions of elicitors prepared in different laboratories. Mannose and galactose residues were implicated in the elicitor activity of the extracellular elicitor which is thought to originate from peptidogalactomannan on the cell wall surface (De Wit & Kodde, 1981).

(ii)   Others

Other glycoprotein elicitors worthy of mention are the mannose- and galactose-rich glycopeptides extracted from cell walls and culture fluids of Colletotrichum lagenarium which induce ethylene synthesis in melon (Toppan & Esquerré-Tugayé, 1984) and the glycoprotein fractions from Phytophthora megasperma cell wall and Colletotrichum lindemuthianum. The relationship between the carbohydrates in the elicitor-active polysaccharides (Section 1.2.1.1) and glycoproteins is not yet clear.

1.2.1.3   Peptides and enzymes

(i)   Monilicolin A

Monilicolin A is a polypeptide of M_r 8 000 isolated from the mycelia of the stone-fruit pathogen Monilinia fructicola (Cruickshank & Perrin, 1968). It induces phaseollin accumulation in the non-host bean but not in stone-fruit host tissue. It is not known whether this protein posseses enzymic activity, but its elicitor activity appears to be relatively heat-stable (Cruickshank & Perrin, 1968).

(ii)   Cladosporium fulvum

Race-specific elicitors of necrosis and chlorosis were found in the intercellular culture fluids of tomato leaves infected with Cladosporium fulvum (De Wit & Spikman, 1982). This is in contrast to the previously reported non-race specific glycoproteins present in the culture fluids of the fungus (Section 1.2.1.2). Race-specificity

was found to be determined by the fungal race and not the plant cultivar (De Wit et al, 1984) and was attributed to a polypeptide of M_r 5 500 thought to be the product of the fungal avirulence gene A9 (De Wit et al, 1985).

(iii) Nucleases

Ribonuclease and deoxyribonuclease were found to elicit phaseollin accumulation in cell cultures of Phaseolus vulgaris (Dixon & Fuller, 1977). Elicitor activity required high concentrations of the nucleases and was not lost after autoclaving. These enzymes are believed to act by virtue of their polycationic charge which may allow them to interfere with host plasma membrane integrity (Dixon et al, 1983).

(iv) Cell wall-degrading fungal enzymes

In a number of cases microbial enzymes capable of degrading host cell walls were found to possess potent elicitor activity dependent on the maintenance of enzyme activity. Examples include the polygalacturonase from culture fluids of Rhizopus stolonifer which induces synthesis of the phytoalexin casbene in castor bean seedlings (Lee & West, 1981) and the endopolygalacturonic acid lyase from Erwina carotovora which induces synthesis of pterocarpan derivatives in soybean cotyledons (Davis et al, 1984). Both the Erwina and Rhizopus pectic enzymes are believed to act by releasing elicitor-active polysaccarides from plant cell walls. This will be discussed in Section 1.3.

1.2.1.4 Fatty acids

Involvement of lipid elicitors from Phytophthora infestans in the expression of disease resistance in potato comes from experiments in which insoluble glucan and lipid fractions from cell walls of P. infestans resulted in induction of cellular necrosis and rishitin accumulation in potato tubers (Kurantz & Zacharius, 1981). It should

be noted however, that both fractions were insoluble and assayed at high concentrations and that the lipid fraction was inactive on its own. Later work has shown that arachidonic and eicosapentaenoic acids, both components of the fungal mycelia, were host-specific (Bloch et al, 1984), non-race specific (Preisig & Kuc, 1985) elicitors of phytoalexin accumulation. Elicitation by lipids is enhanced by the presence of fungal glucan fractions (Maniara et al, 1984; Preisig & Kúc, 1985).

1.2.2 Molecular determinants of race-specificity

1.2.2.1 Elicitors

A basic pattern which has emerged from genetical analysis of several cultivar-race plant-pathogen interactions is that one gene in the host for resistance is complemented by a single gene in the pathogen for avirulence (Ellingboe, 1981). This relationship is known as the gene-for-gene hypothesis and holds irrespective of the number of loci in the host which may be able to confer resistance. The race-specific elicitor-receptor model is generally accepted as the simplest model to account for induced resistance in gene-for-gene interactions in which the plant 'resistance gene' is envisaged to code for an active receptor molecule which binds to the product of the pathogen 'avirulence gene', proposed to be the elicitor molecule. Thus, following the discovery of elicitor molecules, several workers have attempted to demonstrate the existence of race-specific elicitors, believed to be the primary determinants of avirulence. To date, reports in the literature on race-specific elicitors are often confusing and their existence is somewhat controversial. An early report of a race-specific glycoprotein fraction from culture fluids of Phytophthora megas p erma var glycinea (Wade & Albersheim, 1979) was later withdrawn by the group as a result of variation in the bioassay (Desjardins et al, 1982). Race-specific glycoprotein and glucomannan elicitors have been reported by Keen and his co-workers from P. megasperma cell walls (Keen & Legrand 1980; Keen et al, 1983). Pseudomonas syringae p.v. glycinea (Brueger & Keen, 1979) and

Cladosporium fulvum (De Wit & Spikman, 1982; De Wit et al, 1985) were also reported to produce race-specific elicitors; in the latter case they were only found in intercellular fluids of infected host tissue (Table 1.2).

It should be pointed out that harsh 'unphysiological' methods are often employed in the isolation of elicitor molecules. Fungal elicitors released by more physiological methods, for example from culture fluids of infected plants or by treatment of fungal cell walls with cell wall-degrading host enzymes may be more useful in the search for race-specific elicitors. In this respect it is interesting to note that race-specific elicitors were isolated from fungi by either of these methods but not from fungi grown in culture (De Wit & Spikman, 1982; Keen et al, 1983). Although fluids recovered from infected plants might provide a useful source of possible race-specific elicitors, problems may arise if, for example, no diffusible elicitors were produced by the fungus; elicitors were not detected in fluids of lettuce plants infected with the lettuce downy mildew fungus Bremia lactucae (Crucefix et al, 1984).

Even if only non-race-specific elicitors involved in resistance determination were isolated, then assuming a physiological role for these elicitors, other molecules may act to determine race-specificty. Such molecules may exert their effect at the elicitor receptor or at other sites. In the former case, they would be predicted to affect the overall induced metabolic changes in a quantitative but not qualitative manner.

1.2.2.2 Specificity factors

The term specificity factor is used to describe a microbial molecule with the ability to modify one or more disease resistance responses but which does not, on its own, have an effect on the resistance repsonse. The operation of a specificity factor in the Phytophthora megasperma f.sp. glycinea-soybean interaction was proposed by Albersheim and his co-workers. They argued that as glyceollin

accumulated at identical rates in soybean hypocotyls treated with elicitor or fungal mycelia from virulent or avirulent races of the pathogen, the decrease in the rate of fungal growth in incompatible interactions was a result of a primary response triggered by a 'specificity factor' (Ayers et al, 1976 b).

1.2.2.3 Enhancers and suppressors

Enhancers and suppressors are types of specificity factor whose effects on elevated or decreased phytoalexin levels are thought to have a direct bearing on the outcome of the host-pathogen interaction. The most studied examples of enhancers and suppressors have been isolated form Phytophthora infestans. Evidence for the production of suppressor molecules was obtained from dual inoculation experiments in which prior inoculation with a virulent race of the fungus suppressed resistance to a subsequent challenge with a non-pathogen or virulent race (Heath, 1980). Water-soluble glucans from compatible and incompatible races suppressed terpenoid accumulation in potato tuber discs following infection with an incompatible race or treatment with crude elicitor preparations (mycelial sonicates) from compatible or incompatible races of the fungus (Garas et al, 1979). The glucans from the compatible race were more active in suppressing the HR than those from the incompatible race (Doke et al, 1979; Garas et al, 1979). Later work showed that agglutination of potato protoplasts by a glucan elicitor from P. infestans was suppressed by race-specific glucan components from the fungus (Doke & Tomiyama, 1980), although they were required at 400 times the elicitor concentration for suppression of agglutination.

In addition to glucan elicitors (Section 1.2.1.1 (iii)) and glucan suppressors, the picture is further complicated by reports of glucan enhancers from P. infestans. Elicitor-inactive, branched chain glucan components enhanced the activity of the elicitor arachidonic acid (Maniara et al, 1984). The molecular sites of action and the relationships between elicitor, suppressor and inhibitor preparations in the P. infestans-potato interaction remain to be elucidated.

1.3   Mode of action of fungal elicitors

The gene-for-gene hypothesis, widely accepted to explain coevolution of host-pathogen systems, suggests that race-specific elicitor molecules or race-specific enhancer of suppressor molecules which may render elicitor activity race-specific, should be products of avirulence genes of the pathogen.   Receptors for race-specific or race-specifically produced microbial elicitor molecules are therefore predicted to be candidates for the products of host resistance genes (Ellingboe, 1981).   However, there is as yet no conclusive data on the location and nature of these putative elicitor-binding sites and much of the available evidence in favour is circumstantial and indirect. Thus elicitor activity was shown to be inhibited by a number of methyl-sugar derivatives which were proposed to act by competing for elicitor binding (Ayers et al, 1976 c; Marcan et al, 1979) and elicitor preparations from Phytophthora infestans were shown to cause rapid agglutination of potato protoplasts (Peters et al, 1978). More recently, the presence of elicitor-specific receptors has been suggested by work using radiolabelled elicitor-active ligands for membrane binding studies using membrane preparations from plants. Thus in soybean cotyledons, maximum specific binding of ^{14}C-labelled mycolaminarin per mg protein was found to be associated with a fraction tentatively identified as containing plasma membranes, although binding was observed throughout membrane fractions (Yoshikawa et al, 1983).   Also, binding of ^{3}H-labelled partially purified glucan elicitor from P. megasperma to a plasma membrane fraction from soybean cotyledons was displaced by treatment with elicitor-active, but not elicitor-inactive preparations (J. Ebel, unpublished results).   The use of pure homogenous elicitor preparations such as the structurally defined heptaglucoside elicitor from Phytophthora megasperma f.sp. glycinea cell walls (Sharp et al, 1984) and the development of improved methods for plasma membrane fractionation and identification should circumvent the problems so far encountered as a result of the use of crude elicitor preparations and facilitate further work on the analysis of putative elicitor-binding sites.

Even in the absence of data on elicitor receptors, it is clear that elicitors can exert profound effects on plant plasma membranes such as stimulation of electrolyte leakage (Dow & Callow, 1979 a) and rapid depolarisation of the trans-membrane potential (Katou et al, 1982). However, a causal sequence of events between these early effects of elicitor and phytoalexin accumulation has not been established. It is also not clear whether elicitor acts as a trigger or is continuously required for maximal enzyme or phytoalexin induction. In bean cell cultures a requirement for continual presence of elicitor and reversible binding rather than rapid triggering has been suggested, the extent of PAL induction was relatively independent of the concentration of a crude elicitor preparation from cell wall of Colletotrichum lindemuthianum during the lag phase preceding increased enzyme appearance, whereas alteration of elicitor concentration 2 h after elicitation yielded a response 5 h later which was proportional to the final elicitor concentration (Dixon et al, 1981).

Early reports on the release of dialysable molecules from autoclaved (Hargreaves & Bailey, 1978) or freeze-thawed (Hargreaves & Selby, 1978) bean hypocotyls which were capable of inducing phytoalexin accumulation in bean cell cultures and hypocotyl sections indicated that molecules of host origin may act as intercellular transmitters of elicitation. The main candidates so far proposed as endogenous elicitors are host cell wall fragments. Albersheim's group demonstrated that pectic fragments obtained by partial acid hydrolysis of soybean cell walls and citrus pectin had similar elicitor activity (Hahn et al, 1981; Albersheim et al, 1981; Nothnagel et al, 1983) and that a dodeca-α-1,4-D-galacturonide was the most active elicitor in both these preparations (Nothnagel et al, 1983). Further indirect proof of a causal role for pectic fragments in phytoalexin accumulation came from studies on the release of elicitor-active molecules from soybean cell walls (Davis et al, 1984, 1985 a,b) and from sodium polypectate (Davis et al, 1986 a) by polygalacturonic acid lyase from Erwina carotovora and from castor bean cell walls by polygalacturonase from Rhizopus stolonifer (Bruce

& West, 1982). The highest specific elicitor activity released from the sodium polypectate by Erwina endo-polygalacturonic acid lyase resided in a linear α-1,4-D-decagalacturonide (Davis et al, 1986 a) which appears to be structurally closely related to the α-1,4-linked tridecagalacturonide elicitor from Rhizopus endo-polygalacturonase digests of commercial polygalacturonic acid (Jin & West, 1984). Additional evidence demonstrating a synergistic effect between the endogenous elicitor of soybean cell wall and the β-glucan elicitor of P. megasperma (Davis & Albersheim, 1984; Davis et al, 1986 b) supports the proposed physiological role of oligogalacturonide elicitors in induction of phytoalexins during microbial attack.

Highly active elicitors of lignification released from cucumber cell walls by autoclaving were found to be oligomers of polygalacturonic acid with the most active oligomer consisting of eleven galacturonosyl residues (Robertsen, 1986). Elicitor-active pectic fragments were also reported from carrot cell walls (Kurosaki & Nishi, 1984). These were later shown to consist of two active molecular species in which the participation of a peptide as well as oligouronide were suggested (Kurosaki et al, 1985). Plant pectic polysaccharide fragments also appeared to play a role as endogenous elicitors in the induction of protease inhibitors in wounded tomato plants (Ryan et al, 1981) and ageing tomato, tobacco and alfalfa cell cultures (Walker-Simmons & Ryan, 1986).

Characterisation of a peptide or pectic polysaccharide fragment as an endogenous elicitor does not establish its role as a 'second messenger' and other candidates have been considered, notably the gaseous plant hormone ethylene. Induction of ethylene production preceded induction of hydroxyproline-rich glycoprotein synthesis in melon tissue treated with glycopeptide elicitors from mycelium, cell walls and culture fluids of Colletotrichum lagenarium (Toppan & Esquerré-Tugayé, 1984) or with endogenous elicitor released from autoclaved melon cell walls (Roby et al, 1985). However, inconclusive results on the role of ethylene as an endogenous elicitor were reported in a number of host-pathogen interactions (Dixon, 1986).

Nuclear DNA has been proposed as the direct site of action of the biotic elicitor chitosan (Hadwiger & Beckman, 1980). Such a proposal excludes the need for specific intracellular response-couplers but is thought unlikely to apply to the majority of known biotic and abiotic elicitors as it is difficult to reconcile with the observation that a range of compounds with remarkably different structures could all possess the ability to switch on gene expression directly in a highly specific manner (see Section 1.4.1). Further study of the events between initial recognition of elicitor molecules and subsequent changes in host gene expression is clearly needed.

1.4 Molecular biology of induced resistance

1.4.1 Selectivity of end-product accumulation, enzyme induction and mRNA appearance in infected or elicitor-treated plant cells

A variety of metabolically unrelated biochemical responses known to be specifically induced in plant tissue in response to microbial stress or treatment with microbial elicitors are now recieving considerable attention. These responses include the accumulation of lignin-like phenolic material in the host cell wall (Bolwell et al, 1985 b), synthesis of hydroxyproline-rich glycoproteins which may act as bacterial agglutinins (Leach et al, 1982; Bolwell et al, 1985 b) and rapid induction of ethylene synthesis (Chappell et al, 1984). By far the most known and studied is the phytoalexin response.

An early study on the effects of both biotic and abiotic elicitors on bean hypocotyls has indicated that, within the area of phenylpropanoid metabolism, isoflavonoid accumulation is induced specifically in the absence of changes in the levels of biosynthetically related phenylpropanoids (Rathmell & Bendall, 1971). This also appears to be the case in bean cell cultures treated with the abiotic elicitor denatured ribonuclease (Dixon & Bendall, 1978 a) or with elicitor heat-released from the cell walls of Colletotrichum lindemuthianum (Bolwell et al, 1985 a), although here cell wall-bound phenolics also increased on elicitation.   In addition to phenylpropanoid accumulation, increased levels of wall bound hydroxyproline-rich glycoproteins (HGRPs) were also observed in elicitor-treated bean cell cultures (Bolwell et al, 1985 a).

A survey of the available literature shows that two main approaches have been adopted to investigate changes in host cell metabolism related to the induction of resistance-related end-product accumulation. The first approach employs two-dimensional isoelectric focussing: SDS-polyacrylamide gel electrophoretic (2D IEF: SDS-PAGE) analysis of changes in the total patterns of proteins newly synthesised in vivo or in vitro from isolated mRNA, whereas the

second approach involves screening for changes in the extractable activities and rates of synthesis of a number of strategically selected enzymes in plant cells undergoing induced resistance responses.

The use of 2D IEF: SDS-PAGE analysis of protein synthesis has identified a number of newly synthesised polypeptides, whose appearance may be correlated with resistance expression, in pea endocarp challenged with Fusarium solani f.sp. phaseoli (Wagoner et al,1982), in bean cell cultures exposed to elicitor from the cell walls of Colletotrichum lindemuthianum (Cramer et al, 1985 b), in soybean cell cultures exposed to a β-glucan elicitor from the cell walls of Phytophthora megasperma (Grab et al, 1985) and in parsley cell cultures treated with elicitor from P. megasperma (Kuhn et al, 1984; Somssich et al, 1986). Two-dimensional gel analysis of translation products from mRNA isolated by organomercurial affinity chromatography from elicitor treated bean cells pulse-labelled with thiouridine in vivo has demonstrated that many of the induced polypeptides are the products of newly synthesised mRNA (Cramer et al, 1985 b). Although these types of experiments do not themselves lead to the actual identification of induced gene products, they may prove useful in directing attention to polypeptides suitable for further study. For example, DNA sequences specific for the induced state were selected from poly A mRNA from pea pods infected with Fusarium solani f.sp. phaseoli by differential hybridisation to cDNAs complementary to induced and non-induced message (Riggleman et al, 1985). These clones were used in hybridisation experiments to detect the appearance of specific gene transcripts following induction. The extent of induction of the mRNAs corresponding to the above clones was shown to vary between 2 to 20 fold and one mRNA appeared to be inducible by excision alone (Riggleman et al, 1985). Although none of the cDNAs specific for the induced state has yet been identified, hybrid-release translation experiments would allow these clones to be related to the induced polypeptides observed on two-dimensional gels (Wagoner et al,1982). In this manner, the elicitor-induced appearance of two low M_r, low pI proteins in parsley cells was shown to be

associated with rapid increases in the transcription rates of their genes following elicitation (Somssich et al, 1986).

The picture that emerges from studies on specific enzyme activities is one of induction of a limited number of enzymes directly related to the formation of the specifically induced end-product. Thus, induction of isoflavonoid biosynthesis in elicitor-treated bean cell cultures involves the induction of enzymes specifically involved in channeling of primary metabolites e.g. phenylalanine into the induced secondary pathway (see Section 1.4.2.1) (Dixon & Bendall, 1978 b; Bolwell et al, 1985 a; Robbins et al,1985). Induction of these enzymes occurs in the absence of significant changes in the extractable activities of a number of other enzymes metabolically peripheral to phytoalexin biosynthesis (Robbins et al, 1985).

It is also clear, however, that microbial stress can induce changes in enzymes other than those directly involved in phytoalexin synthesis; soybean hypocotyls and cell cultures showed marked induction of acetyl Coenzyme A carboxylase, glutamate dehydrogenase and glucose-6-phosphate dehydrogenase together with elicitor from the same fungus respectively (Börner & Grisebach,1982; Ebel et al, 1984). The three former enzymes are not induced in elicitor-treated bean cell cultures or infected hypocotyls (Robbins et al, 1985). Interestingly, in cultured bean cells the activities of glutamate dehydrogenase (Robbins et al, 1985) and 4-coumarate hydroxylase (Bolwell et al, 1985 a) which were increasing in untreated control cultures, showed no increase following addition of elicitor. The suppression of these enzymes, which are not involved in phytoalexin biosynthesis in bean, by elicitor is reminiscent of the effects of P. megasperma elicitor on light-induced CHS and acetyl CoA carboxylase activities in parsley cell cultures (Hahlbrock et al, 1981). The latter enzymes are involved in the synthesis of flavonoids and not in the biosynthesis of furanocoumarin phytoalexins (Tietjen et al, 1983) produced by parsley.

Although the above results are not exhaustive, they suggest that phytoalexin induction is associated with selective gene expression. In addition, such studies have revealed that different elicitor preparations may induce different patterns of phytoalexin accumulation (Tietjen et al, 1983) and that elicitors may also switch off the appearance of enzymes not directly related to phytoalexin accumulation (Tietjen & Matern, 1983; Kombrink & Hahlbrock, 1986).

1.4.2 Mechanisms underlying the transient appearance of induced gene products
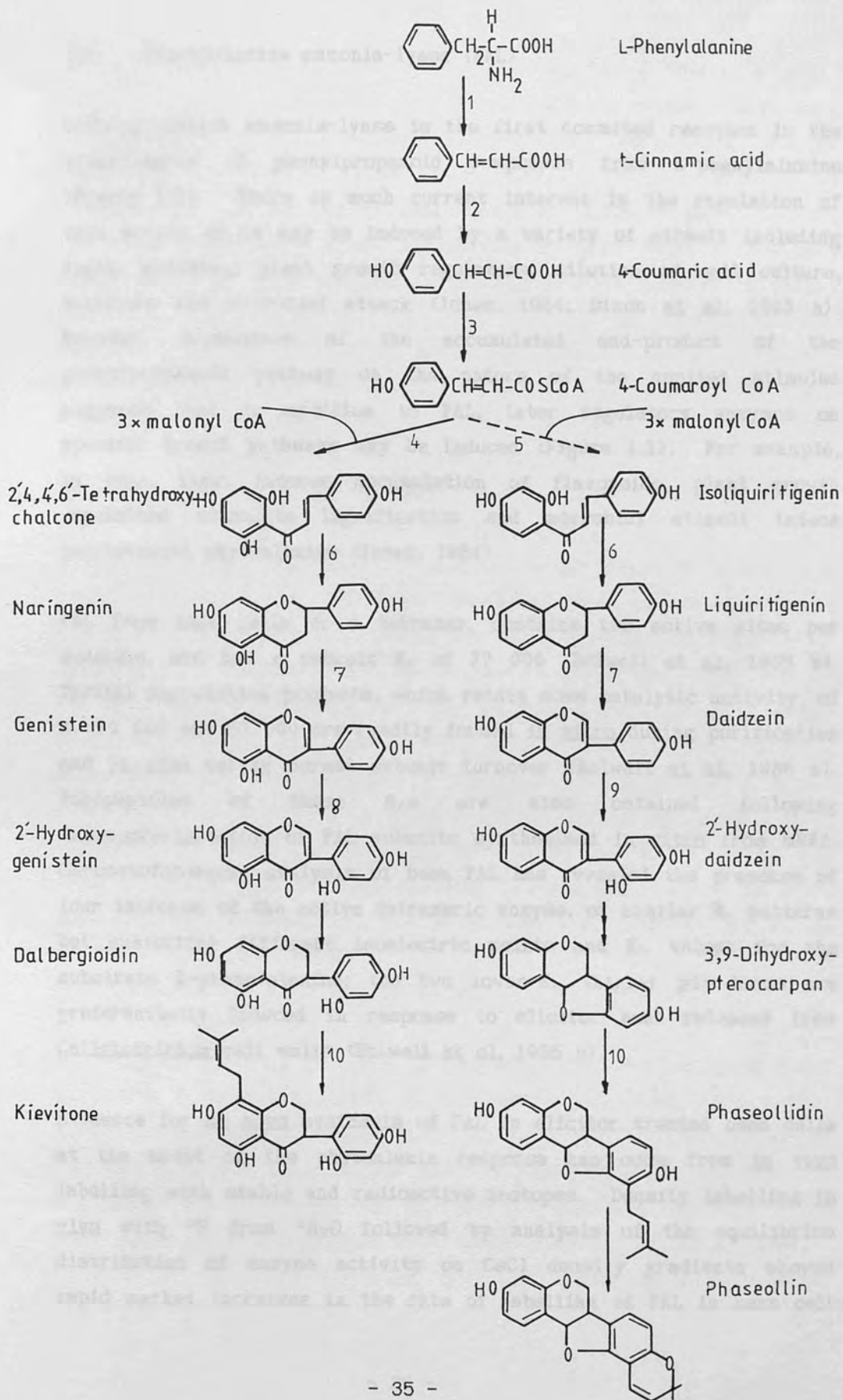
Much effort has recently been expended on the study of the induction of genes encoding enzymes involved in the accumulation of end-products related to active defence. Although these genes cannot themselves represent resistance genes in the sense of the gene-for-gene hypothesis, studies on their induction are important as they may lead to an understanding of the genetic regulatory sequences characteristic of stress-induced products and the molecular signals which link expression of such genes to the early recognitional events which determine incompatibility. The most studied active defence response in plants is the accumulation of phytoalexins although attention has also recently been focussed on the defence-related accumulation of wall bound hydroxyproline-rich glycoproteins.

1.4.2.1 Enzymes of isoflavonoid phytoalexin biosynthesis

A large body of work is available in the literature on the enzymology of the phenylpropanoid-derived isoflavonoid phytoalexin biosynthetic pathway (Figure 1.2). The metabolic pathway of isoflavonoid phytoalexin biosynthesis from L-phenylalanine in bean is well elucidated as far as the chalcone isomerase (CHI) reaction (Figure 1.2). The enzymes of the central phenylpropanoid pathway are also involved in the synthesis of wall-bound phenolic components. Later reactions specific for isoflavonoid formation have so far only been investigated in soybean (Glycine max).

Figure 1.2 -<u>Biosynthesis of isoflavonoid phytoalexins in *Phaseolus*</u>
vulgaris

(1) L-Phenylalanine ammonia-lyase; (2) Cinnamic acid 4-hydroxylase;
(3) 4-Coumarate: CoA ligase; (4) 6'-Hydroxychalcone synthase; (5) 6'-
Deoxychalcone synthase; (6) Chalcone isomerase; (7) Isoflavone synthetase;
(8) Genistein 2'-hydroxylase; (9) Daidzein 2'-hydroxylase;
(10) Dimethylallyl transferase(s)

L-Phenylalanine

t-Cinnamic acid

4-Coumaric acid

4-Coumaroyl CoA

3 × malonyl CoA

3 × malonyl CoA

2',4,4',6'-Tetrahydroxy chalcone

Isoliquiritigenin

Naringenin

Liquiritigenin

Genistein

Daidzein

2'-Hydroxy-genistein

2'-Hydroxy-daidzein

Dalbergioidin

3,9-Dihydroxy-pterocarpan

Kievitone

Phaseollidin

Phaseollin

(i)   Phenylalanine ammonia-lyase (PAL)

L-Phenylalanine ammonia-lyase is the first commited reaction in the
biosynthesis of phenylpropanoid compounds from L-phenylalanine
(Figure 1.2). There is much current interest in the regulation of
this enzyme as it may be induced by a variety of stimuli including
light, wounding, plant growth regulators, dilution of cell culture,
elicitors and microbial attack (Jones, 1984; Dixon et al, 1983 a).
However, dependance of the accumulated end-product of the
phenylpropanoid pathway on the nature of the applied stimulus
suggests that in addition to PAL, later regulatory enzymes on
specific branch pathways may be induced (Figure 1.1). For example,
in bean, light induces accumulation of flavonoids, plant growth
regulators stimulate lignification and microbial stimuli induce
isoflavonoid phytoalexins (Jones, 1984).

PAL from bean cells is a tetramer, contains two active sites per
molecule, and has a subunit M_r of 77 000 (Bolwell et al, 1985 b).
Partial degradation products, which retain some catalytic activity, of
M_r 70 000 and 53 000 are readily formed in vitro during purification
and in vivo during normal subunit turnover (Bolwell et al, 1986 a).
Polypeptides of these M_rs are also obtained following
immunoprecipitation of PAL subunits synthesised in vitro from mRNA.
Chromatofocussing analysis of bean PAL has revealed the presence of
four isoforms of the active tetrameric enzyme, of similar M_r patterns
but exhibiting different isoelectric points and K_m values for the
substrate L-phenylalanine; the two lower-K_m (higher pI) forms are
preferentially induced in response to elicitor heat released from
Colletotrichum cell walls (Bolwell et al, 1985 b).

Evidence for de novo synthesis of PAL in elicitor treated bean cells
at the onset of the phytoalexin response has come from in vivo
labelling with stable and radioactive isotopes. Density labelling in
vivo with ^2H from ^2H_2O followed by analysis of the equilibrium
distribution of enzyme activity on CsCl density gradients showed
rapid marked increases in the rate of labelling of PAL in bean cell

cultures treated with biotic or abiotic elicitors (Dixon & Lamb, 1979). Later work in which CsCl gradients were replaced with high resolution KBr density gradients enabled newly synthesised labelled enzyme to be resolved from pre-existing unlabelled enzyme and demonstrated that at low concentrations of Colletotrichum elicitor, increased PAL activity arises exclusively by stimulation of the rate of de novo synthesis against a constant rate of removal of active enzyme (Lawton et al, 1980). In contrast, at higher elicitor concentrations the increase in enzyme activity was accompanied by a marked apparent stabilisation of the enzyme in vivo, and the rapid but transient increase in enzyme activity was achieved by a program of reciprocal change in the rate constant for de novo enzyme production and the rate constant for removal of enzyme activity (Lawton et al, 1980). However, the information obtained from density-labelling methods is kinetic rather than molecular and putative inactive or processed forms of the enzyme cannot be monitored. More detailed information had to await the availability of specific anti-(PAL)sera. Hence, the rapid de novo synthesis of PAL in response to elicitor treatment or fungal infection in a number of systems was confirmed by in vivo ^{35}S-methionine labelling experiments and the increased synthetic rate was shown to result from increased translatable mRNA activity (Hahlbrock et al, 1981; Löschke et al, 1983; Lawton et al, 1983 a, b; Ebel et al, 1984). In bean, increased mRNA activity encoding PAL was observed in the total cellular RNA, polysomal RNA and polyA RNA fractions (Lawton et al, 1983 b) and was therefore not simply the result of selective recruitment of PAL mRNA into polysomes.

The elicitor-mediated increase in PAL mRNA activity has been shown to result from increased PAL gene transcription. PAL cDNA probes have recently been produced and characterised and their use in Northern blot hybridisation has shown a rapid transient induction of PAL mRNA level (Edwards et al, 1985). Analysis of translation products of newly synthesised mRNA isolated from thiouridine-labelled bean cells by organomercurial affinity chromatography provided

corroborating evidence for increased PAL gene transcription following elicitation.

In addition to transcriptional and translational regulation it is now clear that the levels of PAL activity in elicitor treated bean cell cultures may also be modulated post-translationally (Lawton et al,1980; 1983 a).   A number of studies have indicated that cinnamic acid (CA), the product of the PAL reaction (Figure 1.2) may act as a modulator of PAL turnover.   Thus, treatments which prevent the accumulation of CA were found to bring about prevention of PAL induction (Dixon et al, 1980).   By the use of density-labelling techniques it has been shown that the effect of CA on PAL activity in pea epicotyl sections is mediated by a dual mechanism involving inhibition of enzyme synthesis and stimulation of the rate of removal of active enzyme (Shields et al, 1982).   More recently, Northern blotting and nuclear transcript run-off analyses have shown rapid cessation of PAL transcription and/or removal of hybridisable mRNA following addition of CA to elicitor- induced cells during the initial period of increased enzyme synthesis(Bolwell et al, 1986 b).   Pulse-chase and immune-blotting experiments have indicated that CA does not affect the rate of turnover of PAL subunits in vivo, but rather mediates irreversible inactivation of the enzyme by active site modification (Bolwell et al, 1986 b).

Analysis of ^{35}S-methionine labelled PAL immunoprecipitates by two-dimensional gel electrophoresis has revealed complex subunit multiplicity for the enzyme synthesised both in vivo and in vitro from mRNA.   At least 11 subunit isoforms of identical M_r but differing pI exist in vivo whereas up to 5 charge forms were observed following in vitro translation of bean mRNA (Bolwell et al, 1985 b; 1986 a).   Analysis of five PAL cDNA clones isolated from a library of sequences complimentary to elicitor-induced bean PAL mRNA has however, failed to reveal differences in open reading frame sequences which could generate the observed polymorphism (Edwards et al, 1985).   None of these clones however was full length.   As PAL partial degradation products were observed to exhibit greatly reduced

subunit polymorphism (11 forms of M_r 77 000, 2 forms of M_r 70 000 and one form of M_r 53 000) (Bolwell et al, 1986 a), loss of N-terminal fragments during PAL partial degradation was suggested as a possible explanation for the decreased subunit multiplicity with decreasing M_r of the PAL partial degradation products (Dixon et al, 1986 b). Three PAL cDNAs differed in their 3'-untranslated regions with respect to the distance between the translation stop signal and the polyadenylation signal yielding total 3'-untranslated region lengths of from 132 to 129 base pairs (Edwards et al, 1985). More recently, analysis of bean PAL genomic clones has indicated the presence of multiple PAL genes which may encode subunits of altered amino acid composition (K. Edwards et al, unpublished results). The design of highly specific PAL sequence probes should facilitate studies on the differential expression of PAL at the molecular genetic level.

The molecular mechanisms underlying induction of PAL activity appeared to be different between UV-irradiated and elicitor-treated parsley cell cultures; analysis of PAL mRNA levels in parsley cell cultures has shown that the kinetics of appearance of PAL mRNA levels correlated with the observed changes in rates of PAL synthesis, mRNA activity and enzyme activiy in UV-irradiated but not elicitor-treated cells (Kuhn et al, 1984). This lack of correlation was further confirmed when rates of PAL mRNA synthesis were measured in nuclear transcription run-off experiments (Chappell & Hahlbrock, 1984) and it was suggested that the parsley PAL cDNA clone may cross-hybridise to other elicitor-induced mRNAs of similar size. Interestingly however, use of a different parsley cell culture line or a more purified elicitor preparation resulted in co-ordinate changes in the kinetics of PAL mRNA activity and levels (Schmelzer et al, 1986). It is not yet known whether the different experimental conditions resulted in induction of different forms of PAL.

(ii)   Cinnamic acid 4-hydroxylase (CA4H)

The second enzyme of the phenylpropanoid pathway, cinnamic acid 4-hydroxylase (CA4H) (Figure 1.2) is a cytochrome P_{450}-dependent microsomal mixed function oxygenase and its activity is induced in response to biotic and abiotic elicitor treatment of bean cell cultures (Dixon & Bendall, 1978 b; Bolwell et al, 1985 a). Use of an anti-(rat P_{450}) monoclonal antibody originally raised against a rat cytochrome P_{450} but which recognises a highly conserved epitope, has identified an M_r 48 000 membrane-bound polypeptide in bean. Dot blot, pulse-labelling and in vitro translation analyses showed that the M_r 48 000 polypeptide level, rate of synthesis and mRNA activity were rapidly and co-ordinately induced with the increase in CA4H activity (Bolwell et al, 1985 a; Bolwell & Dixon, 1986).

(iii)   4-Coumarate Coenzyme A ligase (4CL)

4-Coumarate Coenzyme A ligase (4CL) was found to exist in isozymic forms in some plants (Hahlbrock & Grisebach, 1979). Phytoalexin induction in suspension cultured cells of bean (Dixon & Bendall, 1978 b), soybean (Hahlbrock et al, 1981; Hille et al,1982) and parsley (Tietjen & Matern, 1983) was associated with increased activity of 4CL. Temporally co-ordinate induction of mRNAs encoding PAL and 4CL preceeded the accumulation of furanocoumarin phytoalexins in elicitor-treated parsley cell cultures (Hahlbrock et al, 1981; Ragg et al, 1981) and of glyceollin accumulation in elicitor treated soybean cell cultures and infected hypocotyls (Schmelzer et al, 1984).

It appears that more than one 4CL mRNA species may be induced following elicitor treatment of parsley cell cultures and two polypeptides of the same M_r but different pI were detected on two-dimensional gel analysis of in vitro translational products from RNA hybrid-released from a 4CL cDNA clone (Kuhn et al, 1984). Although differential rates of PAL and 4CL gene transcription were observed in nuclei isolated from elicitor-treated parsley cells (Chappell & Hahlbrock, 1984), the kinetics of induction of 4CL mRNA levels were

co-ordinate with induced levels of mRNA species encoding two as yet unidentified low M_r acidic proteins (Somssich et al, 1986).

(iv)   Chalcone synthase (CHS)

Chalcone synthase (CHS) catalyses the formation of the C_{15} intermediate 2',4,4',6'-tetrahydroxychalcone by condensation of three molecules of malonyl CoA with one molecule of 4-coumaroyl CoA (Figure 1.2). This is the first commited step in the biosynthesis of flavonoids/isoflavonoids from the central phenylpropanoid pathway. The A-ring hydroxylation pattern of the chalcone product reflects the hydroxylation pattern of 5-hydroxyisoflavonoids such as kievitone. The existence of a separate 6'-deoxy CHS necessary for the formation of 5-deoxyisoflavonoid derivatives such as phaseollin and glyceollin was postulated on the basis of ^{13}C-acetate labelling experiments (Dewick et al, 1982), although it is possible that 6'-hydroxy CHS may serve as a condensing enzyme in both cases. To date there has been no report of the direct characterisation of a 6'-deoxy CHS in a cell-free system. The bean 6'-hydroxy CHS is a dimer whose subunit appears on one-dimensional SDS-PAGE as a doublet of approximate M_r 42 000 and 43 000 (Bell et al, 1984). CHS subunits synthsised in vivo and in vitro from mRNA from both bean and soybean have recently been shown to exhibit charge multiplicity following two-dimensional isoelectric focussing: SDS-polyacrylamide gel electrophoretic analysis (T.B. Ryder, personal communications; Grab et al, 1985).

Accumulation of isoflavonoid phytoalexins was preceeded by increased CHS activity in both bean (Dixon & Bendall, 1978 b; Dixon et al, 1981) and soybean (Ebel et al, 1984). It was subsequently shown by in vivo labelling and in vitro translational studies using a specific anti-(parsley CHS)serum that de novo synthesis and increased mRNA activity of CHS were associated with the phytoalexin response in cell cultures of both bean (Lawton et al, 1983 a,b) and soybean (Ebel et al, 1984; Schmelzer et al, 1984). Bean hypocotyls infected with Colletotrichum lindemuthianum showed clear temporal and spatial

differences in the induction pattern of CHS mRNA activity between compatible and incompatible interactions prior to the onset of phytoalexin accumulation (Bell et al, 1984). In bean cell cultures, CHS activity was efficiently induced only at high elicitor concentrations, although marked induction of CHS synthesis occured at lower concentrations suggesting the operation of post-translational control over the rate of accumulation of CHS activity (Lawton et al, 1983 b). In contrast to the rapid loss of PAL activity however, elicitor-induced CHS activity was relatively unaffected by exogenous additions of cinnamic acid to bean cells (Dixon et al, 1986 c).

Use of a cDNA probe complementary to CHS mRNA from UV-irradiated parsley cells in Northern hybridisation analyses has demonstrated rapid accumulation of CHS mRNA in both infected and elicitor-treated soybean cell cultures (Schmelzer et al, 1984). The parsley CHS cDNA probe was used to identify a clone from a cDNA library containing sequences from elicitor-treated bean cells and the cloned bean CHS cDNA sequences were used to measure the induction kinetics of CHS mRNA in elicitor-treated bean cells by Northern blot hybridisation analysis (Ryder et al, 1984). More recently, the increase in bean CHS mRNA levels was shown to reflect increased de novo synthesis of the message (Cramer et al, 1985 b).

(v)   Chalcone isomerase (CHI)

Chalcone isomerase (CHI) catalyses the stereospecific isomerisation of chalcones to their corresponding flavanones (Figure 1.2). The enzyme from bean is active against both 6'-hydroxy and 6'-deoxy chalcones (Dixon et al, 1982) indicating that differential regulation of the appearance of 5-hydroxy and 5-deoxy substituted phytoalexins in bean (Whitehead et al, 1982) is not regulated at the CHI level. Although CHI activity is usually high in unelicited bean cell cultures and its overall response is slower and of a smaller magnitude compared to those of PAL and CHS (Dixon & Bendall, 1978 b), it is nevertheless believed to play a role in isoflavonoid phytoalexin accumulation as (a) its activity is strongly inhibited by the

isoflavonoids kievitone and coumestrol (Dixon et al, 1982), (b) its activity increases prior to phytoalexin accumulation in elicitor-treated bean cell cultures (Dixon & Lamb, 1979; Dixon & Bendall, 1978 b) and (c) the magnitude and timing of its induction parallels those of the preceeding PAL and CHS in infected hypocotyls (Dixon et al, 1986 b). The bean isomerase is a monomeric enzyme of subunit M_r 27 000 and does not exhibit charge polymorphism (Robbins & Dixon, 1984).

Both infection and elicitor treatment of bean cells result in de novo synthesis of the enzyme as shown by density labelling with ^2H from ^2H_2O (Dixon et al, 1983 b), in vivo pulse-labelling with ^{35}S-methionine followed by specific immunoprecipitation (Robbins & Dixon, 1984) and by in vitro translation of polysomal mRNA (Cramer et al, 1985 a, b). Results from density labelling and immune-blotting experiments however suggest that a proportion of increased activity levels arises from enzyme activation (Dixon et al, 1983 b; Robbins & Dixon, 1984). Whether activation of CHI is a direct result of elicitor action or increased cinnamic acid levels following elicitor induction of PAL activity is not yet clear; exogenous cinnamic acid has been shown to induce CHI activity in bean cell cultures (Gerrish et al, 1985). It is clear however that increased appearance of CHI message in elicitor-treated bean cell cultures is a result of activated gene expression as CHI mRNA was present in the newly synthesised RNA fraction (Cramer et al, 1985 b).

1.4.2.2 Hydroxyproline-rich glycoprotien synthesis

The induction of hydroxyproline-rich glycoproteins (HRGPs) in response to both fungal infection and host cell wall elicitor treatment has been observed in a number of systems (Bolwell, 1984; Esquerré-Tugayé et al, 1979; Hammerschmidt et al, 1984) and was suggested to be correlated with expression of disease resistance. HRGPs are major structural components of plant cell walls and may function in defence as specific agglutinins of microbial pathogens (Leach et al, 1982) and/or as structural barriers. HGRPs are

initially synthesised as proline-rich polypeptide precursors whose polypeptide residues are post-translationally hydroxylated in the endomembrane system by the enzyme prolyl hydroxylase (peptide proline: 2-oxoglutarate dioxygenase). Further modification leads to glycosylation of a significant number of hydroxylated amino acids predominantly with arabinose-rich side chains.

In bean cell cultures, the elicitor-induced accumulation of HRGPs in the cell walls was more rapid than that of phytoalexins and was preceeded by a rapid but transient increase in prolyl hydroxylase and arabinosyl transferase activities, cessation of transfer of arabinose to polysaccharide acceptors and increased levels of an M_r 42 500 hydroxyproline-rich arabinosylated glycoprotein (Bolwell, 1984; Bolwell et al, 1985 a). Labelling in vivo and in vitro translation experiments followed by immunoprecipitaton with antiserum raised against bean prolyl hydroxylase have demonstrated that in bean cell cultures, elicitor induces de novo synthesis of this membrane-bound hydroxylase more rapidly than the induction of PAL synthesis (Bolwell & Dixon, 1986). Rapid initial transient increase in prolyl hydroxylase activity was closely related to the increase in the M_r 42 500 glycoprotein although a later less rapid increase in the hydroxylase activity was not accompanied by increases in the arabinose acceptor protein (Bolwell et al, 1985 a). The later response may be related to the appearance of the extensin class of HGRPs which is distinct from the M_r 42 500 glycoprotein. The appearance of extensin mRNA in elicitor-treated bean cell cultures, measured by Northern hybridisation analysis using a tomato extensin genomic clone, was less rapid but more prolonged compared to the appearance of mRNAs encoding phytoalexin biosynthetic enzymes (Showalter et al, 1985). In Colletotrichum-infected bean hypocotyls however, mRNAs encoding the HRGPs of the extensin family accumulated with similar kinetics to the appearance of the mRNAs encoding the phytoalexin biosynthetic enzymes (Showalter et al, 1985).

1.4.3 Integration and co-ordination of induced responses

The picture that emerges from the above studies of induced changes in _de novo_ appearance of gene products during expression of resistance in plants is that elicitation or infection results in rapid transient induction of resistance-related proteins, usually as a consequence of elevated mRNA activities or levels and that a degree of co-ordination exists with respect to the timing of such changes. Thus, in elicitor-treated bean cell cultures, under certain conditions the induction of PAL, CHS, and CHI appears to be highly co-ordinated with respect to the kinetics of changes in enzyme activities, rates of synthesis _in vivo_ and mRNA activities measured in the total and polysomal mRNA fractions (Cramer _et al_, 1985 a). Co-ordinate induction of enzyme synthesis is also observed in bean hypocotyls during race-cultivar specific interaction with _Colletotrichum lindemuthianum_ (Cramer _et al_, 1985 a). This is suggestive of a similar mechanism of induction of these enzymes presumably involving rapid induced gene transcription (Ryder _et al_, 1985; Cramer _et al_, 1985 a, b; Edwards _et al_, 1985). Co-ordinated kinetics of induction of PAL and CHS extractable activities and mRNA activities are similarly observed in elicitor-treated soybean cultures (Ebel _et al_, 1984; Hille _et al_, 1982).

Similar patterns of protein synthesis and mRNA activity changes, demonstrated in the bean cell culture system for elicitor-induced CHS, prolyl hydroxylase and cytochrome P_{450} activities (Bolwell & Dixon, 1986; Bolwell _et al_, 1985 a; Dixon, 1986) suggest a common mechanism underlying the initial induction of synthesis of components which may be functionally related but metabolically unrelated such as phytoalexins, HGRPs and wall-bound phenolics. Other genes, for example those encoding the extensin family of HRGPs in elicitor-treated bean cell cultures and whose mRNA levels increase less rapidly but over a more prolonged period than observed for mRNAs encoding enzymes of phytoalexin biosynthesis (Showalter _et al_, 1985), may be switched on later in response to different secondary signals.

Although increased synthesis, reflecting mRNA changes, is involved in the induction of all the enzymes so far studied in the elicitor-bean cell culture system, subsequent post-transcriptional events may alter significantly the kinetics of appearance of gene products whose initial synthesis was induced co-ordinately. Thus, although in elicitor-treated bean cell cultures, increased synthesis of CHI is rapidly induced with that of PAL and CHS, CHI activity is induced much less rapidly and over a longer time period than is that of PAL or CHS (Robbins & Dixon, 1984). Post-translational modification of de novo synthesised CHI, e.g. via the effects of phenylpropanoid pathway intermediates such as cinnamic acid (Gerrish et al, 1985), may be responsible for the observed differences in the characteristic shapes of the enzyme activity profiles.

The pattern of co-ordination of changes in expression of phenylpropanoid pathway enzymes in elicited bean and soybean cells is however different from that observed during light induction of flavonoid accumulation in parsley cells where enzymes of the central phenylpropanoid pathway (PAL, CA4H and 4-coumarate: Coenzyme A ligase) are induced together but earlier than enzymes of the flavonoid branch pathway (CHS and CHI) which themselves form a co-ordinately induced group (Hahlbrock & Grisebach, 1979). The induction of co-ordinately regulated groups of enzymes in UV-irradiated parsley cell cultures (Shröder et al, 1979; Hahlbrock et al, 1982; Ragg et al, 1981) is based on rapid changes in amounts and activities of the corresponding mRNAs (Kreuzaler et al, 1983).

One of the important features related to induction of disease resistance concerns the hierarchy of expression of stress-related responses. Thus in pea pods, heat shock treatments resulted in increased mRNA activities encoding specific 'heat shock' proteins while at the same time suppressing the synthesis of 'resistance-related' proteins and rendering the pods susceptible to attack by Fusarium solani f.sp. phaseoli (Hadwiger & Wagoner, 1983). In UV-irradiated parsley cell cultures on the other hand, elicitor suppressed induced CHS activity (Hahlbrock et al, 1981). Also, in

bean cell cultures, the increase in extractable activities of glutamate dehydrogenase and 4-coumarate hydroxylase during normal culture growth is prevented by addition of elicitor (Robbins et al, 1982; Bolwell et al, 1985 a). Elicitor treatment of bean cell cultures results in cessation of transfer of arabinose residues onto polysaccharide acceptors and a switch to transfer only onto HRGP acceptors (Bolwell, 1984). Taken together, these phenomena reflect complex integrated control mechanisms at the molecular level.

Although the appearance of resistance-related gene products, where studied, follows similar kinetics, their induction may under some conditions appear to be differentially regulated. Hence, within the overall co-ordination of PAL and CHS synthesis in bean cell cultures, small but distinct differences between the enzymes were observed in the elicitor concentrations giving maximum induction of enzyme activity, the elicitor concentrations giving maximum induction of enzyme synthesis and the precise timing of maximum enzyme synthesis (Lawton et al, 1983 a). Further evidence for possible differential regulation of enzymes in the same metabolic pathway comes from work on PAL and 4CL induction in parsley cell cultures treated with elicitor from Phytophthora megasperma f. sp. glycinea, where co-ordinate (Schmelzer et al, 1986) or unco-ordinate (Kuhn et al, 1984; Chappell & Hahlbrock, 1984; Hahlbrock et al, 1981) changes in transcription rates and mRNA amounts were observed. Also, a clear temporal distinction is observed in both bean hypocotyls and cell cultures between the appearance of the 5-hydroxy and 5-deoxy isoflavonoid derived phytoalexins (Whitehead et al, 1985; Robbins et al, 1985). Such temporal differentiation of expression probably reflects differential control of two distinct CHS activities whose mechanisms of action have been predicted from ^{13}C-labelling studies (Dewick et al, 1982). Similarly, the operation of differential, though possibly not unrelated, signal-reaction chains is reflected by the differential inducton of chitinase, PAL and 4CL on the one hand, and of 1,3-β-glucanase and the enzymes of the furanocoumarin pathway on the other hand, in parsley cell cultures treated with P. megasperma f. sp. glycinea cell wall elicitor (Kombrink & Hahlbrock, 1986) and by

the delayed appearance of HRGP mRNA in elicitor-treated cell cultures (Showalter et al, 1985) relative to that of phytoalexin biosynthetic enzymes (Ryder et al, 1984).

The mechanisms of signal transduction during elicitation are at present unknown. This is largely a result of our, as yet, incomplete knowledge of the nature and involvement of elicitor receptors, intracellular response-couplers, sequence-specific gene activation factors and promotor-regions of elicitor-induced genes. Nevertheless, it appears that co-ordination of resistance-related host gene-expression is a complex event involving the operation of multiple mechanisms such as alteration of gene-expression, changes in enzyme synthesis and changes in post-translational effects on enzyme turnover and activation. The next few years should see rapid progress in our understanding of these and other events related to plant gene-expression.

1.5   Scope and objectives of the project

From a review of the available literature, it can be summarised that
much of the work on fungal elicitors to date may be divided into two
main areas, namely (a) structural studies on active components
following purification from cell walls or culture filtrates (e.g.
Anderson-Prouty & Albersheim, 1975; Ayers et al, 1976 a; Sharp et al,
1984) and (b) work in which elicitor preparations, either crude or
partially purified, are used in the study of the mechanisms
underlying induction of phytoalexins or phytoalexin biosynthetic
enzymes (e.g. Cramer et al, 1985 a, b; Ryder et al, 1984). Generally
in the former case the bioassay of elicitor activity, and therefore
the criterion for purification of elicitors, has relied upon the
assessment of a single parameter, while in the second case selected
specific areas of gene expression have been studied in response to a
single elicitor preparation.

