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Some Physiological Aspects Of The Soybean - Phytophthora Megasperma F Sp Glycinea Interaction, With Special Reference To The Phytoalexin, Glyceollin

Madan Kumar Bhattacharyya

SOME PHYSIOLOGICAL ASPECTS OF THE SOYBEAN-*PHYTOPHTHORA MEGASPERMA* F. SP. *GLYCINEA* INTERACTION, WITH SPECIAL REFERENCE TO THE PHYTOALEXIN, GLYCEOLLIN.

by

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ABSTRACT

The interaction of soybean cultivars (*Glycine max*) with *Phytophthora megasperma* f.sp. *glycinea* (*Pmg*) races is governed by single host genes (*Rps*) and is consistent with the "gene-for-gene" hypothesis. Using the near isogenic cultivars Harosoy (rps_1, susceptible to race 1) and Harosoy 63 (Rps_1, resistant) and *Pmg* race 1 it was demonstrated that the Rps_1 gene is expressed in hypocotyls and roots of light and dark grown seedlings, in green cotyledons and leaves but not in immature leaves. Leaves of cv. Harosoy became resistant with age. The phytoalexins, glyceollin isomers I, II and III, accumulated rapidly in resistant responses. Proportions of the glyceollin isomers varied with the organ, exposure to light, interaction type and incubation period. The three isomers, also differed in their toxicities to *Pmg*. Glyceollin I was almost twice as inhibitory as glyceollin II and III to mycelial growth *in vitro*. An isolate of *Pmg* race 1 (Isolate 1.1) was obtained that was twice as tolerant to glyceollin I and III as race 1. Evidence was obtained for variability in morphology, growth, tolerance to glyceollin I and aggressiveness of single-zoospore progeny of race 1 and Isolate 1.1, that may be accounted for by cytoplasmic factors.

From pulse and pulse-chase experiments using L-[U^{14}C]-phenylalanine as precursor it is concluded that accumulation of higher levels of glyceollin I in resistant than in susceptible responses is due to differences in rates of biosynthesis. Rapid metabolism, which was not constitutive, was demonstrated in all interactions and controls. This is consistent with differences in phenylalanine ammonia-lyase (PAL) activity demonstrated in resistant and susceptible responses. PAL activity also was correlated with changes in glyceollin production and suscep-

tibility at elevated temperatures. Differential effects of temperature on growth and glyceollin I sensitivity of Pmg races were demonstrated also. In some race-cultivar combinations reaction types may be related to the effect of temperature on production and sensitivity to glyceollin I.

A model is presented that attempts to accommodate the physiological data obtained in this study with the requirements of the "gene-for-gene" relationship of host-pathogen interactions.

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INTRODUCTION

1.1 Plant Diseases

Plant disease can be defined as any physiological or morphological state that differs from that of a normal or healthy plant [Walker, 1957]. Plant diseases can be caused by one or a combination of environmental factors e.g. deficiency or excess of mineral nutrients, extremes in soil moisture, temperature, light etc., or by infection with parasitic organisms [Walker, 1957]. The degree of change in the physiology or morphology of a diseased plant is obvious and can be visualized easily. The external signs or observable morphological changes in diseased plants that are characteristic of a particular disease are termed **symptoms**. **Parasitic organisms** are those whose development and growth takes place in or on a living organism. Disease-causing parasitic organisms or parasites are termed **pathogens** [Day, 1974]. Although the term in its broadest sense may include any disease-causing agent e.g., toxic chemicals, it is most commonly applied to viruses, mycoplasma, bacteria, fungi and nematodes. Among these, fungi cause the majority of diseases. Annual crop losses in terms of both yield and quality due to fungal diseases, especially under bad weather conditions, are enormous.

1.1.1 Definitions and Terminology

Considering the number of species of both plants and microorganisms, the number of economically important parasitic diseases are limited. Most plant species are not infected by most plant parasitic microorganisms. Plant species of this kind are termed **non-hosts** and the corresponding microorganisms **non-pathogens**. The plant species

that are infected by microorganisms and develop characteristic diseases are **hosts** and the microorganisms, **pathogens**. A particular plant species can be a host for one microorganism and a non-host for others.

Following infection, plants may fight the attack of an invading organism and in a majority of cases are successful in preventing invasion and disease development. This inherent ability of plants to resist attack by microorganisms is termed resistance, the response of the plants to the microorganisms in such a situation is a **resistance response** and the process by which it is achieved is termed a **resistance mechanism**. The failure of host plants to fight against attack by pathogens may result in disease with loss of yield, quality and finally death of plants. The response of plants to the pathogens in this case is termed a **susceptible response** and the phenomenon **susceptibility**. The equivalent phenomenon in the pathogen i.e. the ability to penetrate, establish and subsequently grow inside the host is termed **pathogenicity**. The interaction between host and pathogen that results in a susceptible response in the host is termed a **compatible interaction** and the interaction that produces a resistant response is known as an **incompatible interaction**.

1.2 Resistance

Resistance can be classified into i) non-host resistance, ii) host resistance or cultivar-specific resistance [Heath, 1981] or race-specific resistance [Mansfield, 1983] and race-non-specific or age-related resistance [Mansfield, 1983] or organ-specific resistance [Graniti, 1976].

Non-host resistance is the response of a non-host to any non-pathogen of that plant species. The responses may vary from immune, in which no macroscopic symptom is observed [Day, 1974], to resistant, in which very few plant

cells die following penetration, with very small but observable flecks of dead tissues. In all these cases non-pathogens are unsuccessful in establishing themselves inside the plant.

Host-resistance, cultivar-specific resistance or race-specific resistance, as the name implies, is the inherent ability of individual cultivars of host plants to prevent attack by specific races of a pathogen. Resistance is cultivar- as well as race-specific. Each pathogenic species may exist as many different physiological races, each of which can be differentiated on the basis of their interactions with **differential host cultivars** [Stakman, 1914]. Differential host cultivars are those that carry different combinations of genes for resistance. Physiological races, commonly referred to simply as races, of a pathogenic species that are unable to cause a susceptible response in their host are termed **avirulent races** and the phenomenon, **avirulence**. On the other hand, races of a pathogenic species that are successful in causing a disease or a susceptible response in the host are termed **virulent races** and the phenomenon, **virulence**. From the study of flax rust resistance to Melampsora lini Flor [1956] put forward the hypothesis that for each gene conditioning resistance in the host there is a specific gene for pathogenicity in the parasite. This hypothesis, later popularly known as the **'gene-for-gene'** hypothesis, explains most examples of cultivar or race-specific resistance. In most host-pathogen systems studied, avirulence is phenotypically dominant over virulence and resistance is dominant over susceptibility [Sidhu, 1975]. A host cultivar carrying a gene for resistance is resistant to those races of a pathogen that carry the corresponding dominant gene for avirulence.

Race-non-specific or age-related resistance as the name implies is the acquired ability of the plant with age

to prevent the attack by any race of a pathogen: while, organ specific resistance indicates that some organs in a susceptible host cultivar may be resistant to a virulent race.

1.3 Resistance Mechanisms

Possible resistance mechanism may be separated into the following categories: i) physical barriers, ii) preformed chemical factors and iii) active plant defence mechanisms.

1.3.1 Physical Barriers

A waxy cuticle on the surface of the epidermis is the first line of defence against those microorganisms that do not produce cutinase [Dickman and Patil, 1986; Kolattukudy and Koller, 1983] and do not penetrate the epidermis by mechanical force. The primary cell-wall impregnated with cutin, suberin, lignin, low molecular weight phenolic compounds, silicon and calcium may be resistant to degradation by microbial enzymes and play a role in resistance [Heath, 1986; Ride, 1983]. Arrangement of stomata, leaf hairs, lenticels and the timing of opening and closing of stomata are other examples of physical barriers that may provide resistance to penetration by micro- organisms. Plugging of the xylem vessels with tyloses is considered to be effective for restricting the spread of pathogens that inhabitat vascular systems [Heath, 1986].

1.3.2 Preformed Chemical Factors

Preformed chemical factors, inhibitory to pathogens, are chemically diverse and found in a wide range of plant families [Mansfield, 1983], as for example, the phenolic compounds that are present in healthy tissues. Following infection phenols are decompartmentalized and oxidised by phenolase to form more toxic substances that cause inhibi-

tion of spore germination, germtube growth, mycelial growth and microbial cell-wall degrading enzymes [Friend, 1981]. For example, prenylated isoflavones e.g., luteone and weighteone of surface wax of lupin leaves [Harborne et al., 1976, Ingham et al., 1977], phenolic compounds e.g., catechol and protocatechuic acid of coloured onion scales [Link et al., 1929, Link and Walker, 1933] are inhibitory to spore germination and/or germtube development.

1.3.3 Active Plant Defences

Active plant defences are the mechanisms or means of defence against invading microorganisms that are initiated by the association of plants with the invading microorganisms [Wood, 1978]. Proposed mechanisms of active plant defence that have been studied include i) cellular barriers, ii) tissue barriers, iii) deposition of silicon, iv) lignification, v) accumulation of hydroxyproline-rich glycoproteins and vi) accumulation of phytoalexins.

1.3.3.1 Cellular Barriers

Cellular barriers include i) cytoplasmic aggregations, ii) haloes and iii) papillae [Aist, 1983].

i) Cytoplasmic aggregations: Masses of cytoplasm that accumulate at the sites of attack in quick response to zoospore cysts or appressoria are termed **cytoplasmic aggregations** [Aist, 1983]. They are reported to form within 23 seconds in cabbage root hairs in response to zoospore cysts of Plasmodiophora brassicae [Aist, 1976]. They are thought to give rise to intercalated materials in some cell wall haloes and also to papillae.

ii) Haloes: A circular region, surrounding the point of infection, in which there are changes in cell-wall proper-

ties and affinity for heavy metal stains, is termed a halo. Earlier studies suggested that haloes are the result of degradation of cell walls by pathogen enzymes [e.g. McKeen et al., 1969]. However, subsequent work indicated that haloes were due to the accumulation of silicon, lipid material and lignins [Heath, 1979; Ride and Pearce, 1979; Sargent and Gay, 1977] that make the cell walls in the halo region more resistant to enzymatic degradation by pathogens.

iii) Papillae: A structure, composed of heterogenous materials, formed following infection between host plasmamembrane and host cell wall is termed a papilla [Aist, 1983]. Formation of papillae has been claimed to be associated with the expression of resistance by inhibiting penetration, but there are also reports indicating that papillae are penetrated to some extent. For example, when papilla-formation was inhibited by low speed centrifugation the penetration efficiency was not increased. However, in the cytoplasm rich ends of epidermal cells of centrifuged tissues, papillae of enhanced sizes formed and were associated with marked reduction in penetration efficiency [Waterman et al., 1978].

1.3.3.2 Tissue Barriers

Tissue barriers may be either of the following types [Aist, 1983]: i) Wall appositions and ii) Suberized walls.

i) Wall apposition: In response to infection in some host species massive or extensive deposition of electron dense materials referred to by Aist [1983] as wall appositions occur in many host cells in the infection court as opposed to cytoplasmic aggregation or papillae formation, which occurs in a specific site of a single infected cell. In some cases, a clearcut coincidence of the extent and timing of cell wall

apposition formation with restriction of pathogen development has been observed [Beckman et al., 1982; Lazarovits and Higgins, 1976a, 1976b].

ii) Suberized walls: Suberization of wounds is an important process to seal-off cut surfaces [Kolattukudy, 1980] by depositing suberins and, thereby, to protect plants from possible infectious agents.

1.3.3.3 Deposition of Silicon

The first report about the role of silicic acid on the expression of resistance mechanisms of wheat plant to Erysiphe graminis was made by Germar [1935] who observed that mildew infection was reduced when wheat plants were grown in soils supplimented with silicon dioxide [see. Clifford et al., 1985]. Kunoh et al. [1975] demonstrated for the first time by X-ray microanalysis that silicon accumulated around penetration pores of Erysiphe gramines hordei on barley leaves. Heath and Stumpf [1986] speculated that the primary role of silicified walls is to act as a permeability barrier to prevent signals from the plant reaching the fungus (Uromyces vignae) that are essential for normal growth of the penetration peg. In addition, silicified walls are possibly also permeability barriers for nutrient transport into haustorial mother cells of that fungus and act as a physical barrier if the penetration peg reaches the silica deposits [Heath and Stumpf, 1986]. It is considered that deposition of silicon confers non-specific resistance against invading microorganisms[Clifford et al., 1985].

1.3.3.4 Lignification

Lignification or deposition of lignins in the infected and surrounding tissues is considered to play an important role in the expression of disease resistance. Many different kinds of lignins are elaborated from three kinds of

hydroxycinnamyl alcohol i.e., i) coniferyl alcohol, ii) coumaryl alcohol and iii) sinapyl alcohol [Ride, 1983]. Lignification makes the cell walls resistant to enzymatic degradation by microorganisms [Ride and Pearce, 1979] and also to penetration by mechanical force. Precursors of lignins e.g., coniferyl alcohol, are toxic to fungi [Hammerschmidt and Kuc, 1982]. Rapid lignification is associated with the expression of cultivar specific resistance of potatoes [Friend, 1976; Friend et al., 1973], and non-host resistance of wheat [Ride, 1975]. Enhanced lignification was reported also to correlate with the induction of systemic resistance in cucumber cultivars [Hammerschmidt and Kuc, 1982].

1.3.3.5 Hydroxyproline-rich Glycoproteins

Hydroxyproline-rich glycoproteins (HRGPs) are major structural components of cell walls. Accumulation of HRGPs in response to infection is correlated with the expression of cultivar specific resistance in a number of host-pathogen systems [Esquerre-Tugaye et al., 1979; Hammerschmidt et al., 1984; Showalter et al., 1986]. Following infection HRGPs are reported to increase from 0.5% in control to 5-15% of the wall [Mazau et al., 1986]. The precise role of HRGPs in plant defence is not well known, but it is suggested [Showalter et al., 1986] that i) they provide sites for lignification [Whitmore, 1978] and ii) they act as agglutinins towards negatively charged pathogens [Leach et al., 1982; Mellon and Helgeson, 1982; Van Holst and Vance, 1984].

1.3.3.6 Phytoalexins

Phytoalexins are low molecular weight antimicrobial compounds that are both synthesized by and accumulated in plants after their exposure to microorganisms [Paxton, 1980]. The phytoalexins are non-specific in their toxicities

towards various microorganisms and they are also synthesized by plants in response to different environmental stresses and treatment with biotic and abiotic elicitors. Phytoalexins are considered to play an important role in the expression of cultivar specific resistance. The evidence so far accumulated, however, is circumstantial [Tegtmeier and VanEtten, 1982]. High levels of phytoalexins that rapidly accumulate in resistant, but not in susceptible reactions, are positively associated with the inhibition of mycelial growth of avirulent races in many host-pathogen systems [Hahn et al., 1985; Yoshikawa et al., 1978; Bailey et al., 1980; Mayama et al., 1982].

In all these active defence mechanisms, the formation or accumulation of defence-related structures or compounds although associated with resistant responses also occur to a lesser degree in susceptible responses. The evidence for the role of all these mechanisms in the expression of cultivar specific or host resistance or non-host resistance is circumstantial. The present study was undertaken to provide further information on resistance and susceptibility of soybeans to Phytophthora megasperma f. sp. glycinea and the role of soybean phytoalexins in this host-pathogen interaction.

Phytophthora rot of soybeans (Glycine max(L.) Merr.) caused by the fungus, Phytophthora megasperma Drechs. f. sp. glycinea (Hildeb.) Kuan and Erwin was chosen as the model disease because,

1) resistant and susceptible reactions of soybean cultivars to 24 races of the pathogen are well defined and their expression is governed by a series of genes(Rps) [Schmitthenner, 1985] in the host and the interaction follows the classical gene-for-gene hypothesis of Flor [1956].

2) Methods of inoculation that mimic the natural infection

process and do not require wounding are available [Ward et al., 1979; Hahn et al., 1985].

3) Near isogenic lines that carry single Rps genes for resistance are available in which many differences between genetic backgrounds of host cultivars have been eliminated.

4) Growing seedlings and disease development are relatively less time consuming than in some other systems and extraction and measurement of phytoalexins from etiolated plants is fairly routine.

5) The pathogen can be maintained in vitro easily without loss of virulence for a long period of time.

1.4 Phytophthora Rot of Soybean: Symptoms, the Causal Organism and Disease Cycle, Host Resistance and Accumulation of Phytoalexins

1.4.1 Symptoms

Phytophthora rot can occur at any stage of growth of the soybean plant. As a pre-emergence damping-off and seed rot, it causes poor stands of the crop. During the post-emergence stage, stem and root rots cause wilting and death of the seedlings. Older plants are killed more gradually. A brown girdling rot may extend up the stem as high as 10 nodes before the plant finally wilts and dies [Schmitthenner, 1985]. After heavy rains, infection may occur also through the leaves and stems [Sinclair, 1982].

Incorporation of genes (Rps) for resistance into commercial cultivars is considered an important method of controlling the disease. Plant breeders have evaluated green seedlings for resistance routinely by inserting mycelium into wounds that are made in the hypocotyl below the cotyledons [Keeling, 1976]. Inoculation of unwounded etiolated hypocotyls [Ward et al., 1979] or roots [Hahn et al.,

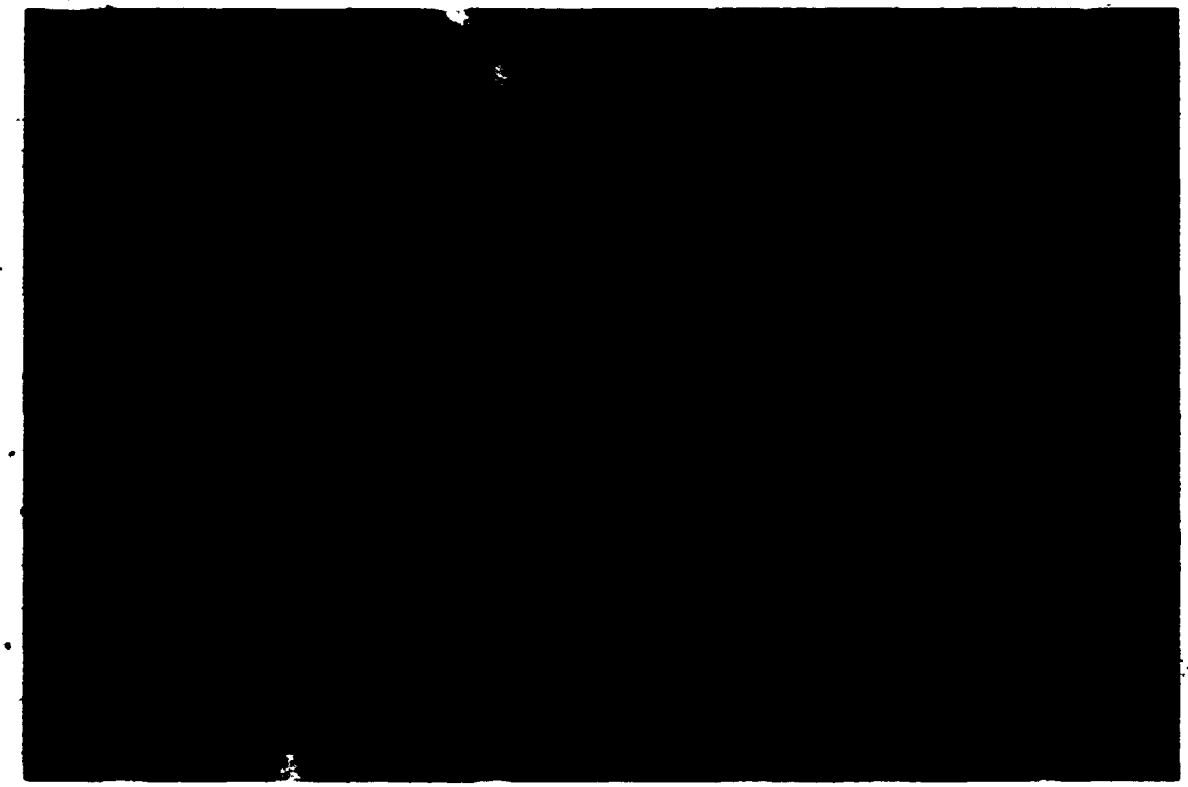
1985] with zoospores suspensions has received attention in biochemical studies of resistance because it avoids complications caused by wounding.

The resistant response, that develops following inoculation of 6-day old etiolated hypocotyls with drops of zoospore suspensions is characterized by restricted brown lesions, approximately the size of the inoculum drops, and with rapid death of host cells close to the hypocotyl surface. In the susceptible response an almost clear or pale brown water-soaked lesion develops that spreads rapidly throughout the hypocotyl [Fig. 1.1; Ward, 1984a].

1.4.2 Casual Organism and Disease Cycle

Phytophthora megasperma f. sp. glycinea is a hemibiotroph i.e., the virulent race on a susceptible cultivar can establish a short compatible period in which no damage to the host cell is detected, and thereafter it can cause necrosis and death of the cells [Bailey, 1982]. The hyphae are coenocytic when young. They become septate with age. Branching of hyphae occurs at right angles. Sporangia are obpyriform in shape and with inconspicuous papillae (Fig. 1.2). Zoospores are ovoid, bluntly pointed at one or both ends and flattened on the sides. One of their flagella is directed anteriorly and the other, four to five times longer than the first, is directed posteriorly. Less frequently chlamydospores also occur. They are intercallary, often acrogenous, irregular or spherical in shape. Spherical ones are as large as oogonia [Hilderband, 1959]. Sexual structures, oogonia and antheridia, are formed in this homothallic fungus. Antheridia are mostly paragynous or occasionally amphigynous [Schmitthenner, 1985]. Meiosis occurs in oogonia and antheridia and nuclear fusion of these gametangia leads to the development of thick smooth-walled, distinctly yellowish diploid oospores [Schmitthenner, 1985;

Fig. 1.1
The symptoms of disease development in etiolated hypocotyls of soybean following inoculation with zoospores of *Phytophthora megasperma* f.sp. *glycinea*. R = Resistant; S = Susceptible response.



Sinclair, 1982].

The disease cycle of the fungus is presented in Fig. 1.2. The fungus overwinters in soil as dormant oospores. Oospores are endogenously dormant and remain dormant for variable lengths of time [Schmitthenner, 1985]. When dormancy is broken, under favourable temperature and moisture conditions oospores germinate through either germtubes or the formation of sporangia. Sporangia can germinate directly through germtubes or by the release of biflagellate zoospores. After a motility period, which may last several days, zoospores encyst by forming a cell wall. The cysts germinate by a germtube, sometimes by secondary zoospores and rarely by a miniature sporangium [Sinclair, 1982]. Chlamydospores, perhaps like other Phytophthora spp. (Ribeiro, 1983), germinate through forming either germtubes or sporangia. Germtubes from any of the above structures form appressoria when they contact host surfaces e.g. roots, hypocotyls, leaves etc. Penetration pegs or hyphae penetrate between anticlinal walls of the epidermal cells or directly through the cuticle into the outer wall of epidermal cells [Stossel et al., 1980].

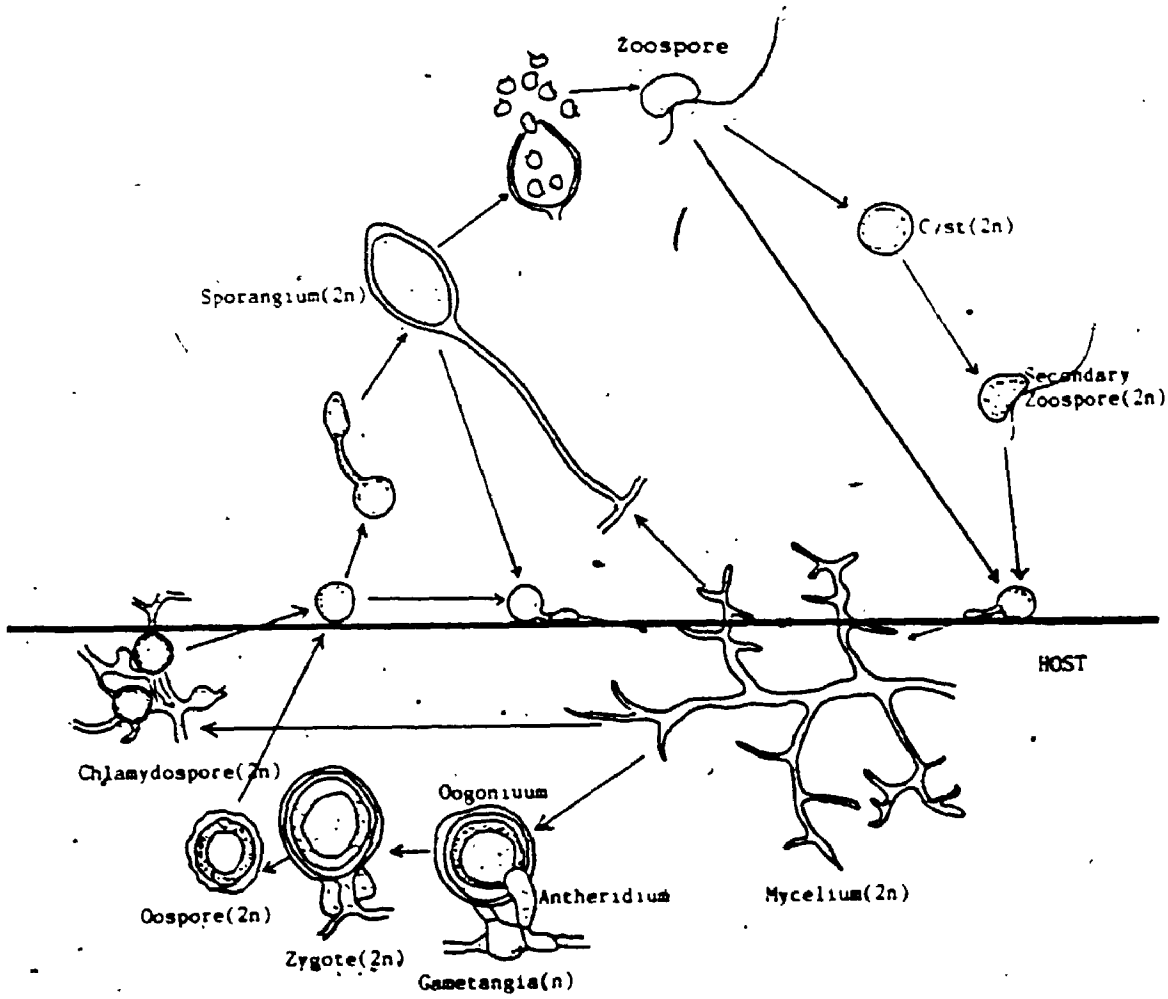
1.4.3 Resistance

Resistance of soybeans to Phytophthora megasperma f. sp. glycinea is governed by a series of major genes (Rps) and their respective multiple alleles. So far seven resistance genes (Rps_1 - Rps_7), four alleles at Rps_1 and a few alleles at Rps_3 have been identified [Schmitthenner, 1985]. In addition to resistance that is governed by Rps genes, field resistance or tolerance in some susceptible cultivars has been reported also [Olah and Schmitthenner, 1985].

1.4.4 Soybean Phytoalexins

The production of post-infectional antifungal metabo-

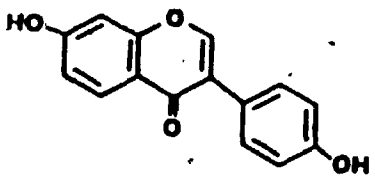
Fig. 1.2
Life cycle of Phytophthora megasperma f.sp. glycinea


Zoospore
Cyst(2n)
Sporangium(2n)
Secondary
Zoospore(2n)
HOST
Chlamydospore(2n)
Oogonium
Antheridium
Mycelium(2n)
Oospore(2n)
Zygote(2n)
Gametangia(n)

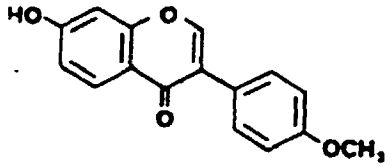
lites by soybean pods in response to infection by Fusarium sp. was reported first by Uehara [1958]. Klarman and Sanford [1968] obtained the first evidence indicating the soybean phytoalexin to be a pterocarpan related to phaseollin, a phytoalexin of beans [Cruickshank and Perrin, 1963]. Later, it was characterized as 6a-hydroxyphaseollin by Sims et al. [1972]. The structure was subsequently revised [Burden and Bailey, 1975]. It was known from gas chromatography that the soybean phytoalexin occurs in three isomeric forms [Keen et al., 1971] and these were later referred to as glyceollins I, II and III [Partridge and Keen, 1977]. In addition to these three isomers that are commonly isolated, a fourth isomer, glyceollin IV and other isoflavonoids: isoflavones, pterocarpans, coumestans have been reported to accumulate also in smaller amounts either as precursor to glyceollin isomers or as soybean phytoalexins (Table 1.1).

Table 1.1

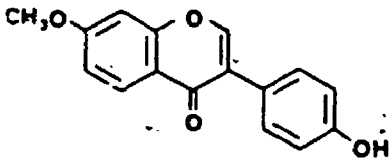
Phytoalexins and related compounds reported to accumulate following infection or elicitor treatment of soybean organs.

PHYTOALEXIN/RELATED COMPOUND	ORGAN	AUTHOR
ISOFLAVONES:		
Daidzein 	Hypocotyls	Keen et al.,1972
	Leaves	Keen and Kennedy,1974
		Fett,1984
	Roots	Porter et al.,1985

Formononetin — Leaves — Fett, 1984

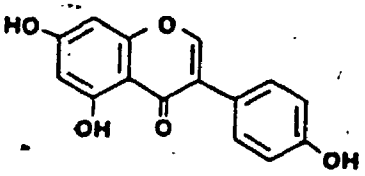


Isoformononetin — Leaves — Ingham et al., 1981



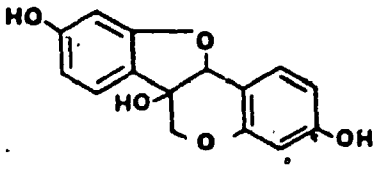
Genistein — Leaves — Fett, 1984

Porter et al., 1985



PTEROCARPANS:

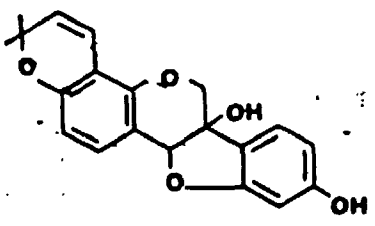
Glycinol — Cotyledons — Ingham et al., 1981
Lyne and Mulheirn, 1978
Weinstein et al., 1981



Glyceollin I — Cotyledons — Burden and Bailey, 1975
Ingham et al., 1981
Lyne et al., 1976
Keen and Horsch, 1972

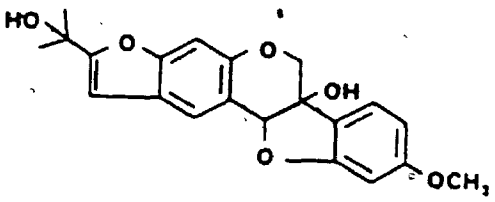
Callus tissues — Keen and Horsch, 1972

Pods — Keen and Horsch, 1972



	Hypocotyls	Keen and Horsch, 1972
		Sims et al., 1972
		Stossel and Magnolato, 1983
	Roots	Keen and Horsch, 1972
		Hahn et al., 1985
	Leaves	Keen and Kennedey, 1974
		Ingham et al., 1981
Glyceollin II	Cotyledons	Ingham et al., 1981
		Lyne et al., 1976
	Leaves	Ingham et al., 1981
	Hypocotyls	Moesta and Grisebach, 1981
		Stossel and Magnolato, 1983
Glyceollin III	Cotyledons	Lyne and Mulheirn, 1978
	Leaves	Ingham et al., 1981
	Hypocotyls	Moesta and Grisebach, 1981
		Stossel and Magnolato, 1983
Glyceollin IV	Cotyledons	Lyne and Mulheirn, 1978
Glyceofuran	Cotyledons	Ingham et al., 1981
	Leaves	Ingham et al., 1981

(-)-9-O-Methylglyceofuran — Leaves — Ingham et al.,1981

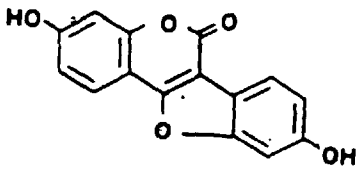


COUMESTANS:

Coumestrol — Hypocotyls — Keen et al.,1972

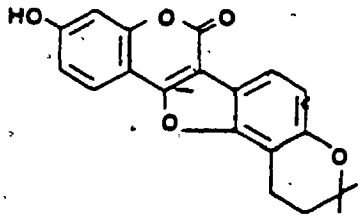
Leaves — Keen and Kennedy,1974; Fett,1984

Roots — Porter et al.,1985



Sojagol — Hypocotyls — Keen et al.,1972

Leaves — Keen and Kennedy,1974



CHAPTER 2

MATERIALS AND METHODS

This chapter describes general materials and methods used throughout this thesis. Each subsequent chapter has a section for materials and methods specifically used in that chapter.

2.1 Host

The cultivars of soybean used, genes(*Rps*) for resistance, responses to *Phytophthora megasperma* f.sp.*glycinea* and sources, are presented in table 2.1. In most of the experiments the near isogenic lines Harosoy(rps_1) and Harosoy 63(Rps_1) were used.

2.1.1 Growing of Seedlings

Seeds were sown in vermiculite and soaked overnight in 15-30-15 fertilizer solution (2.5 g/L) and then they were transferred to growth cabinets. The seedlings were grown for 6 days in the dark on a temperature cycle of 7 h at 16°C, 5 h increasing at 2.2°C/h, 7 h at 27°C and 5 h decreasing at 2.2°C/h. The plants were fertilized again on the fifth day (4.5 g/L). The seedlings were watered every morning [Ward *et al.*, 1979].

2.1.2 Arrangement of Seedlings for Inoculation

The 6 day old etiolated seedlings were carefully uprooted and washed under running tap water so that vermiculite and seed coats were removed. They were blotted dry with cellucotton and arranged horizontally in glass trays (20 seedlings/tray) and held in place by slotted Plexiglass brackets. The roots of the seedlings were covered with a layer of cellucotton soaked in water [Ward *et al.*,1979].

2.2 Pathogen

Phytophthora megasperma f.sp.glycinea(Hildeb) Kuan and Erwin race 1 [Ward and Buzzell, 1983] was used in most of the experiments. In the later part of the study [Chapter 7 and 8], however, a single-zoospore isolate (R1.19) derived from race 1 was used. All the races used in various experiments and their interactions with a set of differential cul

Table 2.1

Soybean cultivars, their source and genes for resistance (Rps) and responses to Phytophthora megasperma f.sp. glycinea races, used in different experiments.

Cultivar[1]	Rps gene	Response[2] to Race		
		1	4	6
Altona	Rps_6	R	R	S
Corosoy 79	$Rps_1{}^c$	R	S	R
Harosoy	$Rps_?$	S	S	S
Harosoy 63	Rps_1	R	S	S
L-70-6494	Rps_2	R	R	R
PRX8-122-1	Rps_3	R	R	R
Wayne	___	S	S	S

[1] All cultivars were provided by Dr R.I.Buzzell, Research Station, Agriculture Canada, Harrow, Ontario.

[2] Responses of etiolated hypocotyls of the soybean cultivars to zoospores of races 1, 4 and 6 that were used for inoculataion in different experiments were obtained from Ward and Buzzell, [1983].

tivars of soybeans, are presented in table 2.2.

2.2.1 Culture Media

The fungus was routinely grown in V8 juice agar medium. Deionized water containing calcium carbonate and Difco agar was autoclaved at 15 PSI (121°C) for 10 minutes and then the volume was adjusted with V8 juice to obtain a final concentration of 0.2% calcium carbonate, 1.5% agar and 20% V8 juice [Riberio, 1978]. The mixture was autoclaved at 15 PSI (121°C) for 25 minutes. In the later part of the study (Chapter VII, VIII, IX) 26% V8 juice agar instead of 20% was used because production of zoospores was better in 26% V8 juice agar than in 20%.

When needed, 2% agar in deionized water (autoclaved as above) was used as a medium to elimihate bacterial contamination from fungal colonies.

2.2.2 Culture Methods

2.2.2.1 Culture Maintenance

Cultures were routinely maintained in 20 or 26% V8 juice agar (as in 2.2.1) in disposable petri plates (90 mm diameter). The cultures were incubated in the dark at 25°C.

2.2.2.2 Zoospore Production

The fungus was grown for 5 days on 20 or 26% V8 juice agar in the dark at 25°C and then soaked with sterile distilled water and kept overnight at 18°C. The plates then were washed with sterile water every 30-45 minutes for about 6-8 h under room conditions until sporangia developed and release of a few zoospores commenced. The plates were then incubated overnight in the dark at 18°C with sterile distilled water just sufficient enough to cover the mycelium. The next morning zoospore concentrations were determined using a haemacytometer and suspensions were adjusted to 10^5

Table 2.2

Interactions[1] with a set of differential soybean cultivars of phygiological races of *Phytophthora megasperma* f.sp. *glycinea* used in different experiments.

Differential Cultivar	Physiological race[2]																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	17	18	19	20
Harosoy($Rps_?$)	C	C	C	C	C	C	C	C	C	C	C	I	C	C	C	C	I	I	C
Sanga ($Rps_1{}^b$)	I	C	I	I	I	I	I	I	I	C	C	C	I	I	I	I	I	C	C
Harosoy 63(Rps_1)	I	I	C	C	C	C	C	C	C	I	I	I	I	I	I	I	I	I	C
Mack ($Rps_1{}^c$)	I	I	I	C	C	I	I	I	I	I	I	C	I	C	I	I	C	C	C
Altona (Rps_6)	I	I	I	I	C	C	C	C	C	I	C	I	C	I	I	C	I	I	I
PI103091($Rps_1{}^d$)	I	I	I	I	I	C	I	C	I	I	I	I	I	I	I	C	I	C	I
PI171442 (Rps_3)	I	I	I	I	I	C	C	I	I	C	I	C	I	I	C	C	I	C	C
Tracy ($\frac{Rps_{3b}}{Rps_1}$)	I	I	I	I	I	I	I	I	I	C	I	C	I	I	I	C	I	C	C

C = compatible interaction and I = incompatible interaction

[1] Adapted from Keeling [1982] with a little modification

[2] All races were provided by Dr R.I.Buzzell, Research Station, Agriculture Canada, Harrow, Ontario.

[3] *Rps* genes are presented in parenthesis [Athow, 1984; Athow *et al.*, 1979].

zoospores/ml for inoculation purposes.

2.3 Abiotic Elicitor

In some of the experiments freshly prepared $AgNO_3$ solution (10^{-3} M) was used as abiotic elicitor either for the production of glyceollin isomers [Stossel, 1982] or for the induction of phenylalanine ammonia-lyase activity.

2.4 Inoculation of Hypocotyls

Horizontally arranged etiolated hypocotyls (2.1.2) were inoculated with 10 µl drops of zoospore suspension, $AgNO_3$ solution or sterile water. The drops were put approximately 2 cm below the cotyledons [Ward et al., 1979]. Immediately following inoculation, glass trays were sealed with plastic film and incubated in the dark at 25°C (in most cases) or at 33°C for various periods.

2.5 Preparation of Glyceollin Isomers

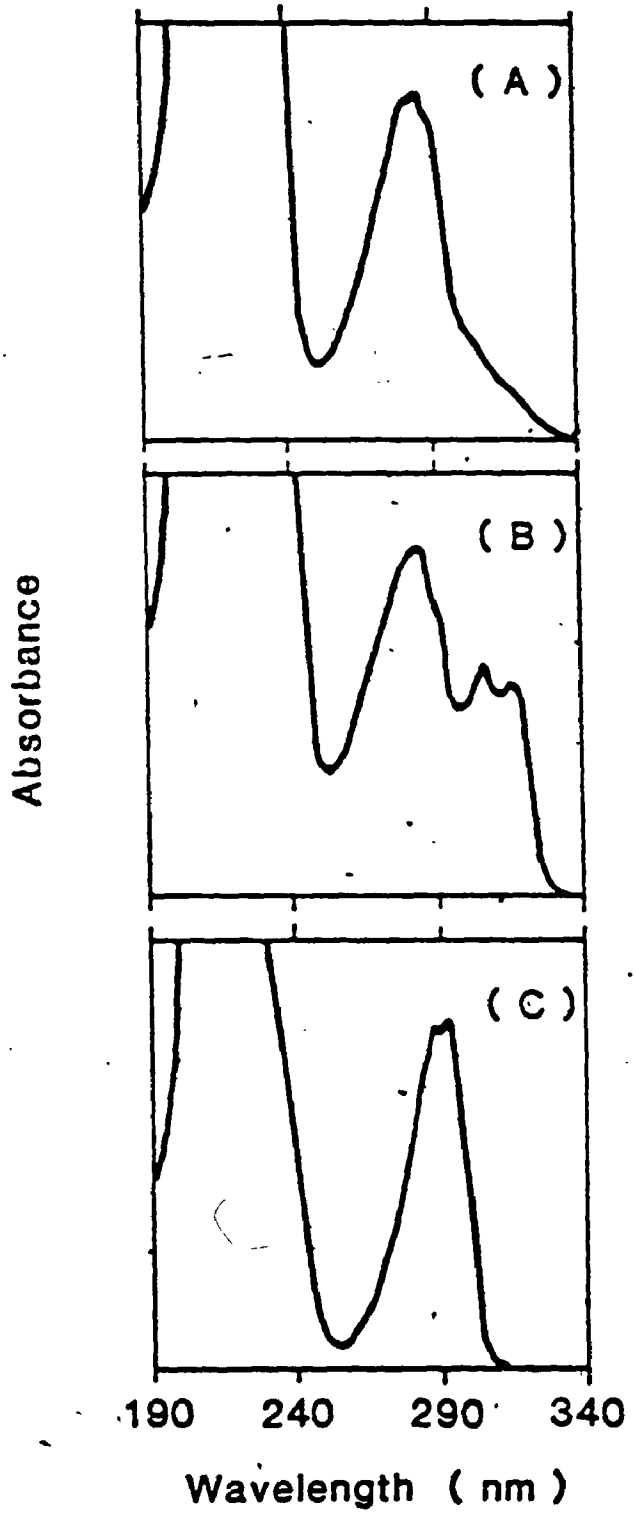
Soybean seedlings (Harosoy 63) were grown for 8 days with a daily cycle of 16 h light (approx. 300 $\mu Em^{-2}/s$) and 8h dark with maximuum temperature 22.5°C in the light and minimum 16°C in the dark. Cotyledons were removed, wounded on the dorsal side, inoculated with 25 µl of a zoospore suspension (10^5/ml) of Phytophthora megasperma f.sp. glycinea race 1 and incubated in the dark for 48h at 25°C and 100% R.H.

