PHYSIOLOGICAL AND BIOCHEMICAL NATURE OF DISEASE RESISTANCE MECHANISMS OF BEANS, *PHASEOLUS VULGARIS*, TO THE ANTHRACNOSE FUNGUS, *COLLETOTRICHUM LINDEMUTHIANUM*

by

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Physiological and biochemical nature of disease resistance mechanisms of beans, Phaseolus vulagaris, to the anthracnose fungus, Colletotrichum lindemuthianum

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ABSTRACT

The herbicide glyphosate, an inhibitor of the shikimic acid pathway and aromatic amino acid biosynthesis in plants, was used to investigate cytological and biochemical aspects of the host-parasite relationship in compatible and incompatible interactions of beans, *Phaseolus vulgaris*, with the anthracnose fungus, *Colletotrichum lindemuthianum*.

In compatible interactions, glyphosate applied prior to lesion development at doses of 2.5 μg per plant or higher suppressed both the delimitation of lesions by the host and the associated accumulation of phytoalexins. Glyphosate did not block delimitation if treatment was delayed until after phytoalexins had accumulated at developing lesions.

The hypersensitive reaction (HR), a cytological response characteristic of an incompatible host-parasite interaction, was not affected by glyphosate, but the accumulation of phytoalexins which accompanies the HR was suppressed. Infection hyphae grew from HR cells near sites of glyphosate application, and eventually girdled the hypocotyl and killed the plant. Glyphosate-associated suppression of phytoalexins and escape of infection hyphae from HR were both enhanced by treatments imposed to deplete the phenylalanine reserves of bean plants. It is concluded that HR does not kill infection hyphae, and is not sufficient of itself to contain the infection hyphae. This conclusion is further supported by the finding that hyphae in HR cells resumed growth even without glyphosate treatment when epidermal strips containing these cells were excised. The phytoalexin content of epidermal strips declined rapidly following excision, and it appears that wounding causes the phytoalexins to diffuse out of HR cells and release infection hyphae from inhibition.

The data obtained suggest that phytoalexins are responsible for containment of the pathogen at infection sites in both compatible and incompatible interactions. This containment is eliminated if the accumulation of phytoalexins is suppressed (by glyphosate) or if phytoalexins are withdrawn from infection sites (wounding).

Expression of the incompatible phenotype was observed only under conditions that allowed development of appressoria. Other methods of pathogen development resulted in either failure to infect or pathogenicity, irrespective of host genotype. From these results, it is inferred that the specificity of interaction (recognition) in this host-parasite system is for resistance.

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INTRODUCTION TO THE THESIS

Research on the physiology and biochemistry of host-parasite interaction has been prolific in recent years. Still, we lack a thorough understanding of the processes whereby plants succeed in remaining healthy despite their constant exposure to potential parasites. In nature, disease is a rare exception and resistance the rule (74).

Conventionally, two forms of resistance are distinguished: 'non-host resistance', where plants are clearly outside the range of hosts a parasite can infect, and 'host resistance', where genetic makeup renders the host resistant to pathogens that would otherwise infect it (25).

The mechanisms by which plants resist infection are diverse and complex. In general, they can be divided into two categories: those that are passive and those that are active (55, 96, 114). Mechanisms are passive when they are independent of the pathogen. Active resistance is due to mechanisms activated in the host plant as a response to infection or attempted infection. It is the resistance mechanisms of the latter type that are of greatest interest to plant pathologists and the subject of this thesis. The elucidation of the biochemical and physiological basis of these mechanisms contributes not only to the understanding of plant parasite relationships, but also to the development of alternative concepts and strategies for plant protection.

The host-parasite interaction between the green bean, *Phaseolus vulgaris* L., and the anthracnose fungus, *Colletotrichum lindemuthianum* (Sacc. and Magn.) Briosi and Cav., was selected as a model system for this study. The main features of this system that make it suitable for detailed biochemical

and physiological analysis are (i) distinct, genetically controlled interaction phenotypes (host and parasite each can exhibit either of resistant or susceptible and virulent or avirulent phenotypes, respectively, depending on the genotype of the other), (ii) the ease with which the fungus can be cultured on nutrient media, and (iii) well defined biology and physiology of the disease (12, 88, 90). In the bean anthracnose pathosystem, as in many plant pathosystems embodying a gene for gene relationship between host resistance and pathogen virulence, a hypersensitive host cellular reaction (HR) and localized accumulation of host-derived antifungal metabolites (phytoalexins) are associated with the resistant (incompatible) interaction phenotype (8, 12, 26, 76, 90). The compatible interaction is characterized by the absence of both the HR and the rapid accumulation of phytoalexins at the infection site (8, 12, 90). Strong evidence suggests that phytoalexins are responsible for the restriction of fungal growth during HR resistance and lesion limitation in susceptible plants (12, 79, 90), though direct proof of such an involvement is lacking. It is also unclear, what relationship, if any, exists between HR and phytoalexins and what the relative contribution of each is towards resistance (12, 26).

An ideal system to test the importance of phytoalexins in HR and disease resistance would be a mutant in which phytoalexin synthesis is blocked at a single step. No such mutants are available at present. An alternative strategy would be the use of chemical inhibitors to produce artificial phytoalexin-minus phenotypes. To this end I used glyphosate, a herbicide which inhibits the enolpyruvyl shikimate phosphate synthase-catalysed step of the shikimic acid pathway (3, 44), thus blocking production of phenylalanine in plants. Phenylalanine is a precursor of phytoalexins produced by beans

(27). Therefore, assuming that glyphosate inhibits phytoalexin synthesis via this mode of action, it might be possible to correlate the degree of inhibition with increased susceptibility.

The question of specificity between the host and parasite was also addressed. The interaction phenotype is thought to be controlled by complementary genes in the host and parasite acting in a one-to-one relationship (25, 39, 67, 87). Until now the molecular nature of this phenomenon has remained both obscure and a subject of much dispute. Two general dogmas have emerged. Either the pathogen actively induces susceptibility in its host plant (specificity resides in susceptibility (16, 41, 49, 108, 110, 116)), or the plant actively defends itself (specificity resides in resistance (1, 12, 36, 64, 67)). There is some evidence for both scenarios, but further research is needed to validate these proposals.

The bean-*C. lindemuthianum* interaction provides an excellent system to study the nature of specificity between the host and parasite. During germination, fungal conidia follow different developmental strategies (putatively, saprophytic or parasitic) depending on the presence or absence of nutrients. Different sets of genes are likely to be induced during different modes of development. It may, therefore, be possible to change the incompatible relation into a compatible one, by changing the source of inoculum, if specificity resides in resistance.

Part of the difficulty in solving the mystery of host-parasite specificity is the lack of a suitable host assay system in which to assess the biological activity of putative specificity factors from the pathogen. The potential of plant cell and tissue cultures for such studies has been

recognized but interaction specificity is typically altered relative to that observed in intact plants and the extrapolation of findings from such systems to the intact host-pathogen interaction is questionable (50, 58). These considerations led me to analyse the interaction of host and parasite in excised bean epidermal strips in the hope of developing a simple host system having the flexibilities of a tissue culture system and the specificity properties of an intact plant.

CHAPTER 1

GLYPHOSATE, HYPERSENSITIVITY, AND PHYTOALEXIN ACCUMULATION IN THE INCOMPATIBLE BEAN ANTHRACNOSE HOST-PARASITE INTERACTION

INTRODUCTION

In a recently published work Johal and Rahe (62) established that the apparent herbicidal activity of glyphosate, a commercial herbicide, on bean seedlings (*Phaseolus vulgaris*) was due in large part to parasitization of the seedlings by soilborne fungi. They reasoned that this glyphosate-induced susceptibility of bean plants to colonization by soilborne fungi may be attributed to the suppression of defence mechanisms of the treated plants by glyphosate, since glyphosate blocks the synthesis of phenylalanine (3, 34, 60), a precursor of the isoflavonoid phytoalexins produced by beans (13, 27, 29, 101).

To test the validity of this hypothesis I selected bean anthracnose disease, caused by *Colletotrichum lindemuthianum* as a model system. The interacting genotypes of this pathosystem follow a gene-for-gene relationship in the expression of the phenotype, which is typically qualitatively susceptible or resistant (8, 12, 90, 97). The mechanisms which maintain cell viability after penetration by a virulent race of the pathogen (*biotrophy*) or cause immediate death of host cells penetrated by an avirulent race (*hypersensitivity*) are the fundamental processes which determine the cultivar specific responses in this host-pathogen system (12, 90). Antifungal host metabolites (phytoalexins) are induced and accumulate in and around hypersensitive host cells and appear to be responsible for the inhibition of fungal growth therein (8, 12, 90).

However, the significance, if any, of the relationship between hypersensitive response and accumulation of phytoalexins, and the extent to which each contributes to resistance is not clear. It has been a continuing controversy over the past 15 years as to whether hypersensitivity is the cause or the result of phytoalexin accumulation (12, 29, 72, 110) and whether the hypersensitive reaction causes death of the invading fungus or is a response to its death (71, 110).

My research on the effects of glyphosate on the resistant phenotype of the bean anthracnose host-parasite interaction provides answers to some of these questions and is the subject of this Chapter. It is shown that hypersensitivity and the phytoalexin response are two distinct phenomena, and that the hypersensitive reaction *per se* does not protect beans against avirulent races of *C. lindemuthianum*. In addition, an apparent interaction of wounding and glyphosate in reversing the resistance of beans to avirulent races of the pathogen is shown.

MATERIALS AND METHODS

Plant material

Two cultivars of beans (*Phaseolus vulgaris*), Topcrop and Perry Marrow (White Marrow), resistant to races γ and β of *C. lindemuthianum*, respectively were used in this study. Seeds of cv. Topcrop were purchased from Dominion Seed House, Georgetown, Ontario and seeds of Perry Marrow were purchased from Stokes Seeds Ltd., St. Catharines, Ontario. The plants were grown in 11 cm-diameter plastic pots (8-10 plants per pot) in vermiculite which was moistened with 25% Hoagland-Arnon nutrient solution and autoclaved before

use. The pots were cleaned before use by immersing in aqueous NaOCl solution containing approximately 0.5% available chlorine for 30 min followed by rinsing with distilled water. Seeds of *P. vulgaris* of uniform weight (350±50 mg for Topcrop and 450±50 mg for Perry Marrow) were surface sterilised for 2 min in 1% NaOCl and then thoroughly washed with distilled water. Seeds with swollen or cracked testas were discarded and the remainder were planted aseptically in vermiculite approximately 3 cm deep. Etiolated plants were produced by keeping pots in the dark at ambient laboratory temperatures (20-23°C) and the green plants were maintained under fluorescent light (14:10 h, day:night photoperiod) at 22-24°C. The dark and the light grown plants were watered with 25% Hoagland-Arnon solution every 6 and 4 days, respectively. Vertical support to etiolated seedlings was provided by tying them to a glass rod anchored in the pot of vermiculite.

Fungal inoculum

The β and γ races of *C. lindemuthianum* (obtained originally from the American Type Culture Collection, Rockville, Maryland, U.S.A., in 1963 and frequently reisolated from beans) were grown on bean juice agar at 23-24°C in the dark. Spore suspensions were prepared from 9- to 12- day old cultures by adding distilled water and rubbing the culture surface with a bent glass rod. The resulting suspensions were filtered through eight layers of cheesecloth to remove mycelial fragments. The spores were washed twice by centrifugation at 800 g for 4 min and finally resuspended in distilled water at a concentration of 2×10^6 spores ml^{-1}.

Inoculation of plants

Topcrop and Perry Marrow bean plants were inoculated with the aqueous suspension of γ and β races of *C. lindemuthianum*, respectively (incompatible combinations) at 10-11-days old, when the hypocotyls were fully elongated and the epicotyl hooks were just beginning to straighten. The inoculum was sprayed evenly as a fine mist onto all sides of the full length of hypocotyls. The inoculated plants were maintained in a growth chamber at high humidity (approx. 95% RH) for 10-12 days. The etiolated plants were incubated in the dark (unless otherwise indicated) and the green plants were always incubated under 14 h of light (fluorescent and incandescent; intensity 16000 LX) and 10 h of darkness. The temperature during the period when light was provided was 24°C and during darkness it was 22°C.

Glyphosate treatment

Roundup®, the commercial formulation of the herbicide glyphosate, was purchased from a local dealer and was the source of glyphosate used in this study. Plants were treated with glyphosate at 24 h after inoculation, except where otherwise stated, by placing a 1 μl drop of Roundup® in distilled water (10 μg a.i. μl^{-1}) near the centre of the hypocotyl. Control plants were treated similarly but with distilled water.

Microscopic and gross observations

The nature and extent of host cellular response to fungal penetration and the development of the fungus in inoculated hypocotyls was assessed microscopically. Epidermal strips were peeled freehand from bean hypocotyls

at 12-24 h intervals following inoculation and were viewed either immediately (live) or fixed in formalin : acetic acid : alcohol (50%), 5:5:90, v/v/v (FAA) containing 0.01% trypan blue stain. Live strips were examined under bright field and Nomarski optics, whereas for fixed strips only bright field microscopy was used. At each interval 12 or more randomly selected strips from two plants were examined for every treatment and cv/race combination. At the end of each experiment (10-12 days after inoculation) all the plants were examined macroscopically for symptom development. Plants with at least one spreading lesion were termed 'susceptible', and others 'resistant'.

Phytoalexin isolation and determination

Ethanol (95%) extracts, each representing five-six plants and about 6-7 gm fresh weight of tissue, were prepared from hypocotyl sections cut 3 cm below the cotyledonary node and about 6 cm above the root zone at 5 days after inoculation. The acidic and neutral lipophilic fraction of these extracts in n-propanol was prepared as described by Rahe (90). Silica gel plates with fluorescent indicator, developed in benzene : ethyl formate : formic acid (75:24:1, V/V/V) for approximately 50 min were used to separate phytoalexins and other components in the n-propanol samples. Semiquantitative estimates of phaseollin in the plant extracts were derived from subjective estimates of the intensity of spots of phaseollin on the developed chromatograms (detected under UV light, 354 nm) compared with those in samples containing 1, 2, 5, 10 and 20 μg of pure phaseollin developed on the same plate.

RESULTS

Development of host-parasite interaction

The development of the incompatible interaction in this host-parasite system proceeded as described by Rahe *et al.* (88) and Elliston *et al.* (35). Spores began to germinate within 4-6 h after inoculation, and produced appressoria either directly or at the ends of short germ tubes within 12-24 h after inoculation. Penetration of host epidermal cells from these appressoria occurred mostly 40-52 h after inoculation. Further development of the fungus before containment and the response of penetrated cells differed only slightly in the two cv/race interactions.

The reaction of Topcrop to penetration by the γ race of *C. lindemuthianum* was very rapid and affected cells became granulated and discolored within 60-70 h after inoculation. This hypersensitive (HR) response was mostly confined to the cell penetrated during the first 24 h following penetration but sometimes extended into 2-3 cells. Generally, the fungus did not develop beyond the first cell penetrated; in a very few instances, however, an infection hypha was seen in a live (non-HR) cell adjacent to the HR cell. The morphology of such hyphae was typical of primary hyphae in compatible combinations, but it could not be established whether such hyphae originated from the adjacent HR cell, or directly from an appressorium. However, further growth of such hyphae was inhibited. The number and extent of browning of the hypersensitively dead cells at individual penetration sites increased with time and after 4 days was visible macroscopically as reddish brown flecking scattered on the surface of hypocotyls. After HR became multicellular, the degree of browning of

individual cells in a hypersensitive fleck differed considerably.

Development of the β race in Perry Marrow was more variable than that of the γ race in Topcrop. Frequently, some hyphae penetrated two-three cells although never beyond the second cell layer. Typically, one to five cells were involved in the (HR) during the first 24 h after penetration.

In addition to granulation and browning, material apposed in localized areas at the sites of penetration (reaction material (83, 97)) was detected in some (2-4%) of the infected cells in both cv/race interactions (Fig. 1). Some epidermal cells containing the reaction material were alive, whereas others were granulated and pigmented by 120 h after penetration. Rarely, as many as three reaction material deposits were seen in the same cell. The deposit was cream color during initial stages of interaction but turned dark brown with time. In certain instances, fungal hyphae were visible in the centre of the deposit (Fig. 1).

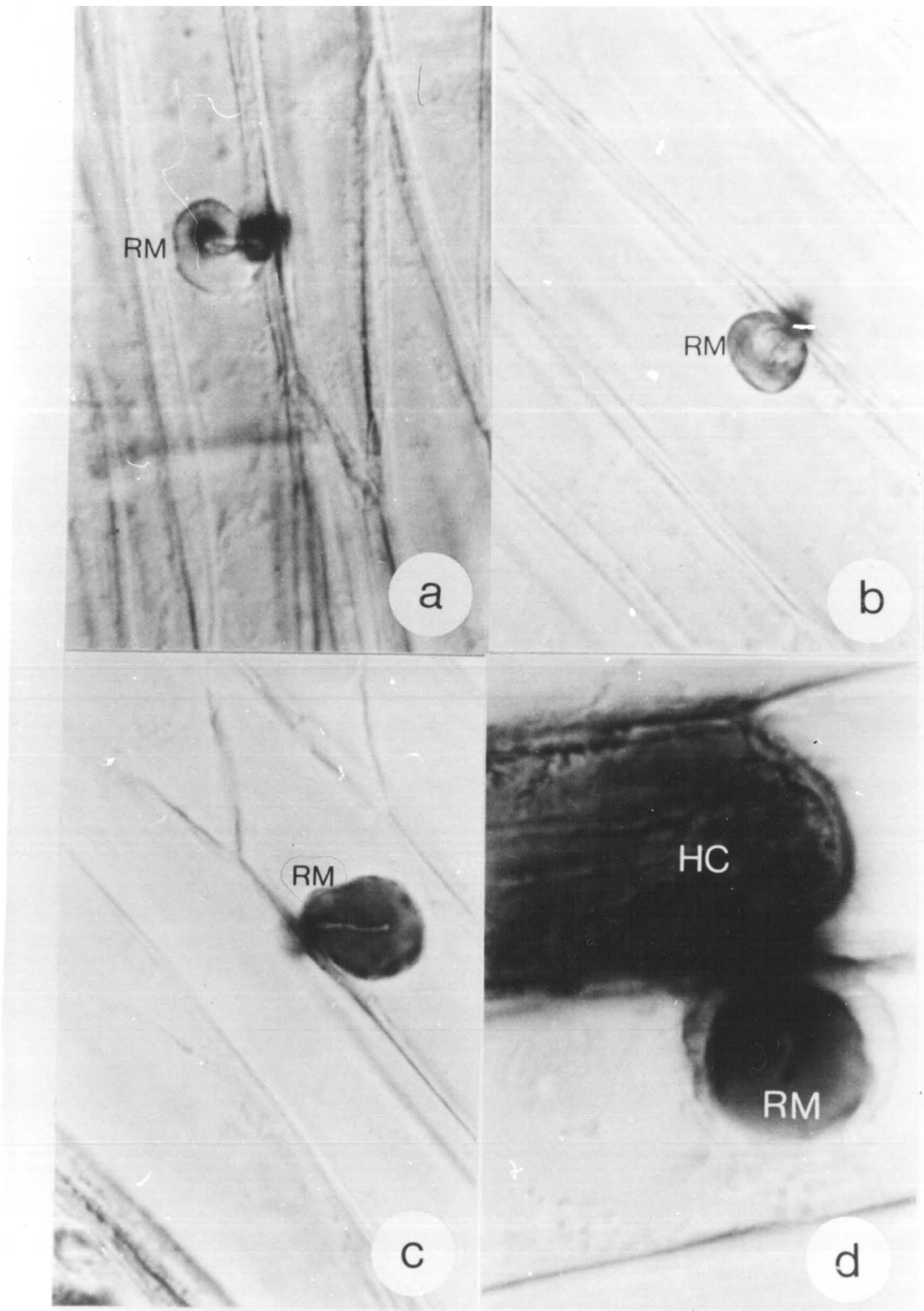
Glyphosate and incompatible host-parasite interaction

Preliminary experiments. In preliminary trials several different sets of experiments were conducted to select the best method, optimal time and ideal dose of glyphosate for treatment. In these empirical attempts to obtain a phenotype reversal with glyphosate, a general reversal of the incompatible phenotype was never obtained and expression of HR was normal. Occasionally, as in the studies reported below, some plants (always $\leq$ 5%) developed a light-colored, spreading lesion which did not become delimited, as is typical of isolated lesions of compatible interactions (90), and resulted in the death of the plant. This suggested that glyphosate had the potential to

Figure 1. Reaction material (RM) deposits and a part of a cell (HC) showing hypersensitive reaction (d) in the bean anthracnose host-parasite interaction.

RM = Reaction material
HC = Hypersensitive cell

Approximate magnification:
(a) 625 (b) 625 (c) 625 (d) 1560.


RM
a
RM
b
RM
c
HC
RM
d

reduce the effectiveness of the resistance mechanism at some sites without affecting the general nature of the interaction phenotype.

The different methods of application of glyphosate that were tried included (a) primary leaf spot treatment (62), (b) single drop treatment of the hypocotyl, (c) root uptake by intact seedlings, (d) uptake through the cut ends of hypocotyls, and (e) spray treatment. Of these methods, hypocotyl treatment (method b) was selected for indepth evaluation.

Glyphosate treatments were applied in preliminary tests at 12 h intervals from 48 h before through 60 h after inoculation. Over this range, the time of application did not appear to affect the occurrence of spreading lesions. The time 24 h post inoculation was selected because it is roughly at the midpoint between appressorium formation and fungal penetration and so would avoid any possible effects of glyphosate on spore germination.

In the dose response experiments, 10 to 100 μg of glyphosate per plant provided similar results. Lower doses did not cause spreading lesions, and higher doses were unacceptably phytotoxic. The dose 10 μg per plant was selected as the optimal compromise between its ability to elicit spreading lesions and low phytotoxicity. As in earlier studies with light-grown *P. vulgaris* (62), glyphosate at this concentration and under the experimental conditions employed in this study, was nonherbicidal. The main phytotoxic symptoms observed in the experiments described here were reduced growth of the apical meristem and a relatively achlorophyllous nature of the leaves of treated etiolated seedlings upon light incubation. In addition, small necrotic scars sometimes developed on the hypocotyls at the points of glyphosate treatment.

In etiolated plants. The development of the HR and the occurrence of reaction material at individual infection sites in glyphosate treated plants appeared identical in timing and intensity to that occurring in inoculated plants not treated with glyphosate (control plants). The HR cells in glyphosate treated plants sometimes appeared dirty brown instead of the reddish brown color typical of HR cells in control plants. Macroscopically, HR in glyphosate treated plants appeared as scattered dark brown flecks over the surface of hypocotyls. In addition, a uniform golden yellow discoloration of the hypocotyl sometimes developed in glyphosate treated plants, especially in areas with large numbers of flecks, by 3 to 4 days after penetration. This discoloration was similar to that observed by Arnold & Rahe (7) on CO_2-treated bean seedlings. Microscopic examination of cross sections of hypocotyls revealed deposits of brown colored substances in both the walls and intercellular spaces of cortical cells. Control (inoculated, non-glyphosate treated) hypocotyls did not show any golden yellow discoloration and also lacked these deposits.

The resistant phenotype, in general, was minimally affected by glyphosate (Table 1), even though phytoalexin accumulation was reduced to 71-75% of that occurring in control plants (Table 4). Occasionally, however, some plants (≤6%) developed a single lesion and were rated 'susceptible' (Table 1). Such lesions typically developed 5-7 days after inoculation, and occurred near the centre of the hypocotyl where the glyphosate drop had been placed. The lesion expanded upwards, downwards and around the stem and ultimately rotted a large portion of the hypocotyl, causing collapse of the plant within an additional 3-4 days. Microscopically, the fungal mycelium associated with spreading lesions appeared to originate from the many HR

TABLE 1

Effect of glyphosate on the reversal of incompatible bean anthracnose interaction phenotype.[a]

Host-parasite combination	Treatment	Total plants	HR expression	'Susceptible' plants	% Reversal
ETIOLATED PLANTS					
TC-γ[b]	Glyphosate	54	Normal	3	5.6
	Control	55	"	0	0
PM-β[b]	Glyphosate	52	"	3	5.8
	Control	53	"	0	0
GREEN PLANTS					
TC-γ	Glyphosate	47	Normal	5	10.6
	Control	44	"	0	0
PM-β	Glyphosate	40	"	4	10
	Control	40	"	0	0

[a] Data from three different experiments were pooled together.

[b] *Phaseolus vulgaris* cvs. Topcrop (TC) and Perry Marrow (PM), inoculated with γ and β races of *Colletotrichum lindemuthianum*, respectively.

cells occurring within and adjacent to the lesion (Fig. 2). Fungal growth, apparently originating from HR cells, was observed up to a distance of approx. 0.5-1.0 cm from the longitudinal margins of the spreading lesion. The extent of growth of these hyphae appeared inversely proportional to the distance of the HR cell from the lesion margin.