A survey of the available literature shows (a) that a vast number of
elicitor molecules with remarkably different structures have been
isolated from different pathogens (see Section 1.2.1), (b) that
treatment of plant tissues with a given elicitor preparation results
in a multicomponent response (see Section 1.2), (c) that for a given
host, different elicitor preparations from the same or different
sources can elicit responses associated with the expression of
disease resistance (e.g. Keenan et al, 1985; Maniara et al, 1985) (see
Section 1.2.2.1) and (d) that the same elicitor preparation may be
active in different plant tissues (e.g. Cline et al, 1978; Ebel et al,
1976) (see Section 1.2.2.1). These observations raise the questions
of whether (a) a single type of elicitor determinant, responsible for
triggering the sum total of responses observed in any particular
interaction, is present in different elicitor preparations from
different fungal sources, or at least in different elicitor
preparations from the same source, or (b) that for a given source of
elicitor, more than one type of elicitor molecule, each responsible
for triggering one or a group of responses, comprising part of the
multicomponent response of the plant to the crude elicitor

preparation, is present. To date, there is little direct evidence linking the action of a single elicitor molecule to the early molecular events underlying expression of resistance either in model systems (tissue cultures) or in intact plant-parasite interactions. This clearly results from the difficulty of being certain that a molecule, usually isolated from the pathogen grown in liquid culture is (a) present in the intact plant-parasite interaction and (b) is the only pathogen-produced molecule involved in induction of resistance. The latter point is of particular relevance with respect to reports of the production of suppressor molecules by phytopathogenic fungi (Ziegler & Pontzen, 1982); equally important however, is the possibility that more than one type of elicitor molecule may have different positive effects in a given interaction.

In view of the above, this project addresses the question of whether one or more elicitor components may be involved in inducing the sum total of resistance-related responses. The overall approach to the problem involved a study of the profiles of metabolic changes in tissues from a single plant, French bean (Phaseolus vulgaris L.), in response to treatment with crude and partially purified elicitor preparations from a single source, the bean pathogen Colletotrichum lindemuthianum. Such an approach, involving screening of different elicitor preparations for their effects on different host responses would facilitate the study of putative multicomponent elicitors as the presence of a single type of elicitor molecule is predicted to affect the overall induced metabolic changes in a quantitative, but not qualitative, manner, whereas qualitative changes might also be likely if more than one class of elicitor molecule were involved. More specifically, work in this project involved:
(a) Comparison of the effects of crude elicitor preparations from cell walls and culture filtrate of Colletotrichum lindemuthianum on (i) viability of suspension cultured bean cells, (ii) electrolyte leakage from leaf slices and isolated mesophyll cells (e.g. Dow & Callow, 1979 a) (see Section 1.3), (iii) induction of extractable activities of phenylpropanoid pathway biosynthetic enzymes (see Section 1.4.1), (iv) induction of accumulation of isoflavonoid

phytoalexins, wall-bound phenolics and wall-associated hydroxyproline (see Section 1.4), (v) changes in the levels of phenylpropanoid pathway intermediates (e.g. Bolwell et al, 1985 a), (vi) profiles of active charge isoforms of phenylpropanoid pathway enzymes (see Section 1.4.2.1).

(b) Fractionation of culture filtrate elicitor on the basis of M_r, charge and affinity for Concanavalin A-Sepharose (e.g. Ayers et al,1979 a, b; Anderson, 1978; Dixon et al, 1981), coupled with screening of the fractions for elicitor activity. Fractions obtained from the culture filtrate elicitor preparation were used for further investigation of multielicitor components as the culture filtrate elicitor is present during normal growth of Colletotrichum cultures and its isolation does not require harsh 'unphysiological' treatments such as autoclaving which is required to release elicitor molecules from Colletotrichum cell walls.

(c) Investigation of possible differential effects of the crude and partially purified elicitor preparations on (i) the overall patterns of proteins synthesised in vivo and in vitro from mRNA and separated by two-dimensional isoelectric focussing: SDS-polyacrylamide gel electrophoresis (e.g. Hadwiger & Wagoner, 1983) (see Section 1.4.3), (ii) post-translational and/or activation/inhibition processes underlying changes in phenylpropanoid pathway enzyme activities by comparison of the ratios of extractable activities, rates of synthesis in vivo and in vitro for phenylalanine ammonia-lyase, chalcone synthase and chalcone isomerase immunoprecipitated with specific antisera (e.g. Lawton et al, 1983 b; Robbins & Dixon, 1984) (see Sections 1.4.2 & 1.4.3), (iii) synthesis in vivo and in vitro of subunit charge isoforms of phenylpropanoid pathway enzymes separated by two-dimensional gel electrophoresis (e.g. Bolwell et al, 1985 b, 1986 a; Grab et al, 1985;T.B Ryder, personal communication) (see Section 1.4.2), (iv) transcription of resistance-related genes by Northern blot hybridisation analysis of mRNA levels using specific cDNA probes (e.g. Kuhn et al, 1984; Ryder et al, 1984).

It is hoped that such studies may be of value in directing attention to possible avenues for further researh in the context of the biochemistry and molecular mechanisms of disease resistance.

CHAPTER 2

MATERIALS AND GENERAL METHODS

Contents   Page No.

MATERIALS AND GENERAL METHODS

2.1 Introduction

This chapter describes general techniques used throughout the thesis and summarises the sources of chemicals used in chapters 3-6. Lists of buffers and methods specific to the work reported in chapters 3-6 are included in separate sections in these chapters.

2.2 Chemicals

2.2.1 Radiochemicals

[2-^{14}C] malonyl CoA (2.0 G Bq/mmol), sodium [2-^{14}C] oxoglutarate (116 MBq/mmol and L-[^{35}S] methionine (43.3 TBq/mmol) were purchased from Amersham International (U.K.)

2.2.2 Chemicals for gel electrophoresis

Polyacrylamide was obtained from Koch-Light. N,N'-methylenebisacrylamide and N,N,N',N', tetramethylethylenediamine (TEMED) were from Eastman-Kodak. Nonidet P-40 (NP-40) and ampholytes pH3-10 were obtained from Sigma (Poole, U.K.). Urea (ultra-pure) was purchased from Polysciences (Warrington, U.K.).

2.2.3. Chemicals for chromatofocussing

Polybuffer 74 and PBE 94 were obtained fom Pharmacia.

2.2.4 Antibodies

Anti-(bean phenylalanine ammonia-lyase) serum and anti-(bean chalcone isomerase) serum were gifts from Dr. G.P. Bolwell and Dr. M.P. Robbins (Royal Holloway and Bedford New College). Anti-(parsley chalcone synthase) serum was a gift from Prof. K. Hahlbrock (Mäx Planck Institut für Züchtungsforschung, Köln).

2.2.5 Chemicals synthesised in the present work

2.2.5.1 p-Coumaroyl CoA

i. Preparation of p-Coumaroyl N-hydroxycinnimide ester

p-Coumaric acid (1.15g) was dissolved in 60ml anhydrous ethyl acetate at 55°C followed by addition of 0.08g N-hydroxysuccinimide. The mixture was allowed to cool to 30°C before addition of 1.65g dicyclohexyl carbodiimide and incubation for 24 h at 30°C. After passage through Whatman No.1 filter paper, the organic filtrate was extracted twice with 100ml of 1 M NaHCO₃. The ethyl acetate layer was collected, dried over anhydrous sodium sulphate, reduced to 2ml under vacuum and applied to 20×20 cm, 0.75 mm thick Sil G-HT thin layer chromatography plates. The plates were then developed in chloroform : methanol (20:1, v/v), compounds visualised under U.V. light, and the p-Coumaroyl N-hydroxcinnimide ester band (a yellow fluorescent band, R꜀ 0.28) was scraped off, eluted with anhydrous ethanol, dried under vacuum and stored under desiccation at -20°C.

ii. Synthesis of p-Coumaroyl CoA

To a test-tube containing 1.25 ml distilled water purged with N₂ gas were added 25 mg coenzyme A, 52.5 mg NaHCO₃ and 32.65 mg p-Coumaroyl N-hydroxycinnimide ester. Acetone was added dropwise until the reactants dissolved, and the mixture was incubated in the dark at 40°C for 24 h. Acetone was evaporated at 4°C under a stream of N₂ gas and the remaining aqueous mixture was desalted with 1.5 g

Dowex 50W × 8 (Sigma) pre-equilibrated in 0.1 $\underline{M}$ KH_2PO_4 buffer pH 8.0, containing 0.7 m$\underline{M}$ 2-mercaptoethanol. The ion-exchanger was removed by centrifugation and the aqueous solution was extracted three times with 10 ml changes of anhydrous ethyl acetate. The aqueous layer was collected, lyophilised, taken up in 2 ml deionised water and the p-Coumaroyl CoA was separated from unreacted coenzyme A by fractionation on a Sephadex G-10 (Sigma) column (Fig. 2.1). Fractions containing unreacted coenzyme A, which absorbs U.V. light at 260 nm but not 333nm, were discarded. Fractions containing p-Coumaroyl CoA, which absorbs at both wavelengths, were pooled, lyophilised, and taken up in 5 ml dilute HC1, pH 3.5. An aliquot was diluted and an absorpotion spectrum read between 220-380 nm. p-Coumaroyl CoA concentration was calculated using the extinction coefficient ϵ_{333}=20892 l mol^{-1} cm^{-1}. The solution was divided into 100µl aliquots and stored at -20°C. The final yield was 8.3 mg of p-Coumaroyl CoA

2.2.5.2 Vanadyl ribonucleosides

These were synthesised as described by Maniatis (Maniatis et al,1984). 1 ml of 2 $\underline{M}$ vanadyl sulphate was added to 8 ml H_2O at 100°C containing 0.5 mmole of each of thymidine, adenosine, guanosine and cytosine while purging with N_2 gas. The pH of the solution was adjusted to pH 6.0 by dropwise addition of 10 $\underline{M}$ NaOH and to pH 7.0 with 1 $\underline{M}$ NaOH under N_2 gas. The solution was adjusted to 10 ml with H_2O to give a final vanadyl ribonucleoside concentration of 200 m$\underline{M}$. The mixture was stored in aliquots at -70°C.

2.2.6 Others

Rabbit reticulocyte lysate and Amplify were purchased from Amersham International (U.K.). Poly-(L-proline) (M_r 30 000) was obtained from Sigma (Poole, U.K.). Macerase was from Calbiochem (Herts, U.K.). Phytoalexin standards (phaseollin, kievitone and phaseollidin) were a
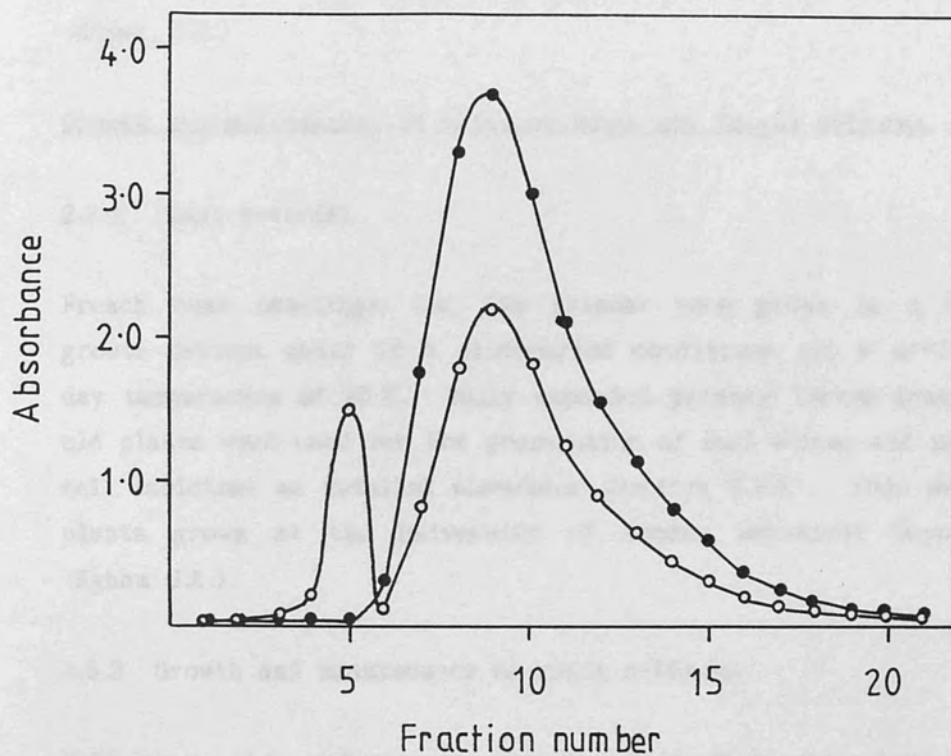
Figure 2.1 - <u>Purification of synthesised p-Coumaroyl CoA</u>

p-Coumaroyl CoA (synthesised as detailed in Section 2.2.5.1) in 2 ml H_2O was applied to a Sephadex G-10 column (70 × 1.5 cm) and eluted with 50 m$\underline{M}$ formic acid at a flow rate of 150 ml h^{-1}. 5ml fractions were collected and their absorbance at 260 nm (●) and 333 nm (o) was measured.

gift from Dr. I. Whitehead (Royal Holloway and Bedford New College).
Other chemicals were obtained from Sigma (Poole, U.K.) and BDH
(Essex, U.K.).

2.3   Growth and maintenance of plant material and fungal cultures.

2.3.1 Plant material

French bean seedlings (cv, The Prince) were grown in a Conviron
growth cabinet under 16 h photoperiod conditions (45 W m^{-2}) with a
day temperature of 25°C. Fully expanded primary leaves from 10-day
old plants were used for the preparation of leaf slices and mesophyll
cell isolation as detailed elsewhere (Section 3.2.5). Pods were from
plants grown at the University of London Botanical Supply Unit
(Egham U.K.).

2.3.2 Growth and maintenance of plant cultures.

Cell suspension cultures of dwarf French bean (cv. Immuna) were
initiated by transfer of callus grown at 25°C in the dark on semi-
solid (6 g agar litre^{-1}) Schenck and Hildebrandt medium (Table 2.1)
into liquid medium of the same composition (minus agar) as
previously described (Dixon and Fuller, 1976). Cell cultures were
maintained by regular subculture at 14-day intervals. Cultures were
grown at 25°C in the dark in a Gallenkamp Model INR-401 orbital
incubator at 110 rev min^{-1}. Four to five day old cells from cultures
in their third to eleventh passage were routinely used. The basal
activities of PAL and CHS increased and their magnitude of induction
with a given elicitor concentration decreased with increasing passage
number. Enzyme elicitation was highly variable past the eleventh
passage and new suspensions had therefore to be established from
callus.

TABLE 2.1

Composition of modified Schenk and Hildebrandt medium

(Dixon & Fuller 1976)

Component	Concentration (gl-1)
Carbon source	
Sucrose	30
Major salts	
KNO_3	1.2625
$MgSO_4 . 7H_2O$	0.1856
$(NH_4)H_2PO_4$	0.1438
$CaCl_2 . 2H_2O$	0.1101
Trace elements	
$MnSO_4 . 4H_2O$	1.3×10^{-2}
H_3BO_3	5.0×10^{-3}
$ZnSO_4 . 7H_2O$	1.0×10^{-3}
KI	1.0×10^{-3}
$CuSO_4 . 5H_2O$	2.0×10^{-4}
$Na_2MoO_4 . 2H_2O$	1.0×10^{-4}
$CoCl_2 . 6H_2O$	1.0×10^{-4}
$FeSO_4 . 7H_2O$	1.5×10^{-3}
EDTA (Na salt)	2.0×10^{-3}
Vitamins	
Thiamine – HCl	5.0×10^{-3}
Nicotinic acid	5.0×10^{-3}
Pyridoxin – HCl	5.0×10^{-4}
Myo-inositol	1.0
Hormones	
p-Chlorophenoxyacetic acid	1.87×10^{-3}
2,4-Dichlorophenoxyacetic acid	4.42×10^{-4}
Kinetin (6-furfurylaminopurine)	1.08×10^{-4}

The pH of the above mixture is adjusted to 5.9 with 0.5 M NaOH

2.3.3 Growth and maintenance of Colletotrichum lindemuthianum

C. lindemuthianum (Commonwealth Mycological Institute isolate IMI 112166) was maintained on a semi-solid glucose neopeptone medium (Mathur et al, 1949) in the dark at 25°C. Conidia were removed from agar cultures by vigorous shaking with sterile distilled water and transferred to 100 ml batches of the glucose neopeptone medium (modified by the addition of 15g glucose per litre). These were incubated on an orbital shaker at 25°C and 120 rev min^{-1} for 8 days. Three of these flasks, 8 days after subculture, were used to inoculate a 9 litre batch fermenter in which the culture was grown for 6 days at 25°C with mixing by aeration (10 l min^{-1}).

2.4   Preparation of elicitors

2.4.1 Cell wall elicitor

Mycelium from a fermenter-grown culture was harvested on 4 layers of muslin and used for mycelial wall preparation as previously described (Anderson-Prouty and Albersheim, 1975). Mycelium was homogenised (5 ml H$_2$O per g mycelium) for 1 min in a Waring blendor and the residue collected by filtration through sintered glass. The homogenisation procedure was repeated three times with H$_2$O, once with chloroform : methanol (1:1, v/v) and finally once with acetone. The mycelial walls were air-dried and autoclaved for 30 min (in 5 ml H$_2$O per g walls) to release elicitor molecules. The filtrate was clarified by filtration through sintered glass followed by centrifugation at 10 000 × g for 10 min and dialysis of the clear supernatant for 24 h in three 10-litre changes of distilled water. The elicitor solution was concentrated approximately 5-fold under vacuum, and stored in 5 ml aliquots at -20°C.

2.4.2 Culture filtrate elicitor

All procedures were carried out at 4°C. 30 l of 95% ethanol were added to 10 l culture filtrate from the fermenter-grown culture with

constant agitation on a magnetic stirrer. The resulting precipitate
was allowed to settle undisturbed for 4 days at 4°C. The clear
supernatant was siphoned off and the residue was centrifuged at
10 000 × g for 10 min. After reconstitution of the pellet in 250 ml
H_2O, the mixture was extensively dialysed against four 25-litre
changes of distilled water, filtered through Whatman No. 1 paper,
lyophilised and stored under desiccation at -20°C.

2.5   Carbohydrate estimation and monosaccharide analysis.

Total carbohydrate was estimated using phenol-sulphuric acid reagent
(Du Bois et al ,1956). Carbohydrate was quantitated using glucose as
standard.

Crude and fractionated elicitor preparations were prepared for
monosaccharide analysis as previously described (Dixon et al 1981);
0.5 ml of 2 M trifluoroacetic acid (TFA) was added to elicitor
solutions (100 μl glucose equivalents in 100 μl H_2O) amd samples
were hydrolysed in capped vials at 100°C for 6 h. Samples were
reduced to dryness over KOH pellets in a vacuum desiccator for 2
days and then taken up in 50 μl H_2O containing lactose (75 mg dl^{-1}).
Quantitative analysis of sugars was by application of 4 μl aliquots
as 1.5 cm bands to 20 × 20 cm F 1500 Schliecher-Schüll TLC plates
(Menzies et al, 1978) together with four separate standard sugar
samples each containing lactose (75 mg dl^{-1}) as internal standard,
and varying concentrations of galactose, glucose, mannose, ribose
and rhamnose. The TLC plates were developed twice with a solvent
rise of 18.5 cm in ethyl acetate : pyridine : acetic acid : H_2O
(6:3:3:1, v/v) and the sugars were located by dipping in PABA reagent
solution (1.4% (w/v) p-aminobenzoic acid, 3.5% (w/v) orthophosphoric
acid in methanol) and heating at 120°C for 10 min. The plates were
scanned using a Chromoscan (Joyce Loebl) densitometer. For a given
sugar concentration, the intensity of the colour reaction was
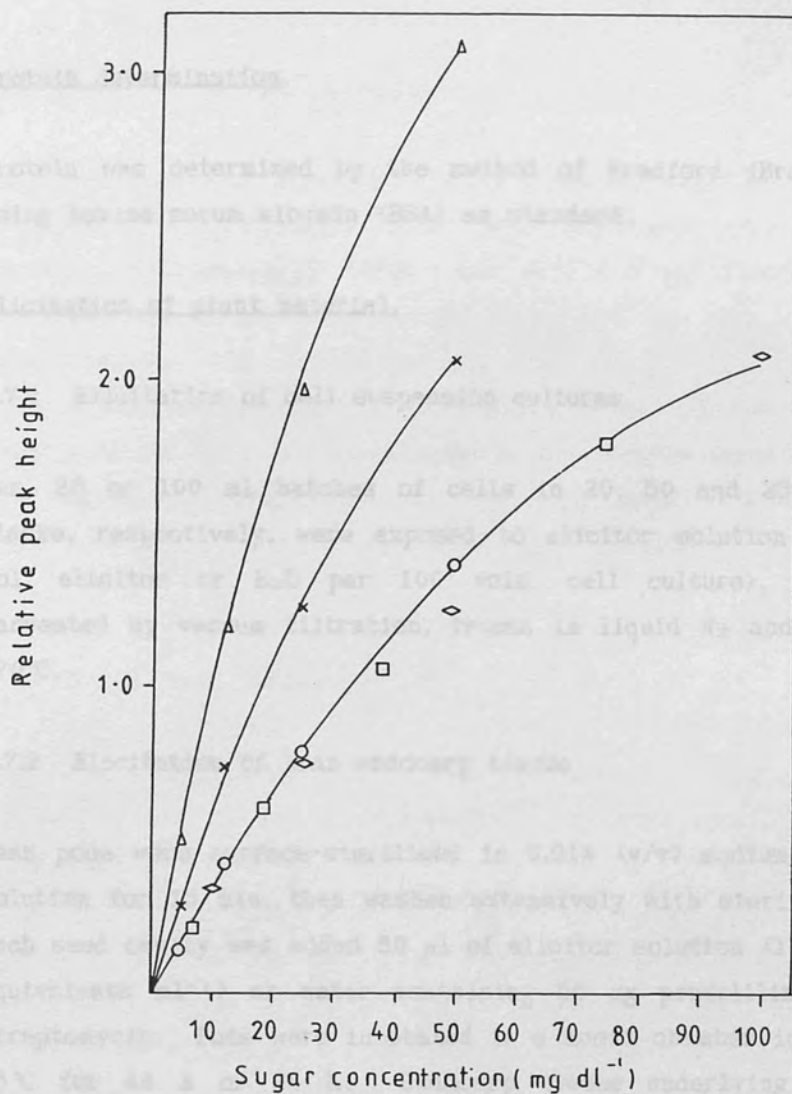dependent on the sugar as seen from the standard curves in
Figure 2.2.

Figure 2.2 – <u>Standard curves for sugars separated by thin-layer chromatography.</u>

Standard sugar solutions containing galactose (◇), glucose (○), mannose (□), ribose (△), rhamnose (×) and 75 mg dl⁻¹ lactose (internal standard) were applied to F-1500 Schliecher-Schüll t.l.c. plates. Plates were developed and reacted with PABA reagent and scanned as described in Section 2.5. Sugar peak height is expressed in arbitrary units relative to that of the internal standard lactose.

2.6   Protein determination.

Protein was determined by the method of Bradford (Bradford, 1976) using bovine serum albumin (BSA) as standard.

2.7   Elicitation of plant material.

2.7.1 Elicitation of cell suspension cultures

Ten, 20 or 100 ml batches of cells in 25, 50 and 250 ml conical flasks, respectively, were exposed to elicitor solution or water (1 vol. elicitor or H_2O per 100 vols. cell culture).   Cells were harvested by vacuum filtration, frozen in liquid N_2 and stored at −70°C.

2.7.2 Elicitation of bean endocarp tissue

Bean pods were surface-sterilised in 0.01% (v/v) sodium hypochlorite solution for 15 min, then washed extensively with sterile water.   To each seed cavity was added 50 µl of elicitor solution (100 µg glucose equivalents ml^{-1}) or water containing 50 µg penicillin and 100 µg streptomycin.   Pods were incubated in a humid chamber in the dark at 25°C for 48 h or 96 h.   Endocarp tissue underlying the applied solutions was excised with a scalpel and stored at −70°C.

2.8   Enzyme extraction and assay.

2.8.1 Phenylpropanoid pathway enzymes

Cells were extracted at 4°C in a pestle and mortar in two volumes of 50 mM KH_2PO_4 buffer pH 8.0, containing 0.01% (v/v) 2-mercaptoethanol. Extracts were centrifuged at 15 000 × g for 10 min in a Sorvall centrifuge or for 5 min at 4°C in a bench microcentrifuge depending on the cell extract volume, and the supernatant was used for enzyme assay.

- 65 -

2.8.1.1. Phenylalanine ammonia-lyase (PAL).

PAL activity was assayed spectrophotometrically by measuring the formation of t-cinnamic acid from L-phenylalanine as described previously (Lamb et al, 1979). For each determination, 100 µl enzyme solution was mixed with 0.9 ml of a 200 mg dl^{-1} solution of L-phenylalanine (for sample cuvette) and 200 µg dl^{-1} D-phenylalanine (for blank cuvette) in 50 mM Tris/HCl buffer pH 8.5. Samples were incubated at 40°C and absorbance of the sample versus the blank at 290 nm was measured at 30 min intervals. Cinnamic acid concentration was calculated using the extinction coeffecient ϵ_{290} = 10 000 1 mol^{-1} cm^{-1}.

2.8.1.2 Chalcone isomerase (CHI)

The conversion of 2',4,4' - trihydroxychalcone to the corresponding flavanone by CHI was monitored spectrophotometrically as previously described (Dixon et al, 1982). Twenty µl of a 1.0 mg ml^{-1} solution of 2',4,4' - trihydroxychalcone in ethanol was added to 2.5 ml of freshly prepared 50 mM KH$_2$PO$_4$ buffer pH 8.0, containing 50 mM potassium cyanide, followed by 50 µl of the enzyme solution. The decrease in absorbance at 30°C was monitored at 400 nm in a Perkin-Elmer recording spectrophotometer against water. The extinction coefficient for 2',4,4' - trihydroxychalcone (ϵ_{400}=31 257 1 mol^{-1}cm^{-1}) was used for calculation of reaction rates.

2.8.1.3 Chalcone synthase (CHS)

A radioactive assay procedure was used to measure the production of radioactive naringenin by the action of CHS on p-Coumaroyl CoA and [2-^{14}C] malonyl CoA as substrates (Whitehead et al, 1982). Freshly prepared 3.7 mM dithiothreitol (DTT) in 0.26 mM KH$_2$PO$_4$ buffer pH 8.0 (35 µl) was added to 15 µl reaction mixture containing 0.05 µCi (3.0 nmol) [2-^{14}C] malonyl CoA and 9 nmol p-Coumaroyl CoA made up in dilute HCl, pH 3.5. The reaction was started by the addition of 50 µl of enzyme solution and the mixture was incubated at 35°C for 30 min,

after which time the reaction was terminated by the addition of 20 μl of freshly prepared naringenin (1.5 mg ml^{-1}) in methanol. The resultant mixture was extracted by vortexing for 1 min with 200 μl ethyl acetate, the aqueous and organic layers were separated by centrifugation, and a 150 μl aliquot of the organic layer dispensed into a scintillation vial and dried under a stream of N$_2$ gas. ^{14}C incorporation was measured by liquid scintillation counting in 5 ml of toluene : PPO scintillant (5 g 2,5-diphenyloxazole /1 toluene). Background counts were obtained from a reaction mixture stopped at zero time with the methanolic naringenin solution.

2.8.2 Prolyl hydroxylase

Hydroxylation of polyproline by microsomal proline 2-oxoglutarate dioxygenase was estimated as the stoichiometric decarboxylation of [2-^{14}C] oxoglutarate using a microscale adaptation of the method of Tanaka (Tanaka et al 1980). Cells (5 g) were extracted 1:1 (w/v) in 50 mM Tris/HCl buffer pH 8.8 containing 25 mM MgCl$_2$, 1 mM dithiothreitol, 0.3 M KCl and 0.4 M sucrose. The extract was centrifuged at 1000 × g for 15 min to remove the crude cell wall pellet. The supernatant was then centrifuged at 15 000 × g for 7 min after which it was used for microsome preparation by centrifugation at 200 000 × g for 1 h. The final microsomal pellet was resuspended in 150 μl of 50 mM HEPES buffer, pH 6.8 containing 10 mM MgCl$_2$, 1 mM dithiothreitol, 50 mM KCl and 0.4 M sucrose, then made up to 0.1% (v/v) with respect to Triton X-100 with 1.0% (v/v) Triton X-100 in H$_2$O to solubilise the microsomal membranes. Twenty μl of freshly prepared 3.75 mM [2-^{14}C] oxoglutarate (375 000 dpm) in 40 mM HEPES buffer, pH 6.8 containing 0.5 mM L-ascorbate was added to duplicate microcentrifuge tubes containing 10 μl of a solution of 4 mg ml^{-1} poly (L-proline) (M$_r$ 30 000) and 2.5 mg ml^{-1} BSA in 40 mM HEPES buffer, pH 6.8. Enzyme preparation (20 μl) was added and a CO$_2$ trap (a 3 × 30 mm Whatman No.1 filter paper wick soaked with 75 μl NCS tissue solubiliser (Amersham International) contained in a 3 × 25-mm plastic tube) was introduced into the reaction tube. Internal controls receiving no polyproline were included. The reaction was allowed to

proceed for 1 h at 30°C after which time it was terminated by the addition of 50 μl of 10% (w/v) trichloroacetic acid to the reaction mixture to flush off residual $^{14}CO_2$; vials were rapidly recapped and incubated for a further 30 min at 30°C. The $^{14}CO_2$ absorbed by the wicks was measured by liquid scintillation counting in toluene : PPO scintillant.

Enzyme activities are expressed as micro or millikatals kg protein^{-1}. One katal is the amount of enzyme required for the formation of 1 mole of product in 1 s under the defined assay conditions.

2.9   Assessment of cell viability

2.9.1   Incubation conditions

As the parameters for efficient reduction of 2,3,5 – triphenyltetrazolium chloride (TTC) had been shown to vary with different tissue cultured cells (Towil & Mazur, 1975), the optimum conditions for TTC reduction by Phaseolus vulgaris cv Immuna cell cultures were investigated as shown in Figures 2.3 A-D. As tissue culture extracts of bean cells were found to contain no materials absorbing significantly at 485 nm (the absorption maximum of the red formazan) all absorbances reported here were measured at 485 nm. Based on the data in Figures 2.3 A-D, cultured bean cells harvested by suction filtration on sintered glass were routinely incubated at 10% packed cell volume in the dark at 20°C for 24 h in 0.6% (w/v) TTC in 50 mM KH_2PO_4 buffer pH 8.0.

2.9.2   Extraction of formazan

At the end of the incubation period, cells were collected by centrifugation, washed with distilled water and the red formazan extracted from the collected cells by heating with 3 ml of 95% ethanol for 3 min at 30°C. The absorbance of the extracts was read at 485 nm.

Figure 2.3 – Optimisation of the triphenyltetrazolium chloride reduction assay as a measure of bean cell viability.

Figure 2.3 A – Effect of pH of the incubation medium on formazan production by Phaseolus vulgaris cell cultures.

Duplicate samples of suspension cultured bean cells (10% packed cell volume) were incubated at 20°C for 24 h in 0.05 (w/v) 2,3,5 – triphenyltetrazolium chloride in 50 mM KH_2PO_4 buffers of varying pH. Formazan extraction was as described in Section 2.5.2.

Figure 2.3 B – Time-course of formazan production by P. vulgaris cell cultures.

Duplicate samples of cultured cells (10% packed cell volume) were incubated at 20°C in 0.05 (w/v) 2,3,5 – triphenyltetrazolium chloride in 50 mM KH_2PO_4 buffer pH 6.0 for various times.

Figure 2.3 - <u>Optimisation of the triphenyltetrazolium chloride reduction</u> <u>assay as a measure of bean cell viability.</u>

Figure 2.3 A - <u>Effect of pH of the incubation medium on formazan</u> <u>production by *Phaseolus vulgaris* cell cultures.</u>

Duplicate samples of suspension cultured bean cells (10% packed cell volume) were incubated at 20°C for 24 h in 0.6% (w/v) 2,3,5 - triphenyltetrazolium chloride in 50 m$\underline{M}$ KH$_2$PO$_4$ buffers of varying pH. Formazan extraction was as described in Section 2.9.2.

Figure 2.3 B - <u>Time-course of formazan production by *P. vulgaris* cell</u> <u>cultures.</u>

Duplicate samples of cultured cells (10% packed cell volume) were incubated at 20°C in 0.6% (w/v) 2,3,5 - triphenyltetrazolium chloride in 50 m$\underline{M}$ KH$_2$PO$_4$ buffer pH 8.0 for various times.
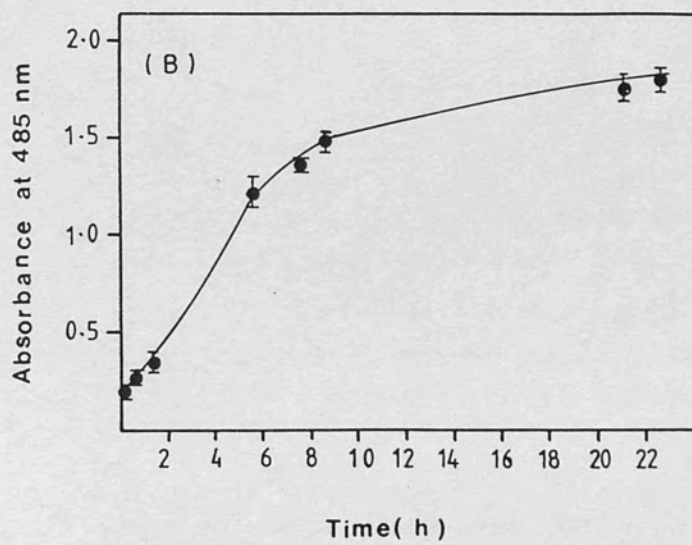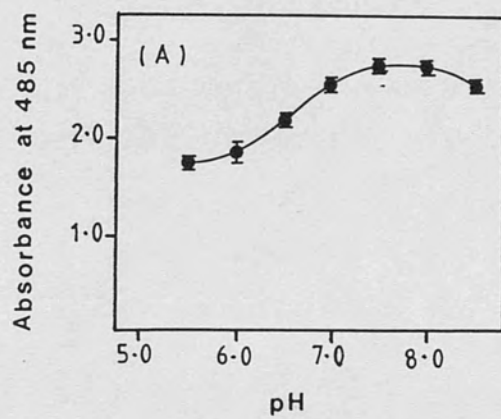
Figure 2.3 C - Effect of 2,3,5 - triphenyltetrazolium chloride concentration on formazan production by *P vulgaris* cell cultures

Cells (10% packed cell volume) were incubated at 20°C for 24 h in varying concentrations of 2,3,5 - triphenyltetrazolium chloride in 50 mM KH$_2$PO$_4$ buffer pH 8.0.

Figure 2.3 D - Formazan production by mixtures of living and heat-killed *P vulgaris* cells

Different proportions of living and heat-killed (100°C for 10 min) cultured bean cells were mixed to give a 10% packed cell volume and were incubated at 20°C for 24 h in 0.6% (w/v) TTC in 50 mM KH$_2$PO$_4$ buffer pH 8.0. Each point is an average of duplicate samples with a spread of values indicated by the bars.

(C)

Absorbance at 485 nm

2·0

1·5

1·0

0·5

0·2 0·4 0·6 0·8 1·0 1·2 1·4 1·6

% TTC concentration (w/v)

(D)

Absorbance at 485 nm

2·0

1·5

1·0

0·5

10 20 30 40 50 60 70 80 90 100

% Living cells

- 72 -

2.9.3 2,3,5,- Triphenyltetrazolium chloride reduction as a measure of cell viability.

As shown in Figure 2.3, D, formazan production was proportional to the number of viable cells when samples containing different proportions of living and heat-killed cells from the same bean culture were tested. Seventy eight and 98% inhibition of formazan production was observed following treatment of cells with 0.1 mM mercuric chloride for 1 and 6 h respectively.

2.10   SDS - Polyacrylamide gel electrophoresis (SDS - PAGE)

2.10.1 Gel preparation

Slab polyacrylamide gels (1.5 mm thick) were prepared according to the method of Laemmli (Laemmli 1970). One volume of the running gel was made by mixing 0.26 volumes of 1.5 M Tris/HCl buffer pH 8.8, containing 0.4% (w/v) SDS with 0.74 volumes of acrylamide stock (29.2% (w/v) acrylamide and 0.8% (w/v) bisacrylamide) plus water. The proportions of acrylamide stock and water were varied to give different gel concentrations. Gels were allowed to polymerise for 45 min after addition of 20 µl N,N,N',N' — tetramethylethylenediamine (TEMED) and 100 µl of 10% (w/v) ammonium persulphate (AMPS) to the degassed solution. The 5% stacking gel mixture was prepared by mixing 0.126 volumes of 1.0 M Tris/HCl buffer pH 6.8 with 0.01 volumes of 10% (w/v) SDS, 0.131 volumes of acrylamide stock (38.5% w/v) acrylamide and 1% (w/v) bisacrylamide) and 0.733 volumes of water. Polymerisation was initiated by the addition of 10 µl of 10% AMPS and 50 µl TEMED.

2.10.2 Sample preparation

Lyophilised protein samples were taken up in SDS-sample buffer (62.6 mM Tris/HCl buffer pH 6.8, containing 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2.0% (w/v) SDS and 0.125% (w/v) bromophenol blue). Protein solutions were diluted at least 1:4 (v/v) with SDS-sample

buffer. Samples were heated at 95°C for 4 min and allowed to cool prior to application to the gel.

2.10.3 Gel electrophoresis

Gels were run in a Bio-Rad Protean gel electrophoresis apparatus at 30 mA constant current per gel in 50 mM Tris base containing 2.88% (w/v) glycine and 0.1% (w/v) SDS until the tracking dye reached the bottom of the slab gel.

2.11   Fluorography and photography

2.11.1 Fluorography

Gels were fixed in 8% acetic acid for 30 min followed by incubation in 50 ml Amplify (Amersham) for a further 30 min. Gels were dried under vacuum in a Bio-Rad gel dryer and exposed to X-ray film (Fuji RX-Medical) at -70°C. The film was pre-sensitised, and the method rendered quantitative, by pre-flashing with a photographic flash gun fitted with an orange filter held 70 cm above the film. Following development, fluorographs were scanned at 410 nm using a Vitatron MPS densitometer.

2.11.2 Photography

Photographs were taken without a filter using transmitted light and Ilford PAN F film, and were developed for maximum contrast.

CHAPTER 3

A COMPARATIVE STUDY OF THE RESPONSES OF BEAN CELLS TO ELICITOR
PREAPARATIONS FROM CELL WALLS AND CULTURE FILTRATE OF *Colletotrichum
lindemuthianum.*

CHAPTER 3

A COMPARATIVE STUDY OF THE RESPONSES OF BEAN CELLS TO ELICITOR
PREPARATIONS FROM CELL WALLS AND CULTURE FILTRATE OF _Colletotrichum_
lindemuthianum

3.1   Introduction

Expression of resistance in a number of model plant-pathogen
interactions has been shown to involve a multicomponent reponse
which may include such biochemical changes as induced electrolyte
leakage (Dow & Callow, 1979a), increased extractable enzyme activities
(Dixon et al, 1981; Tietjen & Matern, 1983; Whitehead et al, 1982) and
levels of phytoalexins (Cline et al, 1978; Dixon et al, 1981), lignin
(Hammerschmidt et al, 1984; Pearce & Ride, 1982), ethylene (Esquerré-
Tugayé et al, 1979; Toppan & Esquerré-Tugayé 1984) and cell wall
hydroxyproline-rich glycoproteins (Esquerré-Tugayé & Mazau, 1974;
Esquerré-Tugayé et al, 1979; Hammerschmidt et al, 1984).   However,
little attention has been paid to the possible requirement of more
than one type of elicitor for the expression of the full spectrum of
events associated with induced resistance, as reliance is usually
placed on the measurement of a single parameter for the bioassay of
elicitor activity.

The presence of and possible interaction between more than one
component in crude cell wall elicitor preparations from the
anthracnose fungus Colletotrichum lindemuthianum is suggested by a
number of observations.   These include a multi-phasic dose-response
curve for PAL induction by the crude elicitor preparation   (Dixon et
al, 1981) and the differential effects of cell wall elicitor fractions
on PAL induction in bean cell suspension cultures (Dixon et al, 1981)
and phytoalexin accumulation in hypocotyls and cotyledons (Whitehead
et al, 1982).   However, there is as yet no direct evidence for the
existence of multiple elicitor types with differing biological
activities in a single source, although different elicitor
preparations from different sources have been shown to have different

effects on enzyme induction (Tietjen & Matern, 1983) and the patterns of phytoalexin accumulation (Tietjen, Hunkler and Matern, 1983) in parsley cell cultures.

Active elicitor preparations from the culture filtrates and autoclaved cell walls of Colletotrichum have been partially purified (Anderson, 1978; Dixon et al, 1981) and it was postulated that elicitor activity in these preparations may reside in a (1→3),(1→4)-linked β-D-glucan (Anderson-Prouty & Albersheim, 1975; Anderson, 1978). Later studies, however, have shown that these preparations also contained high proportions of mannose and galactose (Anderson-Prouty & Albersheim, 1975; Dixon et al, 1981; Whitehead et al, 1982). This chapter reports a comparative study of the biochemical effects of crude elicitor preparations from culture filtrate and cell walls of Colletotrichum on a number of parameters associated with induced host defence. This work was undertaken in order to assess whether different elicitor preparations could induce differential effects on host cell metabolism, thus supporting the presence of functionally non-identical elicitor components.

3.2   Materials and methods

3.2.1 Buffers

The following buffers were used: (A) 0.5 $\underline{M}$ KH_2PO_4 buffer pH 7.8 (B)
0.2 $\underline{M}$ sodium acetate buffer pH 5.0, containing 20 mM $MgCl_2$. (C) 50
mM sodium acetate buffer pH 5.0. (D) 50 mM sodium acetate buffer
pH 4.0. (E) 0.1 $\underline{M}$ MES/KOH buffer pH 6.0, containing 0.55 $\underline{M}$ sorbitol,
20 g l^{-1} soluble polyvinyl pyrrolidone - 40, 2.2 g l^{-1} K_2SO_4 and 10
g l^{-1} macerase. (F) 50 mM KH_2PO_4 buffer pH 8.0, containing for 20 mM
ascorbic acid and 1 mM phenylmethylsulphonyl fluoride (PMSF). (G) 25
mM histidine/HC1 buffer pH 6.2, containing 1.4 mM 2-mercaptoethanol.
(H) 11% (v/v) 'Polybuffer 74'/ HC1 pH 4.0. (I) 50 mM Tris/HC1 pH 8.8,
containing 25 mM $MgCl_2$, 1 mM dithiothreitol, 0.3 $\underline{M}$ KC1 and 0.4 $\underline{M}$
sucrose.

3.2.2   Chemical treatment and enzymic digestion of elicitor
preparations

3.2.2.1 Periodate oxidation

Elicitor (1 mg glucose equivalents ml^{-1}) was incubated in the
presence of 5.8 mM sodium metaperiodate for 36 h in the dark at 20°C.
Excess periodate was then consumed by the addition of 0.43 volumes
of ethylene glycol. Control treatments contained either (a) periodate
pretreated with ethylene glycol prior to addition of elicitor or (b)
ethylene glycol - treated periodate to which water was added in place
of elicitor. Samples were dialysed against water, lyophilised and
taken up in H_2O to a final concentration of 2.5 mg glucose ml^{-1}.
Samples were stored at -20°C until required.

3.2.2.2 Proteinase K digestion

Elicitor (0.8 mg glucose equivalents ml^{-1}) was incubated in the
presence of 0.1 mg ml^{-1} proteinase K (Sigma) in buffer A for 27 h

at 37°C together with a control sample receiving no elicitor. Samples were autoclaved at 120°C for 15 min in order to inactivate the enzyme, dialysed against distilled water, lyophilised, taken up in water to a final elicitor concentration of 2.5 mg glucose equivalents ml^{-1}, and stored at -20°C.

3.2.2.3. Digestion with ribonuclease and deoxyribonuclease

For digestion of possible nucleic acid components, elicitor (2 mg glucose equivalents ml^{-1}) was incubated for 5 h at 25°C with 75 Kunitz of each of DNAase in buffer B (1.9 ng ml^{-1}) or with RNAase in buffer C (10 ng ml^{-1}). Controls receiving no elicitor were also included. Samples were autoclaved at 120°C for 15 min, dialysed against H_2O, taken up in water to a final elicitor concentration of 2.5 mg glucose equivalents ml^{-1}, and stored at -20°C.

3.2.3 Determination of free and esterified phenolic acids.

The soluble and esterified phenolic acid fractions were prepared as previously described (Bolwell et al, 1985a). Cells were extracted in one volume of buffer D and the extract was centrifuged for 1 min in a microcentrifuge. An aliquot of the supernatant (0.5 ml) was extracted twice with an equal volume of diethyl ether and the organic layers were combined, dried under a stream of N_2 gas and taken up in 100 μl ethyl acetate for subsequent determination of free phenolic acids. The aqueous layer remaining after extraction with diethyl ether was mixed with an equal volume of 4 M NaOH and incubated at 4°C for 16 h to hydrolyse the esterified phenolic acids. Samples were extracted twice with an equal volume of ethyl acetate and the organic layers were pooled, dried under N_2 gas, taken up in 100 μl ethyl acetate and used for determiation of esterified phenolic acids. Separation of phenolic acids was by isocratic sample elution with methanol : water : acetic acid (40: 60: 0.1, v/v) on a Partisil 10 ODS1 reverse phase HPLC column. Sample elution was monitored at 254 nm (Figure 3.1) and peak areas were compared with those of standard samples of cinnamic, 4-coumaric, caffeic and ferulic acids.

Figure 3.1 – <u>High-performance liquid chromatographic analysis of phenolic</u>
<u>acids</u>

Esterified phenolic acids from suspension cultured cells of <u>Phaseolus</u>
<u>vulgaris</u> var <u>immuna</u> (A) were prepared as described in Section 3.2.3 and
analysed by comparison with a standard solution (B) containing caffeic
(1), coumaric (2), ferulic (3) and cinnamic (4) acids each at a final
concentration of 35 µg ml^{-1} ethyl acetate. A 20 µl aliquot of the sample
was analysed by isocratic elution from a Partisil 10 ODS1 reverse phase
HPLC column with methanol: water: acetic acid (40: 60: 0.1, v/v) at a flow
rate of 1 ml min^{-1} and monitoring at 254 nm. Retention times of caffeic,
coumaric, ferulic and cinnamic acids were 4 min, 4.5 min, 7 min and 12.5
min respectively.

0·010 — 2

0·005 —

A b s o r b a n c e   a t   2 5 4   n m

0

1 3 4

0   4   8   12   16

A

0·010 — 2 3

0·005 —

1 4

0

0   4   8   12   16

B
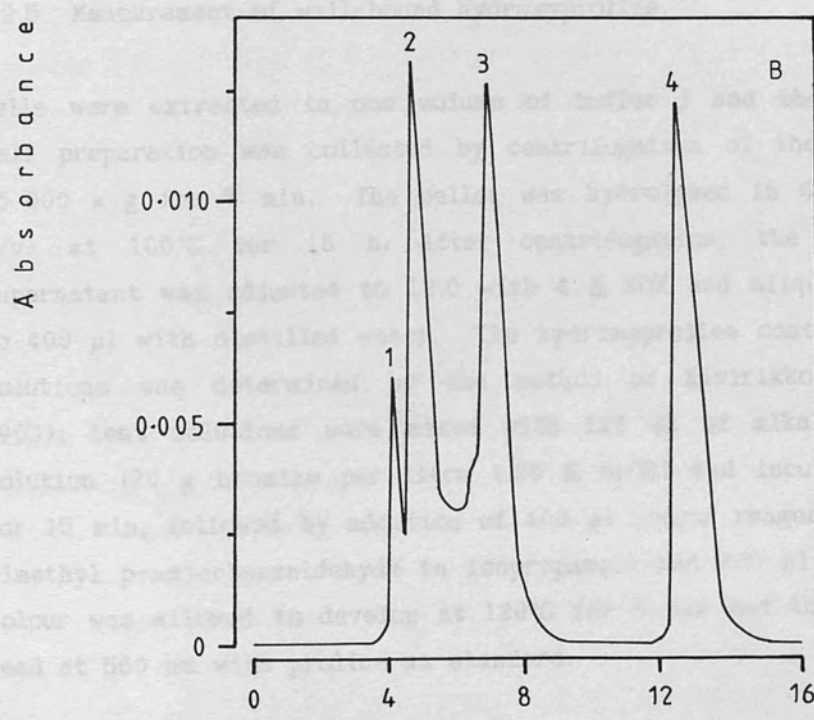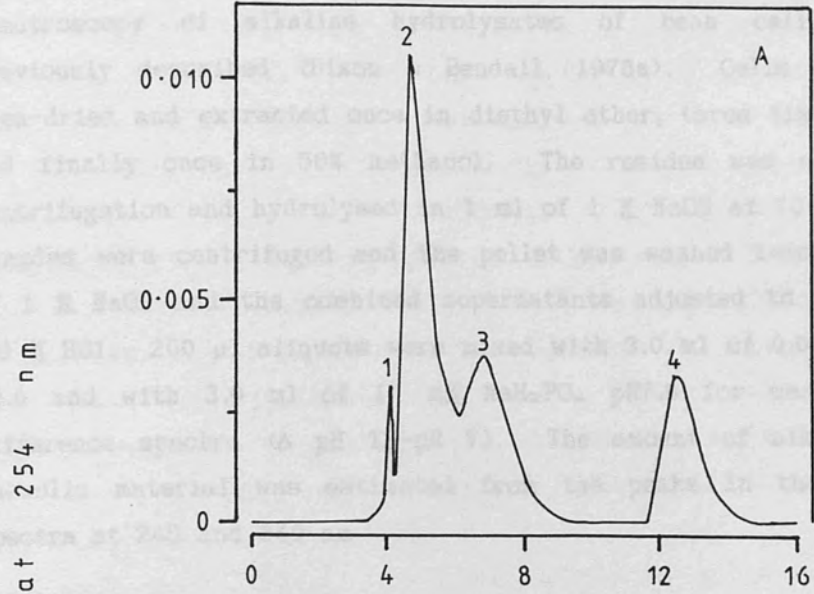
Time after application of sample
to column (min)

3.2.4 Determination of wall-bound phenolic material

Wall-bound phenolic material was determined by difference spectroscopy of alkaline hydrolysates of bean cell walls as previously described (Dixon & Bendall, 1978a). Cells (1 g) were oven-dried and extracted once in diethyl ether, three times in water and finally once in 50% methanol. The residue was collected by centrifugation and hydrolysed in 1 ml of 1 M NaOH at 70°C for 16 h. Samples were centrifuged and the pellet was washed twice in 0.5 ml of 1 M NaOH and the combined supernatants adjusted to pH 8.0 with 3.0 M HCl. 200 µl aliquots were mixed with 3.0 ml of 0.05 M NaOH pH 12.0 and with 3.0 ml of 10 mM NaH$_2$PO$_4$ pH7.0 for measurement of difference spectra (Δ pH 12-pH 7). The amount of alkali-released phenolic material was estimated from the peaks in the difference spectra at 245 and 340 nm.