The cotyledons were extracted with 95% ethanol for 10 min in a water bath (100°C). The extract was filtered through cheese-cloth and reduced to near dryness under reduced pressure. The residue was redissolved in water and extracted three times with equal volumes of ethyl acetate. After concentration, the combined extracts were applied to a column of Sephadex LH 20 (86x2.4 cm i.d., void volume 138 ml) and eluted with methanol. Fractions (6.9 ml, 20 min)

containing glyceollin were detected by scanning from 190-340 nm and reference to published UV spectra for the glyceollin isomers [Ingham, 1982]. Partial separation of the three isomers was achieved at this stage (elution volumes: glyceollin I, 517 ml; glyceollin II, 538 ml; glyceollin III, 559 ml) and, accordingly fractions were combined in three groups and reduced to small volumes.

Further purification was achieved by high performance liquid chromatography (HPLC) (Waters model 510 pump, model U6K injector, model 480 variable wavelength detector and Hewlett Packard, 3390A Integrator) using a semi-preparative column (silica, Whatman Partisil 10 M9/50 50 cmx0.94cm i.d.) with 3% or 5% isopropanol in hexane as the mobile phase. The glyceollin isomers were detected by the absorbance at 285 nm. Elution times for isomers I, II and III respectively were 52.1-55.8, 59.6-60.9, 65.6-67.0 min with 3% isopropanol in hexane (flow rate 5ml/min) and 35.3-39.3, 38.8-42.7, 43.5-46.3 min with 5% isopropanol in hexane (flow rate 3 ml/min). Peaks from the individual isomers were cut and recycled 4-6 times before fractions were collected and their UV spectra compared with published reports [Ingham, 1982; Fig. 2.1]. The separated isomers were then subjected to preparative thin layer chromatography (TLC; Whatman PLK5F silica gel; benzene:methanol, 95:8, Rf=0.35) and detected by fluorescence quenching under UV light. No compound other than the glyceollin isomers could be detected on sample chromatograms sprayed with H_2SO_4: methanol (75:25) and heated. Silica-gel in bands at Rfs corresponding to glyceollin isomers was scraped from the plates and eluted with 3% isopropanol in hexane. Suspended silica was removed by filtration and centrifugation and the solvents were evaporated under reduced pressure yielding white powders.

Fig. 2.1
Ethanolic UV spectra of the soybean phytoalexins, glyceollin I (A), glyceollin II (B) and glyceollin III (C).


(A)
(B)
(C)
Absorbance
190
240
290
340
Wavelength (nm)

2.6 Quantification of Glyceollin Isomers

Quantification of the glyceollin isomers was based upon their UV absorbance maxima [286, 285, 287 nm for glyceollin I, II and III respectively; Ingham, 1982] and molar extinction coefficents. However, the published extinction coefficient for glyceollin I, λ_{285} log ϵ = 3.92 [Sims et al., 1972] was found to be inaccurate. From the evidently purer material, the value was determined to be 10 800(λ_{286}) in ethanol [Chapter 5]. Determination of the extinction coefficients for glyceollin II and III were in agreement with published values of 8 700 and 9 400 respectively [Lyne et al., 1976].

Standard graphs for concentrations of each of the three isomers were prepared for HPLC analyses, by injecting known concentrations of the isomers onto an analytical column (silica, Whatman Partisil 5, 250x4.6 mm i.d.) with 5% isopropanol in hexane (flow rate, 1.5%/min) as the mobile phase. Standard graphs are presented in Appendix I.

2.7 Determination of Glyceollin Isomers

The lesions developed following inoculation with zoospores or $AgNO_3$ treatment were excised and transferred into test tubes containing 5 ml ethanol (95%). The ethanol and tissues were then boiled for 2 min, the ethanol extract was decanted, combined with two ethanol rinses (2 ml) of the tissue and reduced to dryness under reduced pressure. The dried residue was extracted three times with 2 ml of ethyl acetate and the combined extracts were subjected either first to TLC or directly to HPLC. The ethyl acetate soluble fraction was dried and redissolved in 100 µl of ethyl acetate and together with two 100 µl rinses applied to a TLC plate (Whatman LK6DF, 250 µm thick). The plates were developed in benzene:methanol::95:8 and glyceollin was detected by fluoroscence quenching under UV light. Silica

bands containing glyceollin were eluted with ethyl acetate. After evaporating the ethyl acetate and redissolving the residue in ethanol, glyceollin was determined from its absorbance at 285 nm and the extinction coefficient [Ayer et al., 1976]. The tissue remaining in the test tubes was dried to constant weight at 65°C. Glyceollin concentrations were expressed as µg per g fresh weight. In some experiments glyceollin partially purified by TLC method was subjected to HPLC as follows.

The glyceollin obtained from TLC or the residue of the ethyl acetate extract was dissolved in 100-300 µl of ethyl acetate, centrifuged at 15000 r.p.m. in a microcentrifuge for 3 minutes. 25-50 µl of the supernatant was used to separate and quantitate the glyceollin isomers by HPLC (Waters model 510 pump, model U6K injector, model 480 variable wavelength detector and Hewlett Packard 3390A, Integrator) using an analytical column (silica, Whatman Partisil 5, 250x4.6 mm i.d.) with 5% isopropanol in hexane (flow rate, 1.5ml min^{-1}) as the mobile phase. The isomers were detected by their absorbance at 285 nm. Retention times (min) for glyceollin I, II and III were 13.4-13.5, 14.7-14.9 and 16.0-16.1 respectively. They were identified from their UV spectra by comparison with published reports [Ingham, 1982; Fig.2.1]. Amounts were determined by reference to standard curves (Appendix I) and expressed as µg glyceollin isomer/g fresh weight.

CHAPTER 3

RESISTANCE, SUSCEPTIBILITY AND ACCUMULATION OF GLYCEOLLINS I-III IN SOYBEAN ORGANS INOCULATED WITH *Phytophthora megasperma* f.sp. *glycinea*

3.1 Summary

The expression of resistance and susceptibility to inoculation with zoospores of *Phytophthora megasperma* f.sp. *glycinea* race 1 was determined in roots, hypocotyls, and cotyledons of etiolated and green seedlings and in leaves of soybean cvs Harosoy (rps_1) and Harosoy 63 (Rps_1). Gene-specific resistance was demonstrated in all organs tested, except for cotyledons of etiolated seedlings. In each case higher concentrations of the glyceollins accumulated in resistant than in susceptible reactions; the differential being greatest in hypocotyls and smallest in roots. The relative proportions of glyceollin I, II and III varied with the organ, exposure of the seedlings to light, the interaction type and the incubation period. Glyceollin I was relatively the most abundant isomer in roots and to a lesser extent in hypocotyls. Glyceollin III was relatively the most abundant isomer in leaves. Major differences in accumulation rates were observed in time-course experiments and, after reaching a maximum, all three isomers decreased in leaves and glyceollin I decreased in hypocotyls, suggesting that concentrations and hence isomeric proportions were finely controlled by rates of biosynthesis and metabolism.

3.2 Introduction

Evidence that glyceollin, the pterocarpan phytoalexin from soybeans, occurs in several isomeric forms was provided by Keen *et al*. [1971] for preparations obtained from soybean

hypocotyls inoculated with Phytophthora megasperma f.sp. glycinea. Subsequently the structures of four isomers (glyceollin I-IV) were established by Burden and Bailey [1975] and Lyne and co-workers [1976, 1978]. Of these, glyceollin IV has been isolated in minor amounts only, from cotyledons treated with $CuCl_2$ [Lyne et al., 1978], and no evidence that it may play role in the resistant response has been provided. Glyceollin I-III (Fig. 3.1) are all inhibitory to mycelial growth and zoospore germination of Phytophthora megasperma f.sp. glycinea [Chapter 5] and have been demonstrated to accumulate in significant amounts in soybean tissues [Hahn et al.,1985; Ingham, 1982; Kaplan et al.,1980; Lyne et al.,1976, Moesta and Grisebach, 1981b]. The proportions of the three isomers reported by different authors have varied considerably. In mycorrhizae or roots treated with $CuSO_4$ [Morandi et al.,1984] or inoculated with Phytophthora megasperma f.sp. glycinea [Hahn et al.,1985], glyceollin I predominated. Glyceollin I was also the main component in preparations from hypocotyls inoculated with Phytophthora megasperma f.sp. glycinea or treated with $AgNO_3$ [Moesta and Grisebach, 1981b; Stossel and Magnolato, 1983]. In cotyledons, glyceollin I and III have been reported to occur in roughly equal amounts following treatment with $CuCl_2$ [Lyne et al.,1976] but not with $AgNO_3$ [Stossel and Magnolato, 1983]. In leaves infiltrated with bacteria or sodium iodoacetate [Ingham et al., 1981] glyceollin III was reported to be the main constituent. Although these reports suggest that there are major differences in the ability of soybean organs to synthesize the three isomers, the possibility remains that the differences result from a combination of different experimental conditions and the use of different eliciting agents. None of these studies has examined the relationship of the accumulation of the three isomers to resistance and susceptibility in the host-

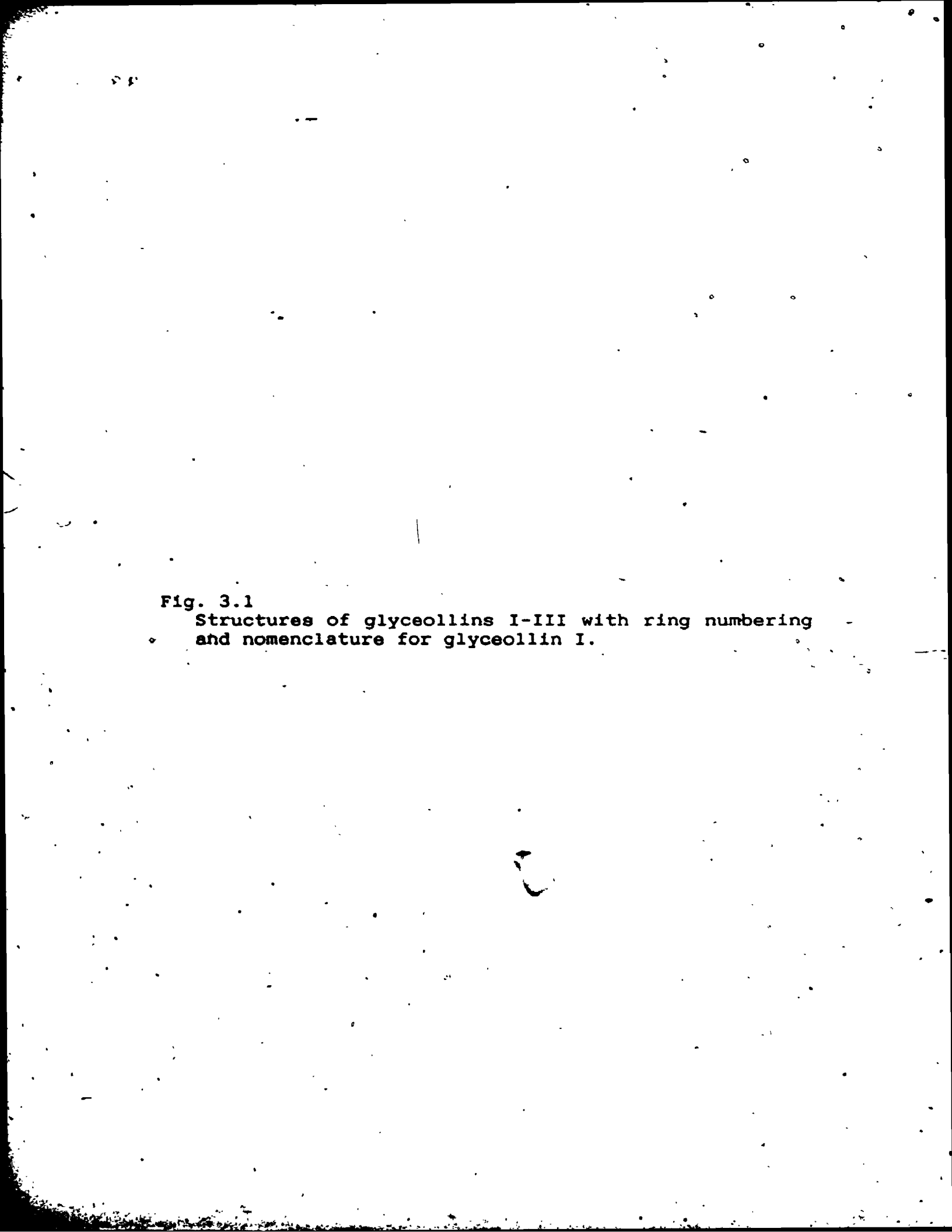
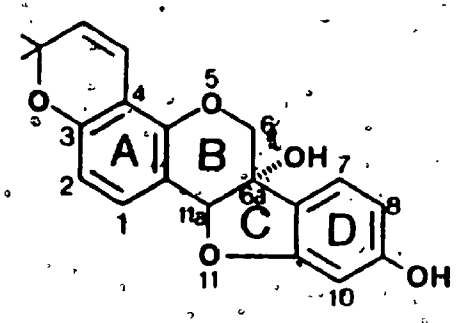
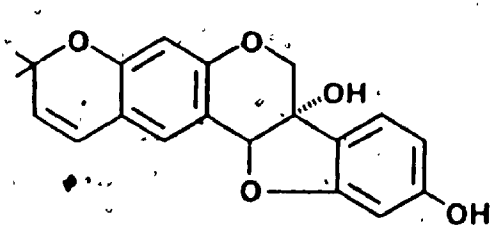


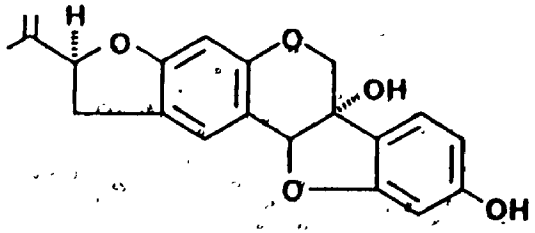
Fig. 3.1
Structures of glyceollins I-III with ring numbering and nomenclature for glyceollin I.



Glyceollin I



Glyceollin II



Glyceollin III

pathogen interaction.

The development of soybean cultivars resistant to Phytophthora megasperma f.sp. glycinea and the demonstration of major genes (Rps_1) for resistance have been achieved using an assay procedure that involved insertion of mycelium of the fungus into hypocotyl wounds [for example, Athow and Laviolette, 1982]. This procedure has also been used widely in studies of the involvement of glyceollin in the resistance of soybeans to Phytophthora megasperma f.sp. glycinea [Keen et al., 1971; Yoshikawa et al., 1978]. Keen and Horsch [1972] concluded that monogenic resistance is expressed only in hypocotyls and that other soybean organs are not suitable for the study of glyceollin production in relation to race specific resistance. This view was re-examined in this chapter, and the accumulation of three glyceollin isomers in roots, hypocotyls, cotyledons and leaves in compatible and incompatible interactions with Phytophthora megasperma f.sp. glycinea was studied.

3.3 Materials and Methods

3.3.1 Host

Soybean cultivars Harosoy and Harosoy 63 were used in the present studies. Etiolated seedlings were grown in trays of vermiculite for 6 days in the dark as described in chapter 2.1.1. Green seedlings were grown similarly but for 8 days and with a daily light period of 16h (300 $\mu Em^{-2}/s$). Unifoliate leaves used in the time course study of glyceollin accumulation were obtained from 12-day-old plants grown in a soil mixture (black muck:peatmoss:sand::5:2:1, pasturized for 30 min at 100°C) with a daily light period of 15 h (150 $\mu Em^{-2}/s$), at 80% RH and temperatures of 24°C in the light and 20°C in the dark.

3.3.2 Pathogen

Phytophthora megasperma f.sp. glycinea race 1 was grown routinely on V-8 juice agar at 25°C and zoospores were produced following methods described previously in chapter 2.2.2, and a 10 µl drop of zoospores suspension (10^5/ml) was used as inoculum.

3.3.3 Abiotic Elicitor

A freshly prepared solution of $AgNO_3$ (10^{-3} M) was used as an abiotic elicitor of glyceollin [Stossel,1982]. It was applied to etiolated hypocotyls in 10 µl drops in the same way as zoospore suspensions.

3.3.4 Inoculation of Soybean Organs

For inoculation of hypocotyls, etiolated or green seedlings were arranged horizontally in glass trays as described in chapter 2.1.2. Etiolated hypocotyls were inoculated by placing a 10 µl drop of zoospore suspension or $AgNO_3$ solution on the hypocotyl surface approximately 2 cm below the cotyledons. The green hypocotyls were wounded (surface wounds, 4 mm long, 0.5 mm deep) at aproximately 2 cm below the cotyledons and inoculated by applying a drop (10 µl) of zoospore suspension to the wound. Unwounded green hypocotyls do not display typical cultivar-race specificity [Ward and Buzzell,1983].

Seedlings for root inoculation were grown from surface sterilized seeds (6% sodium hypochlorite for 30 s, rinsed six to seven times with sterile water) in sterilized vermiculite and watered with sterile water. Seedlings were removed from the vermiculite and the roots were washed gently with sterile water, blotted with cellucotton and the root surface allowed to dry in a stream of air. The seedlings then were arranged in trays lined with moist cellucotton, as for hypocotyls inoculation except that only

the root tips were covered and sterile water was used throughout. Immediately prior to inoculation, a surface wound, as described for green hypocotyls, was made on the tap root below the first secondary root, and then a 10 µl drop of zoospore suspension was applied to the wound.

Cotyledons were removed from the seedlings and placed abaxial surface down on moist filter paper in Petri dishes. The adaxial surfaces were wounded to a depth of approximately 0.5 mm by cutting with a metal cylinder 3 mm in diameter. A drop (10 µl) of zoospore suspension was applied to each wound.

Unifoliate leaves were detached from the 8-day-old seedlings and placed abaxial surface down on moist cellucotton on glass trays. They were inoculated on the upper surface by placing one drop of zoospore suspension on each half of the blade.

All inoculated or $AgNO_3$-treated organs, except for leaves in the time course study below, were incubated in the dark at 25°C and 100% RH.

For the study of the time-course of glyceollin accumulation in leaves, four unifoliates from 12-day-old seedlings were arranged, abaxial side down, on wet filter paper in Petri dishes (14 cm diameter) and inoculated on their adaxial surfaces with two drops of zoospore suspension. Inoculated leaves were incubated at 100% RH with a daily light period of 16 h (33 $\mu Em^{-2}/s$) at 22.5°C, and 16°C in the dark.

3.3.5 Determination of Fresh and Dry Weights of Lesion Tissues and Glyceollin Content

The following procedure was adopted to determine both the fresh weight, and the dry weight after extraction of the tissues of the lesions in the different organs. A volume of 5 ml of 95% ethanol was dispensed into a series of vials and the total weight of each vial plus ethanol was recorded. The

tissue of ten lesions from each organ was excised and immediately dropped into the ethanol in a vial, then the vial and contents were immediately re-weighed to obtain the fresh weight. Parallel control vials, without tissue, were treated in the same way to monitor any weight changes due to ethanol evaporation.

The ethanol and tissues were then boiled for 2 min, the ethanol extract was decanted, combined with two ethanol rinses (2 ml) of the tissue and reduced to dryness under reduced pressure. The dried residue was extracted three times with 2 ml of ethyl acetate and the combined extracts were subjected either first to TLC (leaf extracts in time-course experiments) or directly to HPLC as described in chapter 2.7. The tissue remaining in the vial was dried to constant weight at 65°C and the dry matter content was expressed as a percentage of the fresh weight.

Glyceollin isomers I, II and III in the ethyl acetate extracts or in the glyceollin mixture obtained after TLC were separated and quantitated by HPLC as in chapter 2.7 using an anlytical column (silica, Whatman Partisil 5, 250x4.6 mm i.d.) with 5% isopropanol in hexane (1.5 ml/min) as the mobile phase.

3.4 Results

3.4.1 Symptoms

Race-specific resistance in cv. Harosoy 63 and susceptibility in cv. Harosoy was clearly expressed in leaves, hypocotyls and roots but not in cotyledons (Table 3.1). Wounded cotyledons from etiolated seedlings were resistant in both cultivars (Fig. 3.2) and wounded green cotyledons were resistant in cv. Harosoy 63 and partly resistant in cv. Harosoy (Fig. 3.3).

Resistance responses were generally characterized by dark brown necrotic tissue restricted to the area of contact

Table 3.1

Reaction type, dry matter content, glyceollin accumulation and proportions of glyceollin isomers in lesions in soybean organs inoculated with Phytophthora megasperma f.sp. glycinea race 1

Organ	Cultivar[a]	Reaction[b] type	Dry matter content (%)	Glyceollins[c] (µg/g fresh weight)	Proportions[d] of glyceollin isomers
Roots of etiolated seedlings	Harosoy	S	3.73±0.1[e]	105±9	21.4:1:1.1
	Harosoy 63	R	5.63±0.6	1307±179	51.1:1:1.5
Roots of green seedlings	Harosoy	S	5.17±0.1	65±15	10.9:1:1.2
	Harosoy 63	R	7.25±0.3	443±13	8.6:1:0.7
Etiolated hypocotyls	Harosoy	S	3.59±0.1	201±7	8.7:1:0.6
	Harosoy 63	R	4.75±0.4	3270±419	5.8:1:0.6
Green hypocotyls	Harosoy	S	5.06±0.1	98±4	3.4:1:1.0
	Harosoy 63	R	6.70±0.3	3068±316	1.8:1:0.3
Etiolated cotyledons	Harosoy	R	22.83±1.0	4327±208	4.5:1:2.7
	Harosoy 63	R	22.23±1.2	2374±386	5.1:1:1.7
Green cotyledons	Harosoy	R-S	5.08±0.8	1111±175	2.8:1:2.2
	Harosoy 63	R	5.04±0.2	1894±447	2.9:1:2.1
Green leaves	Harosoy	S	13.06±0.7	499±79	1.9:1:3.1
	Harosoy 63	R	12.02±0.5	1632±205	0.8:1:1.9

[a] Harosoy 63 carries the Rps_1 gene for resistance to race 1 of Phytophthora megasperma f.sp. glycinea.

[b] S = susceptible, spreading lesion; R = resistant, restricted lesion; R-S = a dark brown necrotic lesion similar to R but with continuing spread.

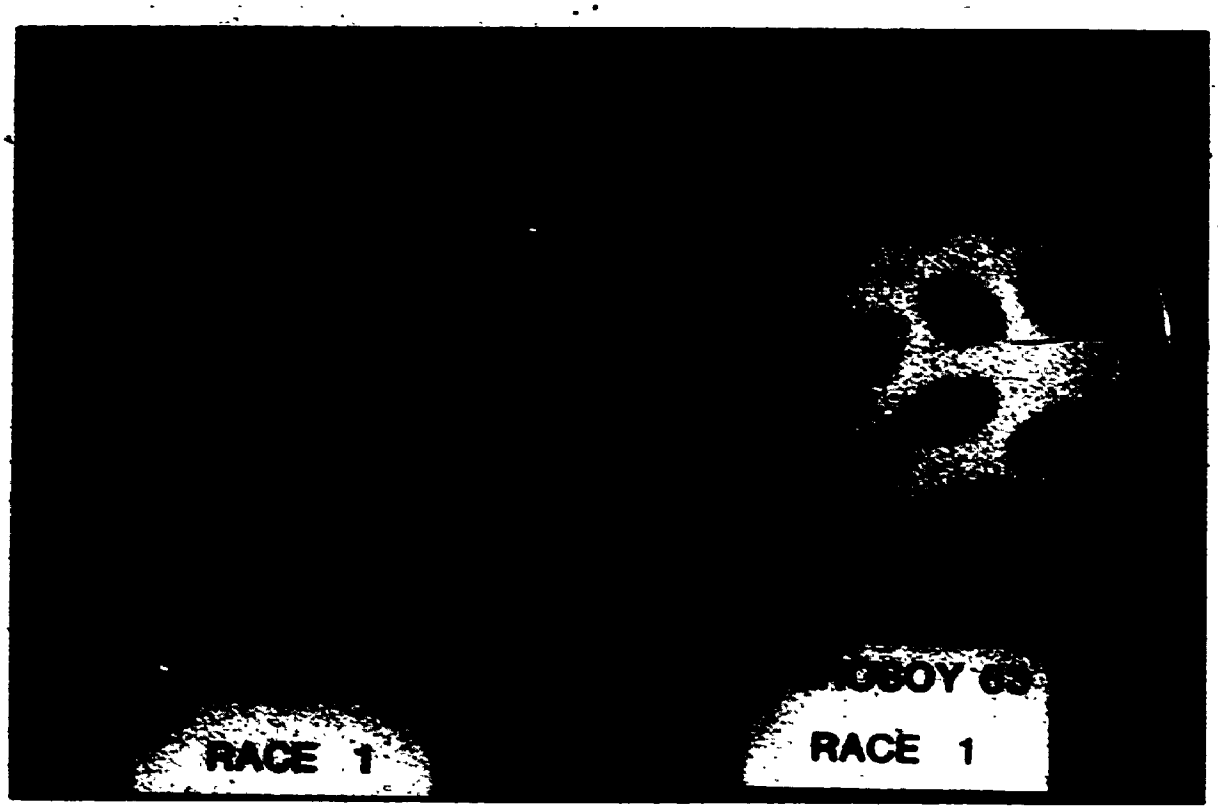
[c] Total of glyceollin I, II and III. Data are means and standard errors from three replications with 10 inoculated sites per treatment.

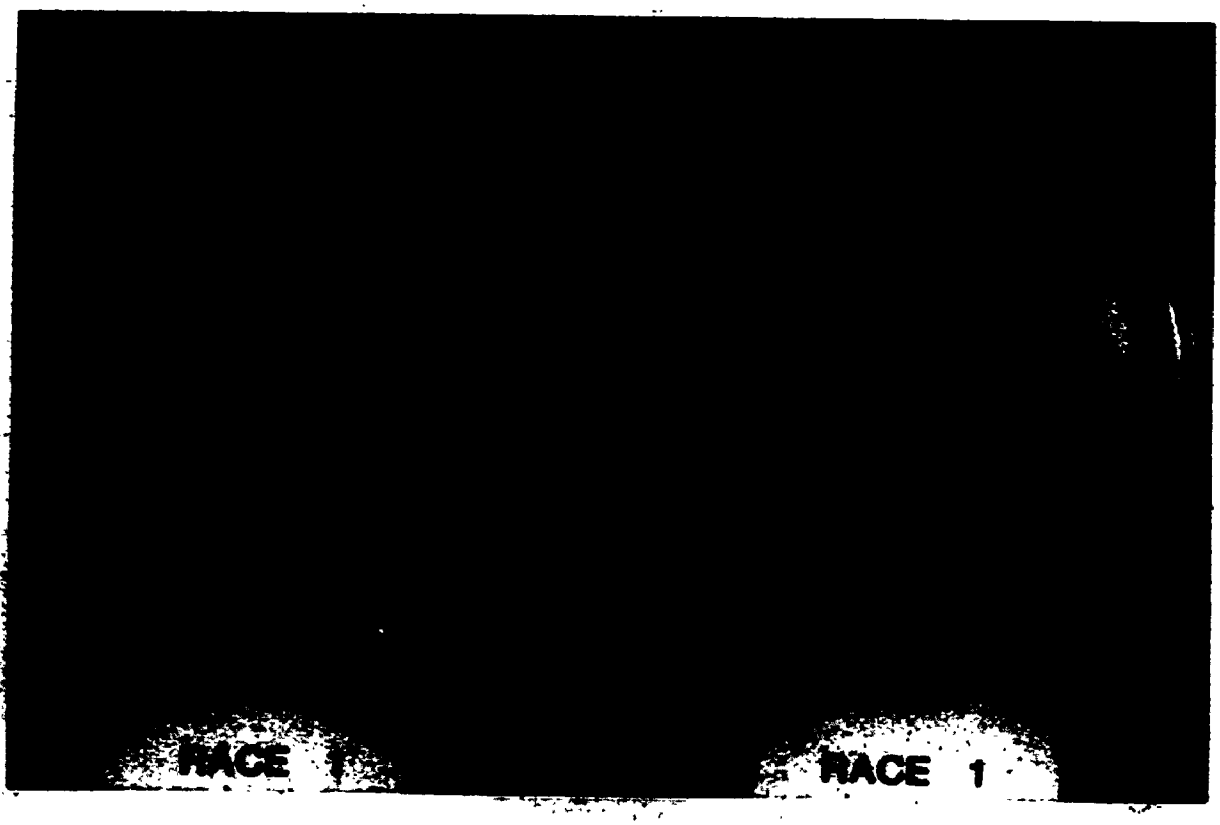
[d] Relative to glyceollin II.

[e] Values for dry matter content after ethanol extraction.

Fig. 3.2
The symptoms of disease development in wounded cotyledons of etiolated seedlings of cvs. Harosoy and Harosoy 63 48 h following inoculation with zoospores of Phytophthora megasperma f.sp. glycinea race 1.

Fig. 3.3
The symptoms of disease development in wounded green cotyledons of cvs. Harosoy and Harosoy 63 48 h following inoculation with zoospores of Phytophthora megasperma f.sp. glycinea race 1.


RACE 1
RACE 1


RACE 1
RACE 1

with the inoculum drop or with the surface of the wound in the tissues. Susceptible responses were more varied. In etiolated hypocotyls of cv. Harosoy, lesions spread rapidly with extensive water soaking and little discolouration (Fig.3.4). Spread of the lesion was similar in green hypocotyls but water soaking was less evident (Fig. 3.5). In roots, susceptible lesions were brown, but much paler than resistant lesions, and expanded slowly. In susceptible leaves, light brown lesions with indistinct margins developed, frequently spreading to cover the entire leaf (Fig. 3.6). Lesions in leaves commonly were bordered by a zone (1-2 mm) of pale green or yellow tissue. Where green cotyledons of cv. Harosoy were susceptible, dark brown, slowly spreading lesions developed (Fig. 3.3).

3.4.2 Dry Matter Content of Lesion Tissues

The dry matter content after ethanol extraction (percentage of fresh weight) varied widely among organs, being highest in cotyledons of etiolated plants and lowest in hypocotyls and roots of etiolated plants (Table 3.1). There was a major decrease in dry matter content of cotyledons in plants exposed to light. In hypocotyls and roots, the dry matter content of tissue from susceptible lesions was significantly less than that from resistant lesions.

3.4.3 Glyceollin Accumulation and Isomeric Proportions

Where resistance and susceptibility were expressed, glyceollin accumulated in higher concentrations in resistant reactions (Table 3.1). Greatest differences occurred in hypocotyls and smallest differences occurred in roots. Surprisingly, much lower concentrations were found in roots of green plants than in roots of etiolated plants.

The relative proportions of the three isomers of

Fig. 3.4
The symptoms of disease development in etiolated hypocotyls of cvs. Harosoy (S = susceptible) and Harosoy 63 (R = resistant) 48 h following inoculation with zoospores of Phytophthora megasperma f.sp. glycinea race 1 and symptoms of necrotic tissues 48 h following treatment with abiotic elicitor of glyceollin, $AgNO_3$ (Harosoy 63 only).

Fig. 3.5
The symptoms of disease development in green hypocotyls of cvs. Harosoy (S = susceptible) and Harosoy 63 (R = resistant) 48 h following wounding and inoculation with zoospores of Phytophthora megasperma f.sp. glycinea race 1.

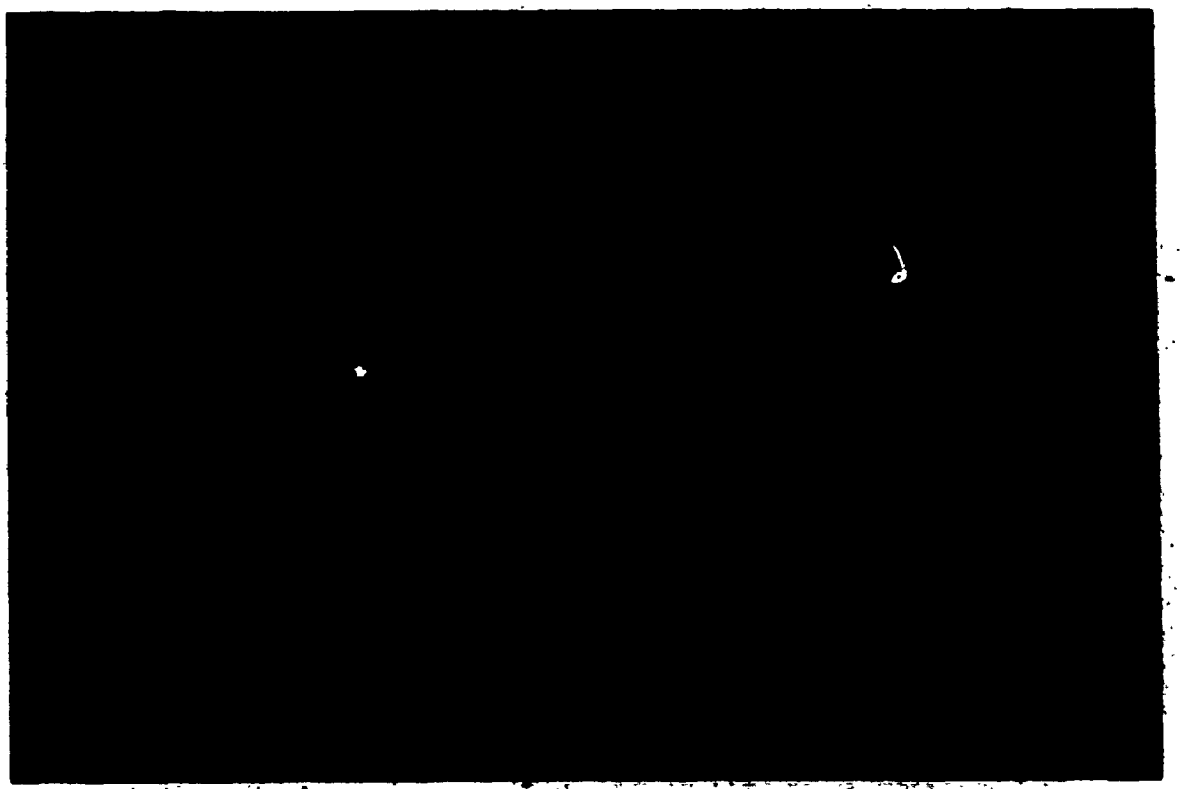
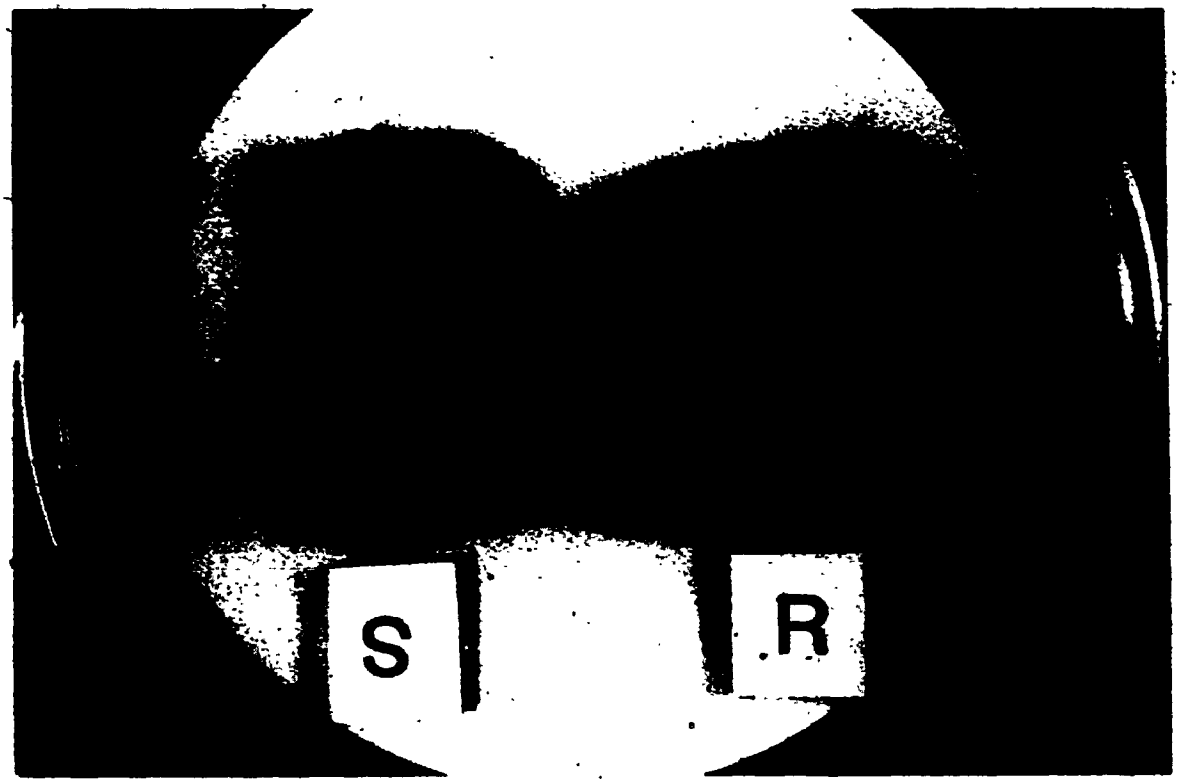


Fig. 3.6
The symptoms of disease development in unifoliates of 8-day-old green seedlings of cvs. Harosoy (S = susceptible) and Harosoy 63 (R = resistant) 48 h following inoculation with zoospores of Phytophthora megasperma f.sp. glycinea race 1.


S
R

glyceollin at 48 h after inoculation varied with reaction -type or cultivar, and organ (Table 3.1, Fig. 3.7). Glyceollin I was predominent in all organs except green cotyledons of cv. Harosoy and leaves of both cultivars. In the latter, glyceollin III was the main component and in green cotyledons, glyceollin I and III occurred in similar amounts. The proportion of glyceollin I to the other isomers varied widely, being highest in roots, especially in roots of etiolated plants where it accumulated almost to the exclusion of the other two isomers. There was a tendency for the relative proportion of glyceollin I in the mixture to be lower in the organs of green plants than in comparable organs of etiolated plants. In organs that developed resistant or susceptible reaction types, the proportions of the three isomers were invariably different in each reaction type.

3.4.4 Time-course of the Accumulation of Glyceollin Isomers

Etiolated hypocotyls and leaves can (1) be inoculated successfully without the complications of wounding, (2) develop distinctive resistant and susceptible reactions, and (3) yield primarily glyceollin I and glyceollin III, respectively. For these reasons they were selected for a time course study of the accumulation of the isomers.

3.4.4.1 Etiolated Hypocotyls

In the resistant reaction of cv. Harosoy 63 to infection *Phytophthora megasperma* f. sp. *glycinea*, glyceollin I was detected after 8 h and had reached a concentration of 600 µg/g fresh weight by 12 h (Fig. 3.8B). In the susceptible reaction of cv. Harosoy (Fig. 3.8A), glyceollin I did not reach this concentration at any time and was not detected until 12 h after inoculation. Maximum levels of glyceollin I in both interaction types were reached at 48 h, whereafter they declined. Glyceollin II and III were first

Fig. 3.7

Accumulation of glyceollin I (□), II (▨) and III (▦) in organs of 6- to 8-day-old etiolated (e) or green (g) seedlings of soybean cultivars Harosoy ($\underline{rps}_1$) and Harosoy 63 ($\underline{Rps}_1$) 48 h after inoculation with zoospores of *Phytophthora megasperma* f.sp. *glycinea* race 1; H= hypocotyls, R= roots, C= cotyledons, L= leaves. Data are the means and standard errors from three replications with 10 inoculated sites per treatment. No glyceollin was detected in water treated controls.