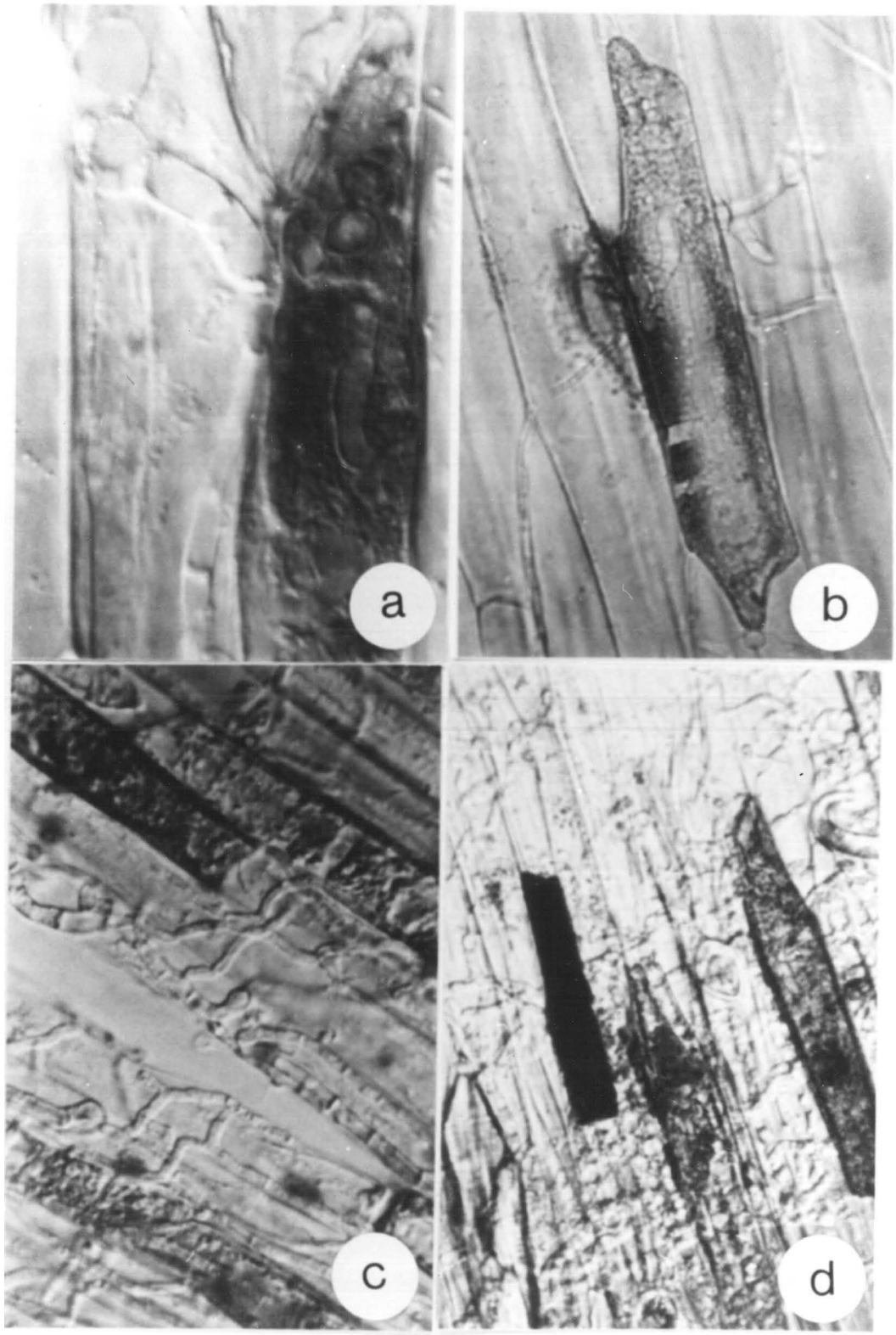
In green plants. As in etiolated plants, HR was unaffected by treatment with glyphosate. Phytoalexin levels in inoculated green plants treated with glyphosate were 65 to 69% of controls (Table 4). The frequency of occurrence of spreading lesions was about 10% as compared to ≤6% in the dark grown plants (Table 1). The 'direction' of this difference is contrary to the general observation that green plants are more resistant than etiolated plants (12, 26, 88).

Effect of light and removal of cotyledons on interaction phenotype in etiolated plants

Two experiments were conducted to evaluate whether exposure of etiolated plants to light after inoculation could amplify the effect of glyphosate on interaction phenotype (e.g., by competition of light-induced protein synthesis and interaction-induced phenolic synthesis for phenylalanine). Etiolated plants, inoculated and treated with glyphosate or water as before, were exposed to light at 40 h post-inoculation (an initial 24 h continuous light treatment was followed by a 14:10 h day:night photoperiod). Two additional experiments were conducted in which cotyledons (the putative reservoir of phenylalanine reserves for etiolated seedlings) were removed from inoculated plants just before treatment with glyphosate or water, and the plants subsequently exposed to light at 40 h post-inoculation.

Figure 2. Escape of infection hyphae of *Colletotrichum lindemuthianum* from hypersensitive cells (a & b) and their growth in the surrounding tissue (c & d) of glyphosate treated bean plants.

Approximate magnification:
(a) 625 (b) 380 (c) 380 (d) 155.


a
b
c
d

The overall incompatible interaction phenotype of HR at individual sites of penetration was not changed from that described for etiolated plants when glyphosate treated plants were exposed to light at 40 h post-inoculation, whether or not cotyledons had been detached. The estimated levels of phytoalexins in glyphosate treated plants were 65-70% and 37-45% of those occurring in control plants for light exposed and detached cotyledon-light exposed treatments, respectively (Table 4). The frequency of occurrence of spreading lesions near the sites of glyphosate application was 16-20% in the light exposed treatment (Table 2), and 40-45% in the detached cotyledon-light exposed treatment (Table 3).

The results obtained to this point showed that, while glyphosate does not interfere with the expression of HR at individual sites of infection of *P. vulgaris* hypocotyls by avirulent races of *C. lindemuthianum*, it can negate, at sites of application, the containment of the fungus normally associated with HR. The frequency of this effect was increased by treatments that presumably compete for or deplete reserves of phenylalanine in the plant, i.e., light exposure (82) and removal of cotyledons (70, 93). In all cases the effect of glyphosate was localized at the sites of application.

Effect of wounding on interaction phenotype

Epidermal and sub-epidermal tissues of etiolated hypocotyls may constitute poor 'sinks' for glyphosate, since it reportedly translocates effectively to only those areas in plants which are metabolically active (18). If this is so, then it should be possible to create localized 'sinks' for glyphosate with treatments that increase metabolic activity, e.g., wounding. To this end I made wounds on hypocotyls by two different methods.

TABLE 2

Effect of glyphosate on the reversal of incompatible interaction phenotype in etiolated bean seedlings exposed to light at 40 h after inoculation[a].

Host-parasite interaction	Treatment	Total plants	'Susceptible' plants	% Reversal	HR expression
EXPERIMENT 1					
TC-γ[b]	Glyphosate	36	6	16.6	Normal
	Water	35	0	0	"
PM-β[b]	Glyphosate	32	6	18.7	"
	Water	32	0	0	"
EXPERIMENT 2					
TC-γ	Glyphosate	32	6	18.7	Normal
	Water	32	0	0	"
PM-β	Glyphosate	31	5	16.1	"
	Water	32	0	0	"

[a] See text for details of light treatment.
[b] *Phaseolus vulgaris* cvs. Topcrop (TC) and Perry Marrow (PM), inoculated with γ and β races of *Colletotrichum lindemuthianum*, respectively.

TABLE 3

Effect of glyphosate on the reversal of incompatible interaction phenotype in etiolated bean seedlings with cotyledons removed[a] and exposed to light[b] at 40 h post-inoculation.

Host-parasite interaction	Treatment	Total plants[c]	'Susceptible' plants	% reversal	HR expression
TC-γ[d]	Glyphosate	36	16	44	Normal
	Control	36	0	0	"
PM-β[d]	Glyphosate	34	13	38	"
	Control	33	0	0	"

[a] Cotyledons were removed at 24 h after inoculation, immediately before glyphosate treatment.

[b] See text for details of light treatment.

[c] The experiment was done twice and the data were pooled.

[d] *Phaseolus vulgaris* cvs. Topcrop (TC) and Perry Marrow (PM), inoculated with γ and β races of *Colletotrichum lindemuthianum*, respectively.

TABLE 4

Effect of glyphosate on the accumulation of phaseollin at 5 days in Colletotrichum lindemuthianum *inoculated* Phaseolus vulgaris *seedlings of two resistant cultivars, under different conditions of growth and incubation.*

Growth/incubation condition of plants	TC-γ Phaseollin levels[a]			PM-β Phaseollin levels[a]		
	Control	Glyphosate		Control	Glyphosate	
	μg g^{-1}[b]	μg g^{-1}[b]	% of control	μg g^{-1}[b]	μg g^{-1}[b]	% of control
Green	42	29	69	38	25	66
Etiolated	52	37	71	44	33	75
Light[c], *+cotyledons*	72	47	65	62	43.5	70
Light[c], *-cotyledons*	49	22	45	53	20	38

[a] These values represent the mean of three different estimates of the same sample, run on separate plates.

[b] Fresh weight of the infected tissue in grams.

[c] Etiolated plants, exposed to light at 24 h post-inoculation.

In the first method (A), an epidermal strip, approx. 30x6 mm, was removed from the hypocotyl. In method B, the epidermal strip was peeled from one end while leaving the other end attached, and then placed back in its original position. Wounds were made two per plant, and located 4-5 cm above and below the site of glyphosate application. Inoculation, removal of cotyledons, aplication of glyphosate or water, and incubation conditions for the etiolated plants were as in the previous experiments. Wounding was done at 40 h post-inoculation, just prior to exposure to light. The experiment was done twice.

Spreading lesions developed at wound sites on almost all (90-97%) of the glyphosate treated plants wounded by method B, and on 70-75% of plants wounded by method A (Table 5). In both cases, the lesion started from the wound margins and spread to adjacent tissue, leading to the eventual collapse of plants 3 to 4 days later. The extent of HR browning on unwounded parts of the hypocotyl in both the treated and control plants was normal, whereas on peeled strips (method B), irrespective of glyphosate treatment, it was quite suppressed. Strips from both the control and treated hypocotyls were successfully invaded by the pathogen. However, in control plants, the fungus did not extend beyond the wound site and no lesion formed.

Increased translocation of glyphosate to the wound sites might have caused the high frequency of spreading lesions observed at such sites. Alternatively, the effect could have resulted from other physiological or biochemical changes induced by wounding. To evaluate these alternatives, wounds were made on glyphosate-treated and nontreated etiolated plant hypocotyls at different times, from 8 h prior- to 130 h post-inoculation. Cotyledons were removed at 24 h post-inoculation and the plants were exposed

TABLE 5

Effect of wounding[a] on the reversal of incompatible interaction phenotype in glyphosate treated etiolated plants[b].

Incompatible interaction	Treatment	Wounding method[d]	Total plants	Susceptible plants	% reversal
TC-γ[c]	Glyphosate	A	31	22	71
	Glyphosate	B	32	31	97
	Control	A	31	0	0
	Control	B	30	0	0
PM-β[c]	Glyphosate	A	28	21	75
	Glyphosate	B	30	27	90
	Control	A	31	0	0
	Control	B	27	0	0

[a] Wounding was done at 40 h after inoculation.
[b] Cotyledons were removed at 24 h and the plants were exposed to light at 40 h after inoculation.
[c] *Phaseolus vulgaris* cvs. Topcrop (TC) and Perry Marrow (PM), inoculated with γ and β races of *Colletotrichum lindemuthianum*, respectively.
[d] A: The epidermal strip (approx. 30x6 mm) was removed from the hypocotyl. B: The epidermal strip was peeled from one end while leaving the other end attached.

to light at 40 h post-inoculation. Observations on the development of lesions associated with various wounding times were made throughout the experiment, starting at day 5 after inoculation. The experiment was done twice and all data were combined.

Spreading lesions developed on 80-100% of wounded, glyphosate treated plants, compared to only 35 to 40% of non-wounded, glyphosate treated plants, and these percentages were independent of the time of wounding (Table 6). Until 60 h after inoculation, the time of wounding did not affect the time of symptom appearance; the lesion appeared and began to spread approximately 5 days after inoculation. It appeared a day later on plants wounded at 84 h. On plants wounded at 130 h, lesions did not appear until the 8th or 9th day post-inoculation, unless they had started already on intact plants by the time of wounding. Glyphosate control (wounded or unwounded) plants did not develop spreading lesions. However, the fungus colonized extensively and produced conidia on the epidermal strips of wounded control plants.

These results indicate that the localized breakdown of resistance in glyphosate-treated plants in response to wounding at 84 and 130 h post-inoculation cannot be explained simply by increased translocation of glyphosate to the wound sites to effectively inhibit phytoalexin production, since phytoalexin accumulation in hypocotyls undergoing incompatible interaction would have already occurred at these times of wounding (8, 12, 90). Some other mechanism induced by wounding may therefore contribute to the increased frequency of spreading lesions caused by wounding in glyphosate treated plants. The observation that the partially detached epidermal strips on plants not treated with glyphosate (Table 6) were colonized by avirulent races of the pathogen also suggests that wounding *per se* is affecting the

TABLE 6

Effect of time of wounding[a] *on the glyphosate-induced development of spreading lesions in bean hypocotyls inoculated with avirulent races of* Colletotrichum lindemuthianum[b].

Time of wounding, before (-) or after (+) inoculation(h)	% plants with spreading lesions: Glyphosate treated TC-γ	Glyphosate treated PM-β	Time of appearance (days)	Glyphosate control TC-γ	Glyphosate control PM-β	Colonizaton of strips (TC-γ & PM-β combined)
8 -	100	100	5	0	0	100
24 +	100	90	5	0	0	100
40 +	100	100	5	0	0	90
48 +	100	90	5	0	0	100
60 +	90	90	5	0	0	90
84 +	80	100	6	0	0	100
130 +	100	90	8-9	0	0	90
Control (not wounded)	35	40	5-7	0	0	-

[a] Method B was used to make the wounds.

[b] Glyphosate treatment and incubation conditions were as in the previous experiment (Table 5).

resistance mechanism of the host.

HR response and pathogen containment

The results from these experiments also indicate that the pathogen can survive in the HR host cell environment for several days. To investigate the viability of the fungus at HR sites and the role of HR in fungal containment, thin epidermal strips (approx. 30x7 mm, in size) containing HR sites were excised from etiolated hypocotyls at 130 and 200 h post-inoculation. Plant hypocotyls were gently wiped with tissue paper and then surface sterilized with 1% NaOCl for 5 min before excising the strips. The strips were either floated onto distilled water containing 100 μg ml^{-1} each of penicillin G and streptomycin sulphate in 60x20 mm disposable plastic Petri plates, or were hung from very fine glass filaments in humid plastic boxes to minimize physical contact of the strips with the surrounding medium. Microscopic observations concerning the resumption of fungal growth in HR cells and its further development in the strip tissue were made at 6-12 h intervals, starting from the time of excision until 96 h later when the experiment was terminated.

Within 10 to 12 h of excision, the fungal hyphae which were originally inside the HR cells resumed growth. All the strips from both the incompatible combinations (i.e., TC-γ and PM-β), whether excised at 130 or 200 h after inoculation and whether floated onto water or hung, were extensively colonized by *C. lindemuthianum* within 4 days of excision (Table 7). Sequential microscopic examination of the same strips at different times post excision revealed that the growth originated from individual hyphae in HR cells, and that fungal 'escape' occurred from 17-23% of HR cells on TC-γ

and 22-29% of HR cells on PM-β plants by 30 h after excision (Table 7). It was not possible at later times to determine the points of origin of fungal growth since there were so many hyphae ramifying through the tissue.

DISCUSSION

Despite considerable research over the past two decades, the interrelationship between hypersensitive cell death, phytoalexin accumulation and the inhibition of infection hyphae in HR remains virtually unresolved (13, 29, 30, 38, 57, 72). One of the major problems in resolving this issue has been the failure to separate these phenomena. The factors which affect production of phytoalexins have a corresponding effect on the HR reaction (12, 29, 31, 33, 66, 85, 101).

There is, however, some indication that glyphosate affects these two processes differently in plants which derive their phytoalexins via the phenylpropanoid biosynthetic pathway. Whereas glyphosate inhibited the production of glyceollin in soybean leaves inoculated with an incompatible race of *Pseudomonas syringae* pv. *glycinea*, necrosis was not inhibited (53). Likewise in this study, glyphosate reduced the levels of phytoalexins in both the etiolated and green plants infected with avirulent races of *C. lindemuthianum*, but HR response was apparently not affected. Additionally, the overall interaction phenotype of individual plants was not changed. However, glyphosate at/or near sites of its application sometimes negated the containment of infection hyphae in HR cells. The frequency of phenotypic reversal was almost zero when considered in terms of absolute percentage of hyphal escapes from individual infection sites. Once growth of hyphae from

TABLE 7

Resumption of fungal growth from HR cells following excision of epidermal strips containing incompatible bean anthracnose interaction sites[a].

Incubation condition of the excised strips	Time after inoculation when strips were taken	% strips[b] showing regrowth after 96 h		% HR cells[c] showing regrowth after 30 h	
		TC-γ	PM-β	TC-γ	PM-β
Floated on water	130 h	100	100	18	25
	200 h	100	100	20	24
Hung from glass wires	130 h	100	100	21	22
	200 h	100	100	17	29

[a] Data from three experiments were pooled.
[b] Forty strips were examined per treatment.
[c] 200 HR cells from four randomly chosen strips were examined.

HR cells resumed, however, it was not subsequently inhibited and a spreading lesion formed which eventually led to collapse of the plant.

It is significant in this regard that reversal of the resistant phenotype by glyphosate, although infrequent, occurred in the presence of apparently normal expression of HR and the initial restriction of infection hyphae. Furthermore, it occurred similarly in both etiolated and green plants, and in two different incompatible cv/race interactions. These results suggest that both the HR *per se* and phytoalexins may have distinct functional roles in this host-parasite interaction. Whereas the HR may be responsible for initial inhibition or at least delay of fungal development (although there is no direct evidence of this function), phytoalexins appear responsible for containment of the pathogen in dead cells.

The mechanism of localized, glyphosate-induced reversal of the resistant phenotype likely involves inhibition of phenylpropanoid synthesis. This contention is supported by the fact that reversal was increased by treatments that presumably compete for or deplete reserves of phenylalanine in the plant (light exposure (82) and removal of cotyledons (70, 93) (Tables 2 & 3). It is further supported by the fact that reduced levels of phytoalexins accumulated in inoculated, glyphosate-treated plants.

Spreading lesions developed at high frequency at wound sites on glyphosate treated plants. Wounding enhances the metabolic activity of adjacent tissue and may result in increased 'sink' activity, thereby increasing translocation of glyphosate to the wound site. This may augment the suppression of phytoalexins by glyphosate in that tissue. However, the results indicate that the increased frequency of spreading lesions caused by

wounding cannot be accounted for solely by the presumed effect of glyphosate on accumulation of phytoalexins. The pathogen inhibited inside the HR cells of plants not treated with glyphosate also resumed growth in many cases after peeling epidermal strips containing these HR cells. Moreover, spreading lesions developed on glyphosate treated plants at wound sites made long after (i.e., 130 h) the time when phytoalexins would have accumulated (12, 90).

These results contradict the general belief that the pathogen restricted inside the HR cells is either severely damaged (dead) or permanently inhibited (13, 57, 71, 72, 108). It was seen that despite being trapped, quite a few of the infection hyphae remained alive for several days within the HR cells. These hyphae resumed growth quickly after excision and colonized the adjacent tissue. This was also observed by Bailey and Rowell (11) and Erb *et al.* (37). The mechanism(s) responsible for the resumption of growth is (are) a matter for conjecture. The possibility that phytoalexins or other substances responsible for containment might dissipate by leaking into the surrounding medium can be excluded because regrowth occurred even when the strips were hung in humid chambers or left on the plant hypocotyls. However, degradation of phytoalexins by the host tissue (12, 13) and/or their delocalization from the HR cells as induced by wounding may be involved.

Collectively, these studies suggest that the HR *per se* is inadequate to contain the pathogen. The fungus sometimes escapes in treated plants and invades the surrounding tissue unimpeded. Generally, however, the pathogen stays inhibited inside the infected cells. It seems that a process other than the HR must cause this inhibition since many infection hyphae resume growth when the tissue is excised. Phytoalexins appear ideally fit for such a role. Detailed studies on the kinetics of phytoalexin accumulation under

different incubation conditions, as influenced by glyphosate, were not attempted during this study. However the semiquantitative data are consistent with the conclusion that accumulation of phytoalexins may be a major co-event with HR, and thus a component of resistance in the bean anthracnose pathosystem.

These results are consistent with earlier reports that glyphosate inhibited both the accumulation of glyceollin and the resistance expression, whether natural (68) or metalaxyl-induced (112) in soybeans to *Phytophthora megasperma* f. sp. *glycinea*. In contrast, the breakdown of resistance was complete in those studies. This could be due to the lack of acute (classic) HR and a clear biotrophic phase in that disease system. While my study provides no direct evidence for the importance of HR, *per se*, in the resistance of *P. vulgaris* to *C. lindemuthianum*, histological evidence suggests that in addition to being a critical marker for the recognition phenomenon (12, 14, 30, 67, 106), the HR *per se* may also initiate inhibition or at least delay the development of pathogen by causing disruption of biotrophy characteristic of the early stages of compatible host-pathogen interaction in this pathosystem (8, 12, 80, 88, 97).

These results raise several possibilities concerning the relationship between host cell death and the production of phytoalexins. The data clearly indicate that the HR and phytoalexin response are two distinct, separable biochemical phenomena. The hypersensitive death of infected host cells does not seem to be due to phytoalexins, although some phytoalexins always accumulated in this study and several reports suggest that phytoalexins are phytotoxic (13, 29, 100). Therefore the possibility that the HR occurs due to accumulation of phytoalexins above a threshold level cannot be ruled out.

However, several independent lines of evidence argue against such a possibility. First, several plants respond hypersensitively to infection without producing phytoalexins (13, 26, 72). Second, live cells can produce and metabolize phytoalexins (13, 15, 29, 46, 78, 86, 101). Third, HR is a complex biochemical phenomenon and reversible to some extent, indicating that it cannot simply be the toxicity symptom of phytoalexins (97). Fourth, parallel studies of the progress of HR and phytoalexins production invariably suggest that the HR response occurs first (12, 13, 29, 97, 106). Fifth, phytoalexin production and cell death may be induced independently (75). My data are in agreement with the assumption that cell death is the trigger for subsequent biosynthesis of phytoalexins (12, 13, 29, 30, 45). Furthermore, the results indicate that HR is not the consequence of fungal death or inhibition as suggested by some workers (71, 72). In fact, the pathogen often survives the HR *per se* and is not even contained by it.

In conclusion, these results demonstrate that glyphosate interferes with the expression of defence in beans by suppressing their ability to produce phytoalexins. The elicitation and magnitude of the HR response are apparently not affected by glyphosate. The data indicate that while HR *per se* may have a role in determining the nature of the interaction, it is unable of itself to provide protection in beans to *C. lindemuthianum*. Glyphosate thus has the potential to separate the effector mechanism(s) from the earlier events which determine the nature of the relationship. This offers an opportunity to dissect and therefore understand better the interaction and functional significance of component mechanisms of disease resistance in *P. vulgaris*.

CHAPTER 2

EFFECT OF GLYPHOSATE ON THE COMPATIBLE BEAN ANTHRACNOSE HOST-PARASITE INTERACTION

INTRODUCTION

During the compatible interaction of beans (*Phaseolus vulgaris*) with the anthracnose fungus (*Colletotrichum lindemuthianum*), a facultative biotroph, the initially infected cells do not die and the pathogen establishes a relatively benign relationship with its host (12, 80, 83, 88). This biotrophic relationship, characterized by a primary mycelial stage, is transient and is followed by a necrotrophic, secondary mycelial stage of fungal development in which host cells adjacent to advancing hyphae are killed before invasion. Under permissive conditions, this leads to extensive damage and, if a number of adjacent infections coalesce, eventual collapse of the plant hypocotyl (8, 12, 80, 90, 94). At present, nothing is known of the mechanisms by which host cells remain alive during infection and subsequent biotrophy, and the nature of trigger(s) which bring about an abrupt change in the mode of parasitism of *C. lindemuthianum* (80, 83).

Although compatibility of the relationship depends on an appropriate combination of pathogen virulence and host susceptibility genes, typical symptom(s) of susceptibility are not always expressed in this interaction (12, 90, 94). The development of symptoms is a complex phenomenon and is influenced by physiological (metabolic) conditions of the host and pathogen (12, 88, 91, 94), as well as numerous other factors including environmental conditions during incubation (6, 12, 43, 88, 91, 94), host age and tissue

differentiation (35, 43, 88), cultivar and particular host-pathogen combination (35, 94). These factors can interfere either with the initial biotrophy or subsequent necrotrophic growth to shift the balance of interaction towards that of resistance. Thus symptoms ranging from flecks, characteristic of hypersensitive reaction (HR) in incompatible interactions, to limited lesions of various sizes are easily produced. How these factors disrupt biotrophy and subsequent fungal growth is unclear, but accumulation of phytoalexins is often associated with the final response (10, 12, 90, 94). Phytoalexins are not detected during the infection process and biotrophic colonization of the host (10, 12, 90). Consequently, Rahe (90) provided strong but indirect evidence that the accumulation of the phytoalexin phaseollin was the functional component of symptom delimitation. Bailey and coworkers (12, 94) later reported that both the extent of biotrophy and phytoalexin accumulation may regulate the expression of symptoms in this host-parasite interaction.

In an earlier study, I established that the apparent herbicidal activity of glyphosate, an inhibitor of aromatic amino acid synthesis in plants, was mainly due to parasitization of bean seedlings by root-rot fungi (62). Subsequently, it was shown that glyphosate suppressed phytoalexin production and induced susceptibility in beans to otherwise avirulent races of *C. lindemuthianum* (Chapter 1). Similar observations of the effect of glyphosate on phytoalexins and loss of resistance were made by Keen *et al.* (68) in the soybean-*Phytophthora megasperma* f. sp. *glycinea* disease system.

The present study was undertaken to determine whether glyphosate could suppress the delimitation of lesions in compatible combinations of bean anthracnose, and to evaluate further the mechanism of glyphosate-induced

susceptibility in plants to diseases.

MATERIALS AND METHODS

Biological material

Etiolated seedlings of *P. vulgaris* c.v. 'Topcrop' were grown as described previously (Chapter 1). The race β of *C. lindemuthianum* was grown on 50% bean juice agar (BJA), and inoculum containing $5x10^5$ conidia ml^{-1} was prepared from 9-10-day old cultures (Chapter 1).