3.2.5 Measurement of wall-bound hydroxyproline.

Cells were extracted in one volume of buffer I and the crude cell wall preparation was collected by centrifugation of the extract at 15 000 × g for 7 min. The pellet was hydrolysed in 6 M HCl (1:1 w/v) at 100°C for 16 h. After centrifugation, the pH of the supernatant was adjusted to 12.0 with 4 M KOH and aliquots made up to 400 µl with distilled water. The hydroxyproline content of these solutions was determined by the method of Kivirikko (Kivirikko, 1963); test solutions were mixed with 120 µl of alkaline bromide solution (20 g bromine per litre 1.25 M NaOH) and incubated on ice for 15 min, followed by addition of 400 µl colour reagent (5%, (w/v) dimethyl p-aminobenzaldehyde in isopropanol) and 200 µl of 6 M HCl. Colour was allowed to develop at 120°C for 3 min and the absorbance read at 560 nm with proline as standard.

3.2.6 Analysis of phytoalexins.

Plant tissue was extracted twice in 95% ethanol (10 ml/g fresh weight). After filtration through sintered glass, the filtrates were

combined, dried under reduced pressure at 40°C and taken up in 1 ml ethanol followed by 50 ml water. The aqueous fractions were extracted twice with successive equal volumes of chloroform, diethyl ether and petroleum ether (BP 40-60°C). The organic layers were combined, dried under vacuum, taken up in 100 μl ethanol: chloroform (3:1, v/v) and analysed by two-dimensional thin layer chromatography as previously described (Wyman & Van Etten, 1982). Samples were applied to 20 × 20 cm, 0.25 mm-thick, Polygram silica gel G/UV$_{254}$ thin layer plates and developed in diethyl ether : hexane (5:1, v/v) in the first dimension and in benzene : methanol (9:1, v/v) in the second dimension. Plates were viewed under U.V. light (254 and 366 nm), fluorescent spots eluted with 1.2 ml ethanol and the absorption spectra recorded in the presence and absence of AlCl$_3$ or sodium acetate. Kievitone and phaseollin were quantitated using the extinction coefficients 16 596 and 10 964 l mol^{-1}cm^{-1} respectively (Bailey & Burden, 1973). The plates were sprayed with a solution of diazotised p-nitroaniline (DpNA) (p-nitroaniline 0.28 g l^{-1}, sodium nitrite 0.5 g l^{-1} in 36 mM HCl) after removal of the fluorescing and absorbing spots to reveal phenolic compounds which developed a yellow-orange colour.

3.2.6.1 Preparation and use of reagent stock solutions for ultraviolet spectroscopy

(i) Aluminium chloride

Anhydrous AlCl$_3$ (50 mg) was dissolved in 1.0 ml of spectroscopic grade ethanol. Three drops of this stock solution were added to a cuvette containing 1 ml of the flavonoid solution and the ultraviolet absorption spectrum recorded 1 min after addition of the AlCl$_3$ stock solution.

(ii) Sodium acetate

Excess anhydrous, coarsely powdered reagent grade NaOAc was added with shaking to a cuvette containing 1 ml flavonoid solution and the

NaOAc spectrum recorded within 2 min after addition of the NaOAc stock solution.

3.2.7 Preparation of bean leaf slices and mesophyll cells

Four washed, primary leaves from 10-day old plants from which the midribs had been removed were surface-sterilised in 0.1% (v/v) sodium hypochlorite for 10 min. The leaves were washed extensively with sterile water, cut into 200 μm-wide strips with a tissue slicer (Mickle Engineering) and vacuum infiltrated for 30 s in 0.55 M sorbitol or in buffer E for preparation of leaf slices or isolation of leaf mesophyll cells repectively. Leaf slices were patted dry on filter paper and divided into 0.75 g batches in cheese cloth bags, ready for electrolyte leakage measurement. Mesophyll cells were prepared by the method of Callow and Dow (Callow & Dow, 1980); strips infiltrated with buffer E were incubated in 15 ml of fresh buffer E with gentle mixing on a magnetic stirrer. The cells were filtered through muslin and isolated cells were collected on 35 μm nylon mesh (Nybolt 25T1). The cells were washed with 0.55 M sorbitol and centrifuged at 200 × g for 3 min. The washing procedure was repeated once and the cells were finally resuspended in 0.55 M sorbitol and left for 1 h before use. The integrity of the cells was checked under a microscope using 2.5% (w/v) Evans blue stain. Viable cells excluded the dye.

3.2.8 Electrolyte leakage assays

Plant material was exposed to 0.55 M sorbitol in the absence and presence of elicitor (100 μg glucose equivalents ml^{-1}) and conductivity was measured using a Gallenkamp conductivity meter. After 1 h, 0.06 volumes of chloroform were added and the mixture incubated for a further 2 h before the final conductance of the solution was recorded. Conductivity was expressed as a percentage of the final conductivity change of the solution after chloroform addition.

3.2.9 Chromatofocussing

Suspension cultured bean cells (60 g) were extracted in 2 volumes of
buffer F (Britsch and Griesebach, 1985), centrifuged twice at
12 000 × g for 15 min and the supernatant fractionated by ammonium
sulphate precipitation. The 40-80% ammonium sulphate fraction was
taken up in 5 ml buffer G, dialysed overnight against two 1-litre
changes of the same buffer and applied to a Pharmacia PBE 94
chromatofocussing column (24 × 1 cm) pre-equilibrated in buffer G.
The sample was eluted with degassed buffer H and fractions were
assayed for PAL, CHS and CHI activities as described in section 2.8.
The pH of the fractions was measured using a micro pH-electrode.

3.3   Results

3.3.1 Preparation and composition of elicitors

The average yield of culture filtrate elicitor from four preparations
from 6-8 day old fermenter-grown Colletotrichum cultures was
0.19 ± 0.04 g l^{-1} culture. All preparations contained protein and the
ratio of protein to carbohydrate varied from between 2 to 6% (w/w)
(Table 3.1). The amount of insoluble material recovered following
dialysis and prior to lyophilisation of the culture filtrate elicitor
preparation varied from between 0.02 to 0.13 g l^{-1} culture.
Approximately 2 mg glucose equivalents of cell wall elicitor was
recovered per gram Colletotrichum hyphal walls, no protein being
detected in cell wall elicitor preparations.

The monosaccharide compositions of the elicitor preparations from the
cell walls and culture filtrates of Colletotrichum showed close
similarities (Table 3.1). Both preparations contained galactose,
glucose, mannose, rhamnose and traces of a pentose (pink band on
thin-layer chromatograms developed in PABA reagent) with the same R_f
as ribose. Galactose accounted for approximately 50% of the
carbohydrate content of both elicitor preparations. The levels of
glucose, however, were four-fold higher in the cell wall elicitor
preparation compared to culture filtrate material from the same
fermenter-grown fungal cultures.

The extractable activities of phenylalanine ammonia-lyase (PAL),
chalcone synthase (CHS) and chalcone isomerase (CHI) were induced in
bean cell suspension cultures in response to treatment with
Colletotrichum cell wall and culture filtrate elicitors (see Section
3.3.7). Autoclaving or proteinase K treatment of elicitor
preparations did not decrease their enzyme-inducing potential (Table
3.2). Similarly, no loss of enzyme induction was observed with
ribonuclease- or deoxyribonuclease-treated culture filtrate elicitor
(Table 3.3). Periodate oxidation, however, destroyed enzyme induction
by both elicitor preparations. The results suggest that the activity

Table 3.1

Composition of crude elicitor preparations from C. lindemuthianum

	Monosaccharide composition[a]					Protein (% w/v)
	Galactose	Glucose	Mannose	Ribose	Rhamnose	
Cell wall elicitor	41 ± 2	16 ± 1	32 ± 3	Trace	10 ± 3	0 ± 0
Culture filtrate elicitor	50 ± 4	5 ± 4	36 ± 3	Trace	9 ± 2	4 ± 2
RL[b]	2.45	3.25	3.70	4.63	4.89	

[a] Percentage composition based on total sugars. Results are the average and spread of values from four elicitor preparations.

[b] TLC mobility relative to the internal standard lactose.

TABLE 3.2

Effects of treatments to Colletotrichum elicitor preparations on their ability to induce phenylpropanoid pathway enzymes in bean cell cultures

ENZYME ACTIVITY[a]

Elicitor[b]	Phenylalanine ammonia-lyase (μkat kg protein^{-1})			Chalcone synthase (μkat kg protein^{-1})			Chalcone isomerase (mkat kg protein^{-1})		
	Water (control)	Cell wall elicitor	Culture filtrate elicitor	Water (control)	Cell wall elicitor	Culture filtrate elicitor	Water (control)	Cell wall elicitor	Culture filtrate elicitor
Treatment to Elicitor[c]									
None	20	70	59	0.06	0.38	0.25	3.77	9.95	7.40
Autoclave, 15 min	-	75	54	-	0.38	0.21	-	9.57	7.60
Proteinase K	11	73	58	0.14	0.45	0.29	4.48	10.00	7.68
Sodium periodate	23	17	29	0.09	0.09	0.13	3.65	3.95	3.66
Periodate, ethylene glycol	17	73	56	0.08	0.37	0.25	3.25	9.52	7.56

[a] Enzyme activities were determined after 6h (phenylalanine ammonia-lyase, chalcone synthase) or 18h (chalcone isomerase) exposure to elicitor.

[b] Elicitors were added to cells at a final concentration of 25 μg glucose equivalents ml^{-1}.

[c] Details of treatments are described in Section 3.22.

Table 3.3

Induction of phenylalanine ammonia-lyase in bean cultures exposed to nuclease-treated culture filtrate elicitor from C. lindemuthianum

Treatment[a]	Phenylalanine ammonia-lyase activity (μkat kg protein^{-1})
Water	9
Culture filtrate elicitor	75
Ribonuclease-treated culture filtrate elicitor[b]	68
Deoxyribonuclease-treated culture filtrate elicitor[c]	72
Ribonuclease[b]	11
Deoxyribonuclease[c]	10

[a] Cells were exposed to elicitor at a final concentration of 50 μg glucose equivalents ml^{-1}.

[b] Ribonuclease was present at a final concentration of 10 ng ml^{-1} in the cell culture.

[c] Deoxyribonuclease was present at a final concentration of 1·9 ng ml^{-1} in the cell culture.

of both cell wall and culture filtrate elicitors resides in a carbohydrate moeity and that possible contaminations in the elicitor preparations, such as fungal enzymes (English et al, 1971) and nucleic acid material, are inactive.

Earlier work has shown that elicitor activity could be inhibited by certain methyl sugar derivatives which were proposed to act by competing for elicitor binding (Ayers et al, 1976 b; Marcan et al, 1979). The effectiveness of α-methyl glycosides in blocking enzyme induction by competing with Colletotrichum culture filtrate elicitor molecules for putative binding sites was investigated using methyl derivatives of mannose, glucose and galactose, sugars which are constituents of the elicitor preparation (Figure 3.2). Induction of PAL and CHI activities by crude culture filtrate elicitor was partially inhibited (by 13% for both PAL and CHI) by a 5 min pre-treatment with 0.1 mM α-methyl mannoside, with inhibition increasing to 35% and 22% for PAL and CHI activities, respectively, with 2 mM α-methyl mannoside. Other methyl sugar derivatives however (α-methyl β-glucosides, α-and β-galactosides) had little effect on elicitor induction of either enzyme at both sugar concentrations tested. These results tentatively suggest that mannose residues may be important for elicitor activity.

Gel filtration of crude and proteinase K-treated culture filtrate elicitor on Sephacryl S-300 showed that although there was no shift in the elution volume of the major carbohydrate peak following proteinase K treatment, a small increase in the amount of low M_r carbohydrate was observed, accompanied by the loss of detectable protein (Figure 3.3). These results suggest that a small proportion of the carbohydrate in the elicitor preparation is associated with protein.

<u>Figure 3.2 - Effect of methyl glycosides on induction of phenylalanine</u>
<u>ammonia-lyase and chalcone isomerase in bean cell suspension cultures</u>
<u>treated with *Colletotrichum* culture filtrate elicitor</u>

Phenylalanine ammonia-lyase (A) and chalcone isomerase (B) activities
were measured in bean cell cultures treated with 0.1 m$\underline{M}$ (a) or 2.0 $\underline{M}$ (b)
α-methyl mannoside (2), α-methyl glucoside (3), β-methyl glucoside (4),
α-methyl galactoside (5) and β-methyl galactoside (6) in the presence,
open histograms, or absence, hatched histograms, of <u>Colletotrichum</u>
culture filtrate elicitor (25 μg glucose equivalents ml^{-1}). Where methyl
glycosides were used, the cells were incubated with the sugar for 5 min
prior to addition of the elicitor preparation. Histograms labelled 1
represent control cultures treated with H_2O in place of methyl sugars.
Cultures were treated for 6 h (PAL) or 18 h (CHI). Each histogram
represents the average result from three separate experiments whose
spread of values is indicated by the bars.

Figure ... - ... tration of crude ... elicitor ... elicitor on Sephacryl S...

Crude culture filtrate elicitor (3) ... ... buffer, pH 7.5) ... ... (Section 4.2.1). Elicito... ... proteinase K digestion as described ... ... were determined for total carbohydrate (●), ... and protein (○) as determined with Bradford ... ... The ... shown in (A) indicate the ... ... markers: (1) blue dextran ... ... M, 35 000, (3) bovine serum ... ... M, 25 000 and (4) ribonuclease A ...
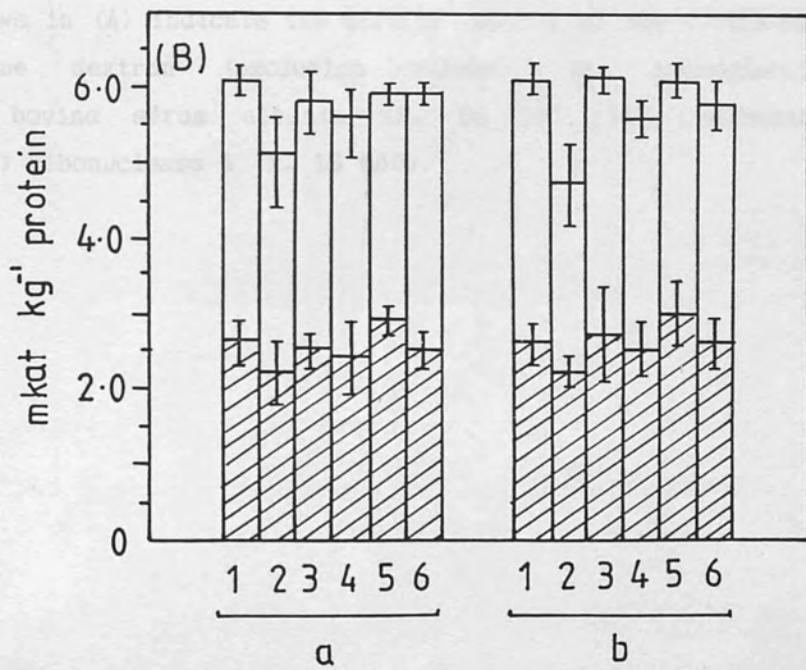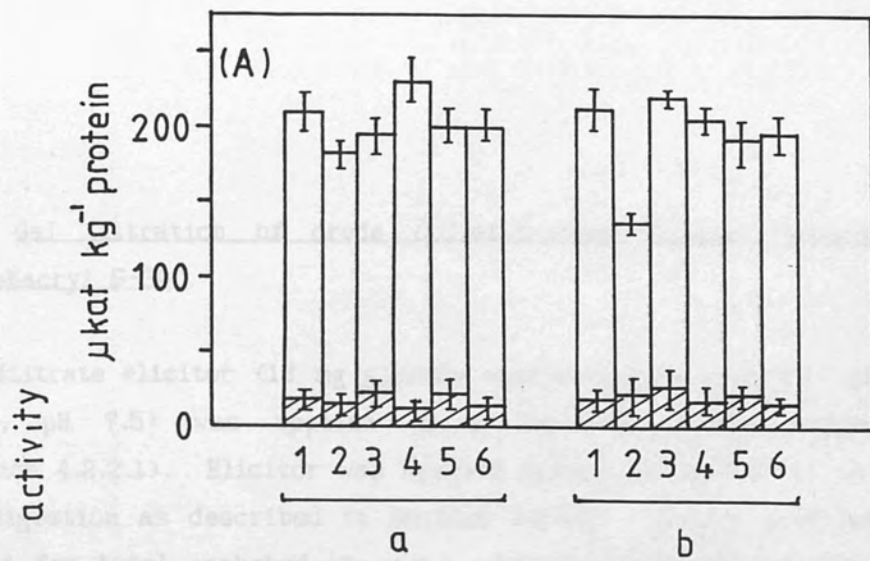
Figure 3.3 – <u>Gel filtration of crude *Colletotrichum* culture filtrate elicitor on Sephacryl S-300</u>

Crude culture filtrate elicitor (13 mg glucose equivalents in 1 ml 50 mM KH_2PO_4 buffer, pH 7.5) was applied to a Sephacryl S-300 column (20x1 cm)(Section 4.2.2.1). Elicitor was applied before (A) and after (B) proteinase K digestion as described in Section 3.2.2.2. Elution profiles were determined for total carbohydrate (●), absorbance at 280 nm (o) and protein (□) as determined with Bradford's reagent (Absorbance at 595 nm). The arrows in (A) indicate the elution volumes of the following markers: (1) blue dextran (exclusion volume), (2) haemoglobin (M_r 68 000), (3) bovine serum albumin (M_r 66 000), (4) ovalbumin (M_r 45 000) and (5) ribonuclease A (M_r 13 680).

3.2.2   Effect of elicitors on electrolyte leakage and apparent viability of bean cells

Culture filtrate and cell wall elicitors at concentrations up to 100 µg glucose equivalent ml^{-1} gave similar profiles for apparent cell viability following exposure of cultured bean cells to elicitor for 6 h and 18 h. A decrease in cell viability, as assessed by TTC reduction, was observed with increasing elicitor concentration (Table 3.4). Elicitor interference with plasma membrane integrity and/or with mitochondrial TTC reduction could account for the observed decrease in formazan production. However, as electrolyte leakage from both isolated leaf mesophyll cells and leaf discs was unaffected by treatment with cell wall or culture filtrate elicitors at a concentration of 100 µg glucose equivalents ml^{-1} (Figure 3.4, A,B), it is unlikely that elicitors cause serious interference with membrane integrity. As the apparent cell viability, at a given elicitor concentration was no less than that seen at 6 h while that at 18 h, when cell cultures had been exposed to elicitor, the effect on bean cell growth over this time period was minimal at 18 h). It appears that the decreasing viability assay is an impairment of TTC reduction rather than any significant effect on cell viability per se.

3.2.3   Induction of phytoalexins

Phytoalexin induction in response to the cell wall elicitor preparations was measured in bean cell suspensions and wounded underground tissue. The high-performance TLC system used for phytoalexin analysis readily separated a number of isomers from one another and from other constituents present in the extracts as fluorescing spots under ultraviolet light (Figure 3.5). In addition to phaseollin and phaseollidin, which were detected in significant quantities, other well-characterised 5-hydroxy and 5-deoxy isoflavones were quantitated including coumestrol and cell suspension culture extracts (Table 3.5; Figure 3.5).

- 97 -

3.3.2   Effect of elicitors on electrolyte leakage and apparent viability of bean cells

Culture filtrate and cell wall elicitors at concentrations up to 100 μg glucose equivalent ml^{-1} gave similar profiles for apparent cell viability following exposure of cultured bean cells to elicitor for 6 h and 18 h. A decrease in cell viability, as measured by TTC reduction, was observed with increasing elicitor concentration (Table 3.4).   Elicitor interference with plasma membrane integrity and/or with mitochondrial TTC reduction could account for the observed decrease in formazan production.   However, as electrolyte leakage from both isolated leaf mesophyll cells and leaf slices was unaffected by treatment with cell wall or culture filtrate elicitors at a concentration of 100 μg glucose equivalents ml^{-1} (Figure 3.4, A,B), it is unlikely that elicitor causes serious interference with membrane integrity.   As the apparent cell viability at any given elicitor concentration was no less at 18 h than at 6 h (Table 3.4), and as cell wall elicitor had been previously shown to have little effect on bean cell growth over these time periods (Bolwell et al, 1985 a), it appears that Colletotrichum elicitors simply cause an impairment of TTC reduction rather than any significant effect on cell viability per se.

3.3.3   Induction of phytoalexins

Phytoalexin induction in response to the two Colletotrichum elicitor preparations was measured in both cell suspension cultures and endocarp tissue.   The two-dimensional TLC system used for phytoalexin analysis readily separated a number of isoflavonoids from one another and from other unidentified compounds which were visible as fluorescing spots under ultraviolet light (Figure 3.5 and Table 3.5).   In addition to phaseollin and kievitone (the only phytoalexins detected in significant quantities), other isoflavonoids such as coumestrol and a number of related 5-deoxy coumestans, and 5-hydroxy and 5-deoxy isoflavones were tentatively identified in endocarp and cell suspension culture extracts (Table 3.5; Figure 3.6).

Table 3.4

Effect of Colletotrichum elicitor preparations on the ability of bean cell suspension cultures to reduce 2,3,5-triphenyltetrazolium chloride

Elicitor concentration (μg glucose equivalents ml^{-1})	Apparent cell viability (Absorbance at 485 nm)[a]			
	6 h		18 h	
	Culture filtrate elicitor	Cell wall elicitor	Culture filtrate elicitor	Cell wall elicitor
0	2.05 ± 0.15	2.05 ± 0.15	2.21 ± 0.25	2.21 ± 0.25
10	1.95 ± 0.05	1.92 ± 0.07	1.80 ± 0.05	1.75 ± 0.12
25	1.62 ± 0.09	1.73 ± 0.13	1.56 ± 0.06	1.75 ± 0.05
50	1.63 ± 0.02	1.57 ± 0.15	1.60 ± 0.15	1.51 ± 0.14
75	1.61 ± 0.09	1.57 ± 0.06	1.47 ± 0.08	1.62 ± 0.08
100	1.59 ± 0.05	1.56 ± 0.04	1.50 ± 0.09	1.53 ± 0.13

[a] Viability was assessed by measurement of the absorption of the red formazan extracted from bean culture batches (0.5 g) after 24 h incubation with 2,3,5-triphenyltetrazolium chloride. Cell cultures had previously been incubated with different concentrations of elicitors for 6 h or 18 h. Results are the average and spread of values from two separate determinations.
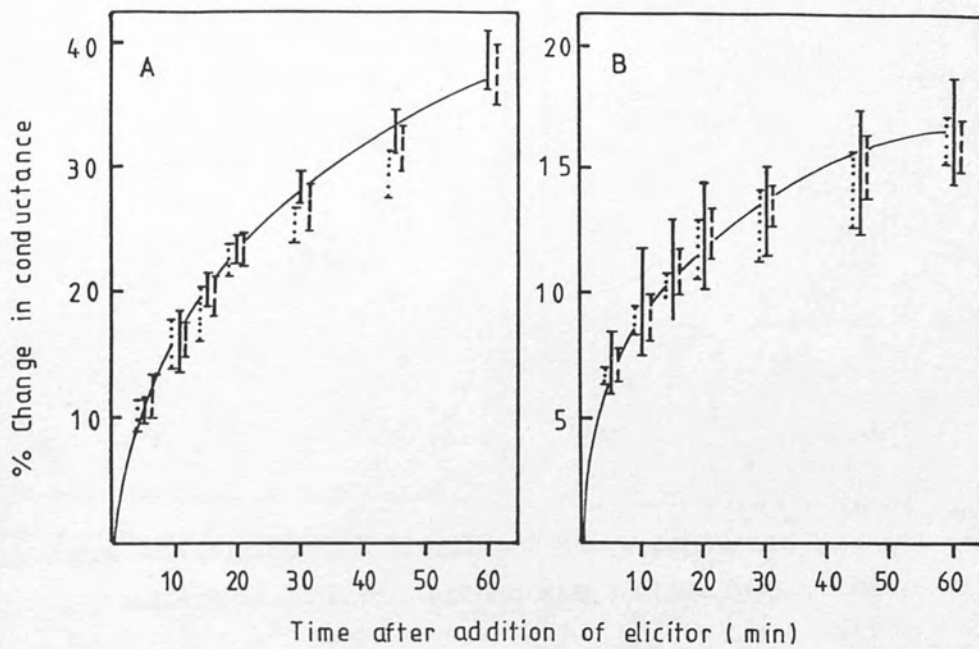
Figure 3.4 - Effect of _Colletotrichum_ elicitors on electrolyte leakage from _P. vulgaris_ tissues

Isolated leaf mesophyll cells (A), or leaf slices (B), were treated with osmoticum alone (.....) or with 100 μg glucose equivalents ml^{-1} cell wall elicitor (___) or culture filtrate elicitor (____) as described in Section 3.2.8. The bars represent the values of two replicate determinations.

Figure 3.5 - <u>Two-dimensional thin-layer chromatographic analysis of phenolics and related compounds in elicitor-treated bean tissue</u>

Bean cell cultures (A-C) and endocarp tissue (D-F) were either untreated (A,D) or exposed to <u>Colletotrichum</u> cell wall elicitor (B,E) or culture filtrate elicitor (C,F) for 48 h as described in Section 2.7. Spots with corresponding R_f values in the first (1) and second (2) dimensions were arbitrarily assigned a number between 1 and 29. Spots from experiment 1 (A,B,C) were numbered separately from those from experiment 2 (D,E,F). Arrows indicate the positions of phaseollin (p) and kievitone (k). See tables 3.6 and 3.7 for quantitative data.

(1) Diethyl ether : hexane (5:1)

(2) Benzene : methanol (9:1)

(A)

28

26

22

20

18

10

19

12 11

7

15

(B)

28

25

23

p

21

13

14 10

16 9

12 8

11

k

6

4

1

2

(C)

27

26 24

28

25

14

9 8

k

15

17

5 3

(D)

29

28

27 26

p

24

23

22

17 15

18

21

16

14

11

7

5

2

(E)

29

26

25

20

24

23

22

18

17 15 12

16 14 11

9

13 10

21

k

8

6

3 2 1

5

4

(F)

29

26

p

20

24

23

22

18

17 15 12

11

14

21

k

7

6

9

13 10

5

2

- 102 -
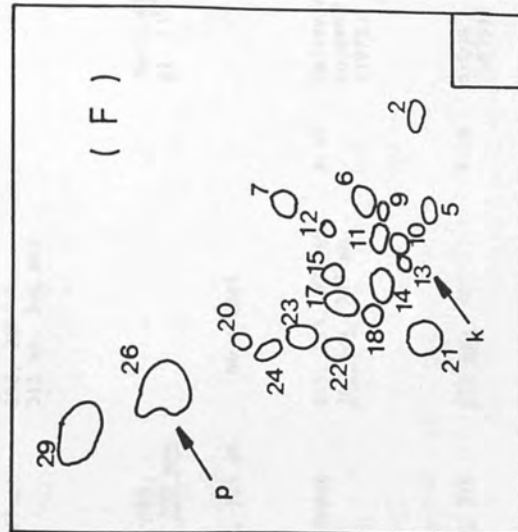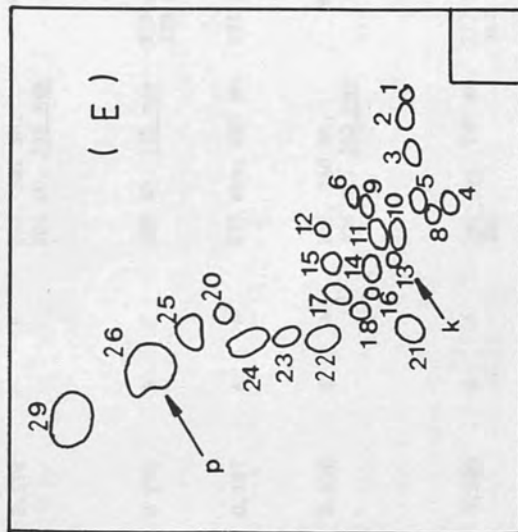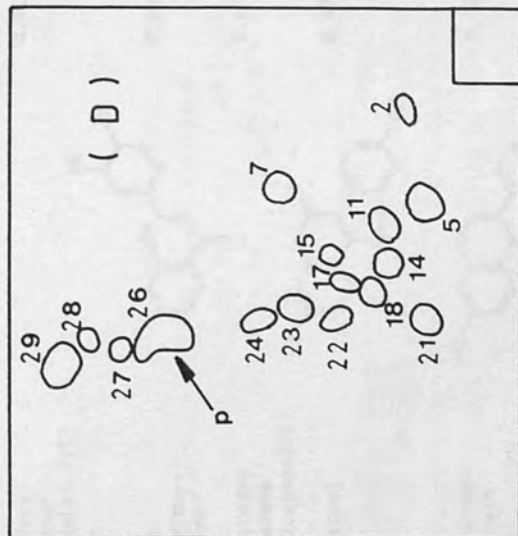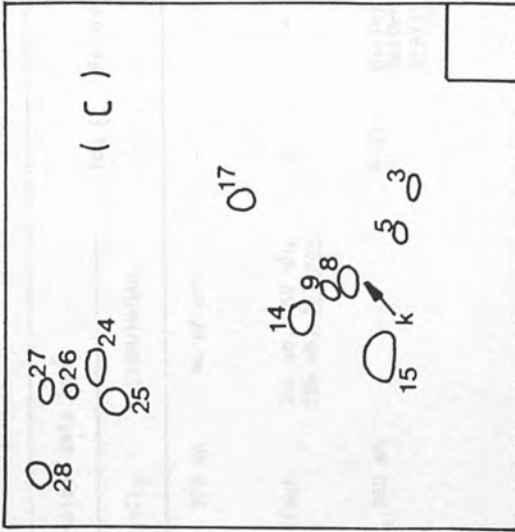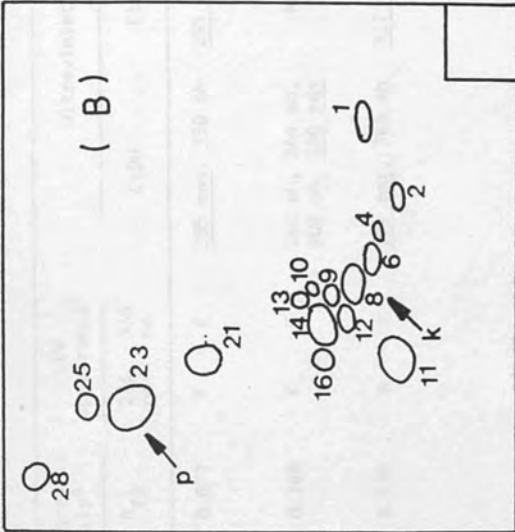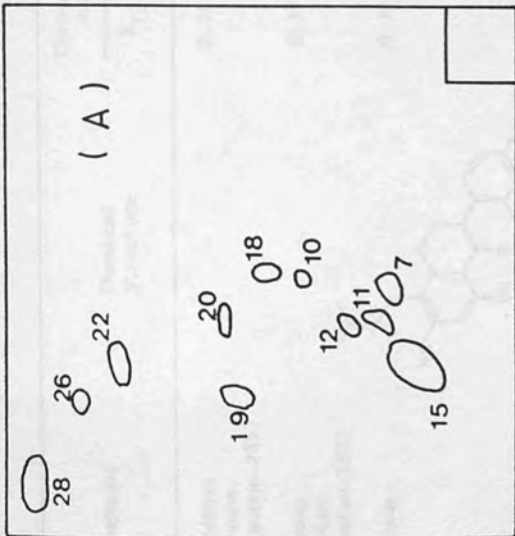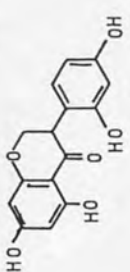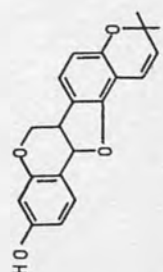
TABLE 3.5

Spectral analysis of flavonoid compounds from elicitor-treated Phaseolus vulgaris tissue

Compound	Chemical Structure	Chromatographic mobility[a]		UV appearance[b]		Ultraviolet spectral data λnm[c]			log ε	Reference
		R_{f1}	R_{f2}	254 nm	366 nm	EtOH	EtOH/AlCl₃	EtOH/NaOAc		
A 5-hydroxy isoflavone, (Isoflavone-285)	-	0.265	0.077	F	F	285 max, 350 sh	295 max, 370 sh	No effect	-	-
A 5-deoxy coumestan (Coumestan-350)	-	0.335	0.168	F	F	240 sh, 264 sh, 280 sh, 350 max	No effect	346 sh, 269 sh, 286 sh, 355 max	-	-
Kievitone		0.394	0.135	A	A	295 max, 345 sh	317 max, 360 sh	-	4.22	Bailey and Burden (1973)
A 5-deoxy coumestan (Coumestan-342)	-	0.419	0.174	F	F	242, 262 sh, 304 sh, 342 max	No effect	250, 269 sh, 311 sh, 346 max	-	-
2',3,4-trihydroxy-chalcone?		0.406	0.271	A	F	306 sh, 338 max	235 sh, 315, 328 sh, 379 max	-	-	Mabry et al. (1970)
A 5-hydroxy isoflavone (Isoflavone-275)		0.470	0.187	A	A	275 max, 325 sh	291 max, 335 sh	No effect	-	-
Coumestrol		0.471	0.258	A	A	245, 260 sh, 304 sh, 343 max	No effect	245, 275, 304 sh, 363 max, 383 sh	4.45	Bailey and Burden (1973)
2'-Hydroxy-genistein		0.503	0.200	A	A	262 max, 285 sh, 340	272 max, 315, 363	275 max, 345	4.14	Biggs (1975)

TABLE 3.5 (continued)

Compound	Chemical Structure	Chromatographic mobility[a]		UV appearance[b]		Ultraviolet spectral data λ_{nm}[c]			log ε	Reference
		R_{f1}	R_{f2}	254 nm	366 nm	ETOH	EtOH/AlCl₃	EtOH/NaOAc		
Dalbergioidin		0.574	0.281	A	A	290 max, 340 sh	240, 271 sh, 315 max, 365	272, 313 max	4.31	Farkas et al. (1971)
A 5-hydroxy isoflavone (Isoflavone-250)	-	0.561	0.372	A	A	250 max, 271 sh, 286 sh, 336	261 max, 272 sh, 303, 371	251 max, 262 sh, 282 sh, 331	-	-
Phaseollin		0.638	0.681	A	A	280 max, 285 sh, 317	No effect	-	4.04	Bailey and Burden (1973)
A 5-deoxy coumestan (Coumestan-239)	-	0.748	0.865	F	F	239 max, 265 sh, 350, 367 sh	No effect	244 max, 270 sh, 355, 372 sh	-	-

[a]Values are the averages of nine determinations in each of the solvent mixtures diethylether:hexane (5:1) (R_{f1}) and benzene:methanol (9:1) (R_{f2}). R_f values varied by ± 0.016 between separate determinations using the same solvent mixture stock solution, and by ± 0.033 between separate experiments in which different solvent mixture stock solutions were used.

[b]F, fluorescence; A. absorbance.

[c]The ultraviolet absorption spectra of the ethanol-eluted spots were determined in the presence of AlCl₃ or NaOAc (Section 3.2.6.1) as shown in Fig. 3.5. Kievitone and phaseollin were identified by comparison with authentic standards of these phytoalexins. Tentative identification of the remaining isoflavonoids was from previously published spectral data and/or comparison with flavonoid spectra reviewed by Mabry et al., 1970; the NaOAc spectrum of 5-deoxy coumestans exhibits bathochromic shifts (i.e. to higher wavelengths) usually for both Band I (associated with absorption due to the B-ring cinnamoyl system, usually between 300-380 nm) with respect to the methanol spectrum. Band II of 5-hydroxy isoflavonoids undergoes a 10-14 nm bathochromic shift, relative to the spectrum in methanol, in the presence of AlCl₃ whereas the spectra of isoflavonoids lacking a 5-hydroxyl group are unaffected by this reagent.

Figure 3.6 - <u>Ultraviolet absorption spectra of flavonoid and isoflavonoid</u> <u>compounds from *Phaseolus vulgaris* tissues treated with *Colletotrichum*</u> <u>elicitor</u>

Compounds separated by two-dimensional thin-layer chromatography (Figure 3.5 Table 3.5) were eluted in ethanol and their absorption spectra recorded in the absence (_____) and presence of $AlCl_3$ (____) or NaOAc (.....) as previously described (Section 3.2.6.1).

Isoflavone-285	Coumestan-350	Kievitone	Coumestan-342
2',3,4-Trihydroxy-chalcone	Isoflavone-275	Coumestrol	2'-Hydroxygenistein
Dalbergioidin	Isoflavone-250	Phaseollin	Coumestan-239

Absorbance

200 300 400   200 300 400   200 300 400   200 300 400

In endocarp tissue, both elicitor preparations induced very similar amounts of isoflavonoids, with higher isoflavonoid levels being detected at 96 h than 48 h (Tables 3.6 and 3.7). In cell cultures the levels of phaseollin and kievitone induced in response to cell wall elicitor after a 48 h incubation were similar to those found in endocarp tissue at 96 h. However, no kievitone was detected in culture filtrate elicitor-treated cell cultures and the induced levels of the deoxy isoflavonoids phaseollin and coumestrol (Table 3.7) were much lower than those observed following treatment with cell wall elicitor.

3.3.4 Changes in the levels of wall bound phenolics

Analysis of wall-bound, lignin-like phenolic material was based on measurement of the ultraviolet absorption difference spectra of alkaline-hydrolysed bean cell walls. The difference spectra of wall extracts from control and elicitor-treated bean cell cultures exhibited a maximum at 245 nm with a shoulder at 300 nm, both arising from non-conjugated phenols (Figure 3.7). An additional maximum at 340 nm, caused by ionisable groups of phenols with conjugated side-chains such as hydroxycinnamic acids, was observed only with extracts from cell wall elicitor-treated cells. Quantitative differences in both the absolute and relative heights of the spectral peaks at 245 and 340 nm were observed in extracts from cell wall elicitor-treated and culture filtrate elicitor-treated cells (Table 3.8). From these results it appears that the walls of bean cells treated with cell wall elicitor have a greater amount of bound simple phenolic groups and a higher number of conjugated side chains, as indicated by the major increase in the 340 nm peak. In addition, it should be noted that the extent of cell browning, which was usually but not always observed on elicitor treatment, was consistently greater in cell wall elicitor-treated cells compared to culture filtrate elicitor-treated cells and that the alkaline hydrolysis procedure used to extract wall-bound phenolics did not result in the extraction of all the brown material from the cell wall elicitor-treated bean cell walls, although all pigmentation was

TABLE 3.6

Levels of phytoalexins and related phenolic compounds extracted from Phaseolus vulgaris tissues treated with Colletotrichum elicitors

O.D.λ_{max} g^{-1} fresh weight tissue[a]

Compound	Spot number[b]	Constitutive	Endocarp 48h Water (control)	Endocarp 48h Cell wall elicitor	Endocarp 48h Culture filtrate elicitor	Endocarp 96h Water (control)	Endocarp 96h Cell wall elicitor	Endocarp 96h Culture filtrate elicitor	Spot number[c]	Cell culture 48h Water (control)	Cell culture 48h Cell wall elicitor	Cell culture 48h Culture filtrate elicitor
Isoflavone-285	5	ND	ND	ND	ND	ND	0.09	0.10	-	ND	ND	ND
Coumestan-350	11	ND	0.01	0.01	0.01	0.04	0.04	0.04	-	ND	ND	ND
Kievitone	13	ND	ND	0.05	0.05	ND	0.16	0.12	8	ND	0.18	ND
Coumestan-342	14	ND	ND	0.01	0.02	0.01	0.02	0.02	9	ND	0.13	0.04
2',3,4-trihydroxy chalcone	15	ND	ND	ND	ND	ND	0.01	0.01	-	ND	ND	ND
Isoflavone-275	16	ND	ND	0.01	ND	ND	0.02	0.01	-	ND	ND	ND
Coumestrol	17	ND	0.01	0.04	0.07	0.02	0.09	0.11	14	ND	0.31	0.02
2'-Hydroxygenistein	18	ND	0.01	0.02	0.02	0.01	0.03	0.02	-	ND	ND	ND
Dalbergioidin	22	ND	ND	0.01	0.02	0.05	0.20	0.10	-	ND	ND	ND
Isoflavone-250	23	ND	0.01	0.03	0.02	0.01	0.05	0.05	-	ND	ND	ND
Phaseollin	26	ND	0.13	0.63	0.57	0.25	1.92	1.90	23	ND	2.49	0.04
Coumestan-239	29	ND	ND	ND	ND	ND	0.01	0.01	-	ND	ND	ND

[a]Bean pods and cell cultures were treated for 48 or 96h with water or elicitor (100 μg glucose equivalents ml^{-1}) as described in Sections 2·7·2 & 2·7·1 respectively.

[b]Numbers refer to spots in Figure 3.4, D - E.

[c]Numbers refer to spots in Figure 3.4, A - C.

[d]Not detected.

- 108 -

TABLE 3.7

Changes in the levels of isoflavonoids in elicitor-treated bean tissues

Plant tissue[a]	Incubation period (h)	Isoflavonoid[b] (n mol g⁻¹ fresh weight)														
		Water (control)					Cell wall elicitor					Culture filtrate elicitor				
		P	K	C	HG	D	P	K	C	HG	D	P	K	C	HG	D
Cell suspension culture	48	ND[c]	ND	ND	ND	ND	283.9	14.9	13.5	ND	ND	4.2	ND	0.9	ND	ND
Endocarp	48	14.8	ND	0.4	0.8	ND	74.2	4.2	1.7	1.7	0.6	64.8	4.2	3.0	1.7	2.9
Endocarp	96	28.4	ND	0.9	0.8	2.9	219.0	12.2	3.8	2.6	11.8	216.8	9.3	4.7	1.7	5.9

[a]Cell suspension cultures were treated with elicitor at 100 μg glucose equivalents ml⁻¹ or water (control). Endocarp was treated with 50 μl droplets of elicitor (100 μg glucose equivalents ml⁻¹) or water per seed cavity.

[b]Isoflavonoids (phaseollin, P; kievitone, K; coumestrol, C; 2'-hydroxygenestein, HG; and dalbergioidin, D) were separated by two-dimensional thin-layer chromatography (Figure 3.5) and identified from their ultraviolet absorption spectra (Figure 3.6).
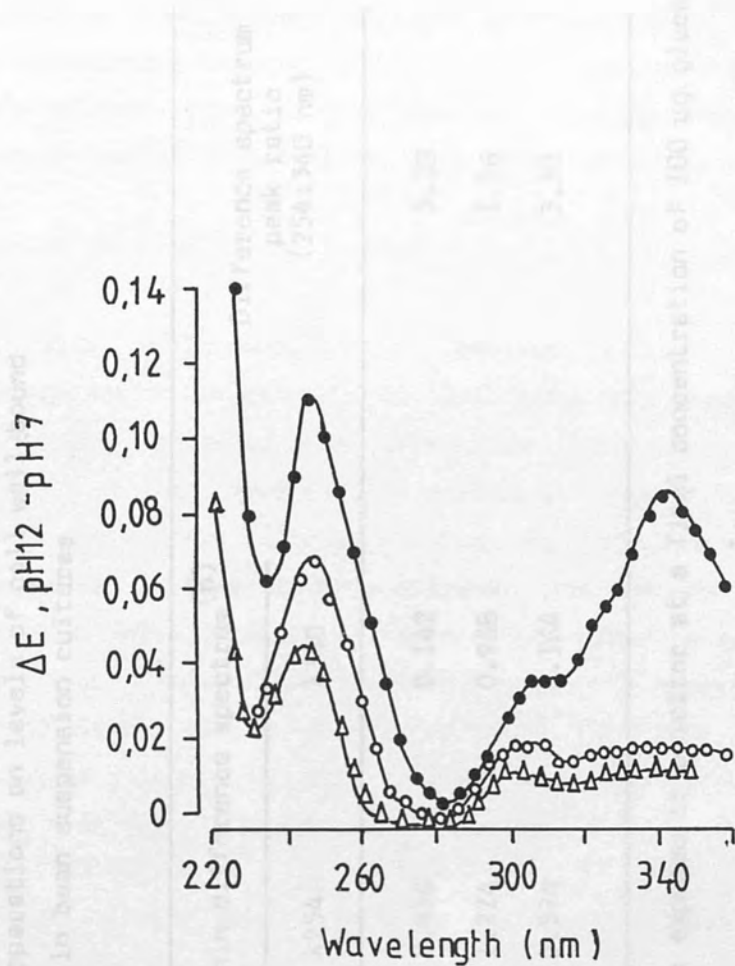
[c]Not detected.

Figure 3.7 - _Difference spectra of alkaline extracts from bean cell cultures treated with Colletotrichum elicitors_

Ultraviolet absorption difference spectra at pH 12.0 and pH 7.0 (ΔE, pH 12-pH 7) correspond to material extracted from 10 mg dry weight of treated cells. Cell cultures were treated for 6 h with 100 μg glucose equivalents ml^{-1} cell wall elicitor (●), culture filtrate elicitor (○), or with water (Δ).

Table 3.8

Effect of elicitor preparations on levels of cell wall-bound phenolics in bean suspension cultures

Treatment(a)	Lignin difference spectrum(b)		Difference spectrum peak ratio (254:340 nm)
	λ254	λ340	
Water (control)	0.456	0.142	3.20
Cell wall elicitor	1.224	0.968	1.26
Culture filtrate elicitor	0.574	0.164	3.50

(a) All treatments were for 6 h. Cells were exposed to elicitor at a final concentration of 100 μg glucose equivalents ml^{-1}.

(b) One aliquot of an alkaline extract from a crude cell wall preparation was adjusted to pH 7.0, another to pH 12.0; the values represent the difference in absorbance (ΔE pH 12-pH 7) at 254 and 340 nm per 10 mg dry weight of cells extracted.

removed from culture filtrate elicitor-treated cell walls. These observations suggest that further qualitative and quantitative differences exist in the phenolic material bound to the walls of cells treated with cell wall and culture filtrate elicitors.

3.3.5 Changes in the levels of free and esterified phenolic acids

Further differences were observed between the two elicitor preparations in relation to their effects on the levels of free and esterified phenolic acid derivatives. Both elicitors had little or no effect on the levels of free caffeic and 4-coumaric acids (Table 3.9). However, culture filtrate elicitor, but not cell wall elicitor, caused a marked increase in free cinnamate and ferulate pools. An increase in the levels of bound caffeate was observed in response to cell wall elicitor treatment as previously reported (Bolwell et al, 1985 a), with little or no change in bound cinnamate or ferulate levels. Culture filtrate elicitor, however, caused a decrease in the levels of all four esterified compounds measured.

3.3.6 Effect of elicitors on wall-bound hydroxyproline and prolyl hydroxylase activity

Increased levels of hydroxyproline have previously been reported in bean cell cultures in reponse to an elicitor preparation from Colletotrichum cell walls (Bolwell et al, 1985 a) and similar changes were observed in the present work (Table 3.10). Prolyl hydroxylase activity was induced to similar levels in cultures treated with cell wall and culture filtrate elicitors, although cell wall elicitor, but not culture filtrate elicitor, induced an increase in wall-bound hydroxyproline.

TABLE 3.9

Changes in the levels of free and esterified cinnamic and hydroxycinnamic acids

in elicitor-treated bean cell suspension cultures

Phenolic acid concentration (nmol g^{-1} fresh weight)[a]

Treatment	Cinnamic acid			4-Coumaric acid			Caffeic acid			Ferulic acid		
	Free	Esterified	Total	Free	Esterified	Total	Free	Esterified	Total	Free	Esterified	Total
Water (control)	2.6 ± 1.6	18.0 ± 2.0	20.6	28.8 ± 1.1	41.5 ± 1.6	70.3	16.9 ± 0.2	26.4 ± 0.3	43.3	2.3 ± 0.7	18.1 ± 0.9	20.4
Cell wall elicitor	3.3 ± 2.1	17.4 ± 2.5	20.7	23.9 ± 0.9	49.3 ± 1.1	73.2	15.8 ± 0.2	36.5 ± 0.5	52.3	2.3 ± 0.6	21.6 ± 0.7	23.9
Culture filtrate elicitor	7.6 ± 1.4	5.1 ± 1.6	12.7	26.4 ± 1.0	28.5 ± 0.7	54.9	17.4 ± 0.2	21.8 ± 0.2	39.2	7.6 ± 1.4	12.4 ± 0.7	19.8

[a]Phenolic acid levels were determined by HPLC analysis of extracts from cells exposed to water (controls), or elicitor (100 µg glucose equivalents ml^{-1}) for 6h. Results are the average and spread of values from two separate determinations.

TABLE 3.10

Induction of prolyl hydroxylase and wall-bound hydroxyproline in elicitor-treated bean cell suspension cultures

Treatment[a]	Prolyl hydroxylase activity (mkat kg protein^{-1})	Wall-bound hydroxyproline (μg g^{-1} fresh weight)
Water (control)	0.0	191
Cell wall elicitor	7.9	297
Culture filtrate elicitor	11.3	155

[a] Cells were treated with water or elicitor (100 μg glucose equivalents ml^{-1} culture) for 6h.

3.3.7   Induction of enzymes of the phenylpropanoid pathway

3.3.7.1   Changes in extractable activities

In view of the previously reported marked dependance of the levels of
phenylalanine ammonia-lyase induction on elicitor concentration in
bean cell cultures treated with Colletotrichum cell wall elicitor, it
was necessary to study the effect of more than a single elicitor
concentration on enzyme induction. Dose-response profiles for the
induction of phenylalanine ammonia-lyase (PAL) , chalcone synthase
(CHS) and chalcone isomerase (CHI) activities were therefore studied
at previously determined times of maximum elicitation response
(Figure 3.8, A-C). Maximal levels of the transiently induced PAL and
CHS enzymes were reached 6 h after treatment of bean cell cultures
with a crude elicitor preparation from Colletotrichum cell walls
(Robbins et al, 1985; Lawton et al, 1983 b), whereas CHI activity
increased slowly to reach maximiun levels 16-24 h following
elicitation (Robbins & Dixon, 1984; Robbins et al, 1985).

The dose-response profiles for the induction of PAL, CHS and CHI
activities by the culture filtrate and cell wall elicitors showed
close similarity although enzyme activity levels induced by the
culture filtrate elicitor were lower than those induced by the cell
wall elicitor at several of the elicitor concentrations tested (Figure
3.8, A-C).   The magnitude of enzyme induction at a given elicitor
concentration varied with cell culture age and passage number.   The
highest levels of PAL induction following incubation with culture
filtrate elicitor at a concentration of 25 µg glucose equivalents
ml^{-1} (ten-fold increase) were obtained with 4-5 day old cells in
their sixth passage.   Generally, increased levels of PAL and CHI
activities were observed with increasing elicitor concentration
(Figure 3.8).   A multiphasic dose-response curve, however, was
observed for CHS induction, with maxima at about 25 and ≥ 180 µg
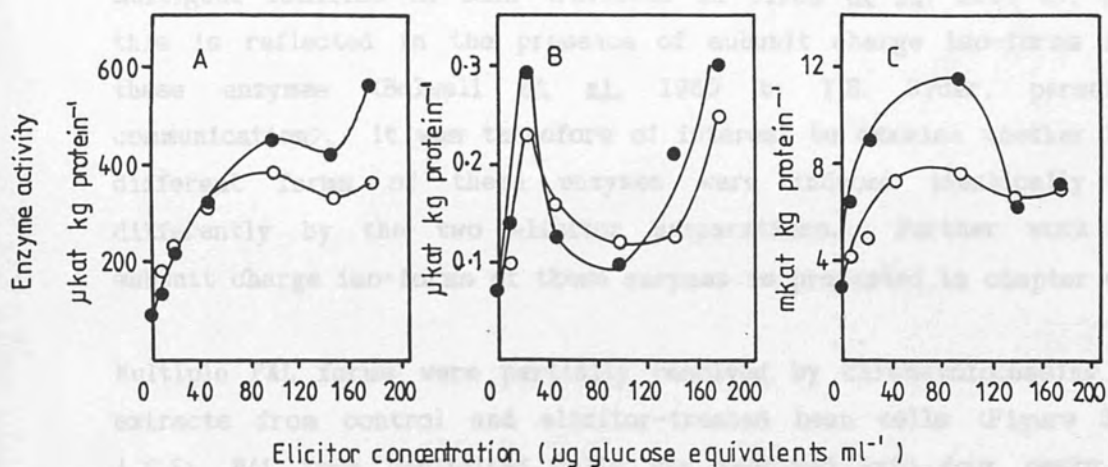glucose equivalents ml^{-1}.

