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The Elicitation of Phytoalexin Accumulation in *Phaseolus vulgaris* by *Rhizoctonia* and *Fusarium*

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LIST OF PLATES

Summary of Thesis submitted for Ph.D. degree

by Andrew James Morris

on

The Elicitation of Phytoalexin Accumulation in

Phaseolus vulgaris by *Rhizoctonia* and *Fusarium*

The phytoalexins kievitone and phaseollin were quantified in hypocotyls of *Phaseolus vulgaris* infected by either *Rhizoctonia solani* or *Fusarium solani* f.sp. *phaseoli*. Infection of intact seedlings produced a pattern of phytoalexin accumulation consistent with earlier reports. In response to *R. solani*, kievitone accumulated rapidly and may have been instrumental in the formation of restricted lesions; little kievitone could be detected in hypocotyls bearing spreading lesions produced by *F. solani* f.sp. *phaseoli*.

These results prompted the development of an artificial inoculation system employing excised, cored hypocotyls to investigate the possibility that the observed disparity in kievitone levels might reflect differential production, by the two fungi, of (an) elicitor(s) of kievitone accumulation. While this system did not exactly reproduce the response of intact plants upon inoculation with mycelium, it did provide a sensitive means for the evaluation of kievitone-eliciting activity in culture filtrates and mycelial fractions.

Material capable of eliciting high levels of kievitone in the artificial system was isolated from cell-free mycelial extracts of *R. solani*; the same procedures failed to demonstrate similar activity in *F. solani* f.sp. *phaseoli*. Kievitone-eliciting activity was also solubilized from cell walls of *R. solani*. Since the active fractions were only partially-purified, a thorough appraisal of the structure and activity of the elicitor(s) was not achieved.

An elicitor preparation from *R. solani*, introduced into bean

hypocotyls prior to inoculation with F. solani f.sp. phaseoli, appeared to delay colonization by this aggressive fungus. This finding raises the possibility of cross-protecting plants through the action of phytoalexins.

Hypotheses to explain the observed differences in the resistance of bean hypocotyls to the two fungi are outlined in the context of current ideas on the elicitation of phytoalexin accumulation and disease resistance.

CONTENTS

SECTION I

INTRODUCTION

Fungi and bacteria are believed to have existed long before higher plants first colonized the land some 400 million years ago. In the likely event, therefore, that plants were subject to continual attack by potentially-pathogenic microorganisms, they must have developed effective means of defence. One can imagine the slow evolution of balanced, yet dynamic, equilibria of plant-parasite interactions. Through the introduction of agriculture about 10,000 years ago, man would undoubtedly have brought about profound changes in the nature of such equilibria.

According to Wilkes (1977), from pre-agriculture to the present day the world's human population has increased from an estimated 5 million (c. 25 km^2/person) to well over 4,000 million (c. 25 persons/km^2). During that period civilisation has selected only a small number of plants upon which its food production is now based. From an estimated 5,000 worldwide, the total number of food plants utilized has dwindled to less than 150; of these, only about 15 account for the basic caloric requirements. Furthermore, many of these plants have become so genetically altered that their survival has become dependent on cultivation. Meanwhile, the human population, following increases in the carrying capacity of the earth made possible by the development of agriculture, many years ago reached a level far in excess of that which could be supported by wild vegetation. Therefore our survival is dependent upon the high yields of cultivated plants.

Faced with an increasing world population, it is not only important to raise the yields of crop plants *per se*, but also to minimize unaffordable losses to plant pathogens. The present-day use of extensive monocultures, widespread cultivation of new varieties, and the introduction of plants (and pathogens) into areas where they did not naturally occur all aid the possibility of serious plant disease outbreaks. Such occurrences are not unknown; the Great Famine in Ireland during the 1840's, where almost the whole potato crop of successive years was lost to late-blight, is a classic example.

To preserve and protect our basic food supplies by helping to define resistance factors for plant breeders to introduce into new crop varieties, and in providing insights into possible disease control measures, the study of plant disease resistance is essential.

Mechanisms of plant disease resistance:

It is not the author's intention to provide a complete appraisal of plant disease resistance mechanisms, since the vast amount of information available on the subject has been adequately reviewed elsewhere (Wood, 1972; Ingham, 1972; 1973; Friend & Threlfall, 1976; Heitefuss & Williams, 1976; Deverall, 1977; Harborne & Ingham, 1978). Rather, the aim is to present an overall, if superficial, view of presumed defence mechanisms in plants, thereby providing a perspective for any role claimed for phytoalexin involvement.

Prior to penetration, the morphology and anatomy of a leaf, for example, may determine whether a fungal spore can adhere, germinate and develop to a stage where it is capable of ingress. Thus, constitutive structural characteristics, such as surface texture, the degree of waxiness, or the number and size of hairs, may be effective in disease resistance, even though perhaps not specifically designed for that purpose (Preece, 1976; Royle, 1976; Harborne & Ingham, 1978).

Antagonism towards microorganisms at this stage may include the presence of toxic substances in the cuticle or surface waxes (Martin & Juniper, 1970; Blakeman & Atkinson, 1976). Also, inhibitory agents may be present for other reasons; the presence of other microorganisms (Blakeman & Brodie, 1976; Fokkema, 1976; Skidmore, 1976) or the leaching of toxic components from the host (Blakeman, 1971; Godfrey, 1976) may be important.

In contrast, leakage from the plant of nutrients and other compounds responsible for the stimulation of germination and chemotactic attraction of microorganisms, particularly in the rhizosphere, must be considered (Blakeman, 1971; Allen, 1976; Coley-Smith, 1976). Such phenomena may have considerable effects in the determination of pathogenicity. Cruickshank (1978) has speculated that, when utilised as substrates for spore germination and growth of potential pathogens, host nutrients in an infection-droplet may play an important part in the qualitative and quantitative formation of fungal components subsequently eliciting a host response.

Resistance to invasion by microbes which must enter through natural openings, such as stomata or lenticels, may be determined by the frequency and size or physiological state of stomata, and by the presence or absence of metabolites leaking from them. On the other hand, where direct penetration is involved, thickness of cuticle may be important (Royle, 1976; Deverall, 1977). However, whether ingress occurs by direct means or via natural openings, at some point the cell wall is generally breached. It has been suggested that resistance may be effected at this level through suppression or lack of induction, by wall-released sugars, of the synthesis of microbial enzymes critical to wall dissolution (Albersheim, Jones & English, 1969). Also, the cell wall may contain components capable of inhibiting the pathogen's degradative enzymes (Albersheim & Anderson, 1971; Fisher, Anderson & Albersheim, 1973). In addition, the host may

deposit material, probably containing callose but in any case resistant to degradation by fungal enzymes, beneath the intended penetration point (Aist, 1976; Ingram, Sarjent & Tommerup, 1976; Ride, 1978).

Following ingress, colonization of the host may be hindered by tissues resistant to invasion due to their lignified or suberized nature; the ratio of sclerenchyma to collenchyma, or the presence of a well-suberized endodermis may be significant (Royle, 1976). Structural barriers of this type may also arise in response to infection, as in the formation of bands of cork cells from a new phellogen. It is thought, however, that induced alterations in cell wall chemistry, such as lignification and the deposition of calcium, silicon or callose, may be more effective through protection of host substrates from microbial enzymes, restriction of transfer of materials between host and parasite or inactivation of microbial enzymes, rather than through the mere presence of a mechanical barrier (Friend, 1976; Ride, 1978).

In the creation of an unfavourable environment in the host tissues immediately surrounding the pathogen following penetration, a role for nutrient availability may exist; hypersensitive cell death in response to infection by biotrophs may represent an extreme form of this type of interaction, effectively removing potential nutrients (Ingram, 1978). The invading microorganism may also encounter a variety of toxic chemicals produced by the host. These can be present upon infection, or synthesized around penetration points in response to infection.

Pre-formed compounds, whether present in active forms or requiring simple enzymic release from inactive "bound" forms, are considered to be involved in delimitation of fungal invasion in several hosts. Phenolics such as protocatechuic acid in onions and phloretin in apples, saponins such as avenacin in oats and cyclamin in Cyclamen spp., and sulphur-containing compounds such as allicin in garlic may all contribute to restricting spread of the pathogen (Schlösser, 1971; Overeem, 1976; Harborne & Ingham, 1978).

Finally, antimicrobial compounds which may not be detectable in healthy tissues but which accumulate in response to infection - phytoalexins - must be considered.

The phytoalexin concept:

Although they were by no means original in their recognition of post-infectional resistance in plants, Müller & Börger (1940) were the first to coin the term phytoalexin (Greek: phyton "a plant" + alexein "to ward off") when they described the then hypothetical factor(s) considered responsible for the inability of a normally-pathogenic race of Phytophthora infestans to infect areas of cut surfaces of potato (Solanum tuberosum) tubers which had previously been inoculated with a non-pathogenic race.

Müller & Börger outlined a number of criteria describing the biological characteristics of these presumed resistance factors. The majority of these criteria (see Cruickshank, 1963) are fulfilled by all of the phytoalexins so far identified, although some modification of the original concept has occurred. Findings necessitating such modification include the existence of abiotic as well as biotic stimulation of phytoalexin accumulation, and the possibility of enhanced as opposed to de novo synthesis. This re-appraisal of the original definition has resulted in the appearance in the literature of a number of alternatives. Therefore it seems appropriate to give a succinct definition which the author believes may best describe a phytoalexin:

" a low molecular weight antimicrobial chemical which undergoes de novo or enhanced synthesis by a plant in partial response to an irritative agent acting upon that plant".

Phytoalexins and disease resistance:

Since the inception of the "Phytoalexin Theory", phytoalexins have been characterized in a great many plants. A number of recent reviews

(Deverall, 1972a; 1972b; 1977; Ingham, 1972; 1973; Kuć, 1972; VanEtten & Pueppke, 1976; Harborne & Ingham, 1978; I.M. Smith, 1978) render a historical resumé of the subject here superfluous. Suffice it to say that phytoalexins are secondary plant metabolites of low molecular weight (mol wt) (generally 200-500) which exhibit great diversity in their chemical structure and which have so far been identified in fourteen plant families (Harborne & Ingham, 1978), although they appear to be most prevalent in the Leguminosae and the Solanaceae. While quite high levels of phytoalexins may be present in some successfully-colonized host tissues, their presence is commonly associated with the expression of a plant's resistance to infection. Thus there is an inferred role for phytoalexins in disease resistance. However, in spite of many investigations into the production of phytoalexins in plants challenged by microorganisms, there is little incontrovertible evidence that they are solely responsible for resistance. Indeed, it has been proposed that phytoalexin accumulation may only occur as a result of resistance (Király, Barna & Ersék, 1972).

Phytoalexins, by definition, exhibit antimicrobial activity; both fungi and bacteria are vulnerable (Bailey & Skipp, 1978; Wyman & VanEtten, 1978). Nonetheless, before any contributions to resistance can be fairly assigned, it seems relevant to consider whether phytoalexins are present in sufficient quantities and in forms accessible to the microorganism at the time resistance is expressed. Generally, these points may be answered in the affirmative.

It is, of course, difficult to obtain very accurate estimates of phytoalexin levels at infection sites since some healthy tissue is invariably extracted along with reacting cells; there are probably dilutions of the localized concentrations. However, the figures obtained often exceed the *in vitro* ED_{50} of the microorganism. Thus it may be assumed that the cellular concentrations at the infection site are greater, and that they may easily be sufficient to cause inhibition of

pathogen development (Bailey & Deverall, 1971). This supposition is borne out by the results of experiments where infected tissue only was excised and analyzed for phytoalexin content in comparison with extracts of adjacent, apparently healthy material or of whole tissues (Rahe, 1973; Smith, VanEtten & Bateman, 1975; Yoshikawa, Yamauchi & Masago, 1978a).

Although some phytoalexins exhibit low water-solubility, many will diffuse in large quantity into challenge droplets on plant surfaces, thus demonstrating an ability to be present at high concentration in the likely environment of pathogens. Further, some fungi are able to metabolize phytoalexins *in vitro* and in some cases the characteristic derivative is also found *in situ*, again demonstrating accessibility to pathogens, or at least pathogen enzymes (Stoessl, Unwin & Ward, 1973; VanEtten & Smith, 1975; Kuhn & Smith, 1978).

Finally, there is often a close temporal association between the accumulation of high levels of phytoalexin and the cessation of pathogen development. A more precise description of this relationship is not easy due to the difficulty experienced in the meaningful determination of both these parameters.

By way of more specific elaboration, two instances where phytoalexin accumulation seems to meet the criteria necessary to allow a role in disease resistance are the *Phytophthora* rot of soybean (*Glycine max*) caused by *P. megasperma* var. *sojae*, and the anthracnose disease of French bean (*Phaseolus vulgaris*) caused by *Colletotrichum lindemuthianum*. These interactions provide examples of two broad classes into which this type of investigation may be divided, namely where different host varieties have been inoculated with the same fungal isolate, or where different varieties of a fungus have been employed on a single host cultivar. The distinction between these two types of investigation may be important when considering any mechanistic explanation of the results (Section VI).

Yoshikawa *et al.* (1978a) compared the accumulation of glyceollin (formerly hydroxphaseollin) (Burden & Bailey, 1975; Partridge & Keen,

1977) in cultivars of soybean susceptible (Harosoy) or resistant (Harosoy 63) to race 1 of P. megasperma var. sojae. Inoculation was achieved by the introduction of mycelium into wounds made just below the hypocotyl surface. Thereafter, glyceollin accumulation and fungal development were monitored in thin tissue sections cut sequentially beneath each inoculation site. Fungal growth was comparable in both cultivars up to c. 8 h after inoculation. From this time onward, however, the advance of hyphae in tissues of Harosoy 63 was retarded while the fungus continued to proliferate in the susceptible host. Analysis of the sections showed that the accumulation of glyceollin in the resistant tissues was well correlated with cessation of fungal development; although some glyceollin accumulated in comparable susceptible tissues, the levels were lower. Further, treatment of Harosoy 63 with Blasticidin S suppressed glyceollin accumulation and rendered the tissues susceptible to the fungus. Keen (1971) had obtained similar results when he used heat treatment to suppress the resistant response of Harosoy 63. Bridge & Klarman (1973) obtained the reverse effect when they subjected the susceptible Harosoy to ultra-violet (UV) irradiation; this treatment caused an increase in resistance together with enhanced glyceollin production. Together, these results suggest that glyceollin is critical for resistance to the disease.

Several reports have been published in which a study has been made of the interaction of one bean cultivar and races of Colletotrichum lindemuthianum exhibiting differential pathogenicity on that variety. The cultivar "Kievitsboon koekoek" is resistant to race β but susceptible to race γ of the fungus (Bailey & Deverall, 1971; Bailey, 1974), and there is a similar distinction between the interaction of race γ (resistant) and race β (susceptible) on cv. "Topcrop" (Rahe, 1973).

Following inoculation with spore suspensions, in the incompatible interaction hypersensitive cell death occurred soon after penetration from appressoria on the hypocotyl surface. This was followed shortly by the accumulation of high levels of phaseollin at the infection sites,

together with the cessation of fungal development. However, at this time, little or no phaseollin could be detected nor cell necrosis observed in tissues of a comparable compatible interaction. Here the fungus continued to invade the tissue, apparently without causing adverse effects. Some days later the developing lesions browned and became delimited, and phytoalexin accumulation was observed.

As with the Phytophthora-soybean interaction above, susceptible plants could be made to develop resistance by manipulation of the environmental conditions. For example, Rahe (1973) observed that in dark-grown hypocotyls the compatible fungus continued to spread as normal at some infection sites, but at others the lesions quickly browned and became delimited. Phytoalexins accumulated rapidly at delimited sites but not in lesions which were unrestricted. Bailey (1974) found that hypocotyls which were completely susceptible at 17°C became resistant at 25°C. Again, differential phytoalexin accumulation was observed; limited lesions contained levels thought to be sufficient to restrict hyphal growth whereas spreading lesions yielded much lower amounts. Thus phytoalexin accumulation was implicated in lesion restriction.

Since phytoalexins may accumulate during an interaction which nevertheless results in successful colonization by the pathogen, some explanation must be proffered for the lack of fungal restriction. As indicated by the work of Yoshikawa et al. (1978a), retarded phytoalexin accumulation in the susceptible interaction might allow sufficient fungal development to occur such that even though a high degree of toxicity may eventually be reached at the infection site, the advancing hyphae have by this time grown out into adjacent tissues and therefore may have succeeded in avoiding a fungitoxic environment. Alternatively, some pathogens may deplete the phytoalexin concentration in the tissues by degradation of these compounds to less toxic products. This has been proposed to operate in the successful colonization of, amongst others, broad bean by Botrytis

fabae (Hargreaves, Mansfield & Rossall, 1977), pepper by Fusarium oxysporum (Stoessl et al., 1973) and alfalfa by Stemphylium botryosum (Higgins, 1972). Further, a separate but possibly related property may be the differential sensitivities of fungal isolates to some phytoalexins (VanEtten & Stein, 1978). Finally, some fungi may somehow be able to avoid coming into contact with these compounds in situ (VanEtten & Pueppke, 1976).

In spite of these possibilities, all consistent with phytoalexins operating as disease resistance factors in appropriate circumstances, one major anomaly has so far defied explanation; this is the interaction between Aphanomyces euteiches and common pea, Pisum sativum. In like manner to the examples described above, Pueppke & VanEtten (1974; 1976) monitored the accumulation of pisatin and growth of the pathogen in inoculated pea epicotyls. They found that pisatin levels in young lesions (1.5 to 2 days after inoculation) were up to forty times that needed to completely inhibit mycelial growth in vitro, yet the lesions continued to expand rapidly. They could find no evidence for degradation of pisatin by the fungus, nor for spatial separation of the phytoalexin from hyphae. Although they were also unable to demonstrate differential sensitivity of the fungus in vitro and in situ, this may yet represent the best explanation of the anomaly. Since it may safely be assumed that factors which may be very important to in situ development are not reproduced in the laboratory, the results of in vitro bioassays may on occasion represent misleading estimates of fungitoxicity (VanEtten & Pueppke, 1976; Smith & Ingham, 1979). Nonetheless, the fact remains that there is no evidence to suggest that pisatin functions as a resistance factor in P. sativum infected with A. euteiches.

Elicitors of phytoalexin accumulation:

From the results of the many studies attempting to consolidate the Phytoalexin Theory, it has become apparent that phytoalexin accumulation may occur in response to many different stimuli. The variety of chemical and other abiotic stimuli is enormous, ranging from anti-malarial drugs, heavy metal salts and fungicides to freezing and UV irradiation (Kuć, 1972; VanEtten & Pueppke, 1976; Deverall, 1977; Cheema & Haard, 1978; Drysdale, 1978).

As suggested by Drysdale (1978), it seems preferable at this stage to avoid the word "induction" in describing processes leading to phytoalexin accumulation in plant tissues, since the term carries mechanistic implications for which there is as yet no evidence. "Elicitation" seems the best alternative, compounds capable of eliciting the response therefore being termed "elicitors" (Keen, Partridge & Zaki, 1972).

In pursuit of elicitors with more relevance to pathogenesis, the discovery that cell-free culture filtrates or mycelial sonicates of several fungi could stimulate phytoalexin accumulation in their respective hosts (Uehara, 1958; Cruickshank & Perrin, 1963a; Frank & Paxton, 1971; Rathmell & Bendall, 1971; Varns, Currier & Kuć, 1971a; Keen et al., 1972; Kim & Uritani, 1974) led to attempts to isolate and characterize the active components. However, there has, as yet, been success in only a limited number of cases.

The first elicitor to be identified was a metabolite from the mycelium of a stone fruit pathogen, Monilinia fructicola. The elicitor, called monilicolin A, was shown to be a polypeptide with a mol wt of c. 8,000, capable of causing phaseollin accumulation in French bean endocarp (Cruickshank & Perrin, 1968). Later, Anderson-Prouty & Albersheim (1975) were able to isolate elicitors from the culture filtrates and mycelial walls of Colletotrichum lindemuthianum. These were high mol wt polysaccharides, comprising mainly 3- and 4- linked glucosyl residues, capable

of eliciting phaseollin production and host tissue browning when applied in only small amounts to the cut surfaces of bean hypocotyls and cotyledons. Similarly, following the work of Keen (1975a), elicitors which appear to resemble fungal wall components have been isolated from culture filtrates and mycelial wall preparations of Phytophthora megasperma var. sojae. Here, the predominantly 3- and 3,6-linked glucans were of a more heterogeneous size distribution, but were still potent elicitors of glyceollin production in hypocotyls, cotyledons and cell suspension cultures of soybean (Ayers, Ebel, Finelli, Berger & Albersheim, 1976a; Ayers, Ebel, Valent & Albersheim, 1976b; Ayers, Valent, Ebel & Albersheim, 1976c; Ebel, Ayers & Albersheim, 1976).

Following the demonstration of elicitors in C. lindemuthianum (Anderson-Prouty & Albersheim, 1975), glucan-containing polysaccharides capable of eliciting browning and phytoalexin production in bean were isolated from a further two Colletotrichum species, C. trifolii and C. destructivum, both non-pathogens of bean (Anderson, 1978a). Despite the differential pathogenicities of these species on P. vulgaris, the elicitors - the most active of which were high mol wt fractions, predominantly glucan in nature - were of similar size, composition and activity (Anderson, 1978a). Anderson (1978b) was also able to mimic the symptoms of resistance in bean cotyledons using isolated hyphal walls and components released from the walls of Fusarium oxysporum f.sp. lycopersici and Fusarium cubense, two non-pathogens of bean; purified preparations comprised carbohydrate, free of protein and lipid. This carbohydrate nature of the components is in agreement with the observed elicitor activity of wall-released glucans from Colletotrichum spp., P. megasperma and yeasts (Anderson-Prouty & Albersheim, 1975; Ayers et al., 1976a, 1976b, 1976c; Anderson, 1978a; Hahn & Albersheim, 1978). Valent & Albersheim (1978) reported that oligosaccharide fragments of the wall glucan, obtained from partial acid hydrolysis and specific enzymolysis

of purified P. megasperma var. sojae mycelial walls, retained eliciting activity down to the level of a nonasaccharide. The presence of naturally-occurring fragments may therefore explain the elicitation of host defence reactions by low mol wt fractions observed in several studies of fungal elicitors (Kim & Uritani, 1974; Ayers et al., 1976a; Stekoll & West, 1978).

Currier (1974; see also Kuć, Currier & Shih, 1976) isolated a component from the mycelium of Phytophthora infestans capable of eliciting the production of rishitin in potato tubers. The suggestion was that the elicitor could be a saponin linked to the cell wall. Subsequently, a group of Russian workers obtained elicitors of rishitin accumulation from cytoplasmic and mycelial wall preparations of P. infestans; the eliciting component(s) appeared to be either glycoprotein or glucan, again bearing structural similarities with elements of the cell wall (Melitskii, Ozeretskovskaya, Yurganova, Savel'eva, Chalova & D'yakov, 1976; Chalova, Ozeretskovskaya, Yurganova, Baramidze, Protsenko, D'yakov & Melitskii, 1977).

Daniels & Hadwiger (1976) fractionated from the culture filtrates of Fusarium solani isolates components capable of causing pisatin accumulation in pea pod endocarp. Later, Stekoll & West (1978) isolated, from culture filtrates of Rhizopus stolonifer, a component which elicited production of the phytoalexin casbene in cell-free extracts of castor bean. In both these cases, at least part of the eliciting activity appeared to be associated with proteinaceous elements since pronase or heat treatment caused partial loss of activity. However, while purification of the pisatin elicitors was not sufficient to indicate further the nature of the active components, the most purified casbene elicitor contained both carbohydrate and protein; treatment with periodate caused complete loss of activity. This information, together with the results of gel filtration studies, indicated that the casbene elicitor was possibly a glycoprotein of

c. 30,000 mol wt, with both carbohydrate and protein elements functioning in phytoalexin elicitation.

Elicitors which may also be glycoproteins have recently been obtained from mycelial walls of P. infestans and P. megasperma var. sojae. Bostock, Henfling & Kuć (1978) successfully solubilized and purified elicitor fractions containing protein and carbohydrate from mycelial wall preparations of two races of P. infestans. Keen (1978a; 1978b) isolated glycoproteins from the cell walls of several races of P. megasperma var. sojae. Elicitor activity was heat stable and was not altered after incubation with pronase; periodate treatment, however, completely destroyed the activity, and it was reduced by α-mannosidase and α-glucosidase. Thus the glycosyl portion appears to be important for activity.

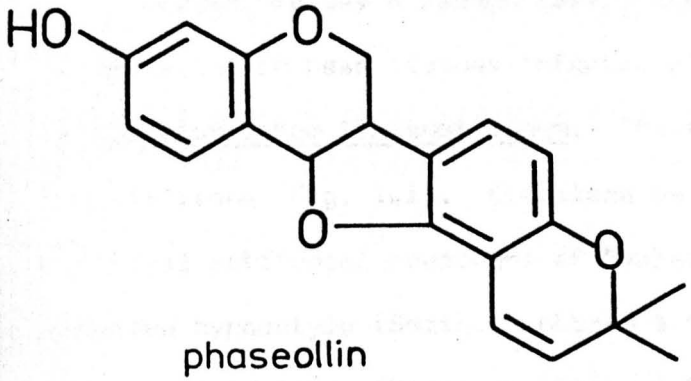
These findings are in agreement with the general observation of H. Smith (1978) that there appear to be two types of fungal elicitor, those comprising only glucosyl residues, and those which contain protein elements but which also require carbohydrate for activity. Whether the carbohydrate in these glycoprotein elicitors resembles (or indeed is the same as) the sugar residues of the glucan elicitors remains unresolved.

Phytoalexins of Phaseolus vulgaris L.:

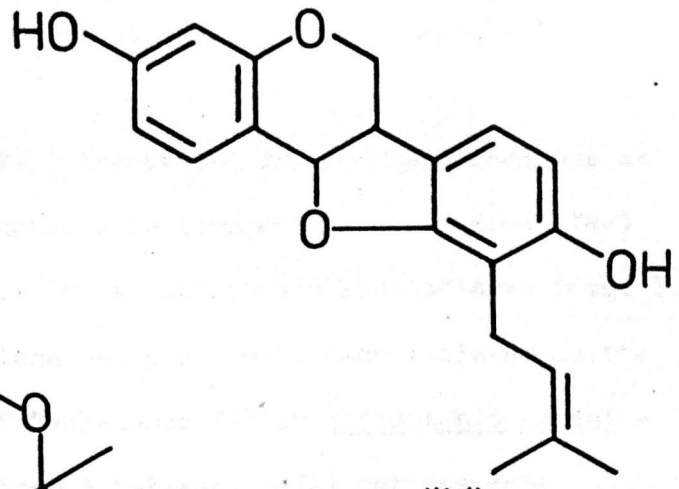
Phytoalexins may be used as taxonomic markers for many species, due to the general consistency of their structure within plant families (Harborne & Ingham, 1978). Apart from the furano-acetylenic compounds of broad bean and some stilbenes and benzofurans, all the phytoalexins so far characterized from legumes are isoflavonoids. These include isoflavones, isoflavanones, isoflavans, pterocarpans and coumestans (VanEtten & Pueppke, 1976; Harborne & Ingham, 1978).

Müller (1958) was undoubtedly the first to demonstrate a phytoalexin presence in P. vulgaris when he inoculated pods with conidial suspensions of M. fructicola or zoospores of P. infestans. Subsequently, the "inhibitory principle" was found to contain phaseollin (Fig. 1.1) (Cruickshank & Perrin,

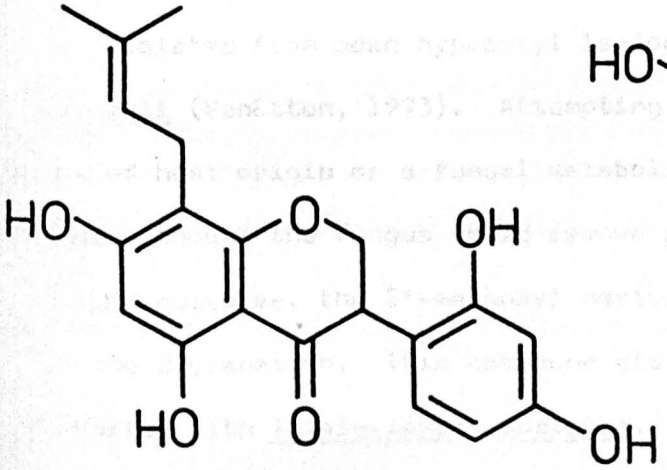
Fig. 1.1. Isoflavonoid phytoalexins of *Phaseolus vulgaris*.



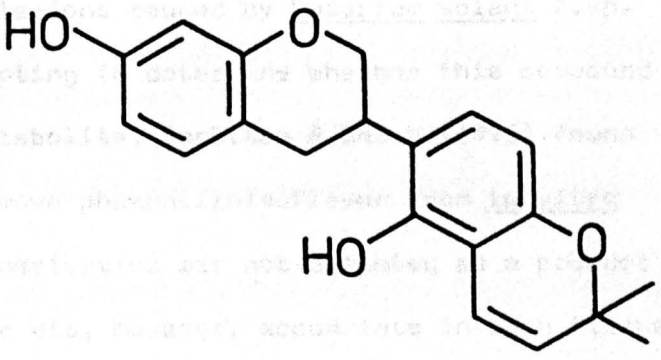
phaseollin



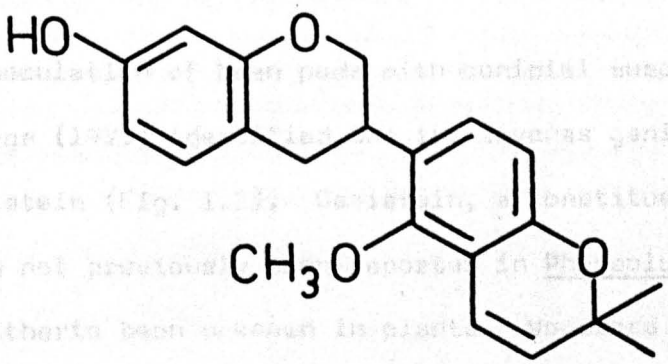
phaseollidin



kievitone



phaseollinisoflavan



2′-methoxyphaseollinisoflavan

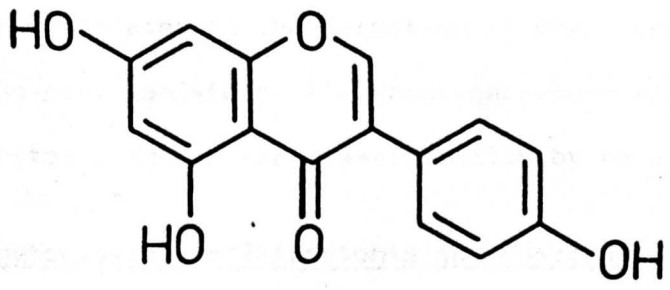
1963b; Perrin 1964) and phaseollidin (Fig. 1.1) (Perrin, Whittle & Batterham, 1972).

Burden, Bailey & Dawson (1972) identified two further compounds as phytoalexins in bean tissues infected with tobacco necrosis virus (TNV) or Colletotrichum lindemuthianum. These were phaseollinisoflavan (Fig. 1.1) and kievitone (Fig. 1.1). Kievitone had previously been isolated as the principal antifungal component of "substance II" in Rhizoctonia solani - infected hypocotyls (Smith, VanEtten & Bateman, 1971) but was only characterized later (Smith, VanEtten & Bateman, 1973a).

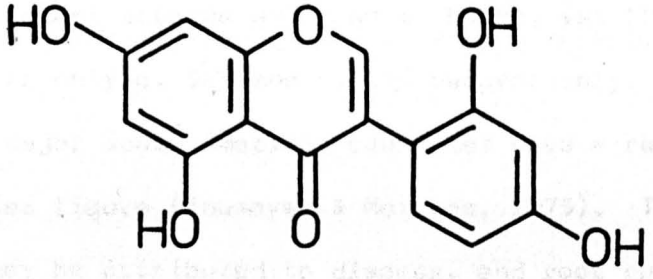
A fifth antifungal compound, 2'-methoxyphaseollinisoflavan (Fig. 1.1), was isolated from bean hypocotyl lesions caused by Fusarium solani f.sp. phaseoli (VanEtten, 1973). Attempting to determine whether this compound was of host origin or a fungal metabolite, VanEtten & Smith (1975) found that although the fungus could remove phaseollinisoflavan from in vitro liquid cultures, the 2'-methoxyl derivative was not detected as a product of the degradation. This compound did, however, accumulate in bean tissues infected with Thielaviopsis basicola. Thus 2'-methoxyphaseollinisoflavan was proposed to be a phytoalexin, possibly resulting from methylation of phaseollinisoflavan by an enzymic system of the host (VanEtten & Smith, 1975).

Following inoculation of bean pods with conidial suspensions of M. fructicola, Biggs (1975) identified the isoflavones genistein (Fig. 1.2) and 2'-hydroxygenistein (Fig. 1.2). Genistein, a constituent of clovers and other legumes, had not previously been reported in Phaseolus spp.; 2'-hydroxygenistein had hitherto been unknown in plants. Woodward (1979) confirmed the presence of both compounds using the same host-parasite system. He was also able to isolate and characterize the isoflavanones kievitone (Fig. 1.1) and dalbergioidin (Fig. 1.2), together with a new isoflavone, phaseoluteone (Fig. 1.2). Kievitone apart, the extent of accumulation and the degree of antifungal activity that these compounds exhibit do not present a strong

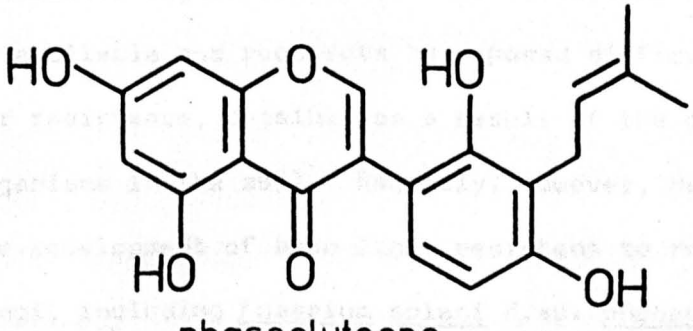
Fig. 1.2. Isoflavonoids of Phaseolus vulgaris with possible roles as phytoalexins.



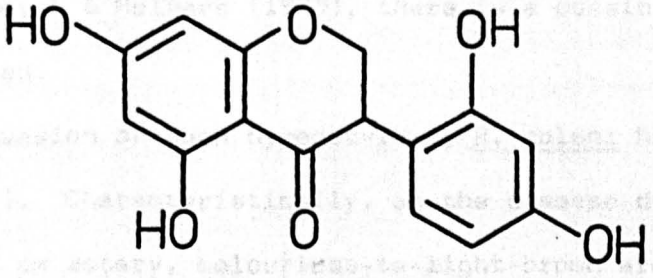
genistein



2′-hydroxygenistein



phaseoluteone



dalbergioidin

case for a role in disease resistance. They may be more significant as intermediates in the formation of phytoalexins such as kievitone. Nonetheless, genistein, 2'-hydroxygenistein and dalbergioidin are considered phytoalexins in the classification by Harborne & Ingham (1978).

Phytoalexins in Rhizoctonia and Fusarium diseases of bean:

Dry bean is the main edible legume grown and consumed in Latin America and Mexico, and is the principal source of dietary protein in many countries. Brazil and Mexico have the largest acreage put down to beans, yet the yield per acre in these countries is only c. 50% and c. 25% respectively, of that of the United States; other major South American countries have a reduction of c. 30% on the United States figure (Zaumeyer & Meiners, 1975). To a large extent, these losses may be attributed to disease, and root rots, caused principally by Fusarium, Rhizoctonia, Pythium and Thielaviopsis spp., contribute significantly to this situation. No satisfactory control measure is available and root rots have posed difficult problems in terms of breeding for resistance, possibly as a result of the complex associations of causal organisms in the soil. Recently, however, Hagedorn & Rand (1978) reported the development of bean lines resistant to root rot disease caused by several fungi, including Fusarium solani f.sp. phaseoli, Rhizoctonia solani and Pythium spp.. The nature of this increased resistance is not reported, but, as suggested earlier by Zaumeyer & Meiners (1975), there is a possibility that phytoalexins are involved.

The penetration and invasion of bean hypocotyls by R. solani has been well-documented (Section III). Characteristically, as the disease develops, young lesions become evident as watery, colourless-to-light-brown areas on the hypocotyl surface. Upon lesion maturation the tissue becomes dark brown in colour and eventually dries out and collapses. Throughout this development, the fungus remains confined to the lesions themselves, which essentially attain a maximum size at an early age and do not appear to spread as maturation

proceeds. Cytological observation has shown that restriction is not effected by the formation of wound periderm or other structural barriers. The suggestion therefore remains that the creation of an antifungal chemical environment in or around the infected tissues restricts spread of the pathogen.

Pierre & Bateman (1967) were the first to demonstrate a possible role for phytoalexins in restricting pathogen development in R. solani-infected bean hypocotyls. They isolated phaseollin and an unidentified component, "substance II", which was later determined to be a mixture of compounds with one principal antifungal component, kievitone (Smith et al., 1971; 1973a; Smith, VanEtten, Serum, Jones, Bateman, Williams & Coffen, 1973b). In addition, phaseollidin and phaseollinisoflavan were isolated from these tissues (Smith et al., 1973a). Further studies revealed that the accumulation of kievitone, in particular, in and around the lesions occurred at such a time and in sufficient magnitude as to be implicated in the inhibition of fungal growth; phaseollin and, to a lesser extent, phaseollidin and phaseollinisoflavan, reached inhibitory concentrations later (Smith, 1974; Smith et al., 1975).

Pierre (1966; 1971) also reported the accumulation of "substance II" in studies on phytoalexin accumulation in bean hypocotyls infected with Fusarium solani f.sp. phaseoli. In such tissues VanEtten & Smith (1975) were able to demonstrate the presence of phaseollin, phaseollinisoflavan, phaseollidin, 2'-methoxyphaseollinisoflavan and a fungal metabolite of phaseollin, 1a-hydroxyphaseollone (Heuvel & VanEtten, 1973), but not kievitone.

Lesions caused by F. solani f.sp. phaseoli do not become restricted in the manner of those caused by R. solani (Section III). Furthermore, the pattern of phytoalexin accumulation in the two disease situations is quite different. Although F. solani f.sp. phaseoli could metabolize phaseollin

in situ and, apparently, transform phaseollinisoflavan *in vitro*, large amounts of both compounds were found in the infected tissue (VanEtten & Smith, 1975). This suggests that the non-appearance of kievitone in *Fusarium*-infected beans may not be explained solely in terms of metabolism by the fungus.

The current investigation, in contrast to related studies on the metabolism of kievitone (Kuhn, 1979), was therefore primarily undertaken in an attempt to determine whether the disparity between observed kievitone accumulation in *Rhizoctonia*- and *Fusarium*-infected bean hypocotyls might be explained in terms of differential elicitor production by the two fungi.