Hypocotyls of etiolated seedlings were inoculated when 9-10 days old (unless specified otherwise). Before inoculation, hypocotyls were marked with India ink at 2 cm intervals at 6-8 places, starting 3-4 cm below the cotyledonary node, in a single vertical line. Drops of inoculum (3 μl) were placed slightly above these marks and the plants were incubated in a growth chamber in the dark at 22°C under conditions of high humidity. Glyphosate treatment was done at 24 h after inoculation (except where otherwise stated), by placing a 1 μl drop of a solution of Roundup® (10 μg a.i. μl^{-1} in distilled water) near the center of the hypocotyl (Chapter 1). In some experiments, glyphosate at concentrations of 10, 5, 2.5, 1.0, 0.5, 0.2, and 0.1 $\mu g\ \mu l^{-1}$ was used. Control plants were treated similarly with water. Incubated plants were exposed to light at 40 h after inoculation (see Chapter 1).

In an experiment to test whether supplementary phenylalanine could alleviate the effects of glyphosate, glyphosate treated and control plants were drop inoculated at 4 sites per plant along the length of the hypocotyl. At 72 h after inoculation, the two uppermost infection sites of some control

and glyphosate treated plants were treated with phenylalanine. This was accomplished by placing a 5 μl drop from an aqueous solution of phenylalanine (50 $\mu g\ \mu l^{-1}$) in distilled water on each infection site (115). The treatment was repeated at 84 h after inoculation. Control sites were treated similarly with distilled water.

Sample plants were removed from the inoculation chamber for gross observations and histological study of the development of interaction. Fungal growth in inoculated seedlings was determined by examining freehand epidermal peels under bright field and Nomarski optics. Plants were harvested 8 days post-inoculation and observations on lesion size, morphology and color were recorded.

Extraction and quantitative analysis of phytoalexins

Duplicate samples representing 40-50 infection sites or lesions, plus 2 mm of healthy tissue at each end (if available), were excised at various intervals and weighed. Ethanol extracts were prepared from tissue samples as described by Rahe (90). Fifty μl of vesidryl (2'-4-4'-trimethoxy chalcone, 0.5 mg ml^{-1}), which was used as an internal standard, was added to each sample before extraction. Phytoalexins from these crude extracts were purified and quantified by HPLC following the method of Goosens and Van Laere (42).

Each sample was finally taken into 500 μl of chloroform. From this a volume of 20 μl was injected and analysed with HPLC equipment (Waters Associates, Milford Massachusettes, USA), comprised of a solvent delivery system M-510, Rheodyne 7125 injector and Lambda-max M-481 variable wavelength detector. Waters 840 data and chromatography control station was used for

recording and integration. The analytical column (Supelcosil LC-Si particle size 5μ, 250X4.6 mm I.D.) was protected by a 5 cm Supelcosil LC-Si guard column.

Separations were carried out by using a linear gradient for 21 min (staring after 1 min) from hexane/chloroform (2:1, v/v) to chloroform/methanol (100:8, v/v) at a flow rate of 2 ml/min. The elusion pattern recorded at 280 nm showed main peaks at retention time (RT)=4.05 min (vesidryl), RT=7.41 min (phaseollin), RT=9.48 min (phaseollidin), RT=10.68 min (phaseollinisoflavan), and RT=15.90 min (kievitone). The identity of these peaks was verified by injecting samples from a solution containing known quantities of pure phaseollin, phaseollidin, phaseollinisoflavan and kievitone in chloroform. Authentic samples of phaseollin and kievitone were kindly provided by C. J. Lamb (Salk Institute, San Diego, CA, USA) and D. A. Smith (University of Kentucky, Lexington, KY, USA). Vesidryl was purchased from Sigma Chemical Co. Phaseollidin and phaseollinisoflavan were purified first by TLC (9) and then HPLC. The identity of phaseollinisoflavan and phaseollidin was confirmed by their UV spectra (56), Rf values in various solvents (10) and RT during HPLC (42). The amounts of phaseollinisoflavan and phaseollidin in the samples were estimated by reference to standard curves prepared using purified preparations and published extinction coefficients (9). Each sample was run twice and the means of four separate values (duplicate samples) are presented in the results.

Effect of glyphosate on C. lindemuthianum in vitro

Toxicity of glyphosate to mycelial growth and spore germination of *C. lindemuthianum* was assayed on bean juice agar (BJA) containing different concentrations of glyphosate. Glyphosate was added to Petri plates immediately before pouring 15 ml of molten BJA and the contents were thoroughly mixed before the agar set. Plugs of mycelium, 5 mm in diameter, were cut from the border of actively growing colonies of *C. lindemuthianum* (6-days old) and placed in the center of the bioassay plates. The cultures were incubated at 22-23°C in the dark. Colony diameters were measured after 9 days to assess fungal growth.

For germination studies, plates were inoculated with approximately $1X10^4$ spores contained in 0.1 ml of spore suspension. The spores were spread evenly on the agar surface with a glass rod. Observations on germination of spores were made after 3 days. A hundred or more spores were examined under the dissecting microscope from five different, randomly selected, locations on each Petri plate and three plates were examined per treatment.

Phenylalanine (100 $\mu g\ ml^{-1}$), was added to some bioassay plates along with glyphosate to assess the possible reversal of glyphosate-induced inhibition of spore germination and fungal growth by the amino acid.

RESULTS

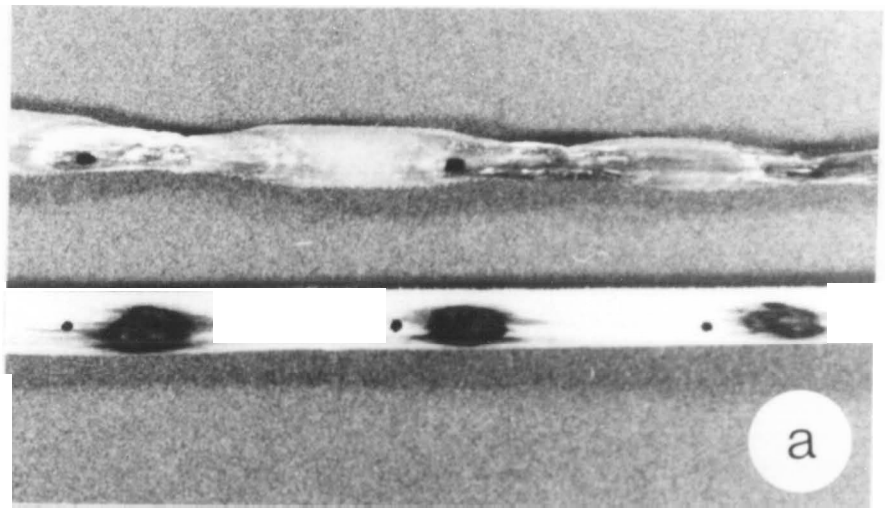
Pathogen development, symptom expression and accumulation of phytoalexins

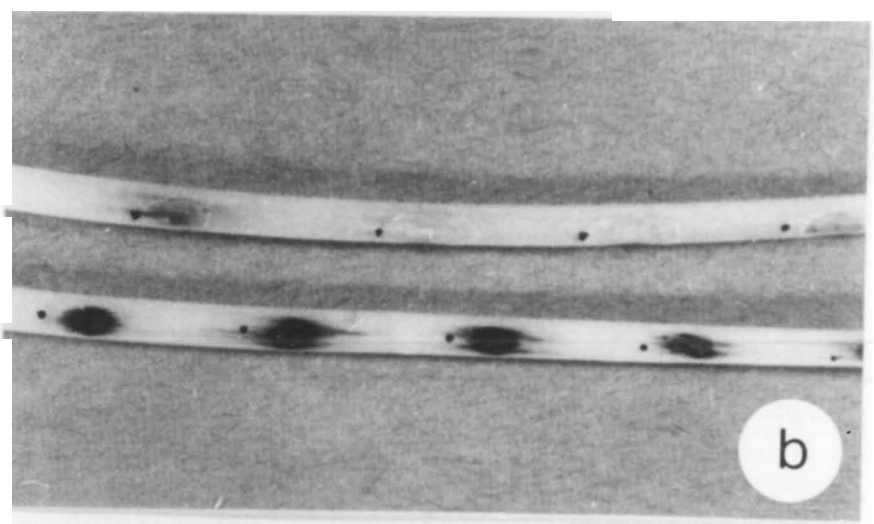
Control plants. Development of the compatible interaction between *P. vulgaris* and *C. lindemuthianum* proceeded as described previously (35, 80, 88). Conidia of *C. lindemuthianum* started germinating within 6-8 h of inoculation and produced darkly pigmented appressoria, mostly attached directly to conidia, by 24 h after inoculation. Penetration of host cells from appressoria occurred between 40 and 55 h after inoculation and was evident by the presence of small globular vesicles within the epidermal cells beneath the appressoria. The infection vesicles enlarged into primary hyphae that continued to grow biotrophically into other epidermal and underlying cortical cells with no apparent disturbance of the integrity of host cells until 84-96 h after inoculation. Secondary mycelium was first observed at infection sites at about 96 h, and diffuse necrotic discolorations became apparent within 5-10 h after the appearance of these necrotrophic hyphae. These discolorations enlarged rapidly during the next 15-20 h and typical brown lesions became sharply delimited from the adjacent healthy tissue by 120 h after inoculation (Fig. 3). After this the lesions expanded little, if at all (Fig. 3). These delimited lesions varied in length from 5 to 7 mm when measured on day 8 after inoculation.

Phytoalexins did not accumulate during the biotrophic phase of parasitism. Their accumulation began about 90-96 h post-inoculation, coincident with the onset of necrosis, and increased slowly during the next 10-15 h and then rapidly during the period 108-144 h post-inoculation (Fig. 4). Infection sites ceased rapid enlargement and became delimited during the

Figure 3. Expression of symptoms in compatible bean anthracnose interaction in control (bottom plant in each photograph) and glyphosate treated plants (top plant in each photograph).

a = 190 h post-inoculation
b = 144 h post-inoculation
c = 120 h post-inoculation


a


b

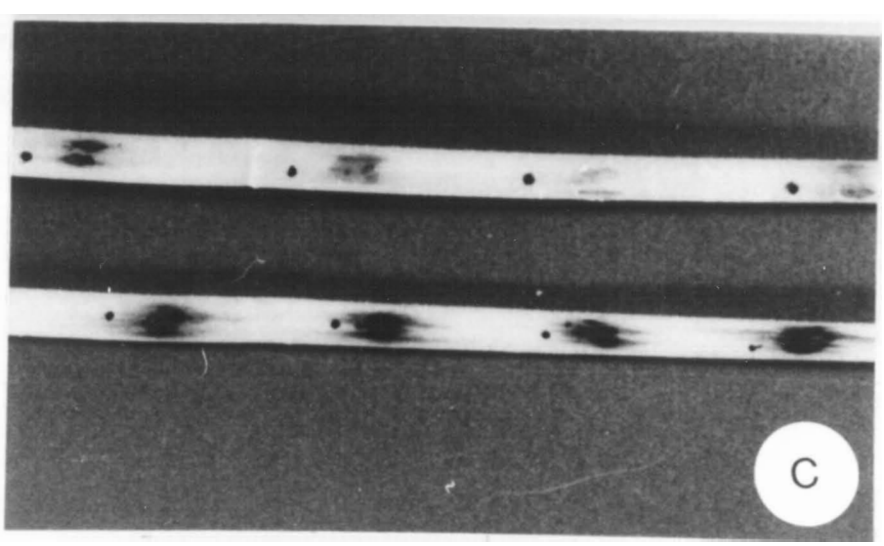

c

Figure 4. Effect of glyphosate on phytoalexin accumulation in compatible bean anthracnose host-parasite interaction.

Total phytoalexins = Sum of phaseollin, phaseollinisoflavan, phaseollidin and kievitone.

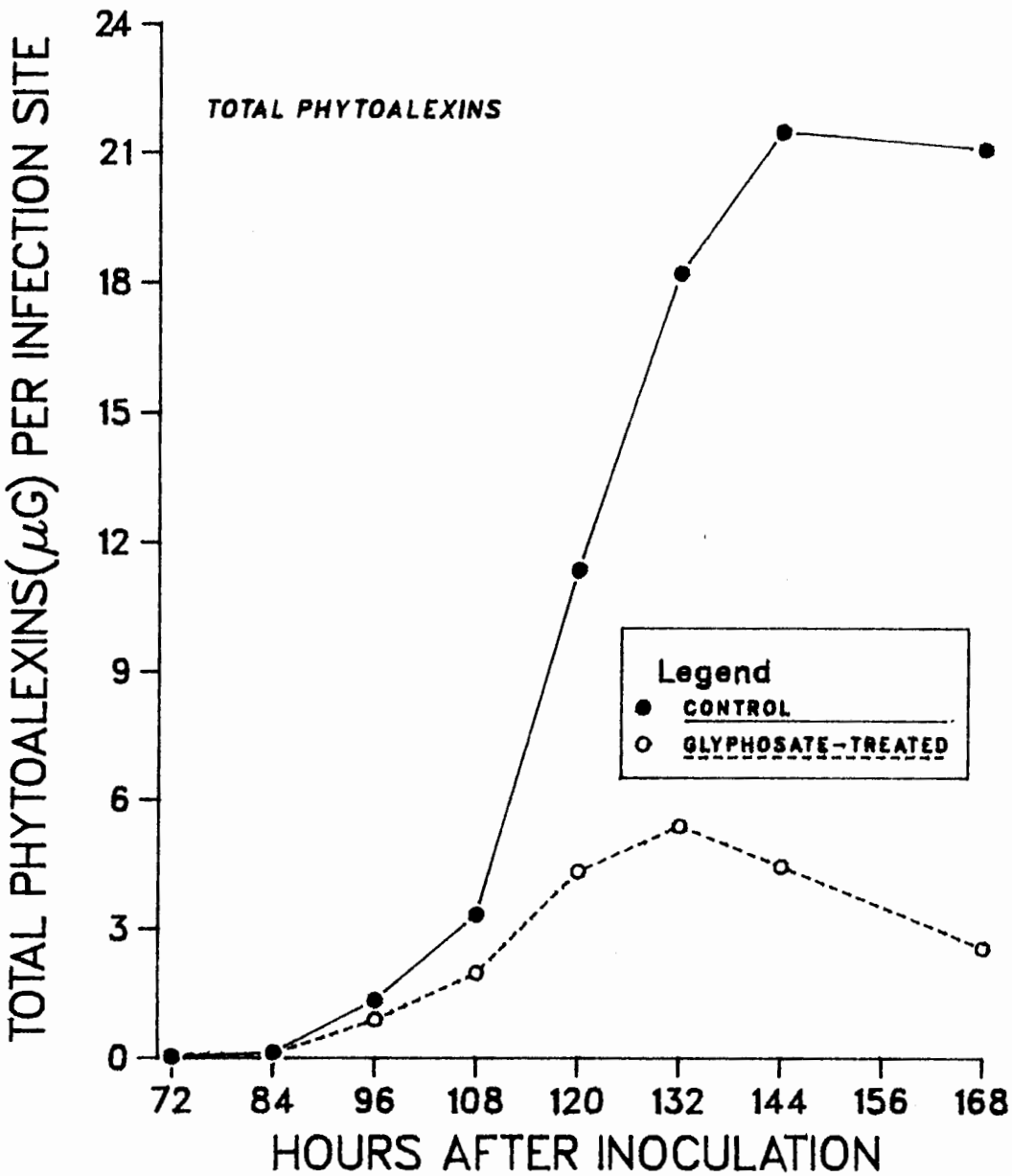
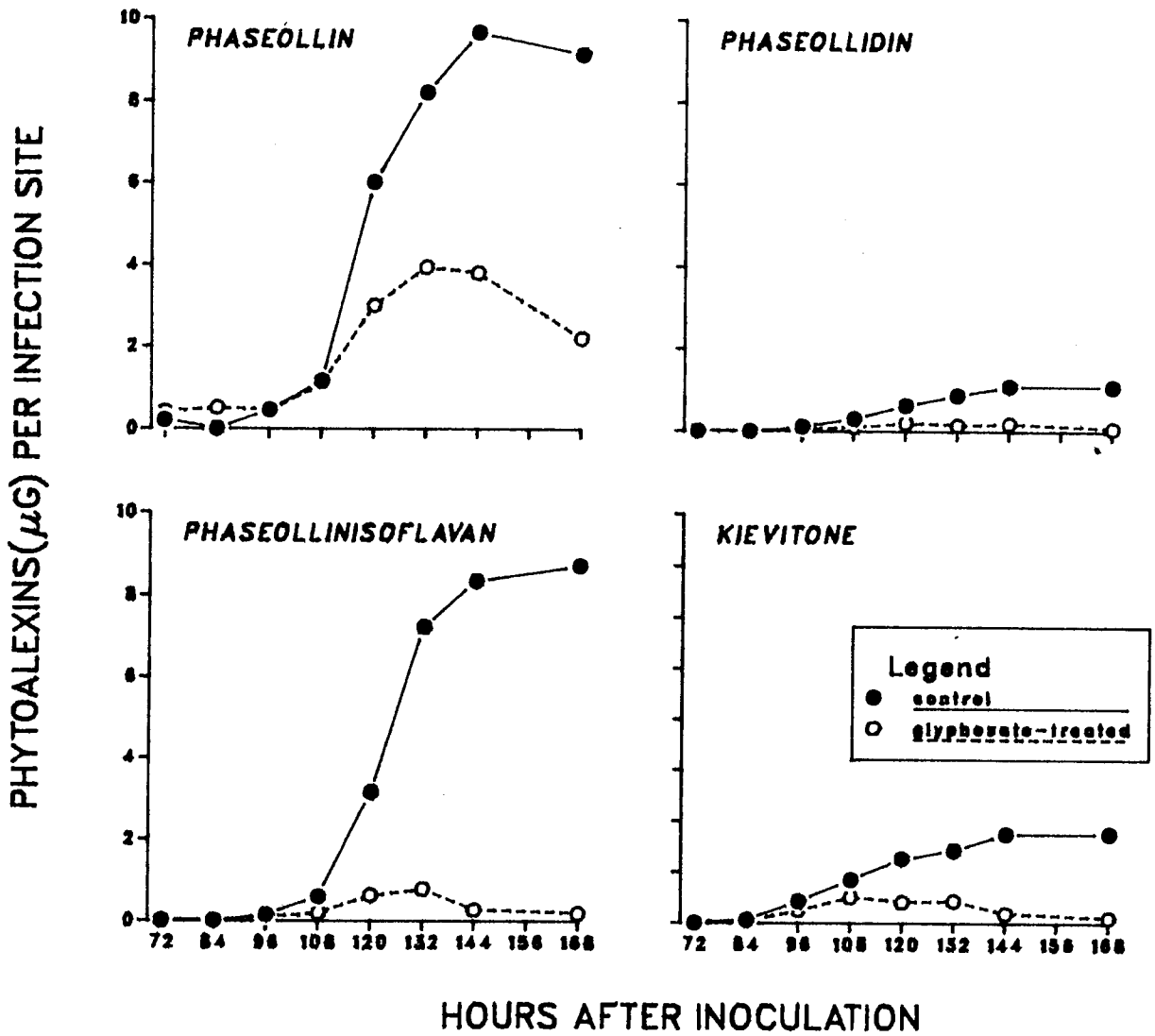

TOTAL PHYTOALEXINS
TOTAL PHYTOALEXINS(μG) PER INFECTION SITE
24
21
18
15
12
9
6
3
0
72
84
96
108
120
132
144
156
168
HOURS AFTER INOCULATION
Legend
CONTROL
GLYPHOSATE-TREATED

Figure 5. Effect of glyphosate on individual components of the phytoalexin response during the compatible bean anthracnose host-parasite interaction.


PHASEOLLIN
PHASEOLLIDIN
PHASEOLLINISOFLAVAN
KIEVITONE
Legend
control
glyphosate-treated
PHYTOALEXINS(μG) PER INFECTION SITE
HOURS AFTER INOCULATION
72 84 96 108 120 132 144 156 168

period of rapid accumulation of phytoalexins. Phaseollin was the major phytoalexin occurring during the early stages of symptom expression; it reached a maximum on the 6th day after inoculation and subsequently declined (Fig. 5). Although it accumulated later in the interaction than did phaseollin, phaseollinisoflavan reached concentrations similar to those of phaseollin, and it continued to accumulate throughout the experiment. Kievitone and phaseollidin were also produced but at much lower levels (Fig. 5).

Treated plants. Development of fungal infection and subsequent biotrophic colonization on plants treated with glyphosate at 24 h after inoculation was closely similar to that on control plants. Host cells were penetrated mostly 40-50 h post-inoculation and the fungus established a biotrophic relationship with the host which lasted until 90-96 h after inoculation. The rate of fungal growth during biotrophic colonization was similar in both the treated and control plants. However, after the onset of the secondary mycelial stage (96-100 h post-inoculation), diffuse discoloration of infection sites typical of control plants did not develop on treated plants (Fig. 3). The fungus continued to grow without being restricted and produced slightly sunken, colorless lesions by 120-125 h post-inoculation. These lesions expanded rapidly in all directions during the next 24 h. Consequently, the lesions girdled, coalesced and rotted the entire hypocotyl within 8 days of inoculation (Fig. 3).

As in control plants, phytoalexins were not detected during biotrophic colonization but started accumulating immediately after the onset of the necrotrophic phase of fungal parasitism (Fig. 4). However, they accumulated at much lower levels in glyphosate treated plants than in controls. At day 6

after inoculation, the amount of total phytoalexins in treated plants was only about 15% of that of the controls. The amount of phaseollin in treated plants was reduced to less than 1/3 of that occurring in control plants, while that of the other phytoalexins was even more strongly suppressed by glyphosate (Fig. 5).

When glyphosate was applied to bean seedlings at 48 h prior to, through 96 h post-inoculation, complete suppression of delimitation was observed (Fig. 6). Administration of glyphosate at 108-120 h post-inoculation led to progressively less suppression of lesion limitation, and none of the lesions became spreading if glyphosate treatment was delayed to 130 or more hours post-inoculation.

When lesions expanded as a result of glyphosate treatment at 108 or 120 h post-inoculation, secondary delimitation did not occur and the area of new colonization did not develop brown pigments. This gave these lesions a characteristic 'halo' appearance showing a dark brown central core surrounded by a colorless periphery (Fig. 7b).

On plants treated with glyphosate at 24 h post-inoculation and which subsequently received phenylalanine, the lesions became delimited and were, in size, color and phytoalexin content, similar to lesions that formed on control plants in the absence or presence of phenylalanine (Fig. 7a and Table 8).

Figure 6. Loss of delimitation of lesions in relation to the time of glyphosate treatment in beans infected with a compatible race of *Colletotrichum lindemuthianum*.