Figure 3.8 - <u>Dose-response curves for enzyme induction by *Colletotrichum*
elicitors</u>

The extractable activities of phenylalanine ammonia-lyase (A), chalcone
synthase (B), and chalcone isomerase (C) were measured in bean cell
suspension cultures treated with varying concentrations of <u>Colletotrichum</u>
cell wall elicitor (●) or culture filtrate elicitor (○). Cells were
harvested at 6 h (A and B) or 18 h (C) after addition of elicitor.

3.3.7.2   Effect of elicitors on relative activities of enzyme charge iso-forms

Recent work has indicated that both PAL and CHS are encoded by small multigene families in bean (reviewed in Dixon et al, 1986 c), and this is reflected in the presence of subunit charge iso-forms for these enzymes (Bolwell et al, 1985 b; T.B. Ryder, personal communication).   It was therefore of interest to examine whether the different forms of these enzymes were induced identically or differently by the two elicitor preparations.   Further work on subunit charge iso-forms of these enzymes is presented in chapter 6.

Multiple PAL forms were partially resolved by chromatofocussing of extracts from control and elicitor-treated bean cells (Figure 3.9, A,C,E). PAL from unelicited cells was resolved into four peaks of approximate pI 4.77, 5.05, 5.20 and 5.40 (Figure 3.9, A) with the predominant pI 4.77 form constituting 74% of total PAL activity (Table 3.11).   Treatment with cell wall elicitor resulted in increased relative appearance of the higher pI forms (Figure 3.9, C) as previously reported (Bolwell et al, 1985 b).   A similar profile was observed for PAL from culture filtrate elicitor-treated bean cultures except that the pI 4.77 form was no longer clearly resolved.

Chalcone synthase activity from unelicited bean cells eluted as a number of components of approximate pI from 4.2-5.3 (Figure 3.9, B). Treatment with cell wall or culture filtrate elicitors resulted in a 20-fold increase in the activities of the pI-5.30 and pI-4.95 peaks, while the overall activity of CHS species of pI less than 4.70 increased only 3-fold (Table 3.11).

Chalcone isomerase activity eluted as a single sharp peak of approximate pI 4.85 in all experiments (Figure 3.9). The peak profile of the monomeric CHI enzyme serves both as an internal marker for PAL (a tetramer) and CHS (a dimer) as well as an indicator of the peak width at half-maximum peak height for a single charge iso-form. Further subunit heterogeneity within the population comprising each

Figure 3.9 - <u>Chromatofocussing profiles for phenylpropanoid pathway</u> <u>enzymes from bean cell cultures exposed to *Colletotrichum* elicitors</u>

Cells were exposed to water (A,B), 25 μg glucose equivalents ml^{-1} cell wall elicitor (C,D) or culture filtrate elicitor (E,F) for 6 h. The 40-80% ammonium sulphate fraction of extracts from 60 g cell batches was dialysed and chromatofocussed as described in Section 3.2.9. Fractions were assayed for phenylalanine ammonia-lyase (● - ● , A,C,E), chalcone synthase (● -● , B,D,F) and chalcone isomerase (o - o , A-F) activities. The three enzymes were assayed in the same column eluates of extracts from cells exposed to the same treatment. Total enzyme activities applied to the column were: PAL (μkat) 27 (A), 148 (C), 150 (E); CHS (μkat) 0.111 (B), 0.960 (D), 1.010 (F); CHI (mkat) 2.3 (A,B), 3.6 (C,D), 3.4 (E,F).

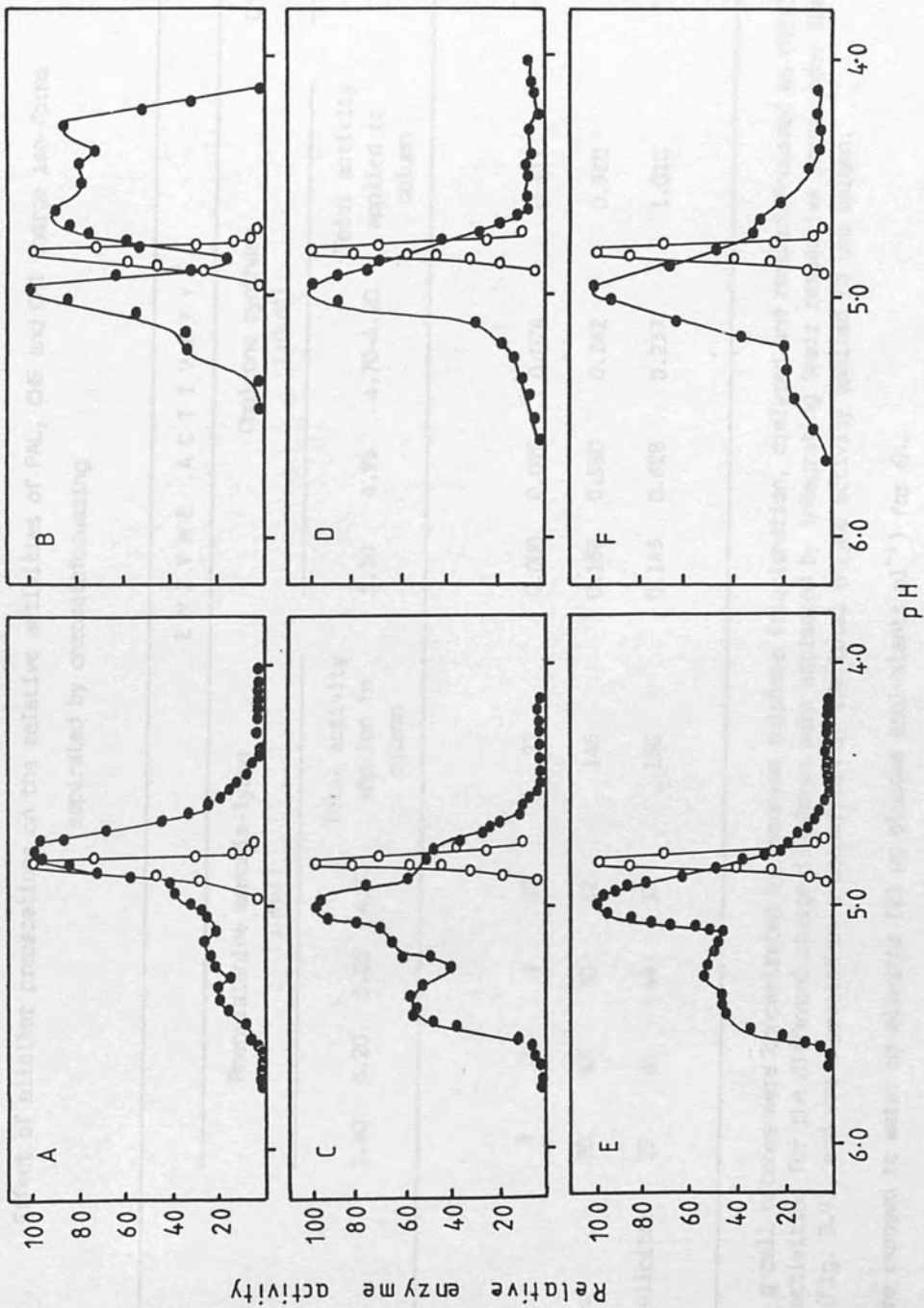## TABLE 3.11

Effect of elicitor preparations on the relative activities of PAL, CHS and CHI charge iso-forms separated by chromatofocussing

	ENZYME ACTIVITY[a]									
	Phenylalanine ammonia-lyase (μkat)					Chalcone synthase (μkat)				Chalcone isomerase ($m$kat)
pI	5.40	5.20	5.05	4.77	Total activity applied to column	5.30	4.95	4.70-4.20	Total activity applied to column	4.85
Treatment[b]										
Water (control)	3	3	1	20	27	0.008	0.029	0.074	0.111	2.3
Cell wall elicitor	30	46	30	42	148	0.158	0.560	0.242	0.960	3.6
Culture filtrate elicitor	22	61	44	33	150	0.145	0.628	0.237	1.010	3.4

[a] Extracts from 60 g cell batches were concentrated by ammonium sulphate fractionation, dialysed and chromatofocussed as detailed in Section 3.2.9. Relative enzyme activities for the different charge iso-forms were estimated by integrating their respective areas under the chromatofocussing elution profile (Fig. 3.9), and are expressed as a proportion of the total enzyme activity applied to the column.

[b] Cell cultures were exposed to water or elicitor (25 μg glucose equivalents ml^{-1}) for 6h.

partially resolved pI form of PAL and CHS is thus further indicated
as the individual PAL and CHS activity peaks were broader than the
CHI peak.

3.4   Discussion

Elicitor activity in preparations from the cell walls and culture
filtrates of Colletotrichum, as determined by the ability of such
preparations to induce PAL, CHS, and CHI activities in bean cell
cultures, appeared to reside in a polysaccharide moeity, although
possible association of some culture filtrate elicitor component(s)
with elicitor-inactive protein could not fully be ruled out. Protein
has also been detected in elicitor preparations from culture filtrates
of three Colletotrichum species (Anderson, 1978).   On the basis of
preliminary observations, mannose residues may be required for
elicitor activity.

Similarity between the monosaccharide composition of the two elicitor
preparations suggests that the culture filtrate elicitor may originate
from the mycelial walls and that it is shed into the culture medium
during hyphal growth.   Although previous preparations of
Colletotrichum cell wall elicitor from the same isolate contained
higher levels of glucose and pentose sugars than observed in this
work (Dixon et al, 1981), this may be a result of differences in
culture medium and/or the relative culture age at which mycelia for
cell wall elicitor preparation were harvested.   Both of these are
factors which have been reported to alter the carbohydrate
composition of elicitor fractions (De Wit & Kodde, 1981).   No
significant differences were observed in the relative sugar
compositions of different preparations of cell wall and culture
filtrate elicitors from fermenter-grown fungal cultures harvested
6-8 days after inoculation.

Although both elicitor preparations had similar effects on cell
viability, electrolyte leakage and the induction of PAL, CHS, CHI and
prolyl hydroxylase activities in bean cell cultures, differences were,
however, observed in their effects on other biochemical responses of
the host tissue. Such differences in the biological activities of the
two elicitor preparations suggest the presence of different
components in the elicitor preparations and this could possibly point

to a requirement for more than one initial stimulus for the overall regulation of induced resistance-related host responses. Earlier work on the induction of PAL activity in bean cell suspension cultures (Dixon et al, 1981) and phytoalexin accumulation in bean hypocotyls and cotyledons (Whitehead et al, 1982) by elicitor fractions from Colletotrichum cell wall elicitor pointed to the possible existence of multicomponent elicitors with different biological activities and suggested that interactions between these individual components may affect the overall activity of the mixture. In addition, different mechanisms of PAL induction in bean cell cultures were observed at low and high concentrations of the cell wall elicitor, with a dual mechanism of induction of synthesis and inhibition of enzyme removal operating at higher elicitor concentrations, whereas lower elicitor concentrations only affected synthesis (Lawton et al, 1980). This again might result from the presence of different elicitor types, with different optimal effective concentrations and ability to interact with other elicitor components.

The most striking differences between the two elicitor treatments were observed in relation to their effects on the induction in bean cell cultures of deoxy- and hydroxy-isoflavonoids and in particular the phytoalexins phaseollin and kievitone. No attempt was made to determine the structure of the tentatively identified 5-hydroxy isoflavones and 5-deoxy coumestans, for example by NMR spectroscopy, as separation and analysis of phytoalexins was undertaken only to show similarities and/or differences between the two elicitor treatments on the patterns of induced phytoalexins. Quantitative differences between the effects of the cell wall and culture filtrate elicitors on a given compound, which had identical spectral profiles in extracts of bean cells treated with either elicitor preparation, could be established from the $O.D._{\lambda max}$ g^{-1} fresh weight values.

The observed failure of the culture filtrate elicitor to induce kievitone accumulation, and its induction of much lower levels of the deoxyisoflavonoids than seen in response to the cell wall elicitor, is unlikely to be a result of differential effects of the two elicitor

preparations on the induction of the overall activities of the regulatory enzymes PAL and CHS or their individual isoforms. Treatment with cell wall or culture filtrate elicitors resulted in similar increased relative appearance of the higher pI, lower K_m forms of PAL from cells in which the low pI PAL forms predominated. Furthermore, broadly similar patterns of induction of the active higher pI CHS forms were observed in response to treatment with cell wall or culture filtrate elicitors.

Treatment of bean cultures with cell wall or culture filtrate elicitors did not appear to result in permanent cell damage or death and no differences were observed between the effects of the two elicitor preparations on apparent viability of cultured cells 24 h after elicitor treatment, a time which coincides with the period of maximal phytoalexin accumulation in cell wall elicitor treated cell suspension cultures (Robbins et al, 1985). This suggests that the different effects of the cell wall and culture filtrate elicitors on phytoalexin accumulation are unlikely to be caused by the lack of suitable accumulation sites; limited cell death has been suggested to be a prerequisite for phytoalexin accumulation in infected bean hypocotyls where dead cells were thought to act as a sink for the accumulation of phytoalexins synthesised by adjacent healthy tissue (Bailey, 1982a). In this respect, the bean cultures used in the present work were very fine suspensions containing a large proportion of single cells, and it is therefore unlikely that death of cells on the outside of aggregates could have acted as the trigger for phytoalexin synthesis and accumulation.

Accumulation of wall-bound phenolics and hydroxyproline may require induction of a wall associated peroxidase activity for oxidative cross-linking to the wall (Fry, 1982), in addition to the possible further requirement of arabinosylation of hydroxyproline-rich protein precursors (Bolwell, 1984; Bolwell et al, 1985 a). No induction of soluble peroxidase activity in cell wall elicitor treated bean cell cultures has been observed (Robbins et al, 1985) although wall-associated peroxidase activity has not yet been investigated.

The major difference between the effects of the two elicitors is, therefore, that end-products of resistance-related host responses, such as phytoalexins, wall-bound phenolics and hydroxyproline, are not induced in response to culture filtrate elicitor treatment, which, however, causes an increase in the levels of enzymes in the early stages of their respective biosynthetic pathways. As all comparative experiments were performed on cells from the same batch culture, using cell wall and culture filtrate elicitors prepared from the same fermenter-grown fungal culture, the observed differences in biological activity therefore most likely reflect differences in components in the elicitor preparation. The observed differences between the two Colletotrichum elicitors may be due to the absence from the culture filtrate elicitor preparation of a component(s) which is responsible for the induction of one or a limited number of enzymes or isoenzymes, such as peroxidases or isoflavonoid synthesis enzymes, further down the biosynthetic pathways from the enzymes here studied (PAL, CHS, CHI). Such a component(s) may either not be released by the fungus into the culture medium or may have been lost during the ethanol-precipitation stage of the culture filtrate elicitor preparation.

The lack of accumulation of end products of pathways in which enzymes in the early stages of biosynthesis are induced following culture filtrate elicitor treatment suggests that induction of the early and late biosynthetic stages is not co-ordinate. A similar lack of correlation between PAL and phytoalexin induction has been described for cell cultures of soybean (Ebel et al, 1984) and parsley (Kombrink & Hahlbrock, 1986). Although co-ordinate induction of phytoalexin biosynthetic enzymes has been demonstrated in bean cultures (Cramer et al, 1985 a), the enzyme activity, rate of synthesis and mRNA activity for CHI have been shown to peak at later times than those for PAL and CHS in bean cultures of cultivars Canadian wonder and Immuna (Dixon and Lamb, 1979; Robbins and Dixon, 1984).

It is difficult on the basis of the above results alone to assign any physiological role for elicitor fractions with different biological activities in intact host-parasite systems in view of the major, and as yet nearly unanswered, question of the availability of elicitors in vivo, and the difficulty of extrapolating results from elicitor-treated cell suspension cultures to infected plants. The latter point is highlighted by the observation that different phytoalexin accumulation patterns were induced by the two elicitor preparations in cell cultures but not in endocarp tissue. However, the possibility that more than one type of elicitor may be involved in switching on the sum total of host responses, and that induction of some metabolic changes related to active defence could be de-coupled from the induction of others, could be of value in elucidating the mechanisms operating in the regulation and coordination of elicitor-induced host gene-expression. Attempts to isolate further, identify and study the effects on host gene expression, of differentially acting components from a crude culture filtrate elicitor preparation are therefore the subject of the remaining chapters of this thesis.

CHAPTER 4

FRACTIONATION AND PROPERTIES OF ELICITOR-ACTIVE COMPONENTS FROM
Colletotrichum CULTURE FILTRATES

Contents

CHAPTER 4

FRACTIONATION AND PROPERTIES OF ELICITOR-ACTIVE COMPONENTS FROM Colletotrichum CULTURE FILTRATES

4.1   Introduction

Previous reports have shown that induced defence reactions in bean tissue exposed to fungal infection or elicitor treatment comprise a multi-component response which includes induction of enzyme and mRNA activities associated with accumulation of isoflavonoid phytoalexins, wall-bound phenolics and hydroxyproline-rich glycoproteins (Bolwell et al, 1985 a; Cramer et al, 1985 a; Dixon et al, 1986 b; Showalter et al, 1985). In Chapter 3, a comparison of defence-related enzyme induction and end-product accumulation in cultured bean cells in response to treatment with crude Colletotrichum cell wall and culture filtrate elicitors demonstrated both qualitative and quantitative differences in the profiles of metabolic changes induced by the two elicitor preparations. These results suggest either that more than one type of elicitor molecule may be necessary to induce the sum total of effects observed in a multicomponent resistance response, or at least that different molecules with the potential differentially to elicit host responses may exist, even though their effects in vivo may be masked by the action of other components with broader biological activity.

Further investigation of the above possibilities would require fractionation of a crude elicitor preparation and examination of whether the fractions can differentially elicit individual components, or groups of components, of the overall induced defence response. Crude culture filtrate elicitor was chosen for the study of the possible presence of multiple elicitor components as it is obtained without the need for harsh unphysiological treatment such as autoclaving which is required for release of elicitors from fungal cell walls and as it elicited some, although not all, of the responses observed with the crude cell wall elicitor. The results in this

chapter, therefore, report the fractionation of crude Colletotrichum culture filtrate elicitor on the basis of size, charge and affinity for Concanvalin A-Sepharose; the effects of the resultant fractions on phytoalexin biosynthetic enzyme induction are compared with the effects of the unfractionated elicitor.

4.2   Materials and methods

4.2.1 Buffers

The following buffers were used : (A) 50 mM KH$_2$PO$_4$ buffer pH 7.5. (B) 20 mM Tris/HCl buffer pH 7.2 containing 10^{-4} M CaCl$_2$ and 10^{-4} M MnCl$_2$. (C) 10 mM Tris/HCl buffer pH 8.0. (D) 10 mM glycine -NaOH buffer pH 4.8.

4.2.2 Column chromatography

4.2.2.1 Gel filtration

Crude culture filtrate elicitor (100 mg glucose equivalents in 4.5 ml buffer A or 13 mg glucose equivalents in 1.0 ml buffer A) was applied to a Sephacryl S-300 superfine (Sigma) column (61 × 1.6 cm or 20 × 1 cm respectively) and eluted with buffer A. Fractions (0.5 ml) were collected at a flow rate of 0.15 ml min^{-1} and assayed for protein, carbohydrate and absorbance at 280 nm. Columns were calibrated with proteins of known M_r : haemoglobin, M_r 68 000; bovine serum albumin, M_r 66 000; ovalbumin, M_r 45 000 and ribonuclease A, M_r 13 700.

4.2.2.2 Affinity chromatography on Concanavalin A-Sepharose

The major carbohydrate peak from the Sephacryl S-300 column (Figure 4.1)(30 mg glucose equivalents in 4.0 ml buffer B) was applied to a Concanavalin A-Sepharose (Sigma) column (2.5 × 1.6 cm) and eluted successively with ten column volumes of buffer B , 0.2 M α-methyl mannoside in buffer B and finally with 0.5 M NaCl in buffer B. Fractions (1.5 ml) were collected at a flow rate 0.2 ml min^{-1}. Carbohydrate content and absorbance at 280 nm were determined. Prior to analysis, fractions eluting with α-methyl mannoside or NaCl were extensively dialysed against H$_2$O until no carbohydrate was detected in control dialysis bags initially containing 0.2 M α-methyl mannoside.

4.2.2.3 Ion-exchange chromatography

(i) Anion-exchange

Crude culture filtrate elicitor (45 mg glucose equivalents in 2.3 ml buffer C) was applied to a DEAE – cellulose (Sigma) column (1.6 × 7.0 cm) and eluted at a flow rate of 0.6 ml min⁻¹ with buffer C followed by 1 M NaCl in buffer C. After analysis of total carbohydrate and absorbance at 280 nm, the bound and unbound fractions were combined separately, dialysed against water, lyophilised and stored at -20°C.

(ii) Cation-exchange

DEAE – cellulose – unbound material (Figure 4.2,A) (5 mg glucose equivalents in 1 ml buffer D) was applied to a CM-cellulose (Whatman) column (1.6 × 8.0 cm) and eluted with buffer D followed by 1 M NaCl in buffer D. After analysis of total carbohydrate and absorbance at 280 nm, the combined CM-cellulose bound and unbound fractions were combined separately, dialysed against distilled water, lyophilised and stored at -20°C.

4.3   Results

4.3.1   Fractionation and composition of culture filtrate elicitor
components

Crude culture filtrate elicitor preparations from a number of
fermenter grown Colletotrichum cultures had closely similar
compositions (Table 4.1). Elicitor preparations were predominantly
carbohydrate with galactose and mannose as the major monosaccharide
components. Protein, which is not required for elicitor activity
(Chapter 3) was present at less than 5% on a weight-for-weight
basis.

Gel filtration of a number of different batches of crude culture
filtrate elicitor on Sephacryl S-300 yielded closely similar elution
profiles (compare Figures 3.3 A and 4.1). Carbohydrate material
eluted as a single, broad peak with M_r at peak maximum of
approximately 110 000. The width of the carbohydrate peak clearly
indicates heterogenous M_r. Material absorbing at 280 nm eluted in
the void volume of the column and as two broad peaks coincident with
the carbohydrate-containing fractions. Absorption at 280 nm is
thought to reflect the presence of pigment and proteinaceous
material. The excluded fraction, which showed high absorbance at 280
nm contained yellow-brown pigmented material. Neither the excluded
nor lower M_r 280 nm- absorbing fractions were destroyed by
proteinase K digestion, which resulted in complete loss of detectable
protein (Figure 3.3 A,B).

Fractions from the Sephacryl S-300 column were pooled as indicated
in Figure 4.1, lyophilised, taken up in distilled H_2O to a final
concentration of 2 mg glucose equivalents ml^{-1} and assayed for their
ability to induce PAL, CHS and CHI activities in bean cell cultures.
A single concentration of 25 µg glucose equivalents ml^{-1} culture was
used for induction of PAL and CHS, a higher concentration of 50 µg
glucose equivalents ml^{-1} culture being used for induction of CHI in
view of the dose-response curve obtained with the Sephacryl S-300

Table 4.1

Composition of _Colletotrichum lindemuthianum_ culture filtrate elicitor fractions

	Monosaccharide composition [a]					Protein (%, w/w)
	Galactose	Glucose	Mannose	Ribose	Rhamnose	
Crude culture filtrate elicitor	50 ± 4	5 ± 2	36 ± 3	trace	9 ± 2	4.0
Sephacryl S-300, fraction B	49 ± 2	7 ± 2	35 ± 5	trace	9 ± 4	4.5
Concanavalin A-Sepharose:						
Unbound	56 ± 5	6 ± 3	30 ± 2	trace	8 ± 2	5.5
α-methyl mannoside eluate	39 ± 11	5 ± 3	50 ± 11	trace	6 ± 1	2.8
NaCl eluate	20 ± 2	6 ± 2	70 ± 3	trace	4 ± 3	6.5
DEAE cellulose:						
Unbound	43 ± 3	8 ± 3	42 ± 2	trace	7 ± 2	0.0
Bound	56 ± 5	4 ± 2	34 ± 2	trace	6 ± 1	3.0
CM cellulose:						
Unbound	42 ± 5	9 ± 4	42 ± 3	trace	7 ± 2	0
Bound	47 ± 6	7 ± 3	39 ± 6	trace	7 ± 2	0

(a) Percentage composition based on total sugars. Results are the average and spread of values from three separate fractionations.
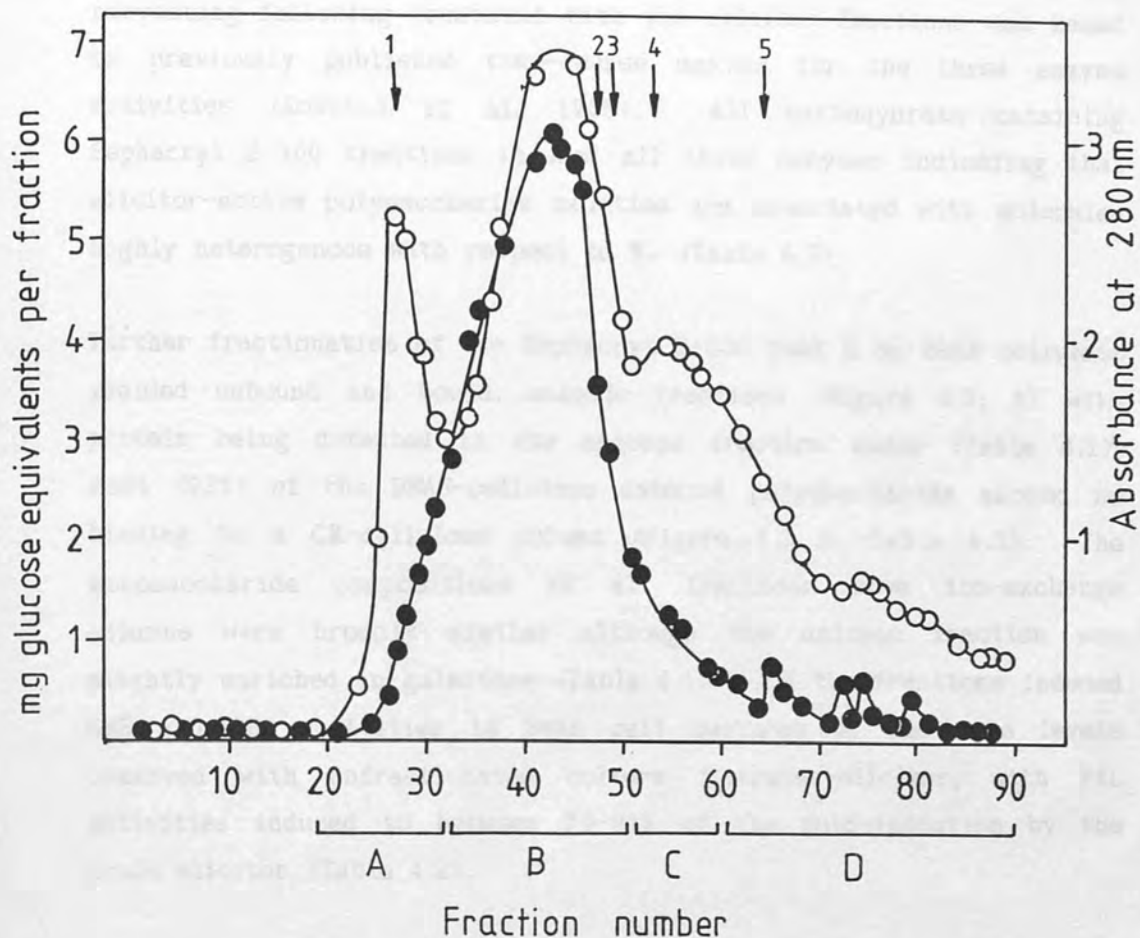
Figure 4.1 - <u>Gel filtration of crude culture filtrate elicitor on</u>
<u>Sephacryl S-300</u>

Crude culture filtrate elicitor (100µg glucose equivalents in 4.5 ml
H₂0) was applied to a Sephacryl S-300 superfine column (61×1.6 cm)
and eluted with distilled H₂0. Fractions (1.5 ml) were collected at
a flow rate of 0.15 ml min⁻¹. Elution profiles were determined for
total carbohydrate (●) and absorbance at 280 nm (○). A-D
represent the fractions pooled, lyophilised and assayed for elicitor
activity (Table 4.3). The arrows indicate the elution volumes of the
following markers: (1) blue dextran (exclusion volume), (2)
haemoglobin (M_r 68 000), (3) bovine serum albumin (M_r 66 000), (4)
ovalbumin (M_r 45 000) and (5) ribonuclease A (M_r 13 680).

fraction B (Figure 4.4 F); concentrations less than 25 μg glucose equivalents ml⁻¹ failed to induce CHI activity. The timing of cell harvesting following treatment with the elicitor fractions was based on previously published time-course maxima for the three enzyme activities (Robbins et al, 1985). All carbohydrate-containing Sephacryl S-300 fractions induced all three enzymes indicating that elicitor-active polysaccharide moieties are associated with molecules highly heterogenous with respect to M_r (Table 4.2).

Further fractionation of the Sephacryl S-300 peak B on DEAE-cellulose yielded unbound and bound, anionic fractions (Figure 4.2, A) with protein being detected in the anionic fraction alone (Table 4.1). Most (93%) of the DEAE-cellulose unbound polysaccharide showed no binding to a CM-cellulose column (Figure 4.2 B, Table 4.3). The monosaccharide compositions of all fractions from ion-exchange columns were broadly similar although the anionic fraction was slightly enriched in galactose (Table 4.1). All the fractions induced CHS and CHI activities in bean cell cultures to the same levels observed with unfractionated culture filtrate elicitor, with PAL activities induced to between 70-97% of the fold-induction by the crude elicitor (Table 4.2).

Peak B from the Sephacryl S-300 column was fractionated by affinity chromatography on Concanavalin-A Sepharose into three distinct fractions. Most of the recovered Concanavalin-A bound material could be eluted from the column with 0.2 M α-methyl mannoside (Figure 4.3). The Sephacryl S-300 peak B obtained from different culture filtrate elicitor preparations contained different proportions of Concanavalin-A binding material ranging between 23-59% of the carbohydrate recovered from the Concanavalin-A Sepharose column (Table 4.3). The third, minor fraction, accounting on average, for less than 1% of the recovered carbohydrate, could be recovered from the α-methyl mannoside-eluted column by elution with buffer B containing 1M NaCl. The total amount of carbohydrate recovered from each of six Concanavalin-A Sepharose column fractionations of Sephacryl S-300 peak B obtained from different batches of crude
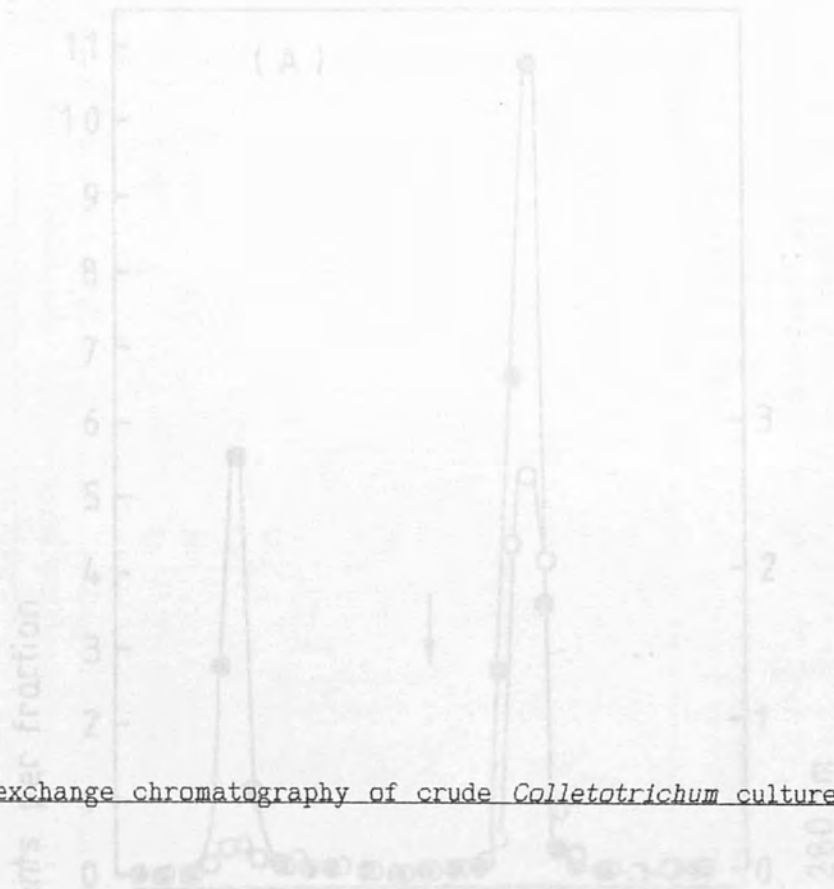
Figure 4.2 - Ion-exchange chromatography of crude *Colletotrichum* culture filtrate elicitor

Fraction B, was fractionated on DEAE-cellulose in 10 mM Tris/HCl, pH 8.0 (A) (Section 4.2.2.3 (i)). Bound material was eluted with the same buffer containing 1 M NaCl (arrow). The unbound fraction was dialysed, lyophilised, taken up in 10 mM glycine-NaOH, pH 4.5, and chromatographed on CM-cellulose (B) (Section 4.2.2.3 (ii)). Bound material was eluted with glycine buffer containing 1 M NaCl (arrow). Elution profiles were determined for total carbohydrate (●) and absorbance at 280 nm (o).
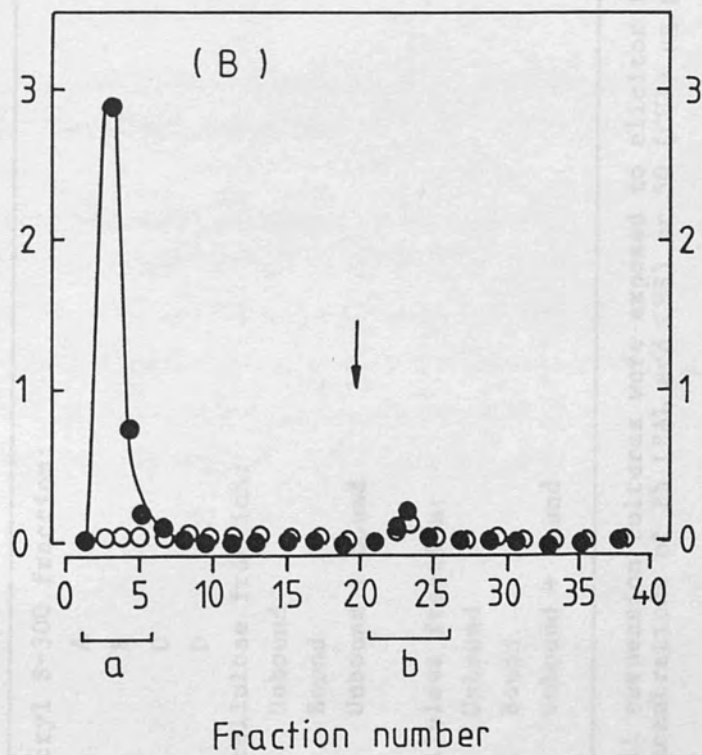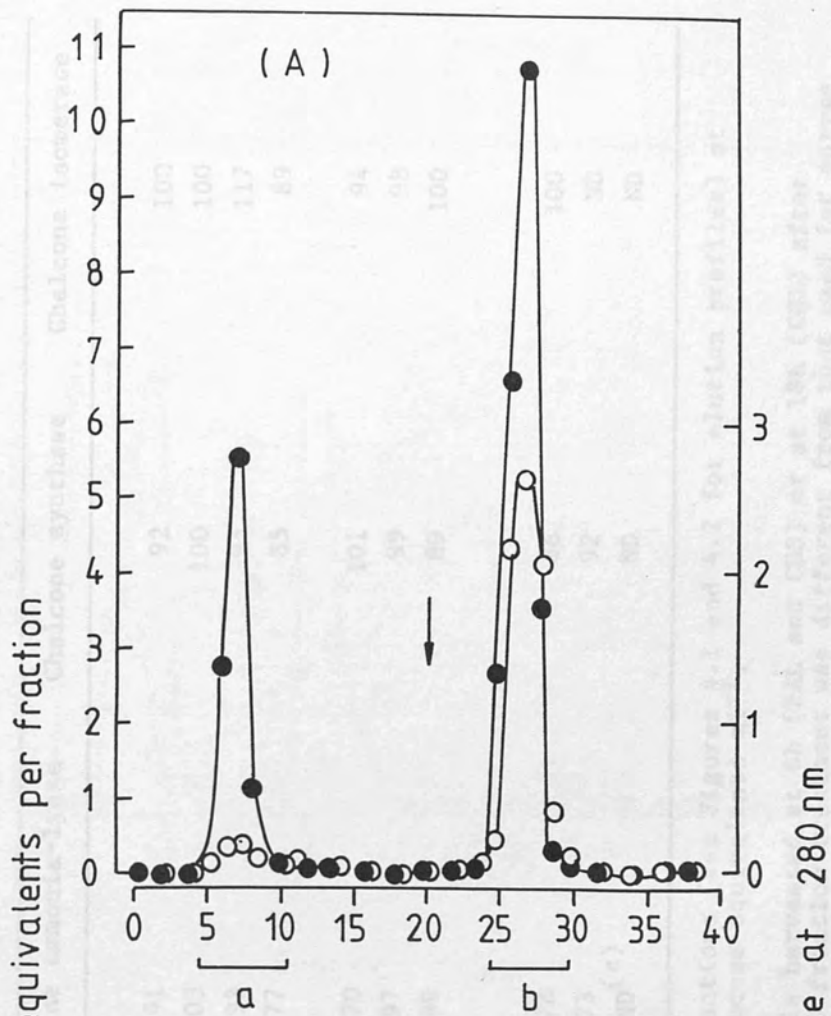
Table 4.2

Elicitor activity of _Colletotrichum_ culture filtrate
components separated on sizing and ion-exchange columns

Fraction(a)	Relative enzyme induction(b)		
	Phenylalanine ammonia-lyase	Chalcone synthase	Chalcone isomerase
Sephacryl S-300 fraction:			
A	91	92	100
B	103	100	100
C	93	95	117
D	77	85	89
DEAE-cellulose fraction:			
Unbound	70	101	94
Bound	97	99	98
Unbound + bound	89	89	100
CM-cellulose fraction:			
Unbound	78	86	100
Bound	73	92	ND
Unbound + bound	ND(c)	ND	ND

(a) Cell suspension cultures were exposed to elicitor fractions (see Figures 4.1 and 4.2 for elution profiles) at concentrations of 25 (PAL and CHS) or 50 (CHI) µg glucose equivalents ml^{-1}.

(b) Enzyme activities were determined in extracts of cells harvested at 6h (PAL and CHS) or at 18h (CHI) after elicitation. The cell batch used for Sephacryl S-300 fraction treatment was different from that used for enzyme assay in cells treated with the ion-exchange column fractions. Enzyme induction levels with the two cell batches with the crude culture filtrate elicitor preparation were 17 and 10 fold for PAL, 4 and 3 fold for CHS, and 2.8 and 1.5 fold for CHI respectively. Values are of fold induction of enzyme activity in the presence of the column fraction relative to that of control (water treated) cells expressed as a percentage of the fold induction of enzyme activity by crude culture filtrate elicitor.

(c) Not determined.
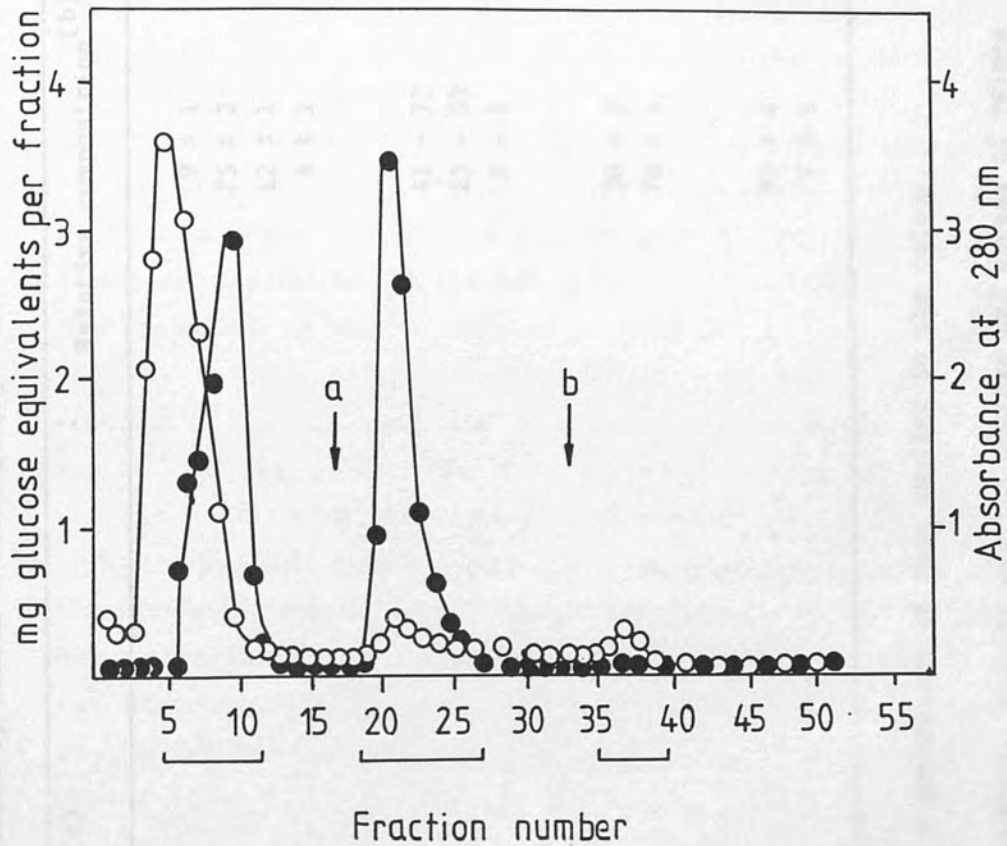
Figure 4.3 – <u>Affinity chromatography of *Colletotrichum* culture filtrate elicitor on Concanavalin-A Sepharose</u>

Culture filtrate Sephacryl fraction B (Figure 4.1) was applied to a Concanavalin-A Sepharose column and eluted with buffer B (Section 4.2.2.2). After elution of the unbound material, the column was eluted in buffer B containing firstly 0.2 $\underline{M}$ α-methyl-D-mannoside (a) and then 1.0 $\underline{M}$ NaCl (b). Total carbohydrate content (●) and absorbance at 280 nm (○) of the fractions were determined.

Table 4.3

Recoveries of *Colletotrichum* culture filtrate elicitor components fractionated by column chromatography

Fraction	Recovery(a)	Relative composition(b)
Sephacryl S-300	100 ± 4	
Fraction A		9 ± 1
Fraction B		75 ± 2
Fraction C		12 ± 1
Fraction D		4 ± 1
Concanavalin A-Sepharose		
Unbound	68 – 96	41 – 77
α-methyl mannoside eluate		23 – 59
NaCl eluate		0 – 1
DEAE-cellulose		
Unbound	96 ± 7	30 ± 2
Bound		70 ± 4
CM-cellulose		
Unbound	100 ± 8	93 ± 4
Bound		7 ± 1

(a) Total carbohydrate (glucose equivalents) recovered as a percentage of that applied to the column. See Figures 4.1 – 4.3 for elution profiles.

(b) Percentage composition based on total carbohydrate recovery. Results are the average and spread of values from at least three separate fractionations.

elicitor varied from 68 to 96% (Table 4.3). Washing of the column with 0.1 $\underline{M}$ Tris/HCl buffer, pH 8.5 followed by 0.1 $\underline{M}$ acetate buffer, pH 4.5 (each containing 1$\underline{M}$ NaCl, 0.1 m$\underline{M}$ MnCl$_2$ and 0,1 m$\underline{M}$ CaCl$_2$) did not result in further elution of carbohydrate material. Non-selective elution methods such as increasing ionic strength by addition of NaCl up to a concentration of 1 $\underline{M}$ and changing the pH, which alters the degree of ionisation of charged groups at the binding sites, have been reported to effect elution of material strongly absorbed to affinity columns (Affinity chromatography, principles and methods, Pharmacia). The reasons for the incomplete recovery of material applied to the column are not understood. Similarly incomplete recoveries following Concanavalin A-Sepharose column chromatography (60% of applied sample) were also reported for culture filtrate elicitor preparations from <u>Cladosporium fulvum</u> (De wit & Roseboom, 1980). Enrichment in the mannose content of the recovered fractions was observed with increasing affinity for Concanavalin-A, with the NaCl-eluted fraction containing 70% mannose and 20% galactose (Table 4.1).

4.3.2   Induction of phenylpropanoid pathway enzymes by culture filtrate elicitor fractions

As outlined above, the activities of the three enzymes PAL, CHS and CHI were induced by all culture filtrate elicitor fractions separated on the basis of M_r or binding to ion-exchange cellulose columns. As induction of PAL, CHS and CHI in bean cell culture treated with <u>Colletotrichum</u> cell wall and culture filtrate elicitors was shown to be dependent on elicitor concentration (Chapter 3), dose-response curves were obtained for the induction of these three enzymes by crude culture filtrate elicitor, Sephacryl S-300 fraction B and the Concanavalin A unbound and bound (α-methyl mannoside-eluted) fractions from Sephacryl S-300 fraction B (Figure 4.4).

Similar dependence of PAL and CHS induction on elicitor concentration was observed in response to treatment with crude and Sephacryl-fractionated elicitor (Figure 4.4) although CHS induction, whose dose-

Figure 4.4 - <u>Dose-response curves for the induction of phenylpropanoid</u> <u>pathway enzymes in cultured bean cells exposed to crude and fractionated</u> *Colletotrichum* <u>culture filtrate elicitor.</u>

The activities of PAL (A,D,G), CHS (B,E,H) and CHI (C,F,I) were measured in extracts from bean cell cultures treated with varying concentrations of crude culture filtrate elicitor (A-C), Sephacryl S-300 fraction B (D-F), and the Concanavalin-A unbound (o) and α-methyl mannoside-eluted (●) fractions (G-I). Cells were harvested for enzyme assay after 6 h (PAL and CHS) or 18 h (CHI) exposure to elicitor components.

Enzyme activity µkat kg⁻¹ protein

Concentration of elicitor fraction

(µg glucose equivalents ml⁻¹ culture)
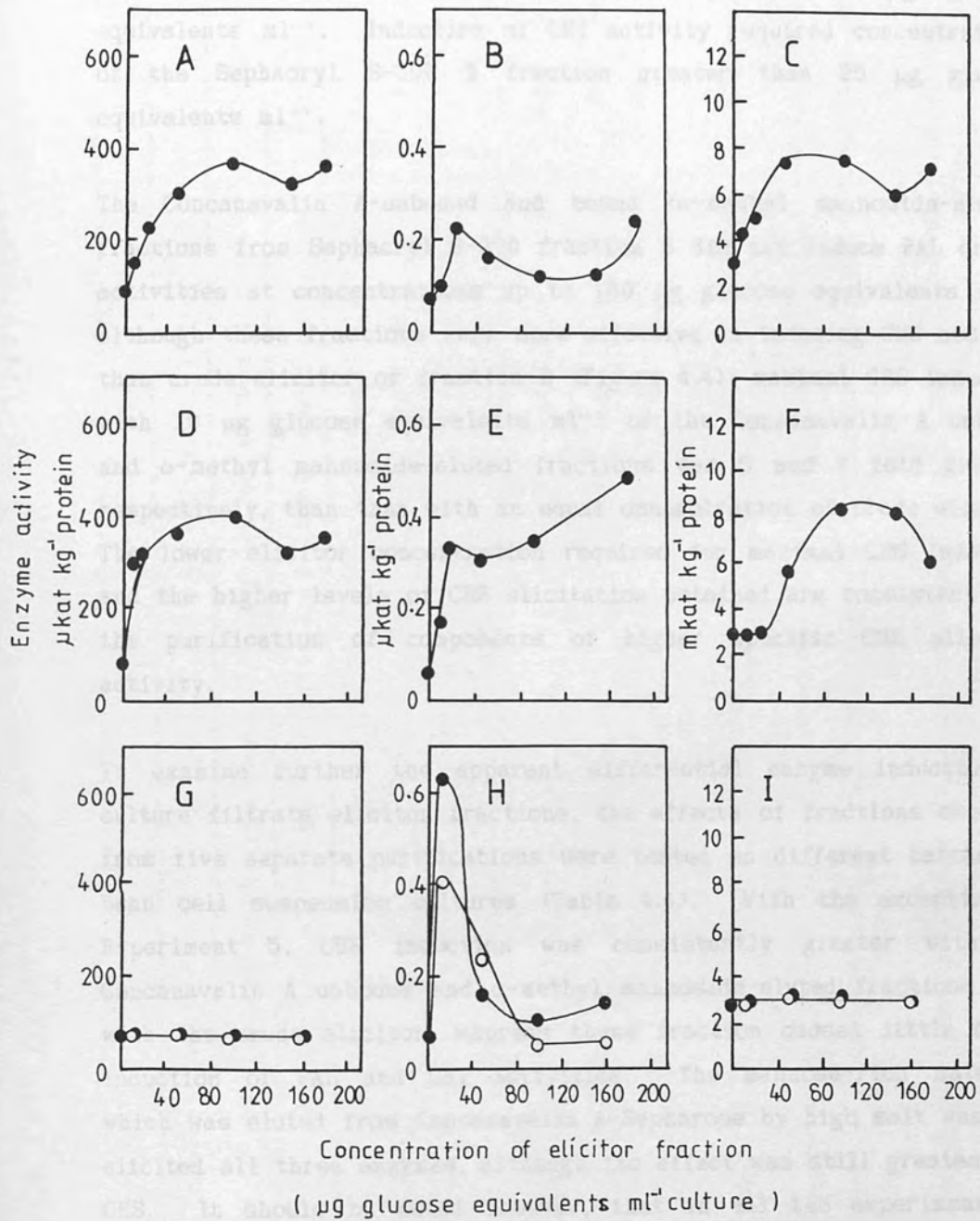
response curve exhibited a peak at 25 µg glucose equivalents ml⁻¹ followed by a second increase at higher concentrations, was higher with Sephacryl S-300 fraction B than the crude elicitor preparation at all elicitor concentrations tested up to 180 µg glucose equivalents ml⁻¹. Induction of CHI activity required concentrations of the Sephacryl S-300 B fraction greater than 25 µg glucose equivalents ml⁻¹.

The Concanavalin A-unbound and bound (α-methyl mannoside-eluted) fractions from Sephacryl S-300 fraction B did not induce PAL or CHI activities at concentrations up to 160 µg glucose equivalents ml⁻¹, although these fractions were more effective in inducing CHS activity than crude elicitor or fraction B (Figure 4.4); maximal CHS induction with 10 µg glucose equivalents ml⁻¹ of the Concanavalin A unbound and α-methyl mannoside-eluted fractions was 5 and 7 fold greater, respectively, than that with an equal concentration of crude elicitor. The lower elicitor concentration required for maximal CHS induction and the higher levels of CHS elicitation obtained are consistent with the purification of components of higher specific CHS eliciting activity.