SECTION II

MATERIALS AND METHODS (GENERAL)

This section describes only those materials and methods routinely employed throughout the period of study. Individual experimental techniques and materials are outlined at the beginning of the appropriate section.

Fungal material:

Rhizoctonia solani Kuhn (isolate RB) and Fusarium solani (Mart.) Sacc. f.sp. phaseoli (Burk.) Snyd. & Hans. (isolate FB) were obtained from Dr. J. van den Heuvel, Phytopathologisch Laboratorium "Willie Commelin Scholten", Baarn, The Netherlands. They were maintained at 25°C on potato-dextrose agar (PDA) (Oxoid Ltd.) and routinely transferred at 1- or 2-weekly intervals, respectively.

Mycelium for inoculation of plant material or for preparation of mycelial extracts was obtained from liquid cultures. Rhizoctonia solani was grown at 25 ± 2°C in 100 ml Erlenmeyer flasks containing 30 ml of an autoclaved medium similar to that described by Smith et al. (1973a); this contained, on a per litre basis, 20 g glucose, 4.6 g casein hydrolysate, 1.0 g KH_2PO_4, 0.5 g $MgSO_4$, 0.01 g aneurine hydrochloride and 10 ml of a minor elements solution containing 0.25 g $FeSO_4.7H_2O$, 0.22 g $ZnSO_4.7H_2O$, 0.04 g $CuSO_4.5H_2O$, 0.04 g $MnSO_4.4H_2O$, 0.05 g $Na_2MoO_4.2H_2O$ and a trace of H_2SO_4 (to clarify the solution) in one litre of water. The inoculum for each flask was a 1 cm-diameter plug bearing mycelium taken from a 4- to 5-day old PDA culture.

Fusarium solani f.sp. phaseoli was grown at 25 ± 2°C in 250 ml Erlenmeyer flasks in shake culture from spore suspensions prepared using methods similar

to those described by Heuvel & VanEtten (1973) and Pueppke & VanEtten (1974). The liquid medium comprised, on a per litre basis, 20 g glucose, 5.0 g L-asparagine, 0.1 g glutathione, 0.87 g K_2HPO_4, 6.12 g KH_2PO_4, 0.4 g $MgCl_2.6H_2O$, 0.02 g $CaCl_2.2H_2O$ and 10 ml of the minor elements solution described above; the pH was adjusted to 6.0 using 1 M KOH or HCl.

Dry weights of mycelium used in the preparation of inocula or of mycelial extracts were estimated by drying aliquots on pre-weighed filter paper discs in an oven at 75°C until constant weights were established.

Plant material:

Phaseolus vulgaris L. (French bean) seed, cultivar "Masterpiece", (obtained from Charles Sharpe & Co. Ltd., Seed Producers, Sleaford, Lincs., UK) was used to provide plant material for all experiments. Seedlings were grown in steam-sterilized John Innes No. 2 compost in 35 x 22 cm seed trays (40-50 seeds/tray) in a glasshouse (mean day temperature 24°C, mean night temperature 20°C) using supplementary lighting as required from mercury discharge lamps (MBF/U, 400W). After seven days, plants were inoculated using procedures outlined elsewhere (Section III), and the inoculated tissues were subsequently incubated for various periods of time in a growth room at 25 ± 1°C under cool fluorescent lights providing a photosynthetically-active radiant flux density of 100 $\mu E/m^2/s$ and a 16 h day.

Routine extraction procedure for phytoalexins from bean tissues:

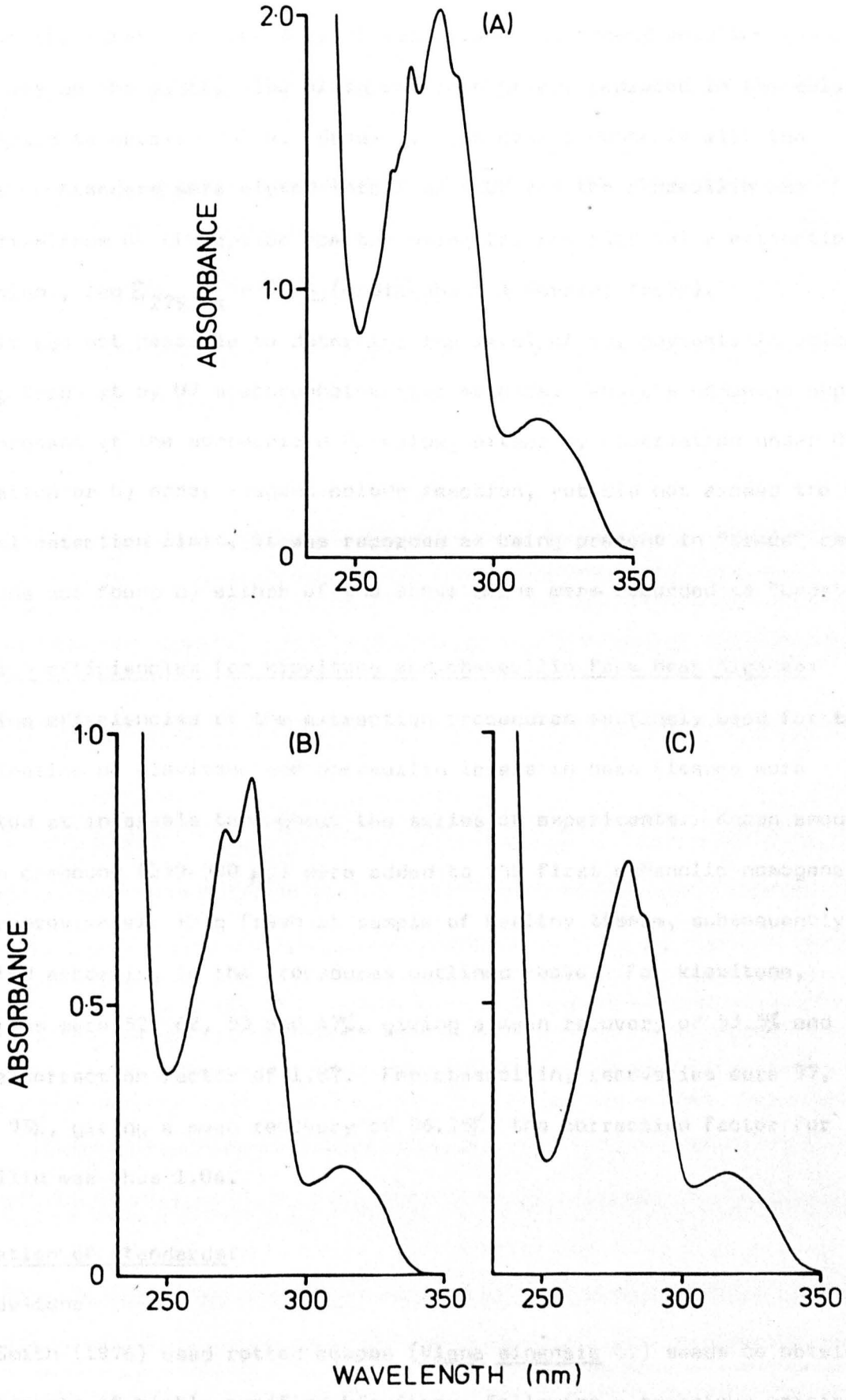
Harvested hypocotyls were weighed to determine the fresh weight (wt) and then lyophilized, usually for 24 h, prior to re-weighing for determination of the dry wt. Quantitation of phytoalexin levels involved a method similar to that of Smith et al. (1975). Freeze-dried tissues (equivalent to 5 - 10 g fresh wt) were homogenized in 95% ethanol (EtOH) (50:1, v/w) using an MSE top-drive mixer (MSE Scientific Instruments) at top speed for 90 s. After filtration through Whatman No. 1 filter paper (W. & R. Balston, Maidstone, UK) under reduced pressure, the tissue debris was re-homogenized in 95%

EtOH (25:1, v/w) and the filtration repeated. The combined filtrates were evaporated to near dryness *in vacuo* at c. 40°C and the residue dissolved in 0.2 M Na_2CO_3 approximately equal to one quarter the original filtrate volume. This basic solution was then adjusted to pH 5.0-5.5 using HCl, and subsequently partitioned twice against chloroform (3:1 and 2:1, v/v). The combined chloroform fractions were evaporated to dryness under reduced pressure and the residue dissolved in 40 ml EtOH. Ten ml aliquots were stored at -16°C prior to resolution by thin layer chromatography (TLC). Samples for TLC were evaporated to dryness *in vacuo* and re-dissolved in a small volume of EtOH/diethyl ether (Et_2O) (1:1, v/v) before streaking on 20 x 20 cm glass TLC plates bearing 1 mm layers of silica gel G (SG) (Type 60; E. Merck, Darmstadt, Germany). Standards, prepared as described below, were applied close to one edge of each of the plates, which were developed in benzene/methanol (9:1, v/v) at room temperature under unsaturated conditions. Bands adjacent to standards, identified by observation under UV irradiation and/or after spraying with diazotized *p*-nitroaniline (DPN) (Stahl, 1965; Dittman, 1968), were scraped from the plates prior to elution in EtOH.

Kievitone, R_f = 0.28, was eluted from the SG by washing three times with 5 ml EtOH, the SG being removed from suspension each time by centrifugation on a bench centrifuge (top speed for 5 min). The combined supernatants were then evaporated to dryness under reduced pressure and re-dissolved in a small volume of EtOH/Et_2O (1:1) before streaking onto 20 x 20 cm TLC plates bearing 0.25 mm layers of SG. After application of standard, the plates were developed in benzene/ethyl acetate/methanol (25:4:2). Bands running co-incidentally with kievitone, R_f = 0.60, were scraped off and eluted into 5 ml EtOH. After centrifugation to remove SG, kievitone was quantified by UV spectroscopy using a Pye-Unicam SP 1800 double-beam recording spectrophotometer and the published molar extinction coefficient, $\log \varepsilon_{294\ nm} = 4.17$ (Smith *et al.*, 1973b).

Phaseollin, R_f = 0.55 in benzene/methanol (9:1), was eluted from the SG into 10 ml EtOH; the resultant suspension was stored at -16°C prior to further purification. Although 2'-methoxyphaseollinisoflavan (VanEtten, 1973) is not produced in bean tissues infected with R. solani (Smith et al, 1975), this antifungal isoflavan does accumulate in similar tissues infected with F. solani f.sp. phaseoli (VanEtten & Smith, 1975). Since 2'-methoxyphaseollinisoflavan exhibits a mobility almost identical to that of phaseollin in several TLC systems (VanEtten, 1973) and may consequently affect the UV absorption spectrum of phaseollin (Cardoso, 1971; VanEtten & Smith, 1975), a procedure for the resolution of these two compounds was necessary. It was found that an authentic sample of 2'-methoxyphaseollinisoflavan (kindly provided by Dr. H.D. VanEtten, Plant Pathology Dept., Cornell University, Ithaca, New York 14853, USA) ran slightly behind an authentic sample of phaseollin when they were chromatographed together on 0.25 mm SG layers developed in toluene/ethyl formate/formic acid (7:2:1). The two compounds were still incompletely separated, however, as determined by spraying with DPN, phaseollin (R_f = 0.65) yielding its characteristic yellow reaction product and 2'-methoxyphaseollinisoflavan (R_f = 0.61) an orange-brown reaction product (VanEtten, 1973). However, if the plates were re-developed in the same solvent, complete separation could be achieved, as observed both by the presence of distinct spots on spraying with DPN and by resolution of an "abnormal" UV absorption spectrum (Fig. 2.1,A) into characteristic UV absorption spectra of 2'-methoxyphaseollinisoflavan (Fig. 2.1,B) and phaseollin (Fig. 2.1,C). Thus, for any routine quantitation of phaseollin from bean tissues, the stored SG/EtOH suspension from the first TLC step was filtered through Whatman GF/C glass fibre paper under reduced pressure and the filtrate, together with three EtOH washes of the SG, evaporated to dryness in vacuo. The residue was dissolved in a small volume of EtOH/Et_2O (1:1) before streaking onto 0.25 mm SG layers which were then developed twice in

Fig. 2.1. Ultra-violet absorption spectra demonstrating the resolution by TLC of a mixture of phaseollin and 2'-methoxyphaseollinisoflavan (A) into the separate components 2'-methoxyphaseollinisoflavan (B) and phaseollin (C).


2·0
1·0
0
ABSORBANCE
(A)
250
300
350
1·0
0·5
0
(B)
(C)
250
300
350
250
300
350
WAVELENGTH (nm)

toluene/ethyl formate/formic acid (7:2:1). The best separation was achieved when, on the first run, the solvent was allowed to ascend only three-quarters of the way up the plate. The plate was then dried, replaced in the solvent and allowed to develop fully. Bands running co-incidentally with the phaseollin standard were eluted into 5 ml EtOH and the phaseollin was quantified from UV absorption spectra using the reported molar extinction coefficient, $\log \varepsilon_{279\ nm} = 3.96$ (Cruickshank & Perrin, 1963b).

It was not possible to determine the level of any phytoalexin below 5 µg/g fresh wt by UV spectrophotometric methods. Where a compound appeared to be present at the appropriate R_f value, either by observation under UV irradiation or by spray reagent colour reaction, yet did not exceed the UV spectral detection limit, it was recorded as being present in "trace" amounts. Compounds not found by either of the above means were recorded as "undetected".

Extraction efficiencies for kievitone and phaseollin from bean tissues:

The efficiencies of the extraction procedures routinely used for the determination of kievitone and phaseollin levels in bean tissues were estimated at intervals throughout the series of experiments. Known amounts of each compound (250-500 µg) were added to the first ethanolic homogenate of an approximately 10 g fresh wt sample of healthy tissue, subsequently extracted according to the procedures outlined above. For kievitone, recoveries were 52, 62, 53 and 47%, giving a mean recovery of 53.5% and hence a correction factor of 1.87. For phaseollin, recoveries were 97, 94, 99 and 95%, giving a mean recovery of 96.25%; the correction factor for phaseollin was thus 1.04.

Preparation of standards:

(a) Kievitone:

Smith (1976) used rotted cowpea (Vigna sinensis L.) seeds to obtain large amounts of highly purified kievitone, following a technique originally outlined by Keen (1975b). One hundred to 150 g dry wt of locally-purchased

seeds were treated in the manner described by Keen. After 4 to 5 days incubation at 25 ± 1°C the seeds were triturated in 95% EtOH (1:10,w/v) in an MSE Ato-mix blender. The resulting suspension was centrifuged at 10,000 x g for 20 min. The supernatant was filtered under reduced pressure through Whatman No. 1 and GF/C glass fibre filters; the pellet was again triturated in an equivalent volume of 95% EtOH and the whole process repeated. The pellet from this second centrifugation was further ground in 95% EtOH (1:2, w/v) before filtration as above. The combined filtrates (c.3.5 l) were evaporated *in vacuo* at c. 40°C; the resultant brown syrup was dissolved in twice its own volume of 0.3 M Na_2CO_3 solution, and then 0.2 M Na_2CO_3 to a total of 400 ml. This basic solution was adjusted to pH 5 using HCl before partitioning twice against chloroform (3:1 and 2:1, v/v) and once against Et_2O (1:1, v/v). The combined chlorform and ether fractions were evaporated to dryness *in vacuo* at c. 40°C and the residue dissolved in a small volume of EtOH. This solution was concentrated by evaporation under a stream of nitrogen prior to the addition of an equal volume of Et_2O in readiness for resolution by TLC.

Kievitone bands running adjacent to authentic samples (kindly furnished by Dr. D.A. Smith, Plant Biology Dept., Hull University) were isolated by sequential TLC in the following solvent systems:

Benzene/methanol (9:1)	R_f = 0.28
Chloroform/EtOH (100:3)	R_f = 0.08
Hexane/acetone (3:1)	R_f = 0.12
Toluene/ethyl formate/formic acid (7:2:1)	R_f = 0.22

The final kievitone eluate was concentrated under a stream of nitrogen and further purified by gel filtration. The material was applied to a 1 x 40 cm column of Sephadex LH-20 (Pharmacia Fine Chemicals) which had been equilibrated in EtOH. The eluant was EtOH and 5 ml fractions were collected at a flow rate of c.0.25 ml/min.

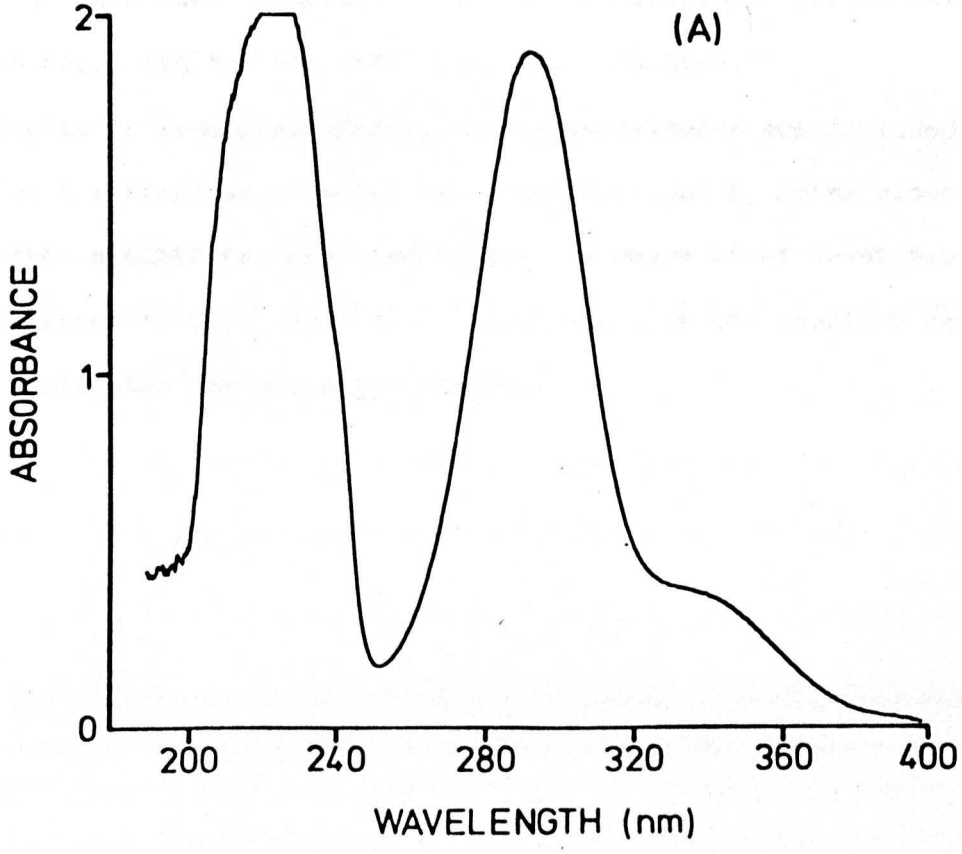
The UV absorption spectra of kievitone in the peak fractions (Fig. 2.2,A) were identical to its reported spectral characteristics (Burden et al., 1972; Smith et al., 1973b). Low and high resolution mass spectral analyses, conducted by Mr. A.D. Roberts, Chemistry Dept., Hull University, using a heated direct insertion probe on an MS 12 (A.E.I. Ltd.) instrument, revealed a molecular ion at m/e 356 (39%), and other major peaks at m/e 221 (55%), 205 (39%), 192 (36%), 177 (52%), 165 (100%) and 136 (57%), all consistent with the fragmentation observed for kievitone (Burden et al., 1972; Smith et al., 1973b).

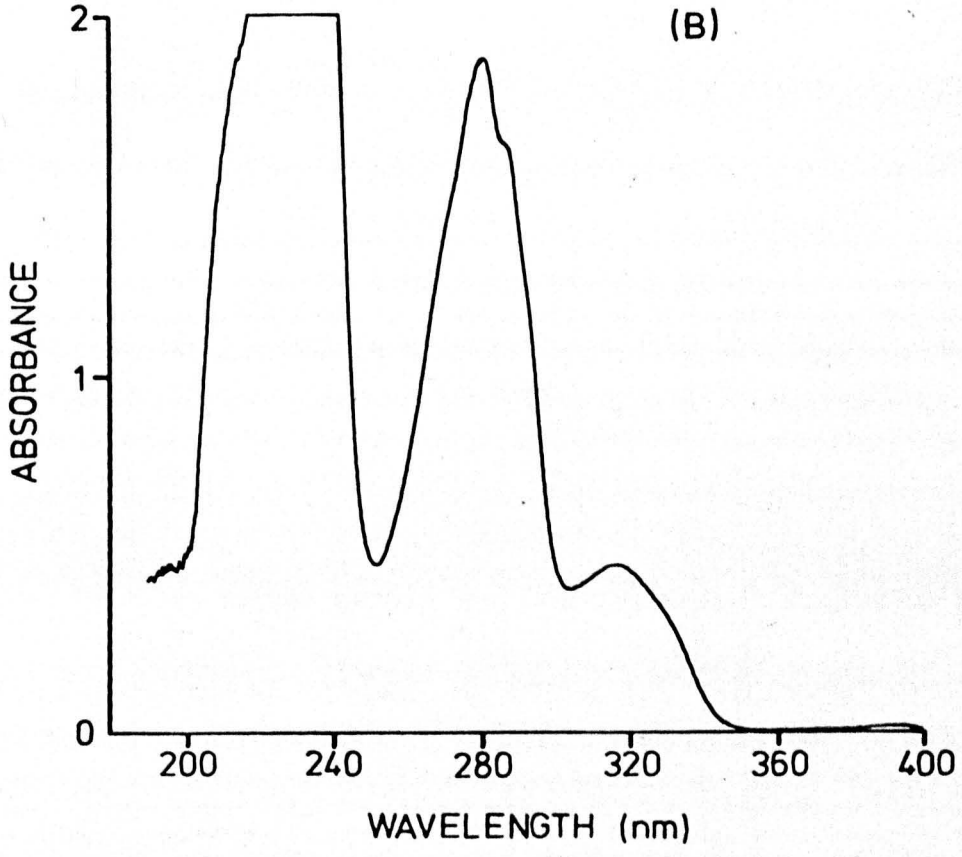
(b) Phaseollin:

Mature lesions of R. solani on bean hypocotyls contain high levels of phaseollin (Pierre & Bateman, 1967; VanEtten, Maxwell & Bateman, 1967; Smith et al., 1975). Therefore, hypocotyls bearing such lesions were harvested and extracted for phaseollin using the routine procedure outlined above. Further purification was achieved by subjecting the combined eluates from the second TLC step to gel filtration on Sephadex LH-20, as described earlier for kievitone. The UV absorption spectra from the peak phaseollin fractions (Fig. 2.2,B) were identical with reported spectral characteristics for this phytoalexin (Cruickshank & Perrin, 1963b; VanEtten & Bateman, 1970). Low and high resolution mass spectral analyses revealed a molecular ion at m/e 322 (55%) and other major peaks at m/e 307 (100%), 279 (36%), 267 (10%), 185 (16%), 173 (22%), 153.5 (13%), 153 (24%) and 147 (27%), consistent with the fragmentation observed for phaseollin (Perrin et al., 1972; D.A. Smith, personal communication).

Thus, samples of kievitone and phaseollin -- identified by co-chromatography with authentic samples in several TLC solvent systems, UV absorption spectral characteristics and mass spectral analyses -- were obtained in milligram quantities for use as TLC standards or for gas-liquid chromatographic analyses (see Appendix).

Fig. 2.2. Ultra-violet absorption spectra of purified kievitone (A) and phaseollin (B).


(A)
ABSORBANCE
2
1
0
200
240
280
320
360
400
WAVELENGTH (nm)


(B)
ABSORBANCE
2
1
0
200
240
280
320
360
400
WAVELENGTH (nm)

In all the results quoted hereafter the levels of phytoalexins given have been corrected for the efficiency of extraction.

Results of some preliminary, single experiments are included; where this is so the findings were generally corroborated by later experiments. In all other situations, the results are the means of at least two replicate experiments. Variation, where shown, is the absolute variation between replicates for any given reading.

SECTION III

ELICITATION BY FUNGAL INFECTION

Initially it was necessary to determine whether, under the experimental conditions available, infection of bean hypocotyls by either R. solani or F. solani f.sp. phaseoli could provide results comparable with those of earlier reports. To this end, the patterns of infection and phytoalexin accumulation were monitored following the inoculation of whole plants with either fungus. Also, in order to investigate the phytoalexin-eliciting potential of culture fluids or mycelial extracts, a system had to be devised whereby such solutions could be maintained in direct contact with bean hypocotyls for substantial time periods.

MATERIALS AND METHODS

Natural infection:

For inoculation of intact plants, a method similar to that described by VanEtten et al. (1967) was employed. Rhizoctonia solani inoculum was prepared by triturating 7-day old mycelial mats, obtained as outlined earlier, in distilled water using an MSE Ato-mix blender at top speed for 30 s. The homogenate was diluted to a total of c.125 ml/mat. Mycelium of F. solani f.sp. phaseoli, developing from spore suspensions ($1.0 - 1.5 \times 10^5$ spores/ml), was separated from the liquid medium of 1- to 2-day old cultures by filtration, washed, and re-suspended in distilled water to a total of c.250 ml/culture. The resultant mycelial suspensions of either fungus were then poured directly on and around the hypocotyls of intact 7-day old bean plants; c.250 ml suspension per tray (c. 50 seedlings) was employed. Since this procedure

represents an analogy to the invasion of plants in the field by soil-borne mycelium it is referred to as the "natural infection" technique, thereby distinguishing it from the inoculation of excised bean hypocotyls. Trays of inoculated plants were incubated under conditions described earlier. At intervals, plants were removed from the trays and washed under running tap water to dislodge adhering soil particles. The majority of roots were removed and the plants decapitated immediately above the level of the highest observable lesion.

Artificial infection:

For maintenance of potential eliciting solutions in direct contact with bean hypocotyls, a method similar to that of VanEtten & Bateman (1971) was used. Sections 3 - 4 cm long were excised from hypocotyls of healthy 7-day old plants, and a central cylinder of tissue was removed from each section by inserting a thin glass tube. In each case, the basal end was sealed with petroleum jelly, providing a central cavity into which test solutions could be applied. Initially, however, experiments were carried out to monitor the response of these excised, cored hypocotyls to living fungal inoculum. The inoculum was prepared by collecting homogenized mycelial mats of R. solani or liquid cultures of F. solani f.sp. phaseoli on Whatman No. 1 filter paper. These tissues, which were separately washed and re-suspended in c. 50 ml sterile distilled water/culture, were injected into the hypocotyl cavities with a sterile hypodermic syringe. This method is hereafter referred to as the "artificial infection" technique.

The hypocotyl sections were then placed upright in moist, autoclaved vermiculite in plastic sandwich boxes, and incubated under growth room conditions as outlined earlier. After various periods of time, hypocotyls were removed from the boxes, wiped to eliminate excess petroleum jelly and adhering vermiculite, and sliced longitudinally with a scalpel in order to observe the internal tissues and to remove the petroleum jelly plug. The

harvested hypocotyls were assayed for kievitone and phaseollin as outlined earlier; generally 24 or 36 hypocotyls were used for phytoalexin determinations.

Photomicroscopy:

Naturally-infected hypocotyl pieces bearing macroscopic symptoms were fixed by vacuum-infiltration with formalin-acetic acid-alcohol and dehydrated through a tertiary butyl alcohol series before embedding in paraffin wax. Sections 10 - 15 µm thick were cut using a Reichert rotary microtome and stained with Pianeze's III B stain, as employed by Simmons & Shoemaker (1952 - described in Gurr, 1965).

Sections of artificially-inoculated hypocotyls were cut freehand with a razor blade and floated on distilled water. Trapped air was removed by vacuum-infiltration, and the sections were mounted unstained in water.

Photographs were taken under a green filter on a Zeiss Photomicroscope II using Panatomic X film.

RESULTS

Natural infection:

Examples of naturally-infected hypocotyls at various time intervals after application of the inoculum are shown in Plate 1.

Rhizoctonia solani-infected plants developed the characteristic delimited, sunken lesions described by Christou (1962) and VanEtten *et al.* (1967). In comparison to uninoculated, healthy plants (Plate 1, A), the treated hypocotyls had developed colourless, water-soaked lesions 24 h after inoculation (Plate 1, B). Individual lesions became more apparent by 48 h as they began to darken in colour (Plate 1, C), such that by 72 h (Plate 1, D) they were already beginning to pass from an intermediate into a more mature stage of development (VanEtten *et al.*, 1967). Ninety-six hours after inoculation, fully mature, dried, sunken lesions were evident (Plate 1, E). Although no

PLATE 1

Hypocotyls naturally-infected with *R. solani* or *F. solani* f.sp. *phaseoli*.

R. solani: (A) Healthy; (B) 24 h; (C) 48 h; (D) 72 h; (E) 96 h. (All x 1.25)

F. solani f.sp. *phaseoli*: (F) Healthy; (G) 24 h; (H) 48 h; (I) 72 h; (J) 96 h; (K) 120 h; (L) 12 days. (All x 1)

A B C D E

F G H I J K L

attempt was made to determine the dimensions of lesions at each stage, individual lesions appeared to remain restricted for at least 120 h after inoculation (the longest time of incubation), apart from some coalescence due to the development of separate lesions in close proximity to one another. Sections taken through lesions caused by R. solani at various times after inoculation are shown in Plate 2. Infection cushion formation preceded direct penetration by several hyphae, and subsequent development, by both inter- and intra-cellular hyphal growth (Christou, 1962; Dodman, Barker & Walker, 1968), continued at least up to 48 h after inoculation. At this time, neither penetrated host cells nor cells immediately adjacent to the invading fungus were seen to have undergone browning, although there was a distinct darkening of cell walls within and immediately surrounding the infected area, and evidence of cytoplasmic granulation (Plate 2, B and C). However, by 72 h (Plate 2, D), the lesions had browned and the tissues deteriorated considerably until by 96 h a large degree of cell collapse and degeneration was observed, with the result that mature lesions assumed their characteristic concave appearance (Plate 2, E).

Disease progression in plants naturally-infected with F. solani f.sp. phaseoli followed a different pattern. Examples of plants at various times after inoculation with this fungus are also shown in Plate 1; a typical healthy example is shown for comparison (Plate 1, F). Twenty-four hours after inoculation (Plate 1, G), small orange-brown flecks began to appear on the hypocotyls and by 48 h (Plate 1, H) these had developed into characteristic orange-brown streaks (Chatterjee, 1958; Christou & Snyder, 1962). These individual lesions continued to spread both longitudinally and horizontally, as seen from further daily observations (Plate 1, I-K), until by 120 h after inoculation (Plate 1, K) almost complete coalescence had occurred and lesions girdled the stem. After 12 days (Plate 1, L), disease was evident further up the hypocotyl and in the roots; much of the cortical tissue had become shrunken and dry, providing the symptoms from

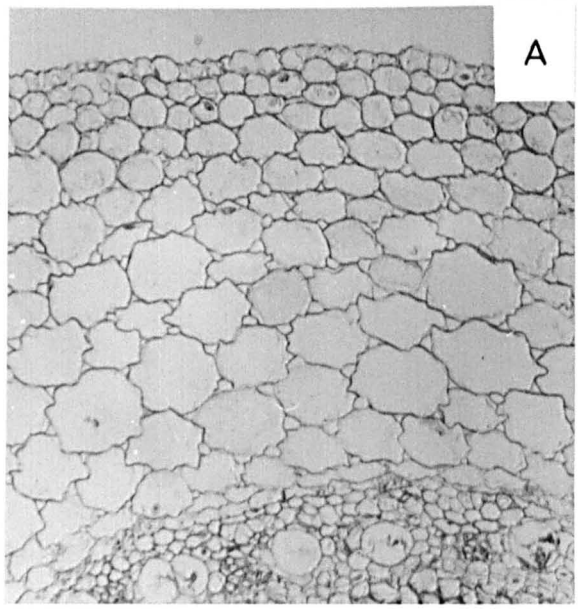
PLATE 2

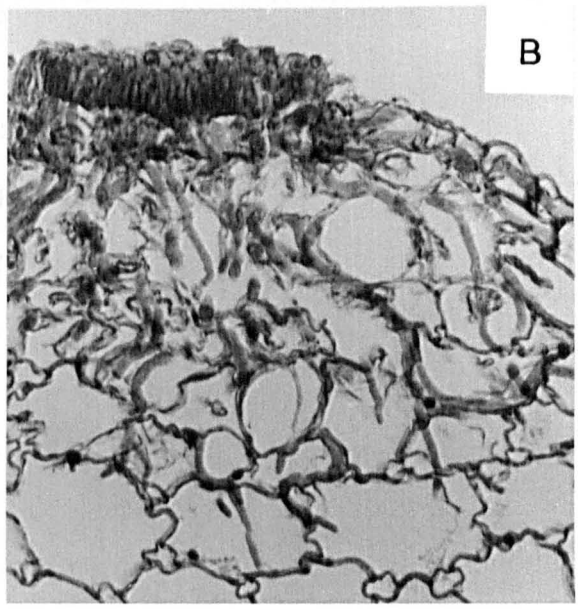
Sections taken from hypocotyls naturally-infected with *R. solani*.

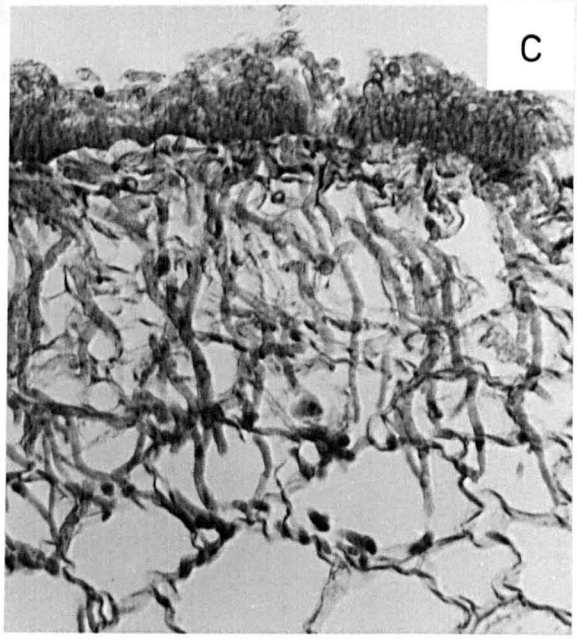
(A) Healthy (x 110); (B) 48 h (x 250);

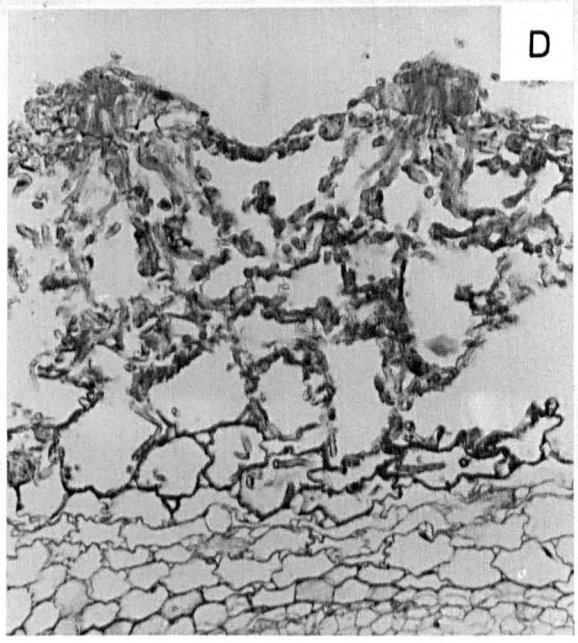
(C) 48 h (x 250); (D) 72 h (x 175);

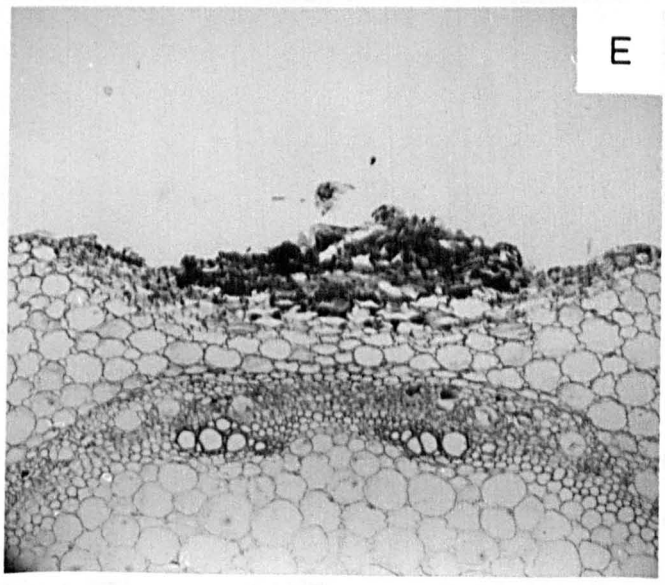
(E) 96 h (x 70).


A


B


C


D


E

which this foot-rot derives its common name (Moore, 1959).

Plate 3 shows sections of hypocotyls at various stages after inoculation with F. solani f.sp. phaseoli. Infection cushions as such were not observed, but penetration could be effected either directly into the epidermis or via stomata after the production of a small thallus (Plate 3, A). Thereafter fungal growth was predominantly inter-cellular and along the longitudinal axis of the plant (Plate 3, B and C). Ramification between the cells was often so intense that they became separated from one another and assumed a more rounded shape (Plate 3, C and D). The cells within the infected area eventually darkened in colour and granulated; this was often accompanied by intra-cellular fungal development and the whole tissue became brown, soft and liable to collapse (Plate 3, E).

Hypocotyls infected with either fungus were harvested at various times after inoculation and extracted for kievitone and phaseollin as described in Section II. The results are presented in Fig. 3.1. In R. solani-infected hypocotyls (Fig. 3.1, A), kievitone accumulated more rapidly and to higher levels than phaseollin up to 96 h. In contrast, the small amounts of kievitone that were initially detectable in tissues inoculated with F. solani f.sp. phaseoli (Fig. 3.1, B) soon fell to trace levels, while phaseollin continued to accumulate up to 120 h after inoculation. Neither kievitone nor phaseollin could be detected in extracts of uninoculated, healthy control plants.

Artificial infection:

Examples of hypocotyls artificially-infected with R. solani or F. solani f.sp. phaseoli are shown in Plate 4.