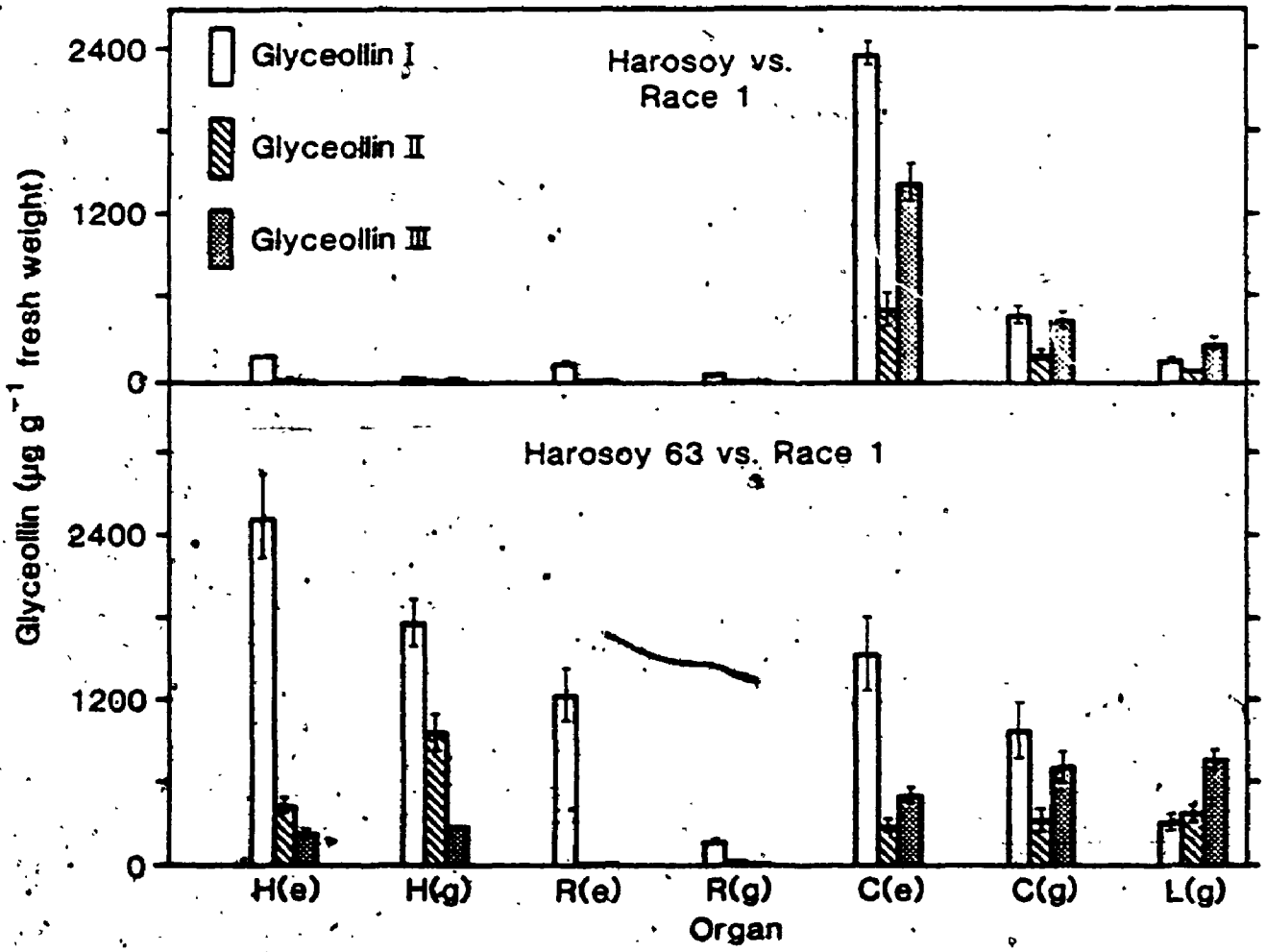
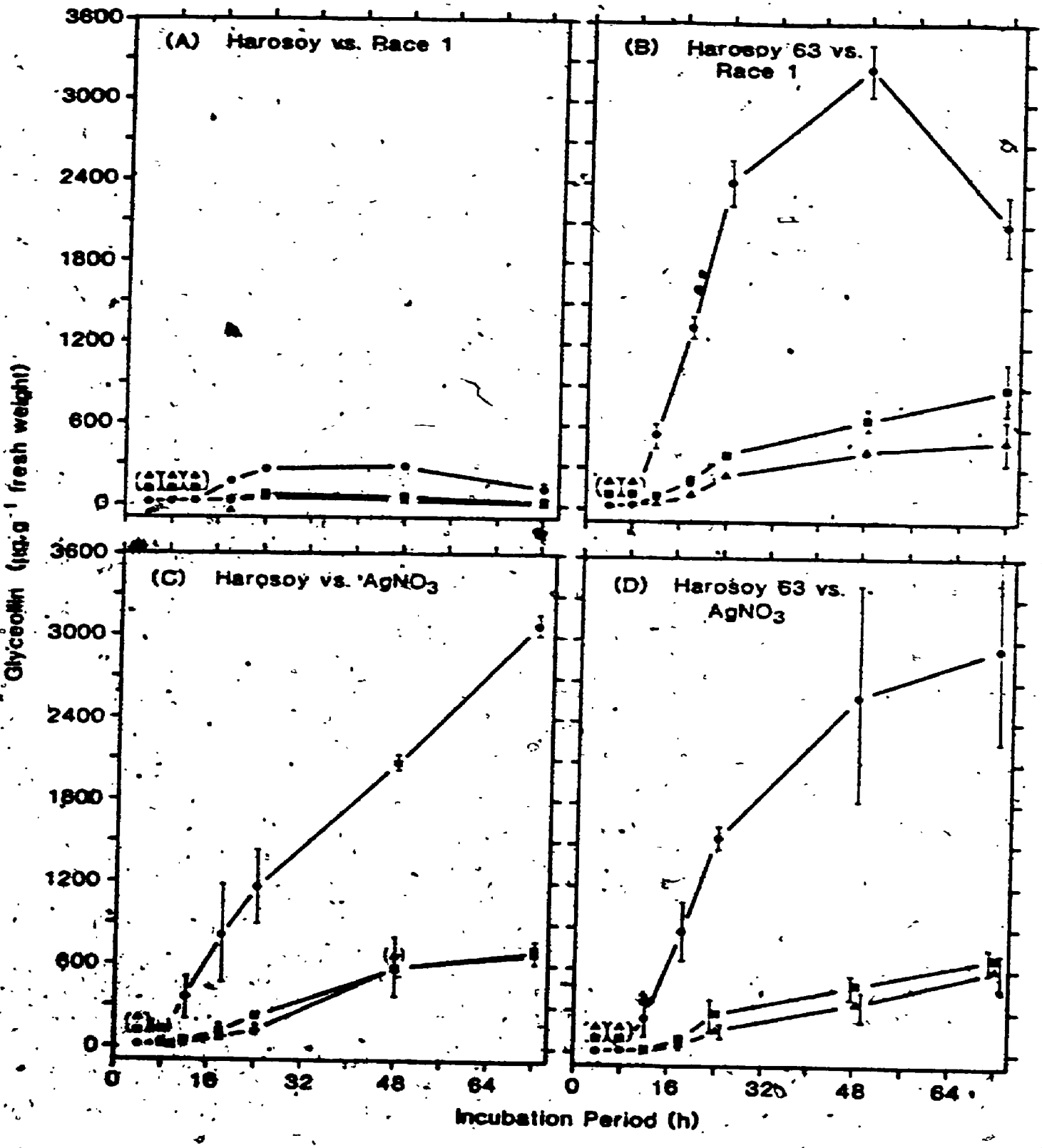

Glyceollin I
Glyceollin II
Glyceollin III
Harosoy vs. Race 1
Harosoy 63 vs. Race 1
2400
1200
0
Glyceollin (μg g⁻¹ fresh weight)
H(e)
H(g)
R(e)
R(g)
C(e)
C(g)
L(g)
Organ

Fig. 3.8

Time-course of accumulation of glyceollin I (●), II (■) and III (▲) in etiolated hypocotyls, of soybean cultivars Harosoy ($\underline{rps_1}$) and Harosoy 63 ($\underline{Rps_1}$) following inoculation with zoospores of *Phytophthora megasperma* f.sp. *glycinea* race 1 or $AgNO_3$ (10^{-3} M). Data are the means and standard errors from two replications with 10 inoculated sites per treatment. No glyceollin was detected in water treated controls.


(A) Harosoy vs. Race 1
(B) Harospy 63 vs. Race 1
(C) Harosoy vs. $AgNO_3$
(D) Harosoy 63 vs. $AgNO_3$
Glyceollin (μg.g⁻¹ fresh weight)
Incubation Period (h)
0
600
1200
1800
2400
3000
3600
16
32
48
64

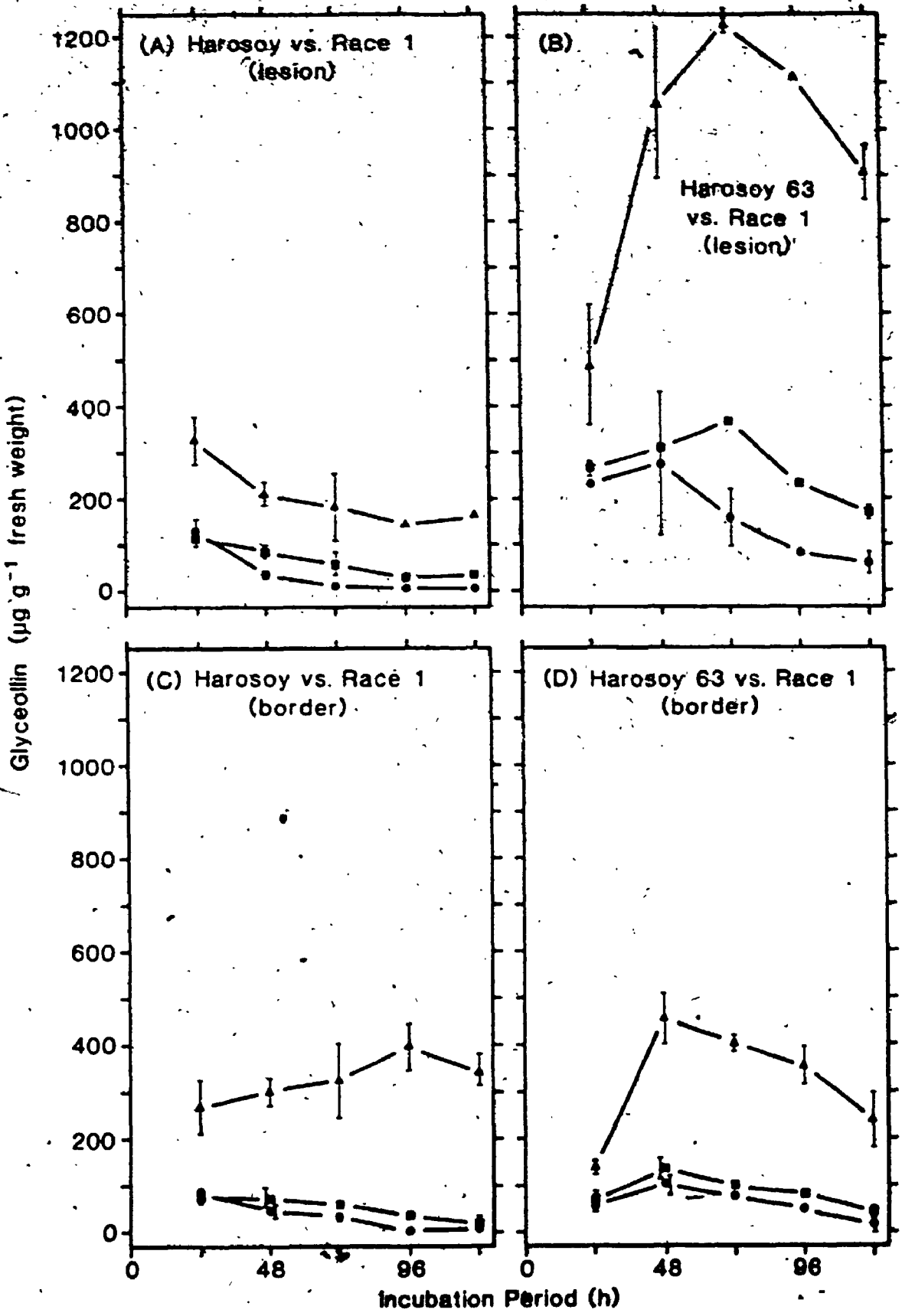
detected at 12 h in the resistant reaction of Harosoy 63 and from about 18 h in the susceptible reaction of cv. Harosoy. In cv. Harosoy 63, they continued to accumulate slowly throughout the course of the experiment (72 h) but changed little in concentration in cv. Harosoy.

Following treatment of hypocotyls with $AgNO_3$ (Fig. 3.4), the pattern of accumulation of the three glyceollin isomers was similar in both cultivars (Fig. 3.8C and 3.8D). In general, it was comparable to that in the resistant reaction of cv. Harosoy 63 to Phytophthora megasperma f.sp. glycinea, except that, unlike the infected tissue, there was no decline in accumulation after 48 h.

3.4.4.2 Leaves

The concentrations of the three glyceollin isomers were determind in lesions on leaves of cv. Harosoy and cv. Harosoy 63 infected with Phytophthora megasperma f.sp. glycinea at 24 h intervals until 120 h after inoculation (Fig. 3.9A and 3.9B). The concentrations of the isomers were determined also in the zone of lighter coloured leaf tissue bordering the lesions (Fig. 3.8C and 3.8D). Within the first 24 h of inoculation, lesions on leaves were too small to excise from uninfected tissue with accuracy. Glyceollin III was the predominent isomer in lesions in both cultivars and in the tissue bordering the lesions. Concentrations of glyceollin I and II were generally similar to each other, declining from the initial concentrations at 24 h in cv. Harosoy and, after small increases, from maximum at 48 or 72 h in cv. Harosoy 63. In the compatible interaction (Harosoy, Fig. 3.9A and 3.9C), the concentrations of these two isomers in the border tissue were generally similar to those in the lesion tissue, whereas in the incompatible interaction (Fig. 3.9B and 3.9D), concentrations were much higher in the lesion tissue than immediately outside.

Fig. 3.9
Time-course of accumulation of glyceollin I (●), II (■) and III (▲) in lesions or the tissue bordering the lesions in leaves of soybean cultivars Harosoy ($\underline{rps}_1$) and Harosoy 63 ($\underline{Rps}_1$) following inoculation with drops of zoospore suspensions of Phytophthora megasperma f.sp. glycinea race 1. Data are the means and standard errors from two experiments with 40 (Harosoy) or 80 (Harosoy 63) inoculated sites per treatment. No glyceollin was detected in water treated controls.


(A) Harosoy vs. Race 1 (lesion)
(B)
Harosoy 63 vs. Race 1 (lesion)
(C) Harosoy vs. Race 1 (border)
(D) Harosoy 63 vs. Race 1 (border)
Glyceollin (µg g⁻¹ fresh weight)
1200
1000
800
600
400
200
0
0
48
96
Incubation Period (h)

Glyceollin III declined from the initial levels at 24 h in lesions in the compatible interaction (Fig. 3.9A) but increased rapidly to a peak at 72 h before declining in the incompatible interaction (Fig. 3.9B). In the tissue bordering the lesion, glyceollin III increased slowly until 96 h in the compatible interaction (Fig. 3.9C) reaching concentrations higher than within the lesion. In the incompatible interaction, concentrations of glyceollin III in the border tissue reached a peak at 48 h (24 h prior to that within the lesion) but were much lower than within the lesion.

3.5 Discussion

With the exception of cotyledons of etiolated seedlings, which were resistant in both cultivars, all organs examined were resistant to Phytophthora megasperma f.sp. glycinea race 1 in cv. Harosoy 63 (Rps1) and susceptible in cv. Harosoy (rps1). Thus the Rps1 gene is expressed in each of these organs. This is consistent with the conclusions of several authors for roots or cotyledons [Hahn et al., 1985; Keeling, 1976; Lockwood and Cohen, 1978; Morrison and Thorne, 1978; Schmitthenner, 1972] but not with those of Keen and Horsch [1972] who considered that roots, cotyledons and pods did not express monogenic resistance. In cotyledons of etiolated seedlings, and also, as reported previously [Lazarovits et al., 1981; Ward and Buzzell, 1983], in mature or intact green hypocotyl tissues, other factors presumably contribute to or duplicate major gene controlled resistance. In cotyledons of etiolated seedlings, one factor may be their very low moisture content, providing an environment unsuitable for growth of the fungus.

Glyceollin concentrations were higher in resistant reactions than in susceptible reactions in each of the organs examined except cotyledons of etiolated plants. The

differential varied widely, however, being greatest in hypocotyls and smallest in roots of green seedlings. There were also wide differences in the concentrations determined in different organs. Lowest concentrations were found in roots, and the concentrations in roots and cotyledons were further influenced by the presence or absence of light during growth of the seedlings.

The activity of glyceollin *in vivo* is not known but estimates of fungitoxicity of undefined mixtures to *Phytophthora megasperma* f.sp. *glycinea* *in vitro* have ranged from ED_{50} values of 25 to 100 µg/ml [Chapter 5; Keen *et al.*, 1971; Lazarovits and Ward,1982; Stossel, 1983; Yoshikawa *et al.*, 1978]. On this basis, concentrations of the isomers combined were sufficient to at least partially restrict spread of the pathogen in lesions in all organs examined (Table 3.1). These included susceptible lesions, however, in which spread of the pathogen was not restricted. Therefore, either fungitoxicity of glyceollin is much lower in infected tissues than *in vitro*, or mechanisms exist in compatible interactions, such as localized compartmentalization or metabolism of glyceollin, that reduce the effectiveness of glyceollin.

The relative proportions of the three glyceollin isomers varied with the organ analysed, the presence or absence of light during seedling growth, the reaction type, and the incubation period. In general they conform to those in other published reports. Thus, the almost exclusive dominance of glyceollin I in roots confirms a recent report by Hahn *et al.* [1985] for *Phytophthora megasperma* f. sp. *glycinea*-infected roots. In hypocotyls, glyceollin I was also the most abundant isomer as reported by Moesta and Grisebach [1981b] and Stossel and Magnolato [1983] although, relatively, much more glyceollin II and III accumulated than in roots. Stossel and Magnolato [1983] also observed, in

agreement with the present findings, that glyceollin I was the main component in hypocotyls following treatment with $AgNO_3$ as well as following infection, and further that proportions were influenced by cultivar and by hypocotyl age. The production of glyceollin I and III in roughly equal amounts in green cotyledons is consistent with the early report of Lyne et al. [1976] for cotyledons treated with $CuCl_2$. The demonstration that glyceollin III is the most abundant isomer in leaves is consistent with similar findings by Ingham et al. [1981] for leaves infiltrated with bacteria or sodium iodoacetate. In general there appeared to be a tendency for more glyceollin III to occur in green tissues whereas glyceollin I predominated in etiolated tissues.

The results reported here and their overall similarities with those from other sources, suggest that each organ has a characteristic pattern of accumulation for the three isomers. The isomers are believed to arise from a common precursor, glycinol [Banks and Dewick, 1983; Zahringer et al., 1979] by prenylation at either the 2- (glyceollin II and III) or 4-position of the A-ring, followed by cyclization (Fig. 3.1, Fig. 7.7). The organ-type, reaction-type and light-related differences in accumulation of the isomers indicate that both these steps come under independent control. Differences in the relative accumulation of the isomers in compatible and incompatible interactions suggest that during infection there is differential control either of these terminal biosynthetic steps or of subsequent metabolism of the isomers in the two interactions.

Evidence that the isomers may be differentially metabolised is provided by the time-course of their accumulation in hypocotyls (Fig. 3.8). In the incompatible interactions, glyceollin I concentration reached a peak at 48 h and declined thereafter, whereas glyceollin II and III continued

to accumulate (Fig. 3.8B). In leaves all three isomers declined in concentration after reaching a peak. This was most noticeable for glyceollin III within lesions of the incompatible interaction, but similar and earlier decreases in concentration occurred in the tissue bordering the lesions (Fig. 3.9B, D). In the compatible interaction in leaves, highest concentrations occurred at 24 h in the lesions (Fig. 3.9A). In the border tissue surrounding these expanding lesions(Fig. 3.9C), the production of glyceollin III evidently occurred at a fairly constant rate as new tissue was invaded. Concentrations of the three isomers in the border tissue in the compatible interaction equalled or exceeded those in the incompatible interaction and, at 24 h, concentrations within the lesions did not differ greatly between the two interactions. This suggests that in leaves, initially, glyceollin accumulation is similar in both interactions and that much of the additional accumulation in the incompatible interaction occurs after the lesion has ceased to spread.

CHAPTER 4

EXPRESSION OF GENE-SPECIFIC AND AGE-RELATED RESISTANCE AND THE ACCUMULATION OF GLYCEOLLIN IN SOYBEAN LEAVES INFECTED WITH *Phytophthora megasperma* f.sp. *glycinea*

4.1 Summary

Detached leaves of soybean cultivars Harosoy 63 (*Rps*$_1$) and Harosoy (*rps*$_1$) were inoculated without wounding, with drops of zoospore suspension of *Phytophthora megasperma* f.sp. *glycinea*, race 1 (avirulent with *Rps*$_1$). Typically where leaves were resistant, lesions were restricted and dark brown with sharply defined margins, whereas where leaves were susceptible, lesions spread to cover the entire blade. Glyceollin accumulated in high concentrations in restricted lesions. Expression of resistance and susceptibility was influenced by leaf maturity as well as by the *Rps*$_1$ gene. Immature leaves of both cultivars were susceptible. When fully expanded, leaves of cv. Harosoy initially were suscetible, those of cv. Harosoy 63 were resistant. As they aged, the leaves of cv. Harosoy became increasingly resistant also. Both resistant and susceptible lesions developed on some leaves that were not fully mature. Age-related resistance and *Rps*$_1$-gene mediated resistance resulted in lesions that were similar in size and colour and contained comparable amounts of glyceollin. The *Rps*$_1$ gene therefore influenced disease expression for only a part of the life of the leaf, extending resistance to younger but not to the youngest leaves. Only during this period can race specific resistance of leaves be assayed or physiological differences between race-specific resistant and susceptible responses assessed.

4.2 Introduction

Phytophthora rot of soybeans caused by Phytophthora megasperma f.sp. glycinea has been reported to develop on all parts of the soybean plant [Sinclair, 1982]. However, it is primarily a disease of the roots and hypocotyls. It can cause both pre and post-emergence damping off resulting in rapid death of the plant and, in older plants, extensive spreading lesions that develop more slowly [Schmitthenner, 1985]. Resistance to the pathogen is provided by a series of dominant major (Rps) genes in seven loci with several allelic forms at two of the loci [Schmitthenner, 1985]. The phytoalexin, glyceollin, accumulates in infection sites in resistant plants and is generally considered to play a role in limiting spread of pathogen [Keen and Yoshikawa, 1983].

Assessment of resistance has been based widely upon a procedure in which mycelium of the pathogen is inserted into wounds made in the hypocotyl of young seedlings [Keeling, 1976]. This procedure, because of its simplicity, has been invaluable in assessing resistant segregants in the development of resistant cultivars. However, evidence has been provided that indicates that the expression of the identified genes for resistance may be influenced by environmental factors [Ward and Buzzell, 1983]. In addition, non-specific age-related resistance has been demonstrated in stems or hypocotyls of "susceptible" soybean plants [Lazarovits et al., 1981; Paxton and Chamberlain, 1969; Stossel et al.,1981; Ward et al., 1981] and race non-specific tolerance or field resistance governed by a limited number of genes occurs in some susceptible cultivars [Walker and Schmitthenner, 1984].

Relatively few studies have been made of resistance in parts of the soybean plant other than the hypocotyls and the results have been inconsistent. Thus, Keen and Horsch [1972] observed a clear distinction between resistance and

susceptibility only in hypocotyls and not in pods, cotyledons or roots. Other authors, however, have found that resistant and susceptible responses can be distinguished satisfactorily in inoculated roots or cotyledons [Hahn et al., 1985; Keeling, 1976; Lockwood and Cohen, 1978; Morrison and Thorne, 1978; Schmitthenner, 1972]. Only one report of infection of soybean leaves by Phytophthora megasperma f.sp. glycinea is available. Morgan [1963] described distinct limited lesions on leaves of resistant plants and spreading watersoaked lesions on leaves of susceptible plants. He also reported that leaves could become infected in the field under favourable weather conditions. Infection of leaves in the field and the development of symptoms is referred to also in a general description of the disease [Sinclair, 1982].

Leaf inoculation would have advantages over other procedures both in the screening of plants for disease resistance and in studies of physiological aspects of disease development. In the former, many races of the pathogen could be assessed on a single plant, which subsequently could be grown to maturity, whereas, in the latter, the pathogen would be more accessible both to observation and to manipulation through the intercellular spaces of the leaf, than in roots or stems.

Glyceollin has been shown to accumulate in soybean leaves in incompatible interactions with bacteria [Keen and Kennedy, 1974], but there have been no reports of its production in response to inoculation with Phytophthora megasperma f.sp. glycinea. In this chapter resistance and susceptibility and the production of glyceollin in leaves of genetically resistant and susceptible plants is examined. The results indicate that race specific resistance is expressed and that this is associatd with glyceollin production but that both are greatly influenced by maturity of the

leaves and of the plant.

4.3 Materials and Methods

4.3.1 Host

Plants of soybean cultivars Harosoy and Harosoy 63 were grown singly in pots (15 cm diameter), for 2, 3 or 4 weeks, or in trays (16x24 cm, 40 seeds per tray; for the experiment reported in Table 4.2), for 12, 13 or 14 days, in a mixture of blackmuck, peat moss and sand (5:2:1), pasturized for 30 min at 100°C. They were grown in a growth room at 95% RH with a daily 15 h light period (approx. 150 $\mu Em^{-2}/S$ of photosynthetically active radiation). The temperature was 23°C for the light period and 16°C for the dark period. Plants of 5 weeks of age and older were also examined in preliminary experiments but the lower leaves of these plants usually were senescent and dropped from the plants.

4.3.2 Pathogen

Phytophthora megasperma f.sp. glycinea race 1 was used throughout. It was grown routinely and for the production of zoospores on V8-juice agar in Petri dishes at 25°C in the dark. Zoospore suspensions (10^5/ml) were prepared following the methods described in chapter 2 [2.2.2.2].

4.3.3 Inoculation and Incubation

Leaves were detached from the plants by cutting the petioles and immediately inserting the cut ends into a layer of wet cellucotton in glass trays, the leaves themselves resting horizontally on the cellucotton, adaxial surface uppermost. Primary leaves (unifoliates) were inoculated by placing 4 drops (each 10 µl) of zoospore suspension on the leaf surface, the trifoliates were inoculated by placing 1-4 drops of zoospore suspension on each of the three leaflets. The trays were closed with plastic films and left undis-

turbed on the laboratory bench under room temperature and light conditions for 3-4 h before transfer for 44 h to a growth cabinet at 100% RH with a daily 16 h light period (fluorescent lamps; approx. 33 $\mu Em^{-2}/s$). The temperature was 22.5°C in the light and 16°C in the dark. Trays of leaves then were returned to the laboratory for examination and left under room conditions for a further 72 h when final observations were made.

Inoculum drops were easily dislodged from the leaf surface during the first five hours following inoculation. Some drops on very small leaves coalesced and occasionally drops remained suspended on hairs and failed to infect. Data were recorded only for inoculum drops that gave rise to visible lesions. Measurements were made of the minimum diameters of the lesions and reactions were classified as resistant (R), a dark brown lesion with little spread, and susceptible (S), spreading lesions with much less browning. Some lesions were intermediate (I) between the two or spread slowly from initially restricted lesions.

4.3.4 Glyceollin Determination

For each determination tissues of lesions from each leaf-type were excised, bulked and extracted first in ethanol and then in ethyl acetate following the methods described in chapter 2.7. Ethyl acetate soluble fractions were combined, dried and redissolved in 100 µl of ethyl acetate. From this, 20 µl was analysed by high performance liquid chromatography [Chapter 2.7] using 6% isopropanol in hexane (flow rate 1.5 ml/min) as mobile phase. Retention times for the glyceollin isomers I, II and III were 9.29, 10.14, 10.82 min respectively. The isomers were quantitated by reference to standard curves for each of the isomers, prepared using published extinction coefficients [Lyne et al., 1976; Chapter 5]. The values obtained were combined and

data were presented as glyceollin µg/g fresh weight of infected leaf tissue (Table 4.2).

4.4 Results

4.4.1 Symptoms

Infection of fully expanded unifoliates and trifoliates of Harosoy 63 typically gave rise to small lesions that spread little from the area of contact with the drop of zoospore suspension. In the most resistant responses, 48 h after inoculation, these were 2-3 mm in diameter, and dark brown with sharply defined margins (Fig. 3.6). In contrast, in leaves of Harosoy that were susceptible, typical lesions were much larger and continued to spread until the entire leaf or leaflet was colonized (Fig. 3.6). These lesions were brown but less intensely so than in the resistant response and usually their margins were much less clearly defined. Frequently the lesions in susceptible leaves were surrounded by a yellow zone that advanced as the lesions spread. Conspicuous small lesions developed in both resistant and susceptible leaves by 24 h following inoculation, however, differences between resistant and susceptible responses could not always be distinguished with the unaided eye at this stage.

4.4.2 Influence of Leaf and Plant Age on Lesion Development and Reaction Type

Soybean plants produce one pair of unifoliate leaves (leaf 0, Fig. 3.6 and 4.1) and a series of trifoliate leaves (leaf 1-5, Fig. 4.1 and 4.2). The effect of leaf age can be assessed for leaves 0 and 1, which were present on plants of the three ages (Fig. 4.1). In Harosoy 63, lesions on leaf 1 were significantly smaller in 3-week-old plants than in 2-week-old plants. There was also a trend towards smaller lesions on leaf 0 as the leaves aged (Fig. 4.3). There was

Fig. 4.1

Influence of plant and leaf age on lesion size in leaves of soybeans, cultivars Harosoy and Harosoy 63, inoculated with zoospores of Phytophthora megasperma f.sp. glycinea race 1. Harosoy 63 carries the Rps_1 gene that conditions race specific resistance to race 1. Plants were 2, 3 or 4-weeks old, and accordingly bore a pair of unifoliates and 1, 3 or 5 trifoliates. Lesion diameters were determined after 48 h (□) and after 120 h (□ + ▦). The data are the means of two experiments each with two replications, each replication consisted of the leaves of a single plant. Standard errors were calculated for the individual observations while LSDs were determined after analysing in a completely randomized block design considering each experiment as a replication.

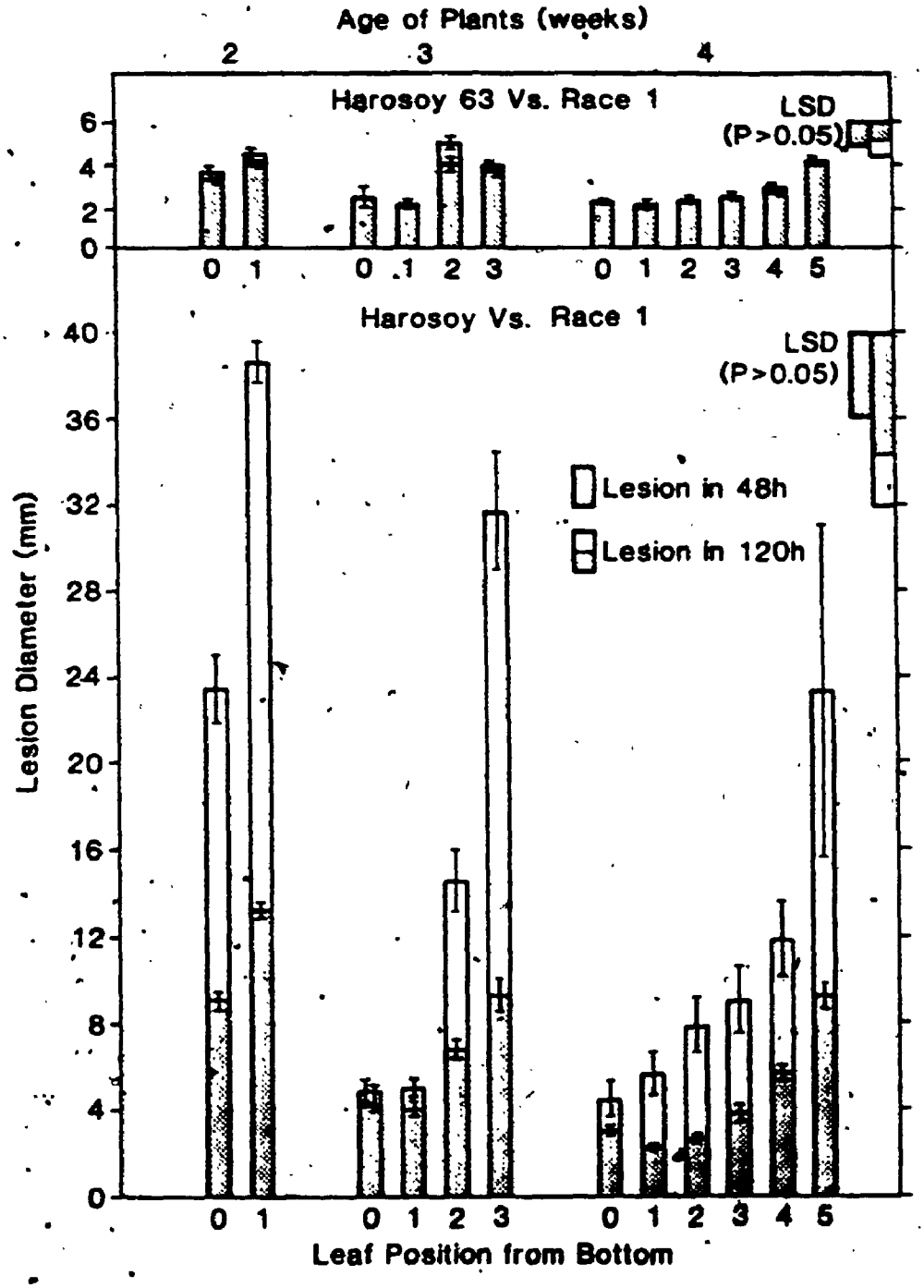

Age of Plants (weeks)
2
3
4
Harosoy 63 Vs. Race 1
LSD
(P>0.05)
6
4
2
0
0 1
0 1 2 3
0 1 2 3 4 5
Harosoy Vs. Race 1
LSD
(P>0.05)
Lesion in 48h
Lesion in 120h
Lesion Diameter (mm)
40
36
32
28
24
20
16
12
8
4
0
0 1
0 1 2 3
0 1 2 3 4 5
Leaf Position from Bottom

Fig. 4.2
The symptoms of disease development on leaves (from left, 0 to 5 as in Fig. 4.1) of 4-week-old plants of cvs. Harosoy 63 (A) and Harosoy (B) 120 h following inoculation with zoospores of Phytophthora megasperma f.sp. glycinea race 1.

Fig. 4.3
The symptoms of disease development on unifoliates (leaf 0 of Fig. 4.1) of (from left) 2-, 3-and 4-week-old plants of cvs. Harosoy 63 (A) and Harosoy (B) 120 h following inoculation with zoospores of Phytophthora megasperma f.sp. glycinea race 1.

very little additional lesion-spread in any of the Harosoy 63 leaves after the initial incubation for 48 h. In the leaves of susceptible cv. Harosoy, age caused much greater differences in lesion size. There were significant reductions in lesion size both on leaf 0 and leaf 1 in the 3-week-old plants and further reductions in the 4-week-old plants. In the latter, the lesions were similar in size and appearance to those produced in Harosoy 63. Similar comparisons can be made for leaves 2 and 3 in the 3- and 4-week-old plants. In both cultivars there was a significant reduction in lesion size in the older leaves.

When the spread of lesions after 48 h was examined (Fig. 4.1, unshaded areas) further differences due to leaf age were evident. Thus in leaves 0 and 1 of both cultivars the spread of the lesions between 48 and 120 h was significantly less in the 3-week-old plants than in the 2-week-old plants. The difference in Harosoy leaves was especially notable. Similar differences occurred in leaves 2 and 3.

The effect of plant age on leaf susceptibility can also be evaluated in this experiment. For example, the youngest leaves in each of the plant age-groups are at the same stage of development, that is they are of the same age although the plant ages are different (Fig. 4.1). Leaf 1 on the 2-week-old plants, leaf 3 on the 3-week-old plants and leaf 5 on the 4-week-old plants can be compared therefore from this viewpoint. Lesion sizes on these leaves after 48 h were similar and were not affected by plant age in either of the cultivars except in Harosoy between the 2-and 3-week-old plants. Similar comparisions between other leaves at the same stage of development lead to similar conclusions (e.g. leaf 0 on 2-week-old plants, leaf 2 on 3-week-old plants and leaf 4 on 4-week-old plants, etc.). However, there were differences related to plant age in the extent to which lesions spread after the initial 48 h incubation period. The amount

of spread significantly decreased with age.

Presumably because of the effects of leaf age on lesion size, there was an overall trend in lesion size within each plant that was related to leaf position (Fig. 4.1). Thus the largest lesions in plants of both cultivars developed in the uppermost or youngest leaves and the smallest lesions developed in the lowest or oldest leaves of each plant.

Data presented in Fig. 4.1 are for fully expanded leaves. However, it was observed also that newly emerging trifoliates were susceptible in both cultivars. In each cultivar, immature trifoliates developed pale brown spreading lesions and entire leaflets were colonized rapidly (Fig. 4.4). Thus, almost all lesions that developed following inoculation of the unfolding first trifoliates of 12-day-old Harosoy 63 plants were of the susceptible reaction type (Table 4.1). Such leaves rapidly developed resistance, however, for in 14-day-old plants lesions were much smaller and a majority were of intermediate or resistant reaction types. Comparable changes did not occur in the fully developed unifoliates of the same plants, although lesions on the unifoliates of 12-day-old plants tended to be larger than on the unifoliates of 13- and 14-day-old plants. It was of interest that lesions of opposite and intermediate reaction-type could develop on the same leaf, both here, on the expanding trifoliates of Harosoy 63 (Table 4.1), and also, on the ageing leaves of Harosoy (Table 4.2, Fig. 4.2).

4.4.3 Accumulation of Glyceollin

In general, glyceollin concentrations in lesions were correlated with the degree of resistance expressed (Table 4.2). On leaves where all lesions were susceptible, for example in 13-day-old Harosoy plants, concentrations were very low, whereas where all lesions were resistant, for example

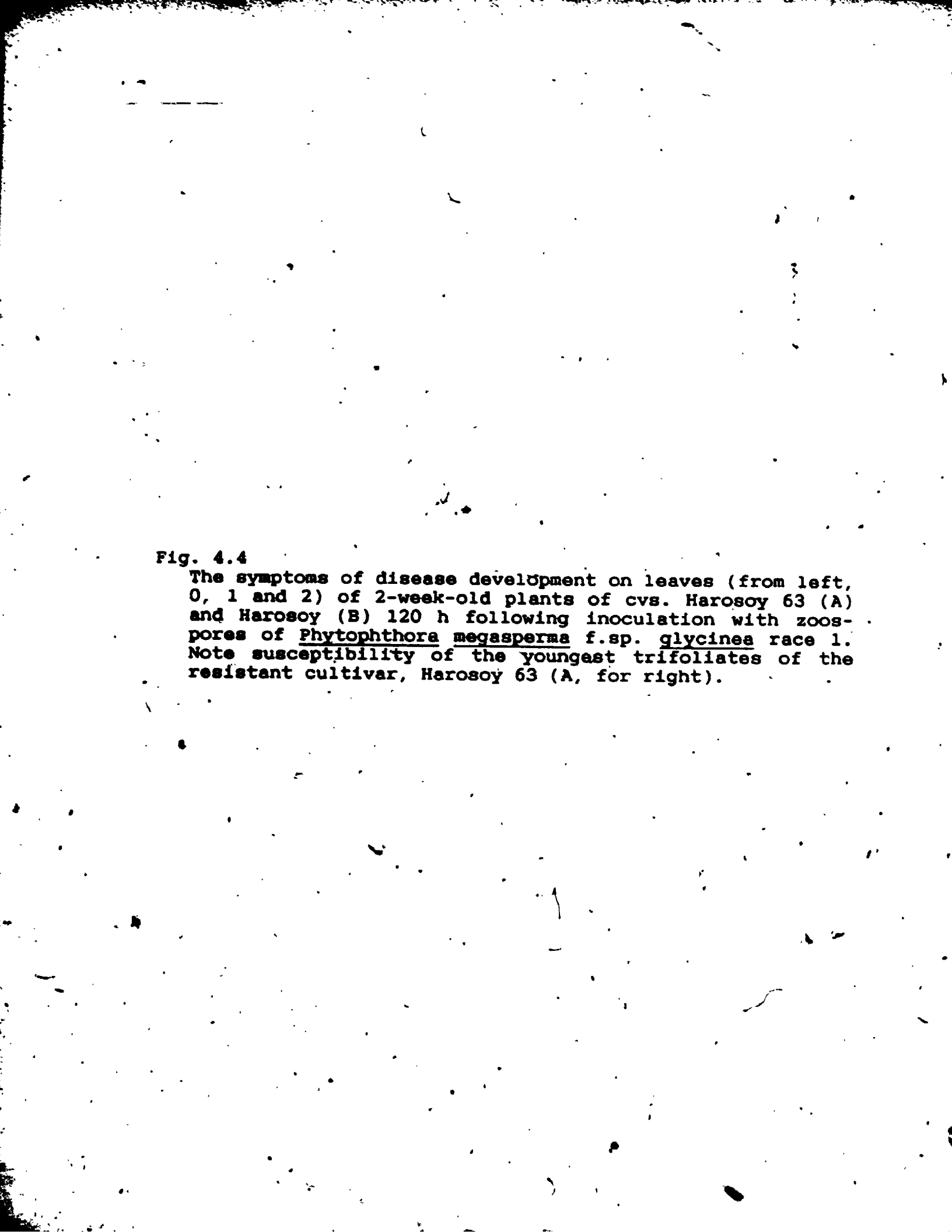
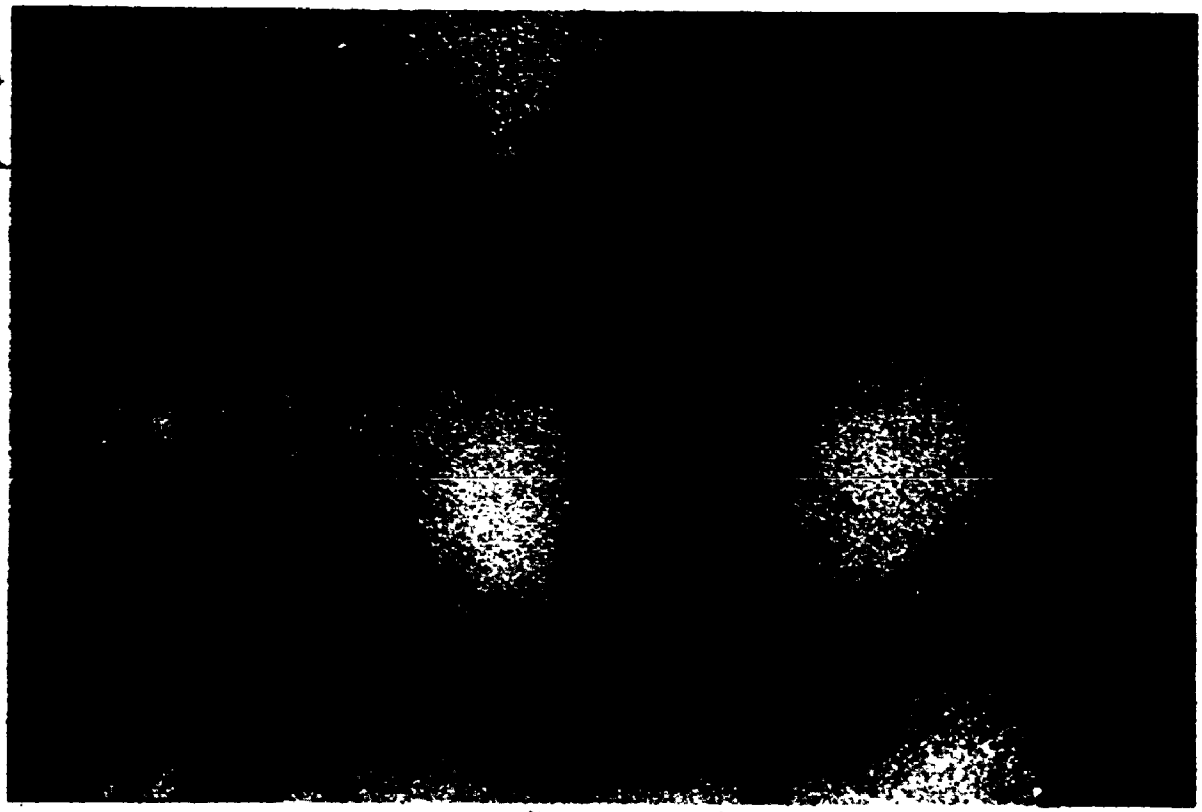


Fig. 4.4
The symptoms of disease development on leaves (from left, 0, 1 and 2) of 2-week-old plants of cvs. Harosoy 63 (A) and Harosoy (B) 120 h following inoculation with zoospores of Phytophthora megasperma f.sp. glycinea race 1. Note susceptibility of the youngest trifoliates of the resistant cultivar, Harosoy 63 (A, for right).



in 21-day-old Harosoy 63 plants, concentrations were very high. Where reaction types were mixed, the determined concentrations were intermediate, frequently with large standard errors. With respect to leaf age and position, the glyceollin concentrations reflect the differences in susceptibility observed in the previous experiments.