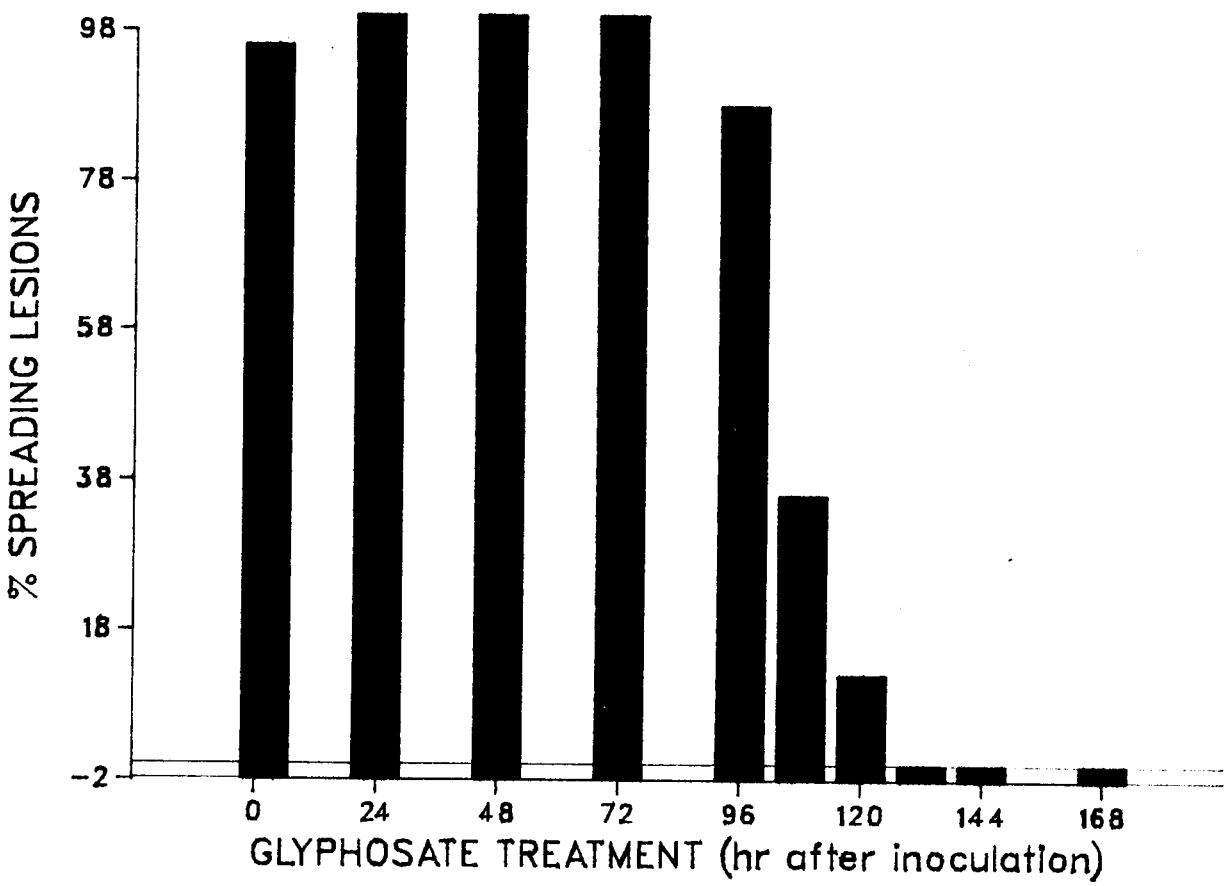
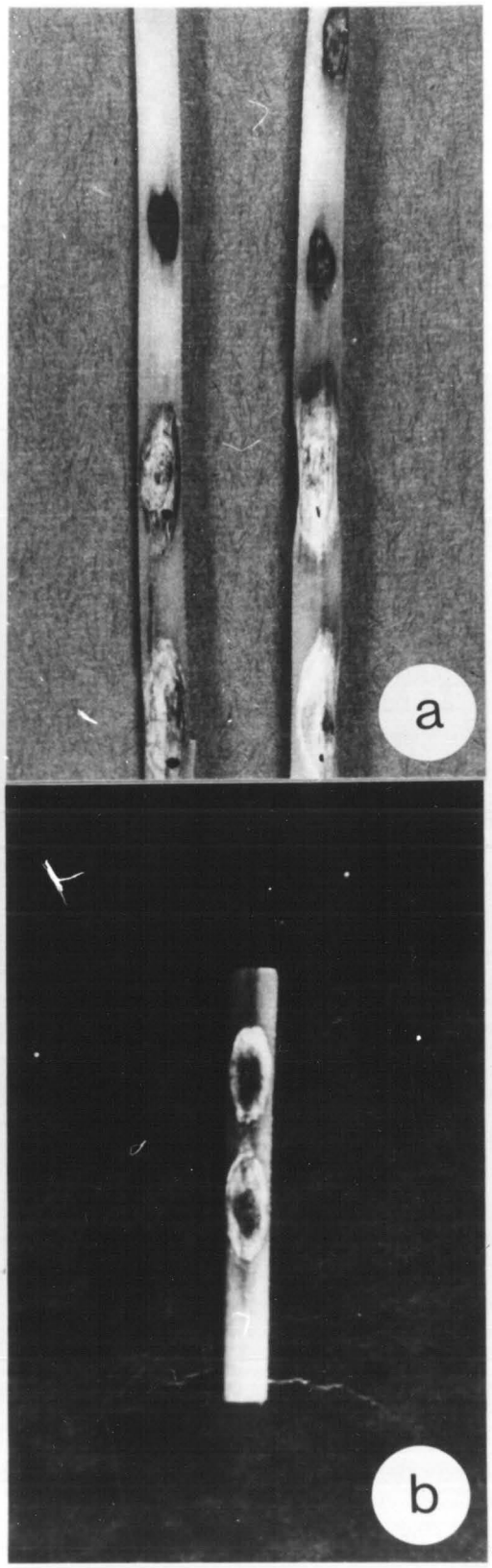

98
78
58
38
18
-2
% SPREADING LESIONS
0
24
48
72
96
120
144
168
GLYPHOSATE TREATMENT (hr after inoculation)

Figure 7. The effect of delayed treatment of glyphosate on lesion expression (b); and reversal of the glyphosate-induced suppression of lesion delimitation by phenylalanine (a) in a compatible bean anthracnose interaction.


a
b

Effect of different concentrations of glyphosate on lesion delimitation

To study further the relationship between phytoalexin accumulation and restriction of fungal growth during lesion limitation, inoculated plants were treated with different concentrations of glyphosate ranging from 0.1 to 10 μg per plant. Doses of 5 and 2.5 μg of glyphosate per plant gave results that were essentially similar to those caused by the 10 μg per plant dose (Table 9). Colorless spreading lesions formed that eventually rotted the entire hypocotyl. In contrast, plants which received doses of 1.0, 0.5 and 0.2 μg of glyphosate showed progressively less suppression of lesion limitation and phytoalexin accumulation. No effects on lesion size or color were noted for the 0.1 μg dose (Table 9).

Glyphosate at the concentrations used in this study was, as reported previously (62 and Chapter 1), non-herbicidal. The only phytotoxicity symptoms observed in uninoculated glyphosate-treated bean plants for the duration of experiments were reduced growth of the apical meristem and relatively achlorophyllous nature of leaves receiving the 10 μg per plant. These toxicity symptoms progressively decreased at the 5, 2.5, and 1.0 μg doses. Treatment with 0.5, 0.2 or 0.1 μg per plant produced no apparent effects on plant growth.

Effect of glyphosate on mature plant resistance

Lesion development on plants inoculated when 16-17-days old was quite limited compared to that occurring on plants inoculated when 9-10-days old. The symptoms varied from small limited lesions (2-4 mm in length) to necrotic flecks typical of hypersensitive resistance of beans to avirulent races of *C. lindemuthianum*. When treated with glyphosate, most of the infection sites on

TABLE 9

Effect of different concentrations of glyphosate on symptom expression and accumulation of phytoalexins in bean anthracnose compatible interaction.

Glyphosate treatment µg per plant	% plants with spreading lesions	% lesions showing expansion	Lesion morphology color	size mm	Total phytoalexins µg per lesion
Control	0	0	dark brown	5.0	21.35
0.1	0	0	" "	5.0	NE
0.2	60	15	mid brown	7.0	NE
0.5	100	30	light brown	8.5	16.50
1.0	100	60	pale brown	12.0	10.85
2.5	100	95	colorless	co	NE
5.0	100	100	"	co	NE
10.0	100	100	"	co	4.60

Glyphosate treatment was done at 24 h. Symptoms were rated and phytoalexins extracted at 144 h after inoculation. Only those lesions which showed expansion in response to glyphosate treatment were recorded for lesion morphology before excision for phytoalexin extraction.

CO = Coalescing lesions

NE = not estimated

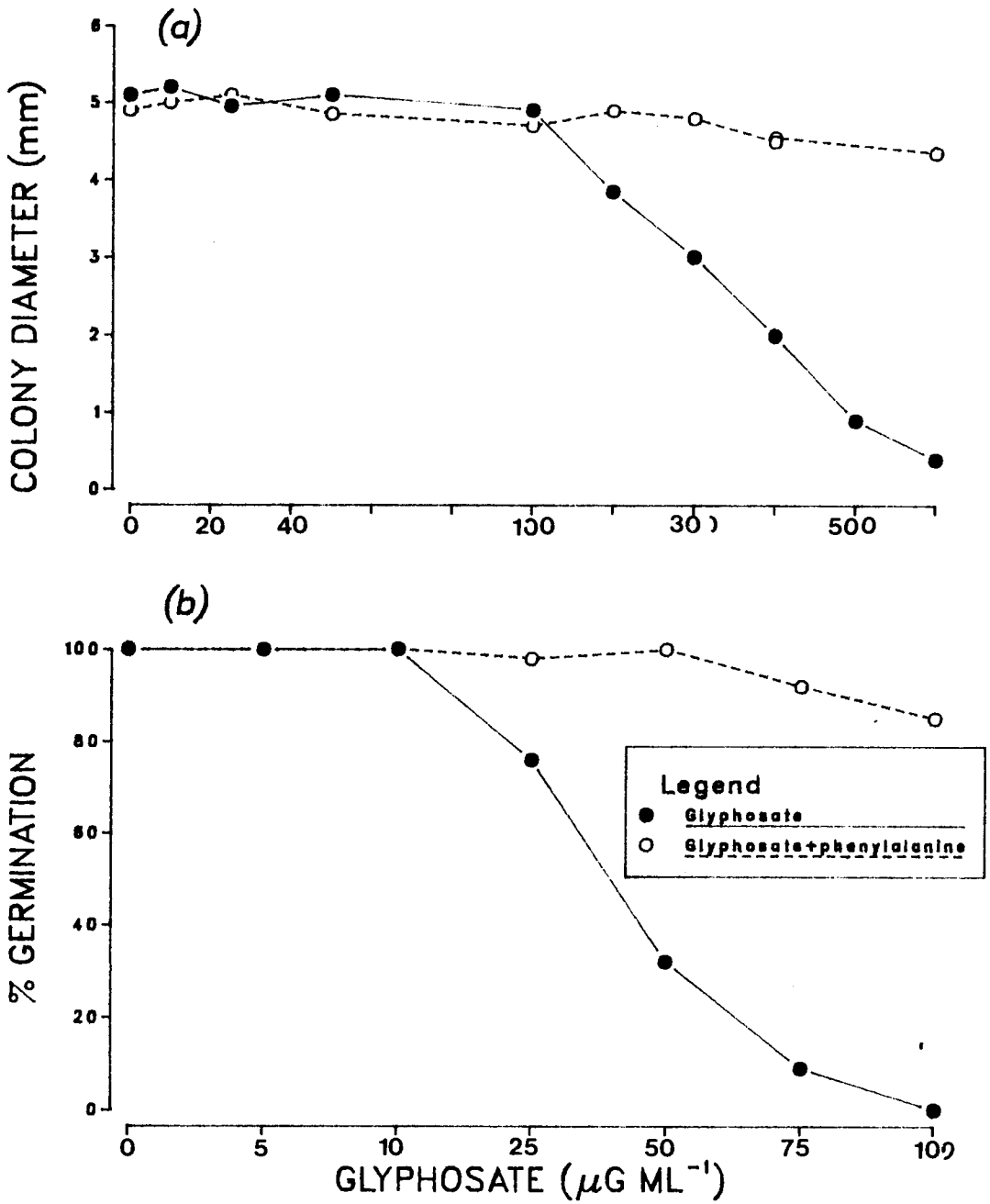
TABLE 10

Effect of glyphosate on mature plant resistance of beans to compatible races of Colletotrichum lindemuthianum.

Treatment	Total plants	Plants with spreading lesions	Suppression of delimitation	
			% plants	% lesions
Glyphosate	16	15	94	65
Control	16	0	0	0

17 day old Topcrop plants were inoculated with β race of *C. lindemuthianum* and observations on symptoms were made after 8 days. Glyphosate (10 µg per plant) treatment was given at 24 h after inoculation.

Figure 8. Effect of glyphosate on the growth (a) and germination (b) of *Colletotrichum lindemuthianum*.


(a)
COLONY DIAMETER (mm)
0
1
2
3
4
5
6
0
20
40
100
300
500
(b)
% GERMINATION
0
20
40
60
80
100
0
5
10
25
50
75
100
GLYPHOSATE (μG ML⁻¹)
Legend
Glyphosate
Glyphosate+phenylalanine

plants inoculated when 16-17-days old continued to enlarge and finally coalesced (Table 10). The sites which did not spread in response to glyphosate treatment were either necrotic flecks or very small (approx. 2 mm) delimited lesions.

Effect of glyphosate on C. lindemuthianum in vitro

Glyphosate was inhibitory to mycelial growth of *C. lindemuthianum* if present at concentrations higher than 100 $\mu g\ ml^{-1}$ of BJA and the growth inhibition was roughly proportional to the concentration of glyphosate in the medium (Fig. 8a). At lower concentrations there was no apparent inhibitory effect of glyphosate on fungal growth; however, the normal greyish-black pigmentation of cultures was markedly suppressed by glyphosate at the tested concentrations of 25 $\mu g\ ml^{-1}$ and higher. Both the loss of pigmentation and reduction in fungal growth induced by glyphosate were reversed by supplementing BJA with phenylalanine.

Spore germination was progressively inhibited by glyphosate from concentrations of 25 to 100 $\mu g\ ml^{-1}$ (Fig. 8b). Addition of phenylalanine to BJA also reversed the inhibition of spore germination by glyphosate.

DISCUSSION

In an earlier study Rahe (90) demonstrated that the timing of lesion limitation in bean anthracnose compatible interactions coincided with the onset of phaseollin accumulation, that phaseollin was localized within 1 mm of delimited lesions, and provided indirect evidence that phaseollin accumulated mainly at the edges of these lesions. Phaseollin did not

accumulate at compatible infection sites that did not become delimited. On the basis of this evidence, it was concluded that inhibition of fungal growth in infected tissues was caused by the accumulating phytoalexins (phaseollin) and consequently the lesions became limited. The results presented here support this conclusion. Glyphosate at 2.5 μg per plant or higher effectively inhibited the accumulation of phytoalexins and also suppressed completely the restriction of lesions. At lower concentrations a close relationship between the decrease in phytoalexin content and the degree of loss of resistance was observed.

A cause and effect relationship between the occurrence of phytoalexins and restricted fungal growth in limited lesions seems apparent from this data. Glyphosate application, at any time prior to accumulation of phytoalexins at infection sites rendered the plants incapable of limiting the size of lesions. However, if phytoalexins had already accumulated prior to application of glyphosate, such treatment had no effect on delimitation at most infection sites.

The negation of the effects of glyphosate on phytoalexin accumulation and lesion limitation by phenylalanine further indicates the association of phytoalexins with delimitation of lesions. This reversal of glyphosate-induced susceptibility of beans to *C. lindemuthianum* by phenylalanine also confirms the inhibition of the shikimic acid pathway (aromatic amino acid biosynthesis) as the putative mode of action for glyphosate in plants (3, 44, 60).

Glyphosate, being an inhibitor of aromatic amino acid biosyntheis, may also interfere with protein synthesis in beans (44). The possibility thus

remains that some mechanism(s), other than phytoalexin production, requiring *de novo* protein synthesis, may be involved in lesion limitation.

In a few instances, lesions started spreading in response to glyphosate treatments applied as late as 120 h after inoculation, even though high levels of phytoalexins were presumably present at most infection sites. Perhaps the levels of phytoalexins at those few sites where spreading occurred were atypically low and the pathogen was able to metabolize phytoalexins in these seemingly delimited lesions in treated plants. The ability of *C. lindemuthianum* to metabolize phytoalexins has been demonstrated (8, 10, 52) and it has also been shown that substantially high concentrations of phaseollin (>50 μg ml^{-1}) must be maintained to keep the pathogen inhibited (10).

Browning of lesions was markedly suppressed by glyphosate and like phytoalexins, this inhibition was subject to reversal by phenylalanine. In contrast, glyphosate had no effect on the necrosis of host cells undergoing hypersensitive reaction (HR) to infection by avirulent races of *C. lindemuthianum* (Chapter 1). Browning has been attributed to oxidation of plant phenols (40, 106). It may be that phenolics, already present in the cells or synthesized *de novo* in response to infection, are oxidized to brown pigments as a result of loss of compartmentalization in cells during both HR and the necrotrophic phase of fungal invasion in compatible interactions. As the demand for precursors (phenolics) during these two responses would be vastly different, it is possible that browning during HR and symptom expression may be affected differently by glyphosate.

Although often associated with plant disease and phytoalexin accumulation, the significance of browning in disease resistance is not clear (40). There are reasons to discount the direct involvement of tissue necrosis in the resistance of bean plants to *C. lindemuthianum*. For example, administration of glyphosate after the onset of necrosis but prior to accumulation of phytoalexins at sites of compatible interaction allows the fungus to escape (Fig. 7b). When infection sites are closely spaced, the entire hypocotyl is colonized and becomes necrotic (10, 88, 90). Additionally, *C. lindemuthianum* sporulates after the lesion in which it occurs becomes necrotic.

Glyphosate, at the concentrations used to treat the plants in this study did not influence the *in vitro* growth of *C. lindemuthianum*. *In vivo*, the establishment and extent of biotrophy were not affected by glyphosate. However, the extent of necrotrophic fungal growth was much enhanced in treated plants. Such growth is normally contained in limited lesions in control plants; there was no apparent defense on the part of treated plants against necrotrophic fungal growth and they eventually collapsed.

The mechanism(s) responsible for physiological resistance of mature plants is not well understood, although resistance of cell walls to penetration and degradation, and rapid dessication of infected tissues have been implicated as factors restricting lesion expansion (12, 43, 88). It has also been suggested (35) that mature plant resistance in beans, like induced systemic resistance, probably results from interference with the transition of *C. lindemuthianum* from primary mycelial (biotrophic) to secondary mycelial (necrotrophic) stage. All these hypotheses suggest that physiological resistance is not an active resistance mechanism, but reflects structural and

physiological changes inherent to the growth and development of bean plants. My results, however, reveal that mature plant resistance also results from active response, which could be due to phytoalexins and/or lignification (103, 107), both of which can be suppressed by glyphosate.

In conclusion, glyphosate effectively inhibits both the general (see also (62)) and specific resistance (68 and Chapter 1) of plants to disease. Both kinds of resistance appear to be mediated largely by phytoalexins. These findings may have some important implications in the field usage of glyphosate as a selective herbicide. Roundup is used as a selective herbicide, especially to control weeds in forest plantations. Concentrations of glyphosate that are non-toxic to seedling trees are herbicidal to the majority of weeds. However, such usage may prove risky since glyphosate at non-phytotoxic levels may increase the damage due to diseases.

CHAPTER 3

DETAILED INVESTIGATIONS ON THE PHYTOALEXIN RESPONSE IN INCOMPATIBLE BEAN ANTHRACNOSE INTERACTION AS INFLUENCED BY GLYPHOSATE AND WOUNDING

INTRODUCTION

In Chapter 1, it was shown that glyphosate reduced the potential of *Phaseolus vulgaris* to effectively defend itself against avirulent races of *Colletotrichum lindemuthianum*, even though the expression of the HR was apparently unchanged. Phytoalexin accumulation in resistant plants was partially suppressed by glyphosate and appeared to be the mechanism for glyphosate-induced loss of resistance. On this basis, it was concluded that phytoalexin accumulation is a major feature of HR and a determinant of resistance. However, phytoalexin comparisons between glyphosate-treated and nontreated plants were semiquantitative, being based on subjective estimates of the intensity of spots of phytoalexins on thin layer chromatograms detected under UV light, and involved only the ultimate levels of phytoalexins without any reference to their rates of accumulation.

During the same study, it was also found that wounding *per se* interferes with the resistance mechanism of the host and enables the infection hyphae to grow from HR cells. This phenomenon was observed by other workers as well (11, 37). The mechanism(s) responsible for wound-induced susceptibility is(are) not clear, although degradation of phytoalexins by the host and/or fungal tissue (11 and Chapter 1) and/or their delocalization from the HR cells (Chapter 1), as induced by wounding, were thought to be involved.

The purpose of this chapter is two fold: firstly, to demonstrate quantitatively how glyphosate affects the kinetics of phytoalexin accumulation during resistant (incompatible) interactions of bean anthracnose; and secondly, to study the possible mechanism(s) of wound-induced escape of infection hyphae from HR sites in resistant tissues.

MATERIALS AND METHODS

Plant and fungal materials

French bean seedlings, cv. Topcrop, were grown in the dark at 24°C in a growth chamber as previously described (Chapter 1). The γ race of *C. lindemuthianum*, non-pathogenic on Topcrop plants, was used to inoculate the plants; its growth and maintenance have been described (Chapter 1). Inoculum was prepared from 9-10-day old cultures, and was adjusted to 2×10^6 spores ml^{-1}.

Inoculation and incubation of bean seedlings

Hypocotyls of etiolated plants were inoculated when 9-days old, by spraying inoculum evenly as a fine mist onto all sides of the full length of hypocotyls. Inoculated plants were incubated at 22°C under high humidity conditions (99% RH) for 12 days either in total darkness or were exposed to light at 40 h post-inoculation as described later. Glyphosate treatment was done at 24 h after inoculation by placing a 1 μl drop of an aqueous solution of Roundup® (10 μg a.i. μl^{-1}) near the center of the hypocotyl. Control plants were treated similarly with distilled water. Cotyledons were excised from some plants (both glyphosate treated and nontreated) immediately prior to glyphosate or similar treatment with water. These plants

(cotyledons-removed) and some more plants (cotyledons intact), both glyphosate treated and nontreated, were exposed to light (16000 LX) at 40 h post-inoculation for an initial 24 h continuous light treatment, followed by a 14:10 h day:night photoperiod.

Analysis of phytoalexins

At various times, starting 72 h after inoculation, six 12-cm sections (5-6 g) cut from the middle of inoculated hypocotyls were used for analysis of phytoalexins. Alcohol extracts were prepared from these tissue samples as described by Rahe (90). Further purification and quantitation of phytoalexins followed the procedure of Goosens and Van Laere (42) with only slight modifications (Chapter 2). Extraction efficiency of phytoalexins during this experiment and the experiments described later ranged from 72-81%. All the values were corrected for losses incurred during extraction and purification.

Effect of wounding on phytoalexins

Three separate experiments were conducted to evaluate the effect of wounding (excision) on the fate of phytoalexins in the resistant tissue. In all these experiments thin epidermal strips (approx. 25x5 mm, in size) were peeled freehand from incompatible hypocotyls at 152 h post-inoculation. The HR reaction and the peak phytoalexin accumulation had already occurred on these plants. Plant hypocotyls were gently wiped with tissue paper and then surface sterilized with 1% NaOCl for 5 min before peeling the strips. There were three replications (3 separate batches of strips from a single experiment) for every treatment.

In the first experiment, epidermal strips (0.4 to 0.5 g) were floated on distilled water containing 100 $\mu g\ ml^{-1}$ each of streptomycin sulphate and penicillin G in 60x20 mm disposable plastic Petri plates and incubated in the dark at 23°C. After different times of incubation, the strips and the floating medium (water) were analysed separately for phytoalexins. Epidermal strips were placed into 10 ml of boiling 95% ethanol and after 5 min the ethanol was filtered through glass wool. The residue was extracted twice more with 10-ml volumes of ethanol and the three fractions were combined. Phytoalexins from such samples were extracted and quantified by HPLC as mentioned above. In the case of the floating medium, an equal volume of ethanol was added before extracting phytoalexins. Control treatments at each time interval consisted of freshly taken epidermal strips floated onto water immediately before being extracted for phytoalexins. Water was analysed separately.

In the second experiment, epidermal strips were either autoclaved before incubation on water or floated directly on 0.03% solution of $HgCl_2$. For autoclaving, excised strips were put in distilled water in 50-ml conical flasks. These autoclaved strips were not allowed to become contaminated except in one treatment in which the strips were exposed to air for 30 min after autoclaving and then incubated in the dark for 48 h. Phytoalexins were extracted from autoclaved strips and their bathing medium together, whereas for $HgCl_2$-floated strips, analysis was done separately for the strips and the medium. Strips for control treatments were directly analyzed for phytoalexins without floating on water or $HgCl_2$ solution.

In the third experiment, epidermal strips were placed on glass filaments in humid Petri plates so as to eliminate contact with liquid during

incubation, and harvested at different intervals for phytoalexin determinations. The glass filaments were washed three times with 3 ml of ethanol each time and these washings were combined with strips for phytoalexin extraction. Strips for control treatments were freshly excised at equivalent times and used directly for extraction of phytoalexins.

Microscopical observations

The development of the interaction and the growth of infection hyphae from HR cells upon excision was followed by light microscopic examination of live epidermal strips under bright field and Nomarski optics.

RESULTS

Effect of glyphosate on HR and phytoalexins

As in chapter 1, the development and magnitude of hypersensitive response of infected host cells was unaffected by glyphosate under any conditions employed during incubation. In all cases, the infected cells were turning brown within 72 h of inoculation and the intensity of this reaction increased during the next couple of days.

Phytoalexin response in this study was characterized by the accumulation of four phytoalexins: phaseollin, phaseollidin, phaseollinisoflavan and kievitone (Figs. 9 to 12). Detectable levels of most of these phytoalexins were present at 72 h after inoculation. As usual, phaseollin was the major phytoalexin. It accumulated rapidly between 72 h to 130 h post-inoculation when the intensity and number of necrotic cells were increasing. Phaseollin reached peak levels by 144 h after inoculation and thereafter its levels

Figure 9. Effect of glyphosate on the accumulation of phytoalexins in etiolated bean hypocotyls infected with an avirulent race of *Colletotrichum lindemuthianum*.

* = Sum of phaseollin, phaseollinisoflavan, phaseollidin and kievitone.

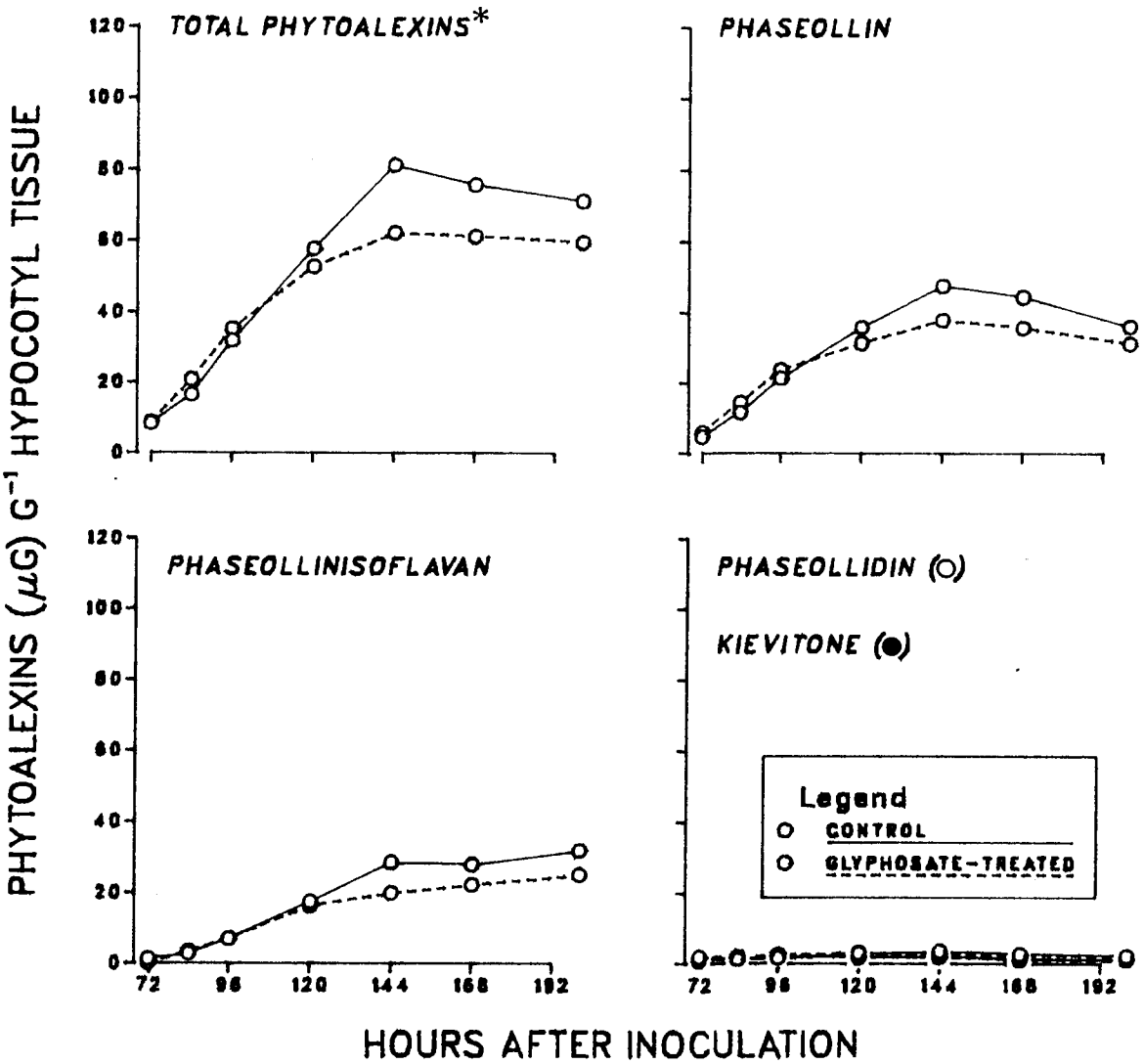

TOTAL PHYTOALEXINS*
PHASEOLLIN
PHASEOLLINISOFLAVAN
PHASEOLLIDIN (○)
KIEVITONE (●)
Legend
CONTROL
GLYPHOSATE-TREATED
PHYTOALEXINS (μG) G⁻¹ HYPOCOTYL TISSUE
HOURS AFTER INOCULATION
0
20
40
60
80
100
120
72
96
120
144
168
192

Figure 10. Effect of glyphosate on the accumulation of phytoalexins in etiolated bean hypocotyls infected with an avirulent race of *Colletotrichum lindemuthianum* and exposed to light beginning 40 h post-inoculation.