In contrast to uninoculated controls (Plate 4, E) or sections treated with sterile distilled water only, hypocotyls inoculated with R. solani demonstrated quite heavy browning of the central cavity walls 24 h after

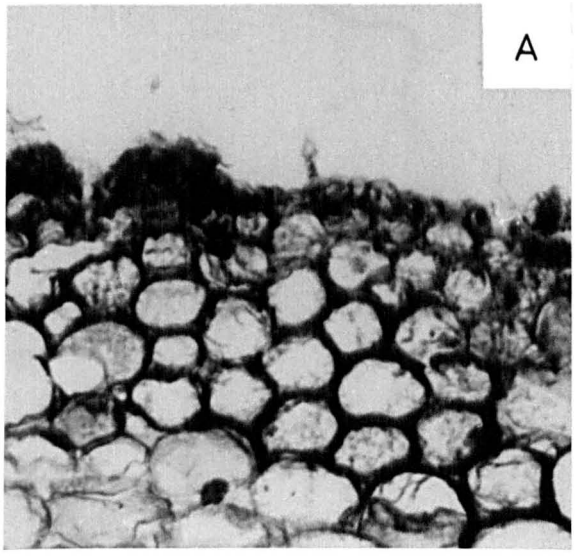
PLATE 3

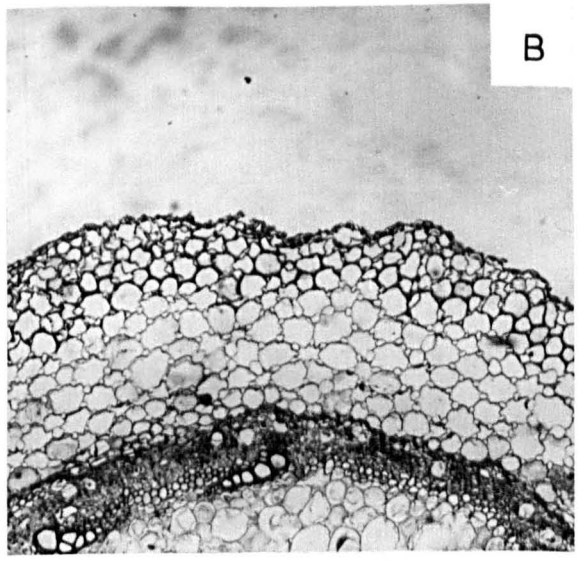
Sections taken from hypocotyls naturally-infected with *F. solani* f.sp. *phaseoli*.

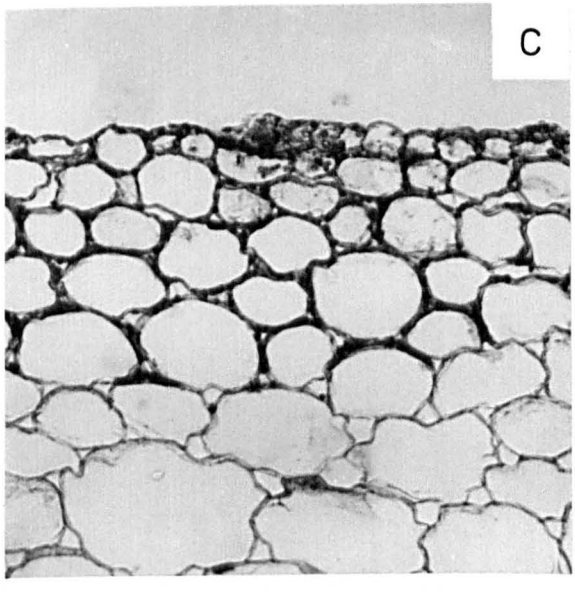
(A) 72 h (x 190); (B) 72 h (x 48);

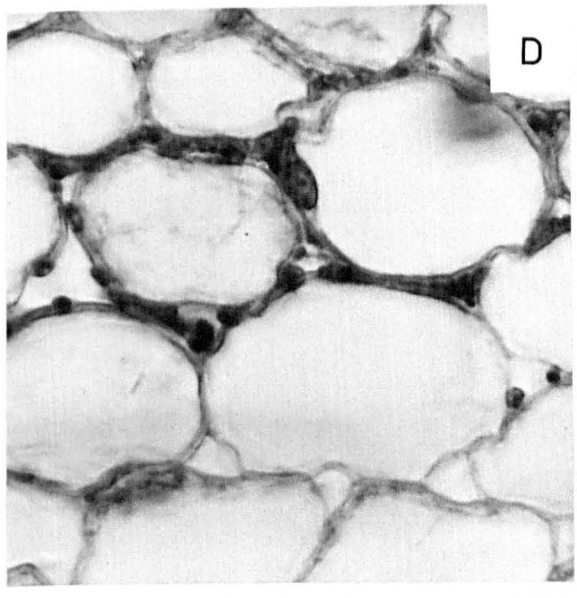
(C) 96 h (x 190); (D) 96 h (x 480);

(E) 120 h (x 85).


A


B


C


D

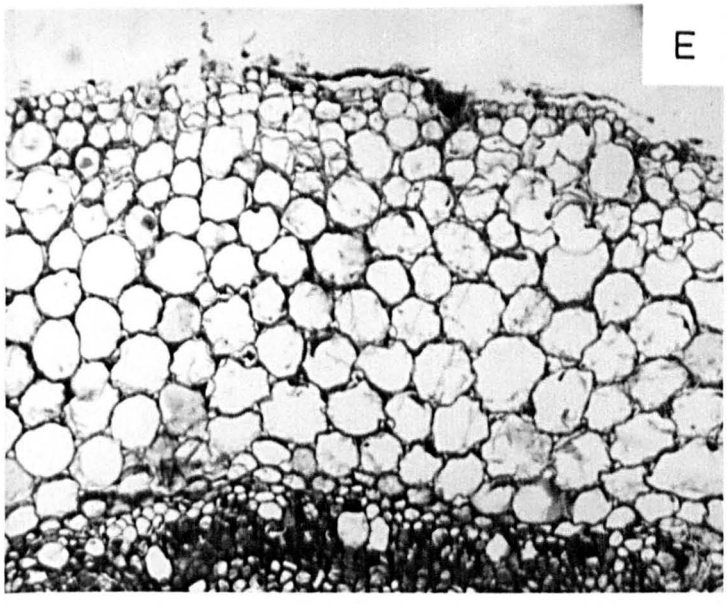

E

Fig. 3.1. Levels of kievitone (▨) and phaseollin (□) in hypocotyls naturally-infected with (A) *R. solani*, or (B) *F. solani* f.sp. *phaseoli*.

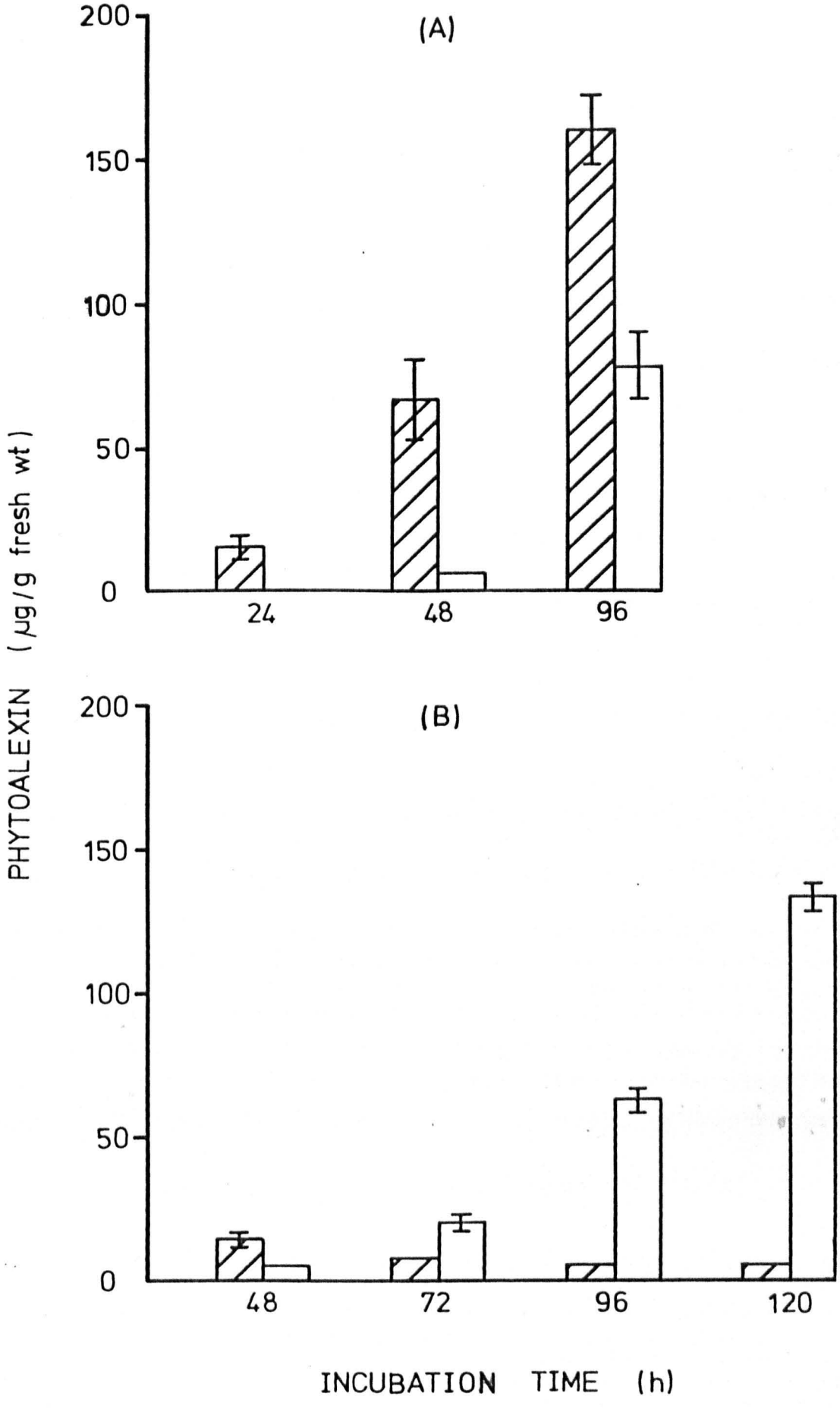

(A)
200
150
100
50
0
24
48
96
(B)
200
150
100
50
0
48
72
96
120
PHYTOALEXIN (µg/g fresh wt)
INCUBATION TIME (h)

PLATE 4

Hypocotyls artificially-infected with *R. solani* or *F. solani* f.sp. *phaseoli*.

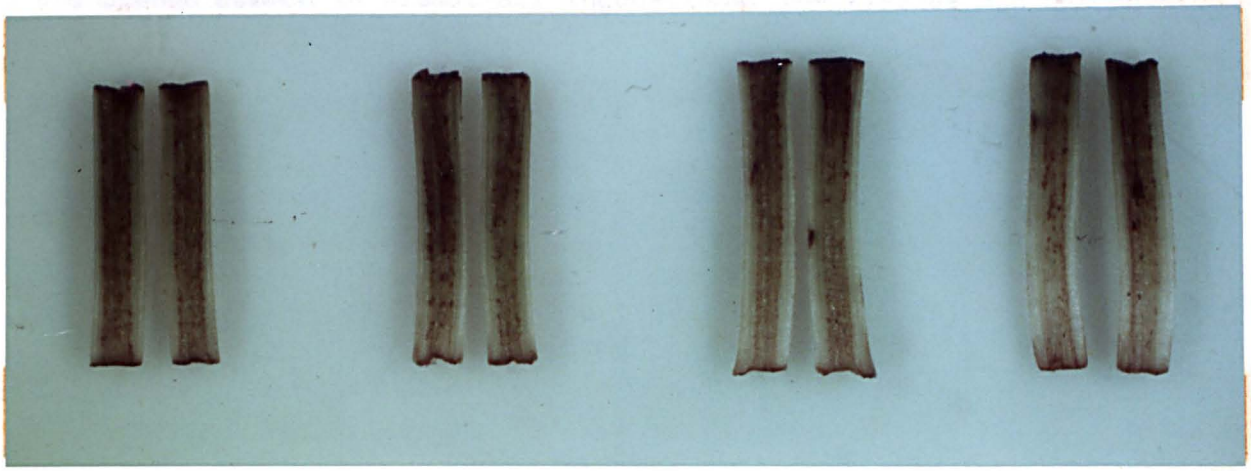
R. solani: (A) 24 h; (B) 48 h; (C) 72 h; (D) 96 h.

(All x 1.25)

F. solani f.sp. *phaseoli*: (E) Healthy; (F) 24 h; (G) 48 h; (H) 72 h; (I) 96 h.

(All x 1.25)

The inoculum contained c.5 or c.1 mg dry wt mycelium/ml for *R. solani* and *F.solani* f.sp. *phaseoli*, respectively.



A B C D

E F G H I

inoculation (Plate 4, A). This browning did not appear to increase in intensity nor spread further through the tissues over the next 72 h (Plate 4, B-D). Apart from some browning of the cut ends of the hypocotyls, a phenomenon common to almost all incubations, the tissues did not develop external symptoms and remained firm throughout the course of the experiment. The restricted development of R. solani in these tissues was evidenced clearly in cross-section. Free-hand sections of hypocotyls at each assay period are shown in Plate 5. Twenty-four hours after application of the inoculum, a ring of browned cells could be observed lining the central cavity of the hypocotyl (Plate 5, A). Subsequent observation showed that the fungus was not able to penetrate more than three cell layers of tissue, even after 96 h incubation (Plate 5, B-F).

The cavity walls of hypocotyls artificially-inoculated with F. solani f.sp. phaseoli had also browned by 24 h after inoculation (Plate 4, F). However, in this case the browning continued to spread rapidly (Plate 4, G-I) until the tissues became quite dark, water-soaked and extensively colonized. Sectioning of these hypocotyls was precluded by the macerated condition of the tissues. An indication of their appearance is provided by the later stages of an incubation time course after hypocotyls had been inoculated with a spore suspension of the fungus (Plate 9, Section V). However, invasion by actively-growing mycelium was more rapid, and the hypocotyls soon exhibited external signs of infection (Plate 10, A, Section V).

At various times after inoculation, tissues infected with either fungus were harvested and extracted for kievitone and phaseollin as described earlier. The results are shown in Fig. 3.2. In R. solani-infected hypocotyls (Fig. 3.2, A), kievitone reached a level exceeding 100 µg/g fresh wt 24 h after inoculation, but declined somewhat thereafter. Phaseollin also accumulated more rapidly than in the natural infection and held steady at about 80 µg/g fresh wt up to 96 h. In the F. solani f.sp. phaseoli situation (Fig. 3.2, B), kievitone attained only low levels and

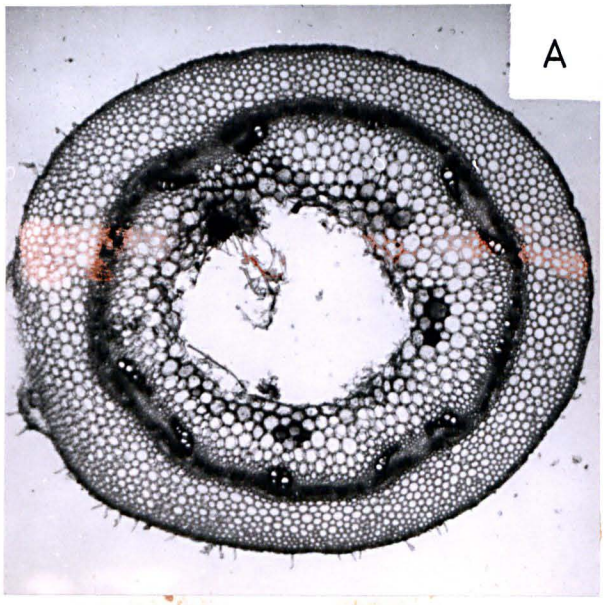
PLATE 5

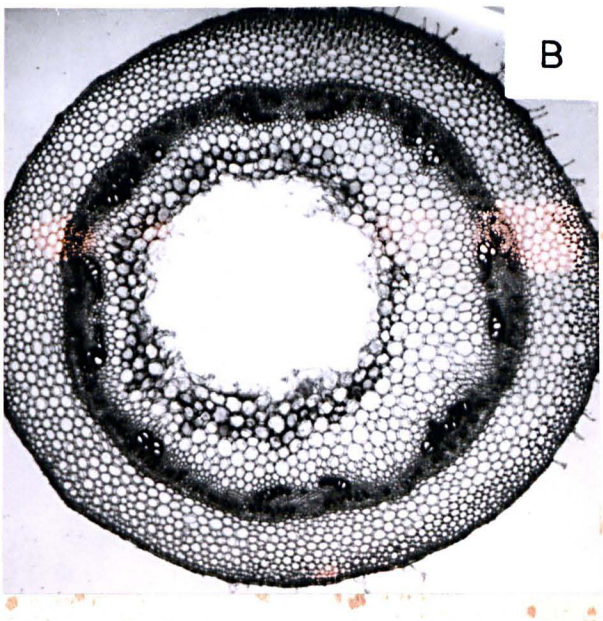
Sections taken from hypocotyls artificially-infected with *R. solani* (c.5 mg dry wt mycelium/ml).

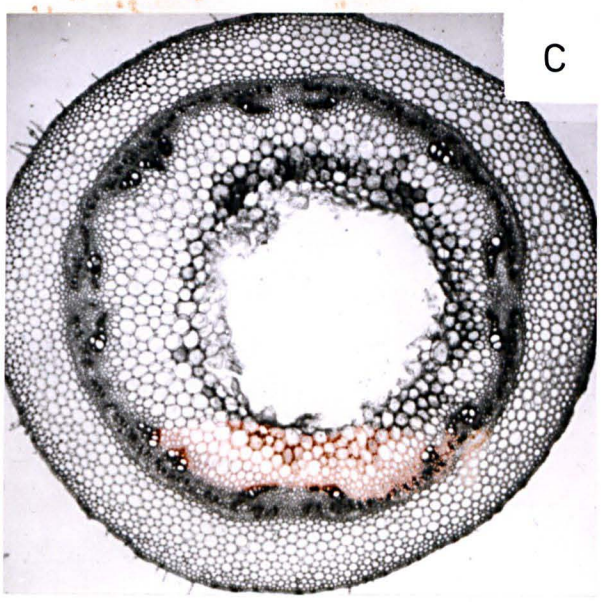
(A) 24 h (x 15); (B) 48 h (x 15);

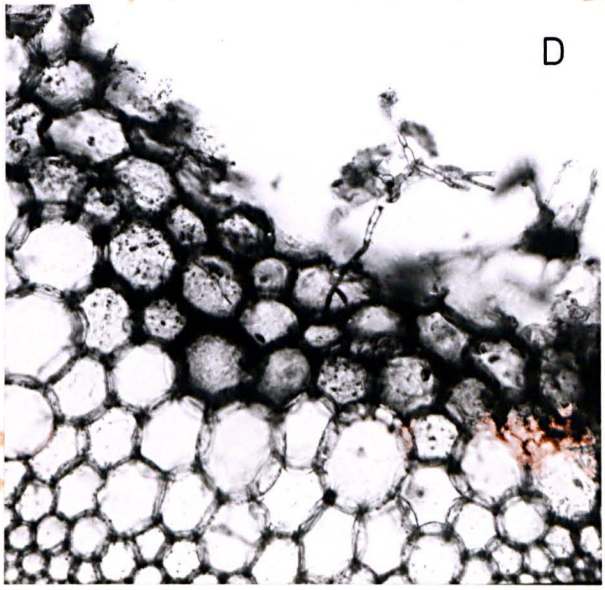
(C) 72 h (x 15); (D) 72 h (x 75);

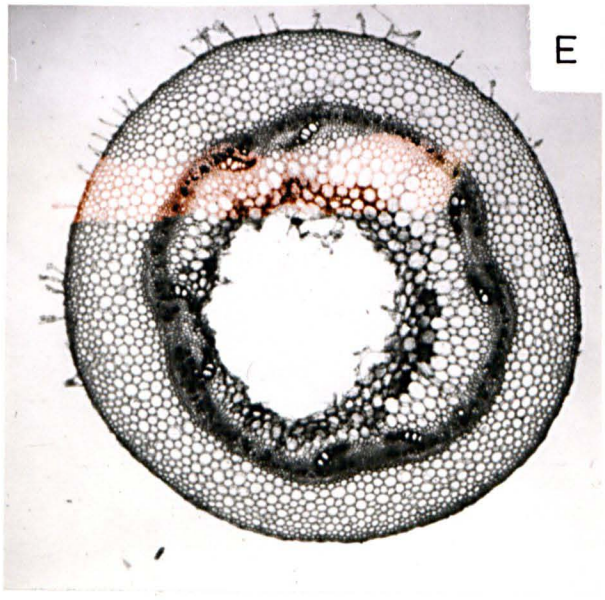
(E) 96 h (x 15); (F) 96 h (x 60).


A


B


C


D


E

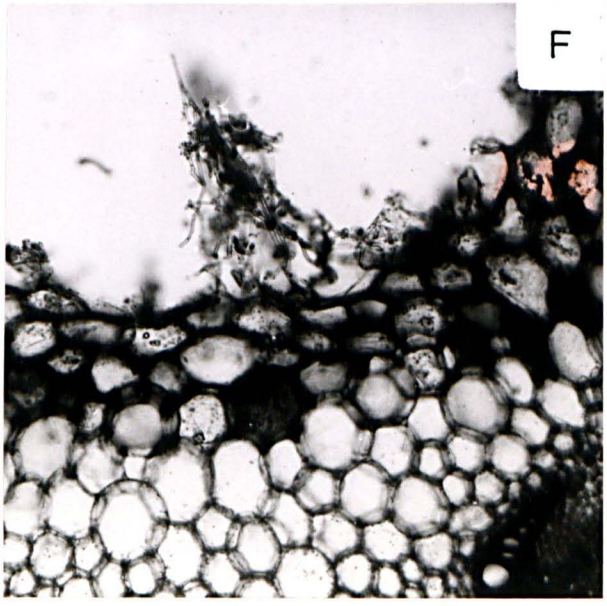
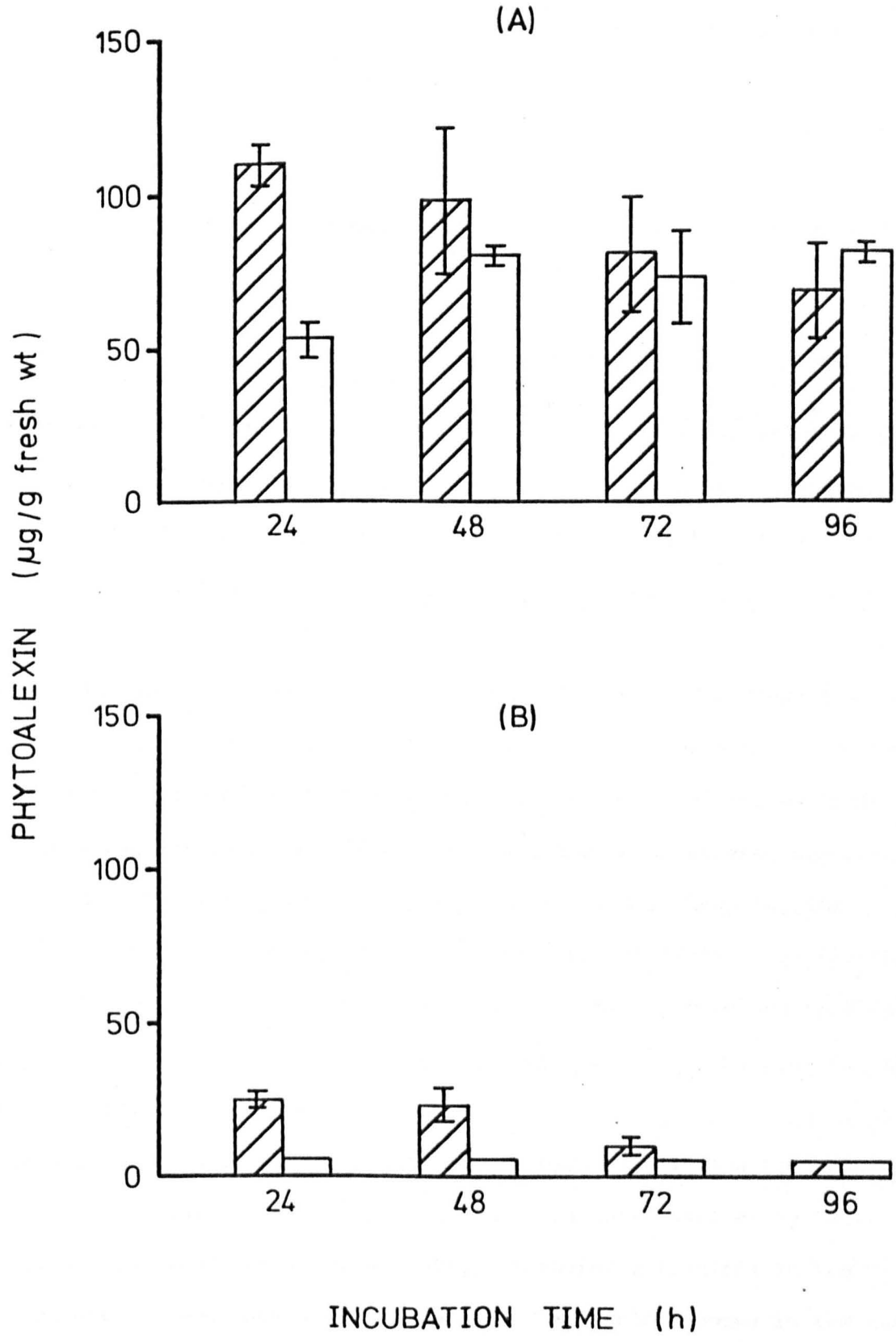

F

Fig. 3.2. Levels of kievitone () and phaseollin () in hypocotyls artificially-infected with (A) R. solani or (B) F. solani f.sp. phaseoli.

The inoculum contained c.5 or c.1 mg dry wt mycelium/ml for R. solani and F. solani f.sp. phaseoli, respectively.


(A)
150
100
50
0
24
48
72
96
(B)
150
100
50
0
24
48
72
96
PHYTOALEXIN (µg/g fresh wt)
INCUBATION TIME (h)

fell to trace amounts by 96 h; phaseollin never exceeded trace levels at any of the time periods assayed. Hypocotyls artificially-inoculated with sterile distilled water and assayed after the same incubation times did not yield either phytoalexin above trace levels.

DISCUSSION

The general pattern of disease development following natural infection of bean hypocotyls with either fungus, described above, was to a large extent consistent with earlier work (Boosalis, 1950; Christou, 1962; VanEtten *et al.*, 1967; Dodman & Flentje, 1970; Chatterjee, 1958; Christou & Snyder, 1962; Pierre, 1966; Pierre & Wilkinson, 1970). It was not initially intended to make a study of host penetration and ingress by either fungus, since they have both been well documented. However, during the search for a system suitable for the fixation, embedding, sectioning and staining of bean tissues to follow the course of infection in excised hypocotyl pieces (Section V), naturally-infected bean tissues were employed. A number of different staining procedures was tried and although limited success was experienced using carbol thionin-orange G (Stoughton, 1930 - described in Gurr, 1965) and periodic acid-decolourised basic fuchsin (Preece, 1959), the most satisfactory results were obtained with the Pianeze III B stain (Gurr, 1965). Even so, the complexity of the procedure was later considered unsatisfactory for routine observations, and since good results could be obtained by freehand sectioning of artificially-infected hypocotyls, this simpler method was adopted for the study of such material.

Likewise, accumulation of kievitone and phaseollin in the two natural disease situations largely confirmed the trends reported earlier by Smith *et al.* (1975) and VanEtten & Smith (1975). Temporal variations in the absolute amounts of each compound may reflect slight differences in the nature of the inoculum, the incubation temperature, and the fact that a different variety of bean was employed. However, the general pattern of events was

the same and the implications of the previous work remain. On the one hand, there was rapid accumulation of kievitone to levels sufficient to inhibit growth of R. solani in vitro at about the time fungal development appeared to cease, while on the other hand kievitone accumulation was either low or non-existent after infection by F. solani f.sp. phaseoli. Thus, whereas kievitone may contribute to the restriction of R. solani to local lesions in bean hypocotyls it can apparently play no role in comparable tissues infected with F. solani f.sp. phaseoli. Since it has now been demonstrated that F. solani f.sp phaseoli can efficiently metabolize kievitone to a less toxic product (Kuhn & Smith, 1976; Kuhn, Smith & Ewing, 1977), any low levels of kievitone present in bean hypocotyls infected by this fungus may soon have fallen below detectable limits. This may explain the inability of VanEtten & Smith (1975) to detect kievitone in F. solani f.sp. phaseoli-infected bean tissues, even after specific extraction for this phytoalexin 16 or 21 days after inoculation.

In the development of an artificial system for monitoring the eliciting potential of test solutions, tissues other than hypocotyls (e.g. seed pod cavities, leaves or cotyledons) could have been used but were discounted due to the possibility of erroneous inferences being drawn from results obtained using host tissues not normally encountered in the disease situation (Bailey & Deverall, 1971; Keen & Horsch, 1972; Kuć, 1972).

Artificial infection by either fungus using the cored hypocotyl system mirrored the observed course of the corresponding natural infection but produced a rather different pattern of phytoalexin accumulation. In response to R. solani, kievitone again accumulated rapidly and phaseollin rather less so. At the earlier time periods, both phytoalexins achieved higher levels than in the corresponding natural infection; thereafter, kievitone levels declined slowly while phaseollin appeared to plateau. Host metabolism could explain the gradual fall in kievitone levels, and may also contribute to the failure of phaseollin to accumulate above about 80 µg/g fresh wt (Stoessl,

Robinson, Rock & Ward, 1977; Glazener & VanEtten, 1978; Hargreaves & Selby, 1978). The fall in kievitone levels might also be attributed to metabolism by the fungus (E.M. McMorrow, P.J. Kuhn & D.A. Smith, personal communication), but the possible role of R. solani in metabolism of phaseollin in the tissues is unclear. Although Heuvel & Glazener (1975) found that phaseollin was removed from culture media of the fungus, the rate of disappearance was slower than that observed for some other bean pathogens and for one non-pathogen. Also, evidence from the natural infection situation would rule out rapid removal of phaseollin in situ by R. solani. It may be, therefore, that the relatively low phaseollin levels attained represent the expression of the maximum potential for phaseollin production in the excised hypocotyls. Alternatively, a deeper penetration than that achieved by the fungus (possibly as a result of the rapid accumulation of high levels of kievitone) may be necessary to stimulate fully phaseollin biosynthesis.

In F. solani f.sp. phaseoli-infected hypocotyls, the low amounts of kievitone that did accumulate declined to trace levels, presumably as a result of host or, more likely, fungal metabolism. The failure of phaseollin to accumulate above trace levels at any of the assay times may be due to the scale and rapidity of fungal invasion into the tissues. Unlike cored hypocotyls inoculated with R. solani, which remained firm throughout the whole incubation period, those inoculated with F. solani f.sp. phaseoli became browned and macerated very quickly. It is conceivable that quantifiable amounts of both phytoalexins were produced initially but that the fungus degraded them very efficiently leaving little of either compound to be detected. However, it seems more likely that despite the apparent disparity in the amount of inoculum applied to each hypocotyl, the inoculum potential, as defined by Garrett (1970), of the germinated F. solani f.sp. phaseoli sporelings exceeded that of the R. solani mycelial clumps, and in fact may have been so high (as evidenced by later use of lower spore concentrations) that widespread host cell death occurred very rapidly and

little phytoalexin biosynthesis was possible. The extent and rapidity of host cell death may explain the low phytoalexin levels observed in some other host-parasite interactions (Rahe, 1973; Hargreaves et al., 1977).

Too much emphasis should not be placed on a comparison of the natural and artificial infections of bean hypocotyls by either fungus. Natural infection produced results comparable with those of earlier studies, both in the pattern of disease development and in levels of phytoalexin accumulation. While this was not exactly mirrored in the artificial infections, this technique nonetheless provided a system whereby individual solutions or suspensions could be tested for phytoalexin-eliciting ability in bean hypocotyl tissues.

SECTION IV

ELICITATION BY CULTURE FILTRATES AND MYCELIAL EXTRACTS

The artificial inoculation system, described earlier, was employed to investigate the phytoalexin-eliciting activities of cell-free mycelial extracts and culture filtrates of *R. solani* and *F. solani* f.sp. *phaseoli*. Positive results were followed by partial purification of appropriate fractions in an attempt to determine the nature of the eliciting component(s). The effect of each purification step was observed not only from the response of inoculated hypocotyls but also by monitoring the levels of protein and carbohydrate in the extracts at each stage. A cell wall extract of *R. solani* was also investigated for phytoalexin-eliciting potential.

MATERIALS AND METHODS

Preparation of crude extracts:

Culture filtrates were prepared by vacuum-filtration of liquid media, from flasks containing mycelial suspensions of *R. solani* or *F. solani* f.sp. *phaseoli*, through 0.8 μm filters (Millipore). The filtrates were concentrated by evaporation under reduced pressure at c. 40°C prior to their introduction into bean hypocotyls. Uninoculated media, concentrated by corresponding amounts, served as controls.

Mycelial extracts of either fungus were prepared by homogenizing mycelia, harvested from liquid cultures, in sterile distilled water (10 ml/culture) using Ultra-turrax and hand-held Teflon homogenizers. Mycelial debris was separated from the resulting suspension by vacuum-filtration employing glass-fibre (Whatman GF/A or GF/C) and 0.8 μm filters. The filtrates (i.e. cell-

free extracts) produced were either used directly for inoculation or, more often, concentrated *in vacuo* at c. 40°C before application to hypocotyls. In either case, sterile distilled water was used as the control.

The total dry wt of homogenized fungus, estimated from the weights of aliquots removed prior to extraction, allowed direct comparisons to be made between the phytoalexin-eliciting abilities of different mycelial extracts. Culture filtrates were concentrated so as to provide preparations which contained, on a volume for volume basis, material derived from an approximately equal dry wt of mycelium. Thus direct comparisons of elicitor activities in culture filtrates and mycelial extracts could reasonably be made.

Random plating of samples of either culture filtrates or mycelial extracts on Oxoid nutrient agar (NA) or PDA, with or without incorporated antibiotics (chloramphenicol and streptomycin sulphate at 100 μg/ml; Gladders, 1976), followed by incubation at 25°C for up to five days, revealed occasional bacterial contamination on the non-antibiotic plates. However, no fungal development was observed at any time. Also, when samples were examined microscopically, neither hyphae nor individual fungal cells were observed.

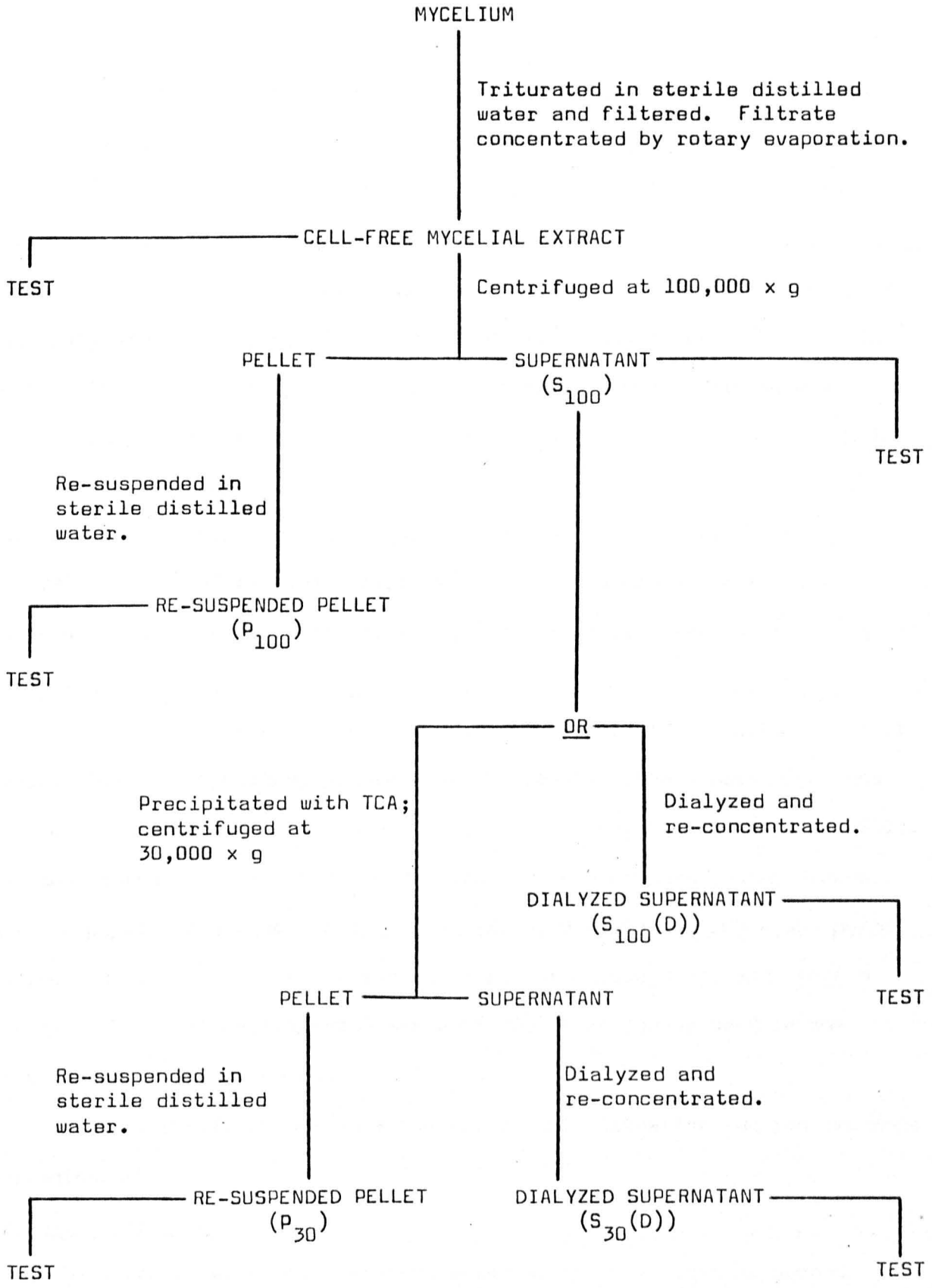
Partial purification of crude mycelial extracts:

To facilitate handling during fractionation, the crude mycelial extract was concentrated to a smaller volume by rotary evaporation at c. 40°C. Thus, aliquots removed for measurement of elicitor activity at each stage of purification were appropriately diluted with sterile distilled water prior to inoculation into bean hypocotyls.

An outline of the procedure used for fractionation of crude extracts from *R. solani* and *F. solani* f.sp. *phaseoli* is given in Fig. 4.1. Cellular fragments and cytoplasmic contents not in solution were removed from the concentrated crude cell-free preparation by centrifugation at 100,000 x g

Fig. 4.1. Procedures for fractionation of mycelial extracts of *R. solani* and *F. solani* f.sp. *phaseoli*.

"TEST" indicates the removal of a small aliquot which was subsequently diluted with sterile distilled water prior to both inoculation into bean hypocotyls and determination of carbohydrate and protein.