To examine further the apparent differential enzyme induction by culture filtrate elicitor fractions, the effects of fractions obtained from five separate purifications were tested on different batches of bean cell suspension cultures (Table 4.4). With the exception of Experiment 5, CHS induction was consistently greater with the Concanavalin A unbound and α-methyl mannoside-eluted fractions than with the crude elicitor, whereas these fraction caused little or no induction of PAL and CHI activities. The mannose-rich material, which was eluted from Concanavalin A-Sepharose by high salt washing, elicited all three enzymes, although its effect was still greatest for CHS. It should be noted however, that in all the experiments in Table 4.4, CHS induction in response to the Concanavalin A-Sepharose fractions is an underestimate, as supraoptimal concentrations of these fractions were used (see Figure 4.4). Reconstitution experiments in which fractions were combined on the basis of the

Table 4.4

Enzyme induction in bean cell cultures in response to _Colletotrichum_ elicitor fractions

Fraction[a]	Enzyme activity[b]														
	Phenylalanine ammonia-lyase (μkat/kg)					Chalcone synthase (μkat/kg)					Chalcone isomerase (mKat/kg)				
	Exp 1	Exp 2	Exp 3	Exp 4	Exp 5	Exp 1	Exp 2	Exp 3	Exp 4	Exp 5	Exp 1	Exp 2	Exp 3	Exp 4	Exp 5
None (H_2O control)	83	15	28	20	62	0.67	ND	ND	0.06	0.71	2.9	1.3	ND	3.8	4.6
Crude culture filtrate elicitor	300	57	162	59	175	2.21	ND	ND	0.31	1.39	7.6	5.1	ND	10.0	8.6
Concanavalin A-Sepharose:															
Unbound	80	15	43	15	140	3.75	ND	ND	0.57	1.41	2.9	2.1	ND	6.0	6.3
α-Methyl mannoside eluate	92	17	67	13	171	4.71	ND	ND	0.57	1.38	3.4	1.9	ND	5.9	6.6
NaCl eluate	ND[c]	ND	ND	68	ND	ND	ND	ND	0.92	ND	ND	ND	ND	10.2	ND
Unbound + α-methyl mannoside eluate	ND	ND	121	33	168	ND	ND	ND	0.34	1.24	ND	ND	ND	6.9	6.7
Unbound + α-methyl mannoside eluate + NaCl eluate	ND	ND	ND	56	ND	ND	ND	ND	0.38		ND	ND	ND	11.0	ND

a   Fractions were applied at final concentrations of 25 (phenylalanine ammonia-lyase and chalcone synthase) or 50 (chalcone isomerase) μg glucose equivalents ml^{-1}.

b   Phenylalanine ammonia-lyase and chalcone synthase activities were determined 6h after elicitation, chalcone isomerase 18h after elicitation.

c   Not determined

Figure 4.5 – <u>Dose-response curves for effects of crude and fractionated</u> *Colletotrichum* <u>culture filtrate elicitors on apparent viability of cultured</u> <u>bean cells</u>

Production of formazan (which absorbs at 485 nm) from triphenyltetrazolium chloride was measured (Section 2.9) in cultured bean cells which had been treated for 6 h (__) or 18 h (....) with varying concentrations of crude <u>Colletotrichum</u> culture filtrate elicitor (A), Sephacryl S-300 fraction B (B), the Concanavalin A-unbound- (C), α-methyl mannoside-eluted-(D) and NaCl-eluted-(E) fractions and the three combined Concanavalin A column fractions (F). Bars represent the spread of results from three separate determinations.

The y-axis is labeled "Absorbance at 485 nm" and the x-axis is labeled "Elicitor concentration (µg glucose equivalents ml⁻¹)"
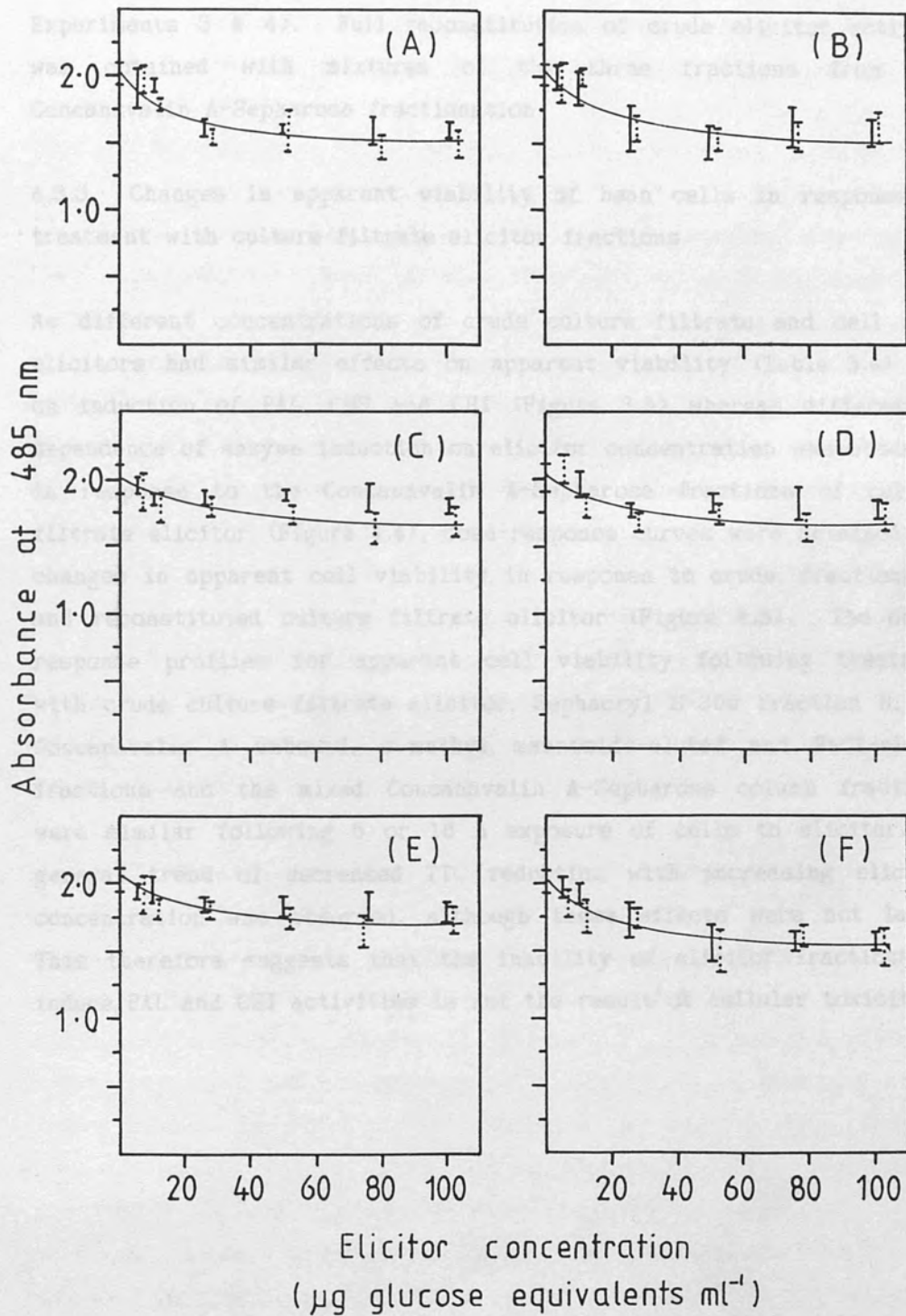
relative weights of glucose equivalents recovered in each fraction demonstrated that mixing of Concanavalin A bound (α-methyl mannoside-eluted) and unbound fractions resulted in partial loss of CHS induction and restoration of PAL and CHI induction (Table 4.4, Experiments 3 & 4). Full reconstitution of crude elicitor activity was obtained with mixtures of the three fractions from the Concanavalin A-Sepharose fractionation.

4.3.3 Changes in apparent viability of bean cells in response to treatment with culture filtrate elicitor fractions

As different concentrations of crude culture filtrate and cell wall elicitors had similar effects on apparent viability (Table 3.4) and on induction of PAL, CHS and CHI (Figure 3.3) whereas differential dependence of enzyme induction on elicitor concentration was observed in response to the Concanavalin A-Sepharose fractions of culture filtrate elicitor (Figure 4.4), dose-response curves were obtained for changes in apparent cell viability in response to crude, fractionated and reconstituted culture filtrate elicitor (Figure 4.5). The dose-response profiles for apparent cell viability following treatment with crude culture filtrate elicitor, Sephacryl S-300 fraction B, the Concanavalin A unbound, α-methyl mannoside-eluted and NaCl-eluted fractions and the mixed Concanavalin A-Sepharose column fractions were similar following 6 or 18 h exposure of cells to elicitor. A general trend of decreased TTC reduction with increasing elicitor concentration was observed, although these effects were not large. This therefore suggests that the inability of elicitor fractions to induce PAL and CHI activities is not the result of cellular toxicity.

4.4   Discussion

The ability of Colletotrichum culture filtrate elicitor components to
induce phenylpropanoid pathway enzymes in bean cell cultures was
found to be associated with fractions which could be separated on the
basis of M_r, charge and affinity for Concanavalin A. These results
suggest that elicitor activity does not reside in a single class of
elicitor molecule. In this respect, it is interesting to note that
more than one elicitor type has been reported from the same pathogen;
for example, glucan (Sharp et al, 1984) and glucomannan (Keen et al,
1983) elicitors have been isolated from cell walls of Phytopohthora
megasperma f. sp. glycinea, and elicitors from Cladosporium fulvum
(syn. Fulvia fulva) have been reported to be glycoproteins (De Wit &
Kodde, 1981) or peptides (De Wit et al, 1985). In the context of the
present work, such considerations do not rule out the possibility,
however, that a single type of elicitor determinant of specific
structural configuration and glycosidic linkage may be associated
with a number of components of differing, but probably closely
related, chemical composition. In this respect, a heptaglucoside
elicitor determinant unit has recently been purified from cell walls
of Phytophthora megasperma f. sp. glycinea (Sharp et al, 1984),
although it is not known whether it is the only elicitor-active
moeity in crude elicitor preparations.

It is not possible at this stage, and without further purification and
structural studies, to speculate on the nature of the structural
unit(s) involved in elicitor activity of preparations from
Colletotrichum culture filtrates. Nevertheless, it appears from
preliminary results in Chapter 3 (Figure 3.1) that mannose residues
may be important for elicitor activity, although involvement of other
sugars cannot be ruled out. The most potent elicitor fraction
obtained in the present work was highly mannose-rich and enzyme
induction by crude culture filtrate elicitor was inhibited by α-
methyl-mannoside. However, caution must by exercised in interpreting
results from inhibition experiments using crude elicitor preparations;
for   example,   α-methyl-mannoside   inhibited   a   crude   elicitor

preparation from the cell walls of P. megasperma f. sp. glycinea far
more effectively than β-methyl glucoside (Ayers et al, 1976 b)
although the smallest elicitor-active component in this preparation
was later found to be a β-glucan (Sharp et al, 1984).

The three fractions isolated from the culture filtrate elicitor
preparation by chromatography on Concanavalin A-Sepharose differed
in their abilities to induce the extractable activities of the
phytoalexin biosynthetic enzymes, PAL, CHS and CHI in bean cells.
These three enzymes are respectively the first commited step in the
biosynthesis of phenylpropanoid derivatives from L-phenylalanine, and
the first and second enzymes of the flavonoid/isoflavonoid branch
pathway which channels phenylpropanoid units into the synthesis of
defence-related secondary products. The Concanavalin-A fractions
showed preferential induction of CHS activity compared to levels
induced with crude culture filtrate elicitor, with partial or complete
loss of PAL and CHI inducing ability. The inability to elicit PAL
and CHI activities does not appear to be the result of differential
effects of these fractions on apparent cell viability (Figure 4.5) or
on supraoptimal concentration effects (Figure 4.4). Rather, it
appears from the results of four experiments in which different cell
culture batches were used (Table 4.4) that differential enzyme
induction may be an inherent property of these elicitor fractions.
The loss of differential enzyme induction in Experiment 5 (Table 4.4)
may have been the result of using a newly established culture line
for this experiment. In suspension cultures of Phaseolus vulgaris
var. immuna, basal PAL, CHS, and CHI activity levels increased and the
extent of enzyme elicitation with a given elicitor concentration
decreased and became highly variable with increasing passage number,
making it necessary to establish new suspensions from callus after
the eleventh passage. This suggests that, in addition to tissue-
specific differences, as observed for phytoalexin accumulation in
bean cell cultures and endocarp tissue (Chapter 3), the effectiveness
of fractions in eliciting a host response may in part depend on the
growth stage or metabolic status of the host cells. The effect of
the physiological state of host tissue on its response to pathogen

products has likewise been demonstrated for maize and potato; young maize leaves from the seedling stage showed significantly greater electrolyte leakage than leaves from adult plants in response to treatment with the host-specific T-toxin from Helminthosporium maydis (Barna et al, 1985) and freshly harvested potato tubers accumulated very low levels of phytoalexin, compared to the levels accumulated in stored tubers, after inoculation with an incompatible race of Phytophthora infestans (Bostock et al, 1983). In the present work in later experiments using later passages of the new culture line newly established in experiment 5 of Table 4.4, the preferential induction of CHS in response to Concanavalin A-fractionated elicitor was again observed (Tables 6.4 & 6.5).

The ability to restore PAL and CHI induction and decrease CHS induction in reconstitution experiments clearly demonstrated that the activity of the unfractionated culture filtrate elicitor is the sum of the additive effects of separate components with different biological activities.

To examine further potential differences in the effects of these elicitor fractions on host cell defence responses, the overall patterns of translational activity of total polysomal mRNA species, and the induced synthesis of separate charge-isoforms of the phenylpropanoid pathway enzymes, have been investigated. These particular approaches, described in chapters 5 and 6 respectively, allow assessments to be made of effects of elicitors on early responses directly related to selective gene expression.

CHAPTER 5

ALTERED PATTERNS OF GENE EXPRESSION IN BEAN CELLS EXPOSED TO CRUDE AND
FRACTIONATED ELICITORS

I. CHANGES IN TOTAL PROTEIN SYNTHESIS AND POLYSOMAL mRNA ACTIVITIES

Contents   Page No.

List of Figures

CHAPTER 5

ALTERED PATTERNS OF GENE EXPRESSION IN BEAN CELLS EXPOSED TO CRUDE AND
FRACTIONATED ELICITORS

I. CHANGES IN TOTAL PROTEIN SYNTHESIS AND POLYSOMAL mRNA ACTIVITIES

5.1   Introduction

In Chapter 3, it was shown that although crude elicitor preparations
from cell walls and culture filtrate of Colletotrichum lindemuthianum
exhibited several similar effects on cultured bean cells, differences
were observed between their effects on a number of individual
parameters including phytoalexin, hydroxycinnamic acid and cell wall
hydroxyproline levels. Three fractions isolated from culture filtrate
elicitor by chromatography on Concanavalin A-Sepharose (unbound
material, bound material eluted with α-methyl mannoside and strongly
bound material eluted with 1M NaCl) showed differential abilities to
induce the extractable activities of phenylalanine ammonia-lyase,
chalcone synthase and chalcone isomerase (Chapter 4). In order to
further extend these observations to a consideration of whether
different elicitor components can exhibit differential effects on host
gene expression, the overall patterns of protein and mRNA induction
in cultured bean cells exposed to unfractionated and Concanavalin A-
Sepharose-fractionated Colletotrichum elicitors were analysed by one-
dimensional and two-dimensional gel electrophoresis. The effects of
proteinase K on patterns of protein synthesis in vivo and in vitro
are also reported in this Chapter as this enzyme, from a non-plant-
pathogenic fungus, Tritirachium album, was found to act as an
elicitor of phenylpropanoid pathway enzyme activity and phytoalexin
accumulation, when not inactivated by autoclaving.

5.2   <u>Materials and methods</u>

5.2.1 Buffers and solutions

The following buffers were used : (A) 1.0 $\underline{M}$ Tris/HC1 buffer pH 8.0.
(B) 0.1 $\underline{M}$ Tris/HC1 buffer pH 8.0 containing 0.2% 2-mercaptoethanol.
(C) 0,1 $\underline{M}$ Tris/acetate buffer pH 8.2, containing 0.05 $\underline{M}$ magnesium
acetate, 10 m$\underline{M}$ potassium acetate, 10 m$\underline{M}$ vanadyl ribonucleosides
(preparaed as described in Section 2.2.5.2), 0.5% (v/v) Triton X-100,
25% (w/v) sucrose, 10 m$\underline{M}$ EDTA and 0.05% (v/v) 2-mercaptoethanol.
(D) 100 m$\underline{M}$ sodium acetate buffer pH 6.0, containing 0.1% (w/v) SDS.
(E) 3 $\underline{M}$ sodium acetate buffer pH 5.5.   (F) 50 m$\underline{M}$ Tris/HC1 buffer pH
7.8, containing 10 m$\underline{M}$ dithiothreitol (DTT), 0.5 m$\underline{M}$ Mg Cl$_2$, 1 m$\underline{M}$
EDTA and 1 m$\underline{M}$ PMSF.   (G) 10 m$\underline{M}$ Tris/HC1 pH 7.4, containing 5 m$\underline{M}$
MgCl$_2$, 1 m$\underline{M}$ PMSF, 0.5 mg ml^{-1} RNAase and 1.0 mg ml^{-1} DNAase.   (H)
9.5 $\underline{M}$ urea (ultra-pure), 8% (v/v) Nonidet P-40 (NP-40), 2% (v/v)
ampholytes pH 3-10, 10 m$\underline{M}$ dithiothreitol and 1 m$\underline{M}$ EDTA.   (I) 62.5 m$\underline{M}$
Tris/HC1 buffer pH 6.8, containing 10% (v/v) glycerol, 5% (v/v) 2-
mercaptoethanol, 2% (w/v) SDS and 0.125% (w/v) bromophenol blue.

5.2.2 Synthesis of proteins <u>in</u> <u>vitro</u>

5.2.2.1 Laboratory techniques for experiments involving RNA

(i) RNAase-free glass and plastic ware

General laboratory glass-ware was siliconised by rinsing with 2%
dichlorodimethyl silane and baking at 180°C overnight.   Sterile
disposable plastic ware was considered essentially RNAase-free.
Disposable gloves were routinely used when handling chemicals and
glassware.

(ii)   Reagents and solutions

a. Water

Distilled water was treated overnight with 0.1% (v/v) diethylpyrocarbonate (DEPC) and autoclaved at 120°C for 15 min. All aqueous solutions used for RNA work were made up using DEPC-treated water.

b. Phenol

8-hydroxyquinoline, an antioxidant and partial RNAase inhibitor, was added to melted, redistilled phenol at a final concentration of 0.1% (w/v). Phenol was extracted once with an equal volume of buffer A followed by several extractions with buffer B until the pH of the aqueous phase was greater than 7.6. The phenol solution was stored at 4°C under buffer B for up to 2 weeks.

c. Chloroform

Chloroform was used as a 24:1 (v/v) mixture of chloroform : isoamyl alcohol.

5.2.2.2 Preparation of polysomal RNA

(i) Polysome preparation

Cells (3 g) were extracted on ice in two volumes of buffer C and the extracts were centrifuged at 10 000 × g for 10 min. After a 10 min recentrifugation at 10 000 × g the supernatant was centrifuged on a 5 ml sucrose cushion (9.4% (w/v) sucrose in buffer C) at 160 000 × g for 2.5 h to obtain the polysomal pellet.

(ii) Extraction of polysomal RNA

Polysomal RNA was purified essentially as described previously
(Maniatis et al, 1984); the polysomal pellet was resuspended in 0.5
ml buffer D, mixed with 50 μl of proteinase K (Sigma) (0.22 mg per
ml of buffer D), and incubated at 37°C for 2 h. Samples were
extracted three times with an equal volume of 1:1 (v/v) phenol :
chloroform mixture, once with chloroform and finally once with water-
saturated diethyl ether. Traces of diethyl ether were removed from
the aqueous phase under a stream of N_2. The aqueous phase was then
mixed with 2.5 volumes of ice-cold ethanol and stored at -20°C
overnight. The ethanolic RNA solution was centrifuged at 8 000 × g
for 20 min, the pellet washed twice with 5 ml buffer E, once with 5
ml 0.3 M NaCl and centrifuged at 8 000 × g for 30 min. The
supernatant was mixed with 2.5 volumes of ice-cold ethanol and left
on dry-ice for 2 h or at -20°C overnight. The final RNA pellet
obtained after centrifugation of the ethanolic RNA solution at
8 000 × g for 1 h was resusupended in 50 μl H_2O and a 3 μl aliquot
was diluted in 1 ml H_2O and the U.V. absorption spectrum recorded
between 240-320 nm. $O.D._{260}/O.D._{280}$ ratio provides an estimation of
the purity of the RNA preparations; pure RNA samples have an
$O.D._{260}/O.D._{280}$ ratio of 2.0. RNA yield was estimated on the basis
that one O.D. unit at 260 nm corresponds to an RNA concentration of
40 μg ml^{-1}.

5.2.2.3 in vitro translation of RNA

Rabbit reticulocyte lysate (Amersham U.K.) was mixed with L-[^{35}S]
methionine to give a final radioactive concentration of 0.5 Ci/l.
Aliquots of this solution (42 μl) were mixed with 8 μl sterile water
or RNA solution containing 30 μg RNA and incubated at 30°C for 1 h.
The reaction was stopped by the addition of 6 μl of buffer G and
incubation on ice for 5 min.

5.2.2.4 Measurement of incorporation of label into total protein

An aliquot of the translation incubation (2 μl) was diluted into 1 ml of 0.33 M NaOH, 0.04% (w/v) methionine and 0.3% (v/v) H_2O_2 and incubated at 30°C for 15 min. Protein was precipitated by the addition of 0.5 ml of 30% (w/v) TCA. Material precipitated after 1 h at 4°C was collected by filtration on 2.5 cm glass-fibre discs (Whatman GF/C) and extensively washed with 8% TCA, 0.5% (w/v) methionine. Dried discs were counted in triton : toluene : PPO scintillant (5 g PPO per litre triton X-100 : toluene (1:1)).

5.2.2.5 Sample preparation for gel analysis

Preparation and translation of polysomal RNA were as previously described (Sections 5.2.2.2 and 5.2.2.3 respectively). A 1 μl aliquot of the translation incubation mixture, containing approximately 20 000 dpm and 15 μg protein, was lyophilised and taken up in 10 μl buffer H ready for two-dimensional gel analysis as described in Section 5.2.4. Sample preparation for one-dimensional gel electrophoresis was as detailed elsewhere (Section 2.10.2).

5.2.3 Synthesis of proteins in vivo

5.2.3.1 Pulse-labelling of cell cultures

Batches of cell culture (5 ml containing approximately 1 g cells) exposed to elicitor (25 μg glucose equivalents ml^{-1}) were labelled with 30 μCi L-[^{35}S] methionine for 1 h between 3 h and 4 h after addition of elicitor. At the end of the labelling period, cells were harvested by suction filtration, frozen in liquid N_2 and stored at -70°C.

5.2.3.2 Sample preparation for gel analysis

Pulse-labelling of cell cultures with L-[^{35}S] methionine was as previously described (Section 5.2.3.1). Cells (0.15 g) were extracted

in two volumes buffer F (Hadwiger & Wagoner, 1983). Following centrifugation, a 50 µl aliquot of the supernatant was mixed with 6 µl of 3% (w/v) SDS and left on ice for 5 min. 6 µl of buffer G (Gabriel & Ellingboe, 1982) was added and the mixture incubated for a further 10 min on ice. Samples were lyophilised, taken up in 20 µl buffer H (Hadwiger & Wagoner, 1983), ready for two-dimensional gel electrophoresis as previously described (Section 5.2.4). Aliquots from the initial extract supernatant were prepared for and subjected to one-dimensional gel electrophoresis as previously described (Section 2.10).

Attempts to apply two-dimensional gel electrophoresis to total in vivo labelled proteins from extracts of cultured bean cells were unsuccessful. Fluorographs of two-dimensional gels to which in vivo labelled protein samples containing 75 000 dpm and 15 µg protein were applied showed extensive streaking in the second dimension SDS-polyacrylamide gel at M_r 20 000 and below (results not shown). Few, faint discrete spots were visually detected at M_r greater than 20 000 in fluorographs which had been left in contact with dried gels for 6 weeks.

It is thought that the problem(s) lies in the preparation of the extracts rather than a failure to use the technique of two-dimensional gel electrophoresis; approximately 90 discrete spots were readily detected visually on fluorographs of 2-D gels of in vitro synthesised proteins to which were applied samples of the same volume (10 µl) and protein content (15 µg) (see Section 5.2.2.5) as the in vivo labelled proteins (Figure 5.5). Streaking was observed although a number of precautions were taken in preparing in vivo labelled protein extracts for 2-D gel analysis. All steps were carried out at 4°C and the time required for extraction was kept to a minimum (30s) to avoid artifacts and a loss of resolution (Gabriel & Ellingboe, 1982). Vigorous grinding was avoided to keep foaming to a minimum and proteinase inhibitor (1 mM PMSF) was included in the extraction medium to reduce protein modification or degradation by cellular enzymes. To avoid artefact formation and protein-ampholyte

interaction, reducing agent (10 mM D T) and metal chelator (1 mM EDTA) were included in the extraction medium (Isoelectric Focussing, principles & methods, Pharmacia). Extracts were treated with ribonuclease and deoxyribonuclease to reduce interference by nucleic acids with the IEF step. Although SDS in the extraction medium is used by a number of workers to solubilise membrane-bound proteins (Garrels, 1979; O'Farrell, 1974; Gabriel & Ellingboe, 1982), SDS was omitted from the extraction medium as it has been reported to cause streaking and interference with the number (Gabriel & Ellingboe, 1982) and intensity (Garrels, 1979) of detected spots. The reasons for streaking of in vivo labelled proteins from bean cell extracts remain unclear.

5.2.4   Two-dimensional gel electrophoresis

5.2.4.1   Isoelectric-focussing (IEF)

Polymerisation of the isoelectric-focussing (IEF) gel solution (9.5 M urea (ultra-pure), 8% (v/v) Nonidet P-40, 2% (v/v) ampholytes pH 3-10, 2.7% (w/v) acrylamide and 0.135% (w/v) bisacrylamide) was initiated by addition of 0.003 volumes of 10% ammonium persulphate (AMPS) and 0.004 volumes of 20% (v/v) TEMED. Polymerisation was allowed to proceed for 3-4 h in glass capillary tubes (Gallenkamp 100 × 1 mm internal diameter). Gels of identical length (80mm) were electrophoresed in a disc electrophoresis apparatus (Shandon) adapted to hold the gel tubes. Gels were overlaid with 3 μl isoelectric focussing overlay solution (buffer H diluted with 0.05 vol. H_2O and 0.05 vol.2-mercaptoethanol) (Garrels, 1979) and run with 0.02 M NaOH and 0.01 M H_3PO_4 in the upper and lower chambers respectively. Prefocussing was at 200v for 15 min followed by successive 30 min periods at 300v, 400v and 500v. Samples (up to 10 μl containing up to 15 μg protein) were then applied and gels were run at 500v for 16 h.

5.2.4.2   Equilibration and application of first dimension IEF-gels to second dimension polyacrylamide gels.

Gels were frozen for 5 min at -20°C prior to extrusion under air pressure into 5 ml buffer I and equilibration for 5-10 min on a rotary shaker at room temperature.   Gels were transferred on to the surface of the polyacrylamide stacking gel (Section 2.10.1) and overlaid with 100 µl of 1% (w/v) agarose in buffer I held at 70°C (O'Farrell, 1974).   The agarose was allowed to set for 5 min and gels were then electrophoresed in the second dimension along with ^{14}C-labelled M_r protein standards (Amersham) as previously described (Section 2.10.3).

5.2.4.3   Measurement of pH gradient

(i) Protein pI markers

Gels were fixed for 1 h in 10% (w/v) TCA, 5% (w/v) sulphosalycilic acid, 33% (v/v) ethanol.   They were washed in 5% (w/v) TCA, 5% (w/v) sulphosalycilic acid, 33% ethanol until all opacity disappeared and then equilibrated in destain solution (7.5% acetic acid, 10% methanol) prior to staining pI markers (Pharmacia) (amyloglucosidase pI 3.5; soybean trypsin inhibitor pI 4.55; β-lactoglobulin A pI 5.20; bovine carbonic anhydrase B pI 5.85; human carbonic anhydrase B pI 6.55; horse myoglobin a (acidic band) pI 6.85, (basic band) pI 7.35; lentil lectins pI 8.15, 8.45, 8.65; trypsinogen pI 9.3) for 1 h in 0.25% (w/v) coomasie blue R-250 in 10% acetic acid : 50% methanol.   Gels were destained in several changes of destain solution.

(ii) Direct pH measurement

The pH gradient was determined using five gels prefocussed and run with 5 µl isoelectric focussing overlay solution as described in Section 5.2.4.1.   Gels were lined up alongside each other on a glass plate, frozen for 5 min at -70°C and cut into 2.5 mm sections. Equivalent sections were placed in 0.25 ml deionised degassed H_2O in

capped vials for 15 min and the pH measured using a micro pH electrode. Five gels were required as the amount of ampholytes in a section of a single gel was not sufficient to alter the pH of the deionised water to that of the corresponding gel section. The pH gradients in gels from two separate experiments were similar (Figure 5.1). The gradient drift, characterised by a flattening of the pH at the anode (Figure 5.1) may have been the result of insufficient cooling of the gels. Gels were not cooled during electrophoresis as urea precipitated at temperatures below 25°C. Attempts to reduce gradient drift included use of high purity reagents, a minimal amount of ammonium persulphate for polymerisation and minimising CO_2 interference by degassing the cathodic NaOH solution and by conducting the isoelectric focussing step in a sealed apparatus.

Measurements of pH gradient relied on direct pH measurement in gel sections rather than on pI markers as conformational changes in the structure of proteins in the presence of urea potentially give rise to a change in surface charge and hence in pI.

5.2.4.4   Analysis of fluorographs of two-dimensional gels

(i)   Computer analysis

Fluorographs were scanned using UltroScan XL Laser Densitometer (LKB) and the data processed with GelScan XL software. The computerised scanning system located and integrated the densities of the spots. Graphic plots of two-dimensional fluorograph scans (isograms) could be overlaid by the computer for matching of isogram patterns.

(ii) Visual analysis

Matching of spots from different fluorographs was done visually by superimposing each fluorograph in turn onto a reference fluorograph (H_2O- treated control) placed on a light box. A plastic transparency was then overlaid onto the superimposed fluorographs and the

Figure 5.1 - <u>pH gradient in the first-dimension isoelectric-focussing gel</u>

Isoelectric-focussing gels (80 mm-long) were pre-focussed and run with isoelectric focussing overlay solution as described in Section 5.2.4.1. Sections of the gels (2.5 mm) were placed in 0.25 ml deionised, degassed H_2O and the pH measured as described in Section 5.2.4.3. Symbols indicate the average values from five gels in each of two separate experiments (o,●).

positions of the spots marked on the transparancy using a colour code. Matching decisions relied on visual comparisons to avoid computer errors which include: a) Two neighbouring spots may be resolved on one scan but not on another. b) Spots may be reported where only streaks or noise are present. c) Faint spots may not be detected due to noise.

5.3   Results

5.3.1   Induction of phenylpropanoid pathway enzymes and phytoalexin accumulation by proteinase K

From initial experiments using proteinase K for elicitor digestion (see Section 3.2.2.2) it was observed that treatment of bean cell cultures with proteinase K alone resulted in cell browning, although no browning was observed when autoclaved proteinase K was used. As cellular browning was found to be associated with induction of phenylpropanoid pathway enzymes in bean cell cultures, the effects of proteinase K were further examined in five independant experiments (Table 5.1). Each experiment involved the use of different batches of cultured cell material. With the exception of experiments 4 and 5, in which the protease inhibitor phenylmethylsulphonylfluoride (PMSF, 1 m$\underline{M}$) was included in the enzyme extraction buffer, PAL levels in proteinase K treated cells were lower than those from water-treated cultures. In contrast, CHS and CHI activities were induced by proteinase K although autoclaving the enzyme destroyed its ability to induce all three enzymes. Phaseollin and kievitone were induced in suspension cultured bean cells in response to a 48 h treatment with proteinase K (Table 5.2). However, protease preparations from bacterial and animal sources had no effect on the levels of the two isoflavonoid phytoalexins. Levels of phaseollin and kievitone in control and cell wall elicitor-treated cells varied considerably between experiments (compare Tables 3.7 and 5.2). This may be the result of differences in the physiological state of the batches of cells used for phytoalexin induction; cell batches (4-day old) were from different cell lines at different passage numbers.

It is unlikely that contaminating   fungal polysaccharides were responsible for proteinase K elicitor activity as no carbohydrate was detected by the phenol-sulphuric method in this enzyme preparation. These results suggest that proteinase K catalytic activity may be required for enzyme induction in bean cultures. In this respect it is interesting to note that protease activity was found to be required

Table 5.1

Enzyme induction in elicitor-treated bean cell suspension cultures

| Treatment[a] | Enzyme activity[b] | | | | | | | | | | | | | | |
| | Phenylalanine ammonia-lyase (µkat kg⁻¹) | | | | | Chalcone synthase (µkat kg⁻¹) | | | | | Chalcone isomerase (mKat kg⁻¹) | | | | |
	Exp 1	Exp 2	Exp 3	Exp 4[c]	Exp 5[c]	Exp 1	Exp 2	Exp 3	Exp 4[c]	Exp 5[c]	Exp 1	Exp 2	Exp 3	Exp 4[c]	Exp 5[c]
H₂O (control)	14	40	75	20	27	0.39	0.04	0.73	0.06	0.01	2.2	2.4	ND	3.8	1.9
Colletotrichum cell wall elicitor	53	143	116	70	170	1.03	0.97	1.11	0.38	0.96	3.6	3.4	ND	7.4	10.0
Proteinase K	9	21	16	55	110	0.72	0.53	0.93	0.30	0.73	2.9	3.1	ND	4.5	6.2
Autoclaved proteinase K	15	51	ND[d]	11	24	ND	0.09	ND	0.14	ND	2.0	ND	ND	4.1	ND

a   Cells were treated with water (control), cell water elicitor at a final concentration of 25 (PAL and CHS) or 50 (CHI) µg glucose equivalents ml⁻¹ or with proteinase K (untreated or autoclaved for 15 min at 120°C) at a final concentration of 6 µg ml⁻¹.

b   PAL and CHS activities were determined 6h after elicitation, CHI 18h after elicitation.

c   In experiments 4 and 5 the protease inhibitor PMSF was included in the enzyme-extraction buffer.

d   Not determined.

- 170 -

Table 5.2

Levels of phytoalexins in suspension cultured bean cells treated with Colletotrichum cell wall elicitor or proteases

Treatment[a]	Phytoalexins (n mol g^{-1} fresh weight)[b]	
	Phaseollin	Kievitone
Buffer	1.6	0.9
Colletotrichum lindemuthianum cell wall elicitor	11.4	7.6
Tritirachium album proteinase (proteinase K)	6.8	2.6
Streptomyces griseus protease	1.4	0.6
Porcine (stomach mucosa) pepsin	1.1	0.8

(a) Cultured cells were treated with 50mM KH_2PO_4 – KOH buffer pH 8.0 (control), cell wall elicitor (25 µg glucose equivalents ml^{-1} in KH_2PH_4 buffer), or protease (6 µg ml^{-1} in KH_2PO_4 buffer) in the presence of penicillin and streptomycin (each at a final concentration of 25 µg ml^{-1}) for 48h.

(b) Phytoalexins were extracted from bean cells as described in section 3.2.6 and separated on 25 x 20 cm Polygram silica gel G/UV$_{254}$ thin-layer plates using diethyl ether : hexane (5 : 1, (v/v)). After visualisation under U.V. light, kievitone (R$_f$ 0.45) and phaseollin (R$_f$ 0.79) were scraped from the plate and eluted in 1.2 ml ethanol. Quantification from the U.V. absorption spectra was as previously described (section 3.2.6).

for induction, by a number of pathogenic fungi, of the phytoalexin benzoic acid in apple tissue (Swinburne, 1973).

In order to compare the effects of a polypeptide and polysaccharide elicitors from non-pathogenic and pathogenic fungi on general patterns of gene expression in bean cultures, treatment with proteinase K was included in parallel in the following experiments using the elicitor fractions from C. lindemuthianum.

5.3.2   Patterns of induced protein synthesis in response to Colletotrichum elicitor fractions

The yield of polysomal RNA extracted from control and elicitor-treated cultures was 25-58 μg/g fresh weight and the O.D.$_{260}$/O.D.$_{280}$ ratio varied from 1.53 to 1.84 (Table 5.3).   In in vivo labelling experiments, both the uptake of [^{35}S] methionine (approximately 25% during a 1 h pulse) and the overall rate of protein synthesis (approximately 46% of label taken up by the cells) were similar in both unelicited and elicitor treated cultured cells.

(i)   One-dimensional SDS-polyacrylamide gel electrophoretic analysis

[^{35}S] methionine-labelled proteins synthesised in vivo or in vitro from isolated polysomal mRNA were analysed by one-dimensional SDS-PAGE and detected by fluorography (Figure 5.2).   The incorporation of [^{35}S] methionine into trichloroacetic acid - precipitable protein represented the synthesis of discrete polypeptides with M_rs up to approximately 150 000 and 100 000 in vivo and in vitro, respectively (Figures 5.2 and 5.4).   The size range of polypeptides synthesised in vivo and in vitro was similar although the M_r distribution of the polypeptides within this range was not identical.

No striking differences in the in vivo labelling of polypeptide subunits were observed in response to the different elicitor treatments, with synthesis of most polypeptides varying between ± 10% relative to control (water-treated) cultures (Figure 5.2, A).

- 172 -

Table 5.3

Changes in the rates of protein synthesis

in vivo and in vitro in elicitor-treated bean cell cultures

Fraction[a]	Synthesis in vivo[b]		Translation in vitro[c]		
	% Uptake of [35S]-methionine[d]	Total protein synthesis[e]	Recovery of polysomal RNA (µg RNA g^{-1} fresh weight)	O.D. 260/O.D. 280 of RNA preparation	Total incorporation (dpm µg^{-1} RNA)
None (H$_2$O control)	26.5	48	37	1.53	2603
Crude cell wall elicitor	27.7	42	32	1.61	2705
Crude culture filtrate elicitor	27.3	44	25	1.74	1881
Concanavalin A-Sepharose fractions:					
Unbound	26.9	49	42	1.57	3156
α-methyl mannoside eluate	26.1	43	58	1.71	3521
NaCl eluate	27.0	45	34	1.84	2879
Proteinase K	26.7	54	27	1.81	2114

(a) Bean cultures were exposed to Colletotrichum elicitor fractions (25 µg glucose equivalents ml^{-1}) and to proteinase K (6µg ml^{-1}) for 4h (in vivo labelling) or 3h (in vitro translation samples).

(b) Cells were exposed to a 1h pulse of [35S] methionine (30µCi) between 3-4h following elicitor treatment as described in section 5.2.3.1.

(c) Polysomal RNA was extracted from bean cell cultures treated with fungal elicitor for 3h as described in section 5.2.2.2 and translated in the presence of [35S] methionine in a rabbit reticulocyte lysate cell-free translation system as described in section 5.2.2.3.

(d) Values were determined as the amount of label present in extracts from 1g cells relative to that used per g cells in the 1h pulse.

(e) Results are of the amount of incorporation of [35S] methionine into protein as a percentage of that taken up by the cells in a 1h pulse.

Figure 5.2 - <u>Fluorographs of one-dimensional SDS-polyacrylamide gel</u> <u>electrophoretic separations of polypeptides newly synthesised in elicitor-</u> <u>treated bean cell cultures</u>

Suspension cultured cells were either labelled with a 1 h pulse of $[^{35}S]$ methionine <u>in vivo</u> between 3-4 h following elicitation (A), or harvested 3 h after elicitation for isolation and translation <u>in vitro</u> of polysomal mRNA (B). Protein samples containing approximately 18 000 and 25 000 dpm were applied to each of the tracks in A and B respectively. Tracks are <u>1</u>, M_r markers; <u>2</u>, no elicitor (H_2O control); <u>3</u>, cell wall elicitor; <u>4</u>, unfractionated culture filtrate elicitor; <u>5</u>, culture filtrate Concanavalin A-unbound fraction: <u>6</u>, Concanavalin A-bound α-methyl mannoside eluate; <u>7</u>, Concanavalin A-bound NaCl eluate; <u>8</u>, proteinase K. Track 9 represents translation products from rabbit reticulocyte lysate incubated with water in place of RNA. Suspension cultures were exposed to <u>Colletotrichum</u> elicitors (tracks 3-7) at a final concentration of 25 μg glucose equivalents ml^{-1}, and to proteinase K (track 8) at a final concentration of 6 μg ml^{-1}. Arrows (a-e) indicate positions of polypeptides whose extent of labelling varies in response to different treatments (refer to text and Figure 5.4).

y-axis: $\log_{10} M_r$ — values 4·0, 4·2, 4·4, 4·6, 4·8, 5·0, 5·2, 5·4

x-axis: Mobility (cm) — values 0, 2, 4, 6, 8, 10, 12
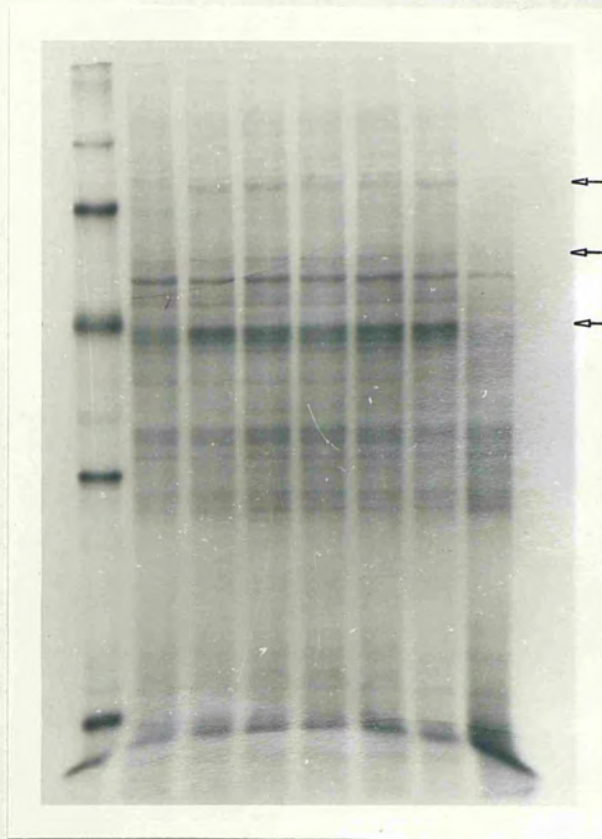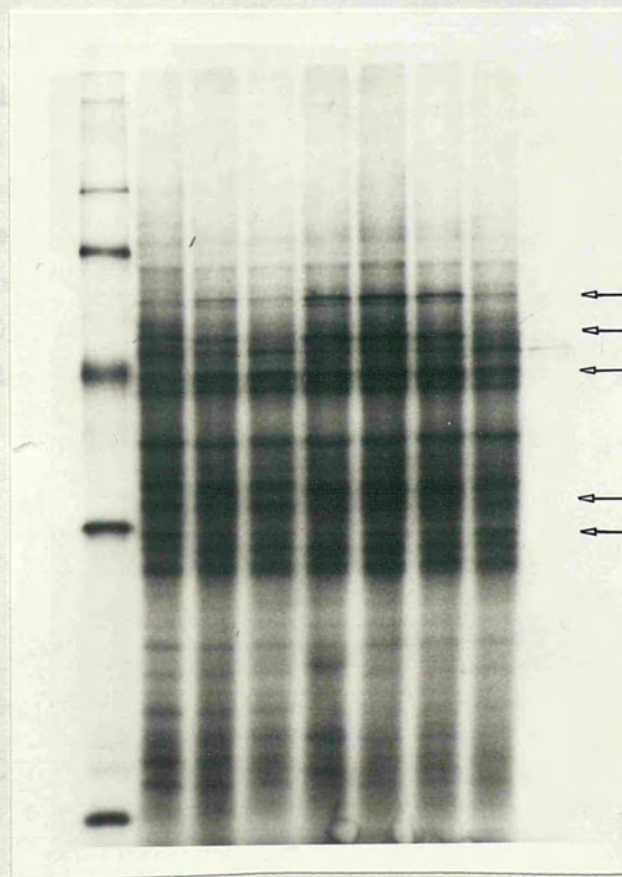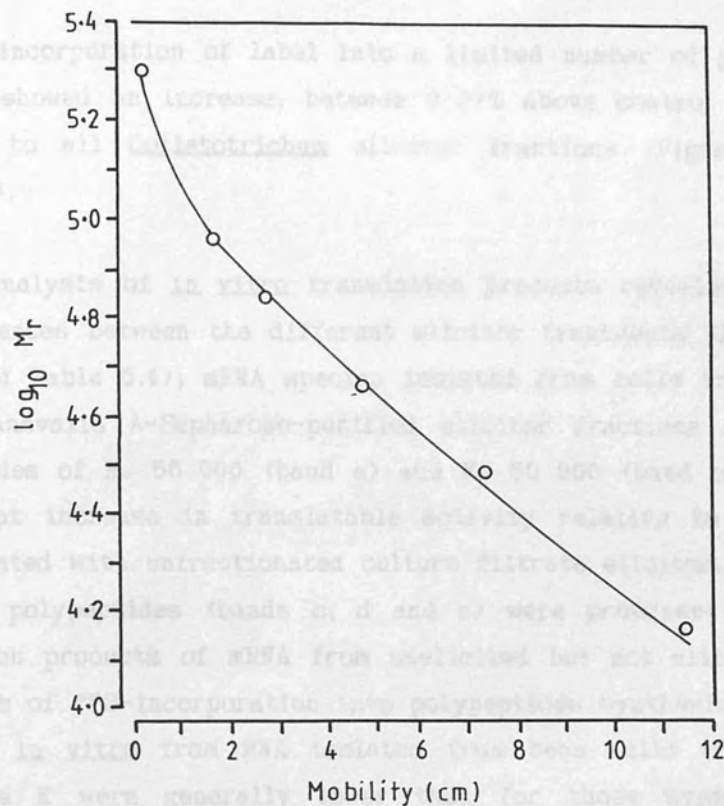
Figure 5.3 - <u>Standard curve of molecular weight distribution in SDS-polyacrylamide gels</u>

[14]C-labelled methylated standard M_r protein markers (Myosin, M_r 200 000; phosphorylase b, M_r 92 500; bovine serum albumin, M_r 69 000; ovalbumin, M_r 46 000; carbonic anhydrase, M_r 30 000 and lysozyme, M_r 14 300) were applied to 10% SDS-polyacrylamide gels, electrophoresed (see Section 2.10) and the mobility of standard proteins measured on fluorographs of the dried gels.

However, incorporation of label into a limited number of polypeptide subunits showed an increase, between 9-27% above control levels, in response to all Colletotrichum elicitor fractions (Figure 5.4 and Table 5.4).

Similar analysis of in vitro translation products revealed a number of differences between the different elicitor treatments (Figures 5.2 B, 5.4 and Table 5.4); mRNA species isolated from cells treated with the Concanavalin A-Sepharose-purified elicitor fractions coding for polypeptides of M_r 56 000 (band a) and M_r 50 000 (band b) showed a significant increase in translatable activity relative to that from cells treated with unfractionated culture filtrate elicitor. A number of other polypeptides (bands c, d and e) were prominent among the translation products of mRNA from unelicited but not elicited cells. The levels of ^{35}S-incorporation into polypeptides synthesised both in vivo and in vitro from RNA isolated from bean cells treated with proteinase K were generally lower than for those synthesised in water-treated cells. It is unlikely that the lower levels of synthesis in vivo are a result of degradation of extracted polypeptides by residual proteinase K as protein extraction was performed in the presence of PMSF (see section 5.2.3.2). Label in a number of low M_r polypeptides (band e and below) appeared in different amounts in the different tracks in Figure 5.2, B; such differences may be a result of differential induction of full length translation products and/or translation of partially degraded mRNA. The general correlation between the patterns of protein synthesis in vivo and in vitro, however, indicates that the polysomal mRNA preparations were not extensively degraded during isolation and suggests accurate translation of exogenous bean mRNA by the reticulocyte lysate preparation.

(ii) Two-dimensional isoelectric focussing - SDS polyacrylamide gel electrophoretic analysis.

In order to examine in more detail any differences between different elicitor treatments in relation to induced protein synthesis,

Figure 5.4 - <u>Densitometric analysis of fluorographs of one-dimensional</u> <u>SDS-polyacrylamide gel electrophoretic separations of polypeptides</u> <u>synthesised in bean cell cultures in response to treatment with</u> <u>*Colletotrichum* cell wall elicitor</u>

Cell suspension cultures were labelled <u>in vivo</u> with a 1 h pulse of [^{35}S] methionine 3 h after treatment with cell wall elicitor at a final concentration of 25 μg glucose equivalents ml^{-1} (A), or harvested 3 h after elicitation for isolation and translation <u>in vitro</u> of polysomal mRNA (B). Fluorographs were recorded by densitometry as described in Section 2.12. Arrows indicate the positions of polypeptides (a-e) whose extent of labelling varies in response to different treatments (see Figure 5.3 and Table 5.4).