* = Sum of phaseollin, phaseollinisoflavan, phaseollidin and kievitone.

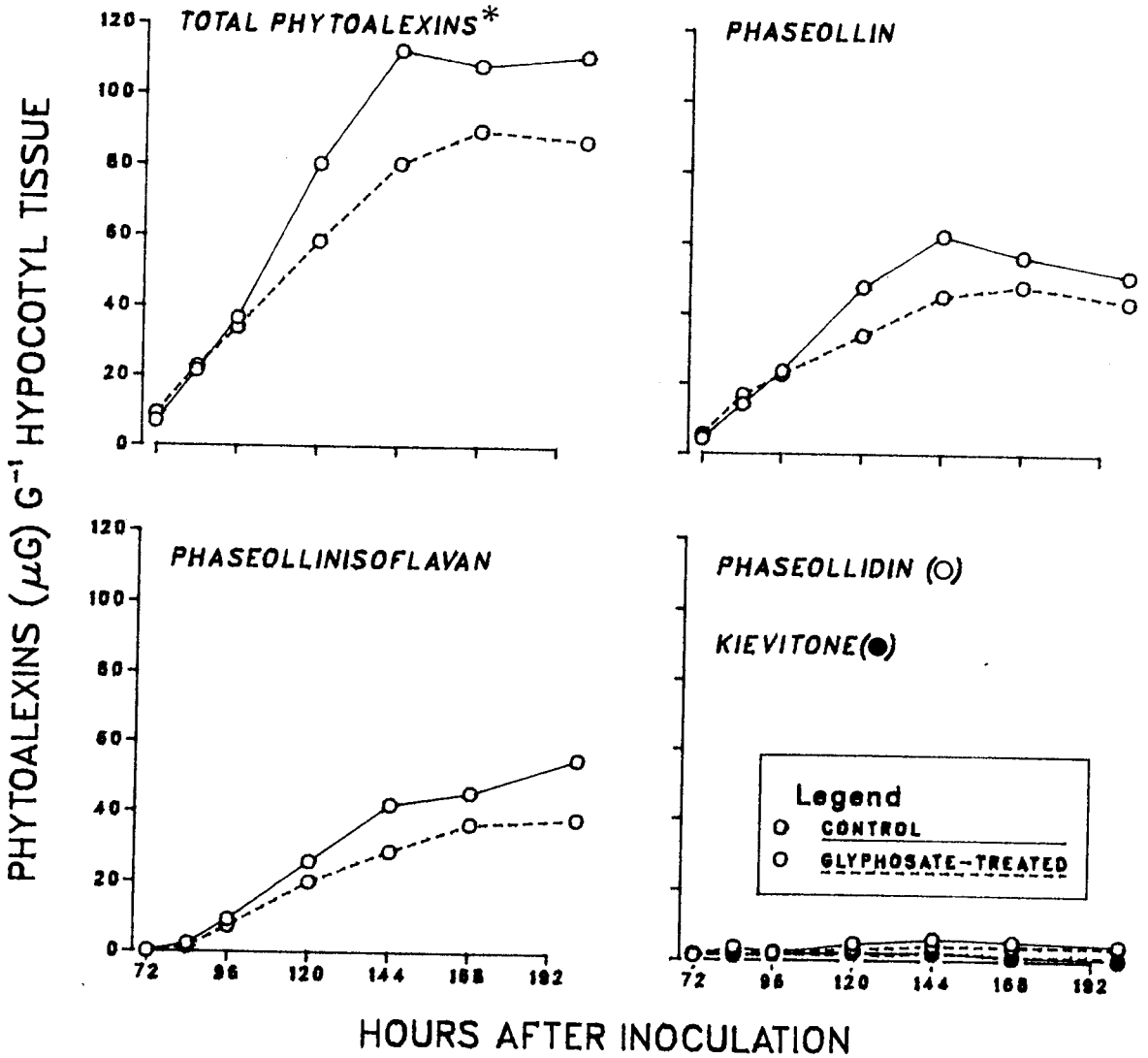

TOTAL PHYTOALEXINS*
PHASEOLLIN
PHASEOLLINISOFLAVAN
PHASEOLLIDIN (○)
KIEVITONE(●)
Legend
CONTROL
GLYPHOSATE-TREATED
PHYTOALEXINS (μG) G⁻¹ HYPOCOTYL TISSUE
HOURS AFTER INOCULATION
0
20
40
60
80
100
120
72
96
120
144
168
192

Figure 11. Effect of glyphosate on the accumulation of phytoalexins in etiolated bean hypocotyls infected with an avirulent race of *Colletotrichum lindemuthianum*. Cotyledons were removed 24 h post-inoculation, and plants were exposed to light 40 h post-inoculation.

* = Sum of phaseollin, phaseollinisoflavan, phaseollidin and kievitone.

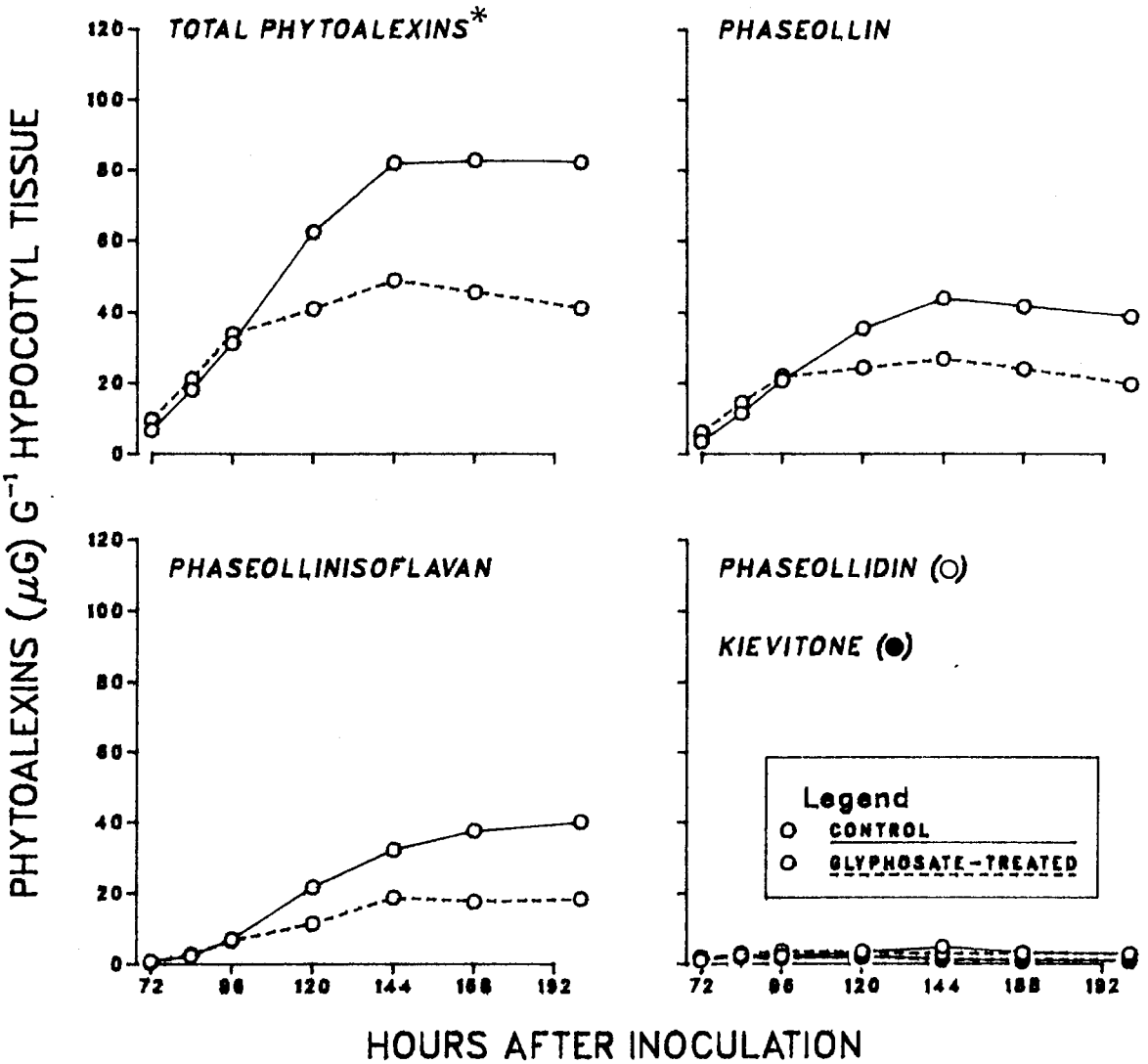
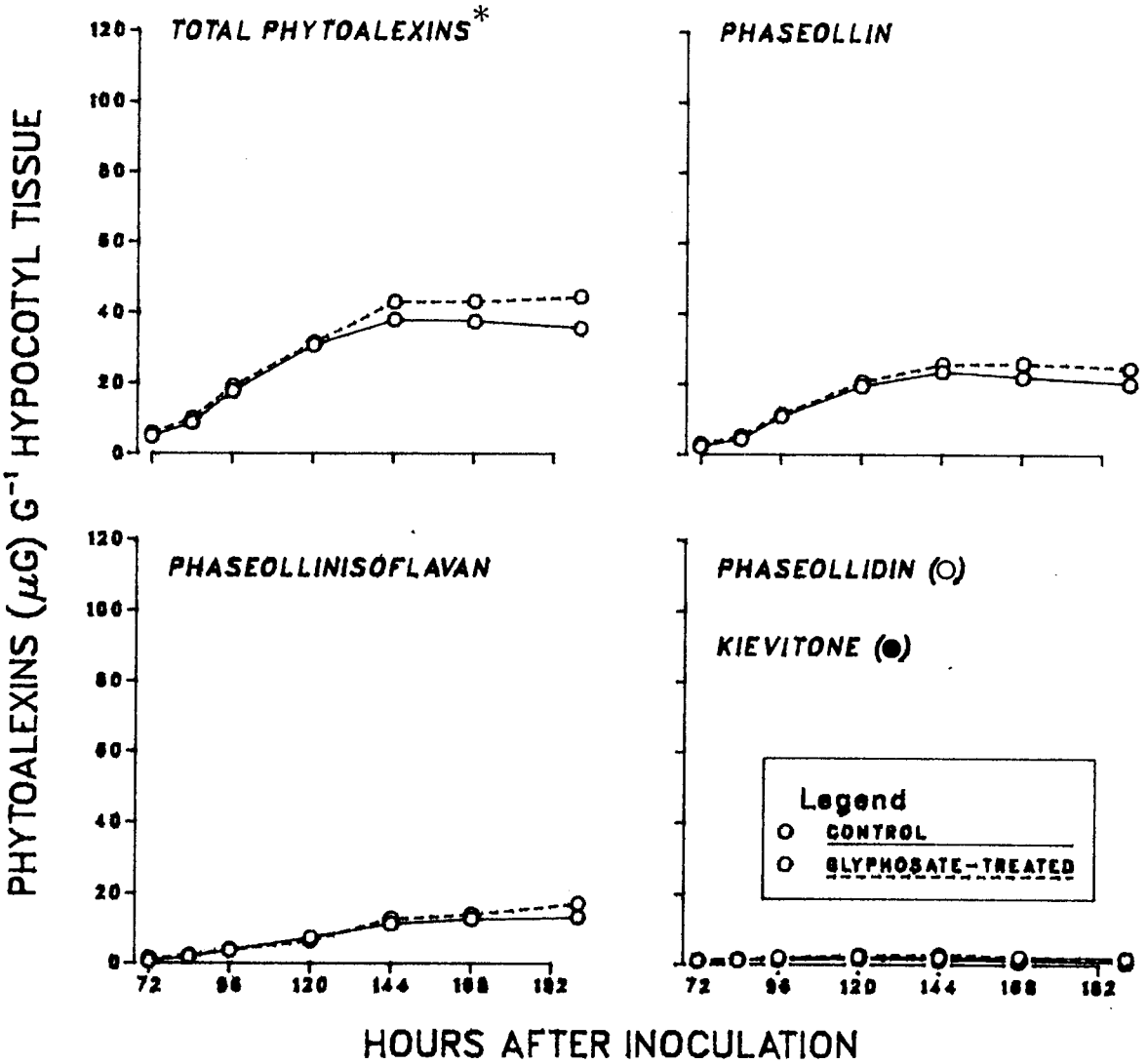

TOTAL PHYTOALEXINS*
PHASEOLLIN
PHASEOLLINISOFLAVAN
PHASEOLLIDIN (○)
KIEVITONE (●)
Legend
CONTROL
GLYPHOSATE-TREATED
PHYTOALEXINS (μG) G⁻¹ HYPOCOTYL TISSUE
HOURS AFTER INOCULATION
0
20
40
60
80
100
120
72
96
120
144
168
192

Figure 12. Effect of glyphosate on the accumulation of phytoalexins in etiolated bean hypocotyls with a low density of infections with an avirulent race of *Colletotrichum lindemuthianum*. Cotyledons were removed 24 h post-inoculation, and plants were exposed to light 40 h post-inoculation.

* = Sum of phaseollin, phaseollinisoflavan, phaseollidin and kievitone.


TOTAL PHYTOALEXINS*
PHASEOLLIN
PHASEOLLINISOFLAVAN
PHASEOLLIDIN (○)
KIEVITONE (●)
Legend
CONTROL
GLYPHOSATE-TREATED
PHYTOALEXINS (μG) G⁻¹ HYPOCOTYL TISSUE
HOURS AFTER INOCULATION
0
20
40
60
80
100
120
72
96
120
144
168
182

declined slightly. Phaseollinisoflavan was the second major component. Compared to phaseollin, the peak of its production was delayed and its levels continued to increase throughout the experiment to reach similarly high concentrations. Phaseollidin and kievitone also accumulated, but their levels remained quite low and did not exceed 10 and 12 $\mu g\ g^{-1}$, respectively, under any of the experimental conditions.

Early in the infection, the pattern of accumulation of different phytoalexins and their relative concentrations were not affected by glyphosate. However, the overall expression of the response was reduced by glyphosate and the degree of this reduction depended upon the incubation condition of inoculated plants. In etiolated plants, the peak level of phytoalexins (combined) after glyphosate treatment was 60 $\mu g\ g^{-1}$, which was about 80% of that accumulating in control (glyphosate-nontreated) plants (Fig. 9). When intact (cotyledon-plus) etiolated plants were exposed to light during incubation, phytoalexins accumulated relatively rapidly and in greater amounts than in the dark inoculated plants (Fig. 10). Again, phytoalexins were only partially suppressed by glyphosate; peak levels in treated plants were 80% of controls (Fig. 10). In contrast, the effect of glyphosate was much more pronounced on plants whose cotyledons were removed prior to light exposure (Fig. 11); treated plants accumulated only 55% of the phytoalexins produced by control plants.

In all these cases, the initial rate of production/accumulation was comparable in magnitude in both the glyphosate-treated and nontreated plants. Only the final accumulation (maximal level) was reduced by glyphosate.

An additional factor, which contributed to the effectiveness of glyphosate in limiting phytoalexin production in resistant plants was the intensity of infection (necrotic cells). In one experiment, the density of infection was visibly lower than in the experiment discussed above. This was achieved by intentional reduction of the inoculum to $5x10^4$ spores ml^{-1}. In this case, the maximum accumulation of phytoalexins was only 24 $\mu g\ g^{-1}$ fresh wt. of infected tissue in control (glyphosate-nontreated) plants and glyphosate did not suppress the overall accumulation of phytoalexins (Fig. 12). In fact, slightly higher levels of phytoalexins accumulated in glyphosate treated than in control plants. Also the expression of resistance in such plants was not affected by glyphosate.

Effect of wounding on phytoalexins in resistant tissues

Epidermal strips used in these experiments were 4-7 cell layers thick and contained all the phytoalexins present in intact hypocotyls. Phytoalexin concentrations as high as 700 $\mu g\ g^{-1}$ wet wt. were detected in these epidermal strips. No phytoalexins were detected from the hypocotyl tissue beneath these strips.

When strips were floated on water, phytoalexins disappeared gradually with time. At 12, 24, and 48 h after excision, the amounts of phytoalexins that remained were only 63, 30, and 16%, respectively, of that present at 0 time after excision (Table 11). In comparison with phaseollin, the more polar phytoalexin, phaseollinisoflavan, disappeared faster. Of the total phaseollin detected at 12, 24, and 48 h after incubation, 36, 30, and 0% was present in the floating medium (water), respectively. Phaseollinisoflavan was not detected from the floating medium at any time after excision. The

TABLE 11

The fate of phytoalexins in excised epidermal strips floated on water.[a]

Time after incubation	Treatment	Total phytoalexins[b] μg g^{-1}[c] strips[d]	Phaseollin μg g^{-1}[c] strips[d]	Phaseollin-isoflavan μg g^{-1}[c] strips[d]
0 h	excised/ control	644.50	349.10	245.85
12 h	excised	405.30	312.11	93.05
	control	652.80	349.65	288.75
24 h	excised	195.00	151.60	43.10
	control	687.05	338.72	331.90
48 h	excised	104.65	66.00	38.65
	control	656.45	321.20	310.80

[a] Epidermal strips were excised from etiolated hypocotyls of *Phaseolus vulgaris*, cv. Topcrop, inoculated with the γ race of *Colletotrichum lindemuthianum* (incompatible interaction) at 152 h post-inoculation.
[b] These values indicate the sum of phaseollin, phaseollidin, phaseollinisoflavan and kievitone detected in strips and water separately.
[c] Fresh wt.
[d] Values reported are the means of three replications (three separate batches of epidermal strips from a single experiment).

concentration of phytoalexins in control strips did not change significantly over this range of time and the pathogen remained inhibited in HR cells. As before (Chapter 1), the infection hyphae, limited in HR cells, resumed growth within 10-12 h of excision. In addition, bacterial growth was observed in the floating medium and in epidermal strips during incubation.

When epidermal strips were autoclaved before incubation under sterile conditions or floated on $HgCl_2$ solution, phytoalexins were not lost (Table 12), although when floated on $HgCl_2$, 10-15% of the phytoalexins leaked into the floating solution. However, when the autoclaved strips were allowed to become contaminated during incubation, phytoalexins disappeared during the subsequent incubation. Bacterial growth was observed in these epidermal strips and their bathing medium.

The experiments in which epidermal strips were placed on glass filaments provided very interesting results. In contrast to strips floated on water, these strips retained the majority of their phytoalexins at least until 24 h post-excision (Table 13). The pathogen, however, outgrew HR cells and even colonized one or two surrounding cells during this time. The levels of phytoalexins were reduced to 34% of the level in control strips by 60 h after incubation but the strips were extensively colonized by the pathogen at this time and some bacterial contamination was also observed.

Occasionally, even the infection hyphae trapped inside reaction material deposits (Chapter 1) resumed growth as a result of excision. This was observed in epidermal strips floated on water as well as in the strips placed on glass filaments.

TABLE 12

The fate of phytoalexins in bean epidermal strips either autoclaved before incubation in water or floated directly on $HgCl_2$ solution.[a]

Time after incubation	Total phytoalexins[b] μg g^{-1}[c] of strips[d]	Phaseollin μg g^{-1}[c] of strips[d]	Phaseollinisoflavan μg g^{-1}[c] of strips[d]
	AUTOCLAVED STRIPS INCUBATED IN WATER		
0 h	708.25	374.10	288.65
24 h	638.85	335.28	275.50
48 h	687.90	358.40	257.50
48 h[e]	84.50	65.35	19.49
	$HgCl_2$-FLOATED STRIPS[f]		
0 h	611.39	357.50	223.76
24 h	652.40	379.22	258.12
48 h	629.85	382.35	237.50
	CONTROL STRIPS		
24 h	682.42	337.10	321.50
48 h	654.90	318.65	324.59

[a] Epidermal strips were excised from etiolated hypocotyls of *Phaseolus vulgaris*, cv. Topcrop, inoculated with the γ race of *Colletotrichum lindemuthianum* (incompatible interaction) at 152 h post-inoculation.
[b] These values indicate the sum of phaseollin, phaseollidin, phaseollinisoflavan and kievitone.
[c] Fresh wt.
[d] Values reported are the means of three replications (three separate batches of epidermal strips from a single experiment).
[e] These autoclaved strips were allowed to become contaminated before incubation.
[f] The values of phytoalexins presented in this experiment are the sum total of phytoalexins detected in strips and $HgCl_2$ solution separately.

TABLE 13

The fate of phytoalexins in bean epidermal strips placed on glass filaments in humid Petri plates.[a]

Time after incubation	Treatment	Total phytoalexins[b] μg g^{-1}[c] strips[d]	Phaseollin μg g^{-1}[c] strips[d]	Phaseollin-isoflavan μg g^{-1}[c] strips[d]
0 h	excised/ control	680.90	360.41	282.35
12 h	excised	612.30	341.85	245.50
	control	632.50	336.94	275.56
24 h	excised	662.50	357.20	274.70
	control	682.42	337.10	321.50
60 h	excised	233.45	152.68	65.95
	control	671.50	306.84	329.25

[a] Epidermal strips were excised from etiolated hypocotyls of *Phaseolus vulgaris*, cv. Topcrop, inoculated with the γ race of *Colletotrichum lindemuthianum* (incompatible interaction) at 152 h post-inoculation.
[b] These values indicate the sum of phaseollin, phaseollidin, phaseollinisoflavan and kievitone detected in strips and water separately.
[c] Fresh wt.
[d] Values reported are the means of three replications (three separate batches of epidermal strips from a single experiment).

DISCUSSION

The data confirm and extend the conclusion of the previous study (Chapter 1) that glyphosate reduces the potential of *P. vulgaris* to effectively defend against avirulent races of *C. lindemuthianum*. Glyphosate does so by suppressing the accumulation of phytoalexins while permitting the normal cytological expression of HR. The time-course analysis shows that the effect of glyphosate on the phytoalexin response can be divided into two phases. During the first phase (initial 40-50 h following the appearance of HR) the rate of accumulation of phytoalexins is insensitive to glyphosate treatment. In the second phase, when peak levels of phytoalexin are attained, the rate of accumulation is relatively low in glyphosate treated plants and consequently, overall levels of phytoalexins are reduced.

In accordance with previous findings (70, 93 and Chapter 1) the present results indicate that the maximum levels of phytoalexin accumulation and the degree of reduction of phytoalexin accumulation by glyphosate are both affected by the physiological status of host plants. Conditions which compete for or deplete indigenous reserves of phenylalanine (exposure to light, and removal of cotyledons, respectively) enhance the suppression of phytoalexins by glyphosate (see also Chapter 1). Additionally, the sensitivity of beans to glyphosate is influenced by the intensity of infection which positively correlates with the amounts of phytoalexins produced during infection (8, 90). At lower infection densities, where the maximal levels of phytoalexins accumulated by the plants remained low, glyphosate failed to interfere with the production of phytoalexins and the expression of resistance. In contrast, glyphosate was a potent inhibitor of

both the accumulation of phytoalexins and the associated restriction of lesions in susceptible interactions of bean anthracnose (Chapter 2). Glyphosate was also very effective in suppressing the accumulation of glyceollin and the resistance of soybeans to *Phytophthora megasperma* f. sp. *glycinea* (68), but as in the present study, the accumulation of glyceollin was only slightly inhibited immediately after infection. In both of these cases, it appears that more phytoalexins per unit infected area than are present in the early stages of accumulation are needed to effectively inhibit the pathogen.

All of the results of this study support the interpretation that glyphosate's ability to suppress phytoalexins depends on the availability and demand for precursors (i.e. phenylalanine) of phytoalexin production. Bean seedlings contain large amounts of free phenylalanine (89) which is derived from cotyledons as well as *de novo* biosynthesis. While glyphosate inhibits the *de novo* production (3, 44, 60), it is unable to affect the phenylalanine already present in plants. Phytoalexin production in glyphosate treated plants probably draws upon these pools of phenylalanine, therefore explaining the lack of a treatment effect during the initial stages of accumulation. Under conditions of high demand for phytoalexins, these pools may become depleted. Consequently, further production of phytoalexins will be hampered, leaving the plants vulnerable to future or secondary infections. This is reflected in the fact that once the pathogen escapes HR, it encounters no further resistance in glyphosate treated plants and eventually rots the entire hypocotyl.