MYCELIUM
Triturated in sterile distilled water and filtered. Filtrate concentrated by rotary evaporation.
CELL-FREE MYCELIAL EXTRACT
TEST
Centrifuged at 100,000 x g
PELLET
SUPERNATANT
(S_{100})
TEST
Re-suspended in sterile distilled water.
RE-SUSPENDED PELLET
(P_{100})
TEST
OR
Precipitated with TCA; centrifuged at 30,000 x g
Dialyzed and re-concentrated.
DIALYZED SUPERNATANT
(S_{100}(D))
TEST
PELLET
SUPERNATANT
TEST
Re-suspended in sterile distilled water.
Dialyzed and re-concentrated.
RE-SUSPENDED PELLET
(P_{30})
DIALYZED SUPERNATANT
(S_{30}(D))
TEST
TEST

for 1 h at 5°C. The resulting supernatant, S_{100}, was carefully decanted and the pellet, P_{100}, re-suspended in a volume of sterile distilled water equal to that of the recovered supernatant. The supernatant was subsequently dialyzed against 5 l distilled water at 4°C for 20 h. Since this generally resulted in an increase in volume of the material inside the dialysis bag, the non-dialyzable solution was concentrated back to the original volume *in vacuo* at c. 40°C. This allowed subsequent comparisons to be made between the dialyzed material, $S_{100}(D)$, and non-dialyzed supernatant (S_{100}). In certain instances, just prior to dialysis, protein precipitation was accomplished using trichloroacetic acid (TCA). An equal volume of 10% TCA was added to the supernatant and the mixture was allowed to stand at 4°C for 20 min. The precipitate formed was removed by centrifugation at 30,000 x g for 20 min.; as before, the pellet, P_{30}, was re-suspended in sterile distilled water and the dialyzed supernatant, $S_{30}(D)$, re-concentrated to the original volume.

Carbohydrate levels were monitored by the phenol-sulphuric acid test (Dubois, Gilles, Hamilton, Rebers & Smith, 1956); protein determinations were carried out by the method of Lowry, Rosebrough, Farr & Randall (1951). Standard curves for carbohydrate and protein were prepared using glucose, 0 - 750 μg/ml, or bovine serum albumen (BSA), 0 - 100 and 100 - 500 μg/ml, respectively. Results are quoted as levels of carbohydrate and protein (in glucose or BSA equivalents) per unit dry wt of fungus used in the preparation of the crude cell-free extract.

Further purification was attempted by gel filtration and ion exchange chromatography:-

(a) Gel filtration

Dialyzed supernatants obtained after either the first or second centrifugation steps were concentrated to small volumes (3 - 4 ml) before application to gel filtration columns. Initial studies involved a 1.6 x 30 cm

column of Sephadex G100 (Pharmacia) equilibrated in distilled water. Later experiments involved a 2 x 50 cm column of Sephadex G75 equilibrated in distilled water; distilled water was also used as the eluant at a flow rate of c. 0.5 ml/min. Either 5 ml or 10 ml fractions were collected and monitored for protein, carbohydrate and phytoalexin-eliciting activity. In a further study, active fractions from G75 columns were combined, concentrated and passed down a 1.6 x 30 cm column of Sephadex G50, equilibrated and run in distilled water. Void volumes (V_0) were determined from the elution profile of Blue Dextran (Pharmacia) measured by absorption at 254 nm (A_{254}). Inclusion volumes (V_I) were determined either by elution of acetone (A_{260}) or potassium dichromate (A_{366}).

(b) Ion exchange

This preliminary study involved batch preparation of DEAE-Sephadex A25 anion exchanger (Pharmacia) in 10 mM Tris-HCl buffer, pH 8.0. A concentrated elicitor preparation, obtained from gel filtration of fractionated extracts of R. solani mycelium on Sephadex G75 and G50, was prepared at 10 mM by the addition of 50 mM buffer (pH 8.0), before application to the anion exchanger. After stirring and allowing to settle, the solution was removed by suction and passed through 0.8 μm filters to preclude Sephadex beads. Thereafter, elution of material from the exchanger was attempted with four 5 ml aliquots of 10 mM buffer (pH 8.0) containing NaCl at 0, 25, 50, 100 and 400 mM. The solution recovered from each step was dialyzed and re-concentrated to the original volume for comparison of carbohydrate and protein content and phytoalexin-eliciting activity with the original preparation.

Preparation of an elicitor from cell walls of R. solani:

Mycelium of R. solani was subjected to a treatment essentially similar to that previously described by Ayers et al. (1976b) and Anderson-Prouty & Albersheim (1975) for the production of phytoalexin elicitors from fungal

cell walls. The residue from the first filtration during the preparation of a mycelial extract of R. solani was re-homogenized in distilled water (5 ml/culture). Insoluble material was collected by vacuum-filtration on a sintered glass funnel, washed with 200 ml distilled water and again collected on the filter. The washing procedure was repeated three times, twice with chloroform/methanol (1:1) and finally with acetone. The final washed preparation was left to dry overnight at 25°C; this preparation is referred to as "mycelial walls". A total of 3.75 g mycelial walls was obtained from approximately 70 g fresh wt of mycelium.

One gram of mycelial walls was suspended in 100 ml sterile distilled water and shaken vigorously to obtain even dispersal. After removal of a small aliquot of this suspended material for introduction into bean hypocotyls, the remainder was autoclaved at 103 kN/m^2 for 1 h. The autoclaved preparation was filtered through sintered glass and 0.8 μm filters. The resulting light brown filtrate was adjusted back to the original volume with sterile distilled water; this preparation is referred to as "solubilized walls". Concentration and dialysis of the solubilized wall preparation was achieved as described for mycelial extracts, the volume after dialysis being re-adjusted back to the original before inoculation into beans.

RESULTS

Phytoalexin accumulation in hypocotyls treated with crude preparations:

The levels of kievitone and phaseollin attained in detached bean hypocotyls 40 h after exposure to various concentrations of culture filtrates of R. solani or liquid medium are shown in Table 4.1. Culture filtrates consistently elicited higher levels of both compounds than did medium alone. Generally, greater amounts of phytoalexin accumulated as the culture filtrate concentration was increased. The medium brought about only modest accumulation of phytoalexins.

Table 4.1. Levels of kievitone and phaseollin in hypocotyls 40 h after treatment with culture filtrates of R. solani or with liquid medium.

Inoculum	Kievitone	Phaseollin
	(µg/g fresh wt)	
Medium (x 4-5)	23 ± 7	16 ± 3
Culture filtrate (x 4-5)	74 ± 3	31 ± 6
Medium (x 10)	54 ± 12	19 ± 2
Culture filtrate (x 10)+	133 ± 1	56 ± 4
Medium (x 50)	24 ± 2	8 ± 1
Culture filtrate (x 50)	172 ± 8	38 ± 1

+ The preparation contained material derived from c. 100 mg dry wt mycelium/ml. Additional solution was added to the hypocotyls 14 and 24 h after the first introduction.

In response to mycelial extracts of R. solani, the levels of kievitone detected were appreciably higher than with culture filtrates (Table 4.2). Phaseollin accumulation, however, remained fairly low. The amounts of both compounds increased as the mycelial extract was concentrated. Although autoclaving seemed to reduce the kievitone-eliciting activity of these extracts by approximately half, phaseollin stimulation seemed unaffected. Distilled water did not cause the accumulation of kievitone or phaseollin above trace levels during this or any subsequent experiment.

Differences in the response of hypocotyls to inoculation with different test materials were manifest not only in observed differences in phytoalexin accumulation, but also in the appearance of the tissues lining the cavity walls. Hypocotyl cavity walls did not generally become discoloured at any time after exposure to distilled water. However, cells lining the cavities of hypocotyls inoculated with culture filtrates, or to a lesser extent liquid medium, exhibited various degrees of browning. Higher concentrations of

Table 4.2. Levels of kievitone and phaseollin in hypocotyls 40 h after treatment with mycelial extracts of R. solani.

Inoculum	Kievitone	Phaseollin
	(μg/g fresh wt)	
Distilled water	Trace	Trace
Mycelial extract*	360 ± 6	48 ± 17
Autoclaved mycelial extract*	173 ± 21	42 ± 16
Mycelial extract+	494 ± 6	61 ± 2
Autoclaved mycelial extract+	294 ± 29	62 ± 3

* The preparation contained material derived from c. 50 mg dry wt mycelium/ml.

\+ The preparation contained material derived from c.100 mg dry wt mycelium/ml. Additional solution was added to the hypocotyls 14 and 24 h after the first introduction.

these preparations produced a more intense discolouration of host cells. Mycelial extracts also caused discolouration of bean tissues, but the response appeared to be less well marked.

The accumulation of kievitone and phaseollin in hypocotyls after treatment with a culture filtrate or mycelial extract of F. solani f.sp. phaseoli is shown in Table 4.3. The amount of kievitone produced in response to the culture filtrate was not appreciably different from that stimulated by medium alone, both levels being low. Likewise, kievitone accumulation after treatment with the mycelial extract was slight. While phaseollin levels exceeded those of kievitone in all cases, the amounts present were nonetheless small.

Phytoalexin levels determined in hypocotyls exposed to mycelial extracts of either fungus and incubated for time periods in excess of 40 h are given in Fig. 4.2. The high levels of kievitone detected initially in response to extracts of R. solani declined slightly up to 120 h after inoculation (Fig. 4.2,A). The accumulation of phaseollin in these tissues remained

Fig. 4.2. Levels of kievitone () and phaseollin () in hypocotyls inoculated with mycelial extracts of (A) R. solani or (B) F. solani f.sp. phaseoli.

The inoculum contained material derived from c.75 or c.50 mg dry wt mycelium/ml of R. solani or F. solani f.sp. phaseoli, respectively.

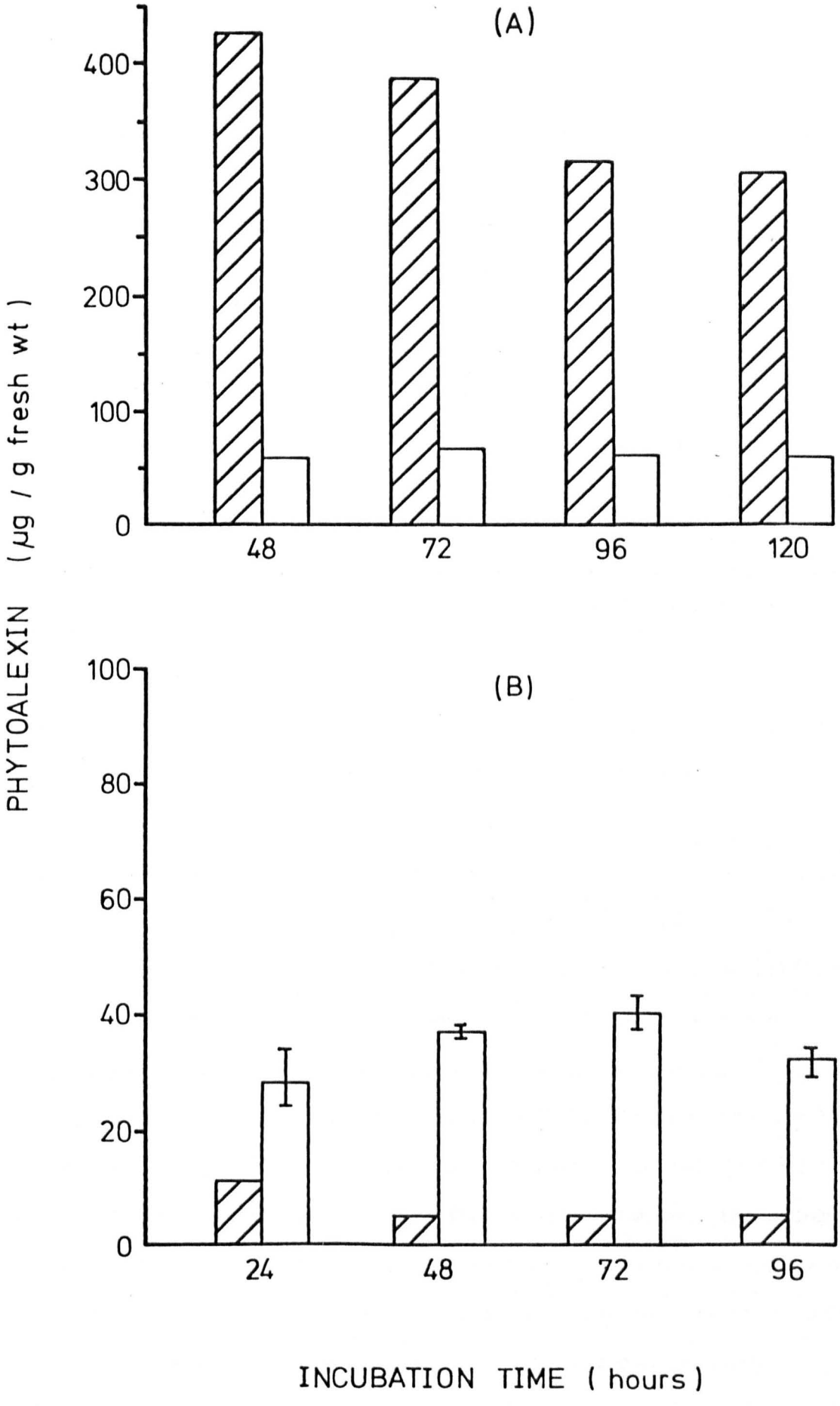

(A)
400
300
200
100
0
48
72
96
120
(B)
100
80
60
40
20
0
24
48
72
96
PHYTOALEXIN (µg / g fresh wt)
INCUBATION TIME (hours)

Table 4.3. Levels of kievitone and phaseollin in hypocotyls 40 h after treatment with a culture filtrate or mycelial extract of *F. solani* f.sp. *phaseoli*.

Inoculum	Kievitone	Phaseollin
	(μg/g fresh wt)	
Distilled water	Trace	Trace
Medium (x 10)	15 ± 1	24 ± 4
Culture filtrate (x 10)[+]	19 ± 1	29 ± 1
Mycelial extract[+]	7 ± 2	29 ± 3

[+] The preparation contained material derived from c. 50 mg dry wt mycelium/ml. Additional solution was added to the hypocotyls 14 and 24 h after the first introduction.

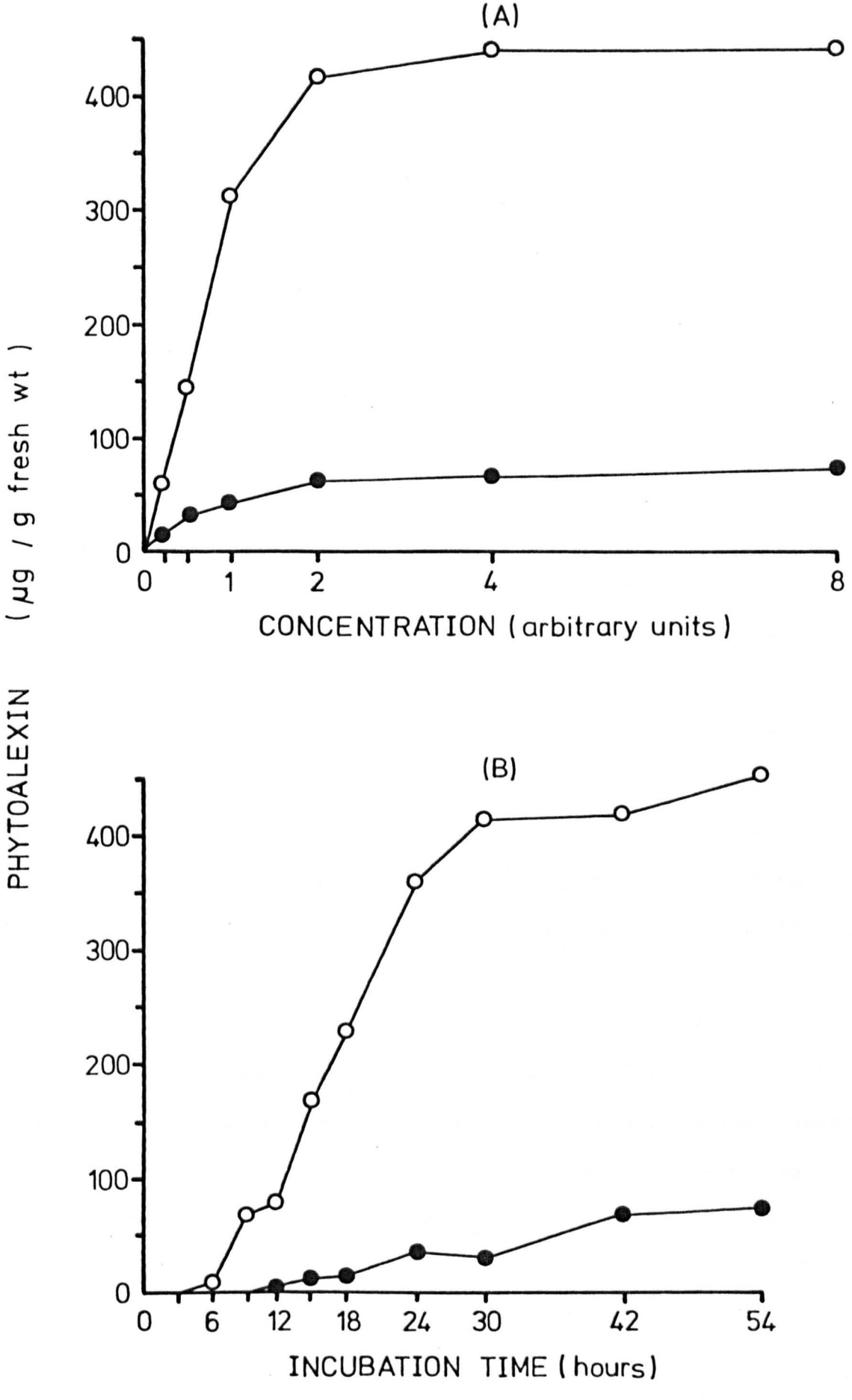
constant, never exceeding 70 μg/g fresh wt. In contrast, hypocotyls inoculated with extracts of *F. solani* f.sp. *phaseoli* contained little or no kievitone and only modest amounts of phaseollin (Fig. 4.2,B).

Phytoalexin formation in hypocotyls after inoculation with a concentration series of *R. solani* mycelial extract is shown in Fig. 4.3. A time course of phytoalexin production in hypocotyls inoculated with a single concentration of a similarly-prepared extract is also shown in this figure. For the concentration series, mycelial extract (prepared as outlined earlier) was either concentrated by rotary evaporation at c. 40°C or diluted with sterile distilled water to give a range of solutions containing material derived from c. 7.5 up to c.250 mg dry wt mycelium/ml. The results show that the accumulation of both kievitone and phaseollin appears to plateau in response to approximately the same concentration of mycelial extract, but that kievitone reaches much higher levels than phaseollin (Fig. 4.3,A). In the time course study (Fig. 4.3,B), kievitone was first detected 6 h after inoculation. Thereafter accumulation was almost linear up to 24-30 h, after which time it levelled off.

Fig. 4.3. Levels of kievitone (—○—) and phaseollin (—●—) in hypocotyls inoculated with mycelial extracts of *R. solani*.

(A) Concentration series: A concentration of 1 arbitrary unit represents material derived from c.30 mg dry wt mycelium/ml. Hypocotyls were incubated for 40 h after inoculation.

(B) Time course: The inoculum contained material derived from c.75 mg dry wt mycelium/ml.


(A)
400
300
200
100
0
0
1
2
4
8
CONCENTRATION (arbitrary units)
PHYTOALEXIN (µg / g fresh wt)
(B)
400
300
200
100
0
0
6
12
18
24
30
42
54
INCUBATION TIME (hours)

Phaseollin was first noted after 12 h; levels increased gradually throughout the incubation period. The maximum phaseollin level attained, however, was only 75 µg/g fresh wt, much lower than that for kievitone.

Phytoalexin accumulation in hypocotyls treated with a cell wall extract of R. solani:

Table 4.4 shows the amounts of phytoalexin occurring in response to a mycelial extract, culture filtrate or cell wall preparation of R. solani. Phytoalexin elicitation by the mycelial extract, culture filtrate or control medium compared favourably with the patterns observed previously (Tables 4.1 and 4.2). While the suspended walls gave rise to moderate levels of kievitone and phaseollin, accumulation of both these compounds was greater in response to the solubilized wall preparation.

Dialysis (against 2 l distilled water at 4°C for 20 h) was carried out on the culture filtrate, mycelial extract and solubilized walls. During dialysis, a precipitate formed in the mycelial extract; this was removed by centrifugation and re-suspended in a volume of sterile distilled water equal to that of the supernatant. All fractions, and their corresponding re-concentrated dialysates were tested for their phytoalexin-eliciting activity; the results are shown in Table 4.5. The mycelial extract again brought about the highest level of kievitone accumulation, although the magnitude of the response was reduced. The culture filtrate and solubilized wall preparation both maintained their earlier activities. Although some phytoalexin accumulation occurred in response to all the dialysates, only the culture filtrate exhibited low mol wt kievitone-eliciting activity which compared to that of the corresponding non-dialyzable material.

Table 4.4. Levels of kievitone and phaseollin in hypocotyls 40 h after treatment with a mycelial extract, culture filtrate or cell wall preparation of R. solani.

Inoculum	Kievitone	Phaseollin
	(μg/g fresh wt)	
Medium (control) (x 12)	22	26
Culture filtrate (x 12)+	111	63
Mycelial extract+	389	68
Suspended walls*	88	39
Solubilized walls*	141	55

+ The preparation contained material derived from c.150 mg dry wt mycelium/ml.

* For explanation see text.

Table 4.5. Levels of kievitone and phaseollin in hypocotyls 40 h after treatment with dialysates or dialyzed preparations of culture filtrate, mycelial extract or solubilized walls of R. solani.

Inoculum	Kievitone	Phaseollin
	(μg/g fresh wt)	
Culture filtrate+	117	41
Culture filtrate (dialysate)+	99	56
Mycelial extract:+		
(a) Supernatant	266	39
(b) Pellet	25	42
Mycelial extract (dialysate)+	63	28
Solubilized walls*	176	39
Solubilized walls (dialysate)*	53	35

+ The preparation contained material derived from c.150 mg dry wt mycelium/ml.

* For explanation see text.

Phytoalexin accumulation in hypocotyls treated with partially-purified fractions:

Preliminary fractionation of a mycelial extract of R. solani yielded preparations with phytoalexin-eliciting abilities as shown in Table 4.6.

Table 4.6. Levels of kievitone and phaseollin in hypocotyls 40 h after treatment with a fractionated mycelial extract of R. solani.

Inoculum	Kievitone	Phaseollin
	(μg/g fresh wt)	
Mycelial extract+	376	74
S_{100}*	287	52
P_{100}*	321	63
$S_{100}(D)$*	216	44

+ The preparation contained material derived from c.125 mg dry wt mycelium/ml.
* For explanation see text.

Gel filtration through Sephadex G100 of the dialyzed supernatant, $S_{100}(D)$, revealed a single broad peak of kievitone-eliciting activity (Fig. 4.4).

Results of similar fractionations, where samples were assayed for total carbohydrate and protein as well as phytoalexin-eliciting ability, are presented in Table 4.7. The supernatant, S_{100}, and re-suspended pellet, P_{100}, both elicited high kievitone levels. Dialysis reduced the kievitone-eliciting ability of the supernatant. However, while kievitone accumulation dropped by approximately 30%, protein content fell by c. 50% and the carbohydrate level by almost 80%. Levels of phaseollin accumulation were again much lower than those for kievitone. When samples of the dialyzed supernatant, $S_{100}(D)$, were subjected to gel filtration on Sephadex G75, elution profiles of total protein, carbohydrate and phytoalexin-eliciting activity were obtained

Fig. 4.4. Elution profile of phytoalexin-eliciting activity after gel filtration of the S_{100}(D) fraction* from a mycelial extract of R. solani.

Kievitone (—●—) and phaseollin (- -△- -) levels were determined 40 h after inoculation of individual 10 ml eluates into bean hypocotyls.

The column comprised 1.6 x 30 cm Sephadex G100 equilibrated and run in distilled water.

* For explanation see text.

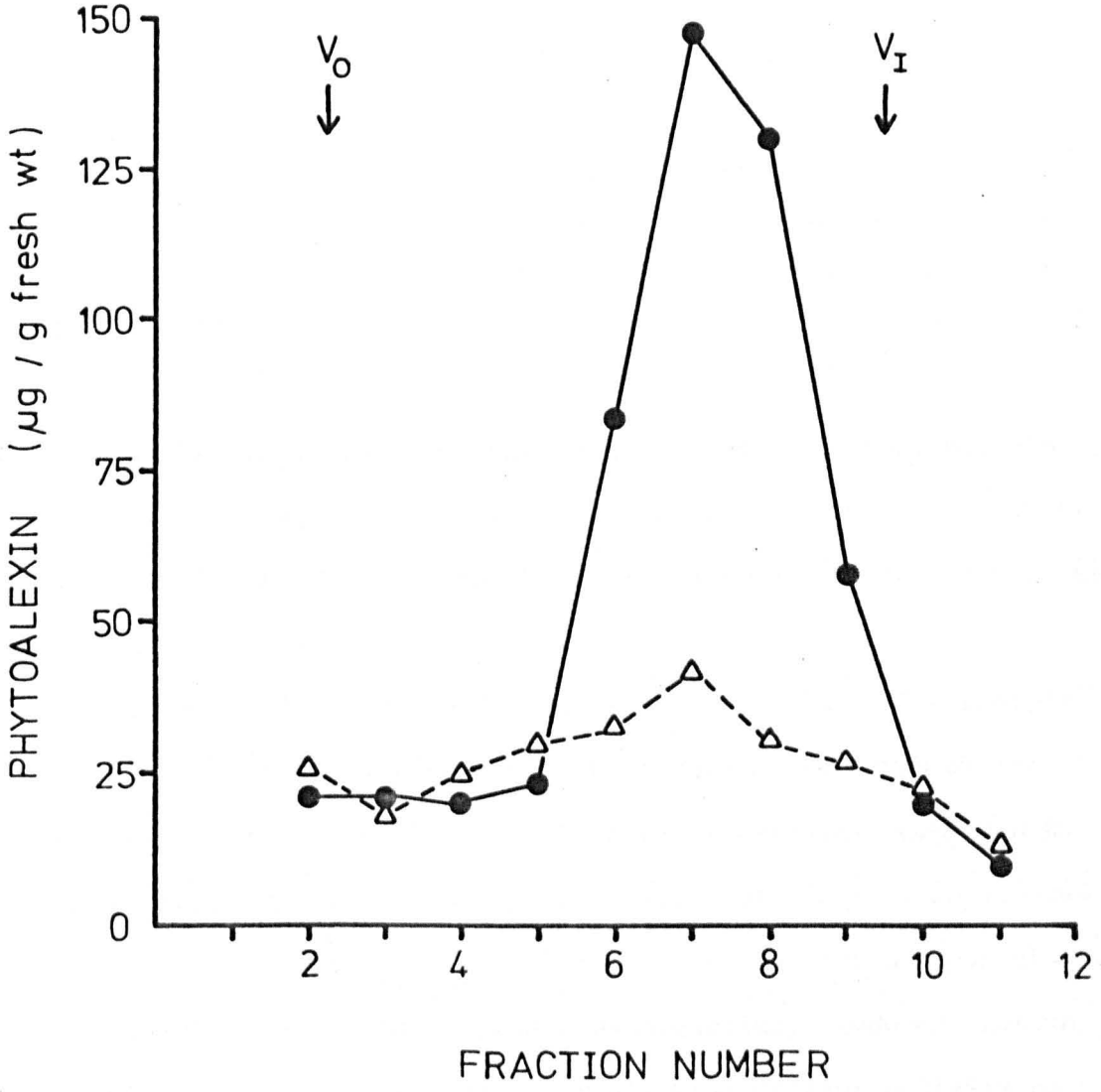

150
125
100
75
50
25
0
V_O
V_I
PHYTOALEXIN (µg / g fresh wt)
2
4
6
8
10
12
FRACTION NUMBER

Table 4.7. Levels of total carbohydrate and protein in fractionated mycelial extracts of *R. solani* and levels of kievitone and phaseollin in hypocotyls 40 h after treatment with these extracts.

Inoculum	Carbohydrate	Protein	Kievitone	Phaseollin
	(mg/g dry wt fungus)		(µg/g fresh wt)	
Mycelial extract+	335 ± 18	54 ± 5	413 ± 19	69 ± 4
S_{100}*	184 ± 23	43 ± 7	337 ± 22	61 ± 8
P_{100}*	231 ± 22	20 ± 2	356 ± 22	61 ± 9
S_{100}(D)*	42 ± 7	24 ± 3	249 ± 15	51 ± 2

+ The preparation contained material derived from c.125 mg dry wt mycelium/ml.
* For explanation see text.

as in Fig. 4.5. Two major peaks of carbohydrate were observed; protein distribution was more regular (Fig. 4.5,A). Kievitone-eliciting activity occurred as a single broad peak, while phaseollin accumulation was more evenly distributed (Fig. 4.5,B).

Results given in Table 4.8 show that the introduction of a protein precipitation step into the fractionation procedure caused much of the Lowry-positive material in the S_{100} fraction to be removed. Compared to material remaining in solution, this precipitated material showed reduced kievitone-eliciting activity. Gel filtration of the dialyzed supernatant, S_{30}(D), under conditions similar to those used earlier, produced elution profiles of carbohydrate, protein and phytoalexin-eliciting activity as shown in Fig. 4.6. Although most of the higher mol wt protein, and a certain amount of carbohydrate, appeared to have been removed, two major peaks of carbohydrate remained (Fig. 4.6,A). Phaseollin accumulation was generally low, but the peak of kievitone-eliciting activity persisted (Fig. 4.6,B).

In all cases, kievitone-eliciting ability and the degree of host tissue discolouration was correlated with brown pigmentation of the eluates.

Fig. 4.5. Elution profiles of protein, carbohydrate and phytoalexin-eliciting activity after gel filtration of the $S_{100}(D)$* fraction from mycelial extracts of R. solani.

(A) Levels of carbohydrate (—○—) and protein (--▲--) in each 10 ml eluate.

(B) Levels of kievitone (—●—) and phaseollin (--△--) in hypocotyls 40 h after treatment with individual 10 ml eluates.

The column comprised 2 x 50 cm Sephadex G75 equilibrated and run in distilled water.

* For explanation see text.

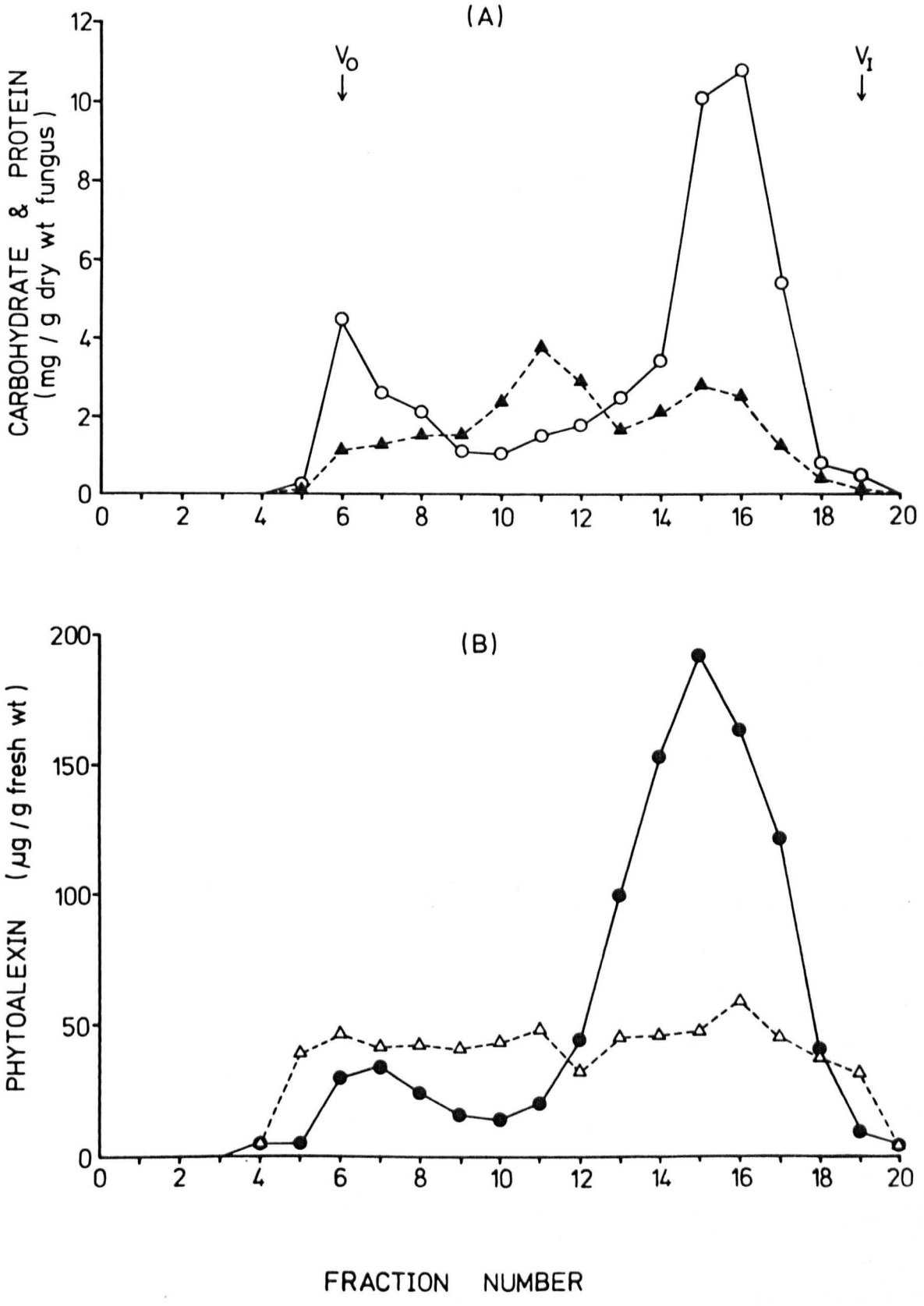

(A)
V_O
V_I
CARBOHYDRATE & PROTEIN
(mg / g dry wt fungus)
0
2
4
6
8
10
12
0
2
4
6
8
10
12
14
16
18
20
(B)
PHYTOALEXIN (µg / g fresh wt)
0
50
100
150
200
0
2
4
6
8
10
12
14
16
18
20
FRACTION NUMBER

Fig. 4.6. Elution profiles of protein, carbohydrate and phytoalexin-eliciting activity after gel filtration of the $S_{30}(D)$* fraction from mycelial extracts of R. solani.

(A) Levels of carbohydrate (—○—) and protein (--▲--) in each 10 ml eluate.

(B) Levels of kievitone (—●—) and phaseollin (--△--) in hypocotyls 40 h after treatment with individual 10 ml eluates.

The column comprised 2 x 50 cm Sephadex G75 equilibrated and run in distilled water.

* For explanation see text.

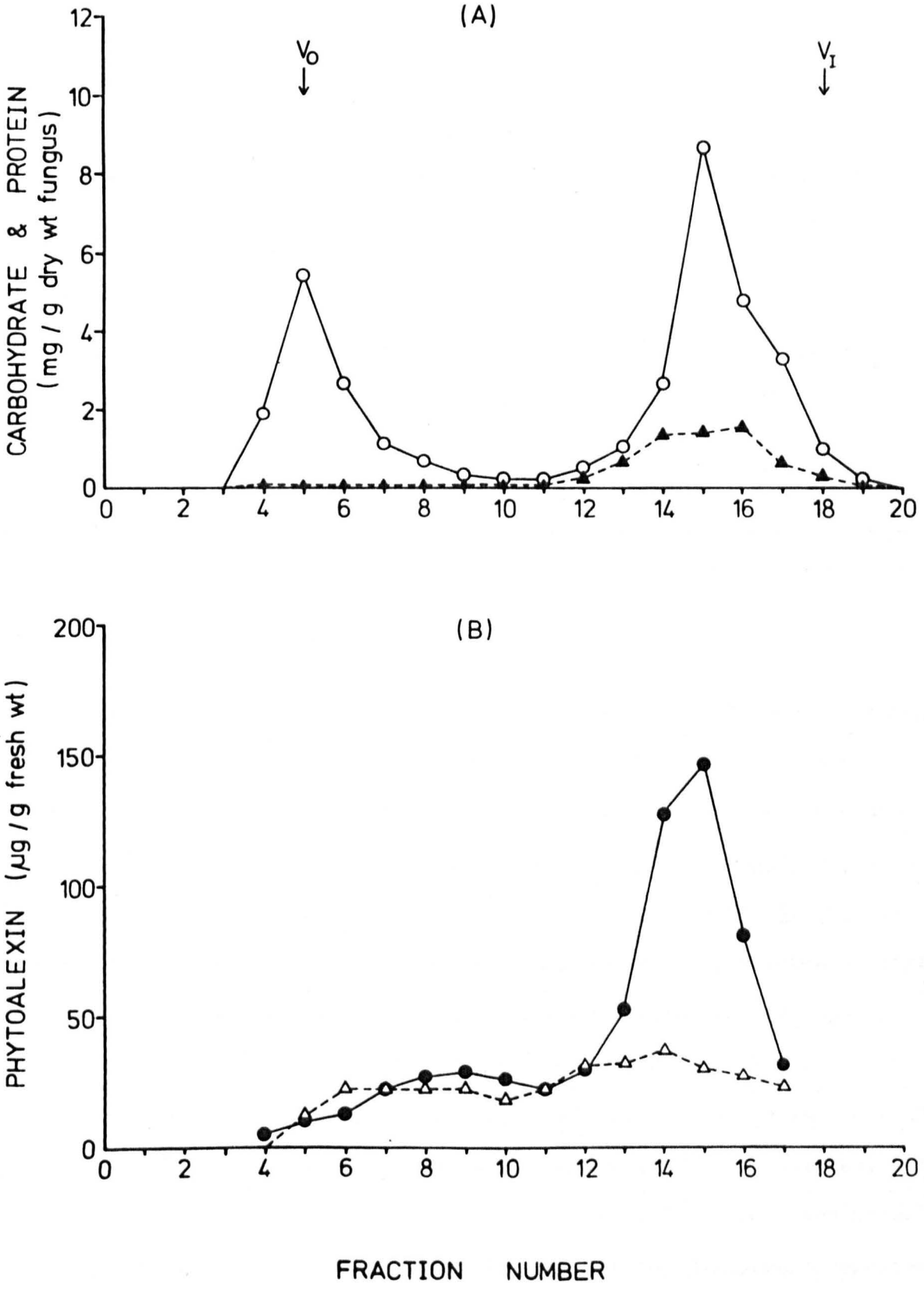

(A)
V_O
V_I
CARBOHYDRATE & PROTEIN
(mg / g dry wt fungus)
0
2
4
6
8
10
12
0
2
4
6
8
10
12
14
16
18
20
(B)
PHYTOALEXIN (µg / g fresh wt)
0
50
100
150
200
0
2
4
6
8
10
12
14
16
18
20
FRACTION NUMBER

Table 4.8. Levels of total carbohydrate and protein in fractionated mycelial extracts of R. solani (including TCA precipitation) and levels of kievitone and phaseollin in hypocotyls 40 h after treatment with these extracts.