Density of fluorograph →

M_r (× 10^{-3})

200   92·5 69   46   30   13·4

A

B
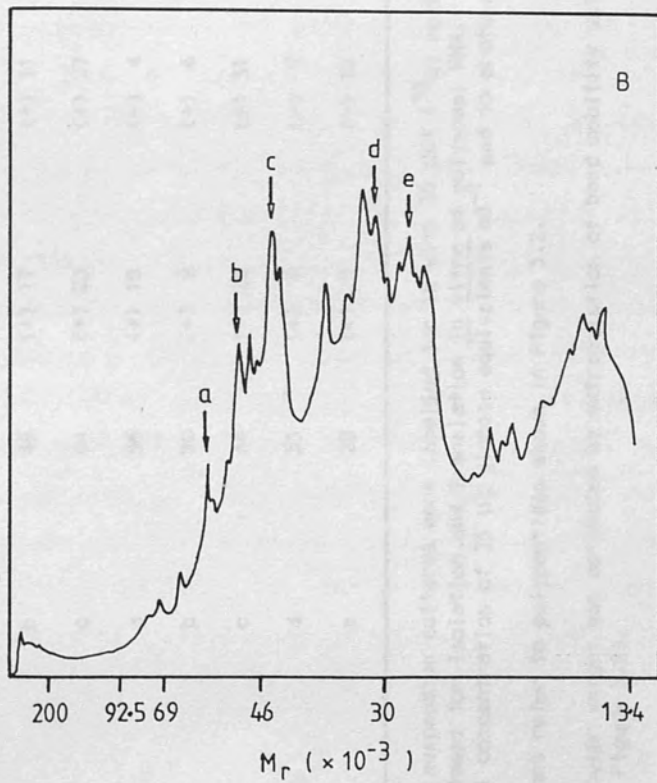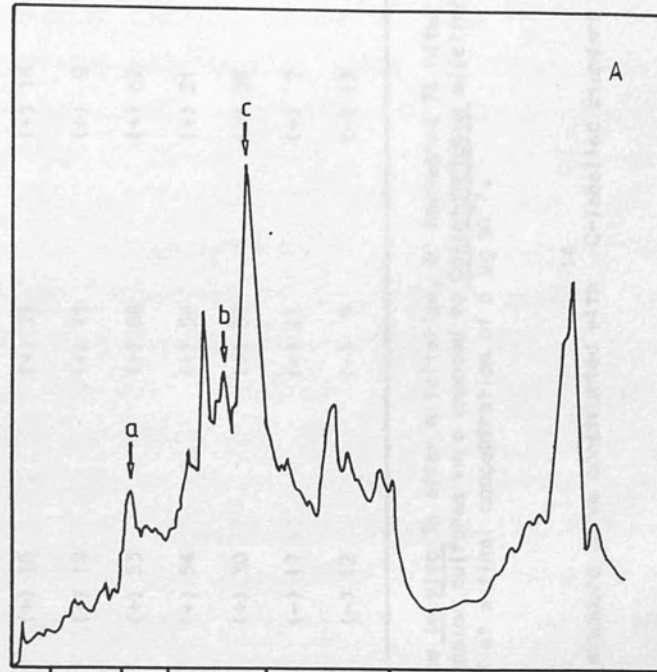
Table 5.4

Effect of elicitor preparations on synthesis
of polypeptides in bean suspension cultures

Protein synthesis [a]	Polypeptide [b]	M_r [c] ($\times 10^{-3}$)	Relative change in protein synthesis [d]					
			Cell wall elicitor	Culture filtrate elicitor	Concanavalin A- unbound fraction	Concanavalin A- α-methyl mannoside eluate	Concanavalin A- NaCl eluate	Proteinase K
In vivo	a	77	(+) 17	(+) 15	(+) 12	(+) 18	(+) 11	(-) 20
	b	49	(+) 17	(+) 11	(+) 18	(+) 21	(+) 16	(-) 31
	c	44	(+) 23	(+) 27	(+) 18	(+) 11	(+) 9	(-) 34
In vitro	a	56	(+) 10	(-) 4	(+) 53	(+) 86	(+) 62	(+) 8
	b	50	(+) 8	(+) 6	(+) 54	(+) 54	(+) 21	(-) 21
	c	44	(+) 29	(+) 31	(+) 30	(+) 37	(+) 36	(+) 3
	d	33	(+) 9	(-) 7	(-) 17	(-) 11	(-) 7	(-) 20
	e	28	(+) 5	(-) 15	(-) 12	(-) 9	(-) 13	(-) 14

(a) Cell suspension cultures were labelled for 1h with 30 µCi [^{35}S] methionine in vivo 3h after elicitation, or harvested 3h after elicitor treatment for isolation and translation in vitro of polysomal RNA. Suspension cultures were exposed to Colletotrichum elicitor preparations at a final concentration of 25 µg glucose equivalents ml^{-1} and to proteinase K at a final concentration of 6 µg ml^{-1}.

(b) Letters refer to polypeptides shown in Figure 5.2.

(c) Molecular weight was estimated by extrapolation of band mobility using a standard curve constructed with ^{14}C-labelled standard protein markers (see Figure 5.2).

(d) Results were calculated using densitometric scans of fluorographs of proteins separated by one-dimensional SDS-polyacrylamide gel electrophoresis (see Figure 5.4). Values represent the amount of label incorporated into total protein, as measured by the total area under the absorption curve expressed as a percentage of that in control (water-treated) cell cultures. Increased and decreased synthesis relative to controls is denoted by (+) and (-) signs, respectively.

polypeptides synthesised both in vivo and in vitro were analysed by two-dimensional gel electrophoresis. Attempts to separate in vivo labelled proteins by two-dimensional gel electrophoresis were unsuccessful (see Section 5.2.3.2). However, good resolution of in vitro translated peptides was obtained using two-dimensional IEF - SDS-PAGE analysis (Figure 5.5). Approximately 90 spots were detected visually on fluorographs of the translation products of polysomal RNA from non-elicited cultures (Figure 5.5, B). Treatment with crude Colletotrichum elicitor preparations and with culture filtrate elicitor Concanavalin A-Sepharose fractions resulted in the appearance of 7 specific polypeptides and disappearance of 9 specific polypeptides common to all treatments (Figure 5.6). The most striking feature was the appearance of a group of polypeptides of apparent M_r approximately 46 000 in response to treatment with all elicitor preparations from Colletotrichum (Figures 5.5, B-G and 5.7, A). Treatment with proteinase K resulted in much lower levels of induction of these polypeptides (Figure 5.5, H). The patterns of mRNA translation products were broadly similar with the three Concanavalin A-Sepharose fractions although they were clearly different from the patterns obtained with crude cell wall or culture filtrate elicitors. Each elicitor or elicitor fraction induced a different overall pattern of mRNA translation products, including the appearance of a limited number of polysomal mRNA activities whose increase from very low or zero basal levels in unelicited cells was unique to each treatment (Figures 5.5, 5.6 and 5.7, A). Identical patterns of polypetide synthesis were obtained following translation of polysomal RNA isolated from equivalent batches of cultured bean cells treated in duplicate with 25 µg glucose equivalents ml^{-1} Concanavalin A-unbound fraction (Figure 5.7, B). This result suggests that the different patterns of mRNA translation products observed in response to treatment of the same batch of cultured cells with different elicitors are unlikely to be artifacts resulting from differential degradation of mRNA during isolation.

Figure 5.5 – Fluorographs of two-dimensional isoelectric focussing: SDS-polyacrylamide gel electrophoretic separations of polypeptides synthesised *in vitro* from polysomal mRNA isolated from elicitor-treated bean cell suspension cultures

Polysomal RNA was isolated from suspension cultured cells treated with elicitor for 3 h, translated in the presence of [^{35}S] methionine in vitro in an mRNA-dependant rabbit reticulocyte lysate cell-free system and the newly synthesised polypeptides separated by two-dimensional IEF: SDS-PAGE. Protein sample containing approximately 20 000 dpm was loaded on each gel. Polypeptides were synthesised in the absence (A) and presence (B-H) of RNA isolated from cells treated with H_2O (B), cell wall elicitor (C), crude culture filtrate elicitor (D), culture filtrate Concanavalin A-unbound fraction (E), Concanavalin A-bound / α-methyl mannoside and NaCl eluate (F,G) and proteinase K (H). Suspension cultures were exposed to Colletotrichum elicitors (C-G) at a final concentration of 25 µg glucose equivalent ml^{-1} and to proteinase K (H) at a final concentration of 6 µg ml^{-1}.
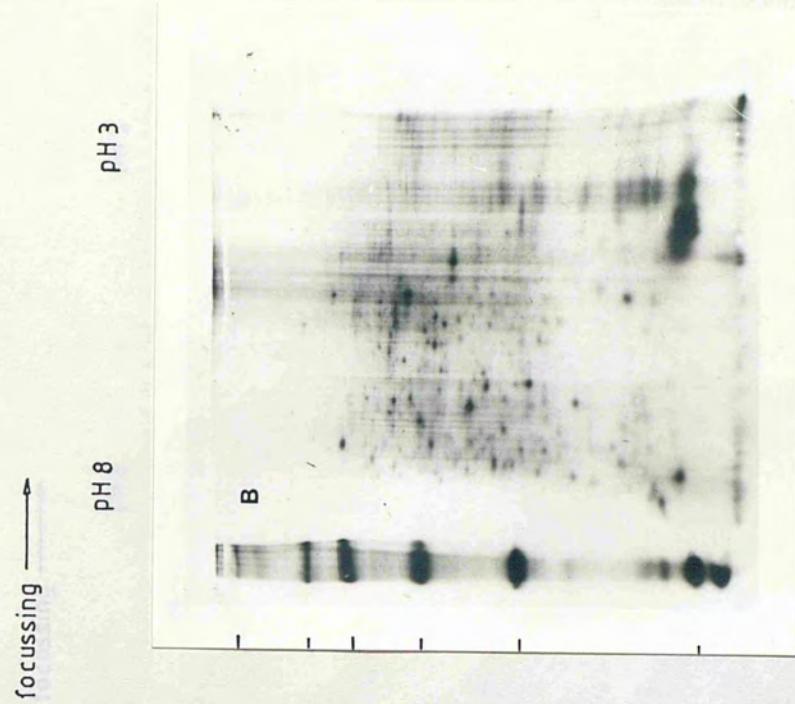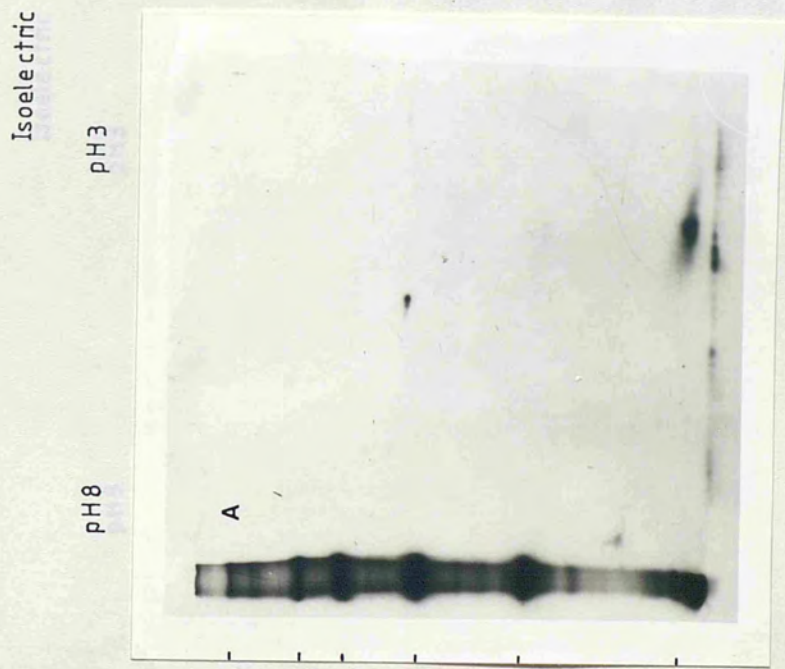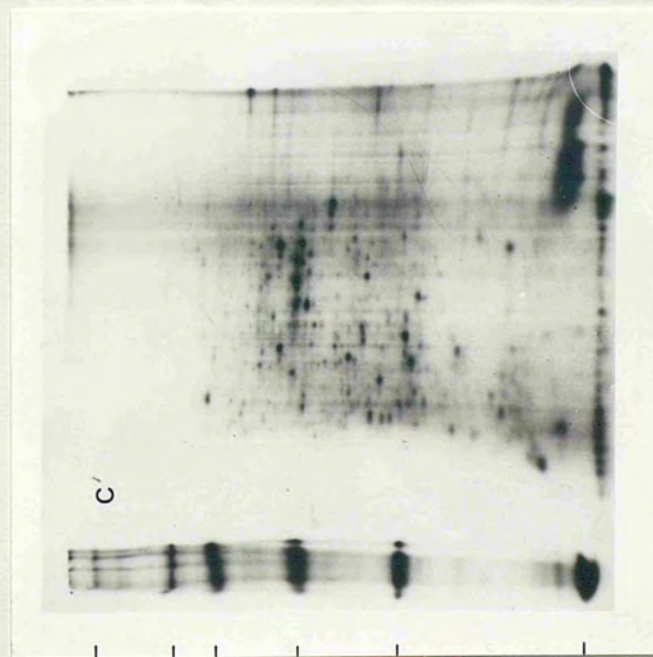
SDS-polyacrylamide gel electrophoresis

Isoelectric focussing

pH 8   pH 3

pH 8   pH 3
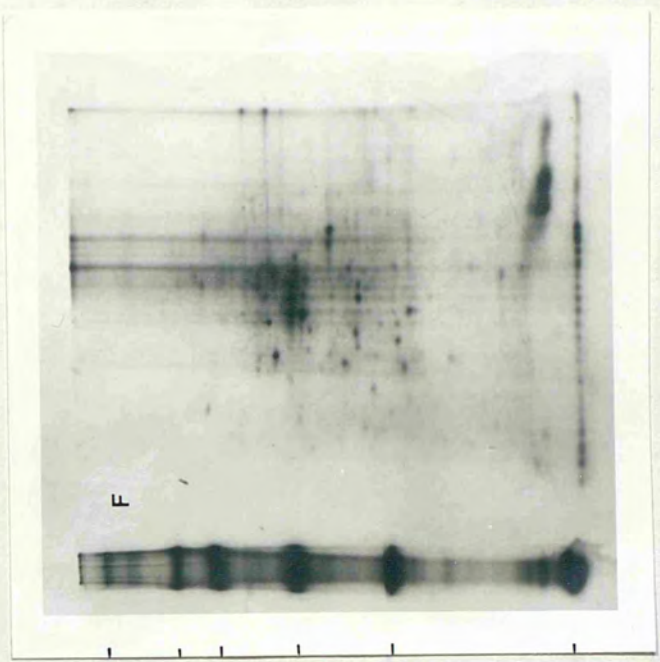
B

A

M_r
$(\times 10^{-3})$

200

92·5

69

46

30

14·3

SDS-polyacrylamide gel electrophoresis →

Isoelectric focussing →

pH 3   pH 8   pH 3   pH 8

D   C'

Mr
(× 10⁻³)

200
92·5
69
46
30
14·3

SDS-polyacrylamide gel electrophoresis →

Isoelectric focussing →

pH 3   pH 8

pH 3   pH 8

F

E

M_r
$(\times 10^{-3})$

200
92·5
69
46
30
14·3

SDS-polyacrylamide gel electrophoresis →

Isoelectric focussing →

pH 3   pH 8

H

G

pH 3   pH 8

M_r
$(\times 10^{-3})$

200

92·5

69

46

30

14·3

M_r
$(\times 10^{-3})$

Isoelectric focussing ⟶

pH 8   pH 3

SDS-polyacrylamide gel electrophoresis

200 —
92·5 —
69 —
46 —
30 —
14·5 —
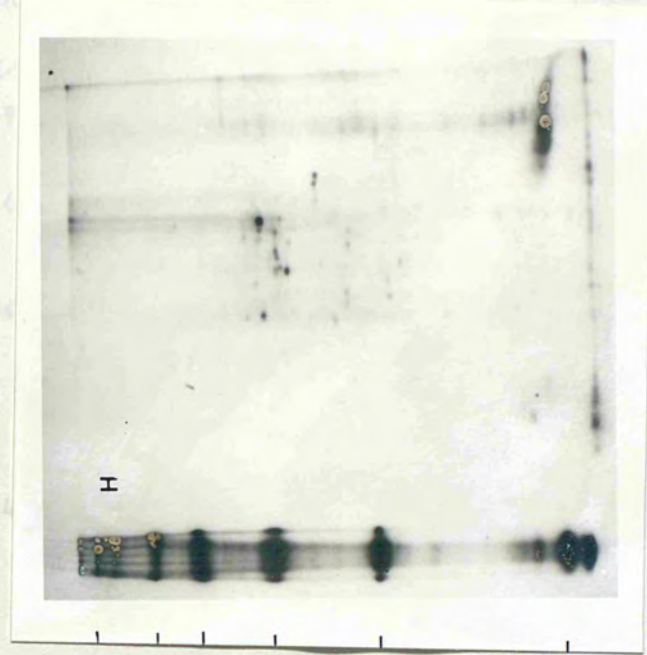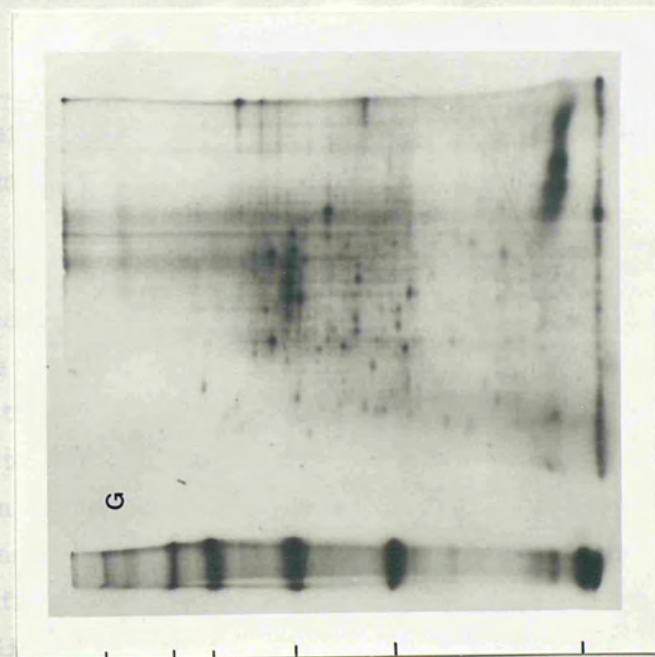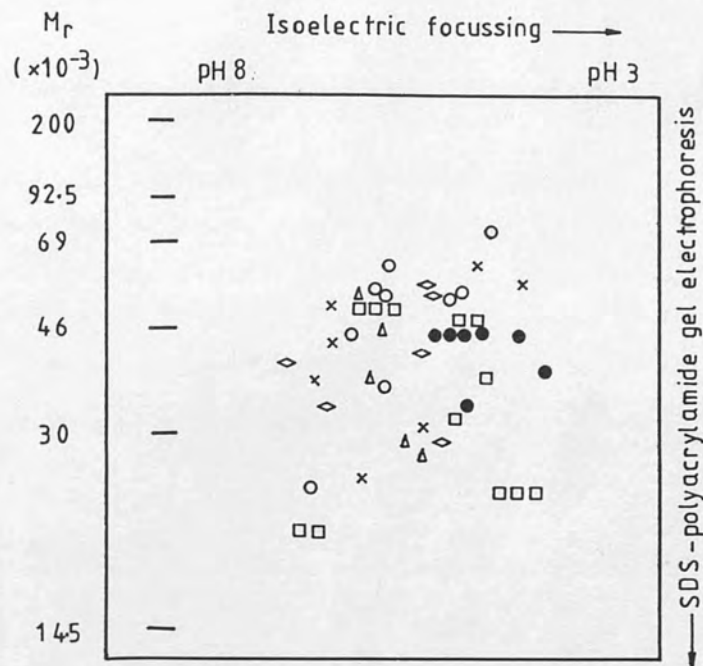
Figure 5.6 - <u>Schematic summary of major differences in the two-dimensional isoelectric focussing: SDS-polyacrylamide gel electrophoretic separation of *in vitro* synthesised polypeptides from elicitor-treated bean cell cultures</u>

The scheme refers to the fluorographs shown in Figure 5.5. Symbols represent polypeptides whose labelling increases (●) or decreases (O) relative to H_2O-treated controls in response to all elicitors, or whose extent of translation from polysomal mRNA isolated from cells treated with culture filtrate elicitor (□), culture filtrate Concanavalin A-unbound fraction (Δ), Concanavalin A- .bound α-methyl mannoside eluate (◇) and Concanavalin A-bound NaCl eluate (×) and differs significantly from that observed with mRNA from cell wall elicitor-treated cells.

Figure 5.7 - _Computer analysis (Section 5.2.4.4.) of patterns of in vitro_ _sythesised polypeptides from elicitor-treated bean cell cultures_

Isograms are of fluorographs shown in Figure 5.5. The region scanned was 50×35 mm in area (magnification ×2.2). Scanning was performed in the SDS-PAGE dimension with a 120 micron step in the IEF dimension. The computer plots in A and B show the superimposed patterns of polypeptides synthesised in the presence of polysomal RNA from bean cell cultures subjected to different treatments. Unlabelled spots are the result of streaks of noise on the isograms.

Figure 5.7, A _ Polysomal RNA isolated from bean cells treated with water (control,■) or Colletotrichum cell wall elicitor (▥) was translated in vitro using batches of the same rabbit reticulocyte translation preparation. Spots which had similar density and location in both fluorographs are circled. Spots which were induced from zero or low levels or which decreased in intensity, relative to H_2O-treated control, following cell wall elicitor treatment are shown by the arrows ___,——,...., respectively.

Figure 5.7, B - Isogram B shows the patterns of polypeptides synthesised in vitro using polysomal RNA preparations from two equivalent batches of bean cells treated, in duplicate, with 25 μg glucose equivalents ml^{-1} Concanavalin A-bound α-methyl mannoside eluate.

Although label uptake and incorporation into protein in both unelicited and elicitor-treated bean cells were measurable (Table 8.2), the possibility that different elicitor treatments may result in differences in the compartmentalisation of exogenous label during the host-elicitor interaction cannot be ruled out.

M_r (×10^{-3})   Isoelectric focussing ⟶

pH 7.5   pH 3.5

(A)

46 —

30 —

(B)

46 —

30 —

SDS – polyacrylamide gel electrophoresis ⟶
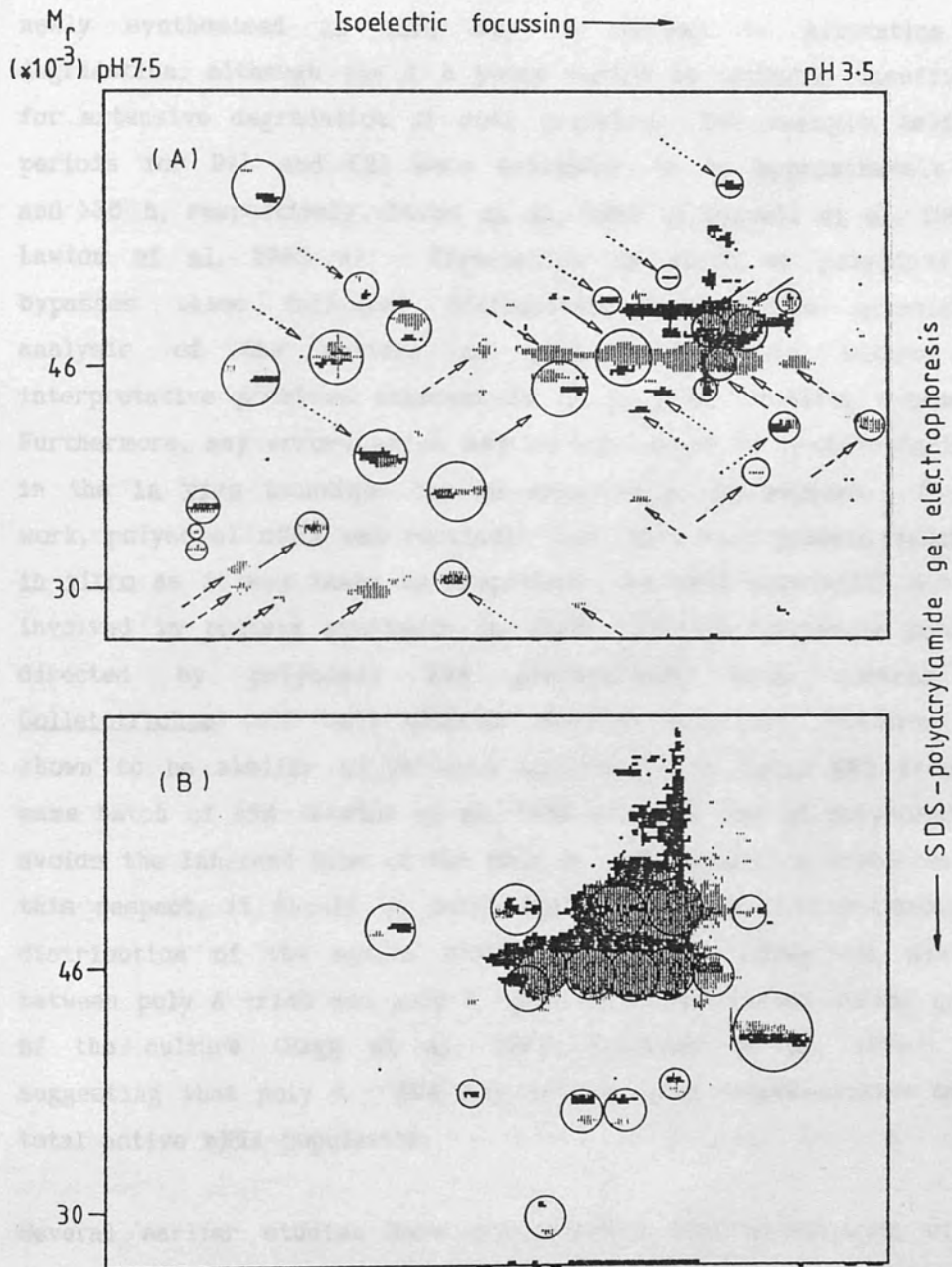
5.4   Discussion

Although label uptake and incorporation into protein in both
unelicited and elicitor-treated bean cells were comparable (Table
5.3), the possibility that different elicitor treatments may result in
differences in the compartmentalisation of exogenous label during the
host-elicitor interaction cannot be ruled out. In addition, proteins
newly synthesised in vivo may be subject to alteration and
degradation, although the 1 h pulse period is probably insufficient
for extensive degradation of most proteins. For example, half-life
periods for PAL and CHI were estimated to be approximately 4 h
and >36 h, respectively (Dixon et al, 1983 a; Bolwell et al, 1986 b;
Lawton et al, 1983 a).   Translation in vitro of polysomal RNA
bypasses these technical difficulties and allows quantitative
analysis of the pattern of protein synthesis without the
interpretative problems inherent in an in vivo labelling approach .
Furthermore, any errors which may be introduced by protein isolation
in the in vivo technique can be expected to be avoided.   In this
work, polysomal mRNA was routinely used to direct protein synthesis
in vitro as it was taken to represent the mRNA population actively
involved in protein synthesis in vivo.   Protein synthesis patterns
directed by polysomal RNA preparations from control and
Colletotrichum cell wall elicitor treated bean cell cultures were
shown to be similar to patterns observed with total RNA from the
same batch of RNA (Lawton et al, 1983 b). The use of polysomal RNA
avoids the inherent bias of the poly A - RNA selection criterion.   In
this respect, it should be noted that in parsley cell cultures, the
distribution of the active mRNA population encoding PAL and CHS
between poly A -rich and poly A -poor fractions varied during growth
of the culture (Ragg et al, 1977; Schröder et al, 1979), thus
suggesting that poly A - RNA may not be fully representative of the
total active mRNA population.

Several earlier studies have demonstrated that elicitation of the
phytoalexin response is the result of selective changes in host gene

expression leading to increased enzyme levels (Cramer et al, 1985 b; Dixon et al, 1986 b; Löschke et al, 1983; Somssich et al, 1986). The 2-D gel analyses of in vitro translation products in this work (Figures 5.5 & 5.6), in which a number of common increased mRNA activities were observed with all elicitor treatments, clearly supports this selective response. Superimposed on this pattern, however, was the appearance of polypeptide patterns specific for different elicitor fractions from Colletotrichum. This result is in agreement with an earlier observation that both qualitative and quantitative differences were exhibited in the metabolic responses of cultured bean cells to elicitors from Colletotrichum cell walls or culture filtrate (Chapter 3). The present differences in induced polypeptide patterns were not limited to the crude cell wall and culture filtrate preparation but included the culture filtrate elicitor Concanavalin A-Sepharose fractions. Broadly similar polypeptide patterns were however observed in response to treatment with each of the three Concanavalin A-Sepharose culture filtrate elicitor fractions although, as a group, they differed from the patterns obtained with crude elicitor preparations. The Concanavalin A-Sepharose fractions were shown earlier to exhibit differential effects on the induction of phytoalexin biosynthetic enzymes in cultured bean cells (Chapter 4).

The patterns of translation products from proteinase K-treated cells were difficult to compare with patterns from water-treated (control) or elicitor-treated cells as fluorographs of polypeptides from proteinase K-treated cells appeared relatively underexposed (Figure 5.5). This highlights one of the problems inherent in the interpretation of qualitative differences visually detected between fluorographs of total proteins separated by 2-D gel electrophoresis. Reliability of visually detected qualitative differences depends on the availability of high quality fluorographs with sharp, well resolved spots of similar intensities. The requirement for fluorographs of polypeptides with similar intensities for visual detection of qualitative differences can be overcome by densitometric scanning.

Enzyme activity of proteinase K appeared to be required for its elicitor activity in bean cell cultures. Lower levels of phenylalanine ammonia-lyase activity in extracts from proteinase K treated cells (Experiments 1-3, Table 5.1) are thought to be the result of PAL degradation by residual proteinase K in the enzyme extracts. The loss of PAL induction by proteinase K in experiments 1-3 (Table 5.1) in which , however, the activities of CHS and CHI were induced, may be a result of differences in the susceptibility of the three enzymes to degradation by proteinase K and/or differences in the interval between enzyme extraction and assay; CHS and CHI activities were assayed within 1 and 2 h of extraction, respectively, whereas PAL activity was determined approximately 4-5 h following extraction, thus allowing more extensive degradation of PAL than CHS or CHI by residual proteinase K activity in the extracts.

It should be noted that Streptomyces griseus protease (Sigma) is an alkaline protease with a pH optimum of 11, whereas porcine pepsin shows maximal activity at pH 2. Thus, even allowing for some change in the pH of the culture medium during growth of cell cultures (culture medium initially adjusted to pH 5.9, see Table 2.1), incubation conditions for phytoalexin induction by the proteases appear not to have been optimal for their proteolytic activity. This may, in part, explain why no phytoalexin induction was observed following treatment of bean cell cultures with these protease preparations. Clearly, choice of proteases active at the pH of the culture medium would have been more appropriate for study of the requirement of proteolytic activity for elicitation.

Several earlier studies have shown that a number of other enzymes possess elicitor activity. These include ribonuclease, deoxyribonuclease (Dixon & Fuller, 1977), the polygalacturonase from culture fluids of Rhizopus stolonifer (Lee & West, 1981) and the endopolygalacturonic acid lyase from Erwina carotovora (Davis et al, 1984). The mode of action of these enzymes is, however, thought to be different, with ribonuclease and deoxyribonuclease acting as elicitors because of their net polycationic nature whereas both

pectic enzyme elicitors from Rhizopus and Erwina act by releasing elicitor-active pectic polysaccharides from host cell walls which serve as endogenous elicitors (Davis et al, 1984; Bruce & West, 1982). With respect to the latter, it is interesting to note that although endogenous elicitors reported to date appear exclusively to be pectic fragments of plant cell walls, a recent report by Kurosaki et al, (1986) suggests that peptides released by tryptic hydrolysis of a pectin preparation from carrot cell walls were elicitor-active. Whether elicitor activity of proteinase K may be the result of its release from bean cell walls of an elicitor-active peptide is not yet known.   Further examination of the effect of proteinase K as an elicitor is required as the present results are insufficient to explain the relationship between the enzymic and elicitor activities of this protein.

The observed increases and decreases in the amounts of specific in vitro translation products in response to crude and fractionated elicitor treatments may be induced through changes in the rate of synthesis of their respective specific mRNAs, changes in the processing of precursors to the mature mRNAs, or through changes in the degradation of their respective specific mRNAs. Although these possibilities cannot at present be distinguished, taken together the results in this chapter indicate that treatment with different Colletotrichum elicitor fractions results in differential changes at the RNA level, reflecting activities directed towards metabolic routes such as phytoalexin biosythesis. Further investigation of the effects of the different elicitor fractions on the synthesis in vivo and in vitro of the phytoalexin biosynthetic enzymes PAL, CHS and CHI is reported in the following chapter.

ALTERED PATTERNS OF GENE EXPRESSION IN BEAN CELLS EXPOSED TO CRUDE AND FRACTIONATED ELICITORS

II. CHANGES IN SYNTHESIS OF PHENYLPROPANOID PATHWAY ENZYMES

CHAPTER 6

ALTERED PATTERNS OF GENE EXPRESSION IN BEAN CELLS EXPOSED TO CRUDE AND FRACTIONATED ELICITORS

II. CHANGES IN SYNTHESIS OF PHENYLPROPANOID PATHWAY ENZYMES

6.1   Introduction

Accumulation of isoflavanoid phytoalexins in bean suspension cultures treated with an elicitor preparation heat-released from cell walls of Colletotrichum lindemuthianum results from a selective induction of enzymes of general phenylpropanoid metabolism (Robbins et al, 1985). Labelling in vivo and translation studies in vitro have demonstrated that induction of enzyme activities in bean tissue involves the de novo synthesis of phenylalanine ammonia-lyase (Cramer et al, 1985a; Lawton et al, 1983 a, b), chalcone synthase (Lawton et al, 1983 a, b; Bell et al, 1984) and chalcone isomerase (Robbins & Dixon, 1984; Cramer et al, 1985 a).   Further studies have shown that increased enzyme synthesis results from increased levels of newly synthesised mRNA encoding both PAL (Edwards et al, 1985; Cramer et al, 1985 b) and CHS (Ryder et al, 1984).

Crude elicitor preparations from cell walls and culture filtrate of Colletotrichum, which had similar effects on the activities and chromatofocussing profiles of phenylpropanoid pathway enzymes, were shown to have differential effects on phytoalexin accumulation (Chapter 3) and on overall patterns of protein synthesis (Chapter 5). Differential effects on synthesis of host gene products were also observed in response to treatment with fractions isolated from culture filtrate elicitor by chromatography on Concanavalin A-Sepharose (unbound fraction, bound α-methyl mannoside-eluate and bound NaCl-eluate) (Chapter 5), and these fractions had different abilities to induce the extractable activities of phenylpropanoid pathway biosynthetic enzymes.   These results raise the questions of whether any of the polypeptides differentially induced by the

different elicitor fractions are enzymes involved in phytoalexin biosynthesis and, conversely, whether the different biological activities of these elicitor fractions are the result of differential modulation of the expression of genes previously shown to be under elicitor control.

In order to address the above questions, in vivo pulse-labelling and in vitro translation studies were undertaken, using available antisera for PAL, CHS and CHI. As both PAL and CHS are encoded by multigene families in bean (Dixon et al, 1986 b), preliminary attempts were made to assess whether the different elicitor fractions affected the overall patterns of the induced PAL and CHS gene transcripts/products induced. Experiments were carried out using bean cell suspension cultures treated with crude Colletotrichum cell wall and culture filtrate elicitors and culture filtrate elicitor fractions obtained by chromatography on Concanavalin A-Sepharose. Treatment with proteinase K was included in parallel with experiments using Colletotrichum elicitor preparations as proteinase K was shown to induce phenylpropanoid enzyme activities and phytoalexin accumulation and to alter the overall pattern of polysomal mRNA activities in bean (Chapter 5). In addition to the use of in vitro translation, changes in the levels of CHS mRNA in response to crude, fractionated elicitor preparations and proteinase K were also examined using hybridisation probes specific for this mRNA.

- 199 -

6.2   Materials and methods

6.2.1 Buffers

The following buffers were used: (A) 50 mM KH$_2$PO$_4$ buffer pH 8.0,
containing 0.15 M NaCl, 2% (v/v) Triton X-100, 1% (w/v) BSA and
1.4 mM 2-mercaptoethanol. (B) 0.2 M Tris/acetate buffer pH 8.2,
containing 0.1 M magnesium acetate, 20 mM potassium acetate, 1% (v/v)
Triton X-100 and 20 mM EDTA. (C) 62.5 mM Tris/HCl buffer pH 6.8,
containing 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2% (w/v)
SDS and 0.125% (w/v) bromophenol blue. (D) 50 mM Tris/acetate buffer
pH 7.8, containing 0.15 M NaCl, 2% (v/v) Triton X-100, 1.4 mM 2-
mercaptoethanol and 4 mM methionine.

6.2.2 Immunoprecipitation and gel analysis of ^{14}C-labelled proteins

6.2.2.1 Proteins synthesised _in vitro_

Preparation and translation of polysomal RNA were as previously
described (sections 5.2.2.2 and 5.2.2.3 respectively).

(i) Treatment with antibody

An aliquot of the reticulocyte lysate translation incubation (22 μl)
was treated with 1 μl anti-(PAL) serum or with 2 μl anti-(CHS) plus
4μl anti-(CHI) sera. Mixtures were incubated at 30°C for 1 h
followed by 16 h at 4°C.

(ii) Direct immunoprecipitation

Reticulocyte translation incubations (22 μl) which had been treated
with antibody as described above (6.2.2.1 (i)) were centrifuged on 4
ml 0.67 M sucrose in buffer B at 25 000 × g for 1 h. The pellet was
washed twice with water and collected by centrifugation in a
microcentrifuge. The pelleted enzyme antibody complex was prepared
for gel analysis as described in Section 6.2.2.1 (iv) below.

- 200 -

(iii) Indirect immunoprecipitation

Indirect immunoprecipitation of the enzyme-antibody complex was by addition of 35 μl or 75 μl of a suspension of pre-swollen protein A-Sepharose (0.1 mg ml^{-1} in buffer A) to mixtures treated with one (PAL) or two (CHS and CHI) antibodies respectively, and rotation on an end-over-end turntable at 4°C for 3 h. The protein A-Sepharose beads were collected by centrifugation, washed three times with 200 μl of 0.15 M NaCl, 1% (v/v) Triton X-100 and finally once with water. The washed beads were heated for 4 min at 90°C in 40 μl of 4.2% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, centrifuged, and the supernatant prepared for gel analysis as described in Section 6.2.2.1 (iv).

(iv) Preparation of immunoprecipitated proteins for gel analysis

Protein pellets (Section 6.2.2.1 (ii)) were taken up in 30 μl buffer C and protein solutions (Section 6.2.2.1 (iii)) were diluted with four volumes of buffer C prior to application to SDS-polyacrylamide gels (Section 2.10). For two-dimensional gel analysis (Section 5.2.4), protein samples were lyophilised, taken up in 10 μl urea-buffer (9.5 M urea (Ultra-pure), 8% (v/v) Nonidet P-40 (NP-40), 2% (v/v) ampholytes pH 3-10, 10 mM DTT and 1 mM EDTA) (Hadwiger & Wagoner, 1983) and stored at -70°C until used.

6.2.2.2 Proteins synthesised in vivo

Pulse-labelling of cell cultures with L-[^{35}S]- methionine was as previously described (Section 5.2.3.1).

(i) Treatment with antibody

Pulse-labelled cells (0.3 g) were extracted in two volumes of buffer D (Bolwell et al, 1985a) and centrifuged in a microcentrifuge for 5 min. An aliquot of the supernatant (200 μl) was treated with 5 μl

anti-(PAL) serum or with 5 μl anti-(CHS) plus 50 μl anti-(CHI) sera. Mixtures were incubated for 2 h at 30 °C followed by 16 h at 4 °C.

(ii) Indirect immunoprecipitatioin with protein-A Sepharose

Treatment of the enzyme-antibody mixtures with protein-A Sepharose and collection of the enzyme-antibody complex was as previously described in Section 6.2.2.1.

(iii) Preparation of immunoprecipitated proteins for gel analysis

Immunoprecipitated protein prepared as described in Section 6.2.2.2 (ii) was divided into two 20 μl aliquots. One aliquot was lyophilised, taken up in 10 μl of urea-buffer (9.5 M urea (Ultra-pure), 8% (v/v) NP-40, 2% (v/v) ampholytes pH 3-10, 10 mM DTT and 1 mM EDTA) (Hadwiger & Wagoner, 1983) ready to apply to the first-dimension isoelectric focussing gel for two-dimensional gel electrophoresis as described in Section 5.2.4. For one-dimensional SDS-polyacrylamide gel electrophoresis (Section 2.10), the other 20 μl aliquot was diluted 1:4 (v/v) with buffer C prior to application to the gel.

6.2.3 Incorporation of label into total protein

A 50 μl aliquot of the supernatant from the initial cell extract obtained in Section 5.2.3.2 was used for measurement of incorporation of ^{14}C into total protein as described in Section 6.2.2.2 (i) and a 2 μl aliquot of the translation incubation from Section 6.2.2.1 was used for measurement of incorporation of ^{14}C into total protein as previously described in Section 5.2.2.4.

6.2.3 Blot hybridisation of CHS mRNA

(i) Northern blotting

Polysomal mRNA preparations (15μg in 5μl H₂0) from control and elicitor-induced bean cells were heated with glyoxal and dimethylsulphoxide prior to electrophoresis on 1% agarose gels (Maniatis et al, 1984). RNA was blotted on to PALL Biodyne nylon hybridisation membranes by a standard blotting procedure (Maniatis et al, 1984). Blots were prehybridised, hybridised with labelled probe (see below) and washed prior to autoradiography according to the ' Protocol Guide for RNA Transfer to PALL Biodyne Membranes'.

(ii) Preparation of chalcone synthase gene-specific probes

Gene-specific CHS subclones in SP6 riboprobe vectors were a gift from Dr. T.B. Ryder (Salk Institute, San Diego). pRP1-3, a sub-clone of the 3'-untranslated region of the CHS 1 gene, and pRP4-3, a subclone of the 3'-untranslated region of the CHS 4 gene, were cut with BamHI, and the linearised plasmids phenol extracted, ethanol precipitated and resuspended at 1 μg μl⁻¹. ³²P-labelled RNA transcripts were synthesised using SP6 RNA polymerase and α-[³²P] UTP according to established protocols (Arrand, 1985).

6.3   Results

6.3.1 Characterisation of immunoprecipitates

Investigation of the synthesis of PAL, CHS and CHI requires the use
of specific antibodies. The source of the antisera used in this
chapter, and the characterisation of polypeptides immunoprecipitated
by them, is therefore discussed.

(i) Immunoprecipitation with anti-(chalcone synthase) and anti-
(chalcone isomerase) sera

Chalcone   synthase   and   chalcone   isomerase   subunits   were
simultaneously immunoprecipitated with anti-(parsley chalcone
synthase) and anti-(bean chalcone isomerase) sera. Anti-(parsley
CHS) serum has been shown to immunoprecipitate subunits of identical
M_r and V8 proteolysis patterns from elicitor-induced bean cell
suspension cultures and from light-induced cells of parsley (Lawton
et al, 1983 a).

Simultaneous immunoprecipitation of CHS and CHI was possible because
of the large difference in the apparent M_r of the enzyme subunits;
fluorographic analysis of ^{35}S-labelled proteins synthesised in vitro
or in vivo from RNA simultaneously immunoprecipitated with anti-
(CHS) and anti-(CHI) sera, and separated by 1-D SDS-polyacrylamide
gel electrophoresis showed the presence of a band at M_r 28 000 and a
doublet at M_r 43 000 (Figures 6.1, B and 6.2, B). The M_r 28 000 and
M_r 43 000 polypeptides have previously been identified as authentic
CHI (Robbins & Dixon, 1984; Figure 6.3) and CHS (Lawton et al, 1983 a
& b) subunits, respectively.

(ii)   Immunoprecipitation with anti-(phenylalanine ammonia-lyase)
serum

Anti-(bean phenylalanine ammonia-lyase) serum used in this work was
raised against PAL purified to apparent homogeneity from bean cell

Figure 6.1 – <u>Fluorographs of one-dimensional SDS-polyacrylamide gel</u> <u>electrophoretic separations of polypeptides synthesised *in vivo* and</u> <u>immunoprecipitated from extracts of elicitor-treated cultured bean cells</u>

Suspension cultured cells were labelled for 1 h with [^{35}S] methionine 3 h following elicitation (Section 5.2.3). [^{35}S] labelled subunits were immunoprecipitated using anti-(phenylalanine ammonia-lyase) serum (A), or a mixture of anti-(chalcone synthase) and anti-(chalcone isomerase) sera (B) (Section 6.2.2.2(i)). Tracks are 1, M$_r$ marker; 2, no elicitor (H$_2$0 control); 3, cell wall elicitor; 4, culture filtrate elicitor; 5, culture filtrate Concanavalin A-unbound fraction; 6, Concanavalin A-bound α-methyl mannoside eluate; 7 Concanavalin A-bound NaCl eluate; 8, proteinase K. Suspension cultures were exposed to <u>Colletotrichum</u> elicitors at a final concentration of 25 μg glucose equivalents ml^{-1} (Tracks 3-7), and to proteinase K at a final concentration of 6 μg ml^{-1} (Track 8).

Figure 6.2 - Fluorographs of one-dimensional SDS-polyacrylamide gel electrophoretic separations of immunoprecipitated proteins synthesised *in vitro* from polysomal mRNA from elicitor treated bean cell cultures

Suspension cultured cells were harvested 3 h after elicitation for isolation and translation *in vitro* of polysomal mRNA (Sections 5.2.2.2 & 5.2.2.3). Indirect immunoprecipitation of [^{35}S]-labelled proteins using protein A-Sepharose (Section 6.2.2.1 (ii)) was performed using anti-(PAL) serum (A), or a mixture of anti-(CHS) and anti-(CHI) sera (B) (Section 6.2.2.1 (i)). Tracks are 1, M_r markers; 2, no elicitor (H$_2$0 control); 3, cell wall elicitor; 4, culture filtrate elicitor; 5, culture filtrate Concanavalin A-unbound fraction; 6, Concanavalin A-bound α-methyl mannoside eluate; 7 Concanavalin A-bound NaCl eluate; 8, proteinase K. Cultures were exposed to Colletotrichum elicitors at a final concentration of 25 μg glucose equivalents ml^{-1} (Tracks 3-7), and to proteinase K at 6 μg ml^{-1} (Track 8).

1   2   3   4   5   6   7   8

M_r ($\times 10^{-3}$)

A

— 77

— 53
— 43

B

- 43

- 28
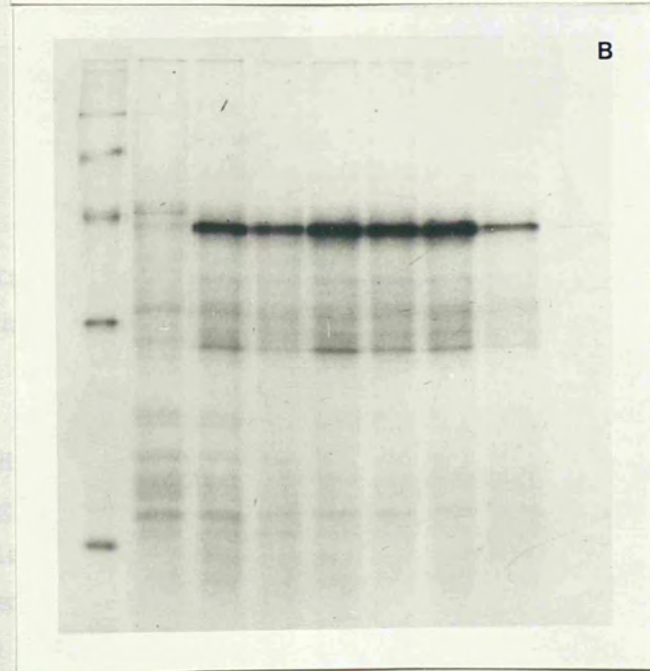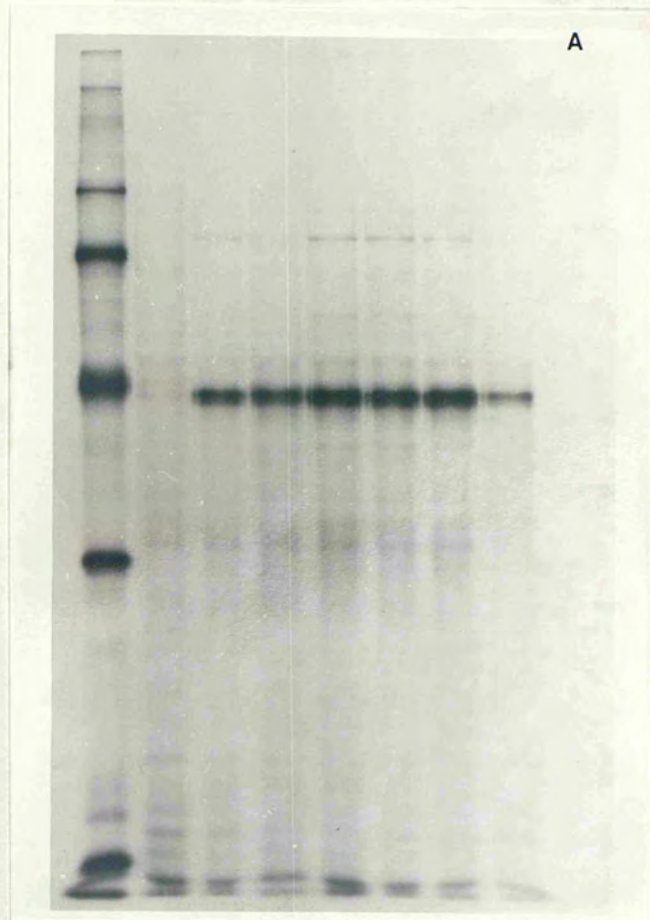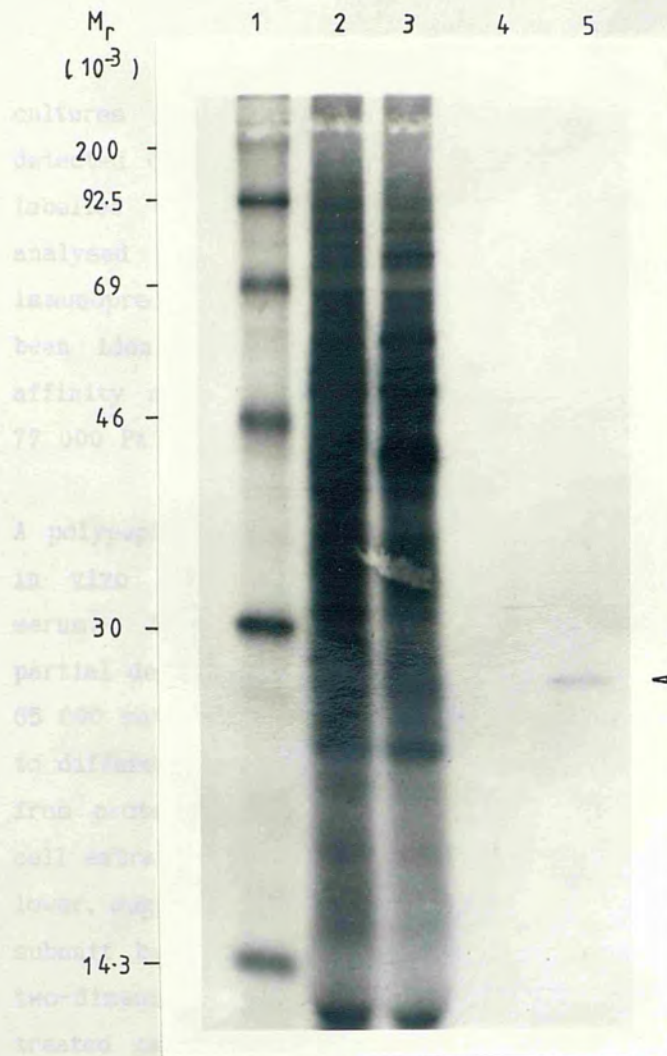
Figure 6.3   Electrophoretic analysis of [³⁵S] methionine-labelled translation products synthesised *in vitro* and immunoprecipitated with antiserum to chalcone isomerase (Courtesy of Dr. M. P. Robbins)

Polysomal RNA was isolated from bean suspension cultured cells treated for 3 h with H₂0 (control) (Tracks 2,4) or with Colletotrichum cell wall elicitor (30 μg glucose equivalents ml⁻¹) (Tracks 3,5). Proteins labelled with ³⁵S-methionine by synthesis in vitro from bean polysomal RNA were either untreated (Tracks 2,3) or indirectly immunoprecipitated with anti-(bean chalcone isomerase) serum using protein A-Sepharose (Tracks 4,5). Arrow indicates position of CHI.

cultures (Bolwell et al, 1985 b). A number of polypeptides were detected on fluorographs of in vivo and in vitro synthesised, ^{35}S-labelled proteins, immunoprecipitated with anti-(PAL) serum and analysed by 1-D SDS-PAGE (Figures 6.1 A & 6.2 A). The immunoprecipitated M_r 70 000 and M_r 53 000 bands have previously been identified by peptide mapping, immunological and active-site affinity methods, as partial degradation products of the native M_r 77 000 PAL subunit (Bolwell et al, 1986 a).