To obtain conclusive evidence regarding the role of HR in disease resistance, it would be desirable to completely eliminate the concomitant

accumulation of phytoalexins. Apparently, this cannot be accomplished with glyphosate, even under conditions which should tentatively deplete the majority of the substrates for phytoalexin production. This may suggest, as pointed out by Ward (112), that there is a special commitment of resources towards plant defense and this may occur at the expense of other possibly essential functions.

Interference with the resistance of beans to *C. lindemuthianum* by wounding (11 and Chapter 1) is confirmed by the results obtained in this study. The study focussed on the effect of the various treatments on phytoalexins, nevertheless recognizing the possibility that resistance of beans to *C. lindemuthianum* could be due to some other, unknown mechanism which wounding renders ineffective.

Wounding caused a reduction in the levels of accumulated phytoalexins in resistant tissues, although definitive evidence of the mechanism of reduction was not obtained. Phytoalexins were presumably localized at infection sites at the time of wounding, as all of the phytoalexins present in hypocotyls were contained in these peels. Phytoalexins diffused from these peels, and also disappeared from the peel/floating medium systems. These findings suggest that both delocalization and catabolism of phytoalexins may contribute to the loss of tissue resistance associated with wounding.

The demonstrated diffusion of phytoalexins from strips into water on which they were floated strongly suggests that wounding causes delocalization of phytoalexins from infection sites. Several other reports lead to a similar conclusion, e.g., the apparent diffusion of phytoalexins into inoculation-droplets (22), removal of phytoalexins from infection sites by

cotton wicks (73), production of phytoalexins by living tissue and their accumulation in dead tissue during incubation of live and dead bean hypocotyl halves (dissected) in close contact (45), and the leakage of phytoalexins from cell suspensions into the medium (28, 46).

Catabolism of phytoalexins, which seems to be a general phenomenon in plants (15, 65, 104, 109, 115) and can be induced by wounding (59), apparently was caused by excision of the epidermal strips from hypocotyls. However, it is unlikely that the degradation of phytoalexins was responsible for the 'escape' of infection hyphae from HR cells in excised strips, since escape occurred prior to substantial reduction in the levels of phytoalexins.

Catabolism of phytoalexins could be due to metabolic process of the host, the pathogen, microbial contaminants or some combination of these. The data obtained in this study suggest that bacteria may have been substantially responsible for the disappearance of phytoalexins from excised epidermal strips, although the evidence for this conclusion is indirect. Evidence for degradation of phytoalexins by host and/or pathogen metabolism was not obtained in this study, although *C. lindemuthianum* has been shown capable of degrading all bean phytoalexins (8, 12, 52, 99).

Of the four compounds that characterized the phytoalexin response in this study, phaseollin and phaseollinisoflavan were predominant under all incubation conditions. Phaseollin accumulated early in the interaction and after peaking around 140 h post-inoculation, its levels slightly declined. Phaseollinisoflavan attained a maximum rate of accumulation approximately 20-30 h later than phaseollin and its levels continued to increase for the duration of the experiment. By using cell suspension cultures of *P.*

vulgaris, the evidence has been presented that phaseollinisoflavan is a metabolic product of phaseollin and that this conversion occurs during accumulation (46). The pattern of accumulation of phaseollin and phaseollinisoflavan here indicates that similar conversion may be occurring in infected hypocotyl tissue.

How and where phytoalexins are synthesized, localized, transported and decomposed are clearly questions of fundamental importance. Our greatest barrier in this regard is the lack of suitable techniques to accurately detect and quantify individual phytoalexins *in situ*. Recent research on phytoalexins has emphasized differential sites for biosynthesis and accumulation (13, 24, 29, 30, 45, 67). It is proposed that phytoalexins are synthesized in healthy cells and then transported to dead or dying HR cells for accumulation so that they may be excluded from further metabolism (67). This concept recognizes that a translocation mechanism for phytoalexins exists in infected tissues. The nature of this mechanism is not clear, although it is often assumed that this probably involves simple diffussion, the net direction of which may be dictated by the presence of deposition sites for phytoalexins (i.e., HR cells). Our results seem to support this hypothesis and further indicate that HR cells are not necessarily the ultimate destination for phytoalexins. These phytoalexins are subject to further mobilization provided new sinks are created. Peeling epidermal strips injures cells at the site of excision and thus may create new 'sinks' for phytoalexins. Consequently, the phytoalexins may diffuse out of HR cells and release the infection hyphae from inhibition.

In conclusion, the present results suggest that wounding causes delocalization of phytoalexins from infection sites, although the evidence

for this is not unequivocal. Nevertheless, these results are of particular interest because they draw attention to regulatory mechanisms governing cellular concentration and activity of phytoalexins *in situ*. It is often taken for granted that phytoalexins, once formed, 'do what they are supposed to do', without being subject to normal cellular regulations. Therefore, critical analysis of the nature of wound-induced loss of resistance may resolve several questions concerning the role of phytoalexins in disease resistance.

CHAPTER 4

THE NATURE OF SPECIFICITY IN BEAN ANTHRACNOSE HOST-PARASITE INTERACTION

INTRODUCTION

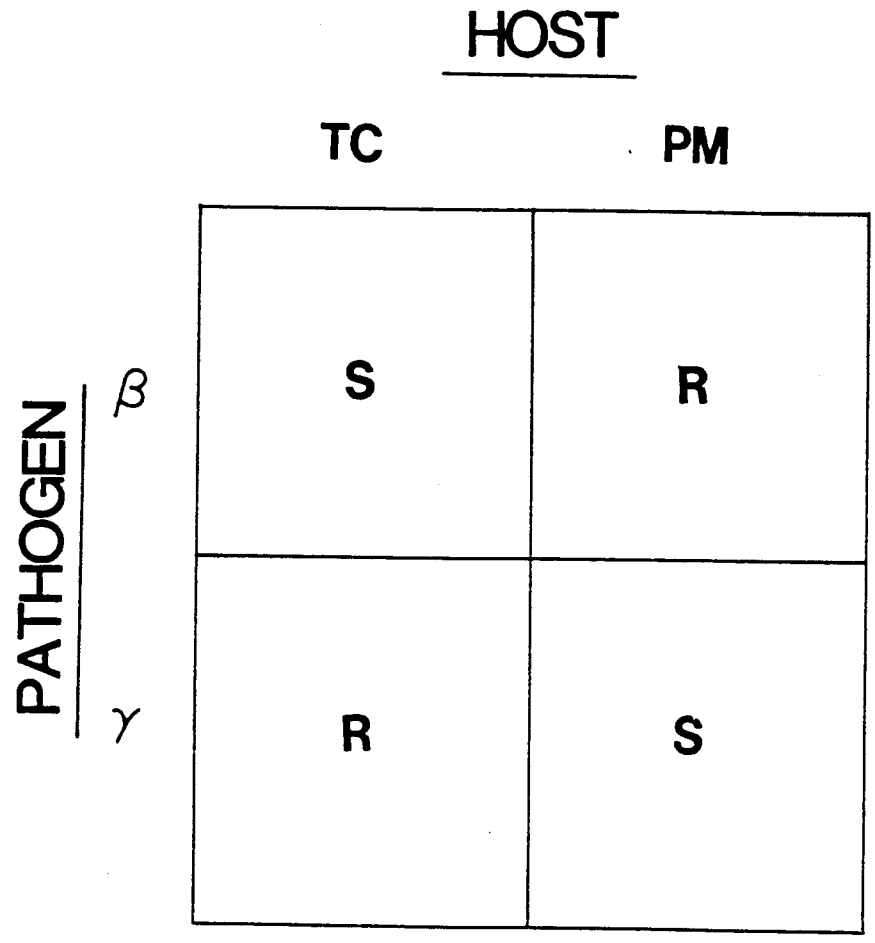
Another question of fundamental importance and much speculation in plant pathology is of the specificity of interaction during the relationship of the host with its parasite. The observed differential interaction of bean cultivars with different races of *Colletotrichum lindemuthianum* (Fig. 13) implies specific recognition between the host and the parasite. But the question that remains is whether this specific recognition leads to resistance or susceptibility.

Based mainly on analogies and results from other related fields of research, it is considered that recognition of a fungus by a plant occurs between complementary molecules on the surface of the host and pathogen which probably are the primary and/or secondary products of corresponding genes of the two partners (1, 17, 25, 49, 64, 108). It is proposed that the recognitional event precedes and in turn leads to subsequent physiological and biochemical changes associated with compatible and incompatible reactions (1, 17, 32, 49, 67). On this basis, there are two opposing views to explain specific interactions of cultivars with fungal pathogens.

(1) The specific elicitor/receptor model: This requires that recognition determines resistance (i.e. hypersensitive cell death and accumulation of phytoalexins) and proposes that the product of a host resistance gene (receptor) interacts with the product of an avirulence gene of the pathogen (elicitor) to induce resistance (1, 36, 67). The lack of either

Figure 13. Differential interaction of bean cultivars, Topcrop (TC) and Perry Marrow (PM), with β and γ races of *Colletotrichum lindemuthianum*.

S = Susceptible
R = Resistant


HOST
TC
PM
PATHOGEN
β
γ
S
R
R
S

or both products results in susceptibility.

(2) The nonspecific elicitor/specific suppressor model: This proposes that recognition determines susceptibility and that resistance is due to a non-specific elicitor from the pathogen causing the death of infected cells and accumulation of phytoalexins, whereas susceptibility is achieved by specific suppressors produced by virulent races which either prevent recognition or the subsequent death of infected cells (16, 23, 32, 41, 49, 108, 116).

Unfortunately, direct evidence to support any of these hypotheses is currently rather thin.

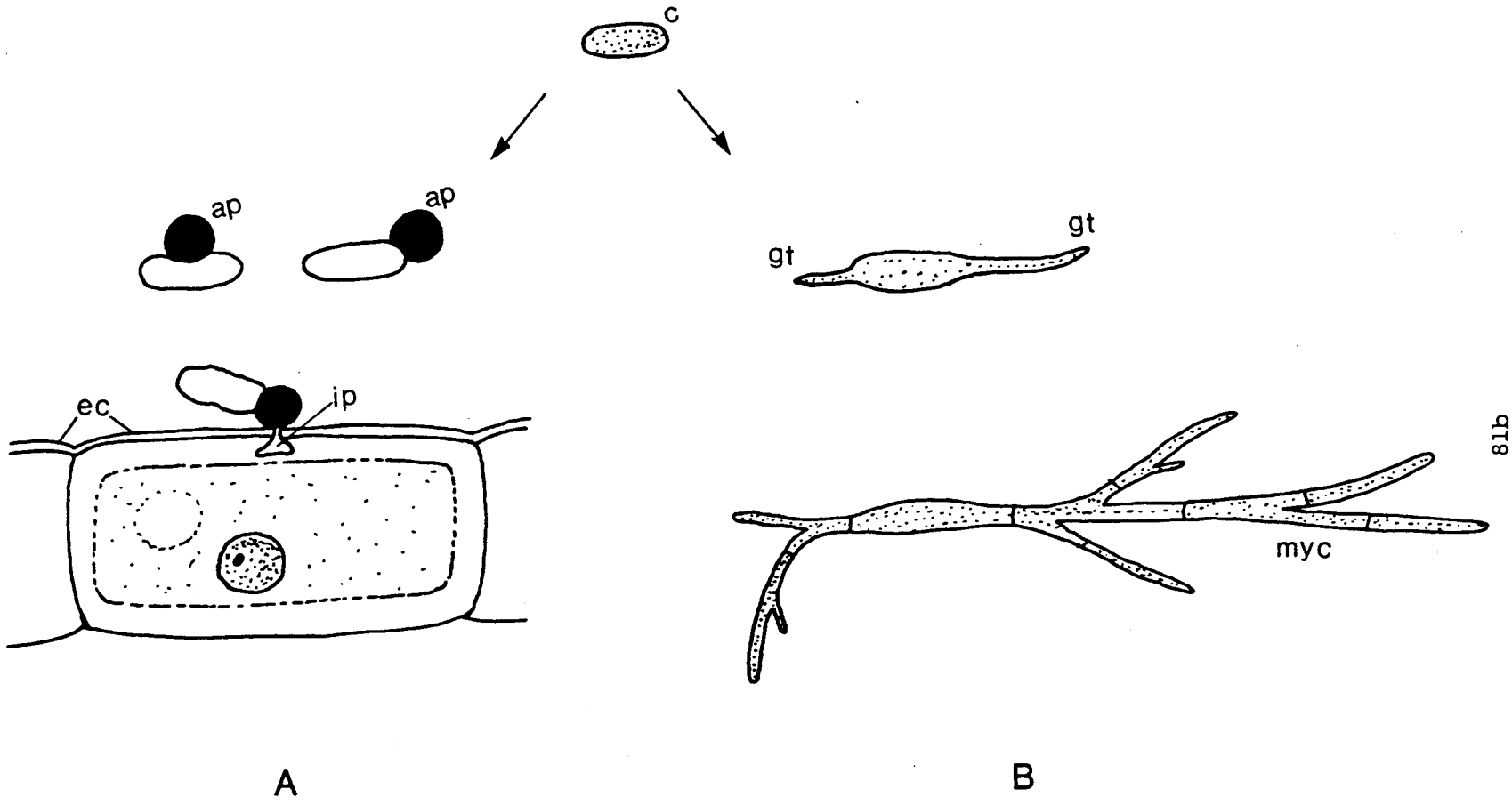
I decided to examine these proposals in the bean anthracnose pathosystem with regard to an unusual epigenetic phenomenon displayed by conidia of *C. lindemuthianum* (Fig. 14). Under natural conditions, a conidium germinates to produce an appressorium directly or at the end of a short germ tube which then penetrates directly into the epidermal cell via a penetration peg. The outcome is either compatibility or incompatibility. In the compatible interaction mycelium of *C. lindemuthianum* develops within the host as a biotroph initially, and then shifts to a necrotrophic mode of growth (12, 80, 88). The pattern of germination differs in the presence of nutrients on a Petri plate. The conidium produces one or more long germ tubes which continue to grow and branch repeatedly to produce a mass of hyphae (mycelium) and no appressorium is produced. It is possible that saprophytic growth occurring on defined media is physiologically similar to necrotrophic development in the host.

The rationale was that if specificity is for resistance, then it might be possible to change the incompatible relationship into a compatible one by

Figure 14. Development of *Colletotrichum lindemuthianum* conidia in the absence (A) or presence (B) of nutrients.

ap = appressorium, c = conidium, ec = epidermal cells

gt = germ tube, ip = infection peg, myc = mycelium


c
ap
ap
gt
gt
ec
ip
myc
A
B
81b

using mycelia instead of conidia as inoculum, because different sets of genes are likely to be expressed during different modes of development. The avirulent gene product which is recognized by the resistance gene product may be expressed selectively and only during the appressorial phase in the infection court.

MATERIALS AND METHODS

Two cultivars of beans, Topcrop (TC) and Perry Marrow (PM), which are reciprocal differentials to the β and γ races of *C. lindemuthianum* (Fig. 13), were used as host plants. Etiolated seedlings were grown as described in Chapter 1. Soyabean (*Glycine max*), broad bean (*Vicia faba*) and pea plants (*Pisum sativum*), which served as non-hosts, were also grown under similar conditions. In preliminary trials, both etiolated and green bean plants were used. Because both types of plants provided similar results, only etiolated plants were employed in the experiments described here.

Isolates of the β and γ races of *C. lindemuthianum* were grown on bean juice agar (BJA) in the dark at room temperature. Four different types or sources of inoculum were used in this study. These included conidia, conidia+BJA, mycelial plugs from BJA plates, and mycelium from infected tissues of susceptible plants. Spore suspensions containing $5X10^5$ conidia ml^{-1} were prepared from 9-to 10-day old cultures (Chapter 1). For conidia+BJA inoculum, 5mm long pieces were removed from 9 cm BJA plates 2 h after inoculation with $1X10^6$ spores in 100 μl of distilled water. Mycelial inocula were obtained by either cutting approximately 5X2 mm size plugs from the borders of actively growing fungal colonies on BJA (4-5-days old) or

peeling epidermal strips of similar size from infected susceptible plants for use in transplant experiments, to be discussed later.

Inoculation and Incubation

Hypocotyls of Topcrop and Perry Marrow bean plants were inoculated when 10 and 11-days old respectively, with either the β or γ race of *C. lindemuthianum*. Inoculation with conidia was achieved by placing a 5 μl drop of spore suspension at two different sites 7-8 cm apart. Drops were placed either directly on the hypocotyl surface (intact) or onto a fresh wound (approx. 7X3 mm) made by peeling away the epidermis. Similar inoculations of wounded and intact sites on other plants were made with conidia+BJA plugs, and with mycelial plugs. The agar plugs were placed on inoculation sites such that the side with mycelia or spores contacted the wound or plant surface.

Soybean, broad bean and pea plants were inoculated similarly when 10, 12, and 20 days old respectively, but with spore suspensions and mycelial plugs only.

In an attempt to find and possibly exploit any inherent difference in the parasitic capabilities of the biotrophic and necrotrophic modes of development of *C. lindemuthianum*, tissue transplant experiments were conducted. Thin epidermal strips (4-6 cell layers thick) were taken from infected susceptible plants (e.g. TC infected with β and PM infected with γ race of *C. lindemuthianum*) at 72 (pathogen biotrophic) and 108 h (pathogen necrotrophic) post-inoculation and used as inoculum. Fresh epidermal wounds on hypocotyls of TC or PM plants were inoculated with these strips. Inoculation of healthy TC and PM plants with epidermal strips from uninfected

TC and PM plants served as controls.

There were 12-15 plants per treatment and each experiment was repeated at least twice. In all experiments, half of the plants were treated with glyphosate (10 μg per plant) immediately after inoculation (see Chapter 1 for details of glyphosate treatment). Inoculated plants were incubated in the dark under conditions of high humidity (99% RH) at 22-23°C.

Microscopic and gross observations

At various times after inoculation, fresh epidermal strips (peeled freehand) or plant cortex tissue excised from underneath the wounds were examined microscopically to assess the growth and development of the pathogen in host tissues. Where spores were placed directly on wounds, their germination behavior was compared with those placed on intact hypocotyls. When the wound tissue became discolored or necrotic, such tissue was cleared in 0.1 N NaOH for 2-3 min before examination.

Observations on lesion size were made on day 6 after inoculation. On wound-inoculated plants, depending on the size of the lesion and histological proof of pathogen development in host tissues, the lesions were categorized as susceptible, intermediate or resistant. Lesions were rated susceptible if their length was 1 cm or more and colonization had occurred. Intermediate lesions were those which were less than 1 cm long but the pathogen had definitely colonized the host tissue. In resistant lesions, there was no evidence of fungal growth in host tissues. Such lesions, unlike susceptible and intermediate lesions, did not spread on glyphosate treated plants. Plants which were inoculated intact were rated only susceptible or resistant.

RESULTS

Response of beans to inoculation of intact hypocotyls with conidia

The reaction of each of the the cultivars to the β and γ races of *C. lindemuthianum* were as expected; TC plants were susceptible to β and resistant to γ, whereas PM was resistant to β and susceptible to the γ race of *C. lindemuthianum*. The development of interaction in resistant and susceptible plants proceeded as described in Chapters 1 and 2, respectively. Lesions, which developed in compatible combinations of host and pathogen, ranged from 8 to 11 mm in length; however, if glyphosate was applied to plants such lesions continued to enlarge. Glyphosate had no effect on symptom development in resistant host-pathogen combinations.

Response of beans to inoculation of wounds with conidia

Regardless of the host-parasite combination, infection did not occur when spores were introduced into wounds (Table 14). Microscopic observations indicated that the germination of spores was drastically affected in wounds. On intact surfaces of bean hypocotyls, 80 to 85% of spores germinated, most of which produced appressoria directly. In contrast, only 40 to 50% of spores germinated in wounds and, of these, only 5 to 10% produced normal appressoria, none of which developed any further. The majority (60-70%) of spores which germinated in wounds produced either swollen or long germ tubes (12 to 18 μm) without any appressoria at their ends, and their further growth was inhibited as well. Another 20-30% of germinated spores produced abortive appressoria which were either unpigmented or very small and deformed. The nature of germination on wound surfaces of plants was not influenced by glyphosate.

Response of beans to inoculation by mycelial plugs from BJA

In contrast to the results of spore inoculation of wound surfaces, all plants in both compatible and incompatible cultivar-race combinations developed susceptible lesions when wounds were inoculated with mycelial plugs (Table 14). The lesions were comparable in size and morphology, in both susceptible and resistant cultivar-race combinations and were typical of anthracnose lesions produced on bean cultivars after drop inoculation of intact hypocotyls with spores. Glyphosate suppressed delimitation and allowed continued expansion of the lesions. The pathogen failed to penetrate and produce any lesions when intact hypocotyl surfaces were inoculated with mycelial plugs.

Response of beans to inoculation by conidia+BJA

The symptoms which developed following inoculation of wounds with conida+BJA were mostly intermediate in reaction type (Table 14) and they occurred in similar frequency (66-75%) on each of the four cultivar-race combinations studied. The remaining sites (25-34%) were of the resistant phenotype. Only the intermediate lesion types spread in glyphosate treated plants. The pathogen followed the necrotrophic mode of growth on the intact surface of hypocotyls and failed to penetrate.

Response of beans to inoculation by hyphae in infected tissue

Plants, whether resistant or susceptible, developed small to normal size lesions after inoculation of wound sites with epidermal strips from infected susceptible plants (Table 15). Strips containing either biotrophic or necrotrophic mycelium were effective inocula, although larger lesions

TABLE 14

Response of bean hypocotyls (cvs. Topcrop (TC) and Perry Marrow (PM)) to wound-inoculation with different types of inoculum of β and γ races of Colletotrichum lindemuthianum.

Host-pathogen combination	Type of inoculum	% lesion type on plants		
		Susceptible	Intermediate	Resistant
PM-β	conidia	0	0	100
	mycelial plugs	96	4	0
	conidia+BJA[a]	0	75	25
PM-γ	conidia	0	0	100
	mycelial plugs	100	0	0
	conidia+BJA[a]	0	66	34
TC-β	conidia	0	0	100
	mycelial plugs	93	7	0
	conidia+BJA[a]	0	70	30
TC-γ	conidia	0	0	100
	mycelial plugs	90	10	0
	conidia+BJA[a]	0	72	28

[a] *C. lindemuthianum* conidia were placed on bean juice agar (BJA) for 2 h before agar discs were cut for inoculation.

TABLE 15

Response of bean hypocotyls (cvs. Topcrop (TC) and Perry Marrow (PM)) to wound-inoculation by epidermal strips excised from Colletotrichum lindemuthianum-*infected susceptible bean plants.*

Source of epidermal strips	Host plant cultivar	% lesion type[a] on plants inoculated with epidermal strips made at[b] 72 h S	I	R	108 h S	I	R
TC-β	TC	16	60	24	44	49	7
TC-β	PM	18	70	12	38	52	10
PM-γ	TC	10	68	22	63	37	0
PM-γ	PM	14	71	15	54	43	3

[a] Lesions were classified as:
S = Susceptible
I = Intermediate
R = Resistant

[b] Epidermal strips were excised from infected susceptible plants either at 72 h or 108 h post-inoculation, and contained biotrophic and necrotrophic phases of fungal growth, respectively.

developed on plants inoculated with necrotrophic mycelium. Epidermal strips excised at 108 h post-inoculation contained extensive necrotrophic growth of the pathogen, particularly in the innermost layers of the cortex. In 72 h inoculation strips, the pathogen development was biotrophic and hyphae were still largely confined in epidermal cells. However, by the time the pathogen came into contact with cortical cells of the inoculated plant, it had already shifted to a necrotrophic mode of parasitism. The nectrotrophic hyphae then rapidly invaded cortical cells at the wound site and frequently colonized hypocotyl tissue (including epidermal cells) beyond the wound margins. Browning at inoculation sites was initially limited to wounded and colonized tissues but later extended to the surrounding uninfected cells as well. Both the intermediate and susceptible lesions spread on glyphosate treated plants. Similar to inoculation of intact hypocotyls by mycelial plugs or conidia+BJA, the fungus from epidermal strips failed to penetrate the intact surface of bean hypocotyls.

Response of non-host plants to infection by C. lindemuthianum

Following inoculation of intact plant tissues, 70, 55 and 60% of conidia produced normal appressoria on soybean, broad bean and pea plants, respectively. Successful penetration (unambiguous presence of infection hyphae in penetrated cells) was not observed on any of the non-host plants, although 20-35% of appressoria-associated epidermal cells in soybean and peas, respectively, developed browning of cell walls near the appressorium. Epidermal cells in broad bean did not show any visible response to conidia or appressoria.

The fungus, applied as mycelial plugs to wounded tissues, also failed to grow in these non-host plants. Inoculated wound sites turned slightly discolored to necrotic in soybean and peas, but in broad bean plants inoculation sites became almost black. There was not any increase in fungal growth on these plants associated with glyphosate treatment.

DISCUSSION

The results indicate that the specificity of host-parasite relationship can be apparently changed by altering the nature of inoculum and the mode of inoculation. The plants which are normally resistant (incompatible) become susceptible when mycelia instead of spores are used as inoculum on freshly exposed subepidermal tissue. Mycelia appear unable to penetrate the intact epidermis, however, even when supplied with a nutrient source.

If it is assumed that wounded subepidermal tissue is capable of resistant response, it may be concluded that the specific interaction in this host-parasite system is for resistance, and was escaped in these experiments by avoiding recognition of the parasite by the host. Available evidence indicates that penetration of the host by the pathogen is the critical time for deciding compatibility or incompatibility of the interaction (12, 83, 89, 97). If this crucial event is bypassed as in the present study, host defense is not provoked and the interaction becomes susceptible. On the contrary, if the specific suppression of host defense mechanisms by the parasite determined specificity, then avirulent races would not be expected to colonize resistant cultivars and lesions of similar size should not develop on both resistant and susceptible cultivars. In fact, evidence for the

involvement of specific elicitors in bean anthracnose has been reported (4, 105).