Inoculum	Carbohydrate	Protein	Kievitone	Phaseollin
	(mg/g dry wt fungus)		(µg/g fresh wt)	
Mycelial extract+	342 ± 20	57 ± 10	392 ± 31	74 ± 6
S_{100}*	204 ± 28	49 ± 11	278 ± 25	51 ± 11
P_{100}*	237 ± 29	24 ± 5	323 ± 17	57 ± 7
S_{30}(D)*	32 ± 10	7 ± 0.5	221 ± 23	39 ± 4
P_{30}*	4 ± 0.5	13 ± 1	87 ± 8	48 ± 5

+ The preparation contained material derived from c.150 mg dry wt mycelium/ml.

* For explanation see text.

In contrast to R. solani, fractions from mycelial extracts of F. solani f.sp. phaseoli did not elicit high levels of kievitone. In an attempt to minimize differences between the fungi arising from the use of different media or culture methods, mycelial discs of F. solani f.sp. phaseoli were grown in the medium and culture conditions routinely used for R. solani. After 10 days, mycelial mats macroscopically similar in appearance to those formed by R. solani had been produced. These were then fractionated and assayed as outlined previously; the results are shown in Table 4.9. Kievitone accumulation in response to all fractions was low; even phaseollin levels were only moderate. While the measured quantity of protein was not too dissimilar from the amounts found in extracts of R. solani, the carbohydrate levels were much lower. Gel filtration of the dialyzed supernatant confirmed this (Fig. 4.7). Low elution profiles of both carbohydrate and protein (Fig. 4.7,A) were reflected in only low levels of kievitone and phaseollin (Fig. 4.7,B).

Fig. 4.7. Elution profiles of protein, carbohydrate and phytoalexin-eliciting activity after gel filtration of the $S_{100}(D)$* fraction from mycelial extract of F. solani f.sp. phaseoli.

(A) Levels of carbohydrate (—○—) and protein (--▲--) in each 10 ml eluate.

(B) Levels of kievitone (—●—) and phaseollin (--△--) in hypocotyls 40 h after treatment with individual 10 ml eluates.

The column comprised 2 x 50 cm Sephadex G75 equilibrated and run in distilled water.

* For explanation see text.

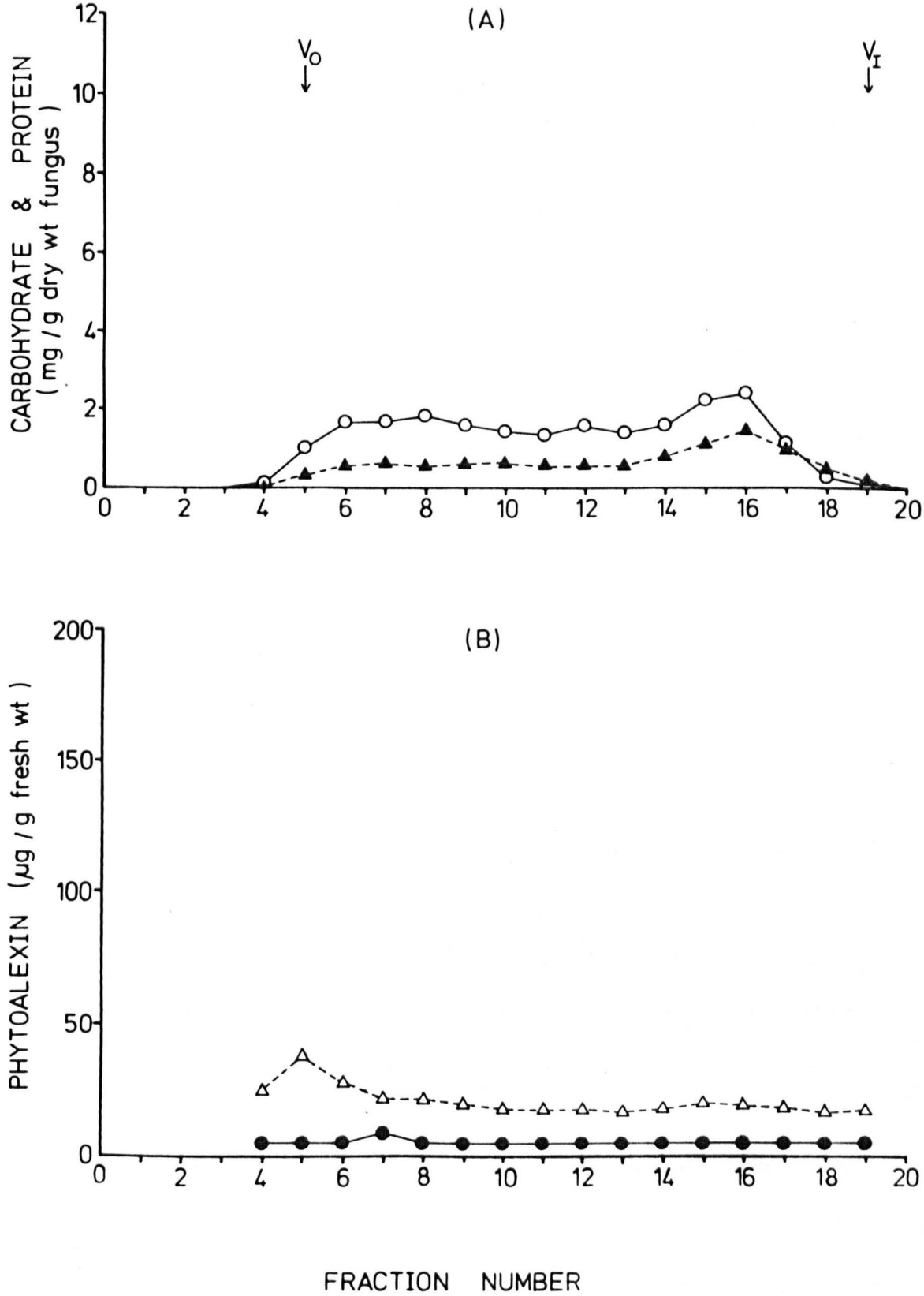

(A)
V_O
V_I
CARBOHYDRATE & PROTEIN
(mg / g dry wt fungus)
0
2
4
6
8
10
12
0
2
4
6
8
10
12
14
16
18
20
(B)
PHYTOALEXIN (µg / g fresh wt)
0
50
100
150
200
0
2
4
6
8
10
12
14
16
18
20
FRACTION NUMBER

Table 4.9. Levels of total carbohydrate and protein in fractionated mycelial extracts of F. solani f.sp. phaseoli and levels of kievitone and phaseollin in hypocotyls 40 h after treatment with these extracts.

Inoculum	Carbohydrate	Protein	Kievitone	Phaseollin
	(mg/g dry wt fungus)		(µg/g fresh wt)	
Mycelial extract+	56 ± 6	40 ± 4	14 ± 3	36 ± 1
S_{100}*	39 ± 7	29 ± 4	7 ± 2	35 ± 6
P_{100}*	18 ± 2	17 ± 3	12 ± 6	42 ± 5
S_{100}(D)*	18.5 ± 0.5	7 ± 2	7 ± 2	29 ± 5

+ The preparation contained material derived from c.75 mg dry wt mycelium/ml.

* For explanation see text.

None of the recovered fractions from F. solani f.sp. phaseoli was noticeably pigmented, nor produced appreciable browning in the hypocotyl tissues.

Ion exchange:

The elicitor preparation used for the ion exchange study initially stimulated kievitone accumulation to c.140 µg/g fresh wt after 40 h. It contained carbohydrate and protein at 6.40 and 1.32 mg/ml, respectively. Two millilitres were used in the experiment, and the results of protein and carbohydrate tests on the recovered fractions are given in Table 4.10. Approximately 40% of the protein and less than 10% of the applied carbohydrate were recovered. None of the fractions stimulated either kievitone or phaseollin accumulation above trace levels after 40 h.

Table 4.10. Levels of protein and carbohydrate remaining in fractions used to elute the elicitor from DEAE-Sephadex A25 in 10 mM Tris-HCl buffer (pH 8.0) after dialysis.

NaCl (mM)	Carbohydrate (μg/ml)	Protein (μg/ml)
0	555	180
25	25	135
50	25	105
100	15	70
400	15	80

DISCUSSION

As observed earlier (Section III), hypocotyls treated with distilled water failed to accumulate quantifiable amounts of kievitone or phaseollin. Thus, despite physical injury and the possibility of contamination, little "background" phytoalexin accumulation is inherent in the artificial inoculation system. The higher levels of phytoalexin determined in response to culture medium (Tables 4.1 and 4.3) might reflect indirect stimulation arising from enhanced growth of bacterial contaminants, due to the increased nutrient status of the inoculum. However, Hargreaves & Bailey (1978), in studies employing a similar inoculation technique, but using hypocotyls grown under sterile conditions or inocula comprising suspensions of cultured bacterial contaminants, failed to find levels of phytoalexin accumulation appreciably different from those obtained when tissues were inoculated with only sterile water. It is also possible that a component in the medium could directly stimulate phytoalexin accumulation; certainly, as discussed earlier, many chemical stimuli can cause this effect. Here, the high concentration of glucose, in particular, suggests the possibility of sugar stimulation. However, separate studies have shown that phytoalexin accumulation and tissue

browning did not occur when host tissues were exposed to a range of different sugars and related compounds (Ayers et al., 1976a; Lisker & Kuć, 1977). It seems more likely, therefore, that media in general might elicit phytoalexin accumulation indirectly by imposing a stress on host cells, e.g. via osmotic potential. Thus, metabolites released from stressed (or indeed dead) cells could lead to phytoalexin production in adjacent tissues (Hargreaves & Bailey, 1978). Wood (1978) hypothesized that elicitors of phytoalexin synthesis could act in this way, their primary action being to damage or kill host cells; synthesis of phytoalexins could then occur as a consequence of cellular disruption.

In this regard, supporting evidence is provided in the report by Hargreaves & Bailey (1978) that a component of bean cells is capable of eliciting phytoalexin production in bean hypocotyls. These workers were able to show that freeze-killed bean tissues could stimulate phytoalexin production in adjacent healthy cells, yet accumulation took place predominantly in the dead tissues. In addition, phytoalexins were removed from solution by dead or living hypocotyl sections and by a bean cell wall preparation; most of the phytoalexin could be recovered from the dead tissues or from cell walls but not from living tissues. Such findings led to the suggestion that during a resistant (hypersensitive) reaction an initial interaction between the fungus and the host might lead to the release from the host of a constitutive elicitor which could stimulate phytoalexin production within and around infected cells. Death of infected cells would provide a site for the accumulation of phytoalexins produced in adjacent living cells, resulting in a localized, highly antifungal environment. That such an elicitor exists was supported by the presence in aqueous hypocotyl extracts of a component capable of causing phytoalexin accumulation in hypocotyl sections. The nature of the component has not been fully established, but clearly the operation of such a system would obviate the necessity for direct

stimulation of phytoalexin synthesis by the pathogen, and may explain the accumulation of phytoalexins in response to any treatment capable of interfering with normal cellular metabolism in the host.

Phytoalexin accumulation in hypocotyls inoculated with culture filtrates of R. solani was consistently higher than the levels produced in response to culture medium alone (Table 4.1). These findings are consistent with the many reports in the literature concerning material in fungal culture filtrates causing phytoalexin accumulation (Section I). The possibility that the phytoalexin-eliciting ability of R. solani culture filtrates could be due to components normally located in the mycelium but leaking out into the culture medium is indicated by the much higher levels of kievitone observed in response to mycelial extracts of the fungus (Table 4.2).

Since maximal kievitone accumulation in hypocotyls artificially-infected with R. solani had occurred at the first day (Section III, Fig. 3.2,A), and since it was also thought best to keep incubation times involving excised tissue pieces to a reasonable minimum, artificially-inoculated hypocotyls were initially incubated for 40 h before harvesting. Time course studies of phytoalexin accumulation in response to mycelial extracts of R. solani later substantiated the propitious nature of this choice. After 40 h incubation, the levels of kievitone and phaseollin had already reached approximate maxima (Fig. 4.3,B), although levels had not yet begun to fall (Fig. 4.2,A). It was fortunate that the mycelial extracts used in the earlier experiments were of a concentration sufficient to produce maximum kievitone levels after 40 h incubation (Fig. 4.3,A).

The failure of kievitone to accumulate to little more than trace levels in hypocotyls treated with mycelial extracts of F. solani f.sp. phaseoli, and the low levels produced in response to culture filtrates of this fungus (Tables 4.3 and 4.9; Fig. 4.2,B) are striking. Nonetheless, they are

consistent with the results obtained when bean tissues become infected with the fungus (Section III, Fig. 3.1,B; VanEtten & Smith, 1975). The appearance of kievitone as part of a general response to the presence of foreign cellular constituents, of the type proposed by Teasdale, Daniels, Davis, Eddy & Hadwiger (1974), might be expected. If so, it might be anticipated that kievitone accumulation in hypocotyls infected with F. solani f.sp. phaseoli would reach higher levels than those observed in R. solani-infected beans, since the former is able to proliferate (and therefore presumably stimulate the resistance response) in a greater amount of tissue. Such an observation has, in fact, been made for phaseollin (and phaseollinisoflavan) but not for kievitone (Section III, Fig. 3.1; Smith et al., 1975; VanEtten & Smith, 1975). From another viewpoint, however, since F. solani f.sp. phaseoli may be regarded as the more successful pathogen in this situation, a reaction between the host and metabolites from this fungus might be expected to result in reduced expression of resistance factors, compared to an interaction involving limited fungal invasion, such as with R. solani.

The resistance response may be significantly altered by the pathogen once it has been initiated, or may never be expressed upon ingress by a compatible parasite. There are several instances in which invading fungi are reported to degrade phytoalexins to less toxic products, thereby presumably reducing the effectiveness of the resistance response (VanEtten & Pueppke, 1976). More specifically, F. solani f.sp. phaseoli can apparently metabolize both kievitone and phaseollin in situ (VanEtten & Smith, 1975; Kuhn & Smith, 1978; Kuhn, 1979). However, as discussed earlier, this may not fully explain the non-appearance of kievitone in bean hypocotyls infected with this fungus (Section I).

As indicated by Daly (1972) and later by Rahe & Arnold (1975), susceptibility rather than resistance might hold the key in determining the nature of an interaction between host and parasite. Resistance would

be expressed unless the microorganism could by some means bring about a compatible interaction. Thus the normal host resistance response might fail to be triggered or may be actively suppressed. There is evidence from a number of host-parasite interactions that products of microbial origin can prevent or delay the host resistance response (Section V). More specifically, Daniels & Hadwiger (1976) showed that metabolites of F. solani f.sp. phaseoli could inhibit production of the phytoalexin pisatin in pea endocarp tissue. However, it seems unlikely that appropriate inhibitors could be present in each fraction of a F. solani f.sp. phaseoli mycelial extract such that kievitone accumulation is prevented when individual fractions are applied to bean hypocotyls.

A comparison of the carbohydrate and protein content of mycelial extracts of R. solani and F. solani f.sp. phaseoli reveals major differences in the amounts of carbohydrate in particular (Tables 4.7 and 4.9). According to the assay system used, mycelial extracts of R. solani contained much greater amounts of carbohydrate than corresponding extracts of F. solani f.sp. phaseoli; observed differences in Lowry-positive material were much smaller. Thus, the ability of mycelial extracts of R. solani to cause much higher levels of kievitone accumulation than mycelial extracts of F. solani f. sp. phaseoli, even when an approximately equal dry wt of mycelium was extracted, may merely reflect the disparity in the absolute amounts of fungal carbohydrate. While the activity of mycelial extracts from both fungi containing equal amounts of measured carbohydrate was not determined per se, a mycelial extract of R. solani, diluted to a level estimated to contain an approximately equal amount of carbohydrate to that in extracts of F. solani f.sp. phaseoli, still caused appreciable kievitone accumulation (compare Fig. 4.3 and Table 4.9). Such comparisons may not be prudent, however, since much of the material present in a crude mycelial extract may well be redundant as far as stimulation of phytoalexin synthesis is concerned. For example, much carbohydrate was lost when fractions from mycelial extracts

of R. solani were dialyzed, yet phytoalexin-eliciting activity was not proportionately reduced (Table 4.7).

Thus it seems that mycelium of F. solani f.sp. phaseoli simply contains less water-extractable carbohydrate than R. solani, and apparently possesses less phytoalexin-eliciting activity. Without purification to homogeneity, any assessment as to whether these two observations are related must, for the time being, remain conjecture.

The disparity in carbohydrate content and phytoalexin-eliciting activity of the respective mycelial extracts could have arisen as a result of employing different media and culture conditions for the preparation of mycelia. However, when F. solani f.sp. phaseoli was grown in the medium and culture conditions normally used for R. solani, the phytoalexin-eliciting activity of the mycelial extract did not appear to be greatly enhanced (Table 4.3, Fig. 4.2,B and Table 4.9).

Melitskii et al. (1976) obtained preparations from the cytoplasm of several races of Phytophthora infestans which, irrespective of their degree of virulence, were capable of eliciting rishitin accumulation in each variety of host potato used. From the results of their experiments they proposed the phytoalexin-eliciting component(s) to be high mol wt, thermally stable and possibly glycoprotein in nature. Subsequently, the same group of workers were also able to isolate two rishitin-eliciting groups of compounds — glucans and mannan-containing glycoproteins — from the cell walls of the fungus (Chalova et al., 1977). When the activity of the preparations was compared to that of the cytoplasmic fraction (based on comparisons of material taken from the same amount of mycelium and adjusted to either the same volume or the same sugar content), the phytoalexin-eliciting activity of the cytoplasmic fraction was always much greater than that of the cell wall preparations. These workers emphasized the structural similarity between the cytoplasmic and wall-released elicitors, and proposed that structural elements of the wall may be formed and

accumulated in the cytoplasm prior to incorporation into the wall. Once present in the wall they may function as activators of the host resistant response, including phytoalexin accumulation.

Zevenhuizen & Bartnicki-Garcia (1970) found that Phytophthora cinnamomi, when grown in a glucose-rich medium, produced a soluble β1,3 and β1,6-linked glucan associated with a small percentage of protein. Since this was not found in mycelium grown in media containing high mol wt carbohydrates as carbon source, they proposed it to be a reserve storage compound. Other workers demonstrated a structural similarity between mycelial wall components and extracellular β1,3 and β1,6-linked glucans released into culture media by Sclerotium rolfsii and certain Claviceps species (Buck, Chen, Dickenson & Chain, 1968). Considering their structural similarity with the very active phytoalexin elicitors purified from the cell walls of other fungi (Section I), and also the results of the Russian workers, it seems reasonable to suggest that compounds like these (whether wall precursors or not), if present in cell-free mycelial extracts or culture filtrates, could effect phytoalexin elicitation in host tissues. Whether the presence of such compounds could be responsible for the phytoalexin-eliciting activity of mycelial extracts and culture filtrates of R. solani is open to speculation, but this is not precluded by the results presented here. Also, although information on the carbohydrate status of R. solani mycelium appears to be slight, reports have included relatively low mol wt carbohydrates, soluble in TCA and comprising up to 71% of the dry wt, as well as other unidentified polysaccharides (Tolmsolf, 1970).

If information on storage components of R. solani is scant, reports concerning the nature of the cell walls are only slightly more numerous. Bartnicki-Garcia (1968) placed both R. solani and F. solani f.sp. phaseoli in the chitin-glucan group of a fungal classification based on cell wall

chemistry. Conveniently, in a series of publications, Skujins, Potgieter & Alexander (1965) and Potgieter & Alexander (1965a; 1965b; 1966) studied the wall composition of both these fungi in terms of susceptibility or resistance to microbial lysis.

A lytic enzyme system containing purified chitinase and β1,3 glucanase readily digested up to 94% of the wall material of F. solani f.sp. phaseoli. From their results, these workers suggested that the hyphal walls of this fungus probably comprise a chitin-containing core masked by predominantly β1,3-linked glucan, and possibly other substances. Thus it is perhaps not surprising that recently Anderson (1978b) was able to solubilize and purify, from cell wall preparations of other Fusaria, carbohydrates capable of eliciting phytoalexin production and browning in French bean hypocotyls. Whether similar factors operate during the infection of bean hypocotyls by F. solani f.sp. phaseoli remains to be seen. However, while undoubtedly the capacity for synthesis of these compounds exists within fungi (Nordin & Kirkwood, 1965), the results here suggest that, unlike R. solani, large amounts of water-extractable phytoalexin-eliciting material may not be present in the cytoplasm of F. solani f.sp. phaseoli.

In contrast to findings with F. solani f.sp. phaseoli, Potgieter & Alexander (1965a; 1966) were unable to obtain significant dissolution of R. solani mycelial walls. Small amounts of glucose and N-acetylglucosamine were released by treatment with β1,3 glucanase or chitinase respectively, but no combination of the enzymes could solubilize the walls. Following chemical analysis of the material, they proposed that melanin-like phenolic polymers could be active in preventing wall lysis. Certainly, as they age, mycelial walls of R. solani become thickened and assume a distinct brown colouration (Butler & Bracker, 1970). It seems likely that such material could be responsible for the brown colouration of the active kievitone-eliciting fractions obtained from R. solani. Whether compounds of this nature might be responsible also for the phytoalexin-eliciting

activity itself is another matter, since they may be purely chance contaminants with a similar molecular size distribution to that of the active component(s). However, it is not impossible that the presence of a random polymer such as melanin on the fungal surface, while perhaps not fulfilling any antigenic requirement, could be recognised by the host and lead to disruption of normal cellular metabolism, for example by the non-specific binding of protein (Nicolaus, Piattelli & Fattorusso, 1964; Blois, 1965). The presence of such compounds, together with their bound protein, might help explain the positive Lowry tests obtained from the active fractions (Fig. 4.6,A).

When a cell wall preparation from R. solani was re-suspended in distilled water and applied to bean hypocotyls, accumulation of kievitone took place (Table 4.4). In this case, the mere presence of fungal wall material in suspension led to kievitone production. Since solubilized walls caused the accumulation of even higher levels of kievitone (Tables 4.4 and 4.5), it may be that this increase reflects greater release from the fungal cell walls, during the solubilization process, of the active moieties. The possibility exists that the wall-released material is similar to the elicitor preparations obtained from the mycelial walls of other fungi (Section I). However, whether the wall-released material and the phytoalexin-eliciting component(s) from the cytoplasm are the same remains unresolved. The light brown pigmentation of the solubilized wall preparation again could indicate the presence of polyphenolic material.

The ion exchange study represented additional attempted purification of the eliciting component(s) from the R. solani mycelial extract. Failure to recover the bulk of the preparation, or indeed the elicitor activity, may have been due to the material remaining bound to the exchanger under the conditions employed. The extracellular elicitor from P. megasperma

var. sojae obtained by Ayers et al. (1976a) had no affinity for DEAE-Sephadex under these conditions, and the majority of the activity associated with wall-released elicitors from this fungus had already been eluted from DEAE-cellulose under the ultimate conditions employed here (Ayers et al., 1976b). Also, pisatin-eliciting components from culture filtrates of Fusarium solani isolates were eluted from DEAE-agarose under similar conditions (Daniels & Hadwiger, 1976). However, the elicitor activity of the cytoplasmic component(s) obtained from P. infestans by Melitskii et al. (1976) required 0.5 M KOH before it could be eluted from DEAE-Sephadex at pH 7.2; the recovered material contained both protein and sugars. Material which may also be glycoprotein in nature, isolated from culture filtrates of Rhizopus stolonifer, and capable of eliciting production of the phytoalexin casbene in cell-free extracts of castor bean, could apparently not be purified by ion exchange chromatography (Stekoll & West, 1978).

Thus, although an accurate picture of the nature of the components present in mycelial extracts of R. solani active in eliciting phytoalexin accumulation in bean hypocotyls has not been achieved, the results so far indicate that the material is likely to resemble more closely the glycoprotein components isolated from P. infestans (Melitskii et al., 1976; Chalova et al., 1977) and from R. stolonifer (Stekoll & West, 1978) than the high mol wt carbohydrates of P. megasperma var. sojae (Ayers et al., 1976a; 1976b; 1976c) or Collectotrichum lindemuthianum (Anderson-Prouty & Albersheim, 1975).

Without any further attempt at purification, the phytoalexin-eliciting activity of the most active gel filtration column eluates was exploited to test the ability of elicited kievitone, in particular, to protect bean hypocotyls from invasion by F. solani f.sp. phaseoli.

SECTION V

CROSS-PROTECTION AGAINST F. SOLANI F.SP. PHASEOLI BY A PHYTOALEXIN ELICITOR FROM R. SOLANI

Experiments employing the artificial inoculation system were conducted to determine whether the presence of high levels of kievitone might increase the resistance of bean hypocotyls to invasion by F. solani f.sp. phaseoli. Spore suspensions of this fungus were introduced into hypocotyls previously or simultaneously treated with a sample of phytoalexin elicitor prepared from mycelial extracts of R. solani. The extent of fungal development in the tissues was monitored by cytological observation and estimation of the chitin content of diseased hypocotyls. These parameters, together with the levels of kievitone, phaseollin and the fungal metabolite of kievitone, kievitone hydrate (Kuhn & Smith, 1976; Kuhn et al., 1977), were compared with those of hypocotyls inoculated with similar spore suspensions but which had not received the elicitor treatment.

MATERIALS AND METHODS

Spore suspensions of F. solani f.sp. phaseoli were prepared as described in Section II, except that the medium used was an aqueous extract of bean hypocotyls. This medium was prepared by homogenizing hypocotyls from 7-day old plants in distilled water (1:10, w/v) and, after boiling for 10 min, filtering the suspension through four layers of cheesecloth and through 0.8 μm filters. The final filtrate was sterilized by autoclaving at 103 kN/m^2 for 15 min; the product is referred to hereafter as "bean medium".

Mycelium of F. solani f.sp. phaseoli was grown in bean medium from spore suspensions incubated at 25 ± 2°C. At intervals, aliquots were removed to pre-weighed 0.8 μm filters for dry wt determinations; further aliquots were monitored for chitin levels. The chitin content, in glucosamine equivalents, was determined by the method of Ride & Drysdale (1972) using standard curves prepared by the incorporation of 0-50 μg glucosamine hydrochloride (Sigma) in the assay procedure. This same method was used to assay inoculated hypocotyls for their fungal content. Controls were treated with bean medium.

The elicitor preparation comprised the combined, concentrated active eluates from gel filtration of material derived from R. solani mycelium, as described earlier. Where application of elicitor preceded that of the spore suspension, the elicitor preparation was removed from hypocotyl cavities via a hypodermic syringe before introduction of medium (control) or F. solani f.sp. phaseoli spores. Where elicitor and spores were applied simultaneously, the concentrations of both components were retained by pelleting spores from a known volume of suspension by centrifugation at 20,000 x g; the supernatant was decanted and the spores re-suspended in an equal volume of elicitor preparation. This mixture was then used for direct inoculation of hypocotyls.

Separate treatments comprised inoculation of hypocotyls with: (a) elicitor for 1 day (24 h pre-incubation period) followed by bean medium for 1-5 days, (b) pre-incubation with elicitor followed by F. solani f.sp. phaseoli spore suspension for 1-5 days, or (c) pre-incubation with bean medium followed by a combined spore suspension/elicitor preparation for 1-5 days. Control hypocotyls again received only bean medium.

Levels of kievitone hydrate in inoculated tissues were monitored by a method similar to that employed for kievitone. Harvested hypocotyls were extracted for phytoalexins as described in Section II, but at the first TLC stage the extracts were also screened for kievitone hydrate.

Bands running coincidentally with authentic samples of this compound (kindly provided by P. J. Kuhn, Plant Biology Dept., Hull University), R_f = 0.18, were eluted in EtOH and re-chromatographed separately on 0.25 mm SG layers developed in benzene/ethyl acetate/methanol (25:4:2). Again, bands running coincidentally with standards, R_f = 0.34, were eluted in EtOH and quantified by UV spectroscopy using the reported molar extinction coefficient, $\log \varepsilon_{293nm}$ = 4.22 (Kuhn et al., 1977).

RESULTS

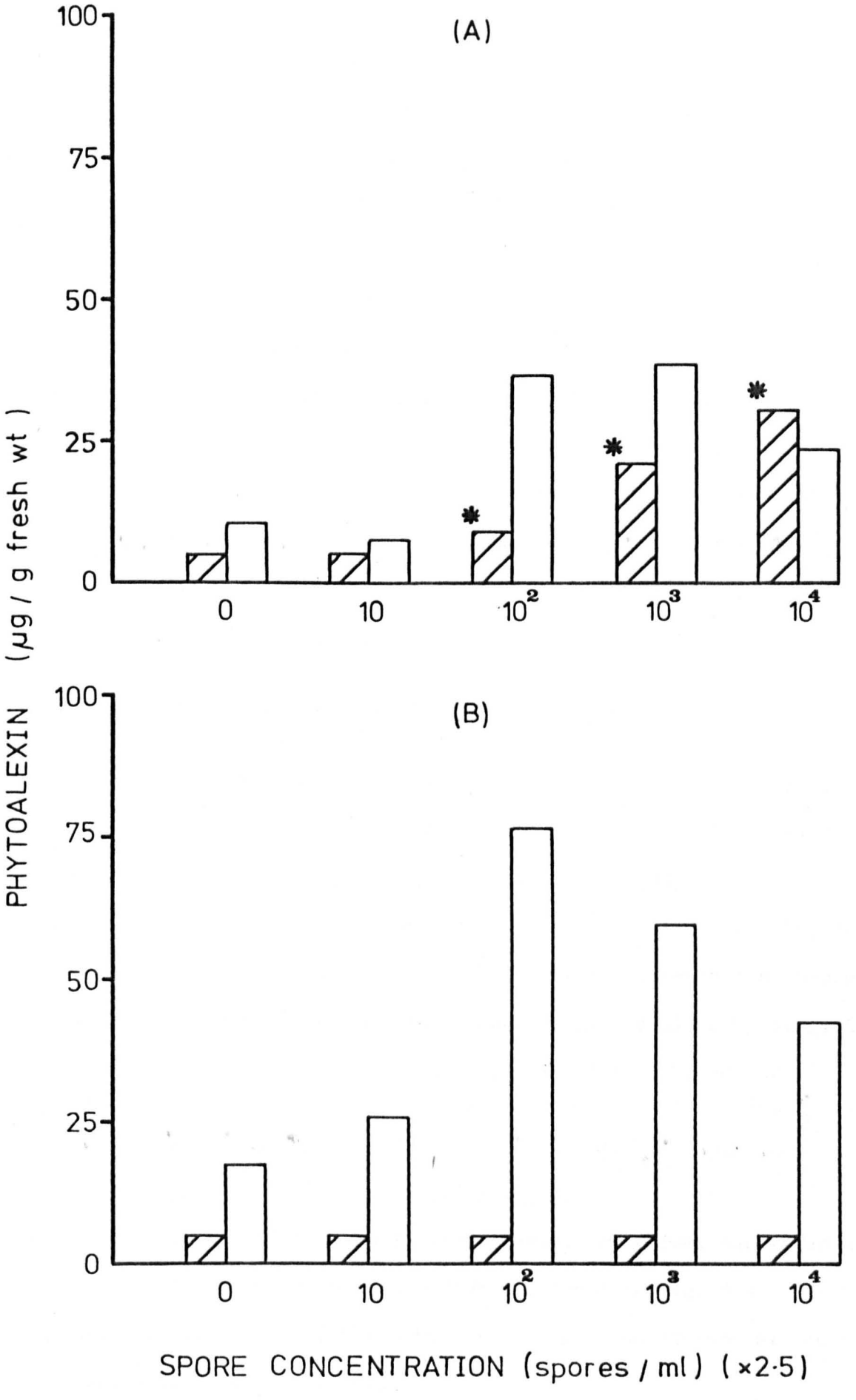
Phytoalexin accumulation and browning in hypocotyls inoculated with spore suspensions of F. solani f.sp. phaseoli:

In order to determine a suitable level of inoculum for cross-protection studies, a preliminary experiment involving the inoculation of bean hypocotyls with a spore concentration series of F. solani f.sp. phaseoli was carried out. Levels of kievitone and phaseollin in hypocotyls inoculated with different spore concentrations are given in Fig. 5.1. Forty-eight hours after inoculation (Fig. 5.1,A), small amounts of kievitone had accumulated in response to the three highest levels of inoculum. After 120 h incubation, however, kievitone could no longer be detected above trace levels (Fig. 5.1,B). In contrast, in response to all treatments, phaseollin accumulation after 120 h reached higher levels than those observed in corresponding tissues at 48 h.

In certain instances (marked * in Fig. 5.1) the UV absorption spectrum obtained after routine purification, although indicative of kievitone, was of insufficient quality to allow accurate quantitation. Therefore samples were re-chromatographed on 0.25 mm SG layers developed in toluene/ethyl formate/formic acid (7:2:1). The efficiency of recovery of material from this third TLC was estimated from that of known amounts of authentic kievitone. However, mass spectral analysis of these samples did not confirm the presence of kievitone, although good fragmentation patterns

Fig. 5.1. Levels of kievitone (▨) and phaseollin (□) in hypocotyls 48 h (A) and 120 h (B) after inoculation with different spore concentrations of F. solani f.sp. phaseoli.

✱ Represents material quantified after three TLC purification steps (See text).


(A)
(B)
100
75
50
25
0
PHYTOALEXIN (µg / g fresh wt)
0
10
10²
10³
10⁴
SPORE CONCENTRATION (spores / ml) (×2·5)

for kievitone have been obtained from F. solani f.sp. phaseoli-infected bean hypocotyls after similar incubation periods (Kuhn, 1979).

The appearance of the host tissues in these fungus-inoculated, cored hypocotyls is shown in Plates 6 and 7. At the earlier time period (Plate 6), the extent of browning approximately corresponded with the level of inoculum; individual points of infection were readily observed at the lower concentrations (Plate 6, A and B). Observation of these macroscopic symptoms at 120 h (Plate 7) revealed that while browning had increased, hypocotyls inoculated with the highest spore concentration had changed most in appearance; these hypocotyls were macerated and prone to collapse upon harvesting (Plate 7,D). Although some decay was evident in response to 2.5×10^3 spores/ml (Plate 7, C), externally these hypocotyls resembled more closely the firm, green tissues inoculated with the lower spore concentrations (Plate 7, A and B).

Phytoalexin accumulation in hypocotyls treated with the R. solani phytoalexin elicitor preparation:

A preliminary experiment was conducted to determine the most satisfactory time for pre-incubation of hypocotyls with R. solani phytoalexin elicitor before introduction of the F. solani f.sp. phaseoli spore suspension. A time course of kievitone and phaseollin production in hypocotyls treated with the elicitor is given in Fig. 5.2. Here, although the absolute levels attained were lower, the pattern of accumulation followed closely that observed earlier in response to crude cell-free mycelial extracts of R. solani (Fig. 4.3,B).

Chitin content of F. solani f.sp. phaseoli mycelium:

The chitin content of F. solani f.sp. phaseoli mycelium, harvested at intervals from cultures grown in bean medium, is shown in Fig. 5.3. The recorded level appeared to vary throughout the incubation period, although a general upward trend was observed.

PLATE 6

Internal appearance of hypocotyls 48 h after inoculation with spore suspensions of *F. solani* f.sp. *phaseoli*. (x 1.5).

The inoculum contained (A) 25, (B) 2.5×10^2, (C) 2.5×10^3 and (D) 2.5×10^4 spores/ml. Hypocotyls each received c.50 µl inoculum.

A

B

C

D

PLATE 7

Internal appearance of hypocotyls 120 h after inoculation with spore suspensions of *F. solani* f.sp. *phaseoli*. (x 1.5).

The inoculum contained (A) 25, (B) 2.5×10^2, (C) 2.5×10^3 and (D) 2.5×10^4 spores/ml. Hypocotyls each received c.50 µl inoculum.

A

B

C

D

Fig. 5.2. Levels of kievitone (—○—) and phaseollin (—●—) in hypocotyls after inoculation with a phytoalexin elicitor preparation from R. solani.