A polypeptide subunit of M_r 65 000 was also detected in samples of in vivo ^{35}S-labelled proteins immunoprecipitated with anti-(PAL) serum. It is possible that this band may also represent a PAL partial degradation product. The amount of label detected in this M_r 65 000 subunit was not constant in samples from bean cells subjected to different treatments. The amount of label in the band in extracts from proteinase K treated cells was 3.5-fold greater than in control cell extracts, while label in the M_r 70 000 band was correspondingly lower, suggesting partial degradation (clipping) of the M_r 70 000 PAL subunit by proteinase K (Table 6.4 & Figure 6.1, A). In addition, two-dimensional IEF: SDS-PAGE analysis of extracts of proteinase K treated cells immunoprecipitated with anti-(PAL) serum showed that the M_r 65 000 subunit appears as multiple spots of identical M_r (Figure 6.4, B). The presence of multiple PAL subunit isoforms has previously been demonstrated in bean cells (Bolwell et al, 1986 a).

A strongly labelled band of M_r 43 000 was detected in both in vivo and in vitro synthesised proteins immunoprecipitated with anti-(PAL) serum (Figures 6.1, A & 6.2, A). This elicitor-inducible polypeptide was present following direct and indirect immunoprecipitation with anti-(PAL) serum, but it was not precipitated with pre-immune serum (Figure 6.5 A & B, respectively). These results suggest that the appearance of the M_r 43 000 subunit is not the result of non-specific adsorption to protein A-Sepharose or to serum proteins. The M_r 43 000 subunit immunoprecipitated with anti-(PAL) serum is probably CHS on the basis of the following observations: a) when immunoprecipitated from in vivo and in vitro synthesised proteins it

Figure 6.4 - <u>Two-dimensional IEF-SDS PAGE of [^{35}S]-labelled polypeptide</u> <u>subunits immunoprecipitated with anti-(phenylalanine . ammonia-lyase)</u> <u>serum</u>

Fluorographs are of polypeptides synthesised <u>in vivo</u> (A, B) or <u>in vitro</u> from mRNA (C), (refer to Figures 6.1, A & 6.2, A). Bean cells were treated with cell wall elicitor (A), proteinase K (B) or culture filtrate elicitor Concanavalin A-unbound fraction (C) for 3 h. CHS subunits are labelled 1 to 6 from the most acidic to the most basic charge isoforms respectiviely. Arrows indicate the positions of PAL polypeptide subunits.
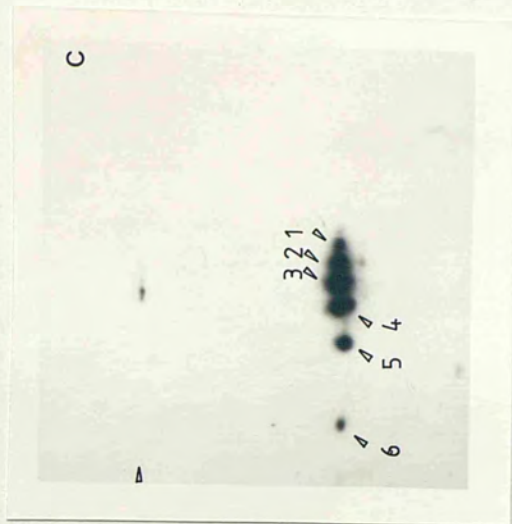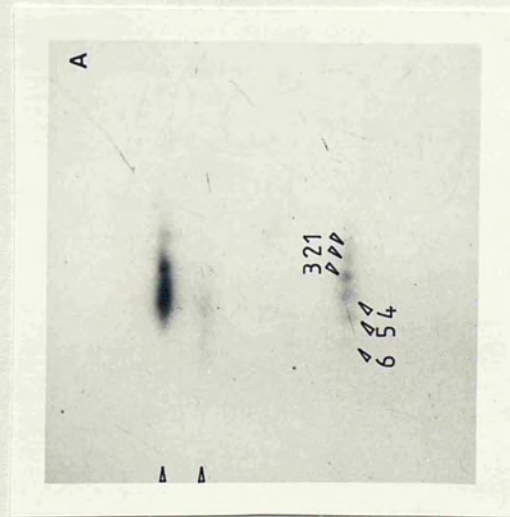
Figure 6.5 - <u>One-dimensional SDS-polyacrylamide gel analysis of polypeptides synthesised <i>in vitro</i> and immunoprecipitated by anti-(bean phenylalanine ammonia-lyase) serum</u>

A. Polysomal mRNA was isolated from bean suspension cultured cells and translated <u>in</u>   <u>vitro</u>   in   the   presence   of   [^{35}S]   methionine. Immunoprecipitation of synthesised polypeptides with anti-(PAL) serum (Section 6.2.2.1 (i)) was either indirect using protein A-Sepharose (Tracks 2-4), or direct (Tracks 5-7) (Sections 6.2.2.1 (iii) & (ii)). Polypeptides were synthesised in the absence (Tracks 2 & 5) and presence of mRNA isolated from cells treated for 3 h with H_2O (Tracks 3 & 6) or <u>Colletotrichum</u> cell wall elicitor (Tracks 4 & 7) at a final concentration of 25 µg glucose equivalents ml^{-1} culture. Track 1 is of M_r standards. N.B. Immunoprecipitated protein applied to track 6 was contaminated with the <u>in vitro</u> translation incubation mixture overlayed on the sucrose cushion (Section 6.2.2.1 (ii)). The period of fluorograph exposure (14 days) to the dried gel was insufficient for the detection of the PAL bands (M_r 77 000 and 53 000) in which label incorporation was approximately 7% of that in the M_r 43 000 band (c.f. Figure 6.2 A).

B. [^{35}S]- labelled polypeptides were synthesised <u>in vitro</u> from polysomal mRNA isolated from <u>Colletotrichum</u> cell wall elicitor-treated (25 µg glucose equivalents ml^{-1} for 3 h) bean cultured cells. Translation mixtures were incubated in the presence of pre-immune serum (Track 2) or anti-(PAL) serum (Track 3). Track 1 is of M_r standards.
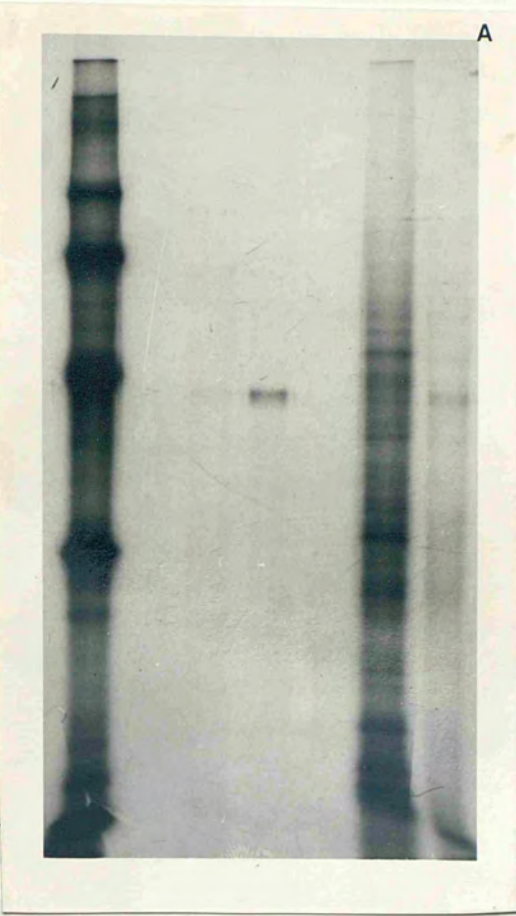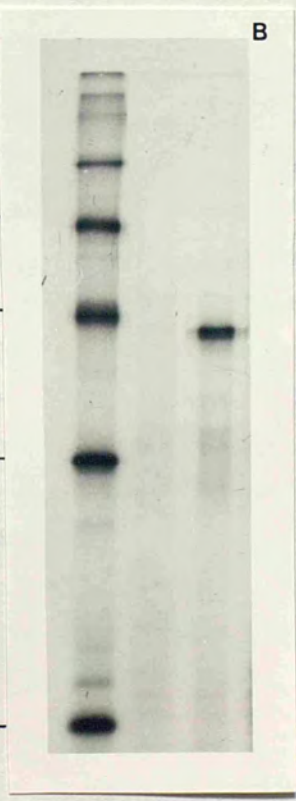
exhibited an identical M_r appearance (doublet) on one-dimensional SDS-polyacrylamide gels as the band immunoprecipitated with anti-(CHS) serum (Figures 6.1 & 6.2); b) identical patterns of spots at M_r 43 000 were observed on two-dimensional IEF: SDS-PAGE analysis of subunits immunoprecipitated with anti-(PAL) or anti-(CHS) sera - six distinct charge species were observed, at least two of which appeared as doublets (Figures 6.4 & 6.6); c) the relative proportions of the six charge species of the M_r 43 000 subunit immunoprecipitated with anti-(PAL) serum (Table 6.1) or anti-(CHS) serum (Table 6.2) from proteins labelled in vivo and in vitro were very closely similar; d) immunoprecipitation of extracts from cell wall elicitor treated bean cells with anti-(PAL) serum resulted in partial loss of CHS activity (Table 6.3). The reasons for immunoprecipitation of CHS subunits by anti-(PAL) serum are not fully understood. The possibility that PAL and CHS undergo a physical interaction, i.e. form a complex, is unlikely as such an interaction would be expected to result in the precipitation of both PAL and CHS subunits following treatment with anti-(PAL) or anti-(CHS) sera. However, PAL subunits were not detected following immunoprecipitation of proteins synthesised either in vivo and in vitro with anti-(CHS) serum (Figures 6.1 B & 6.2 B). An alternative explanation for the immunoprecipitation of PAL and CHS subunits by anti-(PAL) serum would invoke the recognition by anti-(PAL) serum of an amino acid sequence(s) common to PAL and CHS. As the reasons for binding of CHS subunits to anti-(PAL) serum are not clear, and as anti-(PAL) serum appeared only partially to immunoprecipitate CHS, measurement of CHS levels discussed in later sections in this chapter relied solely on quantitative analysis of the 43 000 - M_r CHS subunits immunoprecipitated with anti-(CHS) serum. The M_r 43 000 band detected in immunoprecipitates from samples treated simultaneously with anti-(CHS) and anti-(CHI) sera, and used for quantification of CHS, is immunoprecipitated specifically by anti-(CHS) serum as treatment with anti-(CHI) serum alone only precipitated one band of M_r 28 000 (Figure 6.3).

Figure 6.6 - <u>Two-dimensional IEF-SDS PAGE of [^{35}S]-labelled polypeptide</u> <u>subunits immunoprecipitated with a mixture of anti-(chalcone synthase)</u> <u>and anti-(chalcone isomerase) sera</u>

Fluorographs are of polypeptides synthesised <u>in vivo</u> (A, B & C) or <u>in</u> <u>vitro</u> from mRNA (D) (refer to Figures 6.1, B & 6.2, B). Bean cells were treated with H_2O (A), cell wall elicitor (B), or culture filtrate elicitor Concanavalin-A unbound fraction (C & D) for 3 h. CHS subunits are labelled 1 to 6 from the most acidic to the most basic charge isoforms respectively. Fluorographs were exposed for 28 days (A) and 4 days (B) in order to obtain comparable intensities of signal for gel scanning (see Figure 6.7). The loading of the ^{14}C-M_r markers in (A) was adjusted accordingly.

Isoelectric focussing ⟶

Figure 6·6 (continued)

Isoelectric focussing ⟶

M_r
($\times 10^{-3}$)

pH 6·3

pH 4·0

C

3 2 1

43 —

6   5 4

28 —

D

3 2 1

43 —

6   5 4

28 —

SDS-polyacrylamide gel electrophoresis
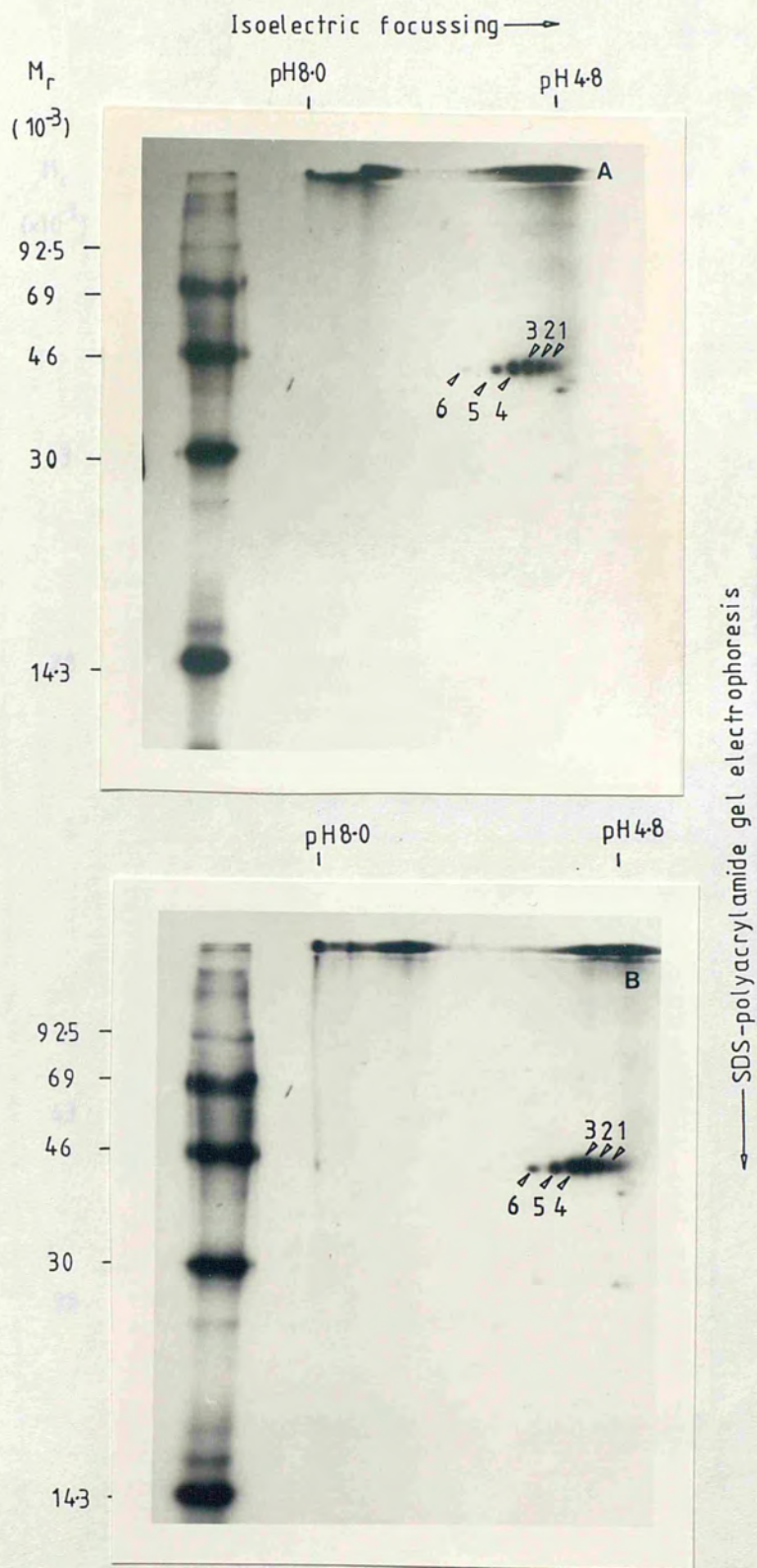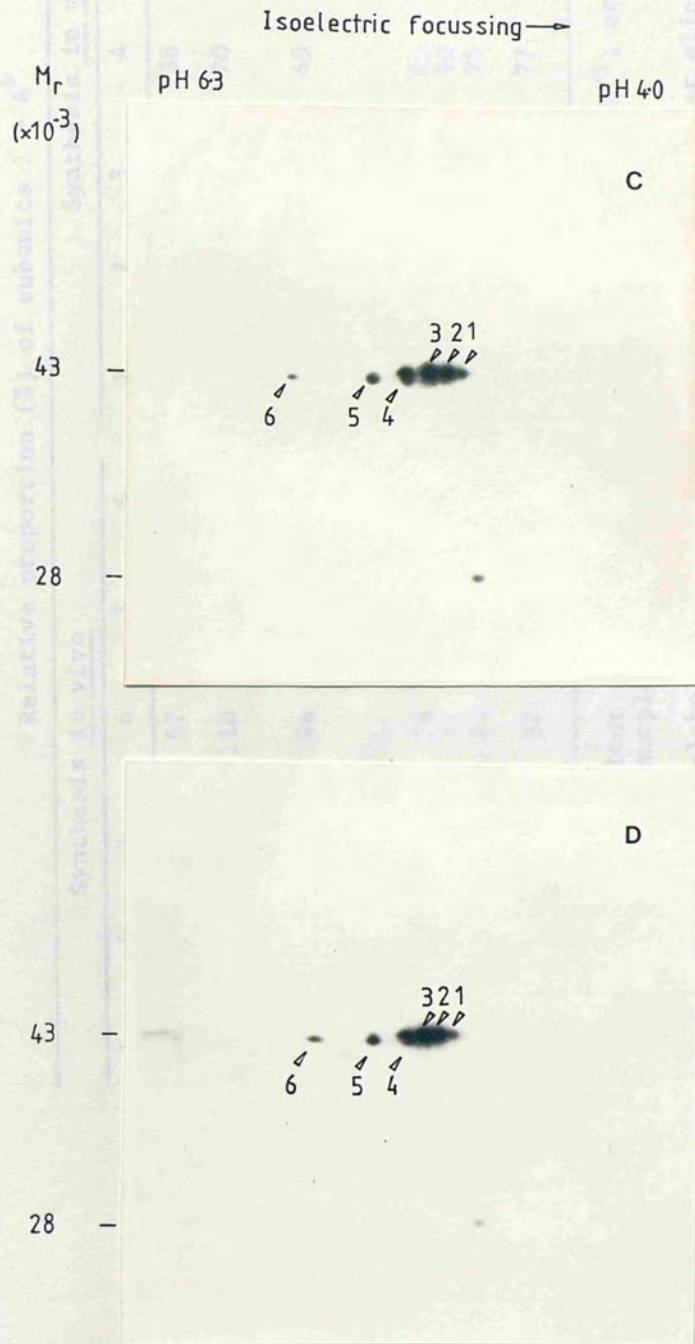
Table 6.1

Relative proportions of the M_r 43 000 polypeptide subunit immunoprecipitated by anti-(phenylalanine ammonia-lyase) serum

| Elicitor fraction[a] | Relative proportion (%) of subunits 1 - 6[b] | | | | | | | | | | | |
| | Synthesis in vivo | | | | | | Synthesis in vitro | | | | | |
	1	2	3	4	5	6	1	2	3	4	5	6
None (H$_2$0 control)	42	40	100	57	58	2	34	63	100	36	30	23
Crude cell wall elicitor	39	58	100	110	56	20	52	81	100	70	39	19
Crude culture filtrate elicitor	38	65	100	94	54	21	42	77	100	49	19	3
Concanavalin A – Sepharose fractions:												
Unbound	35	70	100	78	26	7	47	79	100	85	38	22
α-methyl mannoside eluate	34	70	100	71	49	13	78	97	100	99	77	32
NaCl eluate	32	66	100	84	50	2	43	79	100	95	38	20
Proteinase K	45	78	100	82	41	19	34	72	100	77	23	8

a   Bean cell cultures were exposed to Colletotrichum elicitor fractions (25 μg glucose equivalents ml^{-1}) or to proteinase K (6 μg ml^{-1}) for 3h (in vitro translation samples) or 4h (in vivo labelling).

b   Protein subunits were synthesised in vivo (cells pulse-labelled with ^{35}S-methionine from 3-4h after elicitation) or in vitro from mRNA (legend to Table 6.2). Indirect immunoprecipitation with anti-(PAL) serum used protein A-Sepharose (sections 6.2.2.1 (ii), 6.2.2.2 (ii)). Subunits were analyzed by 2-dimensional IEF-SDS PAGE and the M_r 43 000 polypeptide subunits visualised by fluorography; the subunit numbers are arbitrarily assigned to the subunits ranging from the most acidic to the most basic (numbers 1 to 6 respectively, see Fig. 6.4). After densitometry, the relative subunit proportions were expressed as a % of the peak area of the subunit with the highest incorporation of label (subunit 3). Note that subunits 3 and 4 are clearly doublets (Fig. 6.4).

Table 6.2

Relative proportions of newly synthesised chalcone synthase
subunit charge iso-forms from elicitor treated bean cell cultures

Relative proportion (%) of subunits 1 - 6[b]

Elicitor fraction[a]	Synthesis In vivo[c]						Synthesis In vitro[d]					
	1	2	3	4	5	6	1	2	3	4	5	6
None (H$_2$O control)	45± 3	56±16	100	70±13	47±11	2±0	31±3	58±4	100	56±20	25± 5	8±5
Crude cell wall elicitor	43± 3	66± 8	100	97±13	52± 4	20±1	52±4	88±5	100	79± 9	53±20	14±7
Crude culture filtrate elicitor	50±11	77±11	100	91± 3	49± 5	14±7	45±3	82±5	100	65±13	25± 6	12±9
Concanavalin A - Sepharose fractions:												
Unbound	43± 7	69± 1	100	77± 1	37±11	8±2	48±1	87±6	100	84± 1	38±11	20±5
α-methyl mannoside eluate	36± 2	66± 5	100	83±12	41± 8	10±3	62±4	92±6	100	76± 1	47± 9	29±3
NaCl eluate	42±10	66± 0	100	80± 5	39±11	2±1	40±3	82±3	100	86±10	44± 6	23±2
Proteinase K	59±10	80± 2	100	69±13	38± 3	17±2	36±7	70±5	100	63± 8	24± 2	10±2

a Bean cell cultures were exposed to Colletotrichum elicitor fractions (25 µg glucose equivalents ml^{-1}, final concentration) or proteinase K (6 µg per ml) for 3h (in vitro translation samples) or 4h (in vivo labelling).

b Chalcone synthase subunits were synthesised in vivo (cells pulse-labelled with ^{35}S-methionine from 3h to 4h after elicitation) or in vitro from mRNA (legend to Table 6.2). After indirect immunoprecipitation, using protein A-Sepharose and anti-(CHS) serum (sections 6.2.2.1 and 6.2.2.2), subunits were analysed by 2-dimensional isoelectric focussing: SDS polyacrylamide gel electrophoresis, and chalcone synthase subunit charge iso-forms visualised by fluorography; the subunit numbers are arbitrarily assigned to the subunits ranging from the most acidic to the most basic (numbers 1 and 6 respectively, see Fig. 6.6). After densitometry, the relative subunit proportions were expressed as the peak area of the subunit as a % of the peak area of the subunit with the highest incorporation of label (subunit 3). Note that subunits 3 and 4 are clearly doublets (Fig. 6.6 C, D).

c Results are the average and spread of values from 2 separate determinations.

d Results are the average and spread of values from 3 separate determinations.

Table 6.3

Immunotitration of phenylalanine ammonia-lyase and chalcone synthase activities in extracts from bean cells using anti-(PAL) and anti-(CHS) sera

Treatment[a]	Relative enzyme activity[b] (%)	
	PAL	CHS
None	100	100
Anti-(CHS) serum, protein A-Sepharose in buffer A	106	0
Anti-(PAL) serum, protein A-Sepharose in buffer A	0	52
Protein A-Sepharose in buffer A	100	104
Buffer A	95	100

a   Bean suspension cultured cells were treated with Colletotrichum cell wall elicitor at a final concentration of 25μg glucose equivalents ml^{-1} for 6h. Cells were extracted 1:2 (w/v) in extraction buffer (50 mM KH$_2$PO$_4$/KOH buffer pH 8.0, containing 0.15 M NaCl, 2% (v/v) Triton X-100 and 1.4 mM 2-mercaptoethanol). Following centrifugation in a microcentrifuge for 3 min, 50 μl aliquots of the supernatant were treated with anti-(PAL) or anti-(CHS) sera as described in section 6.2.2.2 (i). The enzyme-antibody complex was collected by indirect immunoprecipitation with protein A-Sepharose pre-swollen in buffer A (extraction buffer, containing 1% (w/v) BSA) as described in section 6.2.2.2 (ii), except that mixtures were left rotating on an end-over-end turntable for 10 min instead of 3h. Protein A-Sepharose beads were removed by centrifugation in a microcentrifuge for 1 min.

b   Values are the amount of enzyme activity remaining in the supernatant after treatment expressed as a percentage of the activity before treatment.

6.3.2   Elicitor-induced changes in enzyme synthesis and mRNA activities

(i) One-dimensional SDS-polyacrylamide gel analysis

The three fractions separated on Concanavalin A-Sepharose from Colletotrichum culture filtrate elicitor (unbound fraction, bound α-methyl mannoside-eluate and bound NaCl-eluate) effectively induced chalcone synthase activity above the level obtained with unfractionated elicitor in two separate experiments using the same bean cell culture line (Tables 6.4 & 6.5) as observed in earlier experiments (Table 4.4).   PAL and CHI activities were also induced, but to levels similar to those attained in response to unfractionated culture filtrate elicitor (Table 6.5), although none of the treatments induced CHI activity in a separate experiment (Table 6.4).   It should be noted that all cell samples analysed in Tables 6.4 and 6.5 were harvested 4 h and 3 h after elicitation, respectively, at the time of maximal rate of label incorporation into PAL and CHS, and the time of attainment of maximal translational activity of PAL and CHS polysomal mRNAs, respectively (Lawton et al, 1983 b; Cramer et al, 1985 a); maximal CHI synthesis occurs 11 h after elicitor treatment (Robbins & Dixon, 1984) and the extractable activities of the three enzymes peak at 6-8 h (PAL and CHS) and 16 h (CHI) (Lawton et al, 1983 a; Robbins & Dixon, 1984).

Similar levels of induction of synthesis of PAL, CHS and CHI subunits were observed in response to treatment with Colletotrichum cell wall and culture filtrate elicitor preparations, and culture filtrate elicitor Concanavalin A-Sepharose fractions, 4 h after treatment (Table 6.4 & Figure 6.1, A, B).   Synthesis of all three enzymes was unaffected by treatment with proteinase K although patterns of immunoprecipitated PAL subunits were altered in response to proteinase K treatment; putative PAL M_r 65 000 subunit constituted 80% of the total immunoprecipitable PAL subunits in proteinase K-treated cells, compared to 22% in untreated cells (Table 6.4 & Figure 6.1, A).   Label incorporation into a 35 000-M_r band

Table 6.4

Changes in the activities and rates of synthesis in vivo of phenylpropanoid pathway enzymes in elicitor treated bean cell cultures

Elicitor fraction[a]	Enzyme activity[b]			in vivo labelling[c] Rate of enzyme subunit synthesis[d]					
	PAL	CHS	CHI	PAL				CHS	CHI
	(μkat kg^{-1})	(μkat kg^{-1})	(mkat kg^{-1})	$M_r(\times 10^{-3})$ 70	65	53	Total[e]	43	28
None (H$_2$O control)	75	0.73	0.15	3.0	0.8	0.3	4.1	5.5	1.5
Crude cell wall elicitor	116	1.11	0.15	5.7	1.8	0.5	8.0	7.7	2.4
Crude culture filtrate elicitor	108	1.06	0.14	5.2	1.4	0.5	7.1	7.5	2.7
Concanavalin A-Sepharose fractions:									
Unbound	103	1.87	0.15	5.6	1.2	0.9	7.7	8.4	2.5
α-methyl mannoside eluate	108	1.95	0.14	5.4	2.0	0.9	8.3	7.7	2.2
NaCl eluate	110	2.13	0.14	6.6	1.9	0.9	9.4	7.5	2.1
Proteinase K	16	0.93	0.14	1.3	2.9	0.2	4.4	5.2	1.6

a   Bean cell cultures were exposed to Colletotrichum elicitor fractions (25 μg glucose equivalents ml^{-1}) or to proteinase K (6 μg ml^{-1}) for 4h.

b   Enzyme activity was measured in extracts of cells treated in parallel with bean cell cultures used for in vivo pulse labelling.

c   Cells were exposed to a 1h pulse of [^{35}S] methionine starting 3h after elicitor treatment (section 5.2.3.1).

d   Expressed in arbitrary units as the peak area of a fluorograph scan of immunoprecipitated enzyme subunit (Figure 6.1 A, B) relative to the incorporation of label into protein (refer to table 5.2).

e   Total rate of synthesis of PAL subunits (M_r 70 000, 65 000 and 53 000).

Table 6.5

Changes in the activities and rates of synthesis in vitro of phenylpropanoid biosynthetic enzymes in elicitor treated bean cell cultures

Elicitor[a] fraction	Enzyme activity[b]			in vitro translation[c] — Rate of enzyme subunit synthesis[d]					Ratio enzyme activity: polysomal mRNA activity[e]	
	PAL (μkat kg^{-1})	CHS (μkat kg^{-1})	CHI (mkat kg^{-1})	M_r(x10^{-3})	PAL 77	PAL 53	Total[f]	CHS	CHI	PAL	CHS
None (H$_2$O control)	41	0.04	2.4		0.1	0.5	0.6	0.9	0.7	68	0.04
Crude cell wall elicitor	143	0.97	3.4		4.2	2.0	6.2	11.9	3.3	23	0.08
Crude cell culture filtrate elicitor	143	0.72	2.9		3.0	1.9	4.9	11.2	3.1	29	0.06
Concanavalin A-Sepharose fractions:											
Unbound	122	1.16	3.2		8.1	5.2	13.3	12.6	2.0	9	0.09
α-methyl mannoside eluate	141	1.30	3.2		7.3	4.7	12.0	13.0	2.3	11	0.10
NaCl eluate	169	1.65	3.5		6.3	3.6	9.9	13.7	2.6	17	0.12
Proteinase K	21	0.53	3.7		1.9	1.4	3.3	5.4	1.4	6	0.10

a Bean cell cultures were exposed to elicitor fractions (25 μg glucose equivalents ml^{-1}) or to proteinase K (6 μg ml^{-1}) for 3h.

b Enzyme activity was assayed in extracts of cells used for polysomal mRNA preparation and translation in vitro.

c Polysomal RNA translated in the presence of ^{35}S-methionine in an mRNA-dependent rabbit reticulocyte lysate cell-free translation system (refer to preparations in Table 5.2). Enzyme subunits were indirectly immunoprecipated with their respective antisera using protein A-Sepharose, analysed by one-dimensional SDS-PAGE (Figure 6.2 A, B) and quantitated by fluorography.

d Expressed in arbitrary units as the peak area of a fluorograph scan of immunoprecipitated enzyme subunit relative to the incorporation of label into total protein (refer to table 5.2).

e Expressed in arbitrary units.

f Total rate of synthesis of PAL subunits (M_r 77 000 and 53 000).

- 224 -

immunoprecipitated by the anti-(CHS) and anti-(CHI) sera mixture from extracts of in vivo labelled cells was much more apparent in proteinase K-treated cells than in cells treated with H_2O or with Colletotrichum elicitor preparations. This band may therefore represent a partially degraded form of CHS.

Induction of PAL,CHS and CHI enzymic activities was accompanied by increased translational activities of their corresponding polysomal mRNAs (Table 6.5 & Figure 6.2 A, B). Although this indicates increased de novo synthesis in response to all Colletotrichum elicitor preparations, the relation between enzyme activity and mRNA activity differed between different treatments for PAL and CHS (the peak in CHI activity 16 h following elicitor treatment, is too delayed to allow comparison with mRNA activity). In particular, the ratio of PAL mRNA translational activity to extractable PAL activity was approximately 3-fold greater in crude culture filtrate elicitor treated cells than in cells treated with a similar concentration of Concanavalin A-Sepharose unbound fraction (Table 6.5). Induction of CHS and CHI activities by proteinase K was also accompanied by a corresponding increase in the translational activities of their polysomal mRNAs (Table 6.5).

(ii)   Two-dimensional isoelectric focussing: SDS-polyacrylamide gel analysis

As all the different elicitor fractions were found to induce synthesis and polysomal activities of the phytoalexin biosynthetic enzymes PAL, CHS and CHI, and as both PAL and CHS were reported to exhibit subunit charge polymorphism when synthesised in cultured bean cells (Bolwell et al, 1985 a, b; Ryder et al, 1984), the effect of the different elicitor treatments on individual enzyme subunit charge isoforms was therefore investigated.

PAL, CHS and CHI subunits labelled with ^{35}S-methionine in vivo or during in vitro translation from polysomal mRNA were immunoprecipitated with their respective antisera and analysed by

two-dimensional IEF: SDS-PAGE (Figures 6.4 & 6.6). Irrespective of inducing agent, CHI appeared as a single subunit of M_r 28 000 at pI 4.8, whereas CHS appeared as a number of subunits exhibiting pI values in the range 4.8-5.5 after synthesis in vivo and in vitro. Six distinct charge isoforms were observed for CHS, at least two of which existed as doublets in the M_r dimension (Figure 6.6 C, D). The relative intensities of individual CHS subunit charge forms, the doublets each being measured as a single charge form, were determined by densitometric scanning of fluorographs in the IEF-dimension (Figure 6.7 and Table 6.2). The results indicate that all 6 CHS subunit charge isoforms were induced in response to elicitor treatment, and that the relative proportions of label in the most basic subunit (subunit 6) was generally increased in response to elicitation after synthesis in vivo and in vitro. However, examination of the relative proportion of label in CHS subunits synthesised in vitro, as compared to in vivo, indicated that the three elicitor fractions purified on Concanavalin A-Sepharose were more effective in inducing polysomal mRNA activity than label incorporation in vivo for subunit 6. This may be the result of inherently different stabilities and/or rates of turnover for the different subunit charge isoforms; for example, isoform 2 constituted a larger proportion of immunoprecipitated CHS subunits in vitro than in vivo with all elicitor preparations. The small differences observed in the rates of synthesis of the CHS charge forms between different elicitor treatments could, however, be related to the type of elicitor used; in particular, the Concanavalin A-Sepharose NaCl eluate was a poor elicitor of label incorporation into subunit 6 in vivo (Table 6.2).

A number of charge isoforms of approximately the same M_r but differing pI values were observed following 2-dimensional gel analysis of immunoprecipitated PAL subunits synthesised both in vivo and in vitro, although these were difficult to resolve (Figure 6.4). The M_r 70 000 and M_r 65 000 PAL subunit charge isoforms synthesised in vivo were detected over the pI range 4.8-5.5 and 5.1-5.9 respectively (Figure 6.4 A, B). Fewer PAL forms, exhibiting pI values

Figure 6.7 - <u>Densitometric analysis of fluorographs of CHS subunit</u>
<u>isoforms separated by 2-dimensional gel electrophoresis</u>

Bean suspension cultured cells were treated for 3 h with H_2O (A) or cell
wall elicitor at a final concentration of 25 µg glucose equivalents ml^{-1}
(B), pulse-labelled with ^{35}S-methionine for 1 h, immunoprecipitated with
anti-(CHS) and anti-(CHI) sera (see Section 6.2.2.2) and the
immunoprecipitated proteins separated by two-dimensional gel
electrophoresis as described in Section 5.2.4. Fluorographs of the dried
gels were photographed using Ilford PAN F 50 film which was then used
for printing on X-ray film (Fuji RX-Medical). Following development, the
X-ray plates were scanned along the iso-electric focussing dimension at
410 nm in a Vitatron MPS densitometer. Scans A and B are of CHS subunit
isoforms of M_r 43 000 shown in Figure 6.6 A & B respectively. Subunits
are labelled 1 to 6 from the most acidic (subunit 1) to the most basic
(subunit 6) isoform respectively.

Relative absorbance (%) at 410 nm

A

100

75

50

25

0

3
4
2
1
5
6

5.5   5.0   4.5

B

100

75

50

25

0

3
4
2
1
5
6

5.5   5.0   4.5

pH
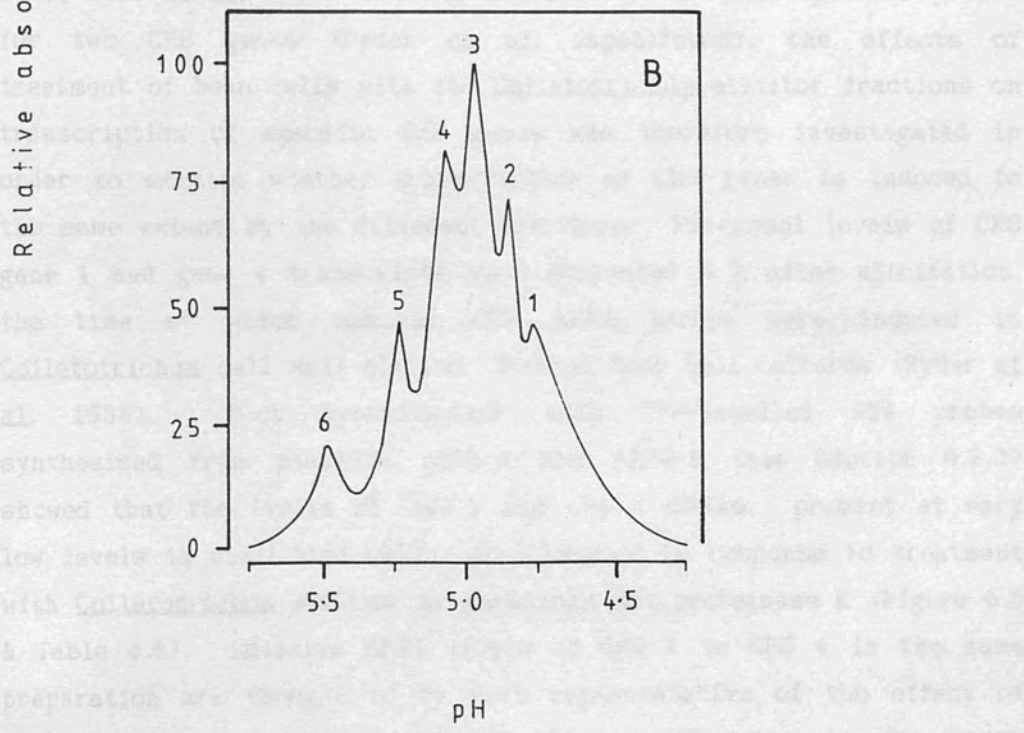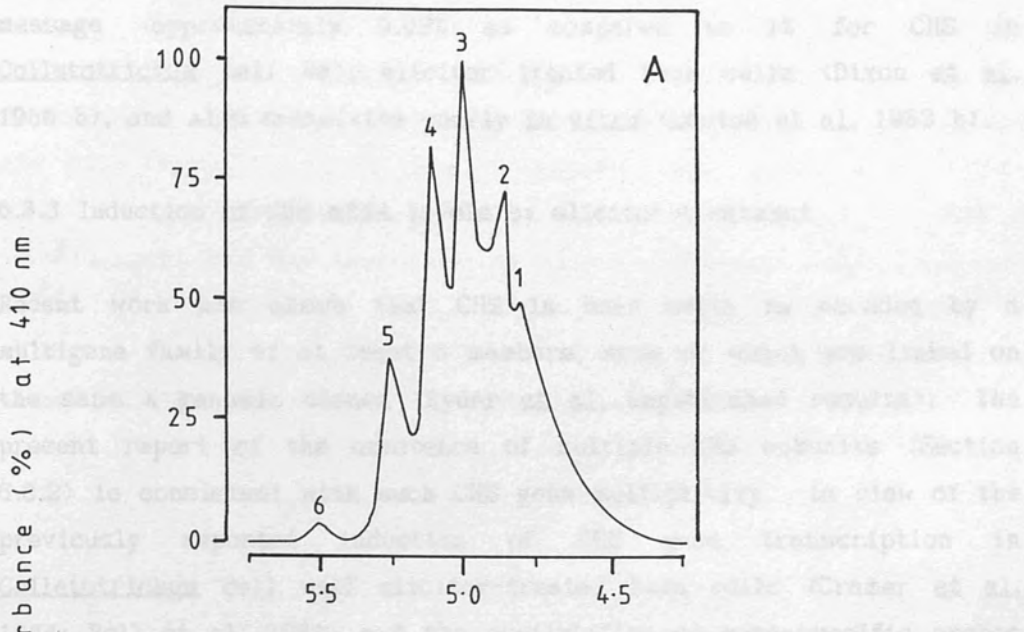
in the range 4.8-5.1, could be discerned in immunoprecipitates of the M_r 77 000 PAL subunit synthesised in vitro. Signals for individual PAL forms were poor on 2-dimensional gels of in vitro synthesised PAL subunits as PAL mRNA constitutes a low percentage of total message (approximately 0.02% as compared to 1% for CHS in Colletotrichum cell wall elicitor treated bean cells (Dixon et al, 1986 b), and also translates poorly in vitro (Lawton et al, 1983 b).

6.3.3 Induction of CHS mRNA levels by elicitor treatment

Recent work has shown that CHS in bean cells is encoded by a multigene family of at least 6 members, some of which are linked on the same λ genomic clones (Ryder et al, unpublished results). The present report of the existence of multiple CHS subunits (Section 6.3.2) is consistent with such CHS gene multiplicity. In view of the previously reported induction of CHS gene transcription in Colletotrichum cell wall elicitor-treated bean cells (Cramer et al, 1984; Bell et al, 1984) and the availability of gene-specific probes for two CHS genes (Ryder et al, unpublished), the effects of treatment of bean cells with the Colletotrichum elicitor fractions on transcription of specific CHS genes was therefore investigated in order to examine whether transcription of CHS genes is induced to the same extent by the different elicitors. Polysomal levels of CHS gene 1 and gene 4 transcripts were estimated 3 h after elicitation, the time at which maximal CHS mRNA levels were induced in Colletotrichum cell wall elicitor treated bean cell cultures (Ryder et al, 1984). Blot hybridisation with ^{32}P-labelled RNA probes synthesised from plasmids pRP1-3 and pRP4-3 (see Section 6.2.3) showed that the levels of CHS 1 and CHS 4 mRNAs, present at very low levels in unelicited cells, were induced in response to treatment with Colletotrichum elicitor preparations and proteinase K (Figure 6.8 & Table 6.6). Relative mRNA levels of CHS 1 to CHS 4 in the same preparation are thought to be more representative of the effect of elicitor treatment on CHS mRNA levels as differences in the purity (Table 5.3, O.D.$_{260}$/O.D.$_{280}$ ratio) and amount of polysomal RNA used for blot hybridisation may be responsible for the observed

- 229 -

differences in the absolute mRNA levels of CHS 1 and CHS 4 between RNA preparations from cells exposed to different elicitor treatments (Table 6.6).   Relative induction of the two mRNA species could be related to the type of elicitor used; crude Colletotrichum cell wall and culture filtrate elicitor preparations had similar effects on the relative induction of CHS 1 and CHS 4 mRNA levels, whereas the Concanavalin A-Sepharose unbound, and α-methyl mannoside-eluted and the NaCl-eluted fractions were respectively approximately 2.5-, 2.7- and 4.7- fold more effective in inducing CHS 1 relative to CHS 4 mRNA levels than the crude culture filtrate elicitor (Figure 6.8 and Table 6.6).   Similarly, treatment with proteinase K resulted in induction of a higher CHS 1: CHS 4 mRNA ratio than treatment with crude Colletotrichum elicitor preparations.

Table 6.6

Changes in the levels of chalcone synthase mRNAs in bean cell
suspension cultures in response to treatment with fungal elicitors

Elicitor fraction[a]	Relative mRNA level (%)[b]		Ratio of mRNA levels
	CHS 1	CHS 4	CHS 1 / CHS 4
None (H$_2$0 control)	4.6	6.9	0.6
Crude cell wall elicitor	27.6	29.3	0.9
Crude culture filtrate elicitor	100.0	100.0	1.0
Concanavalin A-Sepharose fractions:			
Unbound	65.8	26.1	2.5
α-methyl mannoside eluate	21.4	8.0	2.7
NaCl eluate	32.7	6.9	4.7
Proteinase K	26.5	10.1	2.6

(a) Bean cell cultures were treated with <u>Colletotrichum</u> elicitor fractions (25 μg glucose equivalents ml^{-1}) or proteinase K (6 μg ml^{-1}) for 3h.

(b) Polysomal RNA was isolated from cell cultures as described in Section 5.2.2.2 (refer to Table 5.3). RNA (approximately 1.5 kilobases) was detected by autoradiography following hybridisation with 32p-labelled CHS gene specific riboprobes as described in Section 6.2.3 (Figure 6.8). Results represent the levels of mRNA (as measured by the peak areas obtained from densitometric scans of an X-ray print of the autoradiograph) expressed as a % of the level of mRNA from cells treated with culture filtrate elicitor, which hybridised with the CHS 1 and CHS 4 riboprobes.

Figure 6.8 – <u>Autoradiographs of polysomal mRNA from elicitor-treated bean</u> <u>cell cultures hydridised with ^{32}P-labelled CHS gene specific RNA probes</u> (By courtesy of Dr. R.A. Dixon)

Suspension cultured cells were harvested 3 h after elicitation for isolation of polysomal mRNA (refer to Table 5.3). RNA (15µg) was fractionated by agarose gel electrophoresis prior to blot hybridisation with ^{32}P-labelled CHS 1 (A) and CHS 4 (B) gene specific riboprobes (Section 6.2.3). Tracks are <u>1</u>, no elicitor (H$_2$0 control); <u>2</u>, cell wall elicitor; <u>3</u>, culture filtrate elicitor; <u>4</u>, Concanavalin A-Sepharose unbound fraction; <u>5</u>, Concanavalin A-Sepharose-bound α-methyl-mannoside eluate; <u>6</u>, Concanavalin A-Sepharose-bound NaCl-eluate; <u>7</u>, proteinase K. Suspension cultures were treated with <u>Colletotrichum</u> elicitor at a final concentration of 25 µg glucose equivalents ml^{-1} (Tracks 2-6), and with proteinase K at 6 µg ml^{-1} (Track 7).

1 2 3 4 5 6 7

(A)

kb

— 1·5

(B)

— 1·5

6.4   Discussion

6.4.1 Effect of elicitor fractions on induction of PAL, CHS and CHI

In the experiments in this chapter, although Colletotrichum culture
filtrate elicitor fractions obtained by chromatography on
Concanavalin A-Sepharose (unbound fraction, α-methyl mannoside eluate
and NaCl eluate) induced PAL and CHI activities to the same extent as
with the crude culture filtrate elicitor, CHS induction by the
Concanavalin A-Sepharose column fractions was consistently higher
than with the unfractionated elicitor. In particular the NaCl eluate
was the most effective preparation in inducing CHS activity.

Induction of PAL, CHS and CHI activities in response to all
Colletotrichum elicitor fractions was accompanied by corresponding
increased polysomal mRNA activities. This confirms that all
fractions induced de novo synthesis of phytoalexin biosynthetic
enzymes, presumably via increased gene transcription as shown for
CHS (Table 6.6) and as recently demonstrated for PAL and CHS
induction in response to unfractionated Colletotrichum cell wall
elicitor (Edwards et al, 1985; Ryder et al, 1984). However, previous
studies of the effects of cell wall elicitor on the activity, rate of
synthesis in vivo and mRNA activity in vitro for PAL, CHS and CHI in
cultured bean cells have shown that post-translational effects can
contribute significantly to the level of extractable enzyme activity
attained; thus, a dual mechanism involving induction of PAL synthesis
and inhibition of PAL removal was observed at high elicitor
concentration, whereas the lower extent of PAL induction at lower
elicitor concentrations resulted from induction of synthesis alone
(Lawton et al, 1980). Similar effects may also occur with respect to
CHS induction (Lawton et al, 1983 a, b), whereas induction of CHI has
been shown to involve both de novo synthesis and activation of pre-
existing inactive enzyme (Dixon et al, 1983; Robbins & Dixon, 1984).
Differential effects on such post-translational events could explain
the difference between the ratio of polysomal mRNA activity to
resulting extractable enzyme activity observed in response to the

different elicitor preparations, particularly in the case of PAL (Table 6.5). The low ratio of PAL enzyme activity to PAL mRNA activity in cells treated with Concanavalin A-unbound fraction and α-methyl-mannoside-eluate compared to the ratio in crude culture filtrate elicitor-treated cells suggests induction of enzyme synthesis but not a corresponding appearance of enzyme activity. This may explain the complete lack of PAL induction observed in Chapter 5. It should be pointed out however, that conclusions drawn from results using ratios of enzyme activity to mRNA activity at 3 h may be questioned as enzyme activity at 3 h is the result of protein synthetic activity at earlier time points. Thus ratios of enzyme activities at the time of attainment of maximal enzyme induction (6 to 8 h for PAL and CHS, and 16 h for CHI) to enzyme mRNA activities at the time of attainment of maximal translational activity of their respective messages (3 h for PAL and CHS, and 11 h for CHI) would have been more accurate in indicating possible post-translational control.

Ratios of enzyme activity to enzyme synthesis for PAL synthesised in vivo were closely similar in cells treated with crude and Concanavalin A-Sepharose-fractionated culture filtrate elicitor. The results of the in vivo labelling experiment, however, cannot be compared directly to the results of the in vitro translation experiment as these experiments were carried out using different cell batches.

Clearly, a better understanding of the effects of elicitor fractions on enzyme synthesis would be facilitated by the study of differential, preferably all-or-nothing, effects on enzyme induction (as observed in the first four experiments in Table 4.4) using the same cell batch in order to circumvent additional physiological differences. Given more time, the conditions which earlier gave no PAL and/or no CHI induction in the presence of CHS induction would have been reproduced and attempts made to assess whether these effects were on transcription, translation or on post-translational events, for example, turnover and/or enzyme inhibition/activation.

It should be noted that Colletotrichum elicitor fractions were all
tested at the same elicitor concentration (25 µg glucose equivalents
ml⁻¹) irrespective of the degree of purity of the fraction. Thus,
even if the different induction ratios of mRNA activity to
corresponding enzyme activity or of mRNA levels encoding two
different, although related, genes were solely the result of
concentration effects, the data could still reflect the presence of
multiple elicitor components with non-identical biological activities,
as different components in the crude elicitor preparation may exhibit
different dose-response curves (Dixon et al, 1981; Figure 4.4).

6.4.2 Multiple subunits of PAL and CHS

The highly reproducible patterns for CHS subunits obtained following
2-dimensional gel analysis of immunoprecipitates confirms a previous
unpublished observation of subunit charge polymorphism for this
enzyme from bean (T.B. Ryder, personal communication).   The
qualitatively similar patterns of CHS subunits observed after
synthesis in vivo and in vitro indicate that post-translational
events do not result in increased subunit charge polymorphism for
this enzyme. This is in contrast to PAL, for which differences in
subunit number, and the pI ranges over which PAL subunits are spread
after synthesis in vivo and in vitro, confirms previous reports of
post-translational modification of multiple primary products (Bolwell
et al, 1985 b; Bolwell et al, 1986 a). Different rates of synthesis,
stability and turnover of the different CHS forms are likely, and may
underlie the effects of different elicitor preparations on the extent
of labelling of the individual CHS subunits. These effects need to be
tested further, for example by pulse-chase experiments. It should be
pointed out that in the experiments in this chapter, bean CHS is
immunoprecipitated with antibody raised against light-induced parsley
CHS. Thus, the levels of CHS isoforms 1 to 6 immunoprecipitated by
the anti-(CHS)serum may be an underestimate in view of possible
differences in the specificity of the antibody for CHS subunit
isoforms from bean. However, assuming that the specificity of the
anti-(CHS)serum for a given isoform in different in vitro translation

incubation mixtures or cell extracts is the same, changes in the levels of a given isoform following different elicitor treatments are meaningful. Thus, the increase in synthesis in vivo of the most basic CHS subunit isoform, isoform 6, following treatment with crude cell wall or culture filtrate elicitors (Table 6.2) is consistent with the observed increase in the relative proportion of the more basic active CHS forms following elicitation (Figure 3.9).