Table 4.1

Influence of plant age on reaction type and lesion size following inoculation of detached leaves of soybean cultivar Harosoy 63[a] with zoospores of *Phytophthora megasperma* f.sp. *glycinea* race 1[b]

	Unifoliate[c] lesions					Trifoliate lesions				
	Reaction type[d]					Reaction type[d]				
Plant age (days)	R	I	S	Mean diam[e] (mm)	Range (mm)	R	I	S	Mean diam[e] (mm)	Range (mm)
12[f]	146	4	0	1.8±1.0	1.0-11	2	7	94	8.1±0.3	2.0-20
13	135	0	0	1.8±0.1	0.5-3.0	53	37	50	4.7±0.2	0.5-10
14	134	0	0	1.7±0.1	0.5-3.5	110	25	21	3.3±0.3	1.0- 8

[a] Harosoy 63 carries the Rps_1 gene for resistance to race 1.
[b] Data from two experiments.
[c] Plants 12-14 days old have one pair of unifoliates and a single partially expanded trifoliate.
[d] Reaction-type was assessed after 48 h incubation as resistant (R), restricted brown lesion; susceptible (S) spreading lesion; intermediate (I) lesion. Numbers refer to lesions of each type.
[e] Mean diameter and standard errors calculated from all lesions.
[f] Following inoculation, detached leaves were incubated under a 16 h light, 8 h dark, cycle.

Table 4.2

Influence of leaf position, plant age on reaction type and glyceollin accumulation following inoculation of detached soybean leaves with zoospores of *Phytophthora megasperma* f.sp. *glycinea*, race 1[a]

		Harosoy				Harosoy 63			
		Reaction type[c]			Glyceollin[d]	Reaction type[c]			Glyceollin[d]
Plant age (days)	Leaf Position[b]	R	I	S	(µg/g fresh wt)	R	I	S	(µg/g fresh wt)
13[e]	0	0	1	13	1044± 286	14	0	0	4654± 493
	1	0	0	14	81± 1	5	4	8	400± 261
21	0	13	0	0	3736± 310	14	0	0	3906±1396
	1	22	0	0	3747± 753	15	0	0	3602±1136
	2	8	4	12	2318± 176	17	0	0	3811± 533
	3	13	0	12	1937±1344	13	6	0	3790± 233

[a] Harosoy 63 carries the Rps_1 gene for resistance to race 1.
[b] Leaf 0= the unifoliates, leaves 1,2 and 3 are the successively developing trifoliates.
[c] Data for reaction type and glyceollin were obtained after incubation for 48 h and are derived from two replications. Reaction types for individual lesions were resistant (R) restricted brown lesion, susceptible (S) spreading lesion, and intermediate (I) lesions.
[d] Sum of values determined for three glyceollin isomers.
[e] Following inoculation, detached leaves were incubated under a 16 h light, 8 h dark, cycle.

The accumulation of glyceollin was evidently related primarily to the reaction type and not to the cultivar. When all lesions were completely resistant as in leaves 0 and 1 of 21-day-old plants concentrations of glyceollin were virtually the same in both cultivars.

4.5 Discussion

The results reported confirm the early work of Morgan [1963] that leaves of soybean are susceptible to infection by Phytophthora megasperma f.sp. glycinea. They also demonstrated that the Rps_1 gene carried by Harosoy 63 is expressed in leaves, but that race specific differences in resistance and susceptibility can be complicated by age-related factors. For physiological studies of major gene resistance or for the development of assays for the selection of resistant plants, comparable leaves of specific age would be required. For example, the unifoliates (leaf 0) from 2-week-old plants satisfactorily differentiated resistance and susceptibility whereas the same leaves from 3-week-old or older plants did not, due to age-related non-specific resistance.

The increase in resistance in leaves as they age is comparable to similar changes in response reported in stems and hypocotyls of soybeans [Lazarovits et al., 1981; Paxton and Chamberlain, 1969; Stossel et al., 1981; Ward et al., 1981]. Whatever the basis for age-related increases in resistance in the two tissues, it appeares that Phytophthora megasperma f.sp. glycinea has a preference for young tissues. In hypocotyl infections, it rapidly kills plants causing pre- and post-emergence damping off. In older susceptible plants, the pathogen spreads much more slowly producing extensive brown lesions.

Glyceollin production has not been reported previously in soybean leaves following infection by Phytophthora

megasperma f.sp. glycinea, although glyceollin has been reported to accumulate to relatively low concentrations following infection with bacteria [Fett, 1984; Keen and Kennedy, 1974].

In the present study, high levels of glyceollin were invariably associated with resistant responses, although the differential between levels in resistant and susceptible responses was not as well defined as, for example, in hypocotyls [Ward and Buzzell, 1983; Ward and Lazarovits, 1982]. Some of the inconsistencies arise because in some combinations mixed responses developed in which lesion both of resistant and susceptible type were present on the same leaf (Fig. 4.2). The total glyceollin value then obscures the probability that in such mixtures individual lesions of susceptible type may have low glyceollin values and those of the resistant type, high values. The development of both resistant and susceptible reactions on the same leaf suggests that a critical balance between tendencies towards resistance and susceptibility exists. Presumably, local variations in leaf characteristics account for such differences. As these differences evidently duplicate on a local basis the effect of introducing an Rps gene for resistance, these genes also may condition resistance by introducing minor changes that serve to tip the balance in the interaction towards resistance.

It is evident that as individual leaves develop they pass from a stage of complete susceptibility to one of resistance that is independent of race specific resistance. Incorporation of the Rps_1 gene into the Harosoy genome is critical for resistance to race 1 of Phytophthora megasperma f.sp. glycinea for a limited period of leaf development only, for it is ineffective in very young leaves and is duplicated by age-related resistance as the leaves mature. Possibly the two forms of resistance supplement each other;

the solely age-related resistant lesions in Harosoy leaves had a tendency to spread as, to a lesser extent, did the lesions on young Harosoy 63 leaves in which age-related effects had not developed. Age-related resistant lesions and race-specific resistant lesions could not be distinguished from each other visually and the production of glyceollin in comparable lesions of either type was also generally similar. Thus, as in examples from studies in hypocotyls [Lazarovits et al., 1981; Stössel et al., 1981; Ward and Buzzell, 1983; Ward et al., 1981], the Rps genes for resistance are not essential for glyceollin production. The latter evidently is a characteristic of a general biochemical response of the plant that occurs non-specifically and which may be influenced also by competition for precursors [Kimpel and Kosuge, 1985] and differential rates of glyceollin metabolism [Yoshikawa et al., 1979; Chapter 7]. Introduction of the Rps_1 gene extends this response to younger tissues, presumably either by recognising some unique feature of the pathogen not recognised by the slightly older tissue or by interfering with the pathogen's ability to induce susceptibility in the young tissue and hence allowing the basic resistance mechanism of the plant to prevail.

Because of the range of responses that can be obtained, leaves should provide a useful vehicle for the study of mechanisms of resistance and susceptibility of soybeans to Phytophthora megasperma f.sp. glycinea.

CHAPTER 5

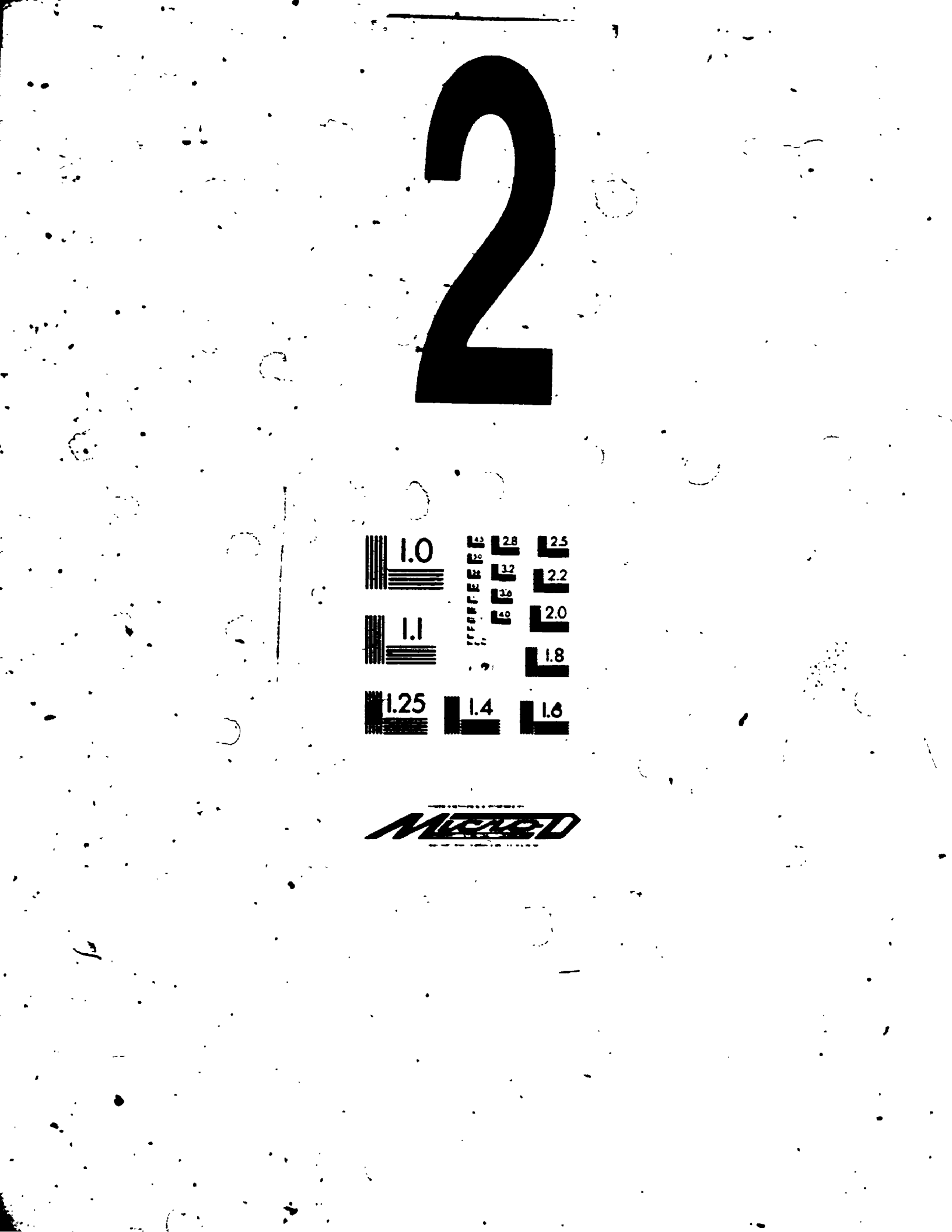
DIFFERENTIAL SENSITIVITY OF *Phytophthora megasperma* f.sp. *glycinea* ISOLATES TO GLYCEOLLIN ISOMERS

5.1 Summary

Glyceollin isomers I, II and III were extracted from soybean cotyledons (cv. Harosoy 63) inoculated with *Phytophthora megasperma* f.sp. *glycinea* race 1, and separated and purified by column, high performance and thin layer chromatography and quantitated by spectrophotometry. The extinction coefficient of glyceollin I was 10800, that of glyceollin II and III conformed to published values. Glyceollin I (ED_{50} approx. 33 µg/ml) was almost twice as inhibitory as glyceollin II and III to growth of *Phytophthora megasperma* f.sp. *glycinea* race 1 on V8 juice agar. All three isomers were less active in soybean hypocotyl extract agar. Glyceollin II (ED_{50} 7 µg/ml) was the most active against zoospore germination, and caused zoospores to burst; ED_{50} values for glyceollin I and III were 12.2 and 13.9 µg/ml respectively. Three isolates (1.1, 1.2, 1.3) were obtained from sectors in colonies on agar medium amended with glyceollin I, II or III respectively. Isolates 1.1 and 1.3 grew more rapidly on medium amended with glyceollin I, III or a natural glyceollin mixture than did wild type *Phytophthora megasperma* f.sp. *glycinea* race 1. All isolates and wild type had similar growth rates on medium amended with glyceollin II. The isolates did not lose their tolerance when grown on control medium and are presumably genetically controlled variants. Tolerance to glyceollin did not increase the agressiveness of the isolates on soybeans.

5.2 Introduction

In a majority of studies of the role of phytoalexins


2
1.0
1.1
1.25
1.4
1.6
2.0
2.2
2.5
2.8
3.2
3.6
4.0
1.8

in the interaction of soybeans with Phytophthora megasperma f.sp. glycinea the three major glyceollin isomers have been treated as a single compound, collectively referred to as glyceollin. Most reported determinations of fungitoxicity have been based on undefined mixtures of the isomers [Lazarovits and Ward,1982; Stossel, 1983; Yoshikawa et al., 1978]. However, the proportion of the three isomers in the glyceollin mixtures extracted from infected tissues vary widely [Banks and Dewick, 1983; Ingham et al.;1981; Lyne et al., 1976; Moesta and Grisebach, 1981; Moesta et al., 1982; Stossel, 1983; Stossel and Magnolato, 1983; Chapter 3] and should the isomers differ in fungitoxicity the level of inhibition of mixtures of glyceollin both in vitro and in infected tissues will presumably reflect the isomeric composition.

Difficulties in obtaining the individual isomers in sufficient quantity and purity restrict the assessment of fungitoxicity. Only one undocumented report of their fungitoxicity has appeared [Lyne et al., 1976], although glyceollin III has been reported to be more toxic than the other isomers to the nematode, Meloidogyne incognita [Kaplan et al., 1980]. Sufficient amounts of the purified isomers were obtained in the present study for limited testing. Contrary to the earlier report, the results indicate that the isomers differ in toxicity to Phytophthora megasperma f.sp. glycinea. Isolates of Phytophthora megasperma f.sp. glycinea that differed from the parent culture in glyceollin sensitivity were also examined.

5.3 Materials and Methods

5.3.1 Preparation of Glyceollin Isomers

Glyceollin isomers I,II and III were extracted from Phytophthora megasperma f.sp. glycinea race 1 infected cotyledons of green plants and purified following the

methods described in chapter 2.5. In addition to glyceollin isomers, a natural glyceollin mixture(M) was obtained from 6-day-old etiolated soybean hypocotyls (cv. Harosoy 63) inoculated with zoospores of Phytophthora megasperma f.sp. glycinea race 1, by procedures described in chapter 2.5 with slight modifications.The eluent from the Sephadex LH 20 column containing the glyceollin isomers was subjected directly to preparative TLC. The proportions of isomers in this mixture was found by HPLC to be 7.5:1:1.3 for glyceollin I, II and III respectively.

5.3.2 Quantification of Glyceollin Isomers

Glyceollin isomers were quantified on the basis of their UV absorbance maxima and their extinction coefficients following the methods described in chapter 2.6. The published extinction coefficient for glyceollin I, λ_{285} logε = 3.92 [Sims et al., 1972] was found to be inaccurate. From the evidently purer material, the value was detemined to be 10 800 (λ_{286}) in ethanol, and this value is consistant with the extinction coefficient of 10 300 (λ_{285}) reported by Ayers et al. [1976] for a mixture of the three isomers and, therefore, used in the present study. The extinction coefficients for glyceollins II and III were those reported by Lyne et al. [1976]. The glyceollin mixture (M) was quantified using the UV absorbance maximum (285 nm) and the extinction coefficient 10 300 (λ_{285}) reported by Ayers et al. [1976].

5.3.3 Bioassays

All bioassays were performed with Phytophthora megasperma f.sp. glycinea race 1 or isolates thereof. Stock cultures were maintained on V8 juice agar at 25^{0}C and procedures for obtaining zoospores were as described in chapter 2 [2.2.2.2].

5.3.3.1 Media

Radial growth assays were performed with 10% clarified V8 juice agar (agar 1.5%) or soybean hypocotyl extract agar. The latter was prepared from 100 g hypocotyls from 6-day-old etiolated soybean seedlings in 500 ml distilled water, autoclaved for 15 min at 121°C . The extract was filtered through cheese-cloth, made upto 1 litre with distilled water containing 15 g agar and re-autoclaved.

5.3.3.2 Preparation of Assay Media

Glyceollin was incorporated into the assay media to give a concentration range from 0-200 µg/ml as follows. Measured concentrations of individual and mixed glyceollin isomers in ethanol were dispensed into 5 ml test tubes and the ethanol gently evaporated on a waterbath (50°C) (Fig. 5.1). Equal volumes of 67% ethanol (20 µl) were dispensed into each tube (including the controls) to dissolve the glyceollin and to ensure that the final concentration of ethanol in the medium was the same (1.78%) in all treatments. In unpublished experiments [Ward, unpublished], ethanol was inhibitory to Phytophthora megasperma f.sp. glycinea at concentrations above 2 per cent.

For the agar media, 0.75 ml molten medium (approx. 65°C) was added to each tube and, after vortexing to ensure thorough mixing, transferred to plastic Petri dishes (35 mm diameter) on a slide warmer. The medium was distributed evenly and the plates were closed and immediately transferred to glass trays lined with moist cellucotton and sealed with plastic film to prevent desiccation of the thin agar layer.

5.3.3.3 Inoculation of Assay Media

Except in the studies of glyceollin tolerance and one

Fig. 5.1
Plate showing the arrangements for preparation of assay media.

other experiment, as indicated below, inoculum for radial growth assays consisted of 5 mm diameter plugs cut from the periphery of the colonies grown on 15 ml V8 juice agar in 9 cm diameter Petri dishes. Inoculum in the tolerance experiments was obtained similarly, but from colonies on 0.75 ml 10% clarified V8 juice agar (either with or without glyceollin 75 µg/ml) in 35 mm diameter Petri dishes. Inoculated plates were incubated at 25°C and 100% RH in the dark.

5.3.3.4 Radial Growth Study

For each concentration of the glyceollin isomers or mixture, two similar but replicate series of plates were set up in each experiment. To eliminate inoculum effects, and compensate for the time required to complete inoculation for one experiment, an initial measurement of colony diameter was made approximately 16 h following inoculation and a second measurement exactly 24 h following the first. The average growth for the two replicates for the 24 h period was used to prepare a dose response curve and to calculate ED_{50} values. For the glyceollin isomers, the ED_{50} values from three different experiments in V8 juice agar were analysed statistically, considering each experiment as an individual block or replicate. Results for two additional experiments in which glyceollin M was included were analysed similarly. In experiments on the tolerance to the glyceollin isomers, colony diameter was measured at intervals as indicated below and data were analysed in a split-plot design [Little and Hills, 1978], with isolates in the main plots and glyceollin isomers in the subplots.

5.3.3.5 Zoospore Germination

The glyceollin isomers and the glyceollin mixture were assayed over the concentration range 0-40 µg/ml (5 µg/ml increments). Ethanol solutions were dispensed into

small test tubes and the ethanol was removed under N_2 at 30-35^{0}C. To each tube, including the control (no glyceollin), 40 µl 20% ethanol was added to dissolve the glyceollin and then, successively, 230 µl sterilized clarified V8 juice (diluted 1:4 with water) and 230 µl zoospore suspension (10^5/ml), vortexing briefly after each addition. From each tube, three drops (15 µl each) were transferred to a microscope slide and incubated in covered glass trays at 100% R.H. for 3 hours, by which time germination in controls was >90%. For each treatment, 100 zoospores were counted and the percentage germination recorded. Dosage response curves for percentage inhibition were prepared and ED_{50} and ED_{90} values determined. The experiment was repeated three times.

5.3.4 Inoculation of Soybean Hypocotyls

Seven-day-old etiolated seedlings of soybean cultivars Harosoy (susceptible to Phytophthora megasperma f.sp. glycinea race 1) and Harosoy 63 (resistant to Phytophthora megasperma f.sp. glycinea race 1) were held horizontally in glass trays and inoculated with a 10 ul drop of a zoospore suspension placed on the unwounded hypocotyl surface, about 2 cm below the cotyledons [Chapter 2.1.2 and 2.4]. Host responses were recorded after 48 h incubation at 25^{0}C in the dark as resistant (R; restricted brown lesions beneath the inoculum drop); intermediate (Rs; a brown lesion but with considerably more spread than a resistant lesion) or susceptible (S; spreading, water-soaked lesion) [Ward and Buzzell, 1983]. Data for lesion lengths are based on measurements of lesions on 30 hypocotyls.

5.4 Results

5.4.1 Radial Growth Assays.

Glyceollin 1 (ED_{50} 32-34 µg/ml) was almost twice as toxic to radial growth on V8 juice agar as either glyceol-

lin II or III (Table 5.1). The mixed glyceollin (M), rich in glyceollin I, had activity similar to glyceollin I alone. All three isomers were much less toxic in soybean extract agar (Table 5.1). The source of the inoculum plug also affected the apparent toxicity (Table 5.2).

5.4.2 Zoospore Germination Assays

Zoospore germination was much more sensitive to the glyceollin isomers than mycelial growth (Table 5.3). Glyceollin II was the most toxic (ED_{50} 7 µg/ml), followed by glyceollin I (ED_{50} 12.2 µg/ml) and glyceollin III (ED_{50} 13.9 µg/ml). The ED_{50} value for glyceollin M was similar to that for glyceollin I. The ED_{90} values followed the same trend, although differences in toxicity between the isomers were less. Glyceollin II caused a majority of non-germinated zoospores to burst.

5.4.3 Isolation of Glyceollin Tolerant Types

The morphology of colonies growing slowly at high concentrations (150 µg/ml) of the glyceollin isomers was frequently irregular (Fig. 5.2). After prolonged incubation, fan-shaped sectors, evidently arising from single hyphae, occasionally extended from the main body of the colony. A representative isolate was taken from a sector developing in response to each of the glyceollin isomers and maintained on half strength clarified V8 juice agar supplemented with 75 µg /ml of the corresponding isomer. The isolates were designated, according to the isomer in the medium from which they were obtained, isolate 1.1, 1.2 and 1.3. Growth rates for the isolates and the original Phytophthora megasperma f.sp. glycinea race 1 (wild type,W) were similar on regular V8 juice agar, but on 10% clarified V8 juice they differed significantly. Thus, 24 h diameter growth rates in mm were: Isolate 1.1, 10.4±0.4; Isolate 1.2, 13.1±0.4; Isolate 1.3,

Table 5.1

Inhibition of radial growth of mycelium of Phytophthora megasperma f.sp. glycinea race 1 by glyceollin isomers I, II and III and by a natural glyceollin mixture(M)

		ED_{50}($\mu g\ ml^{-1}$)			
Medium	control growth(mm)[a]	I	II	III	M
V8 juice	5.5±0.2	34.4[b]	61.6	53.0	
agar	5.2±0.3	31.8[c]	52.1	50.3	34.5
Soybean hypocotyl agar	7.0±0.2	70.0[d]	117.5	90.0	

[a]Radial growth in a 24 h period in unamended media.
[b]From three experiments, LSD(P=0.05)=12.2.
[c]From two experiments, LSD(P=0.05)=4.4.
[d]From a single experiment.

Table 5.2

Influence of inoculum source on inhibition of mycelial growth of Phytophthora megasperma f.sp. glycinea by glyceollin isomers I, II and III

	Inhibition of radial growth (%)		
Inoculum[a]	I[b]	II	III
A	68.8±2.5	56.1±3.7	60.0±1.8
B	85.8±0.9	65.1±1.4	68.3±1.7

[a] In A, inoculum plugs (5 mm diameter) were cut from the periphery of colonies of 15 ml V8 juice agar in 9 cm diameter Petri dishes; in B, similar plugs were obtained from colonies on 0.75 ml half strength V8 juice agar in 3.5 cm diameter Petri dishes.
[b] Glyceollin isomers I, II and III were incorporated into half strength clarified V8 juice agar at 75 µg/ml and inhibition of mycelial growth in a 24 h period relative to growth on unamended agar determined.

Table 5.3

Inhibition of zoospore germination of Phytophthora megasperma f.sp. glycinea race 1 by glyceollin isomers I, II, III and a natural glyceollin mixture(M); values are µg/ml

	I	II	III	M
ED_{50}	12.2±2.3[a]	7.0±1.0	13.9±1.0	12.3±2.3
ED_{90}	16.6±0.9	13.9±1.9	18.3±0.5	14.7±1.7

% germination of controls was 94.4±2.3.
[a] Data are means and standard errors from two experiments.

Table 5.4

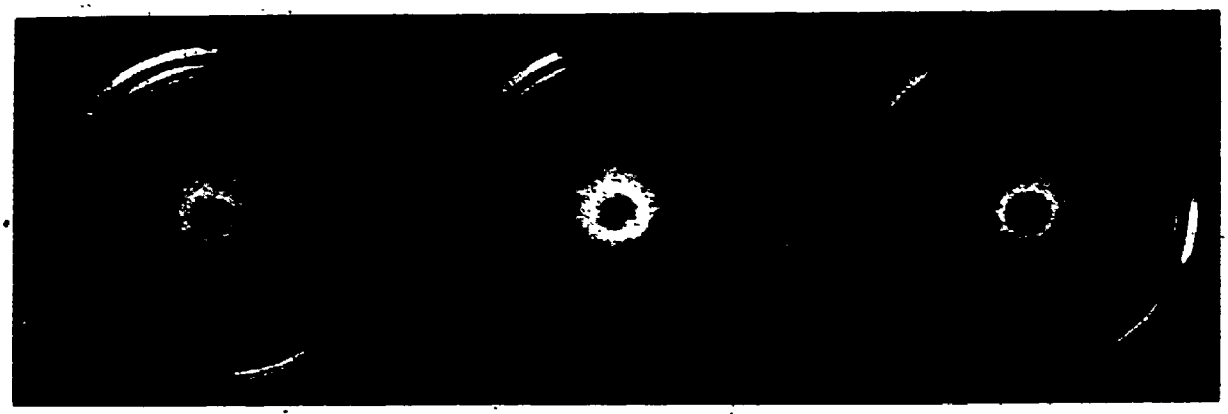
Variation in mycelial growth of Isolates 1.1, 1.2, 1.3[a] and race 1-W of Phytophthora megasperma f.sp. glycinea as affected by glyceollin I, II, III or a natural glyceollin mixture(M) during various incubation periods, randomized in a split-plot design

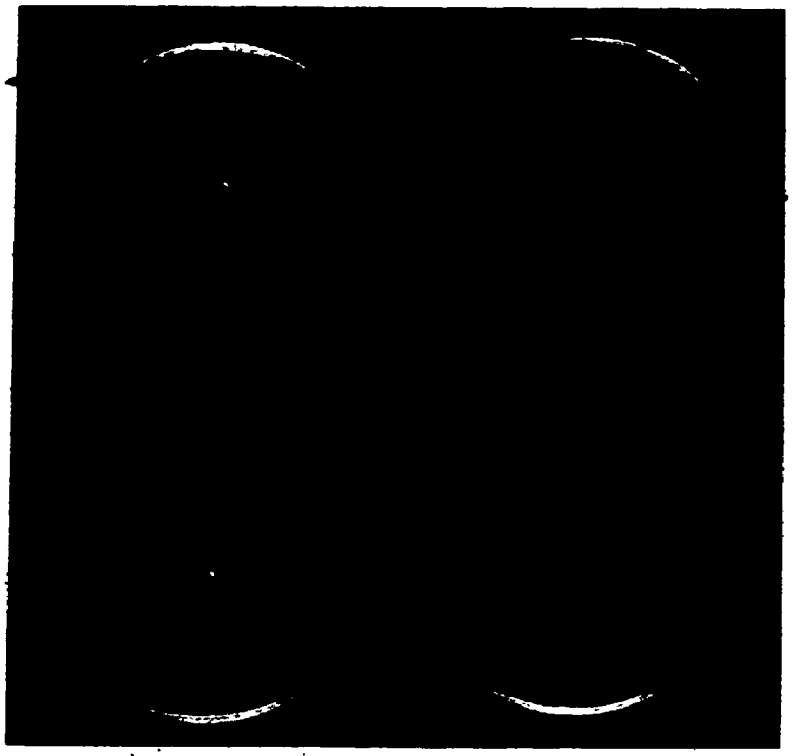
Incubation Period(h)	Grand mean (Growth mm)	Mean sum of squares: Isolates (I)	Glyceollin(G)	Interaction(IxG)	Error (a)	Error (b)
20	2.25	0.75	2.31!	0.31+	0.15	0.10
42	5.18	3.67+	10.74!	1.5!	0.17	0.13
68	9.80	10.21!	37.92!	1.59+	0.29	0.43
92	12.50	32.13!	77.54!	2.57!	0.18	0.55
116	15.29	70.78!	120.54!	3.46!	0.32	1.07

[a] Isolates 1.1, 1.2 and 1.3 were obtained from sectors of race 1-W growing on media enriched with glyceollin isomers I, II and III respectively.
[b] Following inoculation cultures were incubated for 16 h, colony diameters measured and that value used as a zero time value, in order to eliminate variation due to inoculum. The periods indicated are the additional periods starting at 16 h at which colony diameter were measured.
+ Significant at $P=0.05$.
! Significant at $P=0.01$.

Fig. 5.2
Growth of Phytophthora megasperma f.sp. glycinea race 1-W in the presence 150 µg/ml of glyceollin I, II and III (left to right).

Fig. 5.3
Colony morphology of isolates of Phytophthora megasperma f.sp. glycinea growing on half strength V8 juice agar containing 75 µg/ml glyceollin III. Clockwise from top left, Isolate 1.1, Isolate 1.2, race 1-W, Isolate 1.3.





11.7±0.1; race 1-W, 13.6±0.2. Colonies of Isolate 1.1 were much denser, with more uniform margins than those of the other isolates or race 1-W.

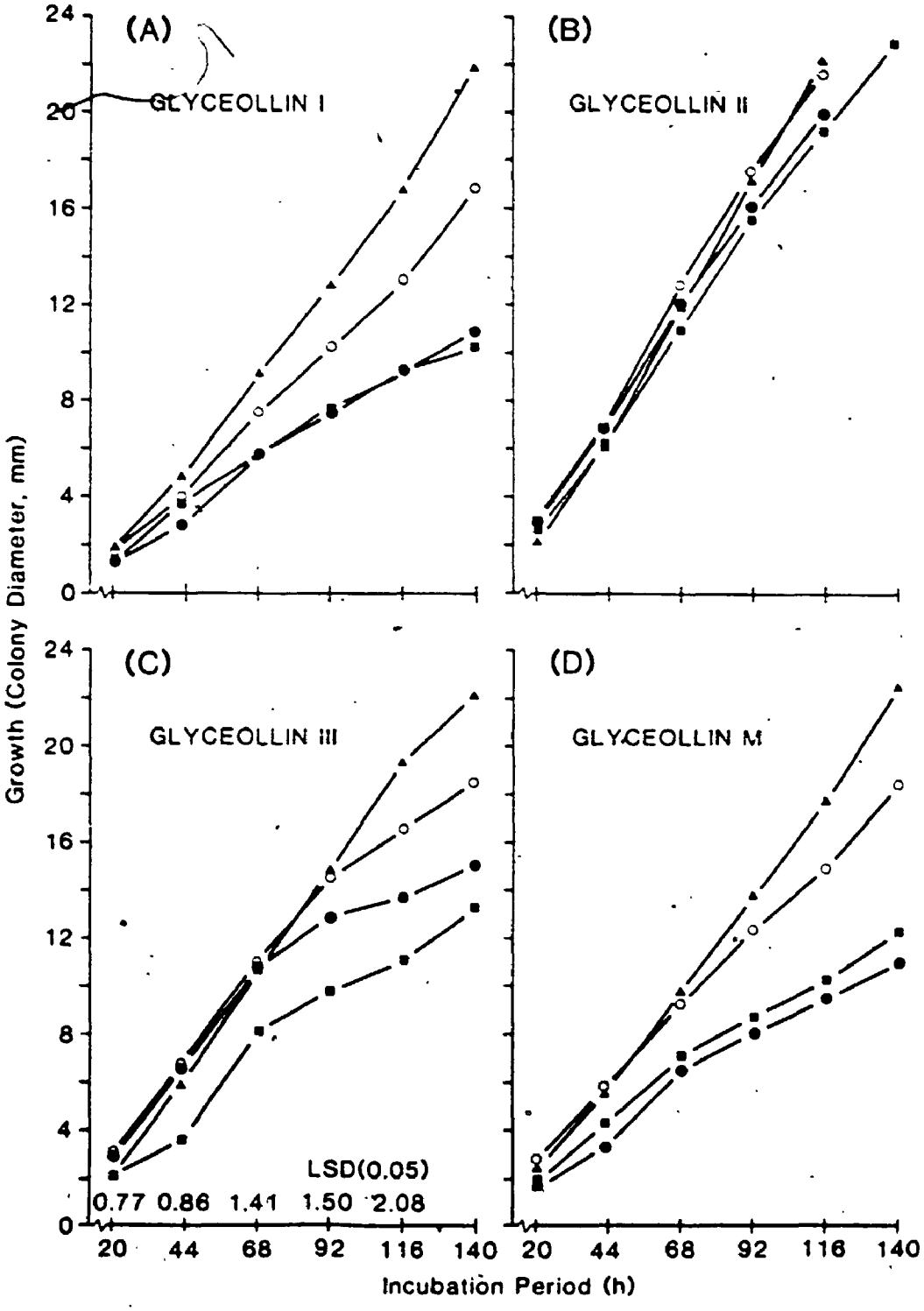
5.4.4 Growth of Race 1 and Glyceollin Tolerant Isolates

Growth rates were determined for each of the isolates and the original Phytophthora megasperma f.sp. glycinea race 1-W, both on medium supplemented with 75 µg/ml of the isomer used to select the isolate and also on medium supplemented with each of the other isomers or the natural mixture of the glyceollin isomers (M). A concentration of 75 µg/ml was used in these experiments because of limitations on the quantities of the isomers available (especially glyceollin II) and the fact that this concentration was sufficiently inhibitory to permit observations of growth over a period of several days.

The isomers had differential effects on the colony morphology of each of the isolates (Fig. 5.3). Differences in growth were significant for the isolates, the isomers and their interactions (Table 5.4). With glyceollin I, the growth rates of Isolate 1.1 and 1.3 were respectively 2x and 1.5x that of the wild type. The growth rate of Isolate 1.2 was similar to that of the wild type (Fig. 5.4A). With glyceollin II growth rates of all isolates were similar (Fig. 5.4B). With isomer III, Isolate 1.2 grew significantly less than the wild type (Fig. 5.4C), Isolates 1.1 and 1.3 grew similarly to the wild type initially, but, subsequently, growth rates of Isolates 1.3 and the wild type declined significantly relative to that of Isolate 1.1. Growth rates with glyceollin M were generally similar to those with glyceollin I, presumably a reflection of the high level of glyceollin I in the mixture (Fig. 5.4). Evidently adaptation to glyceollin I also conferred adaptation to glyceollin III and, similarly, adaptation to glyceollin III conferred adap-

Fig. 5.4

Growth of isolates of Phytophthora megasperma f.sp. glycinea on half strength V8 juice agar containing 75 µg/ml of glyceollin I, II and III or natural mixture, M. Isolates 1.1 (▲—▲), 1.2 (■—■), 1.3 (○—○) were obtained from sectors from colonies of Phytophthora megasperma f.sp. glycinea race 1-W (●—●) growing on medium amended with glyceollin I, II, or III respectively (see Fig. 5.2). LSD values apply to all four graphs.


(A)
GLYCEOLLIN I
(B)
GLYCEOLLIN II
(C)
GLYCEOLLIN III
(D)
GLYCEOLLIN M
Growth (Colony Diameter, mm)
Incubation Period (h)
LSD(0.05)
0.77 0.86 1.41 1.50 2.08
0
4
8
12
16
20
24
20
44
68
92
116
140

tation to glyceollin I.

Isolates 1.1 and 1.3 were transferred to V8 juice agar plates (without glyceollin) and grown for 30 days (six transfers). Plugs removed from the periphery were then returned to agar amended with glyceollin I or III and their growth rates compared with colonies maintained on glyceollin I or III throughout (Fig. 5.5). No loss of tolerance was observed.

5.4.5 Virulence of Glyceollin Tolerant Isolates

All three isolates caused typical brown restricted lesions following inoculation of cv. Harosoy 63 (resistant). On cv. Harosoy (susceptible to Phytophthora megasperma f.sp. glycinea race 1) all three isolates were virulent, but the rate of spread of Isolates 1.1 was less than that of the wild type race 1 (Table 5.5).

5.5 Discussion

Keen et al. [1971] reported the ED_{50} for 6a-hydroxyphaseollin (presumably glyceollin I) for the inhibition of mycelial growth of Phytophthora megasperma f.sp. glycinea on V8 juice agar to be about 25 µg/ml. Using the same medium, higher values were obtained for undefined mixtures of the glyceollin isomers by Yoshikawa et al. [1978], 90 µg/ml, and Lazarovits and Ward [1982], 56 µg/ml. In liquid medium on absorbant pads, Stössel [1983] reported ED_{50} values between 70 and 100 µg/ml. The results obtained in the present study with carefully purified materials are most consistent with the low value originally reported by Keen et al. [1971]. The reason for these differences are not obvious. They do not appear to be related to either the race or the medium used; Stössel [1983] and Yoshikawa et al. [1978] used different races and media but obtained similar ED_{50} values. Nevertheless, both these authors reported that

Fig. 5.5

Persistence of glyceollin tolerance in isolates of Phytophthora megasperma f.sp. glycinea on medium without glyceollin isomers. (A) Growth on half strength V8 juice agar containing 75 µg/ml of glyceollin I of: Isolate 1.1 maintained continuously on medium containing 75 µg/ml glyceollin I (▲——▲); Isolate 1.1 grown for 30 days preceding inoculation on control medium without glyceollin I (▲----▲); Phytophthora megasperma f.sp. glycinea race 1-W (●——●). (B) Growth on half strength V8 juice agar containing 75 µg/ml of glyceollin III of: Isolate 1.3 maintained continuously on medium containing 75 µg/ml glyceollin III (○——○); Isolate 1.3 grown for 30 days preceding-inoculation on control medium without glyceollin III (○-----○); Phytophthora megasperma f.sp. glycinea race 1-W (●——●). LSD values apply to both graphs.