Additionally, these results suggest that specificity factors of the pathogen are developmental-stage specific and probably expressed only in the infection court. The nature of these recognition factors and their role in pathogenesis, if any, is unclear. Specific structures such as appressoria and hyphal infection pegs are differentiated by the pathogen to pierce the cuticle and outer wall of epidermal cells under natural conditions (113). These structures, however, are apparently not needed for invasion of wounded tissue. Therefore, it is likely that the determinants of race-cultivar specificity may also have some role to play during penetration or early establishment of the pathogen in susceptible cultivars.

It has been proposed that to be involved in recognition, specificity factors of interacting organisms must be surface bound and constitutive (1, 17, 67). My results indicate that the specificity factors may not be constitutive, rather they seem to be expressed specifically and exclusively during appressorial phase (penetration) and/or at the level of host epidermis. This may also be evident from studies (48) which have shown that genetically defined incompatibility can occur at any stage in the ontogeny of host-parasite interaction.

There are at least two major objections to the conclusions reached above. First, since plants had to be wounded for mycelial infection, it may mean that determinants of host reaction are localized only in the epidermal cells. Accordingly, the specificity factors in the parasite may or may not be localized or tissue specific.

The second objection concerns the relevance of results obtained from these experiments, in which necrotrophic hyphae were used for inoculation, to the natural situation where successful establishment of the pathogen depends upon its ability to grow as a biotroph, although it behaves as a necrotroph subsequently. Obviously, the mechanisms involved during biotrophic establishment will be quite different form those which enable the pathogen to colonize necrotrophically. Indeed, race specificity in intact plants is mediated by the ability or inability of the pathogen to maintain cell viability and this is in direct contradiction with the necrotrophic mode of parasitism. Hence, even if the specificity factors are still expressed during the necrotrophic phase, their action will be masked by the nonspecific killing of plant cells by necrotrophic hyphae. Attempts to inoculate plants with mycelia, representing the biotrophic phase, were unsuccessful in this study since the pathogen switched to necrotrophic behavior by the time it outgrew the inoculation strip. Therefore, these results may not be meaningfully equated with infections of intact plants with spores.

The success of the pathogen in producing lesions on the susceptible or resistant plants largely depended on the extent of mycelial growth in the agar plugs or the epidermal strips at the time of inoculation. This has also been demonstrated during natural infection of the host by compatible races of the pathogen (88, 94). It was shown that an extensive primary mycelium is necessary to support the subsequent necrotrophic growth, which together with the plant's ability to produce phytoalexins, determines the severity of symptoms (94). That phytoalexins are involved during symptom expression is also evident from these results because glyphosate completely eliminated the bean plant's ability to restrict the pathogen. The reason that spores were

unable to grow in wounds may also be attributed to phytoalexins. Germinating spores non-specifically elicited phytoalexins in bean cotyledon bioassays (2, 5). In addition, germination inhibitors produced in response to wound inoculation by spores may also be responsible for the failure of spores to colonize bean hypocotyls (98).

In view of these findings, the bean anthracnose pathosystem appears to contrast with the soybean-*Phytophthora megasperma* f. sp. *glycinea* host-parasite interaction. In the latter system, irrespective of the nature of inoculum and the method of inoculation, different cultivars of soybean generally exhibit the same differential interaction to different races of the pathogen (111). Clearly there are differences in mechanisms that dictate specificity in these two host-parasite interactions. Therefore, care must be taken not to extrapolate too freely from one system to another since each system likely has unique features.

The gene-for-gene relationship, which exists in many host-parasite systems (25, 39, 108), implies that specific recognition is associated with the incompatible reaction and failure of recognition between corresponding gene products leads to a compatible interaction (36, 67). Some researchers have questioned the validity of this concept because it relies on the assumption that basic host-pathogen compatibility must exist before specificity at the race-cultivar level can develop (23). These results, however, support this assumption and provide experimental evidence for the presence of basic compatibility between *P. vulgaris* and *C. lindemuthianum*. Both virulent and avirulent races of the pathogen colonized bean cultivars equally well once the race specific barriers were bypassed or breached. Such basic compatibility was found to be lacking between *C. lindemuthianum* and the

non-host plants tested. The fungus was unable to grow on these latter species, perhaps because these plants either lack something which *C. lindemuthianum* requires or *C. lindemuthianum* lacks some character essential for its continued development in these non-host plants.

Although far from conclusive, present results provide some evidence for the specificity of the resistant reaction and suggest that expression of specificity factors in the pathogen may be developmentally regulated. This is very significant because lately there has been much emphasis on using tissue cultures of plants and elicitor molecules from sources such as mycelial and cell wall preparations and filtrates from axenic fungal cultures etc. for studying recognition and specificity phenomena (50, 58). According to these results, such molecules may play no role in initial recognition and attention should be focused on the nature of pathogen molecules present in the infection court. Further work on the system clearly is needed.

CHAPTER 5

A MODEL SYSTEM TO STUDY SPECIFICITY AND RESISTANCE/SUSCEPTIBILITY MECHANISMS IN HOST-PATHOGEN INTERACTIONS

INTRODUCTION

In the previous chapter an attempt was made to distinguish between two alternative views on the basis of specificity between the host and parasite in bean anthracnose disease by using the pathogen at different stages of development as inoculum. Although the results obtained were interpreted as supporting the hypothesis that specific recognition is for resistance, the system embodied some assumptions which made this interpretation non-definitive.

The alternative hypothesis, that susceptibility is an active process and therefore specifically determined, has also been tested experimentally (32, 41, 116). However, definitive demonstration of suppressor activity, necessary to support this hypothesis, presents formidable barriers. It should not be hard to isolate and purify a putative suppressor if a suitable assay system were available. The real problem is how to feed this suppressor to host cells in the infection court. Intact plants appear unsuitable because they do not take up these biomolecules efficiently (51) and wounding in the infection court creates artifacts which interfere directly with the test in question (50). The use of tissue or callus cultures for this purpose is inappropriate too, because race-specific resistance, characteristic of intact plants, is not expressed in these tissues (50, 58, 63) and they fail to respond hypersensitively to the pathogen (50).

The ideal assay would combine the ability of an intact plant to interact differentially with the pathogen and the flexibility of a tissue culture system where experimental conditions can be rigorously controlled, and test compounds can be added or removed in a non-destructive manner. The idea that excised epidermal strips from plant hypocotyls might provide such an experimental system came from the previous finding (Chapter 1) that the hypersensitive reaction, as indicated by cellular browning of infected cells, was suppressed in partially excised epidermal strips of bean hypocotyls, and the avirulent pathogen was able to colonize such strips. No attempt, however, was made to evaluate whether or not the infected cells which failed to turn brown died.

The present study investigates the nature of development of *C. lindemuthianum* and the response of infected cells in excised bean epidermal strips. The possible use of this simple host system to explore mechanisms of non-host resistance was also examined.

MATERIAL AND METHODS

Plant and Fungal Material

Etiolated seedlings of *P. vulgaris*, cvs. 'Topcrop' (TC) and 'Perry Marrow' (PM) were grown as described in previous chapters. Spore suspensions of β and γ races of *C. lindemuthianum* were prepared following procedures described in Chapter 1 and adjusted to $1X10^6$ spores ml^{-1} with distilled water.

Specimen preparation and inoculations

Several different experiments were conducted in this study, the majority of which employed epidermal strips of bean hypocotyl approximately 2.0 cm long but differing in thickness. In some experiments hypocotyl segments, intact (IS) or dissected into halves (HS) of similar length, were also used. These strips or segments were excised from 10-11-day old plant hypocotyls cut 2-3 cm below the cotyledonary node and 5-7 cm above the roots. Hypocotyls were surface sterilized by wiping them gently with a cotton swab soaked in 70% ethanol and then dipping in 5% NaOCl solution for 5 min. Following several washings with sterile distilled water, hypocotyls were blotted dry on clean paper towels prior to tissue preparation.

Epidermal strips of 3 different thicknesses were made. These included thin (TN) strips 4-8 cell layers thick, medium (MD) strips 15-20 cell layers thick, and thick (TK) strips 40-60 cell layers thick. Thickness of the strips was determined by microscopic examination of cross sections from 5 different epidermal strips selected from each size-class. These 5 strips represented (subjectively) the entire range of thicknesses, including extremes, in that size-class. With the exception of TN strips, which were peeled freehand from hypocotyls with the help of fine foreceps, all strips or segments were excised with a razor blade. No special technique was designed to cut strips of a particular size; consistency in excising strips of a given class was achieved empirically by rigorous practising and careful selection. All preparations were aseptic.

In the first set of experiments, only TN strips were used. Live strips were either floated on sterile distilled water (epidermis upward) containing

100 μg ml^{-1} each of penicillin G and streptomyocin sulphate in 11 cm plastic Petri plates or suspended in humid air on thin glass filaments in plastic boxes. Strips killed by floating on hot water (60°C) for 5 min or by fixing in 3% glutaraldehyde solution for 20 min at room temperature were also included in these experiments. Glutaraldehyde-fixed strips were thoroughly washed with sterile distilled water by vacuum infiltration before inoculation. Dead strips were only floated on water and not suspended in air. Strips were inoculated with *C. lindemuthianum* by placing two 1 μl drops (approx. 1 cm apart) of spore suspension centrally on their cuticular (intact) side.

In the second series of experiments, epidermal strips of all sizes, as well as the intact (IS) and dissected (HS) hypocotyl segments, were used. These tissues were floated on water, or placed on glass filaments to prevent diffusion of metabolites from strips or segments into water. Another attempt to eliminate leakage was made by sealing the cut sides and ends of all strips (except TN) and segments with wax. Paraffin wax was melted by heating to 60°C and then applied to strips or segments with a camel hair brush. To provide a control for the wax treatment, some strips and segments were first waxed and then dewaxed before inoculation. Inoculation was done as in experiment 1.

In experiments designed to test the importance of phytoalexins in restricting pathogen growth and development in excised bean tissues, MD strips from TC plants were floated on antibiotic supplemented sterile distilled water and inoculated with the β and γ races of *C. lindemuthianum*, by placing six 1 μl drops of inoculum on the upper intact sides of the strips. At 40 h post-inoculation, water was removed from Petri plates and

replaced either with glyphosate (25 μg ml^{-1}) or aminooxy phenylpropionic acid (AOPP) (100 μg ml^{-1}), a specific inhibitor of PALase (81). Phytoalexins in both inoculated and uninoculated (control) strips were assayed at 72, 84 and 96 h post-inoculation. Extraction and quantification of phytoalexins by HPLC were done according to methods described in Chapter 3.

All experiments in this study, including the non-host experiments described below, were repeated at least twice. In most experiments 4 replicate plates were used per treatment, each containing 25 epidermal strips or segments. In experiment 1, where TN strips were suspended in air, a treatment was comprised of a box divided into 3 compartments, each chamber containing 30 strips. The duration of each experiment was 6 days.

Non-host experiments

Two different approaches were used to study aspects of non-host resistance in excised plant tissues. The first approach involved the inoculation of live epidermal strips (TN) from bean (TC) hypocotyls with fungi which are not pathogens of beans. These non-pathogens were *Helminthosporium carbonum* Ullstrup, *Cladosporium fulvum* (Cooke) Ciferri, *Colletotrichum coccodes* (Wallr.) Hughes and *Venturia inaequalis* (Cooke) Winter. *Fusarium solani* (Mart.) Sacc. f. sp. *phaseoli*, (Burk.) Snyd. and Hans. which generally does not directly infect intact bean hypocotyls, was also included in this study. *H. carbonum* and *C. fulvum* were obtained from Dr. Michele Heath (University of Toronto, Canada), *F. solani* f. sp. *phaseoli* from Dr. Robert Hall (University of Guelph, Canada), and *C. coccodes* and *V. inaequalis* were isolated locally from diseased tomato and apple plants, respectively. These fungi were grown under lab conditions in the dark, *V.*

inaequalis on malt agar and others on potato dextrose agar. Spore suspensions were made according to procedures described for *C. lindemuthianum* (Chapter 1) and adjusted to $1X10^6$ spores ml^{-1} for *C. coccodes* and *C. fulvum,* and $1X10^5$ spores ml^{-1} for *H. carbonum, V. inaequalis* and *F. solani* f. sp. *phaseoli* (macroconidia). Inoculations were done as described for *C. lindemuthianum* on strips. Ten-day old etiolated TC plants were also inoculated with 1 μl drops of inoculum and then kept in the dark at 23°C under conditions of high humidity.

The second approach involved the use of etiolated seedlings and epidermal strips from etiolated seedlings of plants which are non-hosts to *C. lindemuthianum.* TN epidermal strips were excised from etiolated stems of 10-day old soybean (*Glycine max*), 12-day-old cucumber (*Cucumis sativus*), and 15-day old mung bean (*Phaseolus aureus*) and adjuki bean (*Phaseolus angularis*) plants and inoculated with the β race of *C. lindemuthianum* as described previously. To differentiate between the active and passive nature of resistance mechanisms in non-host tissues, some strips from all plant species were heat killed before inoculation. Etiolated seedlings of these non-hosts were placed in dark humid chambers held at 22°C after drop (1 μl) inoculation on stems with the β race of *C. lindemuthianum.*

Microscopic observations

At different intervals after inoculation, two strips or segments were randomly picked from each of the replicate plates or compartments for microscopic observations. TN strips were observed directly but from other tissues thin strips of epidermis were peeled and examined under the microscope. Strips were observed before or after fixing and staining with

FAA containing 0.01% trypan blue (Chapter 1). For every treatment, a hundred or more penetrated cells were evaluated. Viability of cells in strips was evaluated by their ability to plasmolyse in a hypertonic salt solution (83) and to accumulate the vital dye neutral red in vacuoles (102). Epidermal strips were immersed in a 0.005M phosphate buffer solution containing 0.85M KNO_3, 0.01% (w/v) neutral red for 30 min, before examining microscopically.

RESULTS

Interaction of TN strips of beans with C. lindemuthianum

Live strips. Development of *C. lindemuthianum* spores on TN strips was similar to that occurring on intact hypocotyls (Chapter 1) and was identical in compatible and incompatible races. Spores started to germinate within 10 h (timing of all events will be expressed in hours after inoculation) and produced dark appressoria, mostly directly without differentiating any germ tubes, within 24 h. Penetration from these appressoria did not occur until 40-48 h.

The development of the compatible interaction proceeded essentially as in intact bean plants (Chapter 2). Immediately after penetration, broad (7-9 μm diameter), spherical or irregular, infection vesicles were formed in epidermal cells from which one or more 'primary hyphae' (4-5 μm) extended (Fig. 15) and grew slowly into adjacent epidermal and cortical cells. During this biotrophic phase, cytoplasm of the infected cells looked and plasmolysed similar to the cytoplasm of adjacent uninfected cells. The fungus started producing 'secondary hyphae' (2-3 μm) and assumed necrotrophic behaviour at about 96-104 h and its rate of growth increased tremendously. Sporulation

was frequently observed after 5 days.

Development of incompatible host-pathogen combination in strips was quite different from that which occurred in intact hypocotyls, and can be divided into 3 phases depending on the rate of fungal growth.

(i) First phase: Following penetration for up to 20-24 h, the growth rate of infection hyphae was comparable to that of the corresponding time period in compatible interaction (Fig. 16). Penetration was successful and an infection vesicle was observed in almost all the penetrated cells. Primary hyphae developed and occasionally extended into 1 or 2 adjacent epidermal or cortical cells. However, unlike compatible combinations, cytoplasm of the infected cells appeared very different from the adjacent unifected cells and failed to plasmolyse and accumulate neutral red. Although browning typical of the cells reacting hypersensitively to incompatible infection in intact hypocotyls was absent in strip cells, the cytoplasm in such cells had pulled away from the cell wall and its contents were granulated (Fig. 15). Without doubt there was cell injury or death.

(ii)Second phase: After the initial development of the pathogen, its growth appeared generally inhibited from about 72 h to 96-108 h.

(iii)Third phase: The inhibition of growth in TN strips was transitory and the pathogen started growing again. The new growth was usually necrotrophic and the fungus colonized the entire strip tissue within 48 hours of regrowth.

Some exceptions to the above mentioned pattern of fungal development in both compatible and incompatible interactions in strips were observed. Sometimes (2-4%) these strips, like the heat killed strips (see below), supported only necrotrophic growth (judged from the morphology and behaviour of infection hyphae originating from appressoria) of the pathogen. In such

Figure 15. Fungal development and the response of infected cells in bean epidermal strips inoculated with virulent (a) and avirulent (b, c, & d) races of *Colletotrichum lindemuthianum.*

Cells in a, b, and c were stained overnight with Trypan blue (0.01% in 50% FAA) prior to photographing.

Approximate magnification:
(a) 380 (b) 380 (c) 380 (d) 625.

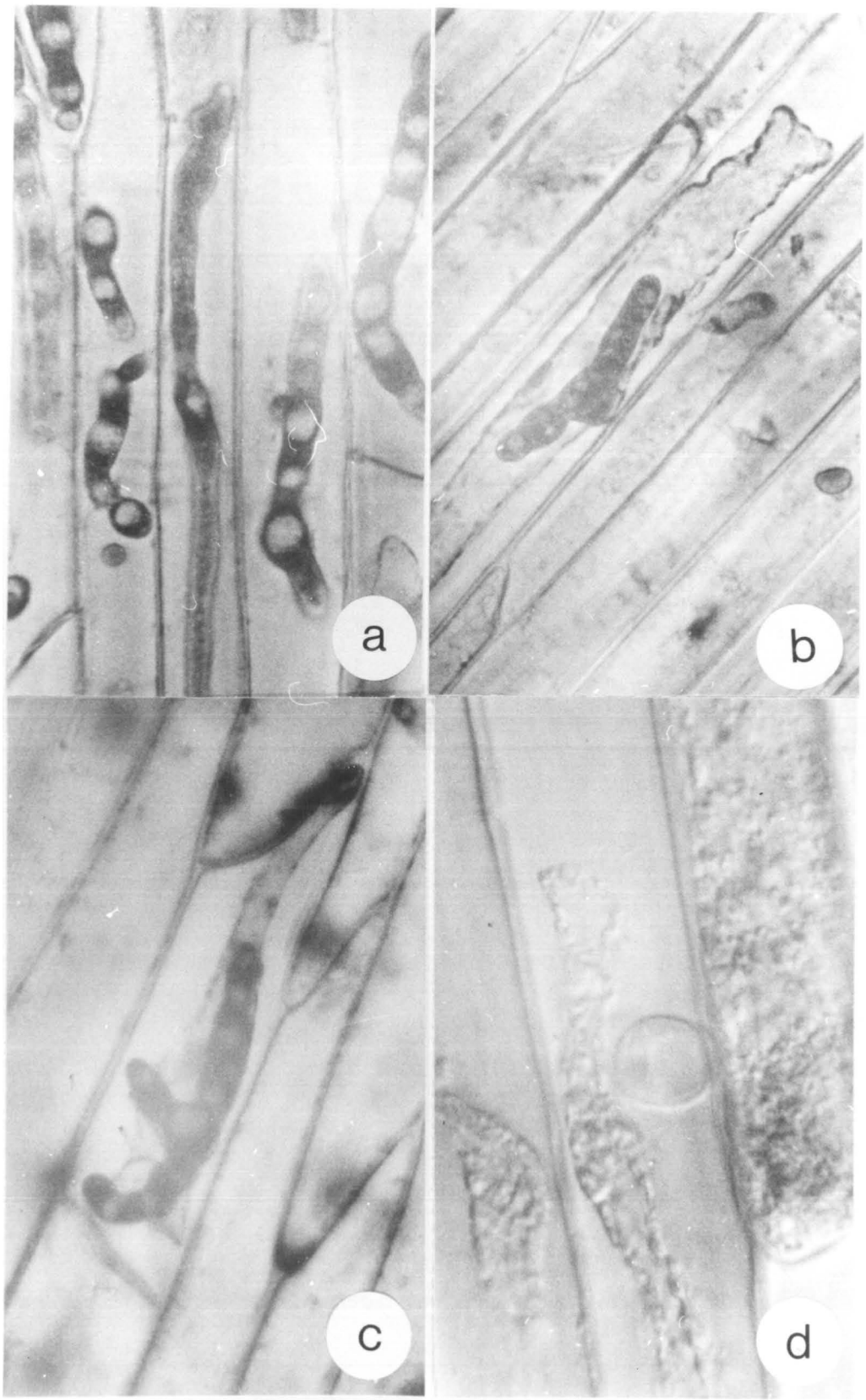

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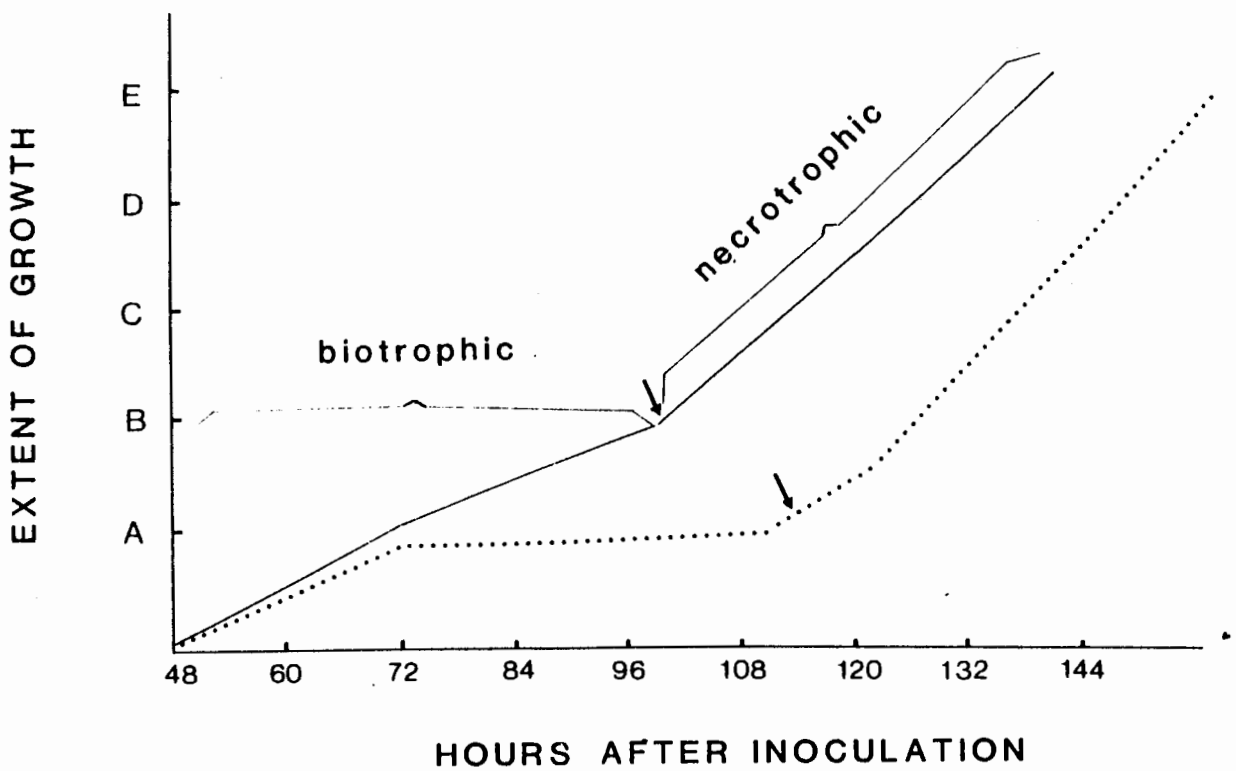
Figure 16. Graphical representation of the nature and extent of growth of *Colletotrichum lindemuthianum* in thin (TN) epidermal strips of compatible and incompatible bean plants.

A = 1-2 cells colonized
B = 10-20 cells colonized
C = ≥ 50 cells colonized
D = extensive secondary mycelial growth
E = sporulation

Arrow indicates the onset of necrotrophic phase of fungal growth



compatible ——————————

incompatible .....

cases, the nature and extent of fungal development was identical in resistant and susceptible strips. The pathogen occasionally (≤ 1%) developed in live TN strips the way it usually did in glutaraldehyde killed strips (see below). Additionally, in 0.5 to 1.0% of penetrated epidermal cells, infection hyphae were completely encased by reaction material (RM) deposits and further growth did not occur. This occurred with similar frequency in both compatible and incompatible host-pathogen combinations. In comparison with dark brown nature of RM deposits in intact plants (Chapters 1 and 2), RM deposits in strips ranged from creamy white to pale brown.

Dead strips. Spore germination, appressorial formation and penetration of host cells proceeded as in live strips. In heat killed strips, both races grew equally well and very extensively. After penetration from appressoria, the fungus did not develop any infection vesicle and primary hyphae; instead narrow hyphae were produced which were similar in size and growth mode to secondary hyphae formed during the necrotrophic phase in live tissues of compatible bean plants. The fungus sporulated within 4-5 days on these strips.

In contrast, both races of *C. lindemuthianum* failed to grow in glutaraldehyde fixed strips. After successful germination (approx. 80% of spores produced appressoria) and penetration (approx. 62%), the fungus produced very thin (about 1 μm) hyphae inside epidermal cells. The growth rate of these hyphae was extremely low and they barely grew from one cell to another during the experiment. Epidermal cells in heat treated or glutaraldehyde fixed strips did not respond hypersensitively or by producing reaction material deposits to penetration by either race of the pathogen.

Interaction of bean epidermal strips and segments of differing thickness with C. lindemuthianum

Fungal development. Normal development of the compatible interaction was observed in all strips and segments and this was not altered by the waxing/dewaxing treatment.

The development of the fungus in incompatible interaction was progressively retarded as the thickness of the strips was increased. As in TN strips, the growth rate of the avirulent race immediately after infection in MD and TK strips was comparable to that of the virulent race. Infection vesicles and primary hyphae were formed; occasionally these hyphae extended as far as the cell adjacent to the penetrated cell. Normally the hyphae were broad (5-7 μm) but narrow hyphae (3-4 μm) were not uncommon, especially in TK strips. Further growth of the pathogen, however, was inhibited in MD and TK strips and it remained inhibited throughout the experiment. Development of the avirulent race in HS segments was generally restricted to a small infection peg and rarely an infection vesicle was observed. In IS segments, as in intact seedlings (Chapter 1), the avirulent pathogen was inhibited immediately after penetration and the infection hyphae were generally not visible in infected cells.