The presence of multiple PAL and CHS subunits is consistent both with the presence of multigene families encoding PAL and CHS in bean·cells (Dixon et al, 1986 b) and with the observation of charge isozymes of native PAL (a tetramer) and CHS (a dimer) (Figure 3.9). Multiple active PAL forms have previously been demonstrated in bean (Bolwell et al, 1985 b) and in mustard (Gupta & Acton, 1979), whereas mulitple CHS forms in bean are reported for the first time in this work (Chapter 3). In contrast, a single active CHS enzyme form has been reported from Tulipa (Kehrl & Wierman, 1985), a single CHS gene identified in Antirrhinum (Kaulen et al, 1986) and at least 6 CHS genes, of which only one however, has so far been shown to be transcriptionally active (in petals), reported in Petunia (Koes et al, 1986); these three species do not require CHS activity for phytoalexin formation.

It is difficult to assign a physiological role for multiple, active CHS forms in bean in the absence of data on their ultimate tissue distribution, kinetics and specificity although crude cell wall and culture filtrate elicitors preferentially induce the higher pI forms (Figure 3.9). Furthermore, it is not possible at this stage to relate subunit patterns in gels to activity patterns observed on chromatofocussing. The observation of fewer polymorphic forms of active CHS following elicitation compared to the active CHS isoforms present in unelicited cells (Figure 3.9) appears to be contradictory to observations at the subunit level of the enzyme (Chapter 6). It should be noted that bean is believed to contain a separate 6'-deoxy-chalcone synthase necessary for the synthesis of 5-deoxyisoflavonoid derivatives such as phaseollin (Dewick et al, 1982) and therefore it

cannot yet be assumed that all the subunits observed are composed of dimers with the 6'-hydroxy-chalcone synthase activity assayed. In the case of PAL, however, it is known that the higher pI forms predominating in cell wall elicitor-treated cells and here also observed in response to culture filtrate elicitor, exhibit considerably lower K_m values than the low pI forms which make up the bulk of the PAL population in unelicited cells.

The observation of a single subunit charge-form of CHI after synthesis *in vivo* and *in vitro* is consistent with the resolution of a single peak of CHI activity on chromatofocussing (Figure 3.9). In contrast, multiple CHI forms have been reported in parsley (Hahlbrock *et al*, 1970), a plant whose flavonoid derivatives are all 5-hydroxysubstituted and whose CHI forms are inactive against 2',4,4'-trihydroxychalcone, the precursor of 5-deoxy-flavonoids/isoflavonoids. Bean isomerase, on the other hand, while existing in a single form, catalyses the isomerisation of both 6'-hydroxy and 6'-deoxy chalcones (Dixon *et al*, 1982).

Treatment with proteinase K resulted in induction of mRNA activities for PAL, CHS and CHI indicating increased *de novo* synthesis of these enzymes in response to proteinase K treatment. The presence of bands of M_r 65 000 and 35 000 in immunoprecipitates of PAL and CHS respectively, from *in vivo* labelled proteinase K-treated cells suggests that proteinase K may cause 'clipping' of PAL and CHS in *in vitro* extracts. The lower M_r bands appear to be partial degradation products of PAL and CHS rather than specific proteinase K-induced bands as they are only detected in immunoprecipitates from *in vivo*, but not *in vitro*, synthesised proteins from proteinase K-treated cells. Increased levels of CHS mRNA in bean cells treated with proteinase K presumably reflect an increase in synthesis of CHS message. Relative induction of the two CHS genes by proteinase K was however markedly different from induction by crude Colletotrichum elicitor preparations.

The apparent differential effects of proteinase K, crude and Concanavalin A-Sepharose fractionated Colletotrichum elicitors on transcription of different CHS genes indicate that different CHS genes or groups of genes may be regulated independently. In this respect it is interesting to note that out of at least 6 CHS genes reported to exist in Petunia hybrida petals, only one CHS gene is transcriptionally active (Koes et al, 1986). Although the significance of differential induction of CHS genes in response to different stimuli is difficult to interpret in the absence of more detailed data on the roles of multigene families, subunit assembly and physiolgical function of different forms of CHS in the induction of CHS activity, use of fractions such as those reported in this work, may prove useful in the study of regulation and co-ordination of plant gene expression.

CHAPTER 7

SUMMARY AND CONCLUDING REMARKS

Contents   Page No.

SUMMARY AND CONCLUDING REMARKS

7.1   Summary of the results of Chapters 3-6

The following conclusions can be drawn from the studies of the
effects of the different elicitors on the defence-related responses in
bean.

1.   Elicitor activity (as measured by the induction of phytoalexin
      biosynthetic enzymes in cultured bean cells) in preparations
      from the bean pathogen Colletotrichum lindemuthianum resides in
      a carbohydrate moeity.

2.   Elicitor activity is present in a number of molecular
      components, separable on the basis of M_r, charge and affinity
      for Concanavalin A.

3.   Unfractionated elicitor preparations from cell walls and culture
      filtrates of Colletotrichum exhibit similar biological effects on
      bean cells with respect to:

      a)   their small negative effect on apparent cell viability of
            suspension cultured cells.

      b)   their lack of induction of electrolyte leakage from leaf
            slices or isolated leaf mesophyll cells.

      c)   their induction of isoflavonoid phytoalexin accumulation
            in bean endocarp tissue.

      d)   their induction of the extractable activities, rates of
            synthesis in vivo and mRNA activities in vitro for PAL,
            CHS and CHI.

      e)   their induction of the active charge iso-forms of PAL and
            CHS.

      f)   their induction of the different CHS subunit charge
            isoforms, measured after synthesis in vivo or in vitro
            from mRNA.

      g)   their relative induction of transcripts encoding CHS
            genes 1 and 4.

4.   Unfractionated elicitor preparations from cell walls and culture
     filtrates of Colletotrichum exhibit different biological effects
     on cultured bean cells with respect to:
     a)   the accumulation of phytoalexins, wall-bound phenolics and
          wall-associated hydroxyproline in response to cell wall
          elicitor but not culture filtrate elicitor.
     b)   the patterns of total polysomal mRNA translation products
          observed from cells treated with the two types of
          elicitor.
5.   Culture filtrate elicitor preparations fractionated on the basis
     of their affinity for Concanavalin A (and therefore differing in
     monosaccharide composition, especially mannose content) exhibit
     similar biological effects on cultured bean cells with respect
     to:
     a)   their small negative effect on apparent cell viability.
     b)   the patterns of many (but not all) polypeptides
          synthesised in vitro or in vivo from mRNA.
     c)   their induction of the different CHS subunit charge
          isoforms, measured after synthesis in vivo or in vitro
          from mRNA.
     d)   their induction of CHS mRNA activity and rate of
          synthesis of CHS in vivo
     e)   their induction of PAL synthesis in vivo
6.   Culture filtrate elicitor preparations fractionated on the basis
     of their affinity for Concanavalin A exhibit different
     biological effects on cultured bean cells with respect to:
     a)   the greater elicitation of CHS activity observed with the
          fraction of hightest mannose content.
     b)   the ratios of PAL enzyme to mRNA activity.
7.   The Concanavalin A unbound and α-methyl mannoside-eluted
     fractions either failed completely to induce PAL and CHI
     activities, or induced them to a level no greater than that
     observed in response to unfractionated culture filtrate elicitor.
8.   Proteinase K was shown to elicit both the accumulation of
     phytoalexins and induction of de novo synthesis of PAL, CHS and

CHI. It appeared to require proteolytic activity for its effects and caused partial degradation of PAL and CHS subunits in vivo.

While investigating the effects of different elicitor preparations on the induction of PAL, CHS and CHI in cultured bean cells, a number of observations were made concerning the nature of these enzymes and their mRNAs. These are sumarised as follows:

1. Results confirm previous reports of the existence of up to four peaks of active PAL enzyme separable by chromatofocussing, of the preferential induction of the higher pI PAL forms by elicitor and of the existence of multiple PAL subunits of differing pI, with more isoforms observed after synthesis in vivo than in vitro from mRNA.

2. CHS exist as multiple charge isoforms of the active enzyme. Up to eight subunit charge isoforms are observed after synthesis both in vivo and in vitro. This indicates that CHS multiplicity does not result from post-translational changes (as presumably occur for PAL), and is consistent with reports of an expressed multigene family for bean CHS.

3. In addition to the presence of a single active CHI charge isoform from bean, there is no multiplicity at the subunit level for this enzyme after synthesis in vivo or in vitro from mRNA.

Most of the above points have been discussed, in the direct context of the other data in the thesis, in the preceeding pages. The following two sections therefore concentrate on the extrapolation of the results to two areas of future interest, namely the value of differentially acting elicitor preparations for studies on host gene expression, and their possible relevance, if any, to the intact plant-parasite interaction.

7.2   <u>Differential responses of bean cells to elicitor fractions from</u>
       <u>*Colletotrichum lindemuthianum*: possible implications for the control</u>
       <u>of host gene expression</u>.

While consistent with the presence of multigene families encoding PAL
and CHS in bean, none of the results presented in this thesis
suggests that crude or fractionated <u>Colletotrichum</u> elicitors can
induce, individually, different members of their active gene products;
the only striking qualitative differences in PAL and CHS expression
are seen when comparing elicited and unelicited treatments. This
result is perhaps not surprising, as all elicitor fractions may be
classified as "stress stimuli": qualitative differences in expression
of these genes may be observed with other stimuli e.g. light,
wounding, culture dilution or plant growth regulators.

The inability of some elicitor fractions to induce PAL and CHI
activities, while efficiently inducing CHS, suggests that the
induction of these early enzymes does not occur via an obligatory
co-ordinate mechanism, and the reconstitution of full elicitor
activity by mixing of fractions indicates that more than one
component may be necessary for the induction of the full response.
This latter point does not, however, imply that single elicitor
molecules cannot induce a complete response on their own; some may
and some may not. In relation to the uncoupling of PAL and CHS
induction, it was unfortunate that later cell culture lines gave a less
clear differential effect with the Concanavalin A-Sepharose fractions.
Nevertheless, it appeared from these later experiments that the
Concanavalin A fractions which had previously failed completely to
elicit PAL were inducing high PAL mRNA activities which were not
subsequently reflected in proportionally increased enzyme activity.
As induction of PAL activity is known to be regulated via induction
of synthesis and inhibition of removal (Lawton <u>et al</u>, 1980), it is
possible that the Concanavalin A fractions lacked a component which
somehow regulated the rate of PAL removal <u>in vivo</u> i.e caused enzyme
stabilisation. This hypothesis, which could be tested with a new
batch of elicitor fractions and with different cell lines, would,

however, imply that PAL synthesis, at least at the level of transcription, was still co-ordinately linked to the synthesis of CHS. In the case of CHI however, any effect on enzyme removal would have to involve accelerated degradation or inactivation (rather than just prevention of turnover inhibition) as the enzyme has a much longer half-life than PAL in cultured cells.

In contrast to the lack of qualitative effects on the induction of PAL, CHS and CHI gene products, different elicitors clearly exhibit different effects on total gene expression patterns. Thus, even if there were no basis for a physiological role in vivo for multi-elicitor components, their use in model systems could be of great value for the elucidation of the mechanisms which operate in the integration and co-ordination of induced defence responses. For example, if it could be demonstrated that cell wall elicitor and culture filtrate elicitor only differ in their effects on host defence at the level of induction of enzymes specific for the later stages of phytoalexin synthesis, differential screening strategies for the isolation of the cDNAs complementary to the mRNAs encoding these enzymes could readily be devised. In addition, the availability of elicitor fractions which quantitatively and/or qualitatively affect the expression of multigene families will be valuable for further studies on the mechanisms underlying differential gene expression, especially in relation to the nature of putative sequence-specific response couplers. It would, for example, be interesting to compare the effects of elicitors of host origin (the so-called endogenous elicitors) and fungal origin on the induction of different PAL and CHS transcripts.

7.3   Differential responses of bean cells to elicitor fractions from _Colletotrichum lindemuthianum_; extrapolation to the intact plant-pathogen interaction

Results presented in this work indicate that more than one type of fungal elicitor may potentially be involved in switching on the sum total of responses observed in microbial elicitor-plant cell interactions. However, although a large body of work exists on elicitation of host-pathogen responses, it is difficult to link a particular resistance response to the initial interaction between host and pathogen largely as a result of our ignorance of plant signalling mechanisms as a whole. As the quintessential implication of a single elicitor/receptor model is that synergists or antagonists of elicitor binding should act to modify the response quantitatively but not qualitatively, the easiest interpretation of the present results would seem to imply the activation of different classes of receptor. The results do not disprove a single elicitor/receptor model however, as it has not been shown that a single elicitor with the ability to induce all the resistance-related responses does not exist.

A model involving multiple signals from the pathogen raises several questions regarding (a) the extent of multiplicity, in terms of the number of signals required to modulate a group of responses which may be functionally related (i.e. help to determine resistance expression) but may be metabolically unrelated (e.g. phytoalexin and hydroxyproline-rich glycoprotein synthesis), (b) the extent to which functionally related host-genes are co-ordinately induced, (c) the nature and number of receptor sites necessary for binding elicitors and/or modifiers of elicitor action (if, in fact all interactions primarily involved in determining induction of resistance take place at the host cell surface) and (d) the number and nature of inter/intra-cellular response couplers necessary to link elicitor binding to altered gene expression or to other physiological effects.

The race-specific elicitor/receptor model is the simplest model to account for induced resistance in gene-for-gene interactions in which resistance expression is conditioned by the interaction between the products of a plant resistance gene (putative elicitor receptor) and a complementary avirulence gene in the pathogen (fungal elicitor). Clearly, glycoprotein and polysaccharide elicitors cannot themselves be products of 'resistance genes', and more than one gene will be involved in their production even if only a single class of elicitor is required for induction of all the characteristics of disease resistance. In the case of polysaccharide elicitors, if the avirulence gene(s) encoded a specific glycosyl transferase(s), for example, then this could render active a number of differentially acting components synthesised by the action of one or more glycosyl transferases on one or more substrates. Therefore, a multicomponent elicitor model is not incompatible with the concept of a single pathogen avirulence gene, or at least no more incompatible than the generally accepted belief that polysaccharides may act as determinants of resistance!

In spite of the large research effort expended, it has not been rigorously proved that elicitor molecules isolated from fungi grown in culture are the response-determining molecules active in the intact plant-pathogen interaction. Thus, it may be argued that the presence of elicitor fractions with differential biological activities has no physiological significance with respect to the intact plant-parasite interaction; for example, such fractions could be artifacts arising from the use of cultured material or the method of elicitor preparation. If this were the case, the present results would nevertheless serve to indicate the potential of plant cells to respond to different biologically active polysacchrides in a manner suggesting subtle differences in reception and/or response-coupling. The elicitor fractions may be acting at host cell receptors which recognise a range of structural determinants, irrespective of whether they are components of natural biological interactions involved in defence. Alternatively, recognition by the host of different elicitor types in the intact plant-pathogen interaction could

potentially increase the flexibility and efficiency of the resulting
induced defence response. Assessment of a possible significance for
multiple elicitor molecules in in vivo plant-parasite interactions
will require the development of suitable techniques for (a) their
isolation, characterisation and detection in situ e.g. by
immunocytochemical methods utilising monoclonal antibodies (c.f.
O'Connell et al, 1986), (b) the identification of fungal avirulence
genes/gene products e.g. by transformation of virulent races with
cloned cDNA fragments from genomic libraries of avirulent races (c.f.
Staskawicz et al, 1984) and (c) the determination of the biological
activities of the corresponding gene products. Several of these
goals may be achieved in the near future.

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Differential biochemical effects of elicitor preparations from *Colletotrichum lindemuthianum*

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Polysaccharide containing elicitor preparations from the culture filtrate and cell walls of *Colletotrichum lindemuthianum* had broadly similar monosaccharide compositions. Both preparations induced phenylalanine ammonia-lyase, chalcone synthase and chalcone isomerase extractable activities in bean (*Phaseolus vulgaris* L.) cell suspension cultures. However, although phytoalexin accumulation was observed in response to the two elicitors in bean endocarp tissue, the culture filtrate elicitor induced only phaseollin in bean cell suspension cultures whereas the cell wall elicitor induced both kievitone and phaseollin, the latter to a concentration 70 times greater than that induced by the culture filtrate elicitor. Only the cell wall elicitor induced deposition of wall-bound phenolics in bean cultures, and differences were also observed in the effects of the two elicitor preparations on levels of free and esterified hydroxycinnamic acids. Induction of prolyl hydroxylase extractable activity was observed in response to both elicitors, although increased accumulation of hydroxyproline in the cell walls of suspension-cultured bean cells was only induced following treatment with cell wall elicitor. The results are discussed in terms of the coordination and regulation of induced resistance responses, and the possible need for more than one elicitor to induce such changes is considered.

INTRODUCTION

The term biotic elicitor is used to describe macromolecules isolated from plant pathogens and their hosts which are able to induce physiological or biochemical responses associated with expression of resistance. A wide range of fungal and bacterial metabolites, including polysaccharides [1, 3, 11, 33], glycoproteins [14, 24, 39] and enzymes [27] have thus been suggested to play a role in host–pathogen interactions. The criteria relied upon for the assessment of elicitor activity include the visual estimation of cellular necrosis [1], measurement of induced electrolyte leakage [18], and the determination of extractable activities of enzymes [16, 35, 38], and levels of phytoalexins [11, 16, 39], lignin [23, 29], ethylene [21, 36] and cell wall hydroxyproline-rich glycoproteins [20, 21, 23], all of which have been implicated in resistance of plants to pathogenic fungi. In most cases, however, the bioassay of elicitor activity, and therefore the criterion for purification of elicitors, has relied upon the measurement of a single parameter.

A picture is now beginning to emerge of the molecular mechanisms underlying the expression of host genes in response to elicitor macromolecules. Thus, use of cDNA

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Abbreviations used in text: CHI, chalcone isomerase; CHS, chalcone synthase; PAL, phenylalanine ammonia-lyase; TTC, 2,3,5-triphenyltetrazolium chloride.

hybridization probes has shown that induction of L-phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS), two key enzymes in isoflavonoid phytoalexin biosynthesis, involves increased appearance of mRNA species encoding these enzymes in bean and parsley tissues [12, 25, 31]. However, in spite of the large research effort expended, there is little direct evidence linking the action of a single elicitor molecule to the early molecular events underlying expression of resistance in either model systems (tissue cultures) or intact plant–parasite interactions. In part, this results from the difficulty of being certain that a molecule, usually isolated from the pathogen grown in liquid culture, is (a) present in the intact plant–parasite interaction and (b) the only pathogen-produced molecule involved in induction of resistance; the latter point is of particular relevance with respect to reports of the production of suppressor molecules by phytopathogenic fungi [41]. Equally important, however, is the possibility, so far relatively unexplored, that more than one type of elicitor molecule may be required for the sum total of events associated directly with induced resistance.

Colletotrichum lindemuthianum, the causal agent of anthracnose disease of bean, has been shown to produce elicitor-active molecules in culture [1], these having been partially purified from both culture filtrates and autoclaved cell wall preparations [2, 16]. It has been suggested that the elicitor activity may reside in a $(1\rightarrow3),(1\rightarrow4)$-linked β-D-glucan [1]. The effects of crude elicitor preparations from the cell walls of this fungus on bean cell suspension cultures include phytoalexin induction [16, 30], a highly selective induction of key enzymes of the phytoalexin pathway [7, 16, 30], increased levels of cytochrome P450 [7], accumulation of wall-bound phenolic compounds [7, 15], and accumulation of hydroxyproline-rich glycoproteins and induction of enzymes involved in their synthesis [6, 7]. In the present paper, we compare the effects of crude elicitor preparations from cell walls and culture filtrates of C. lindemuthianum on a number of parameters associated with induced defence responses. This approach has demonstrated that different elicitor preparations can have differential effects on host cell metabolism, and the results are discussed in terms of the control and co-ordination of the synthesis and accumulation of phytoalexins and cell wall components.

MATERIALS AND METHODS

Growth of plants and maintenance of plant and fungal cultures

French bean seedlings (cv. The Prince) were grown in a Conviron growth cabinet under 16 h photoperiod conditions (45 Wm^{-2}) with a day temperature of 25 °C and a night temperature of 20 °C. Pods were from plants grown at the University of London Botanical Supply Unit, Egham, U.K.

Cell suspension cultures of dwarf French bean cv. Immuna were maintained as described previously [16]. Colletotrichum lindemuthianum (Commonwealth Mycological Institute isolate IMI 112166) was maintained on a semi-solid glucose neopeptone medium [28] in the dark at 25 °C.

Elicitor preparations

Colletotrichum lindemuthianum shake cultures were grown in 100 ml batches of glucose neopeptone medium (modified by the addition of an extra 13 grams of glucose per litre) in 500 ml conical flasks. Three flasks (6 days after their second subculture) were used as

inoculum of a 9 l batch fermenter in which the culture was grown for 6 days in the dark at 25 °C with mixing by aeration $(10 \, \mathrm{lm}^{-1})$. Mycelium was harvested on four layers of muslin and cell walls prepared as described elsewhere [1].

Release of elicitor molecules from cell walls by autoclaving was as described previously [16]. Culture filtrate elicitor was prepared by adding 30 l of 95% (v/v) ethanol to 10 l of culture filtrate from the fermenter grown culture, and the resulting precipitate was allowed to settle for 4 days at 4 °C. Most of the clear supernatant was siphoned off and the residue centrifuged at 10 000 *g* for 10 min. The pellet was dissolved in 250 ml of water and the solution was then extensively dialysed against distilled water, filtered through Whatman No. 1 paper and lyophilized. The residue collected on the filter paper was washed with acetone, air dried at room temperature and stored desiccated at -20 °C.

Periodate oxidation and proteinase K digestion of elicitors

Elicitor preparations (1·3 mg glucose equivalents in 0·8 ml of water) were treated with 0·5 ml of 15 mM sodium metaperiodate for 36 h in the dark at 20 °C. Excess periodate was then consumed by the addition of 1·0 ml of ethylene glycol. Control treatments contained either (a) periodate pretreated with ethylene glycol prior to addition of elicitor or (b) ethylene glycol-treated periodate to which 0·8 ml of water was added in place of elicitor.

For proteinase K digestions, elicitor preparations (2·5 mg glucose equivalents in 1·6 ml of water) were treated with 0·352 mg proteinase K (Sigma Chemical Co.) in 0·5 M potassium phosphate buffer, pH 7·8, for 27 h at 37 °C. Control samples contained no elicitor, and all samples were then autoclaved at 120 °C for 15 min to inactivate proteinase K. Finally samples treated with both periodate and proteinase K were dialysed against water, lyophilized, taken up in distilled water to a final elicitor concentration of 2·5 mg glucose equivalents ml^{-1}, and stored at -20 °C.

Total carbohydrate was estimated using phenol sulphuric acid reagent [5] with glucose as standard.

Monosaccharide analysis

Quantitative TLC analysis of trifluoroacetic acid-hydrolysed elicitor preparations was carried out as described previously [16], except that 20 cm, rather than 10 cm, Merck No. 5748 silica gel 69 TLC plates were used.

Elicitation of plant material

All cell suspension cultures were used 3–4 days after subculture. Batches of cells (20 ml) in 50 ml conical flasks were exposed to elicitor for 6 h (for determination of PAL and CHS induction) or 18 h [for chalcone isomerase (CHI)]. Phytoalexins were estimated in 100 ml batches of cells treated with elicitor $(100 \, \mu\mathrm{g} \text{ glucose equivalents } \mathrm{ml}^{-1})$ for 48 h. Cells were harvested by vacuum filtration, frozen in liquid N_2 and stored at -70 °C.

Bean pods were surface-sterilized in 0·1% sodium hypochlorite solution for 15 min and rinsed several times with sterile water. To each seed cavity was added 50 μl of elicitor solution $(100 \, \mu\mathrm{g} \text{ glucose equivalents } \mathrm{ml}^{-1})$ or water containing 50 μg of penicillin and 100 μg of streptomycin. Pods were incubated in a humid chamber in the dark at

25 °C for 48 or 96 h. Endocarp tissue underlying the applied solutions was excised with a scalpel and stored at −70 °C prior to analysis.

Enzyme assays

Cells were extracted in a pestle and mortar in two volumes of 50 mM potassium phosphate buffer, pH 8·0 containing 0·01% (v/v) 2-mercaptoethanol, prior to assay of PAL [26], CHS [38] and CHI [17] by previously published methods. For assay of prolyl hydroxylase, cells were extracted in one volume of 50 mM Tris–HCl, pH 8·8, containing 25 mM $MgCl_2$, 1 mM dithiothreitol, 0·3 M KCl and 0·4 M sucrose. The extracts were centrifuged at 15 000 **g** for 7 min, and the supernatants re-centrifuged at 200 000 **g** for 1 h. Microsomal pellets were resuspended in 150 µl of 50 mM Hepes, pH 6·8, containing 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM KCl and 0·4 M sucrose. The extracts were made up to a final concentration of 0·1% (v/v) Triton X-100, and prolyl hydroxylase activity assayed by measurement of the poly L-proline-dependent decarboxylation of α-keto [1-^{14}C] glutaric acid as described previously [7].

Protein was determined by the method of Bradford [8]. All enzyme activities are expressed as micro or millikatals kg^{-1} protein, where 1 katal is the amount of enzyme required for the formation of 1 mole of product in 1 s under the assay conditions defined.

Estimation of wall-bound hydroxyproline

Cells were extracted in one volume of 50 mM Tris–HCl, pH 8·8, containing 25 mM $MgCl_2$, 1 mM dithiothreitol, 0·3 M KCl and 0·4 M sucrose, and the extracts were centrifuged at 15 000 **g** for 7 min. The pellets were hydrolysed in 6 M HCl (1:1, w/v) overnight at 100 °C. After centrifugation, the pH of the supernatants was adjusted to 12·0 with 4 M KOH and aliquots made up to 400 µl with distilled water. The hydroxyproline content of these solutions was determined by the method of Kivirikko [24].

Determination of wall-bound phenolic material

Wall-bound phenolic material was determined by difference spectroscopy of alkaline hydrolysates of bean cell walls as described previously [15].

Determination of free and esterified phenolic acids

Cells were extracted in one volume of 50 mM sodium acetate buffer, pH 4·0. The extracts were centrifuged at 1000 **g** for 15 min, 0·5 ml aliquots of the supernatants extracted twice with equal volumes of diethyl ether, and the organic layers combined and dried under a stream of N_2 (soluble phenolic acid fraction). To the aqueous phases remaining after the above extractions were added equal volumes of 4 M NaOH, and the solutions were left overnight at 4 °C. After adjusting the pH to 4·0, the solutions were extracted twice with equal volumes of ethyl acetate and the organic layers pooled and dried under a stream of N_2 (esterified phenolic acid fraction). Dried samples were taken up in HPLC grade methanol, and separation was effected on a Partisil 10 ODS 1 reverse phase HPLC column with isocratic elution with methanol:water:acetic acid (40:60:0·1, v/v). Sample elution was monitored at 254 nm, and peak areas compared with those of standard samples of cinnamic, 4-coumaric, caffeic and ferulic acids.

Analysis of phytoalexins

Plant tissue was extracted twice in 95% ethanol (10 ml g^{-1} fresh weight). After filtration through sintered glass the filtrates were combined, dried under reduced pressure at 40 °C and taken up in 1 ml of ethanol followed by 50 ml of distilled water. Aqueous fractions were extracted twice with successive equal volumes of chloroform, diethyl ether and petroleum ether (BP 40–60 °C). The organic layers were combined, dried under vacuum, taken up in 100 µl of ethanol:chloroform (3:1, v/v) and applied to 20 × 20 cm, 0·25 mm thick, Polygram silica gel G/uv$_{254}$ thin layer plates which were developed in the first dimension in diethyl ether:hexane (5:1, v/v) and in the second dimension in benzene:methanol (9:1, v/v). Plates were viewed under UV light (254 and 366 nm), fluorescent or quenching spots eluted in 1·2 ml of ethanol, and absorption spectra recorded on a Beckman 24 scanning spectrophotometer.

Assessment of cell viability

Assay conditions for the use of 2,3,5-triphenyltetrazolium chloride (TTC) as a viability stain for bean cultures (TTC concentration, pH, time of incubation) were first optimized as described elsewhere [37]. Cells were harvested from suspension cultures by suction filtration on sintered glass and washed with 50 mM potassium phosphate buffer, pH 8·0. To 0·5 g of cell batches was added 1·5 ml of 0·5% (v/v) TTC in 50 mM potassium phosphate buffer, pH 8·0. After incubation for 24 h in the dark at 20 °C, cells were pelleted and washed once with distilled water. The red formazan was extracted from the pelleted cells by heating with 3 ml of 95% ethanol for 3 min at 30 °C. The absorbance of the extracts was read at 485 nm. Formazan production was shown to be proportional to the number of viable cells when samples containing different proportions of living and heat-killed cells from the same bean culture were tested.

Electrolyte leakage assays

Electrolyte leakage from isolated bean leaf mesophyll cells (prepared by the method of Callow & Dow [9]) and from primary leaf slices [32] was determined by measurement of the conductivity of the external medium in control samples and after addition of elicitor preparations (100 µg glucose equivalents ml^{-1}).

RESULTS

Elicitor preparation and composition

The final yield of crude culture filtrate elicitor was typically 0·19 g l^{-1} of 6-day fermenter-grown *Colletotrichum* culture, the ratio of carbohydrate to protein in this preparation being 43:1. The yield of insoluble material (precipitating out during dialysis prior to lyophilisation) varied between preparations, but its carbohydrate and protein contents together accounted for less than 1% of its weight. About 2 mg (glucose equivalents) of cell wall elicitor was recovered per gram of *Colletotrichum* walls, no protein being detected in this preparation.

Both elicitor preparations were shown to contain galactose, glucose, mannose, rhamnose and trace amounts of a pentose (probably ribose) after acid hydrolysis (Table 1). The composition of the two elicitor preparations was similar with respect to all sugars except glucose, which was approximately four times higher in the cell

TABLE 1

Monosaccharide composition (%)[a] of crude elicitor preparations from Colletrochum lindemuthianum

| | Monosaccharide | | | | |
	Galactose	Glucose	Mannose	Ribose	Rhamnose
Cell wall elicitor	41·4	16·4	31·9	Trace	10·8
Culture filtrate elicitor	50·5	4·8	36·0	Trace	9·0
$R_L{}^b$	2·45	3·25	3·70	4·63	4·89

[a]Percentage composition based on total sugars.
[b]TLC mobility relative to the internal standard lactose.

wall elicitor. Culture filtrate elicitor preparations isolated on several occasions always possessed higher galactose:glucose ratios than cell wall elicitor. Galactose was also the major monosaccharide in hydrolysed crude culture filtrate elicitor preparations from the β-race of *C. lindemuthianum* [2], and in preparations released from *C. lindemuthianum* cell walls by short, mild hydrolysis treatments (G. Yousif, personal communication). This suggests that the galactose-rich components in the culture filtrate elicitor preparation may be surface carbohydrates released from fungal walls during hyphal growth.

Elicitor preparations from both cell walls and culture filtrates of *Colletotrichum*-induced increased extractable activities of PAL (Fig. 1) and CHS (Fig. 1) in bean cell suspension cultures. Enzyme induction by both preparations was unaffected by autoclaving for 15 min or treatment with proteinase K, and no loss of induction was observed following treatment of elicitors with DNase or RNase at concentrations which themselves gave no elicitation response (results not shown). However, periodate oxidation destroyed enzyme induction by both elicitor preparations (Fig. 1). These results suggest that elicitor activity in preparations from both cell walls and culture filtrate requires a carbohydrate moiety, and possible minor contaminants in the elicitor preparations, which may include fungal enzymes [19] and nucleic acid material, are inactive.

Effects of elicitors on apparent cell viability
The profiles for apparent cell viability following treatment with different concentrations of cell wall and culture filtrate elicitors were similar after exposure of cells to elicitor for 6 or 18 h (Table 2). A general trend of decreased TTC reduction with increasing elicitor concentration was observed, although these effects were not large. As apparent cell viability at any single elicitor concentration was no less at 18 h than at 6 h, and as cell wall elicitor had been previously shown to have little effect on bean cell growth over these time periods [7], it is possible that the elicitor preparations simply cause an impairment of the availability of electrons for TTC reduction, rather than any effects on cell viability *per se*. Serious interference with plasma membrane integrity does not appear to be occurring in these experiments, as no electrolyte leakage above control levels was observed following treatment of isolated leaf mesophyll cells or leaf slices with
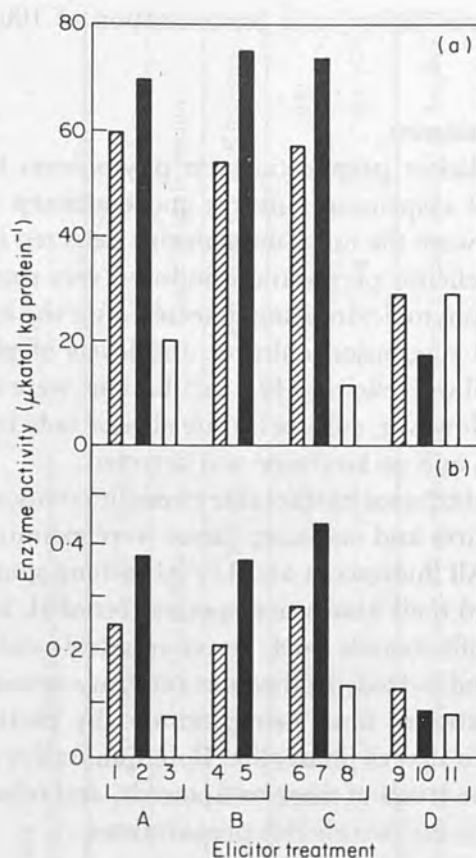
FIG. 1. Effects of *Colletotrichum* elicitor preparations on the induction of (a) phenylalanine ammonia-lyase and (b) chalcone synthase extractable activities in bean cell suspension cultures. Cells (10 ml batches) were treated with water (controls, [3, 8, 11]) cell wall elicitor (2, 5, 7, 10) or culture filtrate elicitor (1, 4, 6, 9). Elicitor (and water) additions were either untreated (A), autoclaved for 15 min (B) treated with proteinase K (C) or treated with periodate (D), as described in Materials and Methods. Elicitors were added to cells at a final concentration of 25 µg glucose equivalents ml^{-1}. Enzyme activities were determined 6 h after exposure of cells to elicitor.

TABLE 2

Effects of Colletotrichum *elicitor preparations on the ability of bean cell suspension cultures to reduce 2,3,5-triphenyltetrazolium chloride*

Elicitor concentration (µg glucose equivalents ml^{-1})	Apparent cell viability (Absorbance at 485 nm)[a]			
	6 h		18 h	
	Culture filtrate elicitor	Cell wall elicitor	Culture filtrate elicitor	Cell wall elicitor
0	2·05±0·15	2·05±0·15	2·21±0·25	2·21±0·25
10	1·95±0·05	1·92±0·07	1·80±0·05	1·75±0·12
25	1·62±0·09	1·73±0·13	1·56±0·06	1·75±0·05
50	1·63±0·02	1·57±0·15	1·60±0·15	1·51±0·14
100	1·59±0·05	1·56±0·04	1·50±0·09	1·53±0·13

[a]Viability was assessed by measurement of the absorption of the red formazan extracted from bean cell culture batches (0·5 g) after 24 h incubation with 2,3,5-triphenyltetrazolium chloride. Cell cultures had previously been incubated with different concentrations of elicitors for 6 h or 18 h. Results are the average and spread of values of two separate determinations.

cell wall or culture filtrate elicitor at a concentration of $100\,\mu g$ glucose equivalents ml^{-1} (results not shown).

Differential induction of phytoalexins

The effects of the two elicitor preparations on phytoalexin induction in bean were measured using both cell suspension cultures and endocarp tissue. In both sources, phaseollin and kievitone were the only phytoalexins detected in significant quantities. In endocarp tissue, both elicitor preparations induced very similar amounts of the two phytoalexins, with more phytoalexin being detected after the longer incubation period (96 h) (Table 3). In cell suspension cultures, the levels of phaseollin and kievitone induced in response to cell wall elicitor (48 h incubation) were similar to those found in endocarp tissue at 96 h. However, culture filtrate elicitor only induced trace amounts of phaseollin in the cultures, and no kievitone was detected.

The overall patterns of ethanol-extractable phenolic compounds induced by the two elicitors in both cell cultures and endocarp tissue were examined by two-dimensional TLC (data not shown). All fluorescent and UV-absorbing spots from thin-layer plates were eluted in ethanol and their absorption spectra recorded. In addition to phaseollin and kievitone, other isoflavonoids such as coumestrol and a number of related coumestans, 5-hydroxy and 5-deoxy isoflavones and trace amounts of phaseollidin were identified in endocarp extracts, most being induced by elicitor treatment, although always to lower levels than that of phaseollin. Both qualitative and quantitative differences were observed in the levels of these compounds, and other unidentified phenolic components, in response to the two elicitor preparations.

Induction of enzymes of the phytoalexin (phenylpropanoid) pathway

The dose response profiles for the induction of PAL, CHS and CHI by the elicitor preparations derived from cell walls or culture filtrate showed close similarity (Fig. 2), although the culture filtrate elicitor induced less enzyme increase than the cell wall elicitor at nearly all concentrations tested. Generally, increasing elicitor concentrations resulted in increased induction of the extractable activities of PAL and CHI over the range $0-100\,\mu g$ glucose equivalents ml^{-1}. In contrast, CHS activity was induced maximally at two concentration ranges of around 25 and $\geqslant 180\,\mu g$ glucose equivalents ml^{-1} in response to either elicitor preparation. Multiphasic dose response curves for enzyme induction in cell wall elicitor-treated cell suspension cultures has been reported previously [16].

Changes in the levels of soluble free and esterified phenolic acids

Changes in the levels of esterified and free cinnamic acid derivatives have been previously reported in bean cell cultures in response to an elicitor preparation from *C. lindemuthianum* cell walls [7], and similar changes were noted in the present work (Table 4). However, significant differences were observed between the effects of the two elicitors on the levels of esterified phenolic acid derivatives. The cell wall elicitor caused an increase in the levels of bound caffeate (as previously observed [7]), with little or no change in bound cinnamate or ferulate levels. On the other hand, culture filtrate elicitor caused a decrease in the levels of all four esterified compounds measured. Although both elicitors had little or no effect on the levels of free caffeic and 4-coumaric acids,
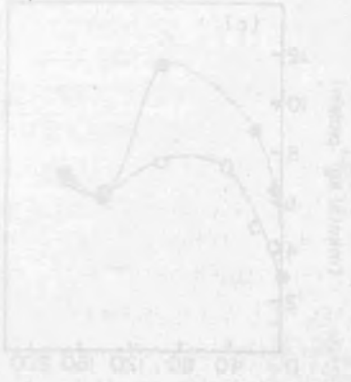
TABLE 3

Levels of phytoalexins in bean tissues treated with Colletotrichum elicitor preparations

Plant tissue	Incubation period (h)	Phytoalexins (nmol g^{-1} fresh wt)[a]					
		Water (control)		Cell wall elicitor		Culture filtrate elicitor	
		Phaseollin	Kievitone	Phaseollin	Kievitone	Phaseollin	Kievitone
Cell suspension culture	48	ND[b]	ND	283·9	14·9	4·2	ND
Endocarp	48	14·8	ND	74·2	4·2	64·8	4·2
Endocarp	96	28·4	ND	219·0	12·2	216·8	9·3

[a] Cell suspension cultures were treated with elicitors at 100 μg glucose equivalents ml^{-1} culture, or water (controls). Endocarp was treated with 50 μl droplets of elicitor (100 μg glucose equivalents ml^{-1}) or water per seed cavity.
[b] ND, not detected.
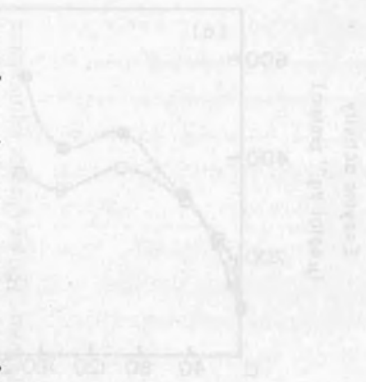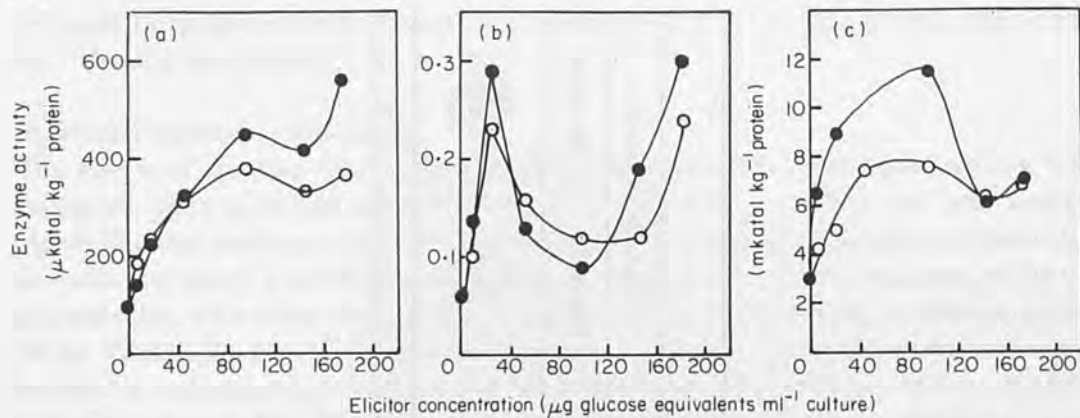
Fig. 2. Dose response curves for the induction of (a) phenylalanine ammonia-lyase, (b) chalcone synthase and (c) chalcone isomerase in bean cell suspension cultures treated with varying concentrations of *Colletotrichum* cell wall elicitor (●) and culture filtrate elicitor (○). Cells were harvested at 6 h [(a) and (b)] or 18 h (c) after addition of elicitor.

culture filtrate elicitor, but not cell wall elicitor, caused a marked increase in free cinnamate and ferulate pools.

Changes in wall-bound phenolic and hydroxyproline levels
Further differences were observed between the elicitor preparations in relation to their effects on induction of wall-bound lignin-like phenolic material in the bean cell cultures. Lignin analyses were based on measurement of the ultraviolet absorption difference spectra of phenolic material solubilized by alkaline hydrolysis of bean cell walls. The difference spectra of extracts from control and treated cells exhibited a maximum at 245 nm with a shoulder at 300 nm, both arising from the ionization of non-conjugated phenols. Only cell wall elicitor-treated cell extracts showed an additional maximum at 340 nm, caused by ionizable groups of phenols with conjugated side chains such as the hydroxycinnamic acids. Both the absolute and relative heights of the spectral peaks were different in extracts from cell wall elicitor-treated and culture filtrate elicitor-treated cells (Table 5). The walls of bean cells treated with cell wall elicitor differed from those treated with culture filtrate elicitor not only in having a greater amount of bound, simple phenolic groups, but also by the number of conjugated side chains as indicated by the major increase in the 340 nm peak.

As previously demonstrated [7], increased levels of hydroxyproline residues were observed in the cell walls of cultured bean cells exposed to *Colletotrichum* wall-released elicitor. However, although prolyl hydroxylase activity was strikingly induced in cultures treated with both elicitor preparations, no corresponding increase in wall-bound hydroxyproline was observed in response to culture filtrate elicitor (Table 6).

DISCUSSION

Although a number of different single parameters have been used as the basis for elicitor bioassay (e.g. phytoalexin induction [11, 16, 39], lignification [29], electrolyte leakage [18], little attention has been paid to the possibility that more than one type of elicitor may be involved in switching on the sum total of responses observed in any

TABLE 4

Changes in the levels of free and esterified cinnamic and hydroxycinnamic acids in elicitor-treated bean cell suspension cultures

| | Phenolic acid concentration (nmol g^{-1} fresh wt)[a] | | | | | | | | | | | |
| | Cinnamic acid | | | 4-Coumaric acid | | | Caffeic acid | | | Ferulic acid | | |
Treatment	Free	Esterified	Total	Free	Esterified	Total	Free	Esterified	Total	Free	Esterified	Total
Water (control)	2·6±1·6	18·0±2·0	20·6	28·8±1·1	41·5±1·6	70·3	16·9±0·2	26·4±0·3	43·3	2·3±0·7	18·1±0·9	20·4
Cell wall elicitor	3·3±2·1	17·4±2·5	20·7	23·9±0·9	49·3±1·1	73·2	15·8±0·2	36·5±0·5	52·3	2·3±0·6	21·6±0·7	23·9
Culture filtrate elicitor	7·6±1·4	5·1±1·6	12·7	26·4±1·0	28·5±0·7	54·9	17·4±0·2	21·8±0·2	39·2	7·6±1·4	12·4±0·7	19·8

[a]Phenolic acid levels were determined by HPLC analysis of extracts from cells exposed to water (controls), or elicitor preparations (100 µg glucose equivalents ml^{-1}) for 6 h. Results are the average and spread of values from two separate determinations.

TABLE 5
Effects of elicitor preparations on levels of cell wall-bound phenolics in bean suspension cultures

Treatment[a]	Lignin difference spectrum[b]		Difference spectrum peak ratio (254:340 nm)
	λ_{254}	λ_{340}	
Water (control)	0·456	0·142	3·20
Cell wall elicitor	1·224	0·968	1·26
Culture filtrate elicitor	0·574	0·164	3·50

[a]All treatments were for 6 h. Cells were exposed to elicitor at a final concentration of 100 µg glucose equivalents ml^{-1}.

[b]One aliquot of an alkaline extract from a crude cell wall preparation was adjusted to pH 7·0, another to pH 12·0; the values represent the difference in absorbance (ΔE pH 12–pH 7) at 254 nm and 340 nm per 10 mg dry wt of cells extracted.

TABLE 6
Induction of prolyl hydroxylase and wall-bound hydroxyproline in elicitor-treated bean cell suspension cultures

Treatment[a]	Prolyl hydroxylase activity (mkatal kg protein^{-1})	Wall-bound hydroxyproline ($\mu g\ g^{-1}$ fresh wt)
Water (control)	0·0	191
Cell wall elicitor	7·9	297
Culture filtrate elicitor	11·3	155

[a]Cells were treated with water or elicitor (100 µg glucose equivalents ml^{-1} culture) for 6 h.

particular interaction. In terms of the host response, an absolute requirement for more than one elicitor would suggest the possibility that, experimentally, induction of some metabolic changes related to active defence could be de-coupled from the induction of other changes. Such an experimental system could be of great value for the elucidation of the mechanisms which operate in the integration and co-ordination of induced defence responses.

Although the present work demonstrates several similarities between the biochemical responses of bean cells to elicitors from cell walls and culture filtrate of *C. lindemuthianum*, the differences observed are sufficient to suggest that the integration of induced host metabolism may require more than one initial stimulus. As all comparative experiments with cell cultures were performed on cells from the same batch culture, the differences in biological activity observed most likely reflect differences in components in the elicitor preparations. Furthermore, the cell wall elicitor used in all experiments was prepared from mycelium whose culture filtrate had been fractionated to yield the culture filtrate elicitor, and the same elicitor preparations were used throughout the work. As the carbohydrate material in the culture filtrate is believed to originate from mycelial walls, the similar monosaccharide composition of the two preparations is perhaps not unexpected. Previous preparations of *Colletotrichum* cell wall elicitor from batches of the same isolate contained significantly more glucose and pentose sugars that observed here [16]. It is, however, known that factors such as

culture medium or relative culture age can significantly alter the carbohydrate composition of elicitor fractions [40]. Earlier work on the effects of fractions from *Colletotrichum* cell wall elicitor on PAL induction in bean cell cultures [16] and phytoalexin induction in hypocotyls and cotyledons [38] was consistent with (a) there being more than one component with biological activity and (b) the possibility that individual components may affect the activity of other components.

Apart from the observation of increased levels of free cinnamate and ferulate in bean cells treated with culture filtrate elicitor as compared to cell wall elicitor, the main differences between the effects of the two elicitor preparations were the absence of the end products of pathways associated with expression of resistance (wall-bound phenols, hydroxyproline and phytoalexins) in cells treated with culture filtrate elicitor. It is therefore possible that the culture filtrate elicitor preparation may lack a component which induced a late event in the accumulation of these compounds in cell cultures, and that such a component could be absent either because it is not released from fungal mycelium or mycelial walls during growth in liquid culture or because it was lost during the precipitation stages in the preparation of the elicitor. It has been proposed that limited cell death may be a prerequisite for phytoalexin accumulation (as opposed to synthesis) in infected bean hypocotyls [4], although in the present work there was little difference in the effects of the two elicitor preparations (at a concentration of $100 \, \mu g$ glucose equivalents ml^{-1}) on the apparent viability of cultured cells between 18 h and 32 h of induction, the period during which maximum rates of phaseollin appearance are observed in cells treated with cell wall elicitor [30]. It is therefore unlikely that the lack of appearance of phytoalexins in culture filtrate elicitor-treated cells simply reflects the lack of a suitable site for their accumulation.

Accumulation of wall-bound phenolics and hydroxyproline may require induction of a wall-associated peroxidase activity for oxidative cross-linking to the wall [22], although arabinosylation of hydroxyproline-rich precursor protein may also be necessary [6, 7]. Total soluble peroxidase activity is not induced in the bean cultures by *Colletotrichum* cell wall elicitor [30]; a wall-bound activity has yet to be investigated in these cells. Lack of induction of one or a limited number of enzymes, for example isoperoxidases or specific later enzymes of isoflavonoid synthesis, may therefore explain what at first appear to be major differences between the effects of the two elicitor preparations.

The inability of the culture filtrate elicitor to induce accumulation of phytoalexins, wall-bound phenolics or wall-bound hydroxyproline in cell cultures, while at the same time eliciting increased activities of the enzymes involved in the early stages in the biosynthesis of these products, indicates that the induction of the early and late stages in these biosynthetic pathways is not co-ordinate. Recent evidence has indicated that, under certain circumstances, the induction of the early enzymes of isoflavonoid phytoalexin biosynthesis (PAL, CHS, CHI) can be co-ordinate with respect to the initial appearance of enzyme synthetic rate and mRNA activity [13]. However, the observation of co-ordinate changes within a closely related group of enzymes does not prove that induced gene expression is of necessity linked mechanistically; for example, the response of cultured bean cells to affinity-purified fractions from *Colletotrichum* culture filtrate elicitor indicates that CHS can be induced in the absence of PAL induction (unpublished results). Thus, even if the presence of elicitor fractions with different

biological activities eventually proves to have no physiological significance with respect to the intact plant–parasite interaction (e.g. if such fractions are artifacts arising from the use of cultured material or the method of elicitor preparation), their use could nevertheless be of great value in elucidating basic biochemical control mechanisms which regulate the co-ordination of host gene expression.

It is important to note that the differential effects of *Colletotrichum* elicitor preparations on phytoalexin accumulation were observed in cell suspension cultures but not in endocarp tissue. The reasons for this are not clear at present. In addition to underlining the difficulty in extrapolating results from cultured cells to the intact plant, this result also suggests that caution should be exercised in the use of cell cultures for the bioassay of potential elicitor molecules. The assessment of a possible significance for a number of differentially acting elicitor molecules in *in vivo* plant parasite interactions will in part depend upon the development of suitable techniques for (a) their isolation, characterization and detection *in situ* (e.g. immunocytochemical methods utilizing monoclonal antibodies) and (b) the identification of fungal avirulence genes (e.g. by the transformation of virulent races with cloned DNA fragments from genomic libraries of avirulent races (cf. Staskawicz *et al.* [*34*]), and the demonstration of the biological activities of the corresponding gene products.

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