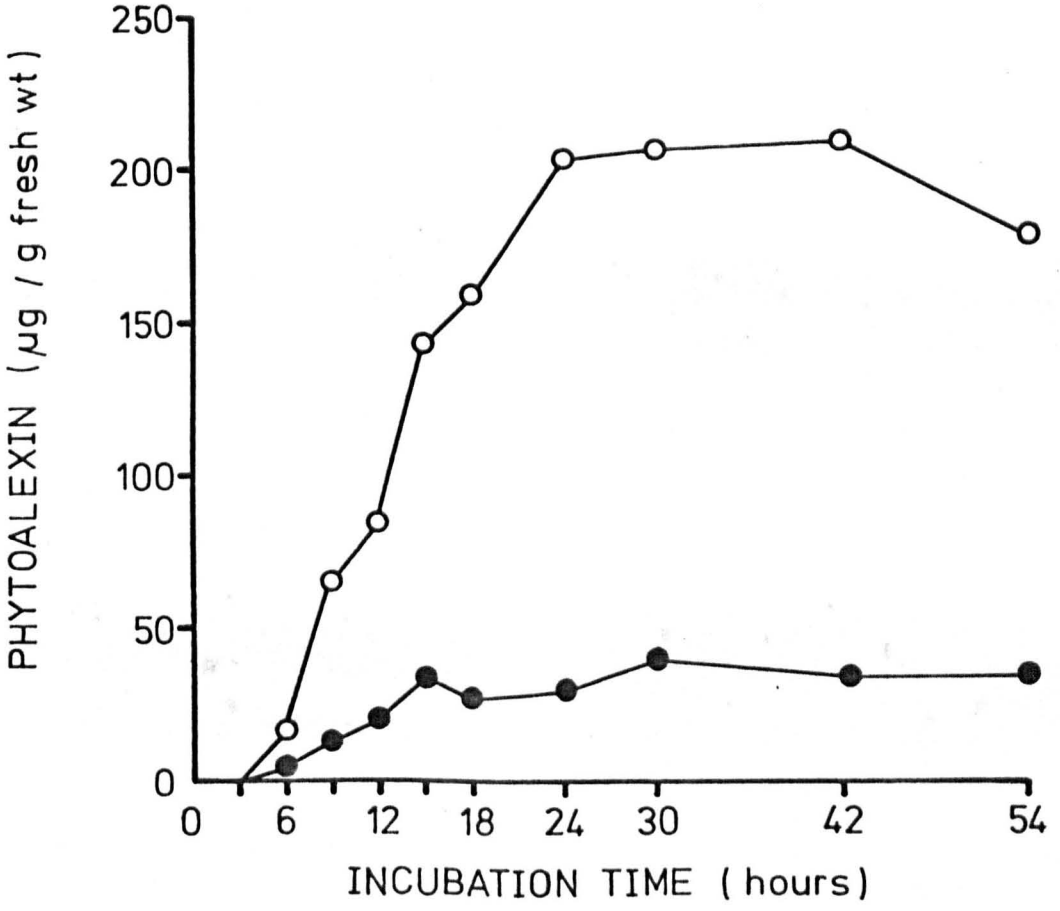
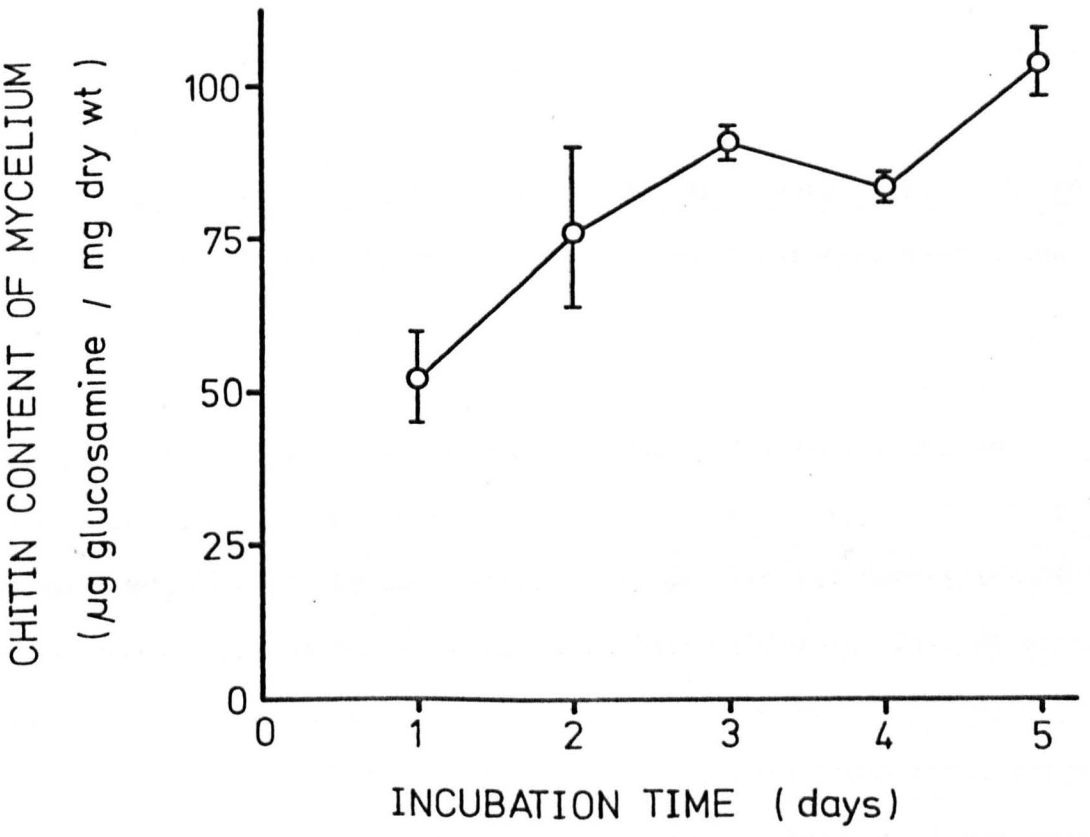

250
200
150
100
50
0
PHYTOALEXIN (µg / g fresh wt)
0
6
12
18
24
30
42
54
INCUBATION TIME (hours)

Fig. 5.3. Chitin content of *F. solani* f.sp. *phaseoli* mycelium in cultures grown from spore suspensions (c.2 x 10^4 spores/ml) in bean medium.


CHITIN CONTENT OF MYCELIUM
(µg glucosamine / mg dry wt)
100
75
50
25
0
0
1
2
3
4
5
INCUBATION TIME (days)

Phytoalexin accumulation and fungal development in hypocotyls inoculated with a spore suspension of F. solani f.sp. phaseoli:

Levels of kievitone and phaseollin in hypocotyls at various times after inoculation with a spore suspension of F. solani f.sp. phaseoli are given in Fig. 5.4,A. The low levels of kievitone that were present initially declined to trace levels by 96 h after inoculation. Phaseollin accumulated more slowly and to higher levels before apparently declining. Kievitone hydrate was not detected at any of the assay periods. Chemical estimation of the fungal content of these tissues revealed low amounts up to 72 h; thereafter a sharp rise occurred, continuing up to 120 h after inoculation (Fig. 5.4,B).

Plates 8 and 9 show sections taken through infected hypocotyls in order to follow cytologically the development of the fungus within the tissues. Material was observed at each time period for which the levels of phytoalexins and chitin in the hypocotyls were measured. Healthy, non-inoculated hypocotyls demonstrated no signs of tissue browning (Plate 8, A and B). After incubation with the spore suspension for 24 h, a ring of discoloured cells developed around the central cavity (Plate 8, C) and hyphae were penetrating the first cell layers (Plate 8, D). After 48 h, although the browning appeared to have spread no further than the initial depth of 2-3 cell layers, the discolouration was more pronounced, providing a sharper distinction between apparently healthy tissues and those reacting to the fungus (Plate 8, E). The ring of brown tissue spread slowly, reaching 3-4 cells deep by 72 h (Plate 8, F). By this time, hyphae could be observed penetrating beyond the brown zone of cells (Plate 9, A). Further observation showed that the fungus rapidly approached and penetrated the vascular tissues such that by 96 h the endodermis was beginning to be breached (Plate 9, B and C). After 120 h, the fungus had penetrated well into the cortex and the tissues began to disintegrate (Plate 9, D and E). Control hypocotyls, inoculated with bean medium, developed only slight

Fig. 5.4. Levels of kievitone, phaseollin and fungal mycelium in hypocotyls after inoculation with a spore suspension (c.2 x 10^4 spores/ml) of F. solani f.sp. phaseoli.

(A) Kievitone (—O—) and phaseollin (-●-).

(B) Fungal mycelium (—O—) (based on chitin estimation).

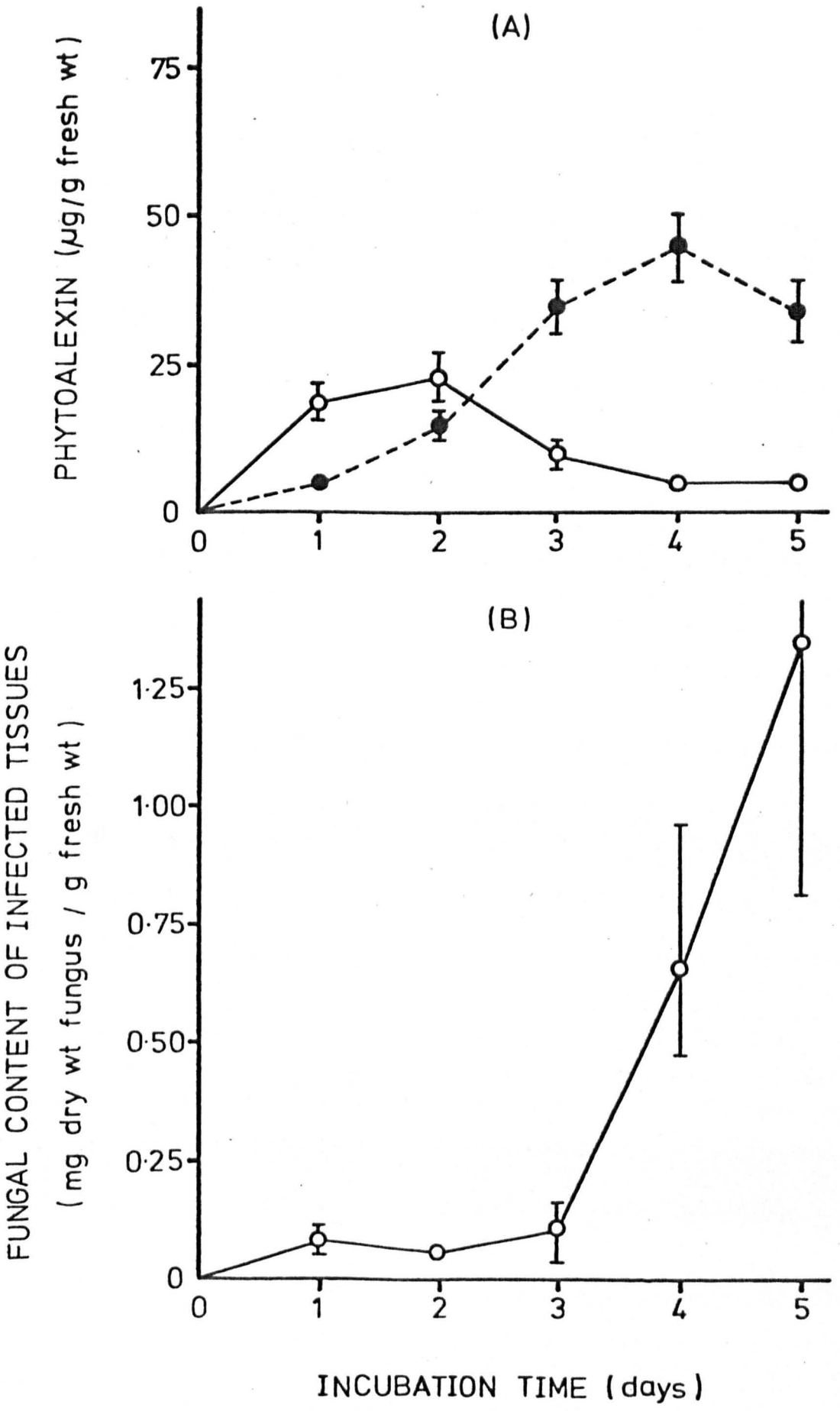

(A)
PHYTOALEXIN (µg/g fresh wt)
75
50
25
0
0
1
2
3
4
5
(B)
FUNGAL CONTENT OF INFECTED TISSUES
(mg dry wt fungus / g fresh wt)
1·25
1·00
0·75
0·50
0·25
0
0
1
2
3
4
5
INCUBATION TIME (days)

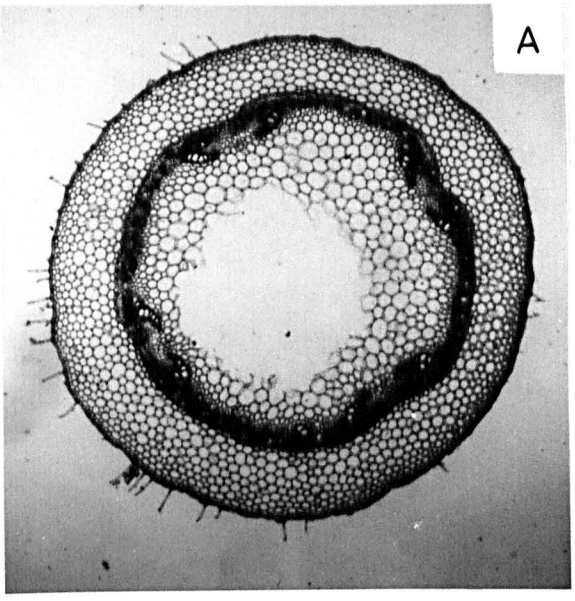
PLATE 8

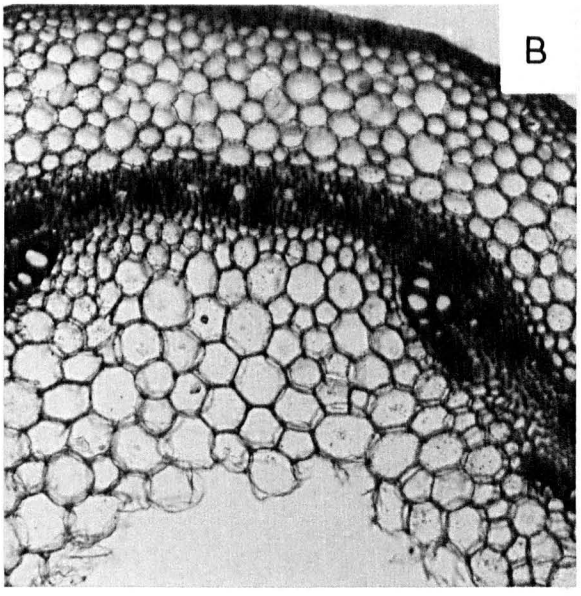
Sections taken through hypocotyls after inoculation with a spore suspension (c.2 x 10^4 spores/ml) of *F. solani* f.sp. *phaseoli*.

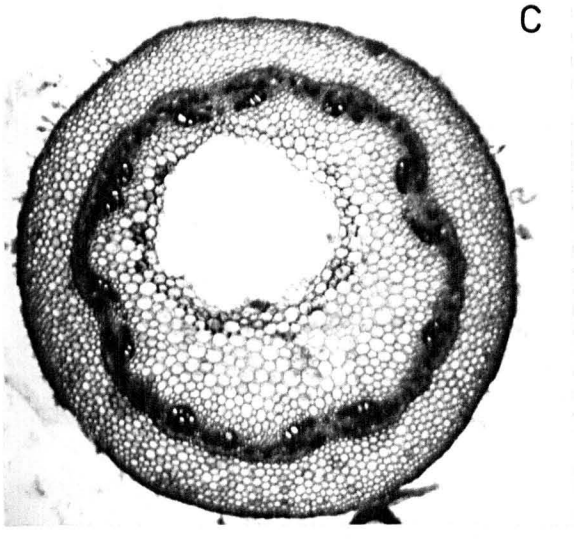
(A) Healthy (x 15); (B) Healthy (x 48);

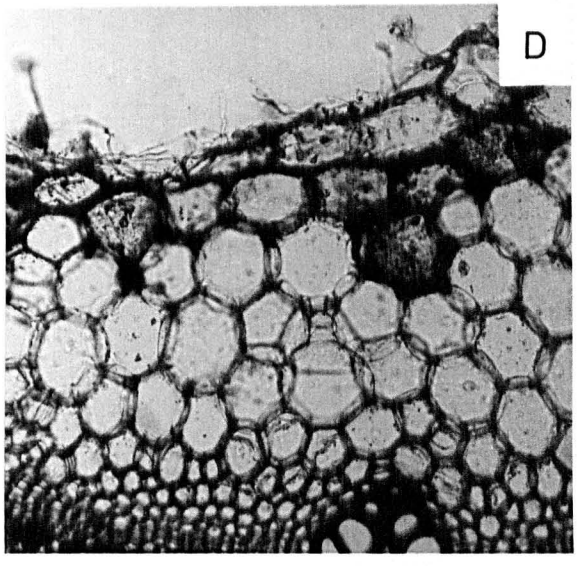
(C) 24 h (x 15); (D) 24 h (x 80);

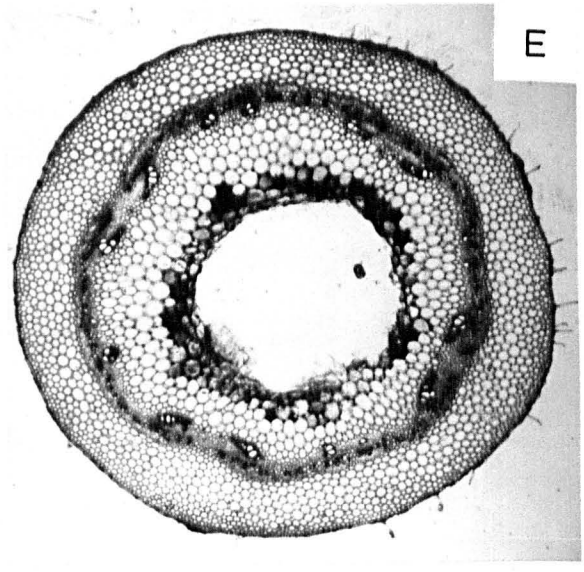
(E) 48 h (x 15); (F) 72 h (x 15).


A


B


C


D


E

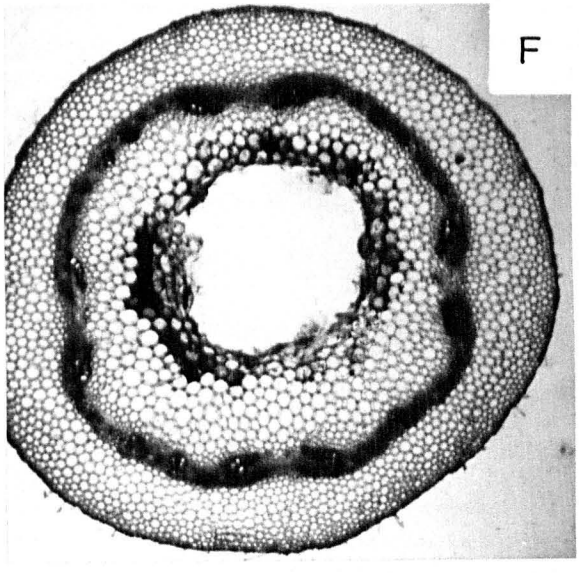
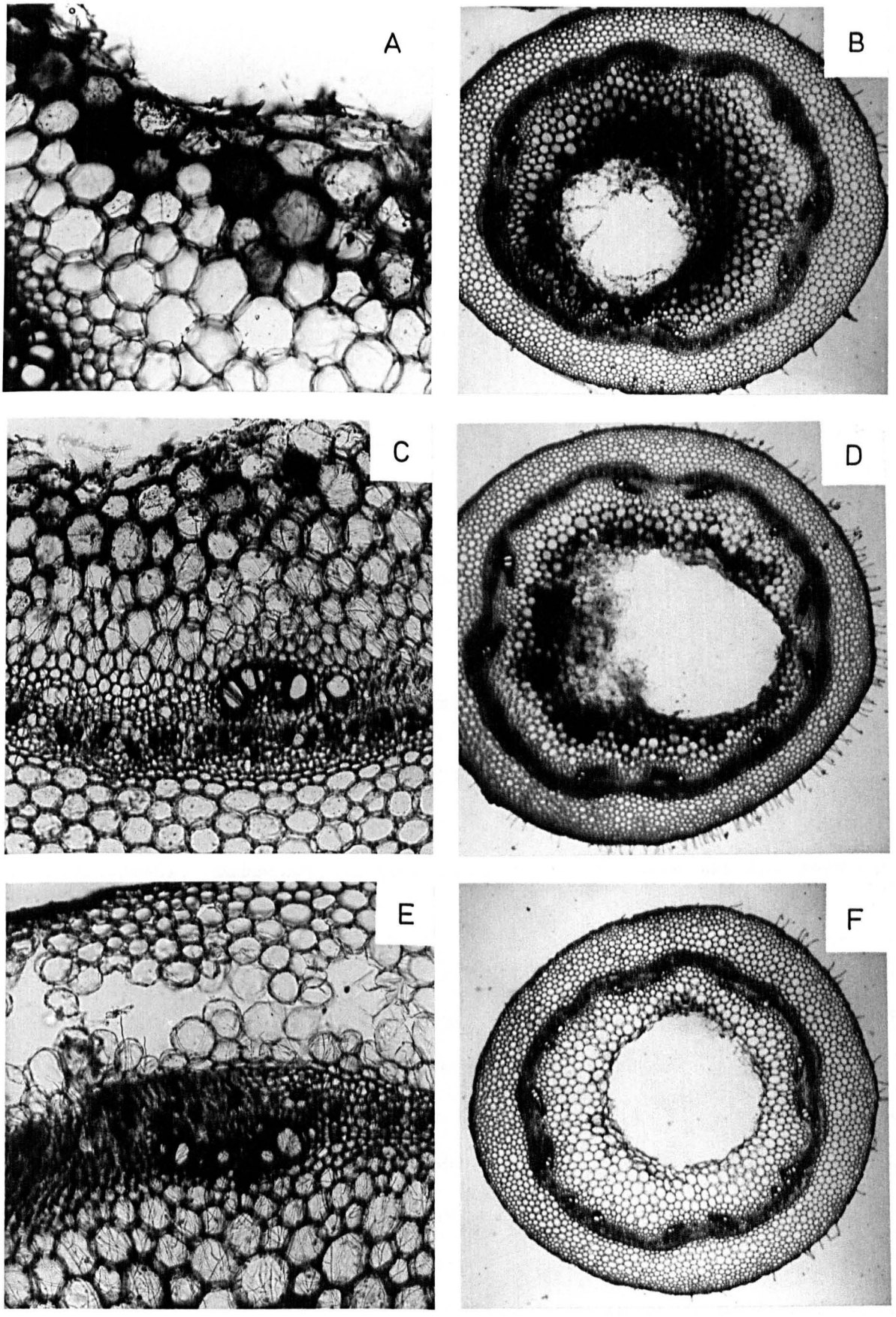

F

PLATE 9

Sections taken through hypocotyls after inoculation with a spore suspension (c.2 x 10^4 spores/ml) of *F. solani* f.sp. *phaseoli*.

(A) 72 h (x 75); (B) 96 h (x 15);
(C) 96 h (x 65); (D) 120 h (x 15);
(E) 120 h (x 65); (F) 120 h after inoculation with bean medium (control) (x 15).


A
B
C
D
E
F

discolouration of the central cavity walls, even after 120 h incubation (Plate 9, F).

Despite the obvious contrast between sections taken from infected and control hypocotyls after 120 h incubation, the photographs are slightly mis-representative since the infected tissues in the later stages of the incubation period became increasingly difficult to handle; reasonable sections could therefore only be obtained from tissues in which the infection was LEAST severe. A more representative picture is perhaps provided by a comparison of the macroscopic appearance of control and infected hypocotyls. Plate 10 shows examples of hypocotyls incubated for 120 h after inoculation with F. solani f.sp. phaseoli or with bean medium; infected hypocotyls were obviously heavily browned and colonized by the fungus (Plate 10, A) whereas the control tissues remained firm and green (Plate 10, B).

Attempted cross-protection of bean hypocotyls against F. solani f.sp. phaseoli with a phytoalexin elicitor from R. solani:

Mean levels of kievitone and phaseollin in hypocotyls 24 h after inoculation with the elicitor preparation were 175 and 30 µg/g fresh wt respectively. Subsequent introduction of bean medium was followed by a steady decline in the level of kievitone, while phaseollin maintained a fairly constant, modest level (Fig. 5.5). However, when the challenge comprised a spore suspension of F. solani f.sp. phaseoli, kievitone levels fell more rapidly, particularly between one and three days incubation (Fig. 5.6); this fall coincided with the appearance of kievitone hydrate. Phaseollin levels increased slowly before apparently declining.

Pre-incubation with bean medium caused the accumulation of no more than trace levels of kievitone or phaseollin. Introduction of the combined spore suspension/elicitor preparation caused the level of kievitone to rise to over 150 µg/g fresh wt after 24 h (Fig. 5.7); thereafter the level fell steadily, kievitone hydrate first appearing three days after inoculation.

PLATE 10

Hypocotyls 120 h after inoculation with a spore suspension (c.2 x 10^4 spores/ml) of *F. solani* f.sp. *phaseoli* (A) or with bean medium (B).

Each hypocotyl is c.3 cm long.

A

B

Fig. 5.5. Levels of kievitone (—○—) and phaseollin (--●--) in hypocotyls inoculated with bean medium after a 24 h pre-incubation with a phytoalexin elicitor preparation from R. solani.

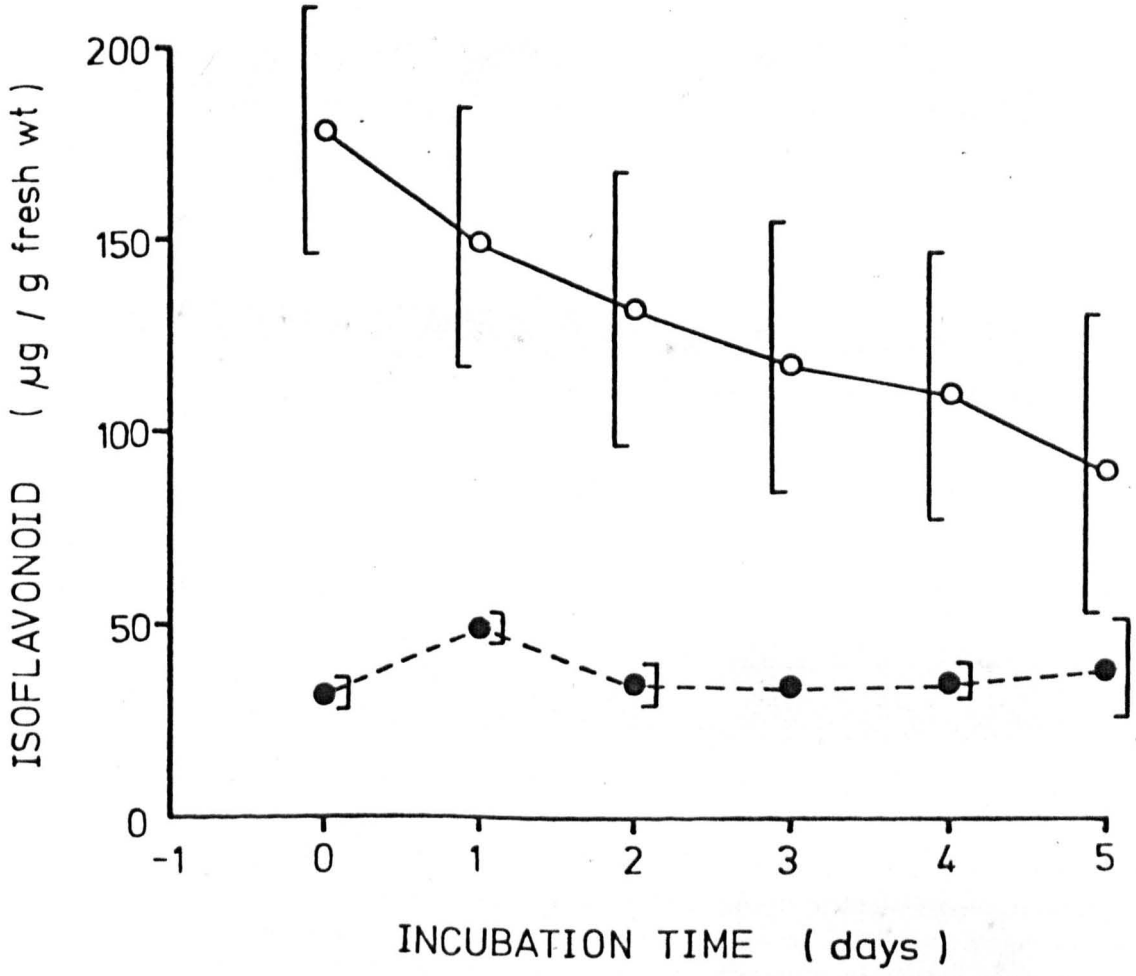

200
150
100
50
0
ISOFLAVONOID (μg / g fresh wt)
-1
0
1
2
3
4
5
INCUBATION TIME (days)

Fig. 5.6. Levels of kievitone (—o—), phaseollin (--●--) and kievitone hydrate (—△—) in hypocotyls inoculated with a spore suspension of F. solani f.sp. phaseoli (c.2 x 10^4 spores/ml) after a 24 h pre-incubation with a phytoalexin elicitor preparation from R. solani.

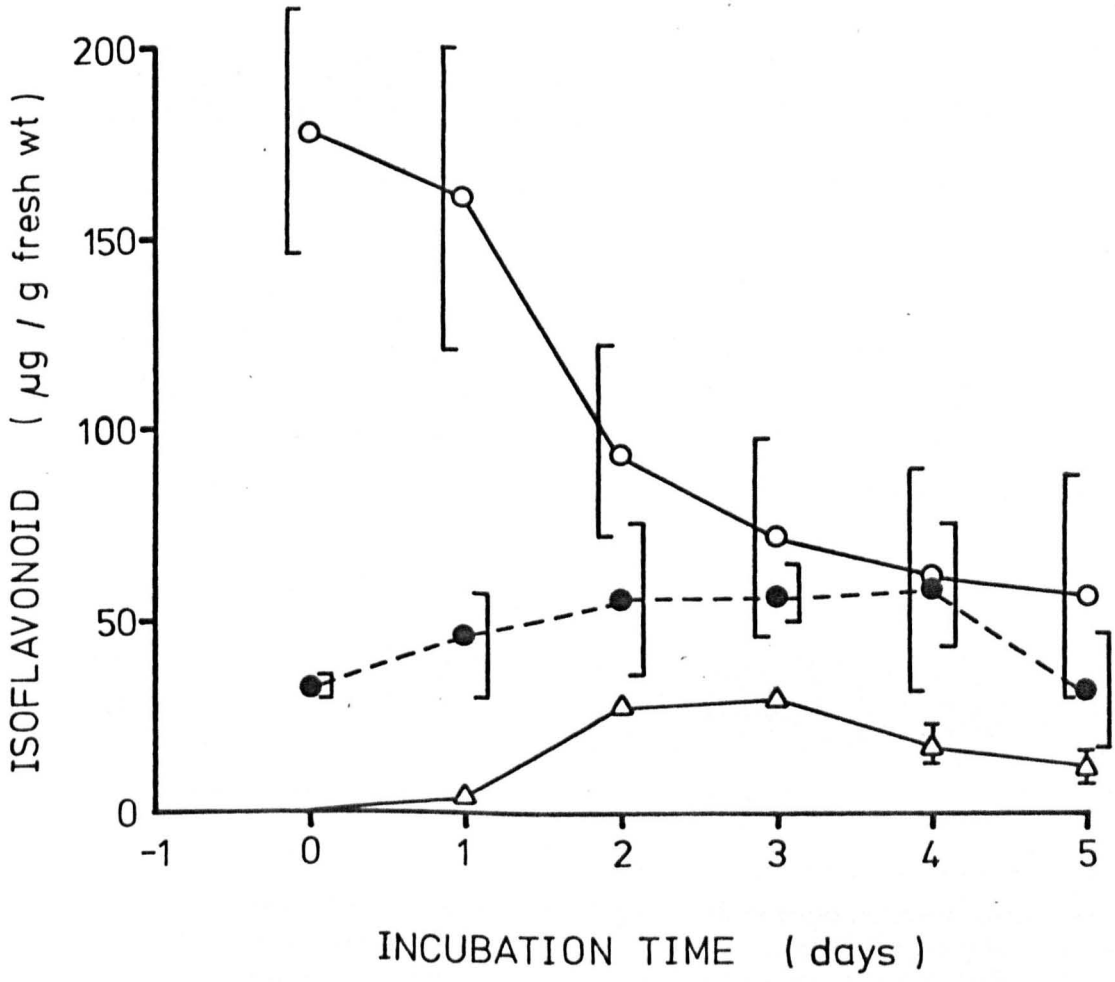
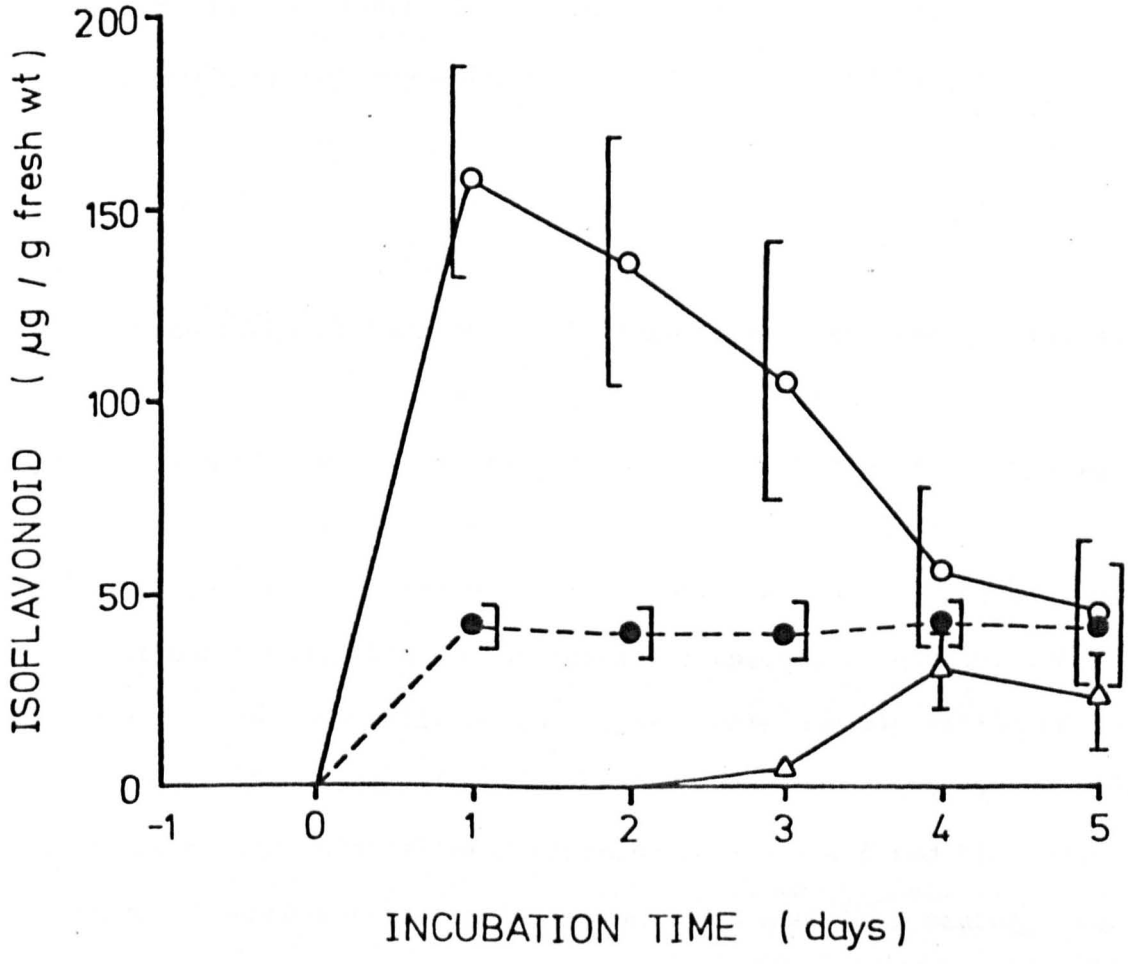

200
150
100
50
0
ISOFLAVONOID (µg / g fresh wt)
-1
0
1
2
3
4
5
INCUBATION TIME (days)

Fig. 5.7. Levels of kievitone (—○—), phaseollin (-●-) and kievitone hydrate (—△—) in hypocotyls inoculated with a combined F. solani f.sp. phaseoli spore suspension/R. solani elicitor preparation after a 24 h pre-incubation with bean medium.


200
150
100
50
0
ISOFLAVONOID (µg / g fresh wt)
-1
0
1
2
3
4
5
INCUBATION TIME (days)

Phaseollin maintained modest levels throughout.

The fungal content of hypocotyls from each treatment is given in Fig. 5.8. Tissues inoculated with bean medium after the elicitor pre-treatment demonstrated the presence of low amounts of assay-positive material after four and five days incubation. In contrast, hypocotyls inoculated with the fungal spore suspension following elicitor pre-treatment showed only a slight increase in fungal content up to four days after inoculation; thereafter the level rose steeply. Hypocotyls pre-incubated with bean medium and then challenged with the combined spore suspension/elicitor preparation demonstrated a gradual rise in fungal content after an apparent 2-day lag.

The appearance of hypocotyls involved in the cross-protection study is shown in Plates 11-14. The effect of pre-incubation with either bean medium or elicitor preparation is demonstrated in Plate 11. Healthy, uninoculated hypocotyls did not exhibit discolouration of the central cavity walls (Plate 11, A and B). Although there was some indication of material being deposited in intercellular spaces, pre-incubation with bean medium did not generally produce appreciable discolouration of host cells (Plate 11, C and D). The elicitor preparation, however, produced distinct intra- and intercellular browning (Plate 11, E and F).

Plate 12 represents hypocotyls inoculated with bean medium after pre-incubation with elicitor. One day after inoculation, the intra- and intercellular browning caused by the elicitor was still apparent (Plate 12, A and B). Successive daily observation did not reveal appreciable changes in the appearance of the tissues except perhaps for a slight increase in the extent and density of the discolouration (Plate 12, C-F).

When a spore suspension of F. solani f.sp. phaseoli was introduced into hypocotyls which had received the elicitor pre-treatment, the fungus was still able to penetrate and colonize the tissues. Discolouration of

Fig. 5.8. Levels of fungal mycelium in hypocotyls after inoculation with:

(i) Bean medium following pre-treatment with a phytoalexin elicitor preparation from *R. solani* (—○—).

(ii) Spore suspension (c.2 x 10^4 spores/ml) of *F. solani* f.sp. *phaseoli* following pre-treatment with a phytoalexin elicitor preparation from *R. solani* (—△—).

(iii) Combined *F. solani* f.sp. *phaseoli* spore suspension/*R. solani* elicitor preparation following pre-treatment with bean medium (-●-).