Both the waxing and dewaxing treatments were effective in enhancing the development of the pathogen in incompatible strips of sizes up to half segments (Table 16). The rate of pathogen growth in such MD and TK strips was only slighly less than the growth observed in compatible strips.

TABLE 16

The extent of browning of infected cells during hypersensitive response and growth of Colletotrichum lindemuthianum *(incompatible host-pathogen combination) in bean epidermal strips of varying thickness subjected to waxing/dewaxing treatments.*

Epidermal strips[a]	Treatment[a]	% brown HR cells[b] TC-γ	PM-β	Fungal growth
TN	control	0	0	extensive[c]
MD	control	2±1.6	3±1.2	inhibited
	waxed	0	0	extensive
	dewaxed	0	0	"
TK	control	8±2.9	11±2.9	inhibited
	waxed	0	0	extensive
	dewaxed	0	0	"
HS	control	23±6.5	28±5.9	inhibited
	waxed	1±1.1	2±1.8	intermediate[d]
	dewaxed	3±2.6	1±1.9	"
IS	control	99±1.4	100	inhibited
	waxed	100	100	"
	dewaxed	97±2.2	100	"

The data were recorded at day 6 after inoculation.

[a] See text for details.

[b] At least 100 infected cells were evaluated from four strips or segments picked from each of four replicate plates. Data represent the mean (± S.D.) of 4 replications.

[c] Extensive colonisation of the strip tissue and fungal sporulation.

[d] Varying extents of both primary and secondary mycelial growth but no sporulation.

Response of infected cells

In compatible interactions, as mentioned earlier, the initially penetrated cell and other cells invaded by primary hyphae gave no indication that they were adversely affected by the pathogen. In contrast, the contents of infected cells in incompatible strips and segments became granulated and/or broken and the cells appeared dead, although they did not always turn brown. The proportion of infected cells (HR) that developed brown pigmentation was related to the thickness of the strip tissue (Table 16). However, the intensity of browning of HR cells in excised tissue was relatively less than in intact segments and seedlings. Waxing and dewaxing treatments had similar effects; they totally eliminated browning of HR cells in excised tissues as thick as half segments. This may be due to the heat shock associated with the waxing treatment. Browning of HR cells in intact segments was not affected by waxing/dewaxing, however.

Whereas the RM deposits were found in about 1% of penetrated cells in TN strips, their frequency increased to 2-3% in MD and TK strips and approached 5-6% in HS and IS segments. Intensity of browning of these deposits also showed a similar pattern of increase with increasing thickness of the hypocotyl tissue. Waxing/dewaxing reduced the RM response of strip tissue, and particularly the intensity of browning of these deposits, relative to these responses in unwaxed MD and TK strips.

*Effect of glyphosate and AOPP on bean-*C. lindemuthianum *interaction in epidermal strips*

Fungal growth and HR response. There was no effect of glyphosate or AOPP on

the development of the pathogen in compatible interactions in MD strips. Resistance of MD strips to the pathogen in incompatible combinations was markedly suppressed by both glyphosate and AOPP. The growth of the fungus was greatly enhanced, approaching the rate with which it grew in compatible combinations. 'Non-brown' HR of these strips to the incompatible race of *C. lindemuthianum*, however, was not blocked by either of these inhibitors.

Phytoalexin response. Phaseollin was the only phytoalexin produced by MD epidermal strips. It accumulated in both inoculated and control strips, but to higher levels in strips undergoing incompatible interaction with *C. lindemuthianum* (Table 17). The levels of phaseollin in strips with compatible infection were the same as in control strips, and were unaffected by glyphosate or AOPP. AOPP, however, reduced the accumulation of phaseollin in strips inoculated by γ race of *C. lindemuthianum* (incompatible), to levels only slighly higher than those in control or β race-inoculated (compatible) strips. Glyphosate also reduced the accumulation of phaseollin in the incompatible interaction, but to a lesser extent than AOPP.

Non-host resistance

Response of bean epidermal strips to non-pathogens. With the exception of *V. inaequalis* and *C. fulvum*, all of the other bean non-pathogens colonized and macerated bean epidermal strips. *H. carbonum* and *C. coccodes* produced appressoria for penetration, but in the case of *F. solani* f. sp. *phaseoli*, penetration was accomplished from loosely defined mats differentiated by germinating conidia. In the case of the *V. inaequalis*-bean epidermal strip interaction, host cellular browning developed which was generally localized within or on the epidermal cell walls at sites where appressoria or spores

TABLE 17

Effect of glyphosate and AOPP on the accumulation of phytoalexins and pathogen development in bean epidermal strips (cv. Topcrop) during infection by Colletotrichum lindemuthianum.

Inoculation and treatment of epidermal strips	Level of phaseollin[a] $\mu g\ g^{-1}$ strips[b] at 72 h[c]	84 h[c]	96 h[c]	Fungal growth in strips
Control + water	34.5±2.2	28.7±6.1	30.2±5.1	
Compatible[d] + water	29.4±4.7	31.5±6.8	35.5±6.8	extensive
" + AOPP	33.8±4.3	26.5±6.2	29.4±3.3	"
" + glyphosate	31.5±3.6	32.5±7.0	35.8±4.7	"
Incompatible[e] + water	79.2±5.5	104.9±11.4	135.3±2.9	inhibited
" + AOPP	37.5±2.8	39.1±4.2	42.4±8.0	extensive
" + glyphosate	41.2±4.9	52.6±6.4	54.5±3.5	"

[a] Values are the mean (± S.D.) of four replications.
[b] Fresh wt. of epidermal strips.
[c] Time after inoculation.
[d] Topcrop epidermal strips infected with β race of *C. lindemuthianum*.
[e] Topcrop epidermal strips infected with γ race of *C. lindemuthianum*.

were located. Further development of the fungus was never seen. Although *C. fulvum* succeeded in penetrating (via appressoria), its development was restricted to a small infection peg even in the absence of any host cellular response. Occasionally, browning of the cell wall at the site of penetration was observed.

On the intact etiolated bean seedlings, none of these fungi was seen to develop inside the non-host tissue. Germination and/or appressorial formation was observed among all of the bean non-pathogens studied but the plant generally responded by browning of the cell wall at the sites of attempted penetration. HR was never observed in bean plants inoculated with *V. inaequalis*, but in plants inoculated with *H. carbonum, C. coccodes, C. fulvum, and F. solani* f. sp. *phaseoli* the frequency of this response was 11, 3, 1.5 and 1% of spore deposition sites, respectively.

Interaction of non-host epidermal strips with C. lindemuthianum

Depending upon the extent of growth of *C. lindemuthianum* in both the live and dead (heat-killed) strips, non-hosts can be divided into two groups. In the first category are plants from the genus *Phaseolus*. Epidermal strips of these non-host plants gave responses which were similar to responses observed in incompatible bean epidermal strips. Successful development of *C. lindemuthianum* was seen in the majority of cells infected. In live strips, both infection vesicles and primary hyphae were formed. Secondary hypae branched off from primary hyphae during later stages of interaction and the strips were extensively colonized within 6 days. Sporulation was also observed frequently. In *P. aureus* and *P. angularis* 20 and 12% of infected cells, respectively, reacted by producing RM deposits. As in *P. vulgaris*,

dead epidermal strips from these non-hosts supported only necrotrophic growth of *C. lindemuthianum.*

In intact tissues of these non-host plants, however, the fungus never developed beyond a small infection peg stage. Infected cells responded hypersensitively and turned dark brown. Reddish-brown RM deposits were also frequent (15-20% of infection sites) in both the *P. aureus* and *P. angularis* plants.

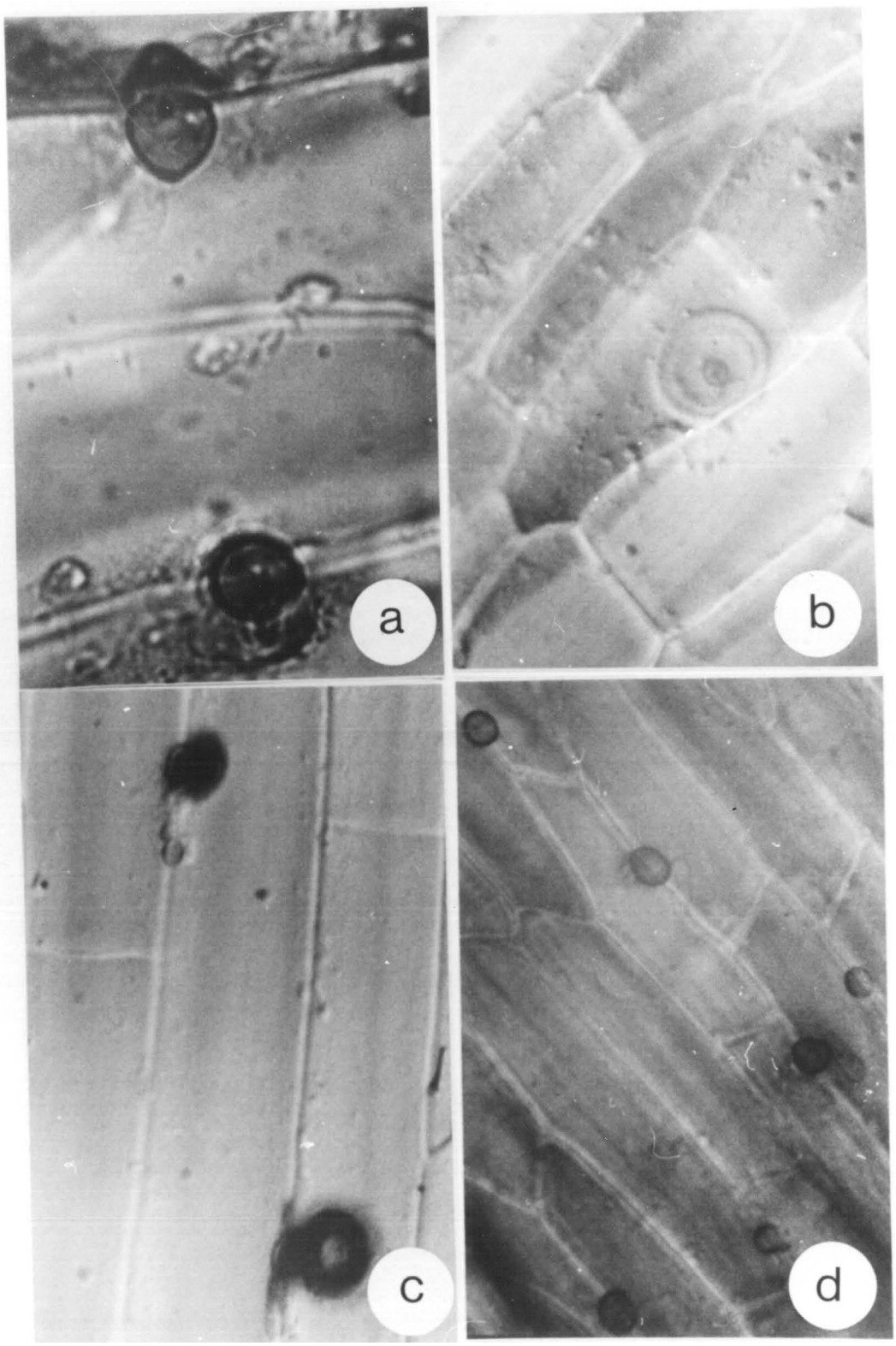
Soybean and cucumber plants fall in the second category. *C. lindemuthianum* germinated and produced appressoria normally on both live and dead epidermal strips from these non-host plants, but cytological evidence of penetration was observed only on dead strips. Live strips responded to attempted penetration by developing localized cell wall browning in soybeans and by cell wall browning and a 'halo' response in cucumbers (Fig. 17). In dead strips, the fungus produced very thin hyphae inside the penetrated cells; their nature and rate of growth was reminiscent of the development of *C. lindemuthianum* in glutaraldehyde killed bean epidermal strips. Intact seedlings of these non-host plants interacted with *C. lindemuthianum* in a manner very similar to that of live epidermal strips.

DISCUSSION

It is apparent that the excised hypocotyl tissue in beans retains the capacity of differential interaction to infection by *C. lindemuthianum* and may, therefore, prove well suited for experimental manipulation to study specificity phenomena. This is both interesting and important: so far all attempts to devise a simple host system have failed because race specific

Figure 17. Response of cells of epidermal strips excised from non-host plants to inoculation with *Colletotrichum lindemuthianum.*

Approximate magnification:
(a) 1560 (b) 625 (c) 625 (d) 380.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resistance was not expressed in those systems (50, 58, 63). My findings, however, contrast those of Skipp and Deverall (97), who found that not only browning but also events which preceded cell death were affected by excision. The reason for this contradiction is not clear but may be related to different handling of the experimental material.

While an incompatible response of host tissue to *C. lindemuthianum* was retained, the ability of epidermal strips to resist colonization by the pathogen was affected and depended on the mass or thickness of the strip tissue. In all excised tissues, the expression of resistance was delayed and initial development of the fungus was comparable to that occurring in compatible strips. Additionally, in TN strips, this resistance was only temporary or insufficient to keep the pathogen inhibited permanently. On the other hand, during the compatible interaction, the pathogen was virtually unaffected and proceeded as in intact seedlings.

The hypersensitive response in intact bean tissues is characterized by the rapid death and browning of infected cells during incompatible interactions. The browning of cells respondingly hypersensitively due to *C. lindemuthianum* infection is suppressed in excised strips. This suggests that a critical mass of host tissue is required for the expression of cellular browning. Phenolic compounds are thought to be the substrates for the browning reaction during HR (40, 106). However, HR browning was not suppressed by glyphosate (Chapter 1), an inhibitor of *de novo* phenol synthesis in plants (34). This may indicate that most of the phenolics which are oxidized or polymerized into brown pigments are present constitutively in host tissues (epidermal cells). The suppression of browning in epidermal strips may, therefore, be due to diffusion of phenols from epidermal cells,

either by direct leakage into the surrounding medium or by consumption during wound repair responses. This explanation is consistent with the work reported by Sakuma and Tomiyama (95) who showed that retardation of cellular browning in thin strips of resistant potato tissue infected with *Phytophthora infestans* was overcome by exogenously applied chlorogenic acid.

The data allows some conclusion to be drawn about the role of browning of HR cells in disease resistance. Most infection hyphae in intact beans are small and often appear dead within dark brown HR cells, suggesting that inhibition of fungal development occurs soon after penetration. However, in epidermal strips where browning of HR cells was suppressed, early pathogen development was apparently unhampered. Taken together this may suggest that events associated with the browning reaction are responsible for the initial inhibition of *C. lindemuthianum* in intact tissues of resistant plants.

Restriction of growth of *C. lindemuthianum* following penetration in incompatible epidermal strips was likely caused by phytoalexin response. This is evident from the pattern of fungal development and inhibition in relation to the time of accumulation of phaseollin. This was further supported by treatments with glyphosate and AOPP, a competitive inhibitor of PAL (81). Both treatments caused a reduction in phaseollin accumulation and an associated increase in fungal growth in strips.

Phaseollin was also produced, but at much lower levels, in uninoculated epidermal strips. Recent studies by Lawton and Lamb (76) have already demonstrated that accumulation of phytoalexins in excision-wounded hypocotyls of *P. vulgaris* is due to specific transcription of "plant defense genes". The pathogen however, developed normally in compatible epidermal strips, in

spite of the presence of levels of phaseollin which should cause inhibition of the fungus (99). This suggests the possible localization of phaseollin in cell layers immediately adjacent to the excision.

The elimination of cellular browning during HR and resistance of epidermal strips to *C. lindemuthianum* in waxing/dewaxing treatments could be due to heat shock associated with the waxing procedure. Heat shock also inhibits HR and resistance of incompatible barley coleoptiles to *Erysiphe graminis* (47). Several investigations indicate that elimination of resistance by heat shock results from interference with the phytoalexin response of the tissue (20, 61, 84). In strip tissue, heat shock may cause depletion of biochemical reserves by inducing the production of heat shock proteins (69), thereby leaving the tissue susceptible to attack by the fungus.

It appears that two factors largely determine whether non-host resistance mechanisms are passive or active. These are (i) the strategy used by the pathogen to cause disease (necrotoph/biotroph), and (ii) the phylogenetic relationship of the host with non-host plants. Active mechanisms, such as HR, RM and phytoalexin accumulation seem to be responsible for protection against necrotrophs. If the defensive activity of the non-host is disabled, the necrotroph may be able to invade and grow in the non-host. It appears that the defense capability of the host is impaired in excised strips.

Against biotrophs and hemibiotrophs, which develop a high degree of specificity in terms of host requirement, the mechanisms of non-host resistance may be active or passive. Their apparent nature in the systems

studied here seemed to be related to the systematic closeness of the non-host with the host. In closely related non-hosts, resistance appeared active, and was subject to elimination by experimental manipulation. In distantly related non-hosts, the mechanisms appeared to be passive, based on the apparent absence of cellular response at the histological level and the inability of the non-pathogen to grow in dead tissues of the non-host.

C. lindemuthianum is a hemibiotrophic parasite and grows intracellularly between the cell wall and plasma membrane without haustoria, and initially without causing apparent tissue damage. The fungus presumably relies on nutrients present already within the host tissue (apoplasm). It is thus possible that *C. lindemuthianum* has a specific nutritional requirement for parasitic development. Such a requirement would not be surprising since the constant association of parasite with the host could make redundant the genetic information for the synthesis of those nutients and growth factors which are readily available from the host (92). Therefore, unavailability of that essential nutrient could restrict colonization and be the reason for non-host resistance.

Fungal structures like infection vesicles and primary hyphae, which typify the biotrophic phase of disease development, are produced only in live, intact or excised tissues. It has been suggested that infection vesicles may be involved in maintaining the survival of penetrated epidermal cells (83). This, however, is not substantiated by my results, since infection vesicles were still formed in resistant epidermal strips where penetration is followed by rapid cytoplasmic incompatibility and apparent host cell death. The biochemical nature of factors that induce biotrophy during bean-*C. lindemuthianum* interaction is not known, but it appears that

cell viability at the time of penetration is vital for biotrophic differentiation of *C. lindemuthianum*.

In conclusion, epidermal strips from bean hypocotyls provide an attractive host system for the analysis of molecular mechanisms involved in resistance/susceptibility, in addition to being ideal for specificity studies. Furthermore, a number of other fundamental pathological problems can be addressed using this system. These include: (i) the basis and possible exploitation of non-host resitance in plant disease, (ii) the phenomenon of organ and tissue specificity in plant-parasite combinations, (iii) the assessment of *in vivo* toxicity of phytoalexins towards fungal pathogens, (iv) the molecular basis for developmental transition from the biotrophic to necrotrophic phase during bean anthracnose disease development and (v) the nutritional requirement for obligate biotrophs for growth in axenic culture etc.

CONCLUSIONS

The concept of active defense in plants being multicomponent in nature has been recognized in recent years (14, 21, 74, 96, 114). This thesis supports this concept by showing that several defense responses may occur simultaneously or consecutively. These responses include, in order of their temporal sequence, cell wall browning at the site of attempted penetration, RM deposits (which resistrict the pathogen from reaching the host cell protoplasm), hypersensitive cell death, browning of these dead cells and phytoalexin accumulation. Of these mechanisms, cell wall browning and RM response are non-specific and probably function against non-pathogens or weak propagules of pathogens. Hypersensitive cell death is a specific response of incompatible interactions. Cellular browning and phytoalexin accumulation probably are consequences of cell death and therefore also occur during symptom expression in susceptible plants.

The study also provides definitive clues as to the interrelationship and relative contribution of component mechanisms in restricting the pathogen in host tissues. In incompatible interactions, glyphosate apparently did not block or diminish the occurrence of HR but the associated accumulation of phytoalexins was suppressed. Glyphosate at its sites of application sometimes allowed the infection hyphae to escape HR cells. Once escaped, these infection hyphae encountered no further resistance in glyphosate treated plants and eventually killed them. Suppression of phytoalexins by glyphosate appears responsible for this. Various treatments, which can be interpreted to deplete phenylalanine pools, tended to increase the glyphosate-induced suppression of phytoalexins and the escape of infection

hyphae from HR cells. It can be concluded that glyphosate reduces the expression of resistance without reversing the incompatible nature of the interaction phenotype. This is very significant because it indicates that the events which determine the nature of the interaction between the host and parasite are functionally different from the events which impart resistance to the plant.

A recurrent theme in many investigations on resistance of plants to fungal parasites is the close association between hypersensitivity and resistance. It has been repeatedly argued whether hypersensitivity is the cause or the consequence of resistance (57, 71, 72, 110). My study resolves this issue in the bean anthracnose system. It is clear from the evidence that the HR is neither the cause nor the effect of fungal inhibition. In fact, by itself, the HR is inadequate to contain the pathogen. Perhaps, the HR and phytoalexin accumulation are two separate biochemical events and the HR is not a result of phytoalexin accumulation.

That phytoalexins are the cause of fungal growth inhibition in HR cells is further evident from the finding that infection hyphae in HR cells resumed growth, even without glyphosate treatment, when epidermal strips containing these cells were excised. Phytoalexin content of epidermal strips declined rapidly following excision. Although definitive evidence of the mechanism of reduction was not obtained, it appears that wounding causes phytoalexins to diffuse and delocalize from HR sites and thus release infection hyphae from inhibition. The phenomenon of loss of fungal containment on wounding is not limited to hemibiotrophs. At least some obligate biotrophs respond similarly when infected tissue from incompatible plants, including necrotic cells, is transferred to compatible tissue (19, 77). From a practical view point this

raises the possibility that an avirulent pathogen genotype inhibited in resistant plants might develop and reproduce in senescing or dead tissue, thus preserving the avirulent genotype in a resistant host population.

A major problem in interpreting the function of hypersensitivity in host-parasite interactions is understanding and defining cell death. The HR, rather than being taken to signify only the death of a cell(s), is more usually used to refer to cells which are both dead and darkly pigmented (necrotic). This tends to confuse the initial determining event, e.g., cell death *per se*, with a final symptom which may occur several hours or days later. The results obtained in this study show that cell death, *per se*, is different from necrosis, and that the two can be separated experimentally (Chapter 5). Browning of hypersensitively dead or dying cells seems to be the consequence of the loss of compartmentalization. The data (Chapter 5) suggest that events associated with the browning reaction contribute to the initial inhibition of pathogen development in HR cells before phytoalexins accumulate.

HR cell death appears to be an obligatory part of the incompatible bean anthracnose host-parasite interaction. If it is not involved in the inhibition of fungal growth, what might its role be? The immediate consequences of recognition in this system are maintained viability of infected cells or immediate cell death. Hypersensitivity would thus be recognized as the primary consequence of specific recognition, although a molecular mechanism to account for this is still lacking. As discussed previously, HR cell death results in cell necrosis which appears to have some function in resistance. HR cell death may also be associated with the elicitation of phytoalexins in adjoining healthy cells (12). Since

phytoalexins are diffusible, and since phytoalexin-degrading enzymes are present in living plant cells (15, 65, 104, 115), it is possible, as pointed out by Keen (65), that the dead cells function as reservoirs for phytoalexin accumulation and therefore indirectly aid in the restriction of parasite development.

In compatible host-parasite interactions, a probable cause and effect relationship between the suppression of phytoalexin accumulation by glyphosate and the loss of the host's ability to restrict the pathogen in delimited lesions was observed (Chapter 2). Glyphosate completely abolished the expression of resistance associated with delimitation of lesions if applied prior to lesion development at doses of 2.5 μg per plant or higher. Glyphosate did not reverse delimitation if treatment was given after phytoalexins had accumulated to concentrations which were presumably inhibitory to the fungus. The association between lesion delimitation and phytoalexin accumulation was further confirmed by the negation of the effect of glyphosate on both processes by phenylalanine.

Specificity (recognition) in this pathosystem appears to be for resistance and a basic compatibility apparently exists between the host and the parasite. This claim relies on the finding that regardless of the host genotype, *C. lindemuthianum* colonized and caused lesions when mycelium, instead of conidia, was used as inoculum. The development of conidia of *C. lindemuthianum* on culture media is different from their development on plants under natural conditions. Since different sets of genes are likely expressed during these different modes of development, it is possible that the avirulent gene product which the plant recognizes to turn on its defense response is produced specifically in the infection court. Therefore, if this

stage is bypassed, as it may be by wound-inoculating plants with fungal mycelia, susceptibility would occur. My results support this interpretation.

There are, however, two problems with the conclusion reached above. One, the plant had to be wounded for colonization to occur from mycelial inoculation and two, the mycelial inoculum was essentially necrotrophic in character, whereas in nature specificity is estabished biotrophically. Conclusive results can only be obtained if intact plants are inoculated with biotrophic hyphae of *C. lindemuthianum.*

A simple model host system, utilizing excised epidermal strips from bean hypocotyls was evaluated. The system appears to offer all the advantages of a tissue culture system, and like intact plants, also gives differential interaction with different races of *C. lindemuthianum.* Besides showing promise for studying specificity phenomenon between plants and their parasites, this system was also useful in delineating component mechanisms of resistance in beans to *C. lindemuthianum.* Although the hypersensitive death of penetrated cells in incompatible strips was not affected, their subsequent necrosis (browning) was suppressed. This apparently allowed the observed initial development of the pathogen in a manner typical of susceptible interactions. Later, however, when phytoalexins accumulated, growth became inhibited. The role of phytoalexins in arresting growth was confirmed because treatment of epidermal strips with glyphosate and AOPP, which inhibited phytoalexin accumulation, also eliminated resistance.

In addition, it was shown that this model system may also facilitate research to understand the mechanistic basis of non-host resistance. The study indicates that mechanisms of resistance to biotrophs and hemibiotrophs

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