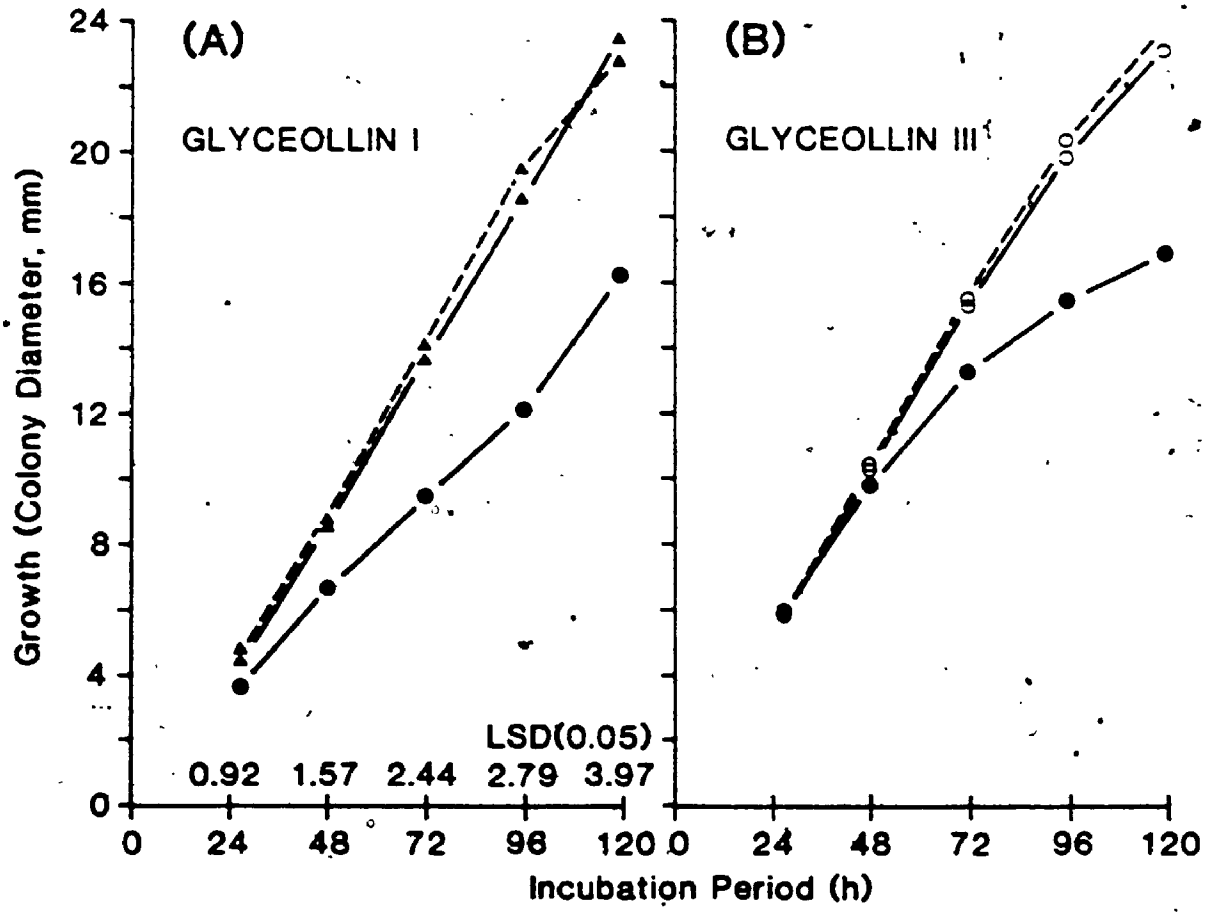

(A)
GLYCEOLLIN I
(B)
GLYCEOLLIN III
Growth (Colony Diameter, mm)
24
20
16
12
8
4
0
LSD(0.05)
0.92 1.57 2.44 2.79 3.97
0 24 48 72 96 120
0 24 48 72 96 120
Incubation Period (h)

Table 5.5

Hypocotyl reaction and lesion size in etiolated soybean seedlings (cultivars Harosoy and Harosoy 63) inoculated with zoospores of *Phytophthora megasperma* f.sp. *glycinea* race 1-W or Isolates 1.1, 1.2 and 1.3.

	Harosoy		Harosoy 63	
Isolate[a]	Reaction type[b]	Lesion length (mm)[c]	Reaction type	Lesion length(mm)
Race 1-W	S	44.2±1.1	R	5.2±0.1
Isolate 1.1	S(24),Rs(6)	31.0±1.4	R	4.5±0.1
Isolate 1.2	S	45.3±0.9	R	4.9±0.1
Isolate 1.3	S	41.1±1.1	R	4.7±0.1

[a] Isolates 1.1, 1.2 and 1.3 were obtained from sectors of race 1-W growing on media enriched with glyceollin isomers I, II and III respectively.

[b] Reaction types were: susceptible (S), a spreading water-soaked lesion; resistant (R), a restricted brown lesion; intermediate (Rs), a brown lesion but with considerably more spread than a typically resistant lesion. Thirty inoculated seedlings were rated after 48 h incubation in each case; the reactions were consistent except where indicated for Isolate 1.1.

[c] Lesion lengths on 30 seedlings were measured after 48 h incubation.

medium commposition can influence the apparent fungitoxicity of glyceollin, and this is supported by the results with the two media used in the present study. The source and size of the inoculum also affected the apparent activity in bioassays, presumably either by diluting the glyceollin or by providing a non-inhibitory nutrient base. Extensive studies with other isoflavanoid phytoalexins have demonstrated that media and assay conditions profoundly influence estimates of fungitoxicity [Bailey and Skipp, 1978; Skipp and Bailey, 1976; Skipp and Bailey, 1977]. Evidence that the fungitoxicity of the glyceollin can be affected by such apparently trivial factors, suggests that conditions in vivo may have even more profound effects.

Although estimates of fungitoxicity evidently vary with assay conditions, the relative fungitoxicities of the glyceollin isomers, either to mycelial growth or to zoospore germination, were consistent in all experiments. The shapes of the mycelial growth curves with each of the isomers were also characteristic: linear with glyceollin I and II, declining following a period of linearity with glyceollin III. Declining growth rate suggests a failing repair mechanism or the accumulation of secondary inhibitors, and may indicate that the mechanism of action of this isomer differs from that of the others. Differences in growth curves and colony morphology, especially in the presence of glyceollin I and III, indicate that the isolates differ distinctly in their response to these compounds. However, when changes occur in colony morphology the possibility that differences in colony diameters may not reflect differences in growth should not be overlooked. The results also suggest that zoospore germination is more sensitive than mycelium growth to inhibition by the glyceollin isomers. Possibly the cell membrane, directly exposed in zoospores, is protected in hyphae by the cell wall. Bursting

of zoospores in glyceollin II is consistent with membrane damage. The effect was not common with the other isomers, however, and this may indicate differences in mechanisms of action.

Previous reports have been based on natural mixtures of glyceollin isomers, usually in unknown proportions. The results of the present study suggest that the isomeric proportions could profoundly influence the fungitoxicity of a mixture. For short incubation periods, mixtures high in glyceollin I would tend to be most inhibitory to mycelial growth, those high in glyceollin II less inhibitory. Over longer periods, glyceollin III would be most inhibitory. Mixtures high in glyceollin II might be most inhibitory to zoospore germination. The isomeric proportions reported for glyceollin extracted from various infected tissues have varied widely [Banks and Dewick, 1983; Ingham et al., 1981; Lyne et al., 1976; Moesta and Grisebach, 1981b; Moesta et al., 1982; Stossel, 1983; Stossel and Magnolato, 1983; Chapter 3]. If relative fungitoxicities in infected tissues are similar to those in vitro, the effectiveness of glyceollin production in restriction of pathogen spread should vary according to the isomeric composition. Some of the differences referred to above in determinations of fungitoxicity may also be accounted for in this way, but not the relatively high ED_{50} values obtained by Stossel [1983], who used glyceollin mixtures high in glyceollin I. The finding, that the three isomers differ widely in fungitoxicity is in disagreement with the undocumented report of Lyne et al. [1976] that they were similar in activity.

Stossel [1983] reported that cysts of Phytophthora megasperma f.sp. glycinea exposed to non-inhibitory concentrations of glyceollin developed a degree of tolerance that permitted germ tube growth at concentrations above the former ED_{100} levels. In addition, in mycelial growth assays,

some growth developed at high glyceollin concentrations, only after long incubation periods. Evidently, a different kind of adaptation was observed in the present study. Isolates 1.1 and 1.3 grew significantly faster than wild type Phytophthora megasperma f.sp. glycinea (race 1-W) in the presence of glyceollin M and glyceollin I and III. As these isolates were obtained as sectors on amended medium and did not lose their tolerance after returning to control medium, it seems probable that they were genetically controlled variants, selected by the conditions applied. Generation of variation through uninucleate zoospores has been observed in this species [Rutherford et al., 1985]. It is possible that single nuclei which condition tolerance to glyceollin are carried in the coenocytic hyphae of Phytophthora megasperma f.sp. glycinea at a very low frequency.

The selection of adapted isolates in vitro suggests that similar selection may occur in vivo. However, in that case it would be expected that strains isolated from infected tissues would already be highly tolerant unless glyceollin exerts no selection pressures in vivo. This may be the situation in susceptible lesions that are presumably the source of most Phytophthora megasperma f.sp. glycinea isolates. The present investigation indicates that the adapted strains had no advantage over the wild type in infection and lesion development in soybean hypocotyls, and Isolate 1.1 was, in fact, less fit. However, if glyceollin does condition resistance to Phytophthora megasperma f.sp. glycinea in soybeans, it is reasonable to assume that tolerance and the higher growth rates associated with it could, under certain circumstances, influence the expression of resistance. Intermediate responses, for example, have been reported in which, in spite of production of necrotic brown lesions and the accumulation of glyceollin, the lesions do not remain restricted but spread slowly [Buzzell et

al., 1982; Ward and Buzzell, 1983]. It is also possible that the isolation of adapted strains could contribute to the discrepancies in toxicity levels reported by various workers.

CHAPTER 6

VARIATION IN MORPHOLOGY, GROWTH, GLYCEOLLIN I TOLERANCE AND AGGRESSIVENESS IN *Phytophthora megasperma* f.sp. *glycinea* RACE 1

6.1 Summary

Single zoospore isolates were obtained from *Phytophthora megasperma* f.sp. *glycinea* race 1 and Isolate 1.1. Isolate 1.1 was obtained earlier [Chapter 5] from an outgrowth of a race 1 colony on a medium containing an inhibitory concentration of the soybean phytoalexin, glyceollin I. Colony morphology of Isolate 1.1 was similar to that of race 1 (normal), however, 98% of single-zoospore progeny of Isolate 1.1 produced were small fluffy colonies. Only 3% of race 1 progeny were of this type. Despite selection of Isolate 1.1 as tolerant to glyceollin I, single-zoospore isolates varied widely in sensitivity to glyceollin I. Single-zoospore isolates of race 1 also varied in growth rate and sensitivity to glyceollin I. Significant variation was still evident after three successive single-zoospore generations. Among 48 single-zoospore progeny of Isolate 1.1 a majority were less aggressive in various degrees than Isolate 1.1 on susceptible cultivar, Harosoy. There was much less variation in aggressiveness of single-zoospore isolates from Isolate 1.1 following inoculation with mycelium on plugs cut from colonies on 20% V8 juice than with zoospores on susceptible cultivars, Harosoy and Wayne. Among 38 single-zoospore progeny of race 1 a majority were more aggressive than the parent and the range in aggressiveness was less than that for Isolate 1.1 progeny. The extent and the quantitative nature of the variation observed are difficult to account for on the basis of nuclear genes and the possible involvement of cytoplasmic factors is discussed.

6.2 Introduction

Phytophthora megasperma f.sp. glycinea, the cause of Phytophthora root and stem rot of soybean is a homothallic fungus and diploid in its vegetative phase [Long and Keen, 1977; Sansome and Brasier, 1974]. It reproduces asexually through zoospores that are reported to be uninucleate [Long et al., 1975]. During periods of vegetative growth and asexual propagation, deleterious recessive mutants may accumulate in the heterozygous condition, and because of the coenocytic nature of the hyphae, homozygous recessive mutants that may arise from somatic crossing over and also deleterious heterozygous dominant mutants may be carried along, without expression, as heterokaryons [Long and Keen, 1977]. These possibilities could account for genetic variability among the first generation progenies obtained through zoospore propagation in this fungus [Hilty and Schimitthenner, 1962; Hobe, 1981; Long et al., 1975; Rutherford et al., 1985] and also in other Phytophthora spp. [Boccas, 1972; MacIntyre and Elliott, 1974]. However, it is difficult to explain the cause of variability in subsequent zoospore generations [Boccas, 1972; Long et al., 1975; MacIntyre and Elliott, 1974 and Rutherford et al., 1985].

In an earlier study [Chapter 5] an isolate (Isolate 1.1) was obtained as an outgrowth from a colony of Phytophthora megasperma f.sp. glycinea race 1 on medium containing an inhibitory concentration of the soybean phytoalexin, glyceollin I. The isolate was less aggressive than its parent for it produced smaller lesions than the parent following zoospore-inoculation of hypocotyls of the soybean cultivar, Harosoy (susceptible to race 1). In this chapter variation in aggressiveness to soybean hypocotyls and also in growth rate, colony morphology and tolerance to glyceollin I in vitro is examined in single zoospore isolates of

Phytophthora megasperma f.sp. glycinea race 1 and Isolate 1.1. Some of the variation is difficult to explain in terms of nuclear genes and the possibility that cytoplasmic factors are involved is discussed.

6.3 Materials and Methods

6.3.1 Pathogen

Phytophthora megasperma f.sp. glycinea race 1 and Isolate 1.1 were those used in a previous study [Chapter 5]. Isolate 1.1 was obtained from race 1 from a faster growing sector on a medium amended with glyceollin I.

6.3.2 Isolation of Hyphal tips from Isolate 1.1

Isolate 1.1 grown in 20% V8 juice agar was transferred to water agar (agar 2% in water) and allowed to grow for 7 days. Thereafter, hyphal tips from individual advancing hyphae were isolated under a dissecting microscope and transferred to 20% V8 juice agar . Two hyphal tip isolates ($I\text{-}1.1\text{-}t_1$, $I\text{-}1.1\text{-}t_2$) were used in the present study.

6.3.3 Production of single-zoospore progeny

Zoospore suspensions prepared aseptically were suitably diluted with sterile water and plated on 10% clarified V8 juice agar (agar 1.5%). After 24 h, plates were examined with a dissecting microscope, and locations of the individual germinating cysts were marked on the bottom of the plates. After incubation for a further 24 h in the dark individual zoospore colonies were transferred to 225 mm Petri dishes containing either 20% V8 juice agar or 10% clarified V8 juice agar.

6.3.4 Host

Etiolated seedlings of Harosoy, Harosoy 63 and Wayne (6-day old) were grown in vermiculite under the conditions

described previously [Chapter 2.1.1].

6.3.5 Inoculation of hypocotyls

Etiolated hypocotyls of 6-day old seedlings of soybean cultivars Harosoy, Harosoy 63 and Wayne were arranged horizontally in glass trays as described previously [Chapter 2.1.2]. They were inoculated approximately 2 cm below the cotyledons with race 1, or Isolate 1.1 or their progenies either with a drop of zoospore suspension (10^5/ml) or with a plug (5 mm diameter) cut from the periphery of a colony grown in 20% V8 juice agar. The plugs were placed with mycelium in contact with the hypocotyls. Inoculated seedlings were incubated in the dark at 25°C and 100% RH for 48 h and then the reaction-type and length of the lesions were recorded. Harosoy ($\underline{Rps}_1$) and Wayne are normally susceptible to race 1 while Harosoy 63 ($\underline{Rps}_1$) is resistant. In a resistant (R) reaction-type the lesion was brown, necrotic and restricted to the area covered by the inoculum. In a susceptible reaction-type the lesion was pale yellow or light brown, water-soaked and unrestricted, spreading extensively up and down the hypocotyl from the inoculated area. Two intermediate classes were also recorded. These were resistant with spreading (Rs), in which a dark brown lesion of the resistant type spread beyond the inoculated area, and susceptible with necrosis (Sn), in which the lesion was initially brown and necrotic but subsequently like the susceptible reaction-type.

6.3.6 Preparation of Glyceollin I

Etiolated seedlings were arranged horizontally in glass trays and sprayed with freshly prepared $AgNO_3$ solution (10^{-3} M)[Stossel, 1982]. After incubation for 48 h in the dark at 25°C and 100% RH, hypocotyls were extracted in 95% ethanol for 10 min in a water bath (100°C). Purification

procedures have been described in detail previously [Chapter 2.5]. The purified glyceollin I was quantified from its UV absorbance maximum (286 nm) and extinction coefficient [10 800, Chapter 5].

6.3.7 Bioassay

Radial growth (mm/day) and colony morphology were determined for colonies in Petri dishes using i) 20% V8 juice agar, ii) 10% clarified V8 juice agar, iii) 10% clarified V8 juice agar amended with 2% ethanol and glyceollin I (75 µg/ml), or iv) 10% clarified V8 juice agar amended with 2% ethanol. For the preparation of media containing glyceollin I (75 µg/ml) a measured amount of glyceollin I in ethanol was mixed with 10% clarified V8 juice agar in a flask in a water bath (50°C) to give a final concentration of 75 µg/ml of glyceollin I and 2% ethanol. Controls contained 2% ethanol only. The mixture was dispensed quickly into Petri dishes (35 mm diameter, 0.8 ml/dish) on a slide warmer, to permit even distribution of the thin layer of medium. The dishes were closed, allowed to cool to permit the agar to solidify and transferred to glass trays. The trays were lined with wet paper towels and sealed with plastic film to prevent desiccation of the agar during incubation. The plates were numbered randomly and divided into two groups. Each group was treated as a replicate.

In bioassays, the medium was inoculated with a plug of mycelium (5 mm diameter) cut from the periphery of a rapidly growing colony in 225 mm Petri dishes containing 6 ml 10% clarified V8 juice agar. Growth rates were determined by measurement of colony diameter, after substraction of an initial measurement made at 24 or 36 h to eliminate variation due to inoculum differences. Measurements usually were made using a dissecting microscope or with the unaided eye and a mm scale. In one large experiment, colonies were

photographed using a polaroid camera, and magnified real images from the negatives were focused on a graphic tablet of an Apple IIe computer. The area of each colony was determined from a series of points on the periphery of the colonies. The results were expressed as radial growth in mm/day.

6.3.8 Statistical analysis

The data presented in Fig. 6.5 were analysed in a completely randomised block design [Little and Hills, 1978]. From the expectation of mean squares (Table 6.1) the genotypic coefficient of variation (GCV) and heritability (in the broad sense) were calculated following the methods of Burton and DeVane [1953] and Allard [1963] respectively. Ranges, differences between the highest and the lowest values, were divided by the respective means and expressed as percentages.

Table 6.1

Analysis of variance table

Source of variation	d.f.	Mean sum of squares	Expectation of mean squares
Replication	(r-1)	MS_E	
Genotype	(g-1)	MS_g	$r\sigma^2_g + \sigma^2_e$
Error	(r-1)(g-1)	MS_e	σ^2_e

r = number of replications, g = number of isolates

From table 6.1:

Genotypic variance = $\sigma^2_g = [MS_g - MS_e]/r$

Phenotypic variance, $\sigma^2_p = \sigma^2_g + \sigma^2_e$

Genotypic coefficient of variation(%) = $[\sigma_g/\bar{X}]x100$

Heritability (in broad sense, %) = $[\sigma^2_g/\sigma^2_p]x100$

6.4 Results

The colony morphology of 314 single zoospore isolates from race 1 and 543 from Isolate 1.1 was examined. Although there were additional variations, colonies were grouped into two categories: those with a low growth rate and abundant aerial mycelium were classified as "fluffy" while those with a high growth rate and only a thin layer of aerial mycelium on 10% V8 juice agar classified as "non-fluffy" or normal. Both parents were non-fluffy. It was recorded that 98.7% of single zoospore isolates from Isolate 1.1 were fluffy while, only 2.5% of isolates from race 1 were fluffy. This low proportion of fluffy types persisted in a third generation of single zoospore isolates of race 1 (Fig. 6.1).

The possibility that fluffy and non-fluffy types of single zoospore progenies arose from a mixture of hyphae differing in the two phenotypes present in the original colony was tested by examining hyphal tip isolates and single-zoospore progeny obtained from them. The isolates were similar to Isolate 1.1 both in colony morphology and glyceollin I tolerance. As with Isolate 1.1, a high proportion of the single-zoospore progeny was of the fluffy type (Fig. 6.2).

Ten isolates that differed in colony morphology or growth rate were selected from the single zoospore progeny of Isolate 1.1 for more detailed examination (Table 6.2). Most of these isolates were fluffy and some of them produced very small dense colonies that grew little in 5 days on 10% clarified V8 juice agar. When transferred to 20% V8 juice agar the growth rate of these isolates increased, but for the first 72 h remained less than that of a normal colony. After 72 h the growth rates increased further and in most cases became comparable to those of normal isolates. Thus, in the first 72 h, growth was correlated ($r = 0.85$) with the

Fig. 6.1
Variation in colony morphology of single-zoospore progeny of Phytophthora megasperma f.sp. glycinea race 1. The plate (10% clarified V8 juice agar) was inoculated with a zoospore suspension from a second generation single- zoospore isolate of race 1. Note the very slow growing flulffy colonies. Photograph was taken 96 h after inocultion.

Fig. 6.2
Variation in colony morphology of single-zoospore progeny of Phytophthora megasperma f.sp. glycinea Isolate 1.1. The vertical row of small dishes contain respectively from the top: race 1, Isolate 1.1, Isolate 1.1-t_2, all growing on 10% clarified V8 juice agar containing 75 µg/ml glyceollin I. Isolate 1.1 was obtained from an out- growth of a colony of race1 growing on glyceollin I amended medium. Isolate 1.1-t_2 was obtained from a hyphal tip of Isolate 1.1. The two larger plates were inoculated with a zoospore suspension of Isolate 1.1-t_2. Note the range of colony morphology; a majority of colonies exhibit the slow growing flulffy habit. Photographs were taken 120 h after inocultion.

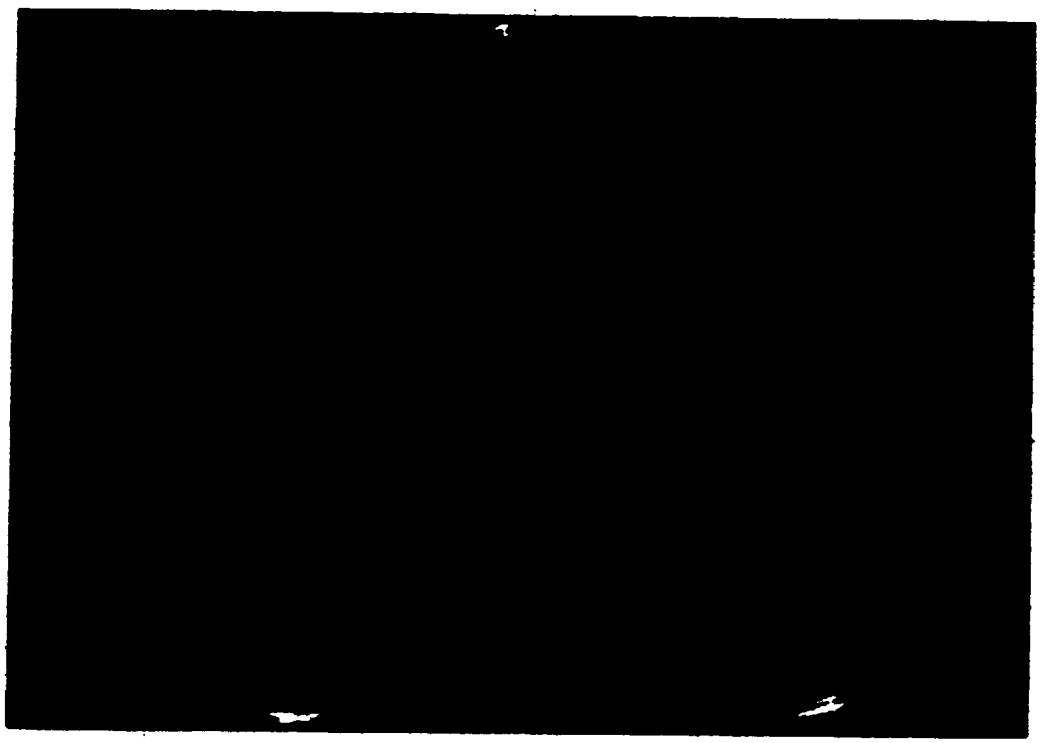


Table 6.2

Variation in colony morphology and growth rate of single-zoospore isolates derived from Isolate 1.1 of *Phytophthora megasperma* f.sp. *glycinea*[1]

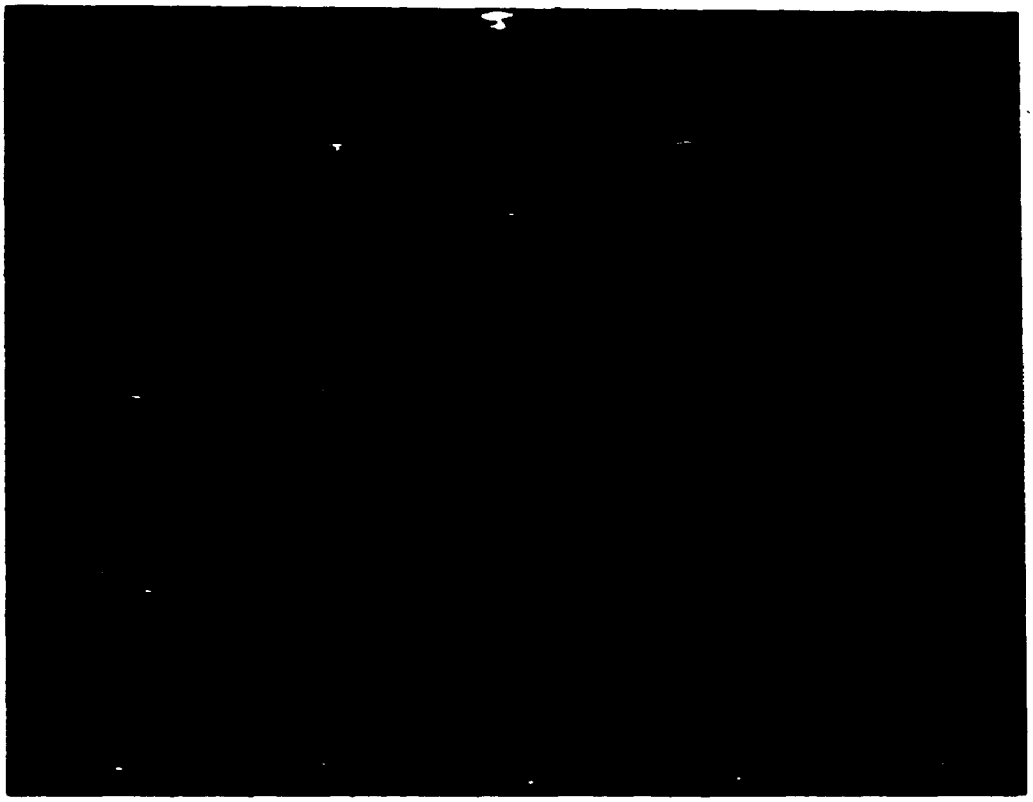
Isolate	Morphology		Radial growth (mm/day)			
	I(a) 10% V8	I(b) 20% V8	I(b) 20% V8		I(c) 10% V8	
			72h	120h	retransfer	
I-1.1-1	Fluffy[2]	Fluffy	0.4	2.6	6.4	4.3
I-1.1-2	Non-fluffy	Non-fluffy	3.0	7.8	7.0	5.5
I-1.1-3	Fluffy	Fluffy	0.7	1.4	5.9	6.2
I-1.1-4	Fluffy	Fluffy	0.2	1.5	4.6	5.1
I-1.1-5	Small dot[3]	Non-fluffy	0.05	2.3	6.8	6.2
I-1.1-6	Fluffy	Fluffy	0.15	3.2	5.8	4.6
I-1.1-7	Big dot[3]	Non-fluffy	0.1	3.9	7.9	3.3
I-1.1-8	Fluffy	Fluffy	3.0	6.8	8.4	5.2
I-1.1-9	Non-fluffy	Non-fluffy	3.7	6.8	7.3	5.2
I-1.1-10	Fluffy	Fluffy	0.7	4.9	6.4	3.6

[1] Single zoospores were isolated on 10% V8 juice agar and grown for 5 days, and average growth in 24 h was calculated from the total (a). They were transferred to 10% V8 juice agar plates and from these transferred (mycelial plugs) to 20% V8 juice agar plates and average growth in 24 h during 0 to 72 and 72 to 120 h periods following transfer were determined (b). They were maintained on 20% V8 juice agar for 28 days and then returned (mycelial plugs) to 10% V8 juice agar and growth rate determined between 36 and 60 h following transfer (c).

[2] Colonies were classified as fluffy (slow growing with abundant aerial mycelium) and non-fluffy or normal (fast-growing with little aerial mycelium).

[3] Colony morphology could not be classified due to small size of the colony.

Fig. 6.3
Differential tolerance of single-zoospore isolates derived from Phytophthora megasperma f.sp. glycinea Isolate 1.1 to glyceollin I. All plates contain 10% clarified V8 juice agar amended with 75 ug/ml glyceollin I. Top, race 1. Row 2, Isolate 1.1 (derived from an outgrowth of race 1 on glyceollin I amended medium) flanked by Isolate 1.1-t_1 and Isolate 1.1-t_2 (hyphal tip cultures from Isolate 1.1). Rows 3 and 4 are 10 single- zoospore isolates obtained from Isolate 1.1. Row 3, left to right: I-1.1-1, -2, -3, -4, -5. Row 4 (bottom) left to right: I-1.1-6, -7, -8, -9, -10. Photograph was taken 120 h after inoculation.



initial growth on 10% clarified V8 juice agar, in the subsequent 48 h, it was not (r = 0.46). When these isolates were returned to 10% clarified V8 juice agar they did not redevelop their original growth rates (r = 0.45) or colony morphology. Slow growing fluffy isolates from a second generation single-zoospore isolate of race 1 (Fig. 6.1) also exhibited delayed changes in growth rate when transferred to 20% V8 juice agar.

Isolate 1.1 was obtained as a sector with tolerance to glyceollin I. The 10 single zoospore isolates derived from Isolate 1.1 were tested, therefore, for their tolerance to glyceollin I (Table 6.3, Fig. 6.3). Among these isolates there were wide differences in sensitivity to glyceollin I and in growth rates on control media. Single-zoospore isolates with normal colony morphology from race 1 were much less variable in these two characters (Table 6.3).

Aggressiveness, as indicated by reaction-type and lesion length, was determined for the 10 isolates selected from single-zoospore progeny of Isolate 1.1 following zoospore- or mycelium-inoculation of hypocotyls of cultivars Harosoy 63, Harosoy and Wayne (Table 6.4). Except for the parent race 1 and I-1.1-3, lesions were longer in hypocotyls inoculated with mycelium than with zoospores. Lesions tended to be shorter and reaction-types more resistant on Harosoy than on the other susceptible cultivar, Wayne. The range of such differences on the two cultivars varied widely among the isolates. Cultivar Harosoy 63 was resistant to all isolates.

Aggressiveness was determined also for random samples of single zoospore isolates derived from either race 1 or Isolate 1.1 (Fig. 6.4). All isolates from both race 1 and Isolate 1.1 were avirulent on Harosoy 63 ($\underline{Rps}_1$), resistant to race 1) (data not presented). On cv. Harosoy ($\underline{rps}_1$,

Table 6.3

Differential sensitivity to glyceollin I of single-zoospore isolates obtained from race 1 and Isolate 1.1 of Phytophthora megasperma f.sp. glycinea

Isolate[1]	Glyceollin I (µg/ml)		% Inhibition
	0[2]	75[3]	
I-1.1-1	4.3±0.8[4]	0.3±0.02	93[5]
I-1.1-2	5.5±0.8	2.1±0.03	62
I-1.1-3	6.2±0.1	1.0±0.08	84
I-1.1-4	5.1±0.0	1.4±0.04	73
I-1.1-5	6.2±0.1	0.9±0.07	85
I-1.1-6	4.6±0.3	0.5±0.13	89
I-1.1-7	3.3±0.4	1.8±0.07	45
I-1.1-8	5.2±0.1	2.0±0.09	62
I-1.1-9	5.2±0.1	2.1±0.02	60
I-1.1-10	3.6±0.1	0.5±0.03	86
I-1.1-t_1	4.8±0.3	1.8±0.00	63
I-1.1-t_2	5.6±0.7	1.9±0.10	66
I-1.1	5.2±0.2	2.1±0.00	60
R_1-6	6.2±0.4	1.2±0.05	81
R_1-10	6.0±0.0	1.4±0.06	77
R_1-13	6.0±0.3	1.3±0.08	78
R_1-17	6.3±0.3	1.2±0.02	81
R_1-23	6.5±0.1	1.4±0.05	78
Race 1	6.4±0.8	1.5±0.2	77

[1] R_1 isolates are single-zoospore isolates obtained from race 1. Isolate 1.1 (I-1.1) was obtained from an outgrowth of race 1 colony on glyceollin I amended mycelium. I-1.1-t_1 and -t_2 are hyphal tip cutures from Isolate 1.1. Isolates(I)-1.1-1, -2, -3, -4, -5, -6, -7, -8, -9, -10 are single-zoospore isolates from Isolate 1.1.

[2] Radial growth rate (mm/day) during 36-60 h following inoculation (5 mm mycelial plug) in Petri dishes (35 mm diameter) containing 0.8 ml 10 % clarified V8 juice agar amended with 2 % alcohol.

[3] As in [2] except that medium was amended with glyceollin I (75 µg/ml).

[4] Data are mean±S.E. from three replications.

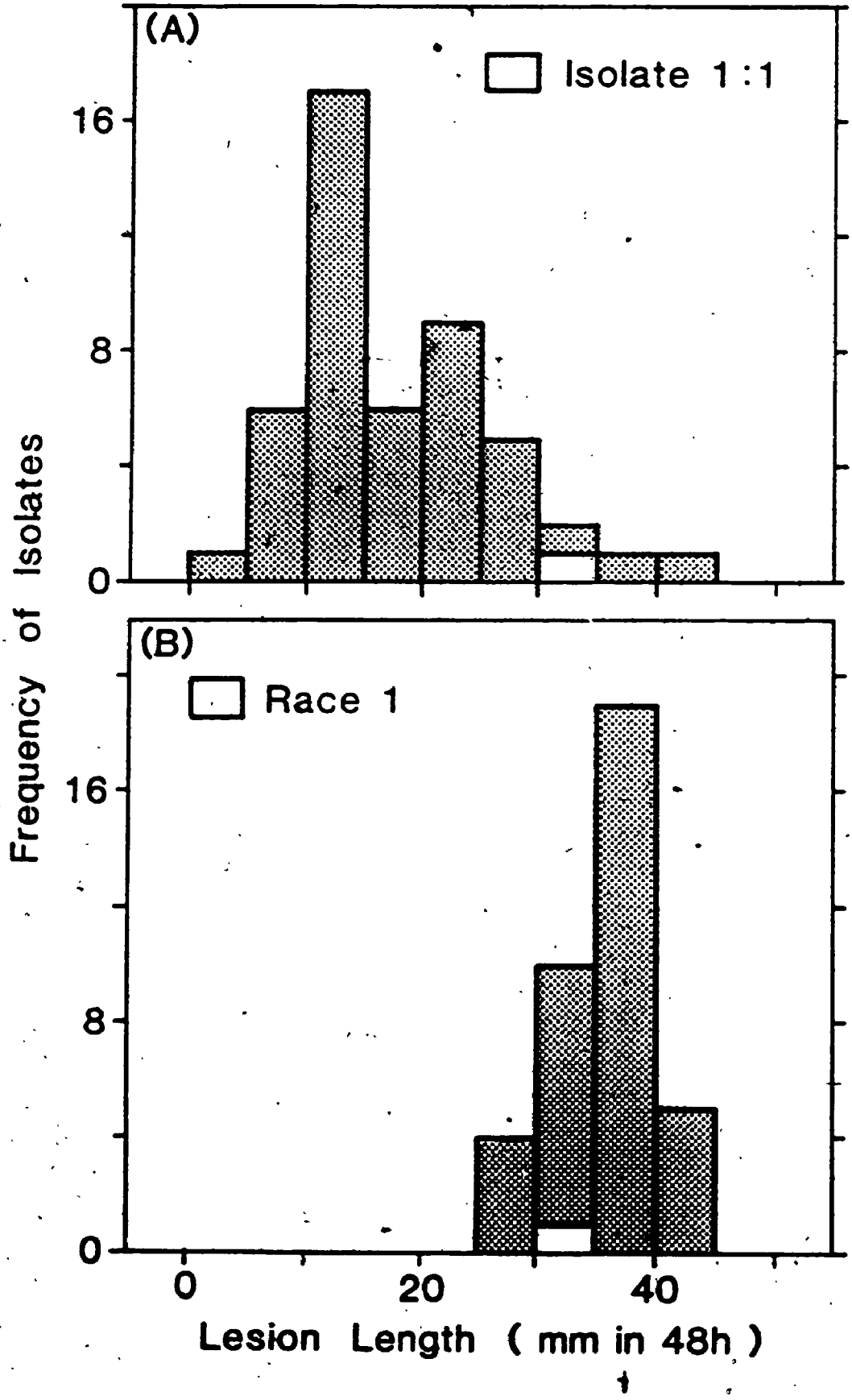
[5] Percent inhibition of growth of the isolates in glyceollin I amended medium over that in control (0 µg/ml) was calculated.

Table 6.4
Aggressiveness on soybean cultivars of single-zoospore isolates obtained from Isolate 1.1 of Phytophthora megasperma f.sp. glycinea

Isolate	Method[1] of Inoculation	Wayne					Harosoy					Harosoy 63
		Lesion length (mm)	Reaction type[2] R	Rs	Sn	S	Lesion length (mm)	Reaction type R	Rs	Sn	S	Lesion length (mm)
I-1.1-1	Z	7±0.4	1	9	0	0	5±0.3	0	10	0	0	3±0.1
	M	30±1.2	0	3	7	0	23±2.1	0	8	2	0	11±0.7
I-1.1-2	Z	27±1.1	0	0	9	1	19±1.1	0	0	10	0	3±0.1
	M	32±1.2	0	0	2	7	30±1.3	0	0	4	6	10±0.2
I-1.1-3	Z	38±2.0	0	1	0	9	15±0.9	0	10	0	0	4±0.2
	M	25±1.9	0	2	1	7	19±1.3	0	10	0	0	6±0.3
I-1.1-4	Z	---[3]	-	-	-	-	---	-	-	-	-	---
	M	11±2.1	1	9	0	0	9±1.8	4	6	0	0	5±0.1
I-1.1-5	Z	8±0.9	3	7	0	0	3±0.1	10	0	0	0	3±0.1
	M	28±1.8	0	1	9	0	21±1.4	0	10	0	0	5±0.3
I-1.1-6	Z	5±0.3	10	0	0	0	5±0.2	10	0	0	0	4±0.2
	M	17±1.7	0	9	1	0	14±1.5	1	9	0	0	7±0.2
I-1.1-7	Z	---[3]	-	-	-	-	---	-	-	-	-	---
	M	20±2.4	0	8	0	0	10±3.2	5	5	0	0	6±0.4
I-1.1-8	Z	13±1.6	0	10	0	0	9±1.4	7	3	0	0	3±0.8
	M	39±2.9	0	3	0	7	28±2.3	0	2	8	0	8±0.6
I-1.1-9	Z	18±1.5	0	10	0	0	17±0.6	0	10	0	0	3±0.1
	M	26±1.2	0	0	0	10	23±1.8	0	10	0	0	6±0.2
I-1.1-10	Z	17±1.4	0	10	0	0	5±0.4	10	0	0	0	5±0.2
	M	33±1.5	0	0	0	10	18±2.6	0	10	0	0	8±0.5
I-1.1	Z	10±0.9	0	10	0	0	8±2.9	9	1	0	0	4±0.0
	M	34±1.2	0	0	0	10	18±2.4	0	10	0	0	8±0.3
Race 1	Z	39±0.8	0	0	0	10	34±2.2	0	0	10	0	5±0.3
	M	29±1.5	0	0	0	10	28±3.4	0	0	0	10	9±0.6

1 Inoculation method: Z = inoculation by 10 µl drop of zoospores (10^5 zoospores/ml); M = inoculation by 5 mm plugs from periphery of colonies grown on 20% V8 juice agar.
2 Reaction type: R = Resistant, Rs = Resistant with spread, Sn = Susceptible with necrosis, S = Susceptible. Cultivars Wayne and Harosoy are susceptible to race 1, cv. Harosoy 63 is resistant to race 1 and was resistant to all isolates.
3 Zoospores were not obtained.

Fig. 6.4
Distribution of aggressiveness (lesion length) among single-zoospore isolates of Phytophthora megasperma f.sp. glycinea A) Isolate 1.1, B) race 1, on soybean cultivar Harosoy (susceptible) following inoculation of intact etiolated hypocotyls with mycelial plugs. Measurements were made 48 h after inoculation and data are means for 5 lesions from 5 hypocotyls.


(A)
Isolate 1:1
16
8
0
(B)
Race 1
16
8
0
Frequency of Isolates
0
20
40
Lesion Length (mm in 48h)

susceptible to race 1) there was, however, considerable variation in aggressiveness as indicated by lesion size. Two single zoospore isolates out of 48 from Isolate 1.1 were more aggressive than their parent, but the majority were less aggressive and reaction-types tended to be resistant with various degrees of spread (Fig. 6.4A). Most of the 38 single zoospore isolates from race 1 were more aggressive than their parent (Fig. 6.4B).