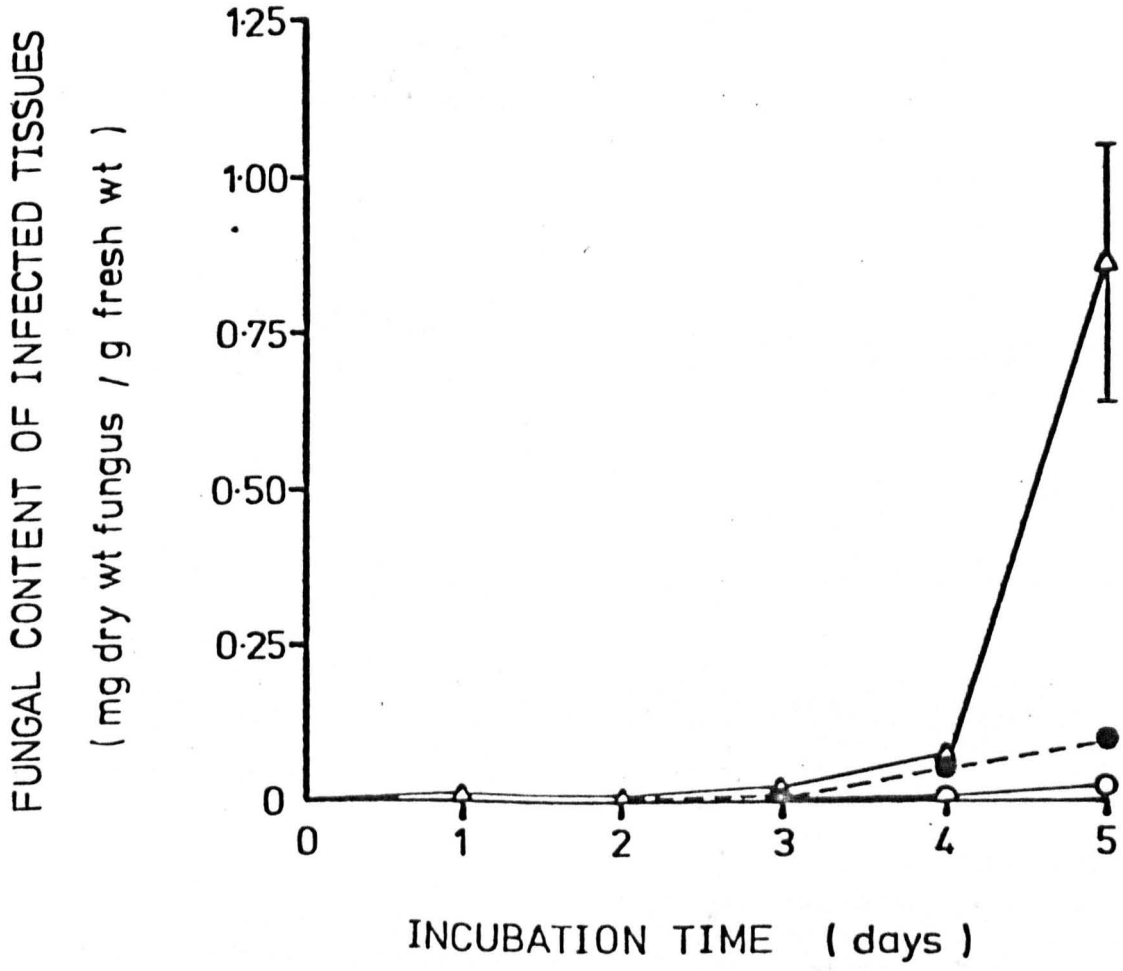

FUNGAL CONTENT OF INFECTED TISSUES
(mg dry wt fungus / g fresh wt)
1·25
1·00
0·75
0·50
0·25
0
0
1
2
3
4
5
INCUBATION TIME (days)

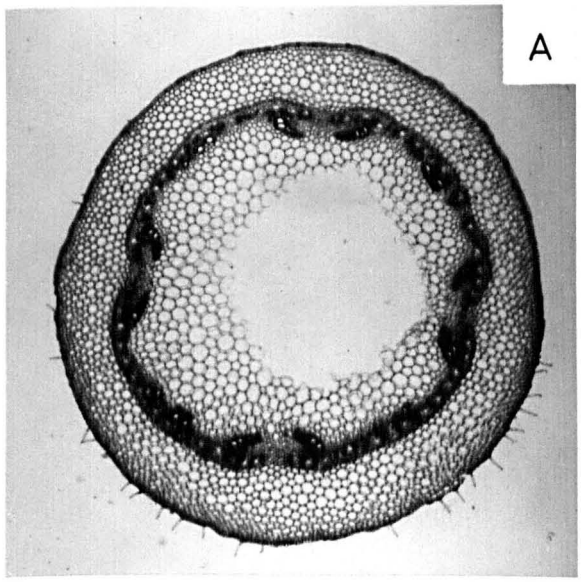
PLATE 11

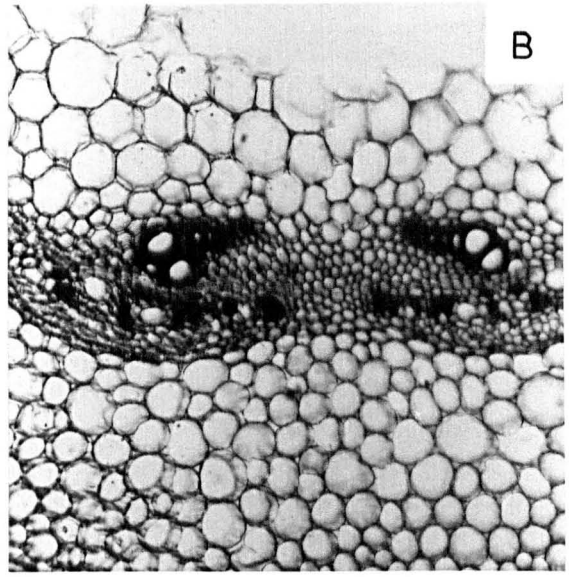
Sections taken from hypocotyls incubated for 24 h after inoculation with bean medium or a phytoalexin elicitor preparation from *R. solani*.

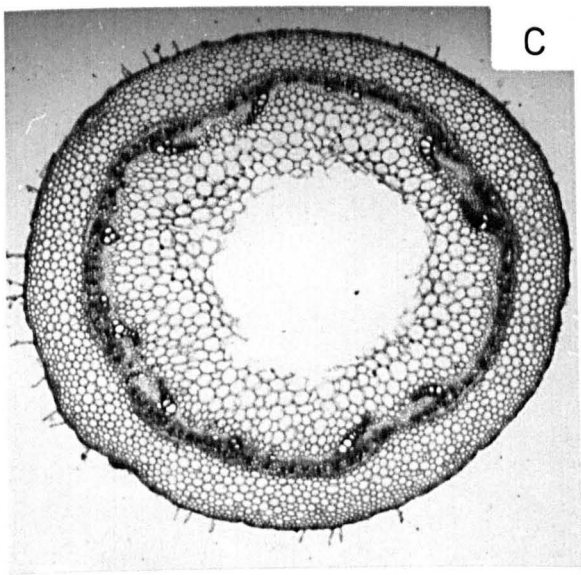
(A) Healthy (x 15); (B) Healthy (x 60);

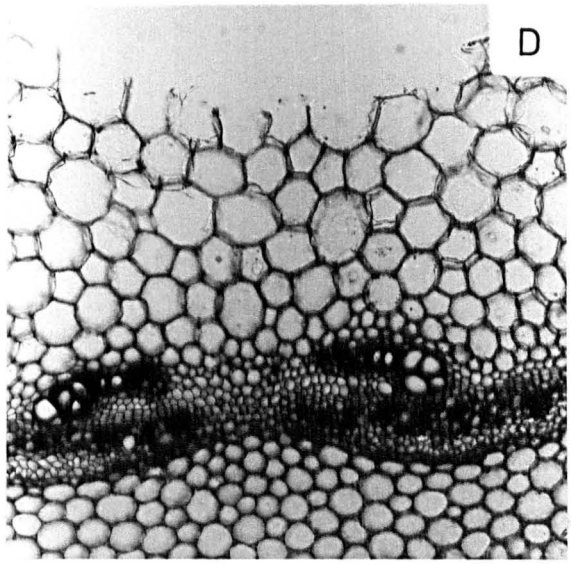
(C) Bean medium (x 15); (D) Bean medium (x 60);

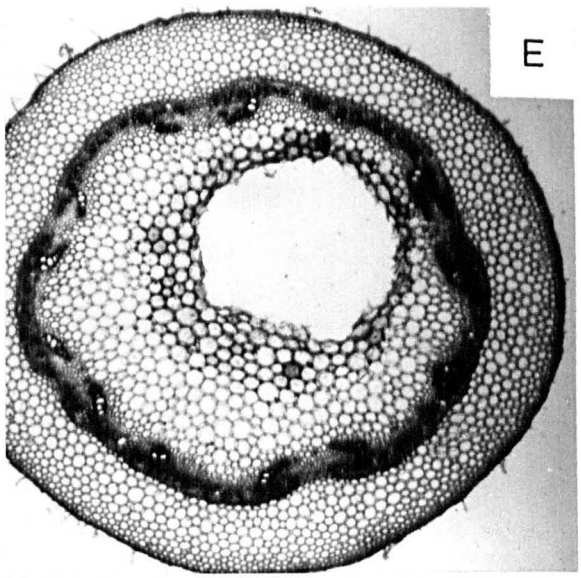
(E) Elicitor (x 15); (F) Elicitor (x 60).


A


B


C


D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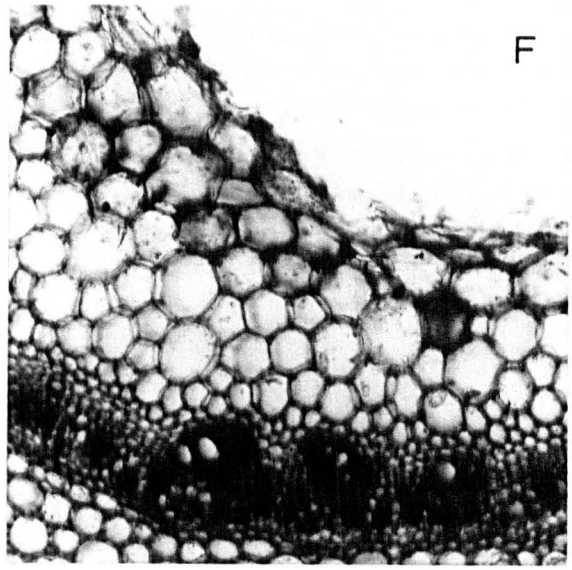

F

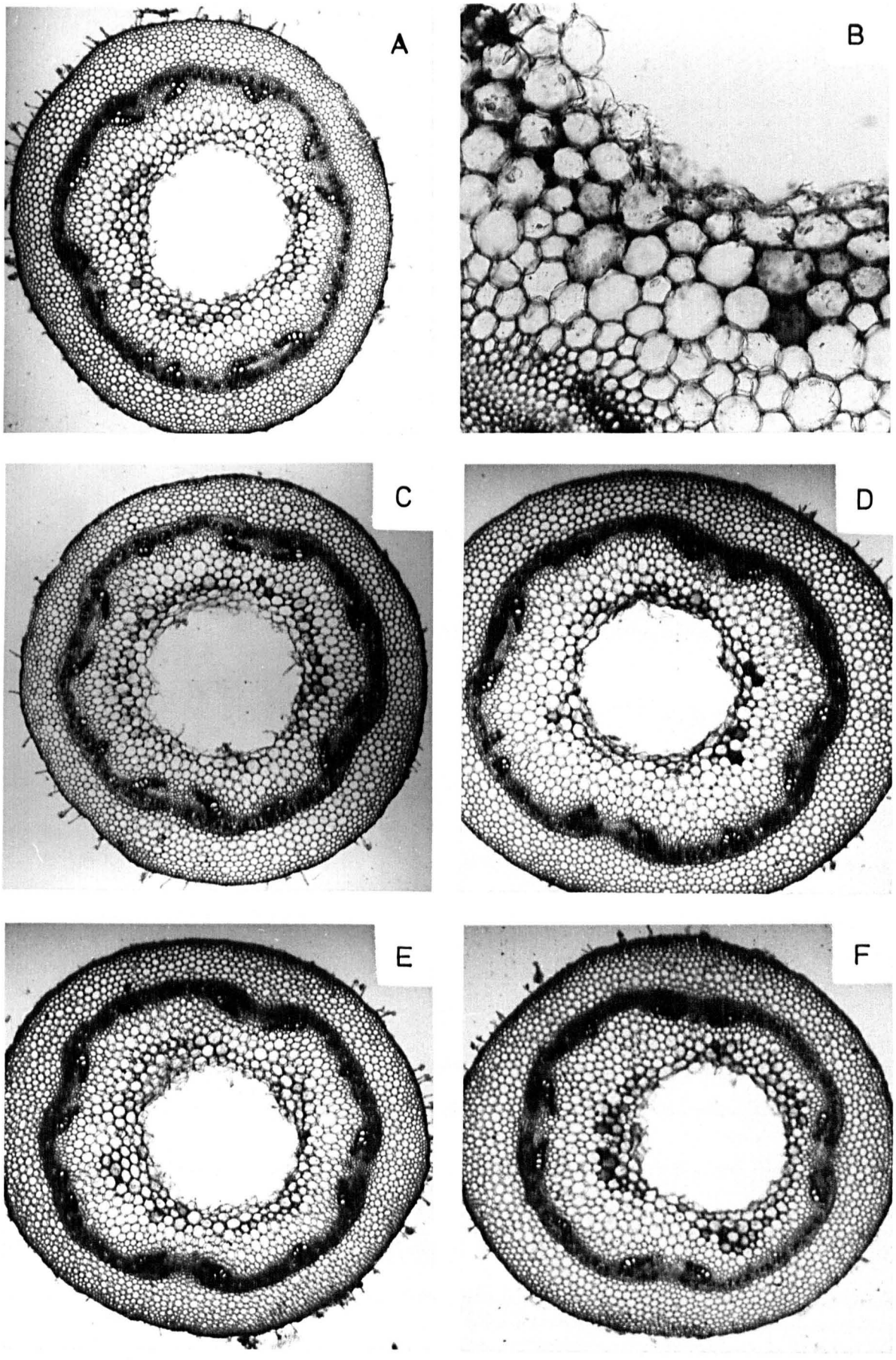
PLATE 12

Sections taken from hypocotyls inoculated with bean medium after pre-incubation for 24 h with a phytoalexin elicitor preparation from R. solani.

(A) 24 h (x 15); (B) 24 h (x 60);

(C) 48 h (x 15); (D) 72 h (x 15);

(E) 96 h (x 15); (F) 120 h (x 15).


A
B
C
D
E
F

the central cavity walls became heavier during the first two days (Plate 13, A and B), and 24 h later had begun to advance slowly through the hypocotyl (Plate 13, C and D). At this time, although many hyphae could be observed penetrating the tissues, there were also granulated, apparently dead, hyphae with attached amorphous material evident in browned cells (Plate 13, E). Four days after inoculation the fungus had begun to advance more rapidly (Plate 13, F) until after five days the hypocotyls had become heavily infected; sections could not be cut at this time due to the macerated condition of the tissues. By now, colonization was only slightly less intense than that in similar tissues which had not received the elicitor pre-treatment.

Inoculation of hypocotyls with the combined spore suspension/ elicitor preparation resulted in the formation of a narrow band of discoloured tissue around the central cavity (Plate 14, A and B). This appeared to increase slowly in area throughout the remainder of the incubation period (Plate 14, C-E). Hypocotyls inoculated with bean medium alone again did not exhibit appreciable browning in cross section, even after five days incubation (Plate 14, F).

DISCUSSION

The response of hypocotyls to inoculation with various spore concentrations of *F. solani* f.sp. *phaseoli* (Fig. 5.1) shows that both kievitone and phaseollin can accumulate in the artificial system following a challenge with this fungus. The extent of accumulation varies with the level of inoculum and the length of the incubation period.

It has already been proposed that the extremely low levels of these compounds in similar tissues inoculated with actively-growing mycelium of *F. solani* f.sp. *phaseoli* reflected such rapid invasion of the hypocotyls that little phytoalexin accumulation was accomplished. In addition, since this fungus can metabolize both kievitone and phaseollin *in situ* (VanEtten

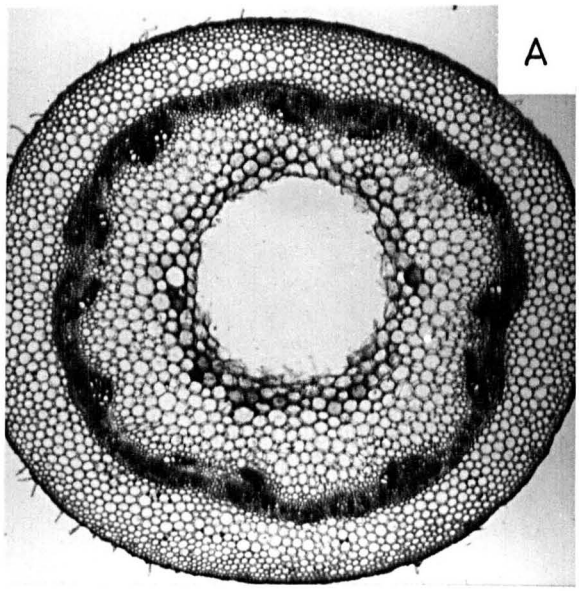
PLATE 13

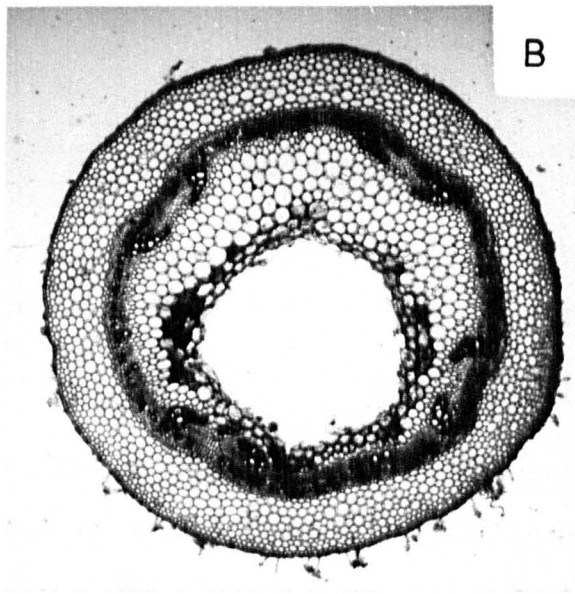
Sections taken from hypocotyls inoculated with a spore suspension of *F. solani* f.sp. *phaseoli* (c.2 x 10^4 spores/ml) after pre-incubation for 24 h with a phytoalexin elicitor preparation from *R. solani*.

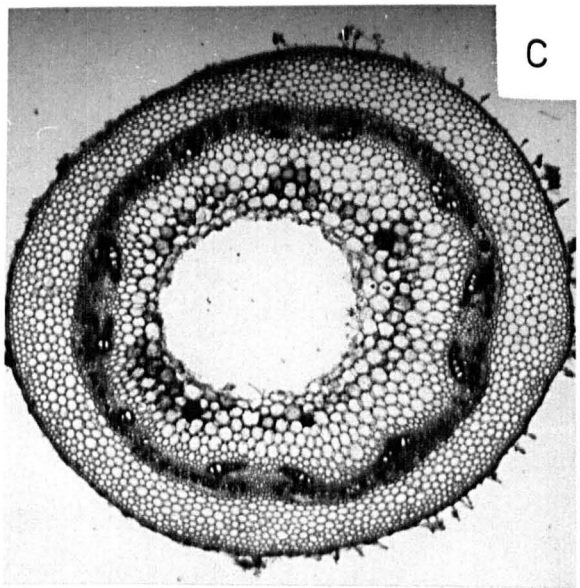
(A) 24 h (x 15); (B) 48 h (x 15);

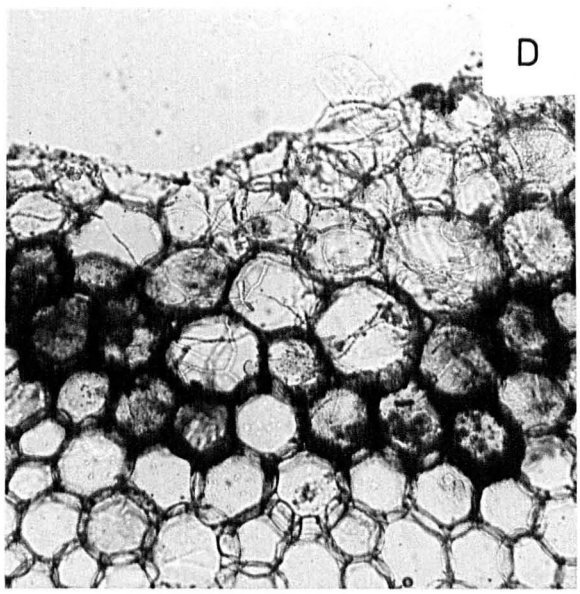
(C) 72 h (x 15); (D) 72 h (x 75);

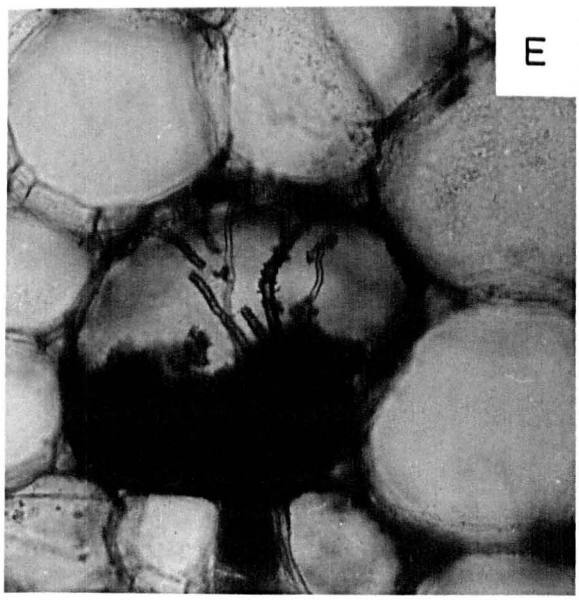
(E) 72 h (x 190); (F) 96 h (x 15).


A


B


C


D


E

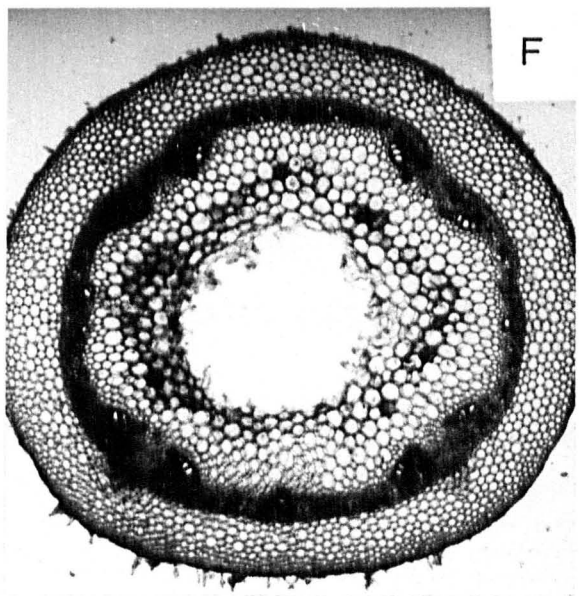
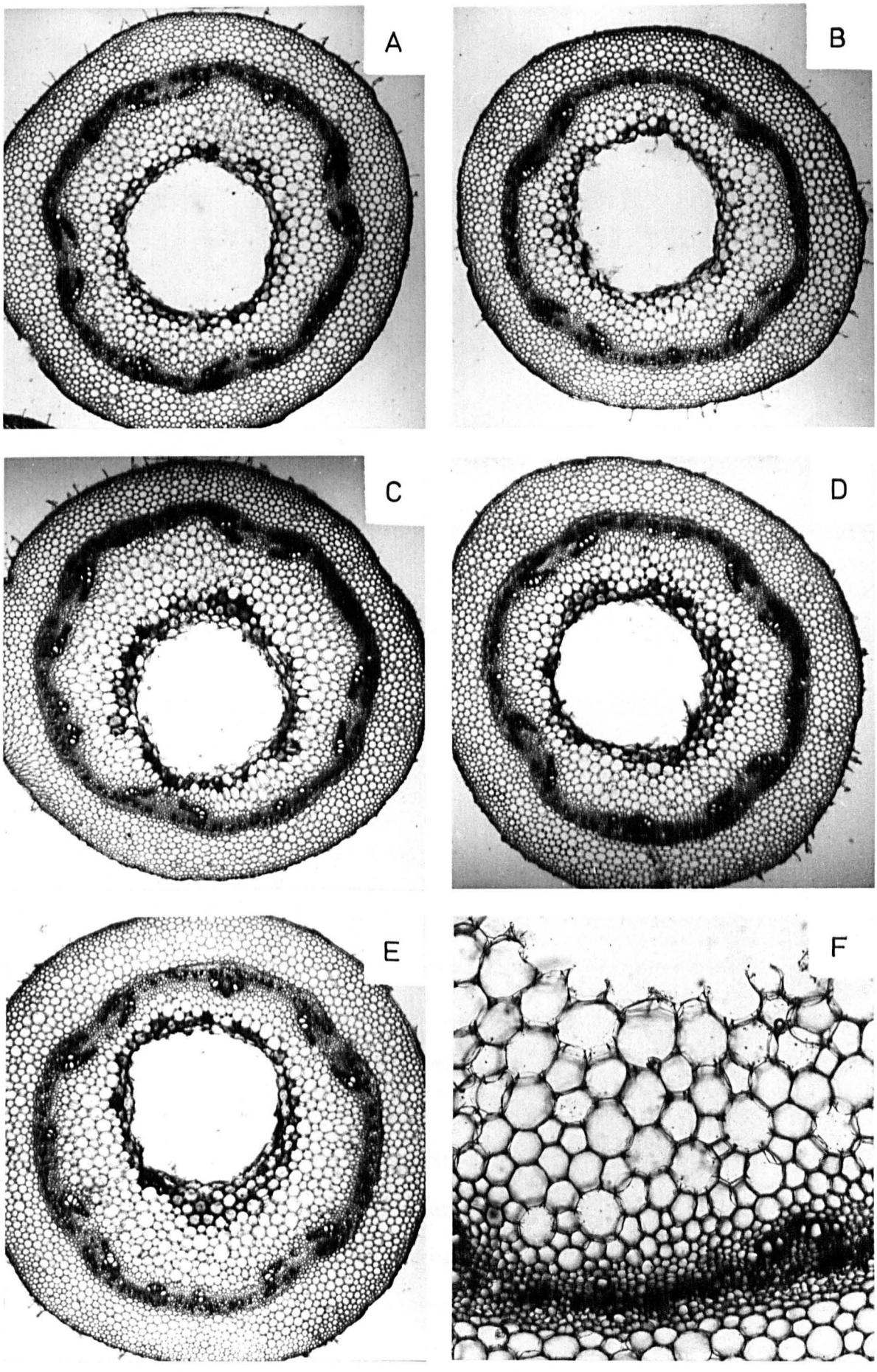

F

PLATE 14

Sections taken from hypocotyls inoculated simultaneously with a spore suspension (c.2 x 10^4 spores/ml) of F. solani f.sp. phaseoli and a phytoalexin elicitor preparation from R. solani after pre-incubation for 24 h with bean medium.

(A) 24 h (x 15); (B) 48 h (x 15);
(C) 72 h (x 15); (D) 96 h (x 15);
(E) 120 h (x 15); (F) 120 h after inoculation with bean medium (control) (x 60).


A
B
C
D
E
F

& Smith, 1975; Kuhn & Smith, 1978; Kuhn, 1979), it may be that phytoalexin-degrading enzymes remove any small amounts of such compounds which arise. However, in the case of a spore suspension, because of the inevitable delay in hyphal penetration of the tissues, the hypocotyls may well have time to synthesize phytoalexins. Nonetheless, once hyphal growth becomes established, the pattern appears to revert to the earlier situation; kievitone declines to trace levels in all treatments and phaseollin falls after a period of accumulation (Fig. 5.1 and Fig. 5.4,A).

Differences in phytoalexin levels arising from inoculation of hypocotyls with a spore suspension as opposed to actively-growing mycelium may, therefore, be significantly influenced by the time taken to penetrate host tissues after initiation of any host response. In addition, vegetative mycelium may be more tolerant to, and more effectively able to reduce, ambient phytoalexin concentrations (Skipp & Bailey, 1976; 1977; Bailey & Skipp, 1978). Adaptation of inocula to the inhibitory effects of phytoalexins may also be considered (Skipp & Carter, 1978). In the experiments outlined here, longer incubation of hypocotyls challenged with lower levels of inoculum (Fig. 5.1 and Plates 6 and 7) might have resulted in an ultimate level of infection similar to that observed after inoculation with the higher spore concentrations. However, the speed of colonization of host tissues and the pattern of phytoalexin accumulation in response to the highest spore concentration were chosen as most satisfactory for the cross-protection study. Therefore a concentration of c.2 x 10^4 spores/ml was used in all the cross-protection experiments.

Since the accumulation of kievitone and phaseollin in response to the elicitor from R. solani reached approximately maximal levels by 24 h after inoculation (Fig. 5.2), this incubation period was used for pre-treatment of hypocotyls during cross-protection experiments.

Variation in the chitin content of fungal mycelium grown in host tissue extracts has been observed previously (Ride & Drysdale, 1972);

there, the widest variation occurred after cessation of active growth. In the present experiment, however, the chitin content of F. solani f.sp. phaseoli mycelium varied more over the initial period of incubation than after active growth (as determined by dry wt measurement) had ceased (3-4 days after inoculation). Therefore, in calculating the amount of fungus present in infected hypocotyls after various periods of incubation, the chitin content of the mycelium at each assay time was used. This, rather than the use of an average figure, hopefully afforded a more accurate estimation of the fungal content of the infected tissues at each harvest.

The fungal content of hypocotyls at various time periods after inoculation with a spore suspension of F. solani f.sp. phaseoli (Fig. 5.4,B) correlated well with the observed development (Plates 8, 9 and 10). Up to three days after inoculation, fungal development was slow and the chitin content of the tissues remained low. Thereafter, rapid ingress by the fungus was reflected in the sharp rise in measured fungal content of the tissues (Fig. 5.4,B).

There was also a good correlation between the level of fungus estimated chemically and the extent of development observed microscopically in hypocotyls during the cross-protection study. Elicitor pre-treatment did not prevent colonization of hypocotyls from a spore suspension of F. solani f.sp. phaseoli. However, extensive growth did not begin until about four days after inoculation (Fig. 5.8 and Plate 13), some 24 h later than the onset of comparable development in unprotected hypocotyls. At this time, the level of kievitone (Fig. 5.6) had been reduced to approximately half that of tissues inoculated with bean medium alone after receiving the elicitor pre-treatment (Fig. 5.5). This, together with the appearance of kievitone hydrate at about the time when the level of kievitone began to fall most steeply (Fig. 5.6), suggests the direct involvement of the pathogen in degradation of the phytoalexin. The results imply that rapid fungal

development is restrained until the host antifungal products have been reduced to less inhibitory levels. The longer delay before rapid invasion by the fungus in cross-protected tissues may reflect the greater amount of kievitone present upon inoculation. Once the high level of kievitone has been reduced, however, the fungus can progress in a manner similar to that in unprotected tissues, where either localized kievitone may be efficiently degraded, or kievitone accumulation directly inhibited.

The apparent inhibition of fungal development observed when spores and elicitor were introduced simultaneously into hypocotyls (Fig. 5.8 and Plate 14), is attributed to the reduced germination of the fungal spores in the elicitor preparation. Separate germination tests of F. solani f.sp. phaseoli spores in the elicitor preparation revealed that germination was reduced to about 10% of that occurring in bean medium. It is perhaps possible, therefore, that further incubation of hypocotyls inoculated with the combined spore suspension/elicitor preparation would have revealed a similar pattern of fungal development to that in elicitor-pre-treated or unprotected tissues, except that the delay before the onset of rapid invasion of the hypocotyls would be even greater. The retarded appearance of kievitone hydrate (Fig. 5.7), compared to that in elicitor- pre-treated hypocotyls (Fig. 5.6) together with the delayed increase in measured fungal content (Fig. 5.8) and slow spread of necrotic tissue (Plate 14) might represent stages immediately preceding further development of the fungus. This possibility, however, was not experimentally verified.

Mechanisms of induced resistance:

A great deal of information is available concerning cross-protection (also commonly described as "acquired immunity" or "induced resistance") of plants against invasion by phytopathogens. This has recently been reviewed by Deverall (1977) and Kuć & Hammerschmidt (1978). If cases involving direct antagonism between the protectant and the challenge organism,

or structural modification of the host tissue in response to the protectant, are discounted, the question as to the nature of the physiological change conferring resistance on the otherwise susceptible host remains unanswered.

The protecting agent may be fungal, bacterial or viral (Goodman, 1967; Rahe, Kuć, Chuang & Williams, 1969; Skipp & Deverall, 1973; McIntyre, Kuć & Williams, 1973). Further, viruses may protect against fungi, or fungi against bacteria, and *vice versa* (Hecht & Bateman, 1964; Lovrekovich, Lovrekovich & Stahmann, 1968; McIntyre & Dodds, 1978). In addition, protection may result from heat treatment after inoculation with a pathogenic fungus (Rahe & Kuć, 1970), or from inoculation with heat-killed bacteria, bacterial or fungal sonicates, DNA or nematodes (Carroll & Lukezic, 1972; Lovrekovich & Farkas, 1965; McIntyre, Kuć & Williams, 1975; McIntyre & Miller, 1978). Thus, while hypersensitivity or cell necrosis occurs during the defence reaction in the majority of cases, the stimulus for response is apparently non-specific.

Stimulation of phytoalexin synthesis and accumulation is also apparently non-specific. Thus the presence of phytoalexins elicited by the protectant in a cross-protection study might seem a plausible explanation for failure of the normally-compatible challenge to develop. Indeed, the results from several studies suggest phytoalexin accumulation as a possible means by which cross-protection may be effected. These have included host-parasite interactions involving potato-*Phytophthora infestans* (Müller & Börger, 1940), soybean-*Phytophthora* spp. (Paxton & Chamberlain, 1967), French bean-*Colletotrichum lindemuthianum* (Berard, Kuć & Williams, 1972; Rahe 1973) and broad bean-*Botrytis* spp. (Hargreaves *et al.*, 1977). The exact relationship between hypersensitive cell death and phytoalexin accumulation is not known, but it is believed that phytoalexins are synthesized in healthy cells adjacent to necrotic areas (Mansfield,

Hargreaves & Boyle, 1974; Paxton, Goodchild & Cruickshank, 1974; Nakajima, Tomiyama & Kinugawa, 1975; Horikawa, Tomiyama & Doke, 1976; Hargreaves & Bailey, 1978). This may explain the observation by Rahe et al. (1969) that while only 5-10% of bean epidermal cells reacted hypersensitively to inoculation with an infection-droplet containing spores of a varietal-non-pathogenic race of C. lindemuthianum, the whole of the inoculated surface was resistant to subsequent infection by a normally-pathogenic race.

However, it appears that the systemic cross-protection operating over short distances on bean hypocotyls (Elliston, Kuć & Williams, 1971) may not be explained in a similar fashion. While it is possible, as suggested by Deverall (1977), that phytoalexins might diffuse from sites of production or follow the same routes taken by systemic fungicides in moving through plant tissue, phytoalexin production in response to infection is characterized by its localized nature. Moreover, phytoalexins could not be detected in tissue that was systemically protected but had not been inoculated with the challenge, yet phytoalexins did accumulate in systemically-protected tissue after application of the challenge (Kuć, 1975). In accord with Skipp & Deverall (1973), Kuć concluded that the induced resistance represented a conditioning of the cells, altering their sensitivity to a normally-pathogenic fungus such that resistance is expressed upon challenge. While phytoalexin accumulation may explain the resistance of the systemically-protected tissue, it is distinct from the conditioning of the cells to accumulate such compounds. This has been further demonstrated in that heat treatment greatly reduced the systemic protection effect, while localized protection and phytoalexin accumulation were not diminished (Elliston & Kuć, 1975). The altered physiological nature of cells adjacent to necrotic areas was demonstrated by heat sensitivity and increased cytoplasmic content, both in bean (Skipp & Deverall, 1973; Mercer, Wood & Greenwood, 1974) and tobacco (Israel & Ross, 1967; Ross & Israel, 1970).

Where induced resistance is apparently systemic, as in many of the cases involving bacteria and viruses, phytoalexins may not be the cause of the effect. Evidence has shown that resistance may be operative through agglutination of the bacterial cells (Kuć & Hammerschmidt, 1978; Sequeira, 1978).

Phytoalexin accumulation per se cannot account for the systemic cross-protection of cucurbits against Colletotrichum lagenarium by prior inoculation with this fungus or with TNV (summarized in Kuć & Hammerschmidt, 1978). Results suggest that a growth inhibitor and an agglutinating factor, both present in infected cucumber leaves, may be active in restricting development of C. lagenarium at the site of infection. Again, translocation (from the leaf inoculated with the protectant) of a factor capable of "sensitizing" the whole plant such that it becomes resistant to a normally-compatible challenge is implied. Such a phenomenon has already been observed in rice plants treated with dichlorocyclopropane derivatives prior to inoculation with the rice blast fungus, Piricularia oryzae. While other fungicides are known to elicit phytoalexin accumulation (Reilly & Klarman, 1972), the systemic chemical WL 28325 (2,2-dichloro-3,3-dimethyl cyclopropane carboxylic acid) appears to act by sensitizing the host such that the resistant response (evidenced by hypersensitive-like browning and production of the diterpene phytoalexins, momilactones A and B) which occurs upon subsequent inoculation with P. oryzae is enhanced (Langcake & Wickins, 1975; Cartwright, Langcake, Pryce, Leworthy & Ride, 1977). Whether other protectants can operate in this way remains to be seen.

Can phytoalexin elicitors effect cross-protection?:

The initiation of hypersensitive cell death and phytoalexin accumulation is far from well understood. Although the nature of a number of fungal phytoalexin elicitors has been determined and their ability to cause host tissue browning and phytoalexin accumulation well-documented (Section I),

apparently in only one case has the presence of an elicitor been shown to confer resistance on host tissues to a normally-pathogenic isolate. This work (Ayers et al., 1976b) formed part of a comprehensive study of the nature, occurrence and biological activity of elicitors purified from culture filtrates and mycelial wall preparations of P. megasperma var. sojae (Ayers et al., 1976a; 1976b; 1976c; Ebel et al., 1976). In their hypocotyl assay system, these workers found that the soybean phytoalexin, glyceollin, accumulated at the same rate whether in response to treatment with elicitor or to inoculation with mycelial suspensions of compatible or incompatible races of the fungus. If elicitor was applied simultaneously with mycelium from a compatible race the infection resulted in a normal, susceptible interaction. But if the hypocotyls were pre-treated for 10 h with elicitor before inoculation with the normally-compatible race, symptoms typical of an incompatible interaction were evident after 48 h incubation. From these findings, and from the fact that elicitors isolated from compatible or incompatible races were equally active in stimulating glyceollin accumulation, it was suggested that elicitors may be active in both compatible and incompatible interactions. Resistance may therefore depend more on the rate at which the fungus can colonize the host tissues.

A criticism of this work may be that observation of hypocotyls was only reported at one time period after inoculation with the compatible fungus. Therefore, the situation could well resemble the results reported here for growth of F. solani f.sp. phaseoli in elicitor-pre-treated as opposed to unprotected bean hypocotyls. The elicited level of kievitone appeared only to delay development of the fungus within the tissues such that if observations were made 96 h after inoculation, the pre-treatment would appear to have protected the bean tissues against spread of the pathogen (Fig. 5.4,B, Fig. 5.8 and Plates 9 and 13). Despite this, the end result was the same in pre-treated or unprotected hypocotyls, the main difference being purely temporal.

Induced susceptibility:

The apparently non-specific distribution of elicitors in varietal-pathogenic and varietal-non-pathogenic isolates of P. megasperma var. sojae is in opposition to the findings of Keen (1975a; 1978a; 1978b). However, the observations of Ayers and his colleagues are confirmed by reports that species of Colletotrichum with different pathogenicities towards Phaseolus vulgaris possess elicitors with equal ability to cause phaseollin accumulation and the hypersensitive response (Anderson, 1978a). Also, components from mycelial walls or zoospores and zoosporangia of Phytophthora infestans are apparently non-specific elicitors of terpenoid accumulation in potato tubers (Lisker & Kuć, 1977; Bostock et al., 1978; Henfling & Bostock, 1978). In fact, non-specific activity has been shown to operate widely in the P. infestans-potato tuber interaction. For example, apart from sonicates of many other fungi (Lisker & Kuć, 1977), cell-free sonicates of all races of P. infestans tested elicited comparable accumulation of terpenoid phytoalexins, whether in susceptible or resistant cultivars (Sato, Tomiyama, Katsui & Masamune, 1968; Varns et al., 1971a; 1971b). Therefore, it is likely that the potential for resistance exists even in an apparently susceptible cultivar, but that during a compatible infection the host resistant reaction is not expressed (Kuć, 1972; Currier, 1974; Kuć et al., 1976).