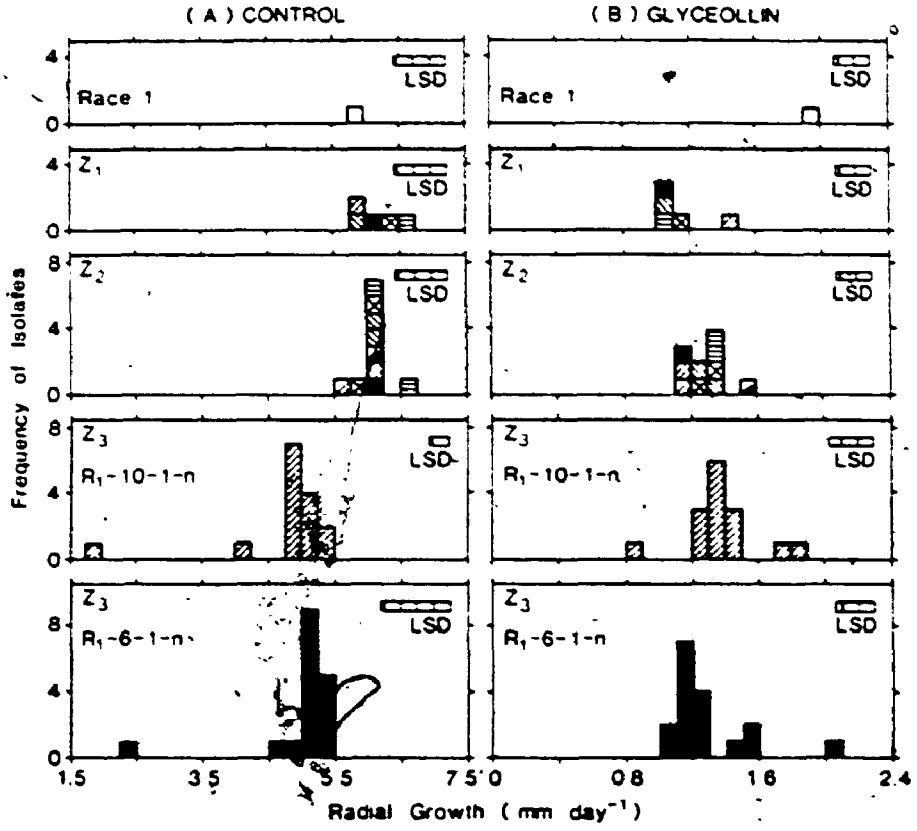
From each of the five most aggressive single zoospore isolates out of the 38 isolates obtained from race 1 (race 1-6, -10, -13, -17, -23) two single zoospore isolates (1 or 2) were selected at random to give a second generation (Fig. 6.5). One of these two again was selected at random to give a third generation of single-zoospore families. These were designated as follows: R_1-6-1-n, -10-1-n, -13-2-n, -17-1-n, -23-2-n (n = number of single-zoospore isolates in the third generation family). Isolates or progenies comprising these five families were tested for their growth along with second generation single-zoospore isolates in 10% clarified V8 juice agar (Table 6.5). No significant variation was recorded among second generation single-zoospore isolates. However, significant variation was recorded among the progenies comprising three out of the five third generation families. Two of these families with the highest estimates of genotypic co-efficient of variation (GCV) were selected and growth rates determined on control (10% clarified V8 juice agar containing 2% ethanol Fig. 6.5A) and glyceollin I amended medium (Fig. 6.5B). In both families there was significant variation in growth both with and without glyceollin I and the GCVs and heritabilities were estimated to be high.

6.5 Discussion

Somatic variation in species of Phytophthora has been

Fig. 6.5

Variation in growth in control (A) and glyceollin I (75 µg/ml) amended medium (B) in successive single-zoospore generations from Phytophthora megasperma f.sp. glycinea race 1. Single-zoospore generations are indicated by Z, thus Z_1, Z_2 and Z_3 for first, second and third single- zoospore generations from the original race 1. Z_1 consists of the 5 most aggressive of the 38 single-zoospore isolates illustrated in Fig. 6.4B. From each of the Z_1 isolates two single-zoospores were selected at random to give the 10 single-zoospore progenies of the second generation illustrated by Z_2. From each of the two single-zoospore lines in Z_2 one was chosen at random to give the third single-zoospore generation Z_3. Two of the 5 families from this generation (R_1-6-1-n and R_1-10-1-n) that are analysed in Table 6.5 are illustrated. Data are from two replicated experiments analysed in a completely randomized block design. Observations were made between 24 and 48 h after transfer for controls (A) and between 36 and 60 h for the glyceollin I treatment (B) because of differences in growth rates.



(C) SCHEME FOR LINEAGES

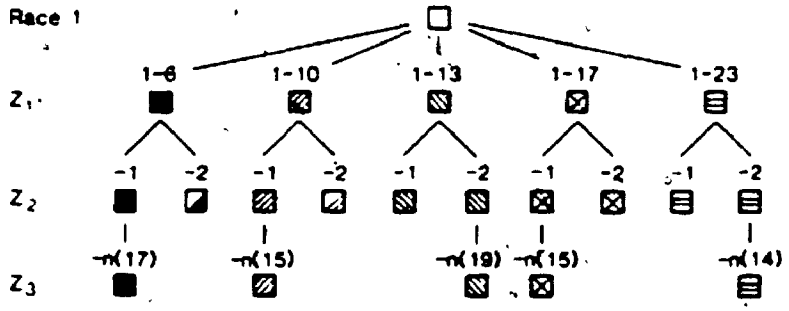


Table 6.5

Genetic variability and heritability of growth rate and sensitivity to glyceollin I of single-zoospore isolates derived from *Phytophthora megasperma* f.sp. *glycinea* race 1

Zoospore Generation	[1]Radial Growth Rate (mm/day)								
	[4]Glyceollin I(µg/ml)								
	[1a]0			[1b]0			[1c]75		
Family	[2]Range	[3]GCV	[4]h^2	Range	GCV	h^2	Range	GCV	h^2
Z_2[5]	5.7	-.-[6]	-.-	15.7	-.-	-.-	17.9	10.0	84.6
Z_3[5]									
R1-6-1-n	34.9	14.7	88.9	59.5	12.0	58.1	75.4	17.7	80.8
R1-10-1-n	56.9	12.6	38.5	70.9	16.7	80.4	61.9	14.3	69.3
R1-13-2-n	13.7	-.-	-.-						
R1-17-1-n	25.6	-.-	-.-						
R1-23-2-n	36.9	6.8	50.9						

[1] Radial growth rate (mm/day) was recorded from growth during the period from 36 to 60 h following inoculation (5 mm mycelial plugs) in, 1a) Petri dishes (90 mm diameter) containing 10 ml of 10% clarified V8 juice agar, 1b) Petri dishes (35 mm diameter) containing 0.8 ml of 10% clarified V8 juice agar containing 2% alcohol and 1c) as in 1b but with 75 µg/ml glyceollin I.

[2] The difference between the highest and the lowest values were divided by respective means and expressed as percentages.

[3] GCV (Genotypic coefficient of variation) = $(\sigma_g/\bar{X})x100$

[4] h^2 (Heritability in broad sense) = $(\sigma_g/\sigma_p)x100$

[5] Z_2, single-zoospore generation number 2; Z_3 means single-zoospore generation number 3.

[6] Zoospore generations or families not showing any significant variation were not considered for calculation of GCV & h^2.

described in a number of reports and these have been summarized and the question of the genetic basis for somatic variation discussed in comprehensive reviews [Brazier, 1983; Erwin, 1983; Shaw, 1983]. The present study has provided additional examples of this phenomenon.

The colony morphology and growth rate of Isolate 1.1 on 20% V8 juice agar was similar to that of the parent culture of race 1, but on 10% clarified V8 juice agar the growth rate was significantly less (about 25%). It was also less aggressive on soybeans. The sector, from which Isolate 1.1 was obtained, presumably arose either as a mutation or by selection of a variant genotype already present within the colony. The latter seems unlikely, for even if the variant was maintained at a very low level in the original colony, a more uniform adaptation of the colony to glyceollin I would be expected than was indicated by the rare fan-shaped outgrowths that actually developed. However, several observations made here argue against the origin of Isolate 1.1 as a mutation in a nuclear gene. Although the association of reduced growth rate (on 10% clarified V8 juice agar) and aggressiveness with development of tolerance to glyceollin I suggests pleitropy, examination of these three characters in the single-zoospore progeny of Isolate 1.1 revealed that they each segregated independently and in a quantitative fashion. Assuming more than one nucleus was present in the hypha from which the sector developed and that a mutation occurred in one of these, then, because each zoospore contains only one nucleus [Long et al., 1975] the three characters examined should have remained associated in the single-zoospore progeny and phenotypes should have conformed either to that of race 1 or to that of Isolate 1.1. The quantitative nature of the variation in phenotype would imply the segregation of many minor genes.

These anomalies would be more readily explained if

based on changes in cytoplasmic rather than nuclear genes. For example, if a mutation occurred in the mitochondrial genome, and mitochondria with or without the mutation were randomly distributed to zoospores, quantitative differences would be expected among single-zoospore progeny, including the occurrence of progeny with greater tolerance than Isolate 1.1 (Table 6.4). Shaw [1983] has discussed the possibilitiy that the mitochondrial genome may be the source of much unexplained somatic variation in Phytophthora spp. Phenotypic effects of changes in the mitochondrial genome or of independently replicating mitochondrial plasmids have been recorded in other species of fungi [Narang, 1985]. It may be of significance that glyceollin has been reported to be an inhibitor of electron transport in mitochondria [Boydston et al., 1983]. Changes in sensitivity to glyceollin, therefore, might occur most directly at the level of the mitochondrion. Furthermore, the possibility that phytoalexins may be DNA-damaging agents has been discussed [Chatterjee and Vidaver, 1986]. Isolate 1.1 could then have arisen as a direct response to glyceollin I. Independent effects throughout the mitochondrial population could explain the simultaneous origin of changes in growth rate, aggressiveness and tolerance to glyceollin I and the quantitative expression and the segregation of these characters in single-zoospore progeny. The demonstration that variation in growth rate and sensitivity to glyceollin I persisted through three single-zoospore generations also suggests that mechanisms other than segregation of heterokaryons must be involved.

Further support for the above arguments for the transmission of variation by cytoplasmic determinants is provided by the differences in colony morphology observed among single-zoospore progeny. Although Isolate 1.1 had a normal colony morphology, similar to race 1, more than 98% of its

single-zoospore progeny gave rise to fluffy colonies. Single-zoospore colonies derived from race 1 were almost all normal, but about 3% were of the fluffy type. The hypha from which the Isolate 1.1 sector arose presumably contained several nuclei, but it is unlikely that there were sufficient to provide ratios of 98:2 or that a mutation could occur simultaneously in such a proportion of the nuclei. The observation that similar ratios were obtained for single-zoospore progeny from hyphal tip cultures of Isolate 1.1 supports this conclusion. By analogy with Pythium ultimum [Grove et al., 1970], nuclei should be absent from the first 20 µm behind the hyphal tip and thereafter be distributed randomly 2-3 per 10 µm of hyphal length. Therefore, few nuclei would be isolated in excised hyphal tips. Mitochondria, however, were very abundant in the sub-apical zone in P. ultimum and the abundance of mitochondria in contrast to the single nucleus in zoospores of Phytophthora infestans has been illustrated by Shaw [1983]. It is unlikely that the high proportion of the fluffy-type isolates derived from Isolate 1.1 was due to selective multiplication of nuclei carrying a mutation for the fluffy character. There was no subsequent selective pressure from glyceollin I and under similar conditions fluffiness in race 1 occurred in only a very small proportion of progeny. From these considerations it is reasonable to conclude that fluffiness is controlled by cytoplasmic elements distributed randomly to zoospores, and mitochondria appear to be good candidates.

Since Isolate 1.1 had a normal colony type, the 98:2 ratio of fluffy to normal colonies in the progeny of Isolate 1.1 indicates that normal colony-type is highly epistatic to fluffiness. Thus, if fluffiness is due to a genomic deficiency only a few normal genetic determinants in coenocytic hyphae may be required for the development of a normal phenotype. The possibility that each of the charac-

ters examined arose due to deficiencies is suggested by the demonstration that increased growth rates were generated by 20% rather than 10% clarified V8 juice agar and that aggressiveness was frequently restored by the use of mycelium on plugs of agar medium instead of zoospores for inoculum. Recovery of growth rates, and their maintenance subsequently in many isolates even when returned to 10% clarified V8 juice agar, suggests some form of repair mechanism, possibly under the control of nuclear genes.

There was some evidence that glyceollin I tolerance was associated with aggressiveness on Harosoy and/or Wayne. Thus three isolates that grew very little in the presence of glyceollin I (I-1.1-1, I-1.1-6, I-1.1-10; Table 6.3) produced small lesions and resistant reaction types following zoospore inoculation (Table 6.4). However, although the opposite was true for two isolates that grew well on glyceollin I (I-1.1-9, I-1.1-2; Table 6.3) there were inconsistencies with other isolates and assumption of a causal relationship would be premature. There was a tendency for cv. Harosoy to be more resistant than cv. Wayne. The differences varied significantly with the isolate, indicating that although neither cultivar carries a gene for resistance to race 1, there is a degree of specificity between these cultivars and individual single-zoospore isolates. Cultivar Harosoy does carry an unidentified gene(s) for resistance to races 12, 16, 18 and 19 [Keeling, 1982] and the possibility that this gene could have residual effects may be considered.

CHAPTER 7

BIOSYNTHESIS AND METABOLISM OF GLYCEOLLIN I IN SOYBEAN HYPOCOTYLS FOLLOWING WOUNDING OR INOCULATION WITH *Phytophthora megasperma* f.sp. *glycinea*.

7.1 Summary

Incorporation of [^{14}C] into glyceollin I during 1 h pulses with L-[U^{14}C]-phenylalanine was determined in soybean hypocotyls of cvs. Harosoy (*rps*$_1$, susceptible) and Harosoy 63 (*Rps*$_1$, resistant) wounded or unwounded and inoculated with zoospore suspensions of *Phytophthora megasperma* f.sp. *glycinea* race 1, and in controls, wounded only. Incorporation of [^{14}C] occurred in all combinations. In unwounded hypocotyls rates of incorporation and accumulation of glyceollin I were higher in resistance than in susceptible responses throughout the time-course of the experiment. In inoculated wounded hypocotyls high rates of [^{14}C] incorporation developed that were similar for the first 11 h in resistance and susceptible responses although accumulation of glyceollin I differed. Evidently, wounding masks the initial responses to infection. High rates of incorporation also developed in uninoculated wounded hypocotyls but only small amounts of glyceollin I of high specific activity were detected. Estimates of phenylalanine ammonia-lyase activity indicated that the rate of flow in the biosynthetic pathway through phenylalanine was limited in the wounded controls but potentially very high in the resistance responses. Thus while [^{14}C]-incorporation clearly demonstrated biosynthesis, differences in rates of incorporation presumably indicated differences in the relative contributions of mobile internal pools and externally applied phenylalanine, rather than differences in rates of biosynthesis. Pulse-chase experiments with [^{14}C]-phenylalanine demonstrated rapid metabolism of

glyceollin I in wounded controls but not in inoculated hypocotyls due to continued [^{14}C]-incorporation during the chase period. Rapid metabolism was demonstrated in all combinations when cinnamic acid was substituted for phenylalanine as the chase. Additional evidence for metabolic activity in all combinations, including wounded controls, was provided by pulse-feeding with [^{14}C]-glyceollin I. Metabolic activity was not constitutive but was induced within 4.5 h of wounding or inoculation and it appears that the stimulus of wounding or inoculation induces a metabolic pathway in which glyceollin I is not an end product. It is concluded that although metabolism is important for the control of glyceollin I levels within the plant, accumulation is governed primarily by rates of biosynthesis and these are much higher in incompatible than in compatible interactions.

7.2 Introduction

The possibility that phytoalexins may be intermediates in constitutive secondary metabolic pathways and that their accumulation may be regulated as much by rates of metabolism as by rates of biosynthesis has been considered by several authors [Ishiguri et al., 1978; Stoessl et al., 1976; Ward and Barrie, 1982; Ward et al., 1977; Yoshikawa et al., 1979]. Inhibition of metabolism rather than elicitation of biosynthesis, then would be the key mechanism controlling phytoalexin accumulation in incompatible interactions. Evidence for phytoalexin metabolism by plants is provided by time-course studies of phytoalexin concentrations in infected or stressed tissue in which phytoalexin levels decline after reaching a maximum [eg. Keen et al., 1972; Moesta and Grisebach, 1981b; Rahe and Arnold, 1975; Sato and Tomiyama, 1969; Chapter 3.4.4.1] and particularly by the disappearance of phytoalexins supplied to or radiolabelled

within plant tissues [Ishiguri et al., 1978; Sakai et al., 1979; Stoessl et al., 1976; Yoshikawa et al., 1979].

In the interaction of soybean hypocotyls with Phytophthora megasperma f.sp. glycinea Yoshikawa et al. [1979] concluded from pulse-labelling experiments with L-[U^{14}C]-phenylalanine as precursor that rates of biosynthesis of glyceollin in compatible and incompatible interactions were similar even though much higher concentrations of glyceollin accumulated in the latter. They also concluded from pulse-chase experiments and from feeding glyceollin to hypocotyl tissues that glyceollin was metabolised in uninoculated tissue but that this activity was inhibited following infection, especially in the incompatible interactions. These results suggested that the differential accumulation of glyceollin in the two interactions primarily reflected differences in the rate of metabolism of glyceollin. Quite different conclusions were reached for the same host-pathogen system by Moesta and Grisebach [1981b]. These authors used [^{14}C]-carbon dioxide as the precursor. They found that although rates of biosynthesis were the same in both compatible and incompatible interactions for the first 12 h following inoculations, biosynthesis subsequently became more rapid in the incompatible interaction. It was only at this stage that differences in glyceollin accumulation between the two interactions were detected. No evidence for significance glyceollin metabolism was obtained by these authors and it was concluded that the differential in glyceollin accumulation was due to the differences in biosynthetic rates that ultimately developed. A difficulty with the conclusions of both groups of investigators is that they imply that elicitation of glyceollin biosynthesis is the same for compatible and incompatible races and that specificity is a second and latter phenomenon. A further inconsistency between the two studies is that differences in

the degree of restriction of the pathogen in compatible and incompatible interactions were detected by 8 h following inoculation by Yoshikawa et al. [1978], a time at which according to Moesta and Grisebach [1981b] but not according to Yoshikawa et al. [1978, 1979], glyceollin concentrations in both types of interactions were the same.

In this chapter biosynthesis and metabolism of glyceollin I (the major isomer accumulating in hypocotyls, [Chapter 3]) were examined in both wounded and intact hypocotyls. The results indicate that glyceollin I biosynthesis and metabolism are induced following wounding and/or infection, that the time-course of both biosynthesis and glyceollin I accumulation in compatible and incompatible interactions are distinctly different, that quantitative differences in biosynthesis rather than metabolism appear to be the major factors leading to differntial glyceollin I accumulation and that in wound-inoculated hypocotyls wound responses initially mask responses to infection. Throughout the chapter the term glyceollin is used with reference to undefined mixtures of the glyceollin isomers.

7.3 Materials and Methods

7.3.1 Pathogen

The culture used was a single zoospore isolate (R1.19) derived from Phytophthora megasperma f.sp. glycinea race 1 [Chapter 6.4]. It was grown routinely on V8 juice agar medium at 25°C and zoospore-suspensions were prepared from 5-day-old cultures on the same medium in Petri dishes following the methods described in chapter 2 [2.2.2.2]. Inoculum in all cases consisted of a 10 µl drop of a standardized zoospore suspension (10^5/ml).

7.3.2 Host

Seeds of soybean, cultivar Harosoy (susceptible to

Phytophthora megasperma f.sp. glycinea race 1) and its isoline Harosoy 63 (resistant) were grown in trays of vermiculite in the dark for 6 days as described previously in chapter 2.1.1.

7.3.3 Inoculation and Incubation

The seedlings were arranged horizontally on moist cellucotton in glass trays [Chapter 2.1.2]. Hypocotyls were inoculated by placing a 10 µl drop of zoospore suspension, or sterile distilled water in controls, onto the surface of the intact hypocotyl about 2 cm below the cotyledons or into a wound at the same position. Wounds were made with a scalpel by removing a strip of tissue from the hypocotyl surface similar in area to that covered by a drop of inoculum (3 mm long and 0.5 mm deep). Wounds were inoculated immediately after wounding except where indicated otherwise. Trays of inoculated seedlings were closed with plastic film to maintain high humidity and incubated in the dark at 25°C.

7.3.4 Radioactive Compounds

L-[$U^{14}C$]-phenylalanine (50 µCi/ml; 0.1 mM or 522 mCi/mM) was obtained from Amersham Corporation. [^{14}C]-Glyceollin I (0.05 µCi/ml; 0.1 mM) was prepared by applying L-[$U^{14}C$]-phenylalanine for 1 h to water-inoculated wounds 25 h after wounding, to obtain glyceollin I of high specific activity. Extraction and purification of glyceollin I is described below.

7.3.5 Feeding of Radioactive Compounds

Routinely, L-[$U^{14}C$]-phenylalanine was supplied to inoculated sites and wounds in 5 µl drops after removing the inoculum drops or water. In an alternative procedure used in one experiment, hypocotyls were cut 2 cm below the inoculated site and 5 µl L-[$U^{14}C$]-phenylalanine was applied to

the cut end. A minimum of 10 hypocotyls were used for each treatment and each hypocotyl received a 5 µl drop of 0.1 mM L-[U^{14}C]-phenylalanine (50 µCi/ml). Except where indicated otherwise, pulse periods were 1 h, after which glyceollin I was isolated and [^{14}C]-incorporation measured. Decline of radioactivity in glyceollin I following pulse feeding was determined for various chase periods. Inoculated sites were washed to remove L-[U^{14}C]-phenylalanine and either [^{12}C]-phenylalanine (10 µl, 1 mM) as used by Yoshikawa et al. [1979] or trans-cinnamic acid (15 µl, 1 mM in 5 mM phosphate buffer, pH 5.5) to inhibit PAL activity [Shields et al., 1982], was applied to the inoculated sites. In the cinnamic acid chase experiment, hypocotyls were kept moist under wet cellucotton and severed about 6.5 cm below the cotyledons. The cotyledons were removed immediately before feeding L-[U^{14}C]-phenylalanine (12.5 µCi/ml, 0.025 mM to wounded hypocotyls; 25 µCi/ml, 0.05 mM to unwounded hypocotyls) to 20 sites per treatment. The pulse period was from 8.5-9.5 h, after inoculation except in wounded control hypocotyls where it was delayed to permit detectable accumulation of glyceollin I and was from 14-15 h after wounding. In addition to chasing with cinnamic acid at the sites of wounding and inoculation, 2 mM cinnamic acid in 10 mM phosphate buffer (pH 5.5) was added also to the wet cellucotton covering the lower cut end of the hypocotyls.

To determine the rate of metabolism of exogenously supplied glyceollin I, 5 µl drops containing [^{14}C]-glyceollin I were applied to infection sites at various times after inoculation and/or wounding and residual glyceollin I in surface fluids and tissue extracts was determined after incubation for 30 min.

7.3.6 Extraction and Determination of Glyceollin I

For each determination, sections (approx. 2 cm long)

containing infected and wounded tissues were excised from 10 or more hypocotyls and the glyceollin isomers were extracted by boiling in 95% ethanol for 2 min. The extract was decanted and together with two ethanol rinses of the tissues evaporated to dryness under reduced pressure. The residue was extracted with ethyl acetate and subjected to TLC as described in chapter 2.7 to obtain glyceollin. Glyceollin I was separated from isomers II and III by HPLC [Chapter 2.7] using an analytical column and 4.5% isopropanol in hexane (flow rate 1.5 ml/min) as the mobile phase. Glyceollin I was detected by its absorption at 286 nm. The retention time was 13.58-14.33 min and this afforded complete separation from isomers II and III. It was quantified from its absorbance at 286 nm reference to a standard curve prepared from purified glyceollin I and the extinction coefficient, 10,800 [Chapter 2.6].

7.3.7 Measurement of [^{14}C]-Incorporation into Glyceollin I

The fraction corresponding to the glyceollin peak detected by HPLC was collected in a scintillation vial, mixed with 10 ml of scintillation fluid (4 g Omnifluor in 1 litre toluene) and radioactivity in d/min measured in a scintillation counter (Beckman LS 9000).

7.3.8 Purity of [^{14}C]-Glyceollin I

This was determined for the glyceollin I preparations obtained in the experiment illustrated in Fig. 7.1. Following the first TLC separation, samples from all the pulse times within each treatment were combined giving bulked samples for wounded-inoculated and intact inoculated cv. Harosoy and Harosoy 63. For the wounded water controls the Harosoy and Harosoy 63 samples were combined also and spiked with purified [^{12}C]-glyceollin I to provide sufficient material for further analysis. The bulked samples were sub-

jected to HPLC, the glyceollin I fractions were collected and aliquots were taken for measurement of radioactivity as described. The remaining portions were subjected to TLC in three solvent systems (Table 7.1), again followed by HPLC and measurement of radioactivity.

7.3.9 Determination of Free L-Phenylalanine

Tissues of 20 lesions and wounds in 10 hypocotyls were excised and ground in methanol with a mortar and pestle. The slurry was centrifuged (microcentrifuge, 15,000 rpm) and the pellet was dried (65°C) and weighed. The methanol was evaporated from the supernatant and the residue dissolved in 3 ml high purity water (Milli-Q system, Millipore). The pH of the solution was adjusted to 2.0 with 1 M HCL and extracted three times with ethyl ether. The aqueous phase was applied to a cation exchange column (Rexyn 101, H^+ form, 0.9 x 7 cm) (Fig. 7.1). The column was washed with 10 ml high purity water and amino acids were eluted with 1 N NH_4OH (12 ml). The eluate was lyophilized and used for the determination of L-phenylalanine by HPLC, in general following procedures described by Bidlingmeyer et al. [1984]. The lyophilized sample was dissolved in 0.5 ml water and 50 µl was taken for analysis. The water was removed under nitrogen at low pressure and the residue was dried a second time after redissolving in 10 µl ethanol:water:triethylamine (2:2:1, v/v). The dried residue was derivatized with 20 µl of freshly prepared derivatizing reagent (ethanol-triethylamine-water-phenylisothiocyanate; 7:1:1:1) at room temperature for 20 min. The reagent was removed by evaporation and the residue redissolved in 100 µl sample diluent (710 mg Na_2HPO_4 mixed with 1 l H_2O, titrated to pH 7.4 with 10% phosphoric acid and mixed with acetonitrile to give a final concentration of 5% by volume). It was injected (15 µl) onto a Waters Pico-Tag column

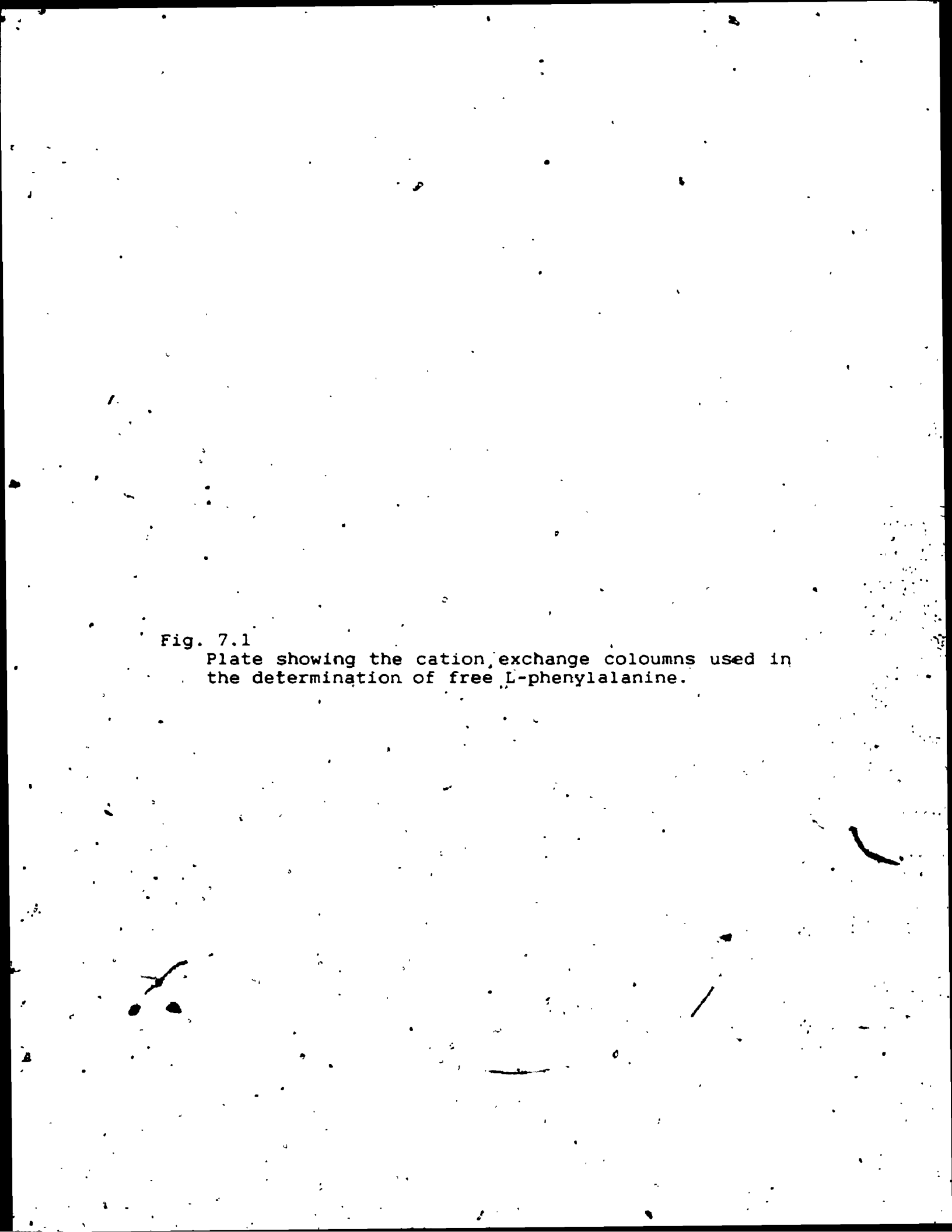


Fig. 7.1
Plate showing the cation exchange coloumns used in the determination of free L-phenylalanine.

Table 7.1

Effect of purification on the specific radioactivity of [^{14}C]-glyceollin I recovered from soybean[a] hypocotyls after wounding or inoculation with Phytophthora megasperma f.sp. glycinea race 1 in pulse-feeding experiments with L-[U^{14}C]-phenylalanine[b].

Cultivar and treatment	Glyceollin I specific radioactivity (d/min/µg)			
	After initial TLC (So) and HPLC	Second TLC		
		S1	S2	S3
Harosoy, Harosoy 63, wounded only[c]	1908±21[d]	2028±22 (106)[e]	1858±17 (97)	1987±32 (104)
Harosoy, wounded, inoculated	346±6	345±3 (100)	361±2 (104)	335±4 (97)
Harosoy 63, wounded, inoculated	480±2	467±4 (97)	480±14 (102)	467±13 (97)
Harosoy, unwounded, inoculated	116±2	104±2 (90)	106±3 (91)	110±1 (95)
Harosoy 63, unwounded, inoculated	275±3	278±4 (101)	290±4 (105)	280±10 (102)

Solvent system		Rf glyceollin I
S0	Benzene: methanol (95:8)	0.35
S1	Diethylether:n-hexane (60:40)	0.48
S2	Chloroform:methanol (100:5)	0.37
S3	n-hexane:diethylether:glacial acetic acid (65:30:3)	0.26

a cv. Harosoy is susceptible and cv. Harosoy 63 is resistant to Phytophthora megasperma f.sp. glycinea race 1.

b Wounded and inoculated sites were pulsed with L-[U-^{14}C]-phenylalanine (5 µl, 50 µCi/ml, 0.1 mM) for 1 h prior to analysis at the times indicated in Fig. 7.2A. Samples for each cultivar and treatment were bulked.

c In wounded controls, glyceollin from both cultivars was combined after the first TLC and spiked with purified glyceollin I.

d Data are means and SEs from 3 replications.

e Specific radioactivity after the second TLC expressed as a percentage of that after HPLC.

(column temperature 38°C, Waters model 710B WISP injector, model 510 pumps, model 480 variable wave length detector and model 840 data system) and L-phenylalanine was determined by the Pico-Tag (Waters) method following instructions provided by the manufacturer. L-phenylalanine concentrations were expressed as n moles/mg d. wt of excised tissue. Data are based on two replications.

7.3.10 Determination of Phenylalanine Ammonia-Lyase Activity

Tissues were excised from 20 lesions or wounds in 10 hypocotyls per treatment, immediately frozen in liquid nitrogen and stored at -70°C. They were ground with a mortar and pestle with 0.1 M sodium borate buffer, pH 8.8, containing 2 mM mercaptoethanol [Lamb et al., 1979]. The slurry was centrifuged in a microcentrifuge at 15000 rpm for 4 minutes. The supernatant was collected, and, after recording its volume, immediately frozen in liquid nitrogen and stored at -70°C until required. PAL activity in the supernatant was determined by measuring the production of cinnamic acid from L-phenylalanine spectrophotometrically [Lamb et al., 1979]. The reaction mixture contained 300 µM sodium borate pH 8.8, 30 µM L-phenylalanine and 1 ml of supernatant in a total volume of 3 ml. Following incubation for 1 h at 40°C the absorbance at 290 nm was read against an identical mixture in which D-phenylalanine was substituted for L-phenylalanine. Amount of cinnamic acid produced from L-phenylalanine was determined by reference to a standard graph prepared from the absorbance of known concentrations of cinnamic acid at 290 nm (Appendix II). The enzyme activity was expressed as n moles cinnamic acid produced in one minute per mg dry weight of tissues.

7.4 Results

Analyses for glyceollin I only are reported here.

Glyceollin II and III, which are produced in hypocotyls in much smaller amounts than glyceollin I, were frequently insufficient to provide reliable estimates of [^{14}C]-incorporation, and could not be purified to constant specific radioactivity. The combination of TLC and HPLC separations (see Methods) yielded glyceollin I sufficiently pure for interpretation of incorporation and metabolic experiments with confidence (Table 7.1).

7.4.1 Estimation of Glyceollin I Rate of Biosynthesis

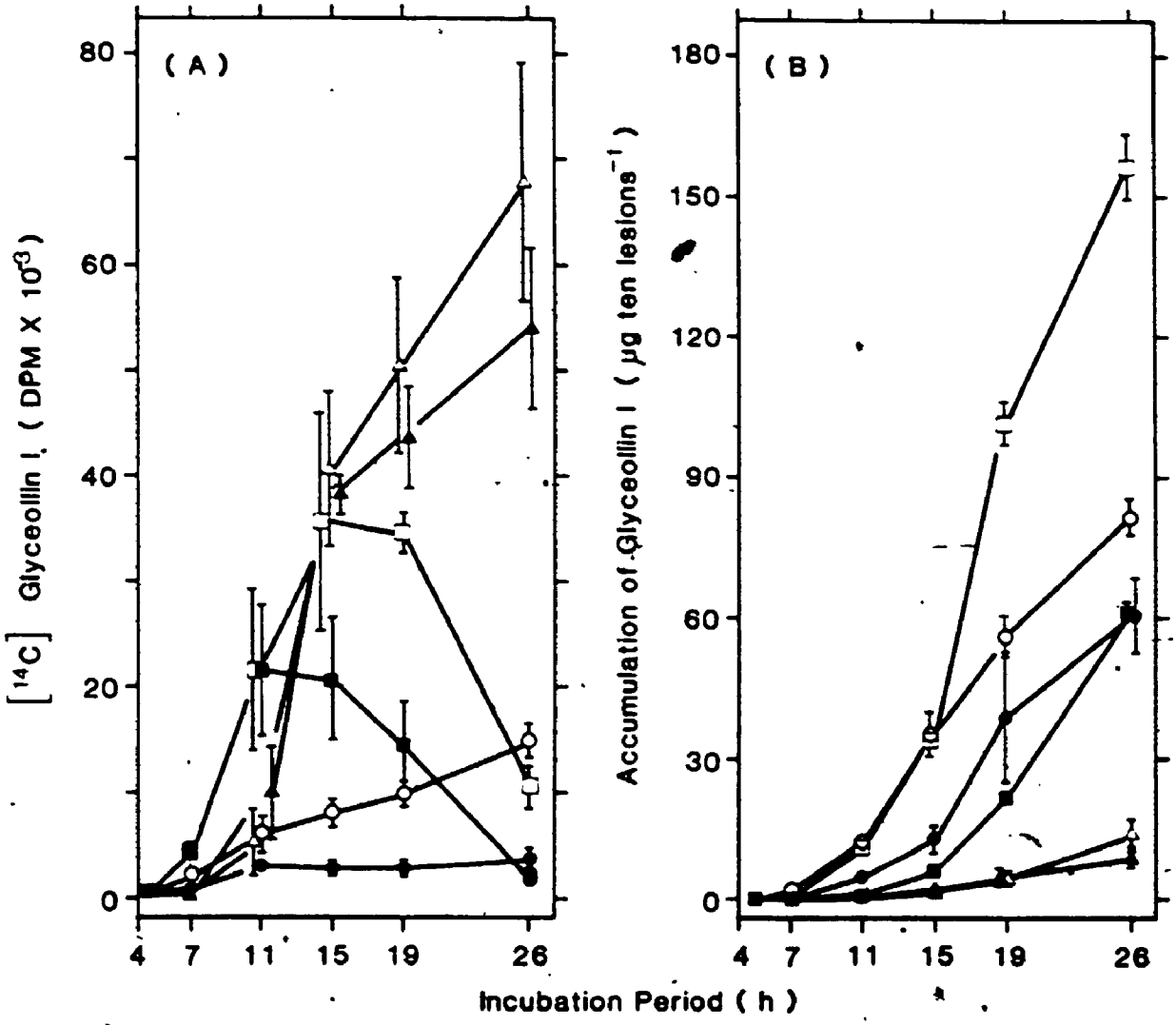
In uninoculated wounded hypocotyls, [^{14}C]-incorporation into glyceollin I following 1 h pulses with L-[U^{14}C]-phenylalanine was demonstrated 11 h following wounding (Fig 7.2A). The rate of incorporation increased very rapidly at subsequent pulse times. However, accumulation of glyceollin I was not demonstrated until 15 h following wounding and only small quantities were detected after longer periods (Fig 7.2B).

In inoculated wounds, [^{14}C]-incorporation into glyceollin I was demonstrated 7 h following inoculation. It increased rapidly and was similar in both cultivars until 11 h (Fig 7.2A). Incorporation then declined in the compatible interaction (Harosoy) but increased in the incompatible interaction (Harosoy 63) until 15 h and then also declined. Accumulation of glyceollin I, however, was distinctly different in the two interactions (Fig 7.2B). In the incompatible interaction it accumulated rapidly from 7 h after inoculation but was not detected until 11 h in the compatible interaction and subsequently accumulated relatively slowly.

In contrast to similarities in early rates of [^{14}C]-incorporation in both interaction types in inoculated wounded hypocotyls, rates of incorporation in the incompatible interaction in unwounded hypocotyls differed from

Fig. 7.2

(A) Incorporation of [^{14}C] into glyceollin I from L-[U^{14}C]-phenylalanine in 1 h pulse periods, and (B) accumulation of glyceollin I in soybean hypocotyls. Intact hypocotyls inoculated with Phytophthora megasperma f.sp. glycinea race 1, cv Harosoy (susceptible) ●—●, cv. Harosoy 63 (resistant) ○—○. Wounded inoculated hypocotyls, cv. Harosoy ■—■, cv. Harosoy 63 □—□. Incubation period indicates the time following inoculation. Wounded control hypocotyls cv. Harosoy ▲—▲, cv. Harosoy 63 △—△.


(A)
(B)
[14C] Glyceollin I (DPM X 10-3)
Accumulation of Glyceollin I (µg ten lesions-1)
Incubation Period (h)
0
20
40
60
80
30
90
120
150
180
4
7
11
15
19
26

those in the compatible interaction when first demonstrated at 7 h following inoculation (Fig 7.2A). The patterns of glyceollin I accumulation in the two interactions in unwounded hypocotyls were consistent with the differences in incorporation rates.

Specific radioactivity of [^{14}C]-glyceollin I differed widely in the different treatments and also varied with time after inoculation and wounding (Table 7.2). Thus specific activities were very high in wounded control hypocotyls and also in the compatible interaction in wounded hypocotyls, although they declined rapidly in the latter. This differed from the incompatible interaction in wounded hypocotyls in which specific activities were low initially and remained lower than in the compatible interaction. In contrast in the unwounded hypocotyls specific activities in both interactions were similar and remained low at all pulse times.

The possibility that wounding or type of interaction might affect access of surface applications of L-[U^{14}C]-phenylalanine to sites of biosynthesis and hence influence incorporation rates was examined in the following experiment. Comparisions were made of [^{14}C]-incorporation from L-[U^{14}C]-phenylalanine applied either to infection sites or through the cut ends of hypocotyls. For the same interaction type much more [^{14}C] was incorporated in wounded than in unwounded hypocotyls following application to infection sites but there were no differences in incorporation following feeding through cut ends of hypocotyls (Table 7.3). Therefore, quantitative comparisions of incorporation rates between inoculated wounded and unwounded hypocotyls cannot be made when precursor is applied to the inoculated site. On the other hand the data also demonstrate that the differential in [^{14}C]-incorporation between compatible and incompatible interactions was not affected by the feeding method. Hence comparisions of incorporation rates in the two

Table 7.2

Specific radioactivity of [^{14}C] glyceollin I recovered folowing pulse feeding with L-[U^{14}C]-phenylalanine from soybean hypocotyls wounded or inoculated with Phytophthora megasperma f.sp. glycinea race 1[a].

Time from inoculation (h)	Glyceollin I specific radioactivity (d/min/µg)					
	unwounded inoculated		wounded inoculated		wounded only	
	Harosoy	Harosoy 63	Harosoy	Harosoy 63	Harosoy	Harosoy 63
11	1927[c]	1755	72,032	4,934	65,281	65,482
15	1509	1875	16,977	5,011	93,146	43,356
19	934	1272	4,508	2,067	62,112	78,563

[a] Wounded and inoculated sites were pulsed with L-[U^{14}C]-phenylalanine (5 µl, 50 µC./ml, 0.1 mM) for 1 h prior to analysis at the times indicated.

[b] cv. Harosoy is susceptible and cv. Harosoy 63 is resistant to Phytophthora megasperma f.sp. glycinea race 1.

[c] Specific radioactivities were calculated from the incorporation of [^{14}C] into glyceollin I during the pulse period and the mean hourly accumulation of glyceollin I.

Table 7.3

Influence of method of feeding L-[U^{14}C]-phenylalanine on incorporation of [^{14}C] into glyceollin I following inoculation of wounded or intact soybean hypocotyls with Phytophthora megasperma f.sp. glycinea race 1.