Control of phytoalexin synthesis might indeed lie with the ability of compatible pathogens to avoid triggering, or to specifically repress, as part of the host defence reaction, the phytoalexin-synthesizing machinery (Section IV). According to Ward & Stoessl (1976), such a scheme fits more closely the available evidence, where compatibility is the exception to the incompatible rule. It also explains the phenomenon of "induced susceptibility" observed by Tomiyama (1966) and Varns & Kuć (1971) in leaf petioles and tubers of potato. In this case, prior inoculation with a compatible race of P. infestans suppressed necrosis and phytoalexin

accumulation in response to subsequent inoculation with an incompatible race or with a cell-free sonicate of the fungus. A mechanistic explanation of this phenomenon, also incorporating an interpretation of the observed differential accumulation of steroid glycoalkaloids in these tissues (Shih & Kuć, 1973; Kuć et al., 1976), has been proposed by Currier (1974 - see also Kuć et al., 1976; and Section VI). As with the "specificity factors" of Ayers et al. (1976c - see also Section VI), the proposed "blocker" of rishitin synthesis in potato tubers has not, however, been identified. Nonetheless, there are a number of recent reports of components from mycelium and zoospores of P. infestans which appear to inhibit the hypersensitive response and terpenoid accumulation in tuber slices (Doke, 1975; Doke, Garas & Kuć, 1977; Garas, Doke & Kuć, 1977).

That this phenomenon is not limited to potatoes is evidenced by the fact that suppressors of hypersensitivity and phytoalexin accumulation occur in spore germination fluids of Mycosphaerella pinodes, a fungus pathogenic to peas, (Oku, Shiraishi & Ouchi, 1978) and in exudates of Pseudomonas phaseolicola, the primary causal agent of halo blight of bean (Patil & Gnanamanickam, 1976). Furthermore, an ability to suppress phytoalexin production may be essential for the infection of grapevines by Plasmopara viticola (Langcake, 1978). Also, the bacterially-induced hypersensitive reaction of tobacco can be suppressed by cytokinin pre-treatment before inoculation with Pseudomonas pisi or, to a lesser extent, P. tabaci (Novacky, 1972). It would certainly be interesting to see whether suppressors from zoospores or mycelium of P. infestans could prevent a resistant reaction by potato tuber tissue in response to elicitor from the same source, or vice versa. From the results of such experiments, a hypothesis based on differential possession of suppressors between fungal races, or differential synthesis or release of such compounds in response to an initial recognition signal, might materialise.

Induced susceptibility in F. solani f.sp. phaseoli infection of bean?:

Although apparent differential synthesis of phytoalexins may reflect metabolism by the pathogen and/or by the host tissues (and both these factors appear to operate in F. solani f.sp. phaseoli-infected bean tissues), it is tempting to suggest that the low level of kievitone produced in response to infection by F. solani f.sp. phaseoli may be due to the production by the fungus of a suppressor of kievitone synthesis. Since kievitone synthesis seems to occur earlier than phaseollin synthesis, possession of a kievitone suppressor would allow the fungus to become established and undergo substantial development before encountering high levels of toxic compounds. Indeed, rapidly-growing hyphae may never experience such levels since they may grow through tissues before phaseollin has accumulated to significantly inhibitory levels.

In this regard, the results of a preliminary "natural" double infection of bean hypocotyls by F. solani f.sp. phaseoli and R. solani are interesting. Five days after inoculation, hypocotyls inoculated with a mycelial suspension of R. solani alone exhibited characteristic dark brown, sunken, restricted lesions; kievitone in these tissues was determined to be c.160 µg/g fresh wt. Hypocotyls inoculated with only a spore suspension of F. solani f.sp. phaseoli had relatively advanced, coalescing orange lesions characteristic of the disease; the kievitone level was c.10 µg/g fresh wt. The amount of kievitone in hypocotyls five days after simultaneous inoculation with R. solani and F. solani f.sp. phaseoli, however, was c.80 µg/g fresh wt, almost exactly half that of hypocotyls inoculated with R. solani alone. This finding may reflect only metabolism of kievitone by F. solani f.sp. phaseoli. However, the symptoms of this double infection appeared much worse than in hypocotyls inoculated with R. solani or F. solani f.sp. phaseoli alone; complete girdling and shrinking of the hypocotyl had taken place. These findings are in opposition to those of Pieczarka & Abawi (1978), who found that there was no synergistic interaction between R. solani and F. solani f.sp.

phaseoli during simultaneous infection of beans.

If *F. solani* f.sp. *phaseoli* produces a suppressor of kievitone synthesis, its action may in part be over-ruled by the presence of an elicitor from *R. solani* such that kievitone levels, perhaps moderated by metabolism, achieve only modest heights. Alternatively, more rapid penetration by *R. solani* could lead to kievitone accumulation before the suppressor from *F. solani* f.sp. *phaseoli* can be released. Finally, the two may be spatially separated, or may be competing for the same cellular sites, such that both are active but the net result is half-way between the two extremes. Further experimentation, possibly involving time courses and/or pre-treatment for various periods with one or other of the fungi, observing cytologically fungal progression in the tissues, and monitoring the phytoalexin metabolites as well as the parent compounds, may prove worthwhile. Certainly the role of phytoalexins in induced resistance or susceptibility cannot be investigated fully until the mechanisms involved in stimulation (or suppression) of the resistant response by recognition of the incitant have been established.

SECTION VI

GENERAL DISCUSSION

This study was undertaken in an attempt to discover whether previously-observed differences in the pattern of phytoalexin, particularly kievitone, accumulation in *Phaseolus vulgaris* hypocotyls infected by either *Rhizoctonia solani* or *Fusarium solani* f.sp. *phaseoli* might be explained in terms of differential production of phytoalexin elicitors by these fungi. Marked differences were found between the two fungi with respect to the ability of their constitutive metabolites to elicit kievitone accumulation in excised bean hypocotyls. Whether such differences contribute directly to the observed disparity in fungal colonization in the natural disease situations was not clearly resolved.

It was foreseen that a possible rate-limiting step in the investigation might be the routine analysis of phytoalexin levels in treated bean tissues. Therefore, time was devoted to the development of a gas-liquid chromatographic (GLC) system to accomplish this more rapidly. Unfortunately, kievitone did not prove amenable to quantitation by any of the GLC procedures tried; details are reported in the Appendix. Later in the investigation, results from preliminary experiments on the use of high performance liquid chromatography (HPLC) to resolve extracts of *R. solani*-infected hypocotyls (carried out by Dr. D.F. Ewing, Chemistry Dept., Hull University), indicated that this technique might provide a more successful rapid method for phytoalexin estimation. No detailed procedure was established, however. Conventional TLC and UV spectrophotometric methods were therefore employed to monitor kievitone and phaseollin levels.

In a host such as P. vulgaris, where a multiple phytoalexin response is known to occur, restricting observations to only two components may seem inadvisable. However, the main interest lay with kievitone, since the pattern of kievitone accumulation in R. solani-infected bean hypocotyls implicated it as the phytoalexin primarily responsible for lesion restriction (Smith et al., 1975). Phaseollin, as the antifungal compound which ultimately reached the highest level in response to this fungus and as the phytoalexin most prevalent during the early stages of infection by F. solani f.sp. phaseoli (Smith et al., 1975; VanEtten & Smith, 1975), was monitored to provide a "reference" for observed levels of kievitone.

Initial experimentation on the inoculation of intact plants provided results which compared favourably with earlier reports. The patterns of disease development and phytoalexin accumulation were as anticipated; hypocotyls infected by R. solani contained high levels of kievitone at about the time lesions became restricted, whereas hypocotyls bearing spreading lesions caused by F. solani f.sp. phaseoli yielded only very small amounts of this phytoalexin (Section III). Since the host tissues were the same in each case, phytoalexin differences may only reasonably be attributed to mediation of the host response by the fungi.

The extraction of whole tissues rather than excised lesion material likely underestimates phytoalexin levels in the immediate environment of the pathogen (Bailey & Deverall, 1971; Section I). However, it is hoped that such figures allow valid comparisons to be drawn between the two disease situations under consideration, particularly during the earlier stages of infection, when the ratio of lesion to healthy material is more likely to be similar.

Studies of many host-parasite interactions have employed excised host tissues to investigate the potential of microorganisms or, more often, their products to elicit a host resistance response. In this respect, the current investigation was no different, but an attempt was

made to develop an artificial system approximating to the natural disease situation (Section III). Although the inoculation procedure employed brought potential elicitors into contact with cells of the central pith rather than the cortex, the response was nonetheless similar. Even so, comparisons of responses produced by excised tissues and by whole plants must be interpreted cautiously, since there must be inherent differences. For example, the apparent limitation of the artificial inoculation system in not allowing substantial phaseollin accumulation, as discussed in Section III, may represent an artefact of the system itself rather than mediation of the host response by a test component. Nonetheless, the artificial system provided a means whereby comparative tests of eliciting potential could be made between different components; it was not considered an exact reproduction of any natural situation.

Phytoalexin elicitors in R. solani and F. solani f.sp. phaseoli:

Cultured mycelia of R. solani and F. solani f.sp. phaseoli contained very different amounts of water-extractable material capable of eliciting kievitone accumulation in excised bean hypocotyls. Kievitone accumulation could apparently be saturated (at c. 450 - 500 μg/g fresh wt) in response to cell-free mycelial extracts of R. solani (Section IV, Table 4.2 and Fig. 4.3); only low levels of the phytoalexin were found in hypocotyls treated with similar extracts from F. solani f.sp. phaseoli (Section IV, Table 4.3 and Fig. 4.2,B).

Partial purification of an elicitor fraction from the cytoplasm of R. solani did not allow any firm conclusions concerning the nature of the active component(s) (Section IV). However, the possibility remains that the active material resembles elicitors reported from the mycelia of other fungi. In addition, a technique which had previously been employed to solubilize elicitors from cell walls of filamentous fungi and yeasts (Anderson-Prouty & Albersheim, 1975; Ayers et al., 1976b) revealed

phytoalexin-eliciting activity in purified cell wall preparations of R. solani. Again, the material was not thoroughly characterized; this awaits further experimentation. Although, as proposed earlier for the cytoplasmic elicitor, wall pigments may be present in the preparation (indeed, they may be the active components), it is possible that the active material resembles other wall-released elicitors. Clearly, there is scope for further investigation into the nature of both the cytoplasmic and wall-released elicitors detected in R. solani. If purification to homogeneity could be achieved, perhaps through the use of ion exchange or electrophoretic methods together with molecular sizing techniques, a more rigorous study of their chemical structures and biological activities could be conducted.

Although the procedure used for the preparation of mycelial extracts from F. solani f.sp. phaseoli was the same as that for R. solani, only comparatively low levels of material, particularly carbohydrate, were isolated from this fungus. It seems likely that this quantitative difference, perhaps more than any qualitative one, could have contributed to the observed disparity in the levels of phytoalexin produced by the host tissues in response to the mycelial extracts.

If elicitor activity is present in extracts of F. solani f.sp. phaseoli, it may be that more concentrated preparations are needed to effect the host reaction. The high activity of elicitors from other fungi, however (Anderson-Prouty & Albersheim, 1975; Ayers et al., 1976a; 1976b; Anderson, 1978a), renders this an unlikely explanation of the low phytoalexin response observed. Any potential elicitor could be inactivated by aggregation with other material in the preparation, or altered in some other way during extraction. One further possibility for the negative results achieved with F. solani f.sp. phaseoli could be that elicitor activity was present in the mycelium but not extracted by the procedure employed. Support for this idea comes from the observation that elicitors

of host resistant responses can be demonstrated in other Fusarium spp. (Anderson, 1978b). The preparations obtained, however, did not cause a defence reaction to develop in all hosts, indicating that there may be some kind of specialization in stimulation of the host response. The fact remains that the current investigation did not provide evidence for the existence of appreciable kievitone-eliciting activity in F. solani f.sp. phaseoli, although it would seem worthwhile investigating whether a kievitone elicitor can be solubilized from the mycelial walls of this fungus.

Phytoalexin biosynthesis and its regulation in infected tissues:

In order to evaluate possible mechanisms operating to exert control over phytoalexin production, it is necessary to gain an understanding of the series of biochemical events leading to phytoalexin biosynthesis.

The enzymic pathways to terpenoid and isoflavonoid phytoalexins are known, at least in outline (Stoessl, Stothers & Ward, 1976; VanEtten & Pueppke, 1976). For isoflavonoids, VanEtten & Pueppke (1976) suggest that the biosynthetic route be considered in stages: (1) early pathways shared with other secondary metabolites, (2) reactions common to flavonoid and isoflavanoid biosynthesis, and (3) biosynthetic steps unique to isoflavonoids. Much less information is available on the latter two stages than for the early pathways.

There are many reports that increased levels of phenylalanine ammonia-lyase (PAL) are associated with phytoalexin production, and isotopic labelling has been used to demonstrate the incorporation of precursors such as phenylalanine, acetate and cinnamic acid into phytoalexins (Hadwiger, 1967; Hess, Hadwiger & Schwochau, 1971; VanEtten & Bateman, 1971; Shih & Kuć, 1973; Dewick, 1975). Also Hadwiger, Hess & Broembsen (1970) showed that compounds which were effective in causing increases in PAL activity in pea and bean tissues did not significantly stimulate PAL in hosts where the presence of phytoalexin-like responses have not been clearly demonstrated.

However, the influence of PAL in the regulation of phytoalexin biosynthesis is doubtful since there are a number of reports where changes in the level of PAL cannot be directly associated with phytoalexin production (Munn & Drysdale, 1975; Partridge & Keen, 1977); in any case, as suggested by Partridge & Keen (1972), the involvement of PAL in many biosynthetic pathways makes it less likely that its regulation can be important in the control of phytoalexin production.

Later steps in isoflavonoid biosynthesis are not completely understood, but VanEtten & Pueppke (1976) present a possible metabolic grid for isoflavonoid interconversion based on the evidence available. This scheme allows the possibility that, in bean hypocotyls infected with F. solani f.sp. phaseoli, kievitone production could be inhibited while other phytoalexins might still be synthesized.

Rathmell & Bendall (1971) found evidence to suggest that phaseollin synthesis in excised bean hypocotyls formed part of a specific stimulation of isoflavonoid metabolism, but they were unable to demonstrate a causal relationship between phytoalexin production and increases in the level of chalcone-flavone isomerase (Rathmell & Bendall, 1972). Zaehringer, Ebel & Grisebach (1978) observed increases in the activity of PAL and two other enzymes related to general phenylpropanoid metabolism, but a decrease in flavanone synthase, the initial enzyme of the flavonoid pathway, when a phytoalexin elicitor from P. megasperma var. sojae was applied to soybean cotyledons, thereby indicating a switch from flavonoid into isoflavonoid biosynthesis. Dixon & Bendall (1978), however, found that increases in the levels of several enzymes of isoflavonoid synthesis in bean cell suspension cultures were not temporally co-ordinated in terms of synthesis of phaseollin and other isoflavonoids. Clearly, as Drysdale (1978) suggests, further investigation of the later enzymes in the pathways of isoflavonoid synthesis may eventually be more fruitful.

A number of studies have attempted to determine whether metabolic control of phytoalexin biosynthesis is dependent upon de novo gene activation and messenger RNA and protein synthesis. Hadwiger and his associates have obtained variable results from their investigations into whether RNA synthesis is a pre-requisite for pisatin production in pea endocarp. Nonetheless, they consider that pisatin synthesis requires transcription of normally-inaccessible regions of DNA, which may be made available as a result of the action of various eliciting treatments (Hadwiger, Jafri, Broembsen & Eddy, 1974). A similar situation may exist for phaseollin in P. vulgaris, although Biggs (1972) found that phaseollin production was not reduced after RNA synthesis had been inhibited by treating bean pods with actinomycin D. Recently, Yoshikawa et al, (1978b) demonstrated, by the use of selective inhibitors, that both RNA and protein synthesis were required for the production of glyceollin in soybean hypocotyls. Their results indicated that the normal expression of resistance to an incompatible isolate of P. megasperma var. sojae or to several non-pathogenic Phytphthora spp. was, together with glyceollin production, dependent upon de novo mRNA and protein synthesis soon after infection.

While fine control of phytoalexin synthesis may be of some influence in determining the particular spectrum of phytoalexins produced by a plant in response to infection by different pathogens, where phytoalexins appear to play a major part in the host defence reaction resistance may rather be dependent upon the outcome of the initial interactions between host and pathogen which determine whether or not phytoalexin accumulation per se takes place.

Elicitors and the determination of disease resistance:

Hadwiger & Schwochau (1969) advanced a hypothesis to explain host resistance responses by proposing that pathogen products could lead to the induction of a specific host system (e.g. phytoalexin production) by

inhibiting the synthesis of a repressor which in healthy plants occupied the operator site of a resistance operon, preventing continuous expression of that particular resistant response. Although various attempts have been made to involve the action of pathogen-produced elicitors in such a system, so far little evidence has been obtained on how such elicitors might effect a host defence reaction.

One of the most-studied interactions has been that between soybean and P. megasperma var. sojae; since several workers have investigated this interaction, their results merit careful consideration.

Keen (1975a) presented evidence for the existence of a race-specific elicitor which could explain varietal resistance of soybean plants to different races of the fungus. The soybean cultivar Harosoy is susceptible to both race 1 and race 3 of P. megasperma var. sojae; the near-isogenic Harosoy 63 is susceptible to race 3 but resistant to race 1. Keen found that culture fluids from race 3, in line with the non-specific elicitor actinomycin D, caused the accumulation of similar amounts of glyceollin in hypocotyls of both cultivars. Culture fluids from race 1, however, elicited higher levels of the phytoalexin in hypocotyls of Harosoy 63 than in those of the susceptible cultivar. Despite the presence of non-specific elicitor activity in the preparations, Keen found evidence of a specific elicitor of glyceollin production which caused the accumulation of higher levels of glyceollin in Harosoy 63 than in Harosoy, thereby explaining the differential resistance of the two cultivars to race 1.

Ayers et al. (1976a; 1976b; 1976c) purified extracellular and wall-released elicitors from races 1, 2 and 3 of P. megasperma var. sojae. They were unable to demonstrate any race-specific activity in these components; active material from the three races was similar in composition and biological activity on Harosoy 63. Pointing out possible qualitative and quantitative limitations of Keen's work, Ayers and his associates produced a hypothesis to explain varietal resistance in terms of differential growth

rates of the fungus in the respective cultivars. Thus, under the influence of a hypothetical "specificity factor" from the fungus, a resistant host might produce other inhibitors capable of slowing pathogen growth such that a build-up of phytoalexin might subsequently restrict further development. A compatible fungus, on the other hand, might avoid triggering the primary inhibitory response and thus, by more rapid growth, avoid the toxic effects of phytoalexin accumulation.

Yoshikawa et al. (1978a) observed closely both fungal development and glyceollin accumulation in resistant and susceptible soybean cultivars. They noted that the initial growth of the pathogen was apparently similar in both cases, but that high levels of glyceollin accumulated earlier at the infection sites in the resistant cultivar. Thereafter, fungal development ceased in resistant tissues, whereas in susceptible hypocotyls the pathogen continued to spread even though highly toxic levels of phytoalexin were ultimately achieved at infection sites. These results may be interpreted simply as slower phytoalexin accumulation in the susceptible interaction allowing the pathogen to invade new tissue before inhibitory levels of antibiotic are reached, i.e. the speed of phytoalexin formation determines resistance. While these findings do not support the 'specificity factor' theory proposed by Ayers et al. (1976c), they are, on the other hand, consistent with the original postulates of Müller & Börger (see Cruickshank, 1963).

The non-specific elicitors isolated by Ayers and his associates may not be the same as the specific elicitor claimed by Keen. Indeed, Keen (1978a; 1978b) has recently reported that by using a milder treatment than the autoclaving procedure of Ayers et al. (1976b), specific elicitors, which may be glycoproteins, can be isolated from the cell walls of P. megasperma var. sojae. He suggests that these specific elicitors may be recognized only by incompatible soybean genotypes, thereby effecting

resistance through enhanced glyceollin production. Although only the glucosyl portion of the glycoprotein appears to be important for specific elicitor activity, the possibility remains that the harsher extraction procedure employed by Ayers et al. altered the elicitors such that race specificity was lost.

Keen's work was the first suggestion of specific elicitors of phytoalexin accumulation operating as determinants of race-specificity in host-parasite interactions. More recently, however, Ziegler & Albersheim (1977) isolated extracellular invertases from races 1, 2 and 3 of P. megasperma var. sojae which proved to be glycoproteins exhibiting race-specificity in terms of their carbohydrate structure. Since such molecules may be among the first to interact with host tissues, they may function as race-specific determinants of resistance. Albersheim and his co-workers have recently claimed that factors from the incompatible but not from compatible races of the fungus can protect otherwise susceptible soybeans from invasion by a compatible race of the pathogen (Albersheim, Valent, Hahn, Wade & Cline, 1978).

Clearly the current investigation does not involve race-specific phenomena, yet the general observations outlined above for the Phytophthora-soybean interaction may well be applicable to interactions at the species level, viz. Rhizoctonia as opposed to Fusarium infections of bean. The evidence indicates that possession of an elicitor capable of causing the accumulation of high phytoalexin levels results in the development of an incompatible interaction. Thus the presence in R. solani of material capable of eliciting high levels of kievitone in bean hypocotyls could result in the formation of localized lesions. Conversely, F. solani f.sp. phaseoli, which may not elicit kievitone accumulation, can extensively colonize similar tissues.

Suppressors of host defence reactions as determinants of disease resistance:

An alternative explanation for varietal resistance associated with differential phytoalexin accumulation is that compatible pathogens may be able to suppress a normal host resistant response. As discussed in Section V, components have been isolated from virulent fungal strains capable of preventing a host defence reaction on subsequent inoculation with an avirulent isolate. In certain cases where suppressors are thought to operate, there may also be a significant amount of elicitor activity present (Varns & Kuć, 1971; Currier, 1974; Oku et al., 1978). An explanation for this may be that during the normal course of infection an aggressive pathogen preferentially secretes metabolites (suppressors) which can "switch off" the host's defences. Mycelial extracts or sonicates, however, since they may contain components capable of elicitor activity, can swamp out the effect of the suppressors, resulting in the expression of a resistant response.

Currier (1974 - see also Kuć et al., 1976) interpreted such results for the potato- Phytophthora infestans interaction by proposing that during a compatible interaction a general defensive reaction may be activated in the host; metabolites of the acetate-mevalonate pathway are shifted from steroid glycoalkaloid into phytoalexin (rishitin) synthesis (Shih, Kuć & Williams, 1973). However, genotypically-determined blockers (suppressors), acting at specific sites, could prevent rishitin synthesis, allowing other, presumably non-toxic, terpenoids to accumulate, thereby negating a resistant response. In an incompatible interaction, the absence of a block in phytoalexin synthesis would allow a full resistant response to develop.

There is evidence to suggest that further investigation of F. solani f.sp. phaseoli for suppressors might prove fruitful. In studies continuing an earlier investigation by Teasdale et al. (1974), Daniels & Hadwiger

(1976) showed that although culture filtrates of this fungus could elicit the accumulation of pisatin in pea endocarp, factors present in concentrated filtrates completely inhibited phytoalexin production. It is therefore possible that inhibitors of the host resistant response may operate in a similar fashion in bean hypocotyls during infection by F. solani f.sp. phaseoli. However, unlike some other situations, where the host resistant response is inhibited, if such components are operative in Fusarium-infected bean hypocotyls, they must exhibit some kind of specificity since lesion browning and the accumulation of phytoalexins other than kievitone are not prevented (Section III). It is conceivable that, as proposed for the potato- Phytophthora interaction, a general response is triggered but that in beans there is no complete block of the defence reaction, merely a suppression of certain facets; the biosynthetic route into isoflavanones (e.g. kievitone) may be blocked while pathways into other isoflavonoids remain operative.

Host-parasite recognition and stimulation of phytoalexin accumulation:

Whether phytoalexin accumulation occurs by specific stimulation of biosynthetic pathways by elicitors or as part of a more general response in the absence of pathogen-produced suppressors, in either case some form of recognition between host and parasite is implied. There is considerably more information available concerning elicitors in this respect.

Phytoalexin elicitors of fungal origin appear for the most part to contain glycosl portions as active components; this is evidenced by direct analysis and by the inhibition of their action by sugars or glycosides (Anderson-Prouty & Albersheim, 1975; Ayers et al., 1976c; Marcan, Jarvis & Friend, 1979). Interactions of these carbohydrate moieties with host receptors such as lectins may therefore be considered in possible determination of disease resistance (Albersheim & Anderson-Prouty, 1975; Callow, 1975; 1977; Sequeira, 1978). The operation of such a mechanism in a specific elicitor

theory of phytoalexin accumulation and disease resistance is not difficult to imagine. However, the idea that varietal resistance might be explained by suppression of a general defensive reaction by a compatible parasite is also tenable - the suppressor may be a hapten which can mask the receptor sites, thereby negating a resistant response.

Neither of these two possibilities is incompatible with a recent report that biotic and abiotic elicitors exert their action by different means (Yoshikawa, 1978). This work confirmed other reports of a possible turnover of phytoalexins in plant tissues (Stoessl, Robinson, Rock & Ward, 1977; Glazener & VanEtten, 1978; Hargreaves & Selby, 1978). However, Yoshikawa was able to show that biotic elicitors, such as cell wall preparations of P. megasperma var. sojae, caused glyceollin accumulation in soybean cotyledons by stimulating synthesis of the phytoalexin. On the other hand, while their effects on phytoalexin synthesis were small, abiotic elicitors, such as heavy metal salts or detergents, inhibited the phytoalexin degrading activity of the tissues. Subsequently, Yoshikawa & Masago (1978) found that glyceollin synthetic activity was the same in soybean hypocotyls infected with an incompatible or a compatible race of P. megasperma var. sojae. Their results showed that glyceollin degrading activity was inhibited more strongly in hypocotyls inoculated with the incompatible race than in those infected with the compatible race, indicating that differential glyceollin degrading activity could be important in determining levels of the phytoalexin in infected hypocotyls and, therefore, disease specificity.

In the present study, as is the case elsewhere, phytoalexin accumulation was associated with host tissue browning. Thus it may be that cell necrosis leads to phytoalexin accumulation, in which case the events determining cell death may be responsible for the determination of disease resistance. Elicitors of phytoalexin accumulation may operate by eliciting cell death and, subsequently and indirectly, phytoalexin accumulation. Such

a theory would fit the results of Hargreaves & Bailey (1978) who found that a constitutive elicitor produced by bean cells could cause phytoalexin accumulation; the bean elicitor might be liberated following cellular reactions in response to abiotic stimuli or viruses, where pathogen-produced elicitors cannot function. These workers also obtained evidence to suggest that phytoalexin synthesis took place in healthy tissue associated with necrotic material, thus shedding some light on the argument as to whether phytoalexins are produced in healthy or dead or dying tissue. Such a decision may well be arbitrary anyway, since cells which produce phytoalexins may rapidly die due either to the phytotoxic effects of the high concentrations produced or to a dysfunction of other cellular processes as a result of a massive switch to phytoalexin synthesis. Thus, phytoalexin accumulation may lead to cell death, although this may be differentiated from the initial interactions (possibly involving cell death) leading to phytoalexin production.

In cases where elicitors of phytoalexin accumulation are operative, even though host tissue necrosis is observed also, phytoalexin presence alone may not be enough to bring about resistance to infection. This is evidenced by the failure of simultaneous application of elicitor (or even short-term pre-incubation with elicitor) from P. megasperma var. sojae to inhibit the growth of a compatible isolate of the fungus in soybean hypocotyls (Ayers et al., 1976b). Also, in the present study, pre-incubation of bean hypocotyls with a phytoalexin elicitor preparation from R. solani did not prevent invasion by F. solani f.sp. phaseoli, although some delay was indicated. Furthermore, in a study of the effects of environmental factors on glyceollin production in Phytophthora-infected soybeans, Murch & Paxton (1978) found that in some tests complete suppression of glyceollin accumulation did not decrease resistance. Clearly other factors must operate here; preliminary results obtained

by Hadwiger, Loschke & Teasdale (1977) and Hadwiger & Adams (1978) have indicated that histones and other basic proteins inhibitory to the growth of Fusarium solani may be involved in the resistance of peas to isolates of this fungus.

On the basis of the evidence so far obtained, little concrete has been learned about the nature of the interactions between host and parasite leading to phytoalexin accumulation. The prospect of the pathogen further altering the manifestation of the host response through metabolizing host products has only briefly been mentioned here, but clearly such mechanisms alter the effectiveness of a defensive reaction. It is to be hoped that continuing studies into the complex nature of recognition and the determination of subsequent events in host-pathogen interactions will provide an understanding sufficient to allow effective protection against disease by manipulating natural mechanisms to become a reality.

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APPENDIX

ATTEMPTED QUANTITATION OF KIEVITONE IN DISEASED BEAN TISSUES BY GAS-LIQUID CHROMATOGRAPHY

Several phytoalexins from diseased tissues of French bean and soybean may be quantified by gas-liquid chromatographic (GLC) analysis of their trimethyl silyl (TMS) ether derivatives (Keen, Sims, Erwin, Rice & Partridge, 1971; Heuvel & VanEtten, 1973; VanEtten & Smith, 1975). Despite the report by VanEtten & Smith (1975) that they were unable to prepare satisfactory derivatives of kievitone, it was felt at the outset that the current investigation would benefit from the development of a GLC technique for quantitation of kievitone in diseased bean hypocotyls. Such a method would, hopefully, prove more rapid than conventional TLC and UV spectrophotometric quantitation for routine tissue analysis, while also allowing more accurate determinations, particularly when the compound was present in only small amounts.

In conjunction with C.F.G. Jones (Plant Biology Dept., Hull University), samples of purified kievitone (Section II) were dried from EtOH under a stream of N_2 and dissolved in tetrahydrofuran (THF: silylation grade) prior to derivatization according to several different silylation procedures. Analysis of the derivatives was accomplished using a Pye 104 Model 64 dual heated flame ionization gas chromatograph. A 2.8 m x 2 mm (i.d.) glass column of 5% OV 25 on Gas Chrom Q (85-100 mesh size) with carrier gas of high purity N_2 (flow rate c. 14 ml/min) was employed, both isothermally at various temperatures and under temperature programme conditions as outlined below. The temperature of

the detector oven was maintained at 320°C. The solvent for sample injection was cyclohexane (silylation grade), and the solvent flush technique was employed.

The initial silylating agent tried was N,O-bis-(TMS)-trifluoroacetamide (BSTFA: Pierce Chemical Company, Rockford, Illinois, USA). This was added to various amounts of kievitone in THF in polythene-capped vials, usually in a proportion of THF:BSTFA of 4:1, and allowed to stand at 60°C for at least 15 min. Isothermal analysis at 300°C on the semi-polar liquid phase employed (McReynolds, 1970) yielded a major peak with a retention time (Rt) of 2.4 min and a minor peak at 3.1 min. This indicated the possibility of incomplete derivatization, which seemed consistent with the sterically-hindered hydroxyl group at C_5 (Fig. I.i,A).

Extended derivatization times and the addition of 1 to 5% trimethyl-chlorosilane (TMCS: Pierce Chem. Co.) as catalyst, however, resulted only in reduction of the major peak and the appearance of a new peak at Rt = 1.1 min. Use of TRI-SIL Z (Pierce Chem. Co.; a prepared formulation of N-TMS-imidazole [TSIM] in pyridine for derivatization of hydroxy and polyhydroxy compounds in the presence of water) at increased derivatization temperatures (up to 100°C) or with addition of BSTFA merely accentuated this trend. The extremely powerful silylating formulation TRI-SIL TBT (Pierce Chem. Co.; from the work of Chambaz & Horning [1967], and capable of silylating even the most sterically-hindered hydroxyl groups in steroid structures) produced a similar result.

A further explanation for the appearance of multiple peaks on the chromatogram could be cylization of the five-carbon side chain of kievitone with the C_7 hydroxyl group under the derivatization conditions employed, yielding the 2,2-dimethylchroman isomer, "cyclized kievitone" (Fig. I.i, B) (D.A. Smith, 1978); assuming consistent derivatization, the presence of only three hydroxyl groups would result in a TMS derivative with a different Rt to that of TMS-kievitone. Therefore, in

an attempt to avoid the possibility of acid conditions arising and causing cylization of the kievitone side-chain, as could have occurred with the silylating agents used, kievitone was acylated using heptafluorobutyryl imidazole (HFBI; Pierce Chem. Co.). However, traces obtained after derivatization using this material were again unsatisfactory; several reaction products appeared on the chromatogram.

To determine whether cyclization of the side chain was in fact occurring during silylation of kievitone, a sample of cyclized kievitone, prepared according to the modification by D.A. Smith (1978) of a general procedure outlined by Burden et al. (1972) and kindly provided by Dr. D.A. Smith, was derivatized using TRI-SIL TBT as above. When chromatographed under the same conditions, however, this resulted in the appearance of two major peaks, neither of which corresponded with the peaks produced by TMS-kievitone. Gas chromatographic and mass spectrometric (GC-MS) analyses of these samples (carried out by Prof. J. MacMillan, Dept. of Organic Chemistry, Bristol University) revealed that the presence of two peaks apparently depends upon enol-TMS ether formation at the carbonyl group. Enol-TMS ether formation gives a derivative with five TMS substitutions (Fig. I.i, C), but if enolization does not take place the difficulty in silylation of the 5-(chelated)-hydroxyl results in a derivative with only three TMS substitutions (Fig. I.i, D). Likewise with cyclized kievitone, enol-TMS ether formation results in the presence of a tetra-TMS derivative (Fig. I.i, E); in the absence of enol-TMS ether formation, a di-TMS derivative is produced (Fig. I.i, F).

It is possible that conversion of the keto group to a methoxime derivative, for example, thereby preventing enol-TMS ether formation, would have allowed reproducible silylation of the hydroxyl groups, but this was not attempted. Rather than introducing further time-consuming manipulations, a return to the use of a less powerful silylating agent

(BSTFA) was investigated in an attempt to achieve consistent, if incomplete, derivatization of kievitone.

It had already been established that under suitable conditions the major peak produced on derivatization of kievitone with BSTFA could be used to identify the phytoalexin in similarly-treated ethanolic extracts of diseased bean tissues, even before any resolution on TLC (Fig. I.ii). However, it was observed that the use of different amounts of purified kievitone in preparation of standards resulted in an alteration of the ratio between the size of the major peak and that of the minor one. Also, this ratio was not always reproducible for samples of the same size. Whether it was due to incomplete derivatization or to the chemical changes on the column, as suggested by Furuya (1965) when TMS ethers of flavanones showed a major peak followed by minor ones, this variation rendered the method unsatisfactory, especially since tissue extracts would contain unknown amounts of material.

In the light of these findings, quantitation by GLC was ultimately abandoned in favour of the established TLC and UV spectrophotometric methods.

Fig. I.ii. Gas chromatograms of material derived from bean hypocotyls:

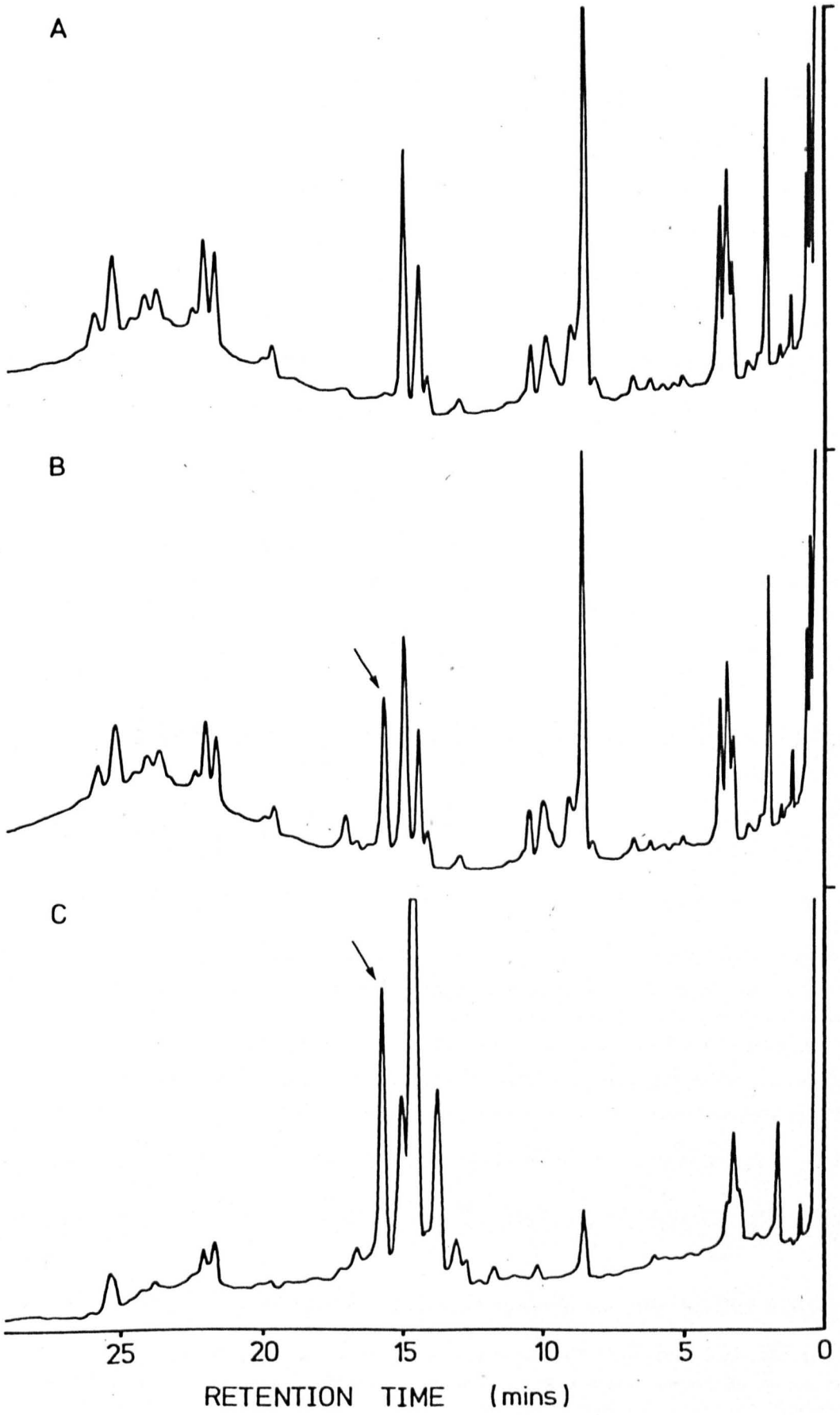
(A) Healthy

(B) Healthy + purified kievitone

(C) 48 h after inoculation with R. solani.

The major kievitone peak is arrowed.

Samples comprised aliquots of ethanolic solutions at the pre-TLC stage of routine extraction for kievitone (Section II). After evaporation to dryness under N_2, they were derivatized with BSTFA as outlined in the text, and run under temperature programme conditions from 200°C @ 6°C/min using the column described earlier.


A
B
C
25
20
15
10
5
0
RETENTION TIME (mins)