Cultivar and treatment[a]	[^{14}C] glyceollin I (d/min)	
	Method 1[b]	Method 2
Pulse period 10-11 h		
Harosoy		
Wounded	17,290±966[c]	130±22
unwounded	1,281±159	94±34
Harosoy 63		
wounded	29,379±1042	1241±323
unwounded	3,800±422	952±107
Pulse period 10-12 h		
Harosoy		
unwounded	1,546±53	1,169±80
Harosoy 63		
unwounded	16,917±1047	12,311±1,109

[a] All plants were inoculated, cv. Harosoy, is susceptible and cv. Harosoy 63 is resistant. L-[U^{14}C]-phenylalanine (50 µCi/ml, 0.1 mM) was applied in 5 µl to each of 10 hypocotyls.

[b] In method 1, inoculum drops were removed from inoculated sites 10 h after inoculation and replaced with L-[U^{14}C]-phenylalanine for 1 or 2 h before analysis. In method 2, at 10 h after inoculation hypocotyls were severed approx. 2.5 cm below inoculation sites and the cotyledons removed. A 5 µl drop of L-[U^{14}C]-phenylalanine was applied to the lower cut surface of each hypocotyl and incubated for 1 or 2 h before analysis.

[c] Mean and standard errors for three replicated experiments.

interaction types are valid when precursor is applied to the inoculated site.

7.4.2 Estimation of Glyceollin I Metabolism

In pulse-chase experiments to determine apparent rates of [^{14}C]-glyceollin I metabolism, a rapid decline in labelled glyceollin I was demonstrated only in the wounded controls (Fig. 7.3). In inoculated hypocotyls radiolabel incorporation continued during the chase period in incompatible interactions and the amount of [^{14}C]-glyceollin I remained more or less constant in the compatible interactions. When cinnamic acid was used as the chase instead of phenylalanine there was a rapid decline in [^{14}C]-glyceollin I in both control and inoculated hypocotyls (Fig. 7.4). Rates of metabolism were similar in compatible and incompatible interactions both in wounded and in intact hypocotyls.

Additional evidence for high rates of glyceollin metabolism were provided by measuring the recovery of [^{14}C]-glyceollin I supplied for 30 min periods at various times after wounding or after inoculation of wounded or intact hypocotyls (Fig. 7.5). Metabolism was negligible shortly following wounding and inoculation but by 4.5-5 h it was very rapid in wounded hypocotyls (inoculated and controls). Thereafter the rate of metabolism continued to increase in the wounded controls but apparently declined in the inoculated wounded hypocotyls, especially in the incompatible interaction. Rates of metabolism were much less in intact hypocotyls than in wounded hypocotyls especially in the incompatible interaction.

7.4.3 Precursor Pools and Phenylalanine Ammonia-lyase Activity

The pulse-labelling experiments (Fig. 7.2) indicated

Fig. 7.3

Fate of [^{14}C] glyceollin I in soybean hypocotyls in a pulse-chase experiment with [^{14}C]-phenylalanine. [^{14}C]-Glyceollin I was synthesized during a 1 h pulse with L-[U^{14}C]-phenylalanine and chased with L-phenylalanine (1mM). The pulse was applied from 10-11 h following inoculation or from 14-15 h after wounding in the wounded controls. Intact hypocotyls inoculated with Phytophthora megasperma f.sp. glycinea race 1, cv. Harosoy (susceptible)●—●, cv. Harosoy 63 (resistant)○—○. Wounded inoculated hypocotyls, cv. Harosoy ■—■, cv. Harosoy 63 □—□. Wounded control hypocotyls cv. Harosoy ▲—▲, cv. Harosoy 63 △—△. Chase period commenced at 0 h (arrow) immediately following the 1 h pulse from -1 h. Data are from one of two similar experiments.

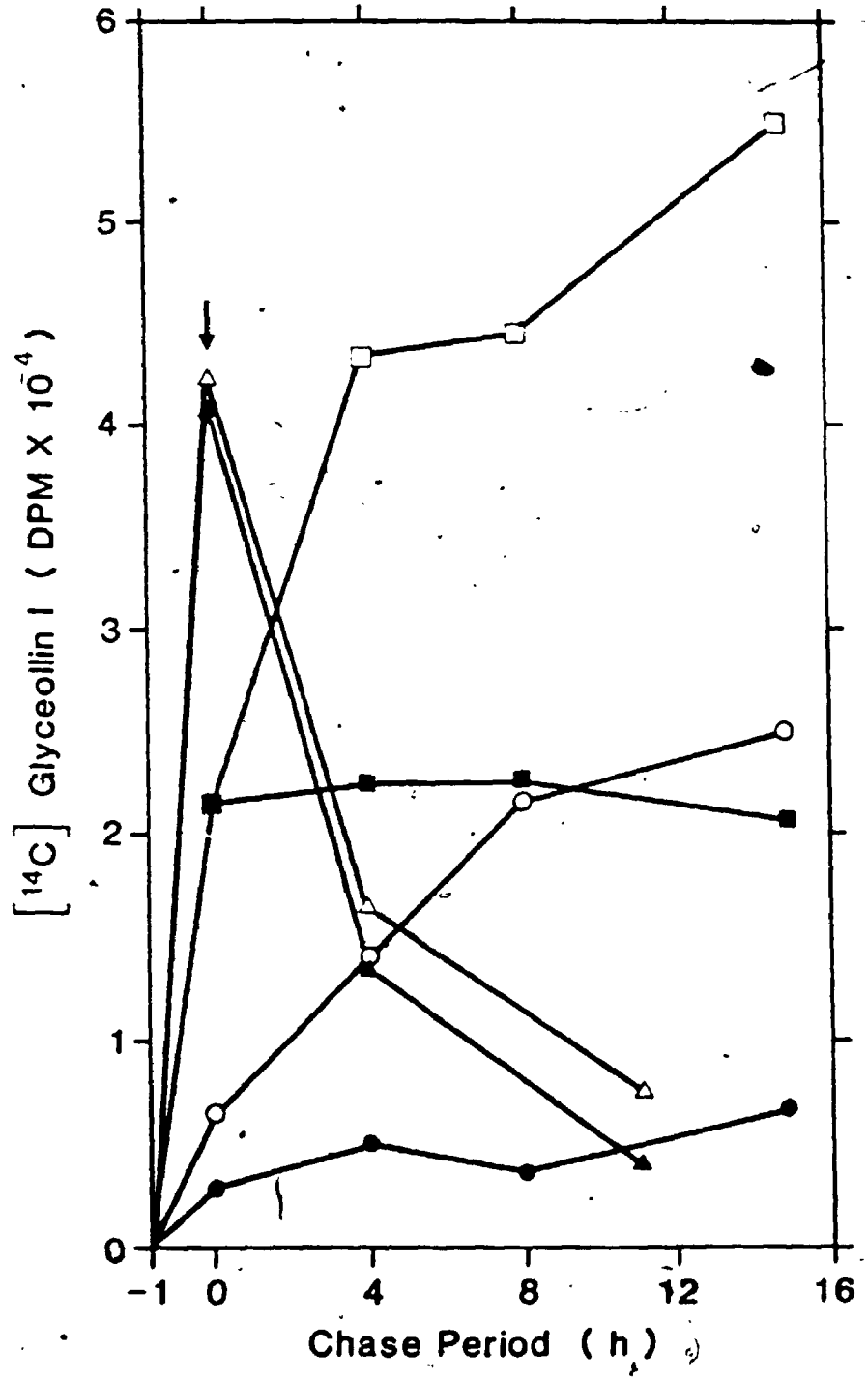

6
5
4
3
2
1
0
[14C] Glyceollin I (DPM X 10-4)
-1
0
4
8
12
16
Chase Period (h)

Fig. 7.4

Metabolism of [^{14}C]-glyceollin I in soybean hypocotyls in a pulse-chase experiment with [^{14}C]-phenylalanine. [^{14}C]-glyceollin I was synthesized during a 1 h pulse with L-[U-^{14}C]-phenylalanine and chased with 1 mM cinnamic acid. The pulse was applied from 8.5-9.5 h following inoculation or 14-15 h following wounding in the wounded control. Intact hypocotyls inoculated with *Phytophthora megasperma* f.sp. *glycinea* race 1, cv. Harosoy (susceptible) ●—●, cv. Harosoy 63 (resistant) ○—○. Wounded inoculated hypocotyls, cv. Harosoy ■—■, cv. Harosoy 63 □—□. Wounded control hypocotyls of cv. Harosoy ▲—▲. Chase period commenced at 0 h (arrow) immediately following the 1 h pulse from -1 h. Data are from one out of three similar experi- ments.

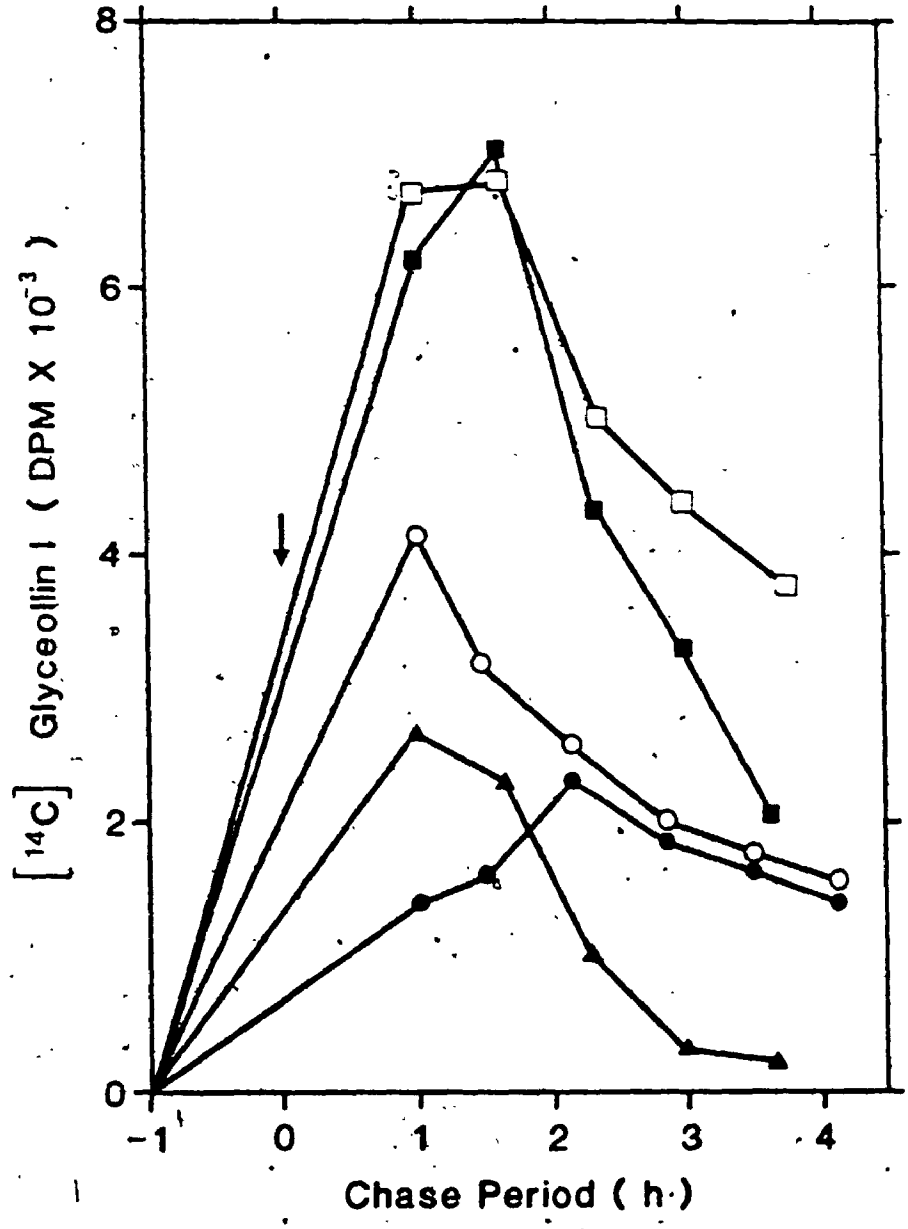
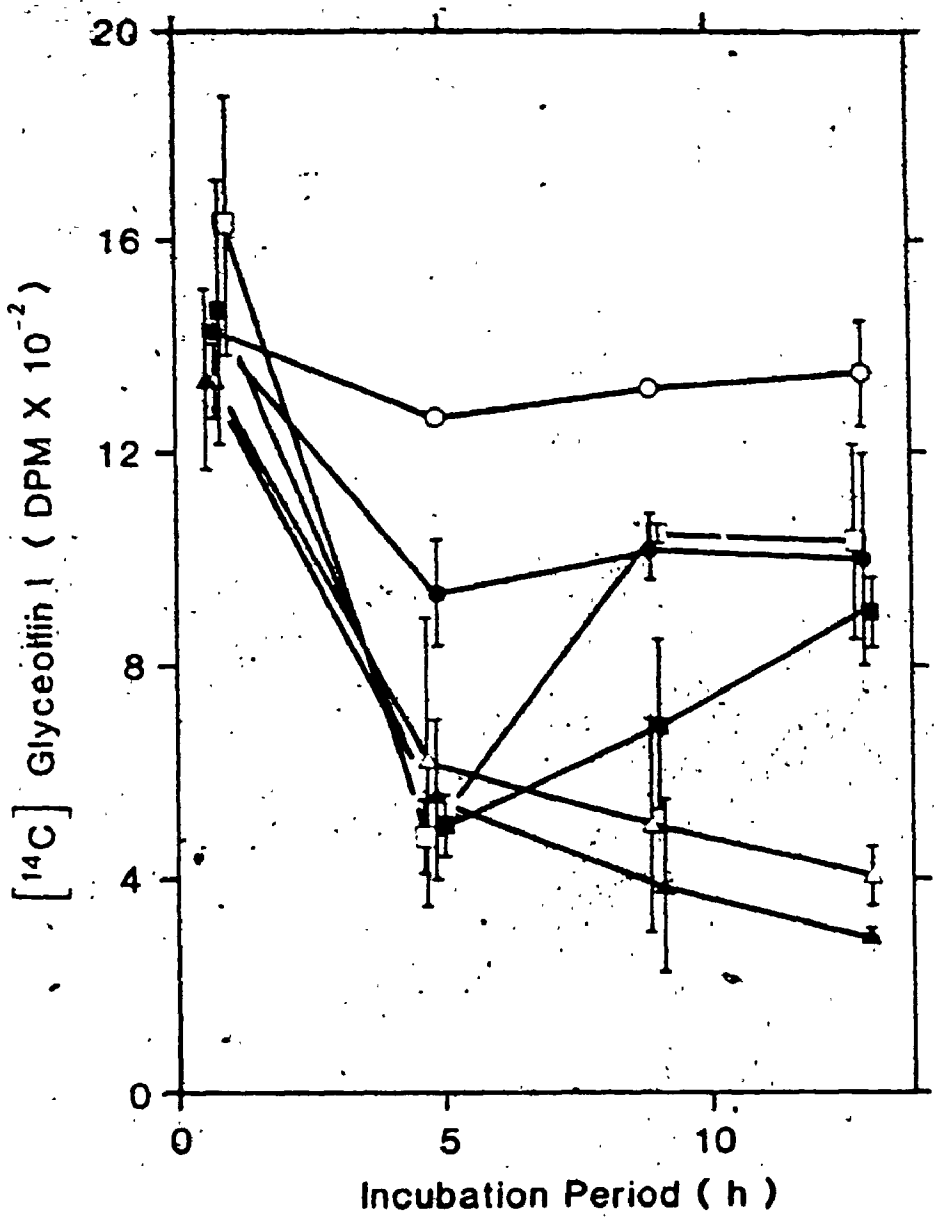

8
6
4
2
0
[14C] Glyceollin I (DPM X 10-3)
-1
0
1
2
3
4
Chase Period (h)

Fig. 7.5

Metabolism of externally fed [^{14}C]-glyceollin I in soybean hypocotyls. [^{14}C]-Glyceollin I was applied to wounds or infection sites for pulse periods of 30 min immediately prior to analysis at the times indicated. Residual glyceollin I was determined following each 30 min pulse. Efficiency of recovery from treated tissue without incubation was 64 percent (1600 d/min). Intact hypocotyls inoculated with Phytophthora megasperma f.sp. glycinea race 1, cv. Harosoy (susceptible) ●—●, cv. Harosoy 63 (resistant) ○—○. Wounded inoculated hypocotyls, cv. Harosoy ■—■, cv. Harosoy 63 □—□. Wounded control hypocotyls cv. Harosoy ▲—▲, and cv. Harosoy 63 △—△. Incubation period refers to time following wounding and inoculation. Data are the mean and standard errors for two replications (5 hypocotyls per replicate).


20
16
12
8
4
0
0
5
10
[14C] Glyceollin I (DPM X 10-2)
Incubation Period (h)

that wounding alone induced glyceollin I biosynthesis. The effect of wounding prior to inoculation on glyceollin I biosynthesis and metabolism was examined, therefore. Inoculation of hypocotyls that had been wounded 12 h previously (after wound-induced biosynthesis had been established Fig. 7.2) resulted in a much earlier onset of glyceollin accumulation than in hypocotyls inoculated at the time of wounding (Fig. 7.6A). However, the pattern of [^{14}C]-incorporation into glyceollin I after pulse feeding with L-[U^{14}C]-phenylalanine (Fig. 7.6A) was entirely different from that in hypocotyls inoculated at the time of wounding (Fig. 7.2). Rapid incorporation occurred in the compatible interaction and no changes in incorporation rate occurred in the incompatible interaction. A pulse-chase experiment (pulsed 12 h following wounding for 1 h, inoculated at 13 h and chased with 1 mM phenylalanine from 15 h) indicated that incorporation of radiolabel into glyceollin I continued during the chase period in the incompatible interaction but declined in the compatible interaction (Fig. 7.6B). The inconsistencies in patterns of glyceollin I accumulation and [^{14}C]-incorporation suggested that the pulse and pulse-chase data might reflect precursor pool sizes rather than true rates of biosynthesis and metabolism.

This possibility was examined by determining the relative incorporation of [^{14}C] into glyceollin I after a 1 h pulse with L-[U^{14}C]-phenylalanine at two specific radioactivities (522 or 50 mCi m/mol) (Table 7.4). The ten-fold difference in specific radioactivity had little influence on [^{14}C]-incorporation in the incompatible interactions but in the compatible interactions and the wounded controls incorporation from the lower of the two L-[U^{14}C]-phenylalanine concentrations was only about 50 percent of that from the higher concentration. This reduction in incorporation suggests that internal pools of phenylalanine were limiting in

Table 7.4

Influence of specific radioactivity of L-[U^{14}C]-phenylalanine on incorporation of [^{14}C] into glyceollin I in pulse feeding experiments in soybean hypocotyls wounded or inoculated with Phytothora megasperma f.sp. glycinea race 1.

Cultivar and treatment	[^{14}C] Glyceollin I (d/min)		B/A x 100
	[^{14}C] Phenylalanine (specific radioactivity)[b]		
	(A) 522 mCi m mol	(B) 50 mCi m mol	
Harosoy wounded only	6539±1777[c] (1.9±0.5)	3206±733 (1.3±0.2)	49
Harosoy 63 wounded only	6147±280 (1.2±0.1)	2770±170 (1.2±0.1)	45
Harosoy wounded inoculated	17,290±966 (2.8±0.03)	8902±2813 (45±0.1)	52
Harosoy 63 wounded inoculated	29,379±1042 (10.7±0.4)	28395±38 (11.3±2.5)	97
Harosoy unwounded inoculated	1281±159 (3.8±0.3)	786±55 (4.5±0.8)	61
Harosoy 63 unwounded inoculated	3801±42.2 (21.8±3.8)	3286±188 (17.4±0.9)	86

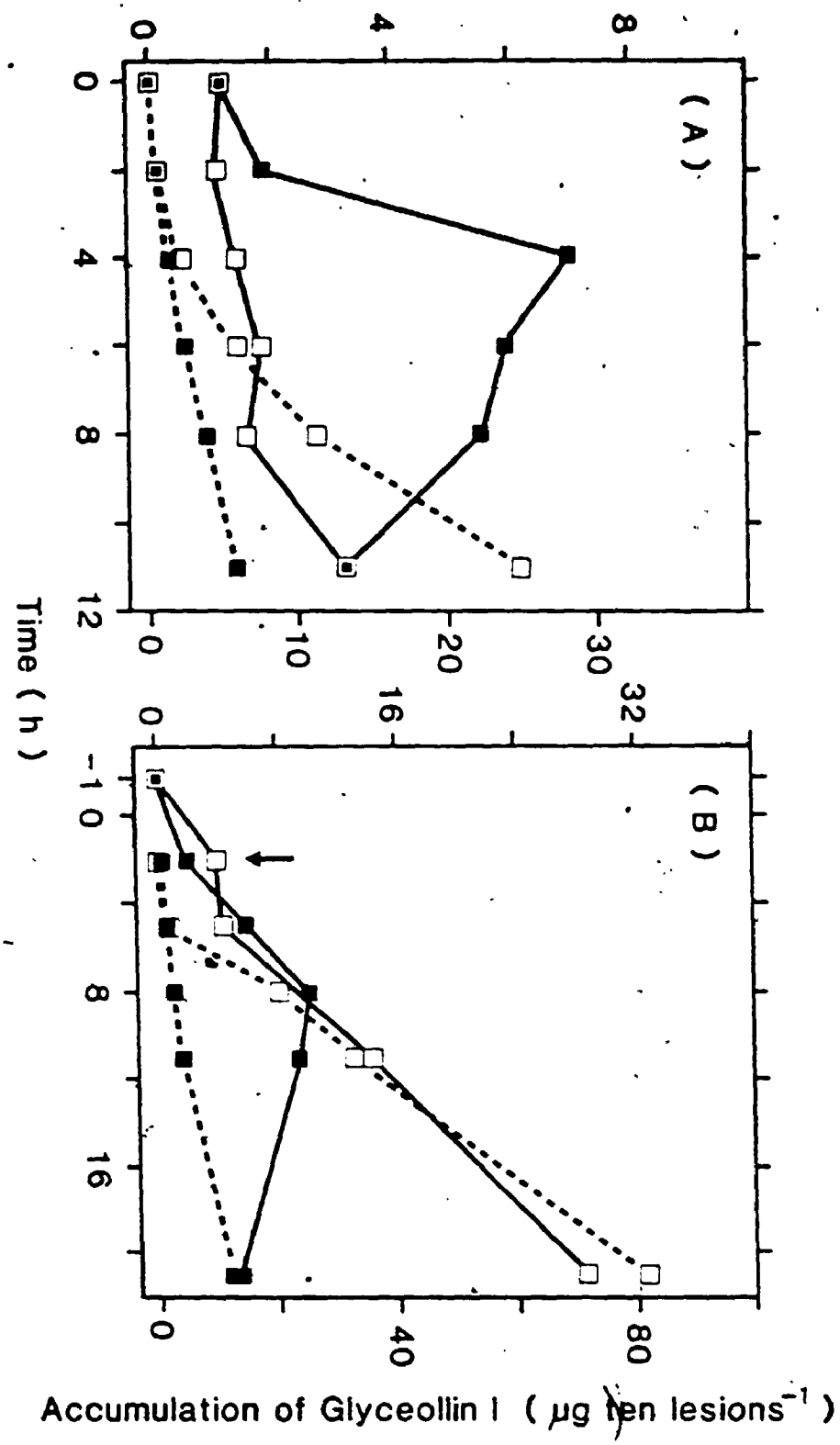
[a] cv. Harosoy is susceptible and cv. Harosoy 63 is resistant to Phytophthora megasperma f.sp. glycinea race 1.

[b] wounded and inoculated sites (10 per treatment) were pulsed with 5 µl 50 µCi/ml L-[U^{14}C]-phenylalanine at two concentrations A and B as above, for 1 h from 10 to 11 h after inoculation or wounding.

[c] [^{14}C] Glyceollin I (d/min), accumulated glyceollin I (µg) in parenthesis. Data are means and SE from three replications.

Fig. 7.6

Effect of wounding soybean hypocotyls 12 h prior to inoculation with Phytophthora megasperma f.sp. glycinea on (A) glyceollin I accumulation and incorporation of [^{14}C] into glyceollin I and (B) the fate of [^{14}C]-glyceollin I in a pulse-chase experiment. In (A) L-[U^{14}C]-phenylalanine (5µl, 50 µCi/ml 0.1 mM, per hypocotyls) was supplied in a 30 min pulse immediately prior to the time of analysis. In (B) L-[U^{14}C]-phenylalanine (5µl, 25 µCi/ml 0.05 mM, per hypocotyls) was supplied for 1 h prior to inoculation and chased with 1 mM phenylalanine from 2 h after inoculation (arrow). In both (A) and (B) hypocotyls cv. Harosoy (susceptible; ■) , cv. Harosoy 63 (resistant; □) were inoculated at 0 h (12 h after wounding). Broken line indicates glyceollin I accumulation. Solid line indicated [^{14}C]-glyceollin I.


[14C] Glyceollin.I (DPM X 10-3)
0
4
8
(A)
0
4
8
12
0
10
20
30
32
16
0
(B)
-1 0
8
16
0
40
80
Time (h)
Accumulation of Glyceollin I (µg ten lesions-1)

the controls and compatible interactions.

Phenylalanine pool sizes were measured at 11 h following inoculation or wounding, a time at which rates of [^{14}C]-incorporation had reached significant levels in control and inoculated hypocotyls (Fig. 7.2A). Measurements in wounded controls were made also after 15 h to coincide with the first detection of glyceollin I accumulation in wounded controls. Phenylalanine concentrations were much lower in inoculated than in control hypocotyls and, in Harosoy 63, lower in the wounded controls than in the intact hypocotyls (Table 7.5). The phenylalanine pool in the wounded hypocotyls decreased significantly between 11 and 15 h. Thus there was in general an inverse correlation between phenylalanine pool size and PAL activity and, from other experiments, glyceollin I accumulation. However, there were striking differences in PAL activity between inoculated and control hypocotyls and activity in incompatible interactions was about three times that in compatible interactions (Table 7.5).

7.5 Discussion

L-[U^{14}C]-Phenylalanine was used in the present study as the experimental precursor of glyceollin I, in general following procedures described by Yoshikawa *et al.* [1979]. Banks and Dewick [1983] demonstrated daidzein (Fig. 7.7) to be a precursor of the glyceollins, but it was found that incorporation of [^{14}C]-daidzein was too low to yield glyceollin I of reliable specific radioactivity for pulse-feeding experiments (data not presented). Similarly, no useful incorporation was obtained with the more immediate precursor [^{14}C]-mevalonate in the pulse periods used (Fig. 7.7), although [^{14}C]-incorporation from this compound in long term feeding experiments has been reported [Zähringer *et al.*, 1978]. Moesta and Grisebach [1981b] used [^{14}C]-carbon

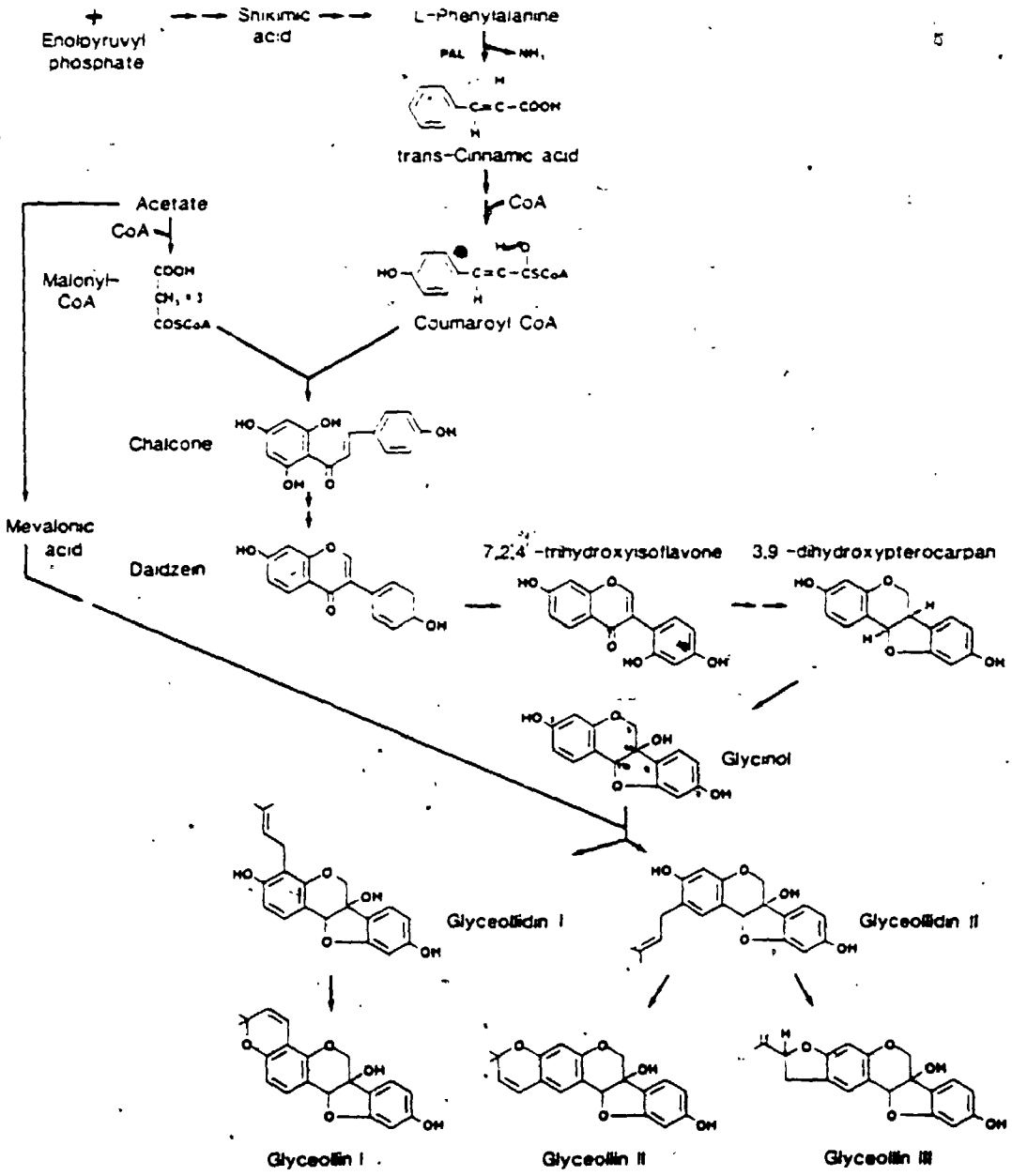
Table 7.5

Concentrations of free L-phenylalanine and activity of phenylalanine ammonia-lyase in soybean hypocotyls folowing wounding or inoculation with Phytophthora megasperma f.sp. glycinea race 1.

Cultivar[a] and treatment	Phenylalanine[b] (n mol/mg d. wt)	PAL activity[b] (n mol cinnamic/min /mg d. wt)
Hypocotyls unwounded (11 h incubation)[c]		
Harosoy inoculated	67±28[d]	0.88±0.23[d]
Harosoy 63 inoculated	56±14	2.61±0.12
Harosoy uninoculated	162±9	0.15±0.04
Harosoy 63 uninoculated	164±8	0.18±0.06
Hypocotyls wounded at time of inoculation -(11 h incubation)		
Harosoy inoculated	85±18	0.92±0.04
Harosoy 63 inoculated	54±20	2.85±0.22
Harosoy uninoculated	146±17	0.20±0.03
Harosoy 63 uninoculated	111±19	0.13±0.06
-(15 h incubation)		
Harosoy uninoculated	66±5	0.16±0.08
Harosoy 63 uninoculated	75±6	0.11±0.01
Hypocotyls wounded[e] 12 h prior to inoculation -(4 h incubation)		
Harosoy inoculated	67±12	0.49±0.17
Harosoy 63 inoculated	49±0	1.06±0.08
-(11 h incubation)		
Harosoy inoculated	25±2	0.88±0.05
Harosoy 63 uninoculated	28±1	3.98±0.35

[a] cv. Harosoy is susceptible and cv. Harosoy 63 is resistant to Phytophthora megasperma f.sp. glycinea race 1.
[b] Based on tissue excised from 20 lesions or wounds from 10 hypocotyls.
[c] Incubation period from the time of inoculation or wounding to the time of analysis.
[d] Mean and standard error from 2 replications.
[e] Hypocotyls in this series were wounded 12 h prior to inoculation, 4 h and 11 h incubation refer to the period after inoculation.

Fig. 7.7
Some major intermediates in phenylpropanoid pathways, starting with deamination of L-phenylalanine by phenylalanine ammonia-lyase into trans-cinnamic acid, leading to glyceollin I.


Enolpyruvyl phosphate
Shikimic acid
L-Phenylalanine
PAL
NH3
trans-Cinnamic acid
CoA
Acetate
CoA
Malonyl-CoA
Coumaroyl CoA
Chalcone
Mevalonic acid
Daidzein
7,2',4'-trihydroxyisoflavone
3,9-dihydroxypterocarpan
Glycinol
Glyceollidin I
Glyceollidin II
Glyceollin I
Glyceollin II
Glyceollin III

dioxide as a precursor of the glyceollins and suggested [Moesta and Grisebach, 1980, 1981a] that phenylalanine might have the disadvantage that uptake might be influenced by experimental conditions. Nevertheless, L-[U^{14}C]-phenylalanine has been successfully employed as a precursor in a number of studies of phenylpropanoid biosynthesis [eg. Banks and Dewick, 1983; Dewick and Martin, 1979; Dewick and Steele, 1982] and the data of Table 7.3 indicate that it is an acceptable precursor for comparisions of [^{14}C]-incorporation into glyceollin I in incompatible and compatible interactions under the experimental conditions used here. In contrast, the drawbacks to the use of [^{14}C]-carbon dioxide appear to be more serious. As will be discussed below, the aims of pulse-chase experiments [Moesta and Grisebach, 1981b] may be frustrated by the remoteness of CO_2 from glyceollin and the large pool of metabolic products that it might be expected to give rise to.

Yoshikawa et al. [1979] concluded from pulse-feeding experiments that high biosynthetic rates were induced by inoculation of wounded hypocotyls and by wounding alone. The rates were similar in both compatible and incompatible interactions, although accumulation of glyceollin differed in the two interactions and was only just detectable in the wounded controls. They concluded from these and pulse-chase experiments that differences in accumulation were a reflection of different rates of glyceollin metabolism. The results in the present study with wounded hypocotyls were broadly similar with the exception that differences in the amount of incorporation in the two types of interaction developed after 11 h following inoculation (Fig. 7.1). However, in unwounded inoculated hypocotyls, rates of biosynthesis and accumulation were distinctly different throughout the time-course in both interaction types. This distinction evidently was masked by wounding, both in the

present study and those of others [Moesta and Grisebach, 1981b; Yoshikawa et al., 1979]. In unwounded inoculated hypocotyls glyceollin accumulation appeared to be governed by biosynthetic rates.

The pulse-chase experiments, with L-phenylalanine as the chase, provided satisfactory evidence for rapid glyceollin metabolism in the wounded controls in both the present study and that of Yoshikawa et al. [1979]. They failed to do so in the inoculated hypocotyls. In both investigations there was continued incorporationn of [^{14}C] both in incompatible and compatible interactions during the chase period and especially in the incompatible interactions. Clearly, extensive pools of radiolabelled precursors were developed during the pulse period and these pools continued to supply the biosynthetic pathway during the chase period. Presumably, the much wider pool of precursors generated from [^{14}C]-carbon dioxide in the experiments of Moesta and Grisebach [1981b] increased this problem. They found [^{14}C]-incorporation continued to increase for 4 to 6 h after the pulse period followed by a limited decline in [^{14}C]-glyceollin in the incompatible interaction only (half life of 28 h). Differences between the phenylalanine chase curves for the two types of interactions, both in the present study and that of Yoshikawa et al. [1979], may indicate differences in sizes of radiolabelled pools as much as differences in metabolic rates. When cinnamic acid was used to feed-back inhibit PAL (Fig. 7.7), following the evidence of Shields et al. [1982], radioactivity in glyceollin I declined rapidly in all interactions, after a lag of between 1 and 2 h, with half lives of about 2 h (Fig. 7.4). Furthermore the rates of metabolism of [^{14}C]-glyceollin I indicated by these data were similar for each of the pairs of compatible and incompatible interactions in either wounded or unwounded hypocotyls. This was in spite of differences in

specific radioactivities of glyceollin I in the two types of interaction (Table 7.2). Hence metabolic rates are related to glyceollin pools rather than directly to interaction-types. On the basis of this experiment it appears that differences in glyceollin I accumulation in the two types of interaction are not controlled by differential metabolic rates.

Additional evidence for glyceollin I metabolism was provided by pulse feeding with [^{14}C]-glyceollin I (Fig. 7.5). The results of the present study were consistent with those of Yoshikawa et al. [1979], obtained with hypocotyl sections in vitro, but demonstrated further that glyceollin I applied to wounds and to inoculated wounded and unwounded hypocotyls was also metabolized. This ability evidently was not constitutive but was induced within 4.5 h of wounding or infection and hence may involve de novo enzyme synthesis comparable to that required for glyceollin biosynthesis [Bonhoff et al., 1986, Esnault et al., 1987, Schmelzer et al., 1984]. Half lives for externally supplied glyceollin I were of the same order as those determined in pulse-chase experiments with cinnamic acid. Such high rates of glyceollin I turn over suggest that a study of the metabolic fate of glyceollin I and enzymic processes involved would be profitable. Although this experiment serves to emphasize that glyceollin I metabolism is induced following wounding and infection, the evidence that it also provides for differential rates of metabolism in compatible and incompatible interactions must be regarded as equivocal. Differences in apparent rates of metabolism may have been due to dilution from endogenous glyceollin I, especially in incompatible interactions. The access of exogenous glyceollin I to sites of biosynthesis may have been influenced by interaction type, being less efficient in intact hypocotyls than in tissues exposed by wounding.

The demonstration (Table 7.4) that [^{14}C]-incorporation into glyceollin I was affected by the specific radioactivity of the phenylalanine fed suggests that pool sizes or metabolic fluxes greatly influence estimates of biosynthesis in pulse-feeding experiments. In controls and compatible interactions the decrease in [^{14}C]-incorporation with the higher of the two phenylalanine concentrations (i.e. low specific radioactivity) would be expected if internal pools of phenylalanine were small relative to quantities provided by the pulse or if endogenous rates of carbon-flow through phenylalanine were low. The much smaller effect of specific radioactivity of phenylalanine on incorporation in the incompatible interactions suggests that internal precursor pools in these interactions were large or that metabolic rates were high relative to quantities provided by the pulse. No evidence for the expansion of free phenylalanine pools was found, concentrations were lowest where demand appeared to be greatest. However, there were major differences in PAL activity (Table 7.5) that were generally correlated with glyceollin I accumulation. Presumably a very high rate of flow of carbon into the glyceollin biosynthetic pathway is generated in the incompatible interaction resulting in large accumulations of glyceollin I of relatively low specific radioactvity (Table 7.2). At the other extreme in the wounded controls the external L-[U^{14}C]phenylalanine pulse presumably makes a major contribution to the very small endogeneous flow, resulting in glyceollin I of high specific radioactivity (Table 7.2). Observations that rates of [^{14}C]-incorporation in wounded controls approach [Yoshikawa *et al.*, 1979] or equal (this study) those in incompatible interactions, therefore, reflect differences in endogeneous flow rates but not similarities in biosynthetic rates.

It might be expected that more uniform labelling of

cursors would be achieved by pulse feeding with [^{14}C]-carbon dioxide as employed by Moesta and Grisebach [1981b], thus avoiding some of the difficulties encountered with [^{14}C]-phenylalanine. That this may not be the case, however, is suggested by evidence that glyceollin biosynthesis in hypocotyls draws heavily on reserves in cotyledons [Kimpel and Kosuge, 1985]. Such reserves, presumably, would not be labelled during [^{14}C]-carbon dioxide pulses and would be tapped differentially as different rates of metabolic fluxes developed in compatible and incompatible interactions. Significantly, incorporation patterns during the early critical hours following inoculation in wounded hypocotyls were similar both following [^{14}C]-carbon dioxide feeding [Moesta and Grisebach, 1981b] and [^{14}C]-phenylalanine feeding [Yoshikawa et al., 1979 and Fig. 7.1a]. In all three studies, rates of incorporation during this period were the same in compatible and incompatible interactions in wounded hypocotyls. Moesta and Grisebach [1981b] considered that their data for glyceollin accumulation were consistent with this, for they failed to find significant differences for the two interactions before 14 h after inoculation (although a trend would appear to have been established earlier). In the previous study by Yoshikawa et al. [1979] and the present study, differences in glyceollin accumulation developed much earlier, leading to the interpretations discussed above. The latter results appear to be the more reasonable. They are consistent with the early differentiation of resistant and susceptible responses in soybean hypocotyls [Ward, 1983; Yoshikawa et al., 1978] and the association of glyceollin with restriction of spread of the pathogen [Lazarovits et al., 1981; Ward et al., 1981; Yoshikawa et al., 1978]. There is evidence that transcription of mRNAs for pathway enzymes takes place within 3 h of inoculation in an incompatible interaction but not in a com-

patible interaction [Esnault et al., 1987]. The data of Table 7.5 indicate also that PAL activity in incompatible interactions was several fold that in compatible interactions at 11 h after inoculation. Furthermore, in roots accumulation of glyceollin I and increases in activity of pathway enzymes occur much earlier in incompatible than in compatible interactions [Bonhoff et al., 1986; Hahn et al., 1985]. Borner and Grisebach [1982], however, had previously failed to find comparable differences in PAL activity in hypocotyls. Possibly, their procedures for inoculation with mycelium introduced non-specific elicitors of glyceollin biosynthesis that, together with the complication due to wounding, obscured any early differences in response between compatible and incompatible interactions.

Results with intact hypocotyls were much less ambiguous than those with wounded hypocotyls. Thus rates of [^{14}C] incorporation were consistent with accumulation of glyceollin 1 and patterns in the two interaction types were distinctly different (Fig. 7.2). Although wounding does not result in the accumulation of significant amounts of glyceollin during the first 12 h, it accelerates the biosynthesis and accumulation of glyceollin following infection (Fig. 7.6) with parallel increases in the activity of PAL (Table 7.5). This suggests that some wound responses may be readily diverted to glyceollin biosynthesis, or that the low level of biosynthesis initiated by wounding can be more rapidly enhanced by inoculation. Such a response may be important in the resistance of wounds to infection. A similar phenomenon was reported for sweet potatoes [Inoue et al., 1984]. Clearly inoculated wounds present a much more complex situation for biochemical analysis than inoculated intact hypocotyls.

It is concluded that differences in rates of glyceollin I accumulation, and presumably of other isomers, are due

to the development of higher rates of biosynthesis in incompatible than in compatible interactions and not to differences in metabolism. This is based on the demonstration that a) rates of [^{14}C]-incorporation into glyceollin I in incompatible interactions either equalled (wounded hypocotyls) or exceeded (unwounded hypocotyls) those in compatible interactions, b) in incompatible interactions specific radioactivity of accumulated glyceollin I was low due to relatively higher contribution from unlabelled internal pools, c) PAL activity was stimulated to a much higher level in incompatible than in compatible interactions, consistent with high metabolic flows through the biosynthetic pathway, d) rapid metabolism of glyceollin I occurred in both interactions (and in uninoculated wounds) and rates were related to the accumulation of glyceollin I and not directly governed by the type of interaction. Accumulation of glyceollin I must depend on differences between rates of biosynthesis and metabolism. Assuming that the products of glyceollin-metabolism are not phytotoxic, it is probably essential that the plant maintains a balance between biosynthesis and metabolism of glyceollin, to avoid harmful accumulation. Glyceollin I should be regarded as an intermediate and not an end product of a secondary metabolic pathway.

Since biosynthesis is stimulated in both wounds and infected tissues it may be a response to cell damage. Major stimulation presumably would require persistent damage that occurs in infected tissues to varying degrees but not in wounds. Evidence that continued stimulation is essential for phytoalexin accumulation in cell-suspension cultures was provided by Dixon et al. [1981]. Requirements for continuous stimulation and high rates of glyceollin metabolism are consistent also with earlier observations that a compatible race may spread from restricted lesions containing high con-

centrations of glyceollin. Growth of a compatible race of Phytophthora megasperma f.sp. glycinea was stopped and glyceollin accumulated in infected hypocotyls placed at high temperatures [Ward and Lazarovits, 1982]. Transfer to normal temperatures evidently removed the stimulus for glyceollin biosynthesis. Metabolism, at least at the periphery of lesions, would presumably reduce glyceollin concentrations sufficiently to permit the pathogen to spread. Similarly, in inoculations with mixtures of compatible and incompatible races [Ward, 1983] low stimulation of biosynthesis together with continued metabolism of glyceollin in the microenvironment of compatible hyphae might allow these hyphae to spread despite the developing toxicity within the lesion as a whole. These observations are generally consistent with a view expressed elsewhere [Ward, 1986] that the basic relationship in host-pathogen interactions of this type is a compatible one and that this persists unless interfered with.

CHAPTER 8

PHENYLALANINE AMMONIA-LYASE ACTIVITY IN SOYBEAN HYPOCOTYLS AND LEAVES FOLLOWING INFECTION WITH *Phytophthora megasperma* f.sp. *glycinea*

8.1 Summary

Phenylalanine ammonia-lyase (PAL) activity increased rapidly after 2 h following inoculation with *Phytophthora megasperma* f.sp. *glycinea* race 1 in unwounded hypocotyls of soybean cv. Harosoy 63 (resistant) but not in cv. Harosoy (susceptible). Small but significant increases in PAL activity were caused by wounding. Compared to unwounded hypocotyls (Harosoy 63) PAL activity increased more slowly in hypocotyls wounded just before inoculation but much faster in hypocotyls wounded 12 h before inoculation. There were comparable effects on symptom development.

Trifoliates of 14-day old cv. Harosoy 63 plants are resistant but trifoliates of 12-day old cv. Harosoy 63 plants and of 14-day old cv. Harosoy plants are susceptible to race 1. Increases in PAL activity following inoculation were demonstrated only in 14-day old Harosoy 63 plants but not until 24-36 h. Significant accumulation of glyceollin occurred by 24 h. Susceptible trifoliates of 12-day old cv. Harosoy 63 plants produced only low levels of glyceollin following either infection or treatment with the abiotic elicitor $AgNO_3$, whereas trifoliates of 14-day old cv. Harosoy plants produced high levels of glyceollin in response to $AgNO_3$. It is concluded that trifoliates of 12-day old, as opposed to 14-day old, cv. Harosoy 63 plants have not developed mechanisms (recognition) for response to either infection or the abiotic elicitor, or they are deficient in metabolic processes that support glyceollin biosynthesis or other defence-related responses.

8.2 Introduction

The association of phenylalanine ammonia-lyase(PAL) activity with the accumulation of phytoalexins was first demonstrated by Hadwiger and Schwochau [1970] for the elicitation of pisatin in peas using abiotic elicitors. The enzyme catalyses the first step of the phenylpropanoid pathway (Fig. 7.7) and hence it would be expected that activity should be correlated with production of isoflavonoid phytoalexins in incompatible host-pathogen interactions. This has been found to be the case for elicitor-treated cell suspensions of beans and soybeans [eg. Cramer et al., 1985; Dixon and Bendall, 1978; Dixon and Lamb, 1979; Ebel et al., 1984; Lawton et al., 1983; Robbins et al., 1985]. Results with whole plants have been less consistent. Recently PAL activity was correlated with glyceollin production in soybean roots [Bonhoff et al., 1986] but Partridge and Keen [1977] concluded earlier that PAL activity was not related to glyceollin synthesis in soybean hypocotyls wounded and inoculated with Phytophthora megasperma f.sp. glycinea. They found little difference between PAL activity in wounded controls, in which glyceollin did not accumulate, and that in inoculated hypocotyls in which it did accumulate. Börner and Grisebach [1982], in a study of the same interaction detected little PAL activity in wounds but reported a gradual increase in activity following inoculation. They also found that activity was similar in both compatible and incompatible interactions for the first 14 h. This was consistent with their data for glyceollin acccumulation but not with those of others. [Yoshikawa et al., 1978, 1979], or with development of early differences in the extent of tissue colonization in compatible and incompatible interactions in soybean hypocotyls or roots in which glyceollin is presumed to play a causal role [Hahn et al., 1985; Yoshikawa et al., 1978]. Furthermore, a recent report [Esnault et al., 1987]

has provided evidence for gene transcription of PAL mRNA in etiolated soybean hypocotyls within 3 h of inoculation in the incompatible but not in the compatible interaction.

It was observed that the Rps_1 gene for resistance to Phytophthora megasperma f.sp. glycinea race 1 carried by cv. Harosoy 63 is expressed in leaves in addition to hypocotyls [Chapter 4]. However, this was influenced greatly by the age of the plant and the maturity of the leaves. Thus, while leaves from 14-day old cv. Harosoy 63 plants were resistant and accumulated glyceollin, leaves from 12-day old plants of the same cultivar were susceptible. In this chapter, therefore, PAL activity was examined in leaves as well as in wounded and intact hypocotyls following inoculation with Phytophthora megasperma f.sp. glycinea.

8.3 Materials and methods

8.3.1 Host

Etiolated seedlings of the near isogenic soybean cultivars Harosoy and Harosoy 63 were grown in trays of vermiculite for 6 days in the dark as described previously [Chapter 2.1.1]. Trifoliates were obtained from 14-day old cv. Harosoy and 12 and 14-day old cv. Harosoy 63 plants grown in soil as described in chapter 4.3.1.

8.3.2 Pathogen

The culture used was a single-zoospore isolate (R1.19) derived from Phytophthora megasperma f.sp. glycinea race 1 [Chapter 6.4]. This race is virulent on cv. Harosoy (rps_1) and avirulent on cv. Harosoy 63 (Rps_1). Zoospores were produced as described previously [Chapter 2.2.2.2]. A 10 µl drop of zoospore suspension (10^5/ml) was used as inoculum.

8.3.3 Elicitor

The abiotic elicitor of glyceollin, $AgNO_3$ (10^{-3}M) [Stossel, 1982] was applied in 10 µl drops to the upper surface of leaves.

8.3.4 Inoculation and Incubation

Etiolated hypocotyls from 6-day old seedlings were arranged horizontally in glass trays as described previously [Chapter 2.1.2] and two drops of zoospore suspension were placed on each hypocotyl about 2 cm below the cotyledons and about 0.7 cm apart. Drops of sterile distilled water were added to control hypocotyls. In hypocotyls that were wounded, two surface wounds (about 3 mm long and 0.5 mm deep) were made about 2 cm below the cotyledons and about 0.7 cm apart immediately prior to inoculation [Chapter 7.3.3], except in one experiment in which hypocotyls were wounded 12 h prior to inoculation. In that experiment sterile distilled water (10 µl) was placed in each wound to prevent desiccation during the 12 h period. Wounds were inoculated with 10 µl of zoospore suspension or sterile distilled water in controls. Ten hypocotyls (20 inoculated or control sites) were used for each treatment. Following inoculation or wounding, hypocotyls were incubated in the dark at 25°C and 100% RH.

Trifoliates were arranged on wet cellucotton in glass trays as described previously [Chapter 4.3.3]. Six or eight drops of zoospore suspension, $AgNO_3$ solution or sterile distilled water were placed on adaxial surfaces of leaves of 12-day old plants, twenty drops were used on leaves of 14-day old plants. Trays were closed immediately with plastic film and left undisturbed on the laboratory bench for 3-4 h. Thereafter, they were incubated in a growth cabinet at 100% RH with a daily 16 h light period (fluorescent lamps; approx. 33 µE m^{-2}/s). The temperature was 23°C in the light

and 16°C in the dark.

8.3.5 Determination of Phenylalanine Ammonia-lyase Activity

Hypocotyl sections approximately 1.75 cm long containing the wounded and inoculated sites and lesion tissues in the case of the leaves were excised, weighed, immediately frozen in liquid nitrogen and stored at -70°C. PAL activity in the excised tissues was determined by following the methods described in chapter 7.3.10. The enzyme activity was expressed as n moles cinnamic acid produced in one minute per g fresh weight of tissue.

8.3.6 Determination of Glyceollin

Excised tissues from lesions in leaves inoculated with zoospores or treated with $AgNO_3$ solution (10^{-3}M) were extracted with 95% ethanol and glyceollin was separated by thin layer chromatography as described in chapter 2.7. Glyceollin (a mixture of three isomers) was determined from its absorption at 285 nm and the extinction coefficient [Ayers et al., 1976]. Glyceollin concentrations are expressed as µg per g fresh weight of tissue.

8.3.7 Determination of Reducing Sugars

Reducing sugars in the supernatant used in the assay for PAL activity were determined following the arsenomolybdate method of Nelson [1944].

8.4 Results

8.4.1 Etiolated Hypocotyls

8.4.1.1 Symptoms

In intact etiolated hypocotyls, symptoms following inoculation were consistent with those reported previously [Ward et al., 1979]. In the incompatible interaction (cv. Harosoy 63) brown spots were visible after 5-6 h, while in

the compatible interaction (cv. Harosoy), surfaces of inoculated sites developed a transparency that preceded the general water soaking with browning that was prevalent after 11-12 h. By comparision the development of symptoms was much delayed in hypocotyls wounded just before inoculation. Browning could not be detected in the resistant response in wounds in cv. Harosoy 63 until 8-9 h following inoculation. There was also a reduction in the amount of browning in the compatible interaction (cv. Harosoy). Hypocotyls wounded 12h prior to inoculation , however, responded very rapidly to infection. In resistant responses in such hypocotyls of Harosoy 63, tissues became brown 4 h following inoculation.

8.4.1.2 Phenylalanine Ammonia-lyase Activity

In intact etiolated hypocotyls PAL activity increased rapidly from 2 h following inoculation in the resistant response (cv. Harosoy 63) to Phytophthora megasperma f.sp. glycinea race 1 (Fig. 8.1) but not in the susceptible response (cv. Harosoy). At 8 h following inoculation PAL activity in the resistant response was more than six times greater than that in the susceptible response or control hypocotyls. Wounding caused a small but significant increase in PAL activity over that in intact hypocotyls (Fig. 8.2). Significant differences in PAL activity between resistant and susceptible responses did not develop until after 4h and stimulation in the resistant response (cv. Harosoy 63) was much smaller than in unwounded hypocotyls. In hypocotyls wounded 12 h before inoculation the trends to increased PAL activity in the controls continued (Fig. 8.3). There was also a rapid increase in activity in the resistant response, possibly greater than that in intact hypocotyls, and significant stimulation of activity in the susceptible response.

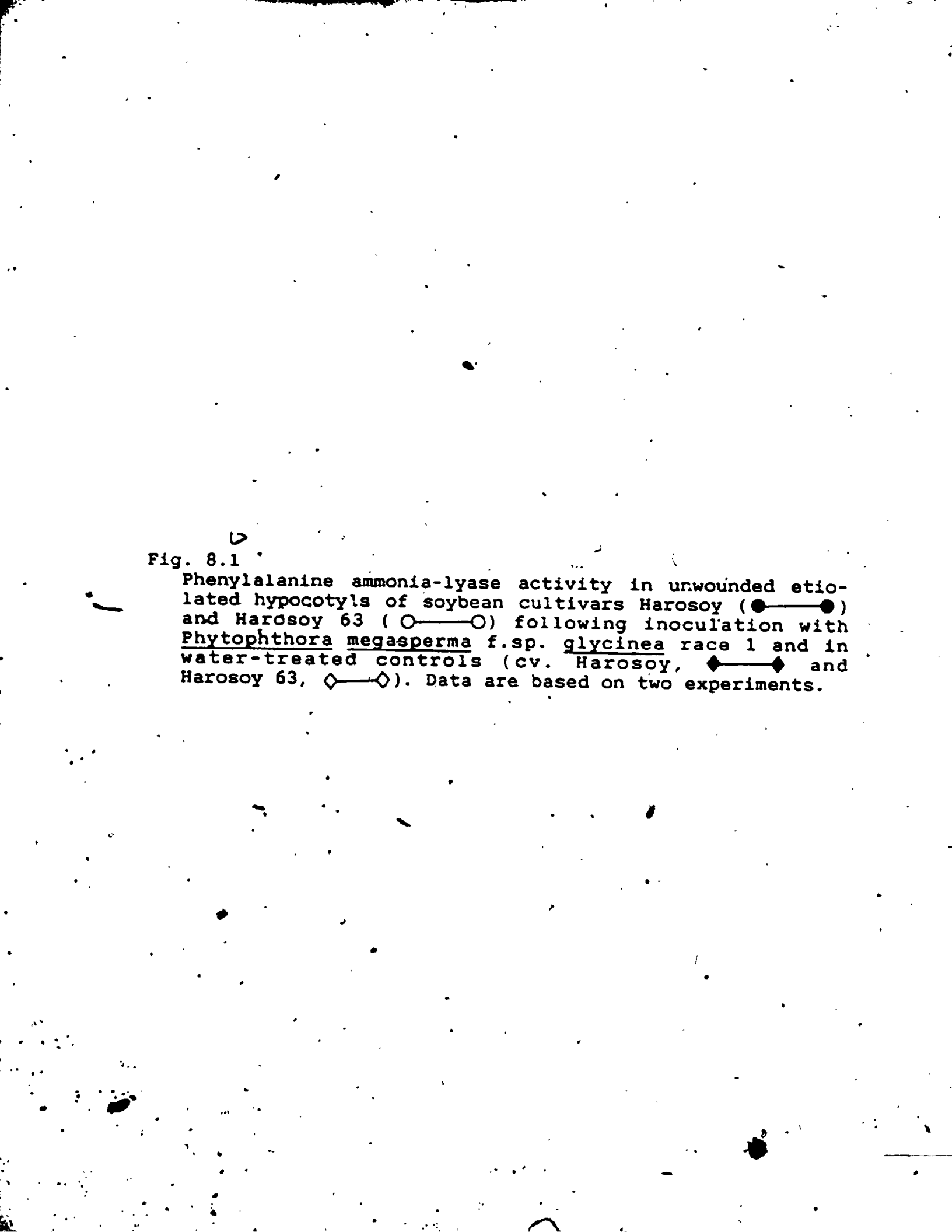
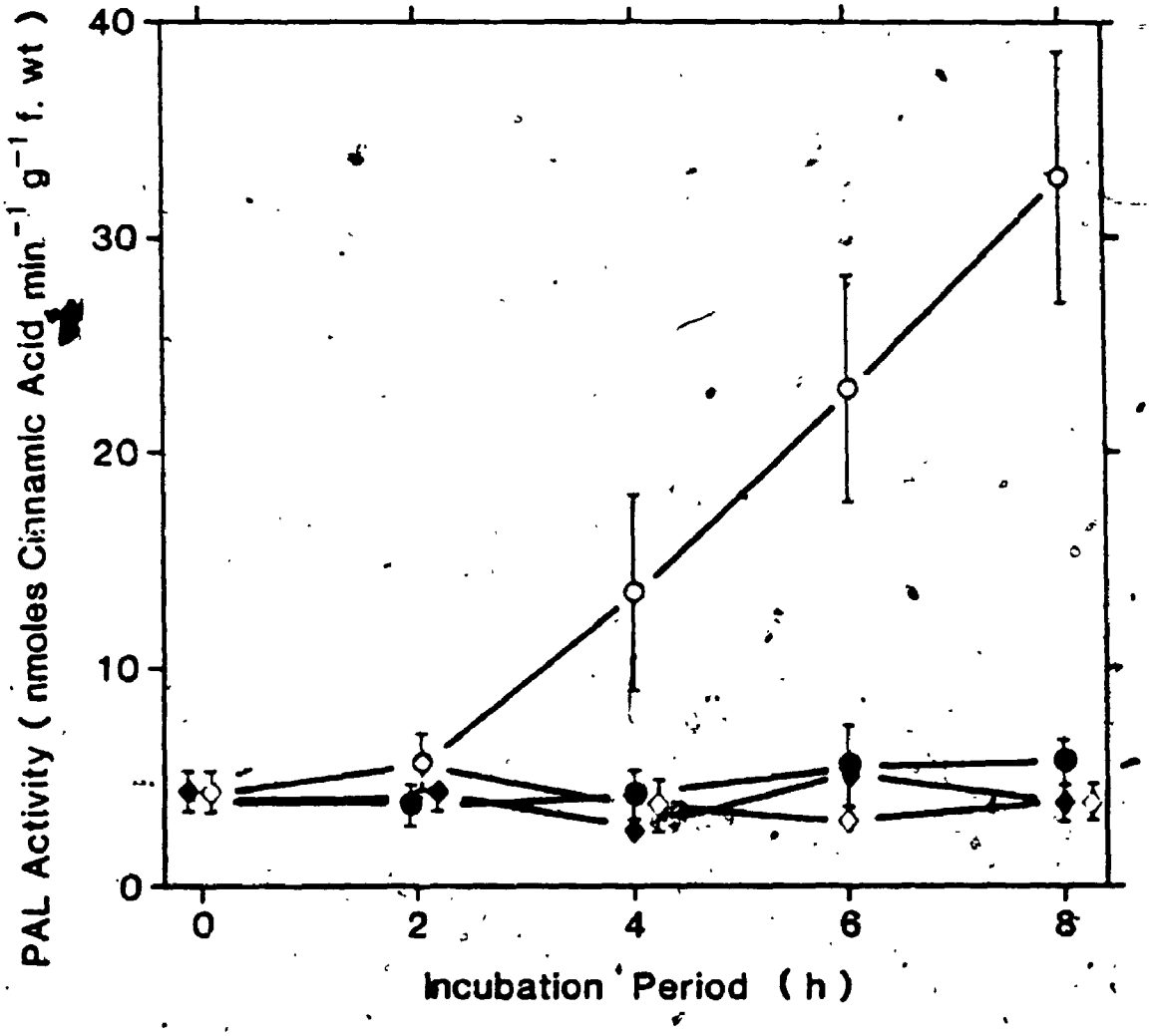
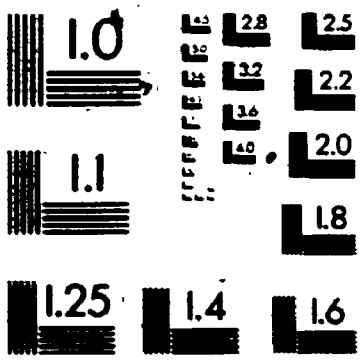


Fig. 8.1
Phenylalanine ammonia-lyase activity in unwounded etiolated hypocotyls of soybean cultivars Harosoy (●——●) and Harosoy 63 (○——○) following inoculation with Phytophthora megasperma f.sp. glycinea race 1 and in water-treated controls (cv. Harosoy, ◆——◆ and Harosoy 63, ◇——◇). Data are based on two experiments.


PAL Activity (nmoles Cinnamic Acid min^{-1} g^{-1} f. wt)
40
30
20
10
0
0
2
4
6
8
Incubation Period (h)



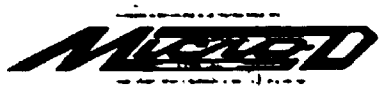
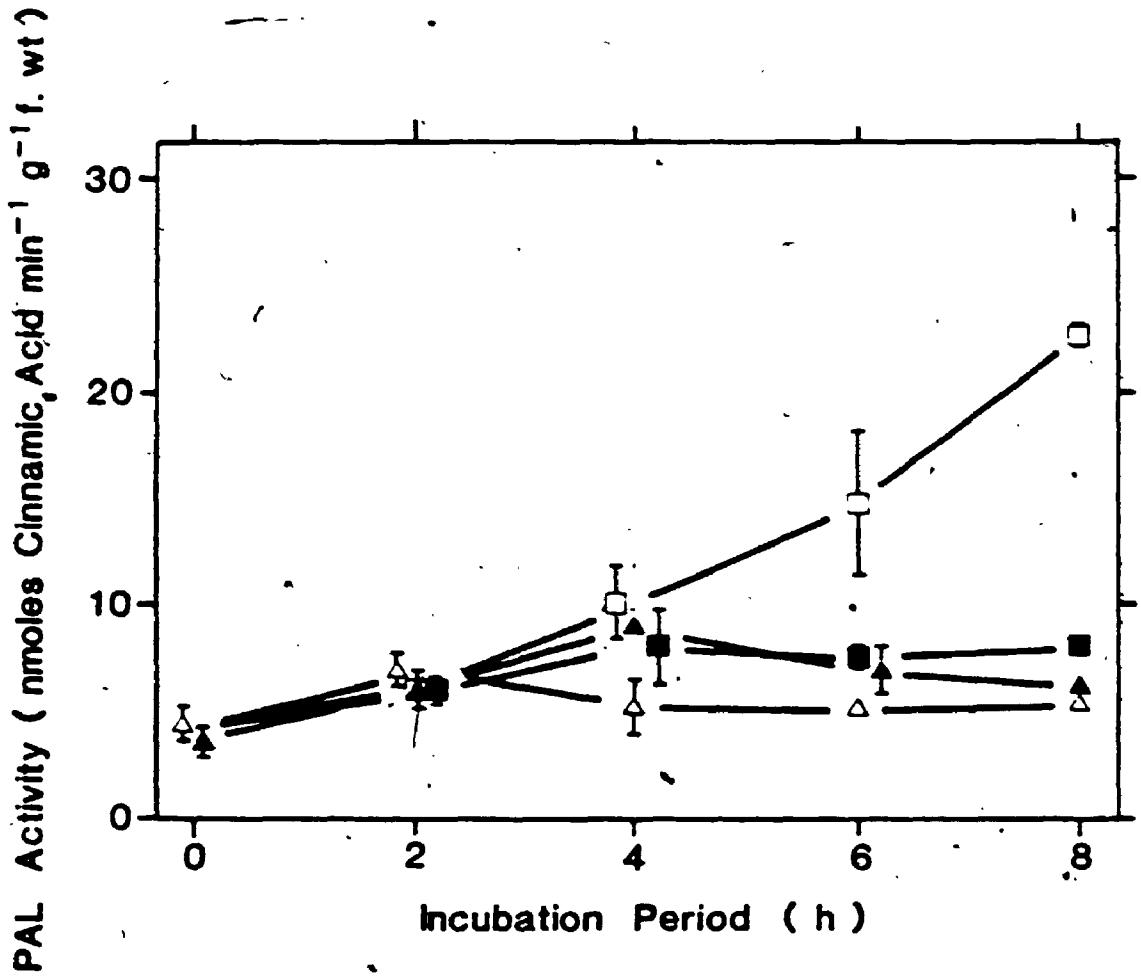


Fig. 8.2

Phenylalanine ammonia-lyase activity in etiolated hypocotyls of soybean cultivars Harosoy (■——■) and Harosoy 63 (□——□) wounded and inoculated immediately with Phytophthora megasperma f.sp. glycinea race 1 and in water-treated wounded controls (cv. Harosoy, ▲——▲ and Harosoy 63, △——△). Data are based on two experiments.


PAL Activity (nmoles Cinnamic Acid min⁻¹ g⁻¹ f. wt)
30
20
10
0
0
2
4
6
8
Incubation Period (h)

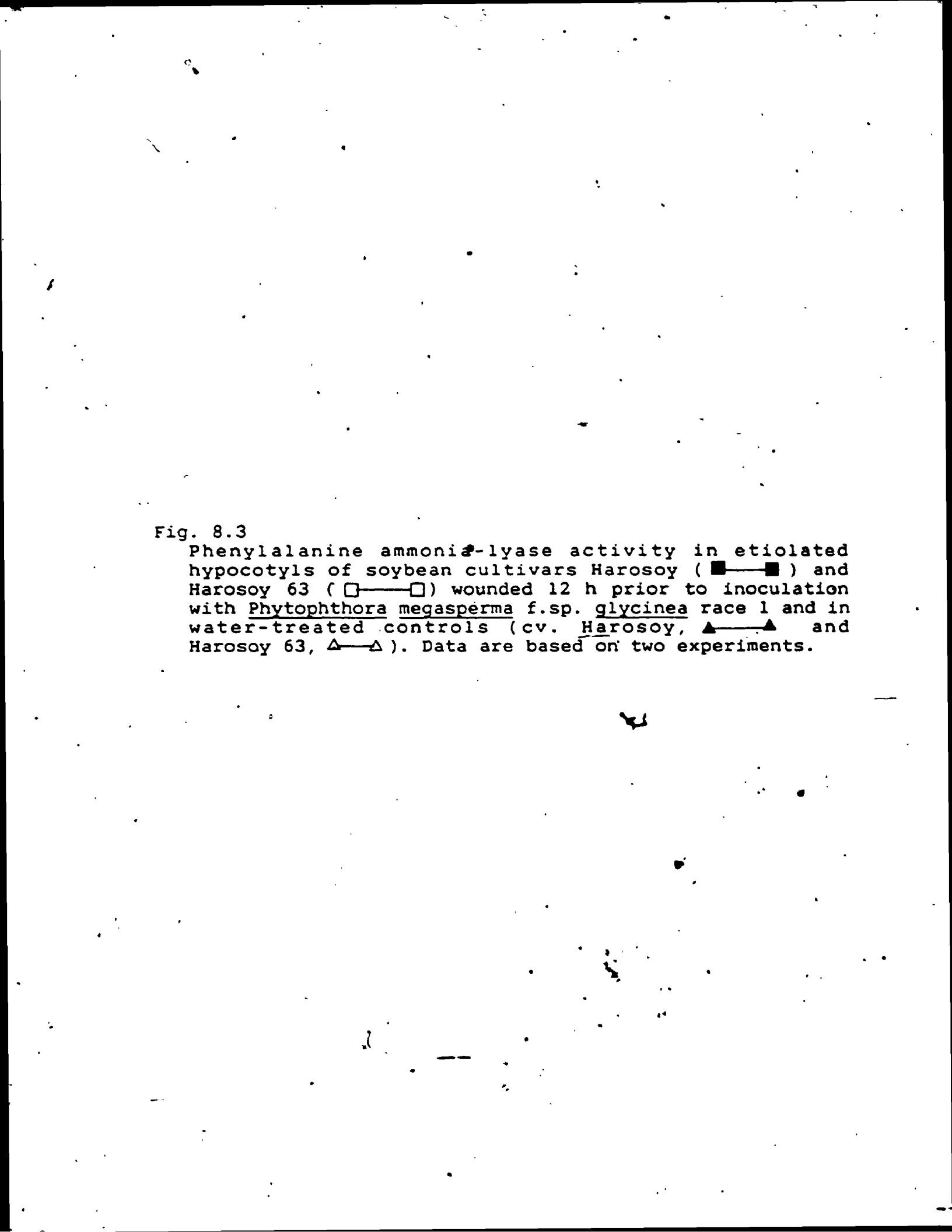
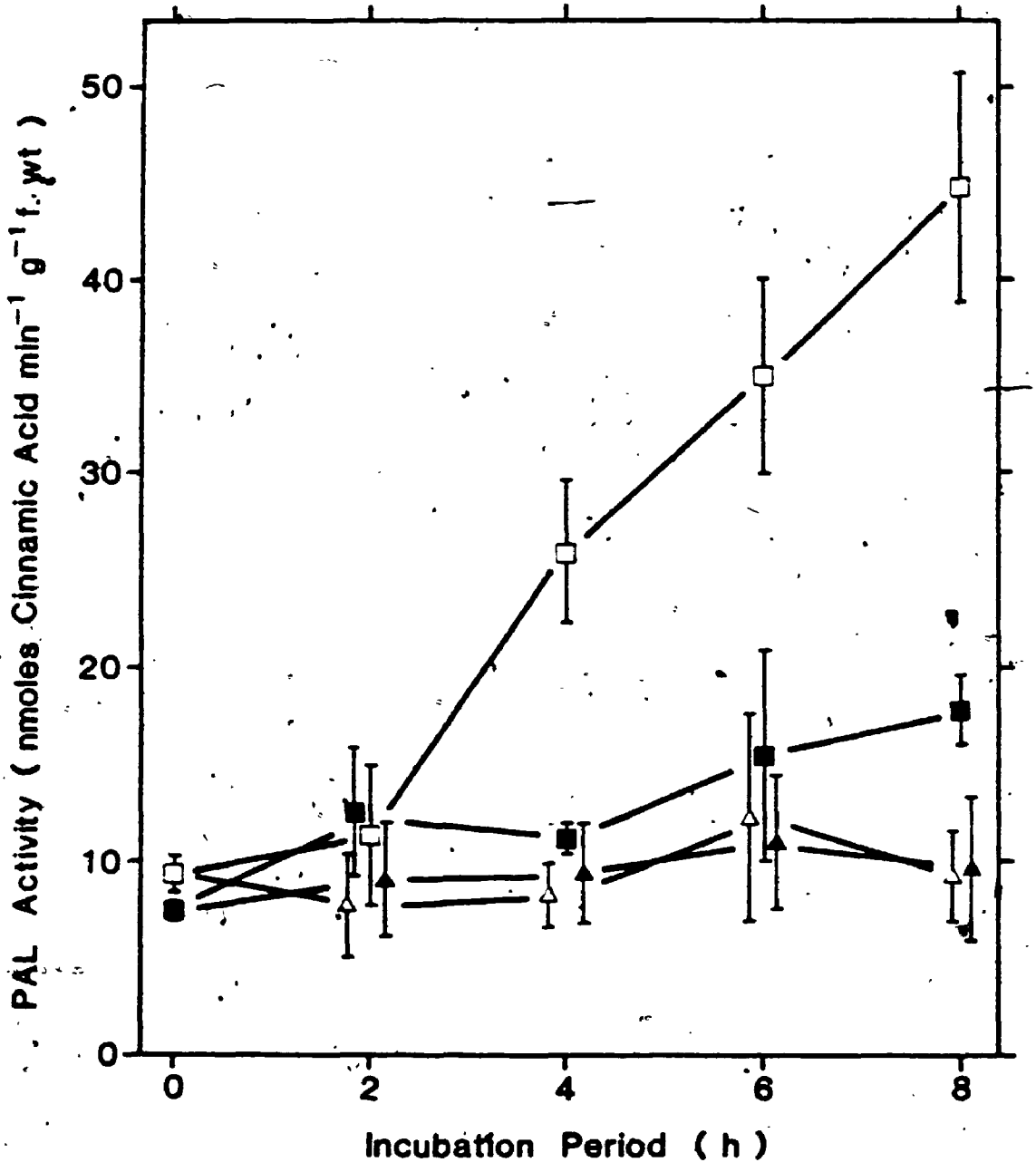


Fig. 8.3
Phenylalanine ammonia-lyase activity in etiolated hypocotyls of soybean cultivars Harosoy (■——■) and Harosoy 63 (□——□) wounded 12 h prior to inoculation with Phytophthora megasperma f.sp. glycinea race 1 and in water-treated controls (cv. Harosoy, ▲——▲ and Harosoy 63, △——△). Data are based on two experiments.


PAL Activity (nmoles Cinnamic Acid min^{-1} g^{-1} f. wt)
0
10
20
30
40
50
0
2
4
6
8
Incubation Period (h)

8.4.2 Leaves

8.4.2.1 Symptoms

The selection of leaves used for determination of PAL activity was based upon observations of symptoms reported previously [Chapter 4.4.2]. Trifoliates from 14-day old cv. Harosoy plants were susceptible and developed pale brown lesions by 24 h that started to spread by 36 h with the development of soft rotten tissues. Trifoliates from 12-day old cv. Harosoy 63 were susceptible also and lesions were similar to those in cv. Harosoy. However, by 14 days trifoliates of cv. Harosoy 63 were resistant. Brown spots appeared beneath inoculum drops 12 h after inoculation and conspicuous dark-brown to reddish-brown lesions, more or less restricted to the inoculated area, developed by 24 h.

8.4.2.2 Phenylalanine Ammonia-lyase Activity

There was no significant change in PAL activity in any of the infected leaves upto 24 h following inoculation (Table 8.1). At 36 h following inoculation there was a major increase in activity in leaves of 14-day old cv. Harosoy 63 plants (resistant) but not in leaves of other plants.

8.4.2.3 Levels of Glyceollin

Following zoospore inoculation much more glyceollin accumulated in trifoliates from 14-day old cv. Harosoy 63 plants than in those from either 12-day old cv. Harosoy 63 plants or 14-day old cv. Harosoy plants (Table 8.2). At 24 h but not 36 h, after inoculation glyceollin accumulation was significantly less in 12-day old cv. Harosoy 63 trifoliates than in cv. Harosoy trifoliates. With $AgNO_3$ treatment (Table 8.2) amounts of glyceollin produced in trifoliates of 14-day old plants of both cultivars were similar but only about one fifth as much was produced in trifoliates of 12-day old plants of cv. Harosoy 63.

Table 8.1

Phenylalanine ammonia-lyase activity in trifoliates of soybean inoculated with Phytophthora megasperma f. sp. glycinea.

Cultivar	Plant Age (days)	PAL activity (nmole cinnamic acid/min/g fresh wt.)			
		Inoculated[1]		Control	
		24h[2]	36h	24h	36h
Harosoy	14	20.6±9.7[3]	9.4±3.7	26.1±10.9	8.2±2.2
Harosoy 63	12	10.5±6.3	16.3±12.2	8.7±1.5	10.1±2.0
Harosoy 63	14	9.9±3.5	84.2±13.3	9.9±3.6	9.2±3.0

[1] Trifoliates were inoculated by placing 10 µl drops of zoospore suspension (10^5/ml) of Phytophthora megasperma f.sp. glycinea race 1 on the adaxial surface. Drops of water (10 µl) were applied to controls.
[2] Incubation period following inoculation until analysis.
[3] Data are based on two experiments.

Table 8.2

Accumulation of glyceollin in leaves of soybeans following inoculation with Phytophthora megasperma f.sp. glycinea race 1 or $AgNO_3$.[1]

Cultivar	Age(day)	Glyceollin (µg/g fresh wt.)		
		Zoospores		$AgNO_3$
		24h	36h	62h
Harosoy	14	593±45[2]	400±4	2876±243
Harosoy 63	12	205±4	336±151	556±2
Harosoy 63	14	928±128	1097±94	2755±301

[1] Trifoliates were inoculated by placing 10 µl drops of a zoospore suspension (10^5/ml) or a $AgNO_3$ solution (1 mM) on the adaxial surface of detached leaves.
[2] Incubation period following inoculation or $AgNO_3$ treatment until analysis.
[3] Data for glyceollin (µg/g fresh wt) are based on two replications.

8.4.2.4 Levels of Reducing Sugars

No differences in reducing sugar levels were found among the three groups of trifoliates (Table 8.3).

8.5 Discussion

The rapid increase in PAL activity in the resistant response but not in the susceptible response of intact etiolated soybean hypocotyls demonstrated here (Fig. 8.1) is comparable to that in intact roots of soybean seedlings reported by Bonhoff et al. [1986]. It is consistent also with early increases in gene transcription for mRNA for PAL in resistant but not in susceptible reactions reported by Esnault et al. [1987] and with the evidence that rates of biosynthesis and accumulation of glyceollin I are higher in resistant than in susceptible inoculated hypocotyls [Chapter 7.4.1].

In hypocotyls, wounded just prior to inoculation, symptom development was delayed compared to that in intact hypocotyls. Similarly, PAL activity in the resistant response in wounded hypocotyls increased at a lower rate than in intact hypocotyls. These differences were due evidently to the removal of the epidermal and a few cortical cell layers by wounding. This indicates that the epidermal cell layer is important for early and rapid stimulation of PAL activity in response to infection.

PAL activity in wounded tissues treated with water only was slightly higher than that of intact hypocotyls. Borner and Grisebach [1982] also observed only a low level of PAL activity, but Patridge and Keen [1977] reported a high level of PAL activity in wounded green hypocotyls of soybeans treated with water. Neither of the two groups, however, observed any difference in PAL activity between resistant and susceptible responses during early stages following inoculation. This contrasts with the present study in

Table 8.3

Total reducing sugar concentrations in soybean leaves following inoculation with Phytophthora megasperma f.sp. glycinea race 1.

Cultivar	Plant Age (days)	Reducing Sugars (mg/g fresh wt)			
		Inoculated		Control	
		24h[2]	36h	24h	36h
Harosoy	14	4.35±0.20	3.96±0.04	3.61±0.54	4.19±0.47
Harosoy 63	12	4.45±0.23	3.85±0.31	4.33±0.54	4.48±0.52
Harosoy 63	14	4.87±0.91	4.47±0.11	4.08±0.20	4.06±0.22

[1] Trifoliates were inoculated by placing a 10 µl drop of zoospore suspension (10^5/ml) of Phytophthora megasperma f.sp. glycinea race 1 on the adaxial surface. Drops of water (10 µl) were applied to controls.
[2] Incubation period following inoculation until analysis.
[3] Data for total reducing sugars (mg/g fresh wt) are based on two replications.

which PAL activity in wounded inoculated hypocotyls was significantly lower in the susceptible response than in the resistant response. Both groups of workers used fragmented mycelium for inoculum. This may have contained non-specific elicitors that caused similar responses in both resistant and susceptible tissues. They also used green hypocotyls and it is possible that, as we observed for leaves, green tissues have a sufficient basal level of PAL to support biosynthesis of glyceollin during the first few hours of the host-pathogen interaction.

Wounding of hypocotyls 12 h prior to inoculation (Fig 8.3) enhanced the development of symptoms and PAL activity significantly compared to wounding immediately before inoculation. This appears to be similar to the effects of wounding on the development of hypersensitivity in potatoes reported by Tomiyama [1960]. There was a gradual increase in PAL activity due to wounding alone and by the time of inoculation this was appreciably higher in hypocotyls wounded 12 h previously than in unwounded hypocotyls. Following inoculation PAL activity reached a higher level in the hypocotyls wounded 12 h before inoculation than in the unwounded hypocotyls. In these hypocotyls, also accelerated production of glyceollin was observed [Chapter 7.4.3]. Possibly, the wound response involves the priming of cellular mechanisms for synthesis of PAL and other enzymes that are rapidly mobilized on infection. Similar conclusions were reached by Inoue et al. [1984] for enhanced activity of enzymes for furanoterpenoid biosynthesis in wounded sweet potato roots. As they suggested, the enhanced response may increase resistance to wound-invading microorganisms.

In leaves, PAL activity increased after 24 h only in the resistant response of 14-day old trifoliates of Harosoy 63. Therefore, accumulation of glyceollin that was demonstrated at 24 h occurred without stimulation of PAL ac-

tivity over background levels. This indicates that background levels of PAL are sufficient for initial accumulations of glyceollin and suggests that the control of pathways after PAL, leading to other phenylpropanoids, may differ in leaves from that in etiolated hypocotyls, e.g., there may be more demands for precursors for lignification in hypocotyls than in leaves. It suggests also, that the Rps gene for resistance in cv. Harosoy 63 does not directly control glyceollin biosynthesis at the PAL step, but that changes in PAL activity occur secondarily in response to demand and may be under allosteric control.

The trifoliates of 12-day old plants of cv. Harosoy 63 were susceptible to race 1 and PAL activity was similar to that in the normal susceptible response of the trifoliates of 14-day old plants of cv. Harosoy. Glyceollin concentrations were reduced also and were comparable after 36 h. However, when treated with $AgNO_3$ solution, an abiotic elicitor of glyceollin [Stossel, 1982], the level of glyceollin accumulation in these leaves was only one fifth of that in $AgNO_3$-treated trifoliates of 14-day old plants of both cultivars. The leaves did not differ in levels of reducing sugars which may be regarded as general precursors for phenylpropanoid biosynthesis as well as for many other processes. It appears that 12-day old leaves have not developed mechanisms for response to either infection or the abiotic elicitor, or they are deficient in metabolic processes to support glyceollin synthesis or other defence-related processes.

CHAPTER 9

TEMPERATURE-INDUCED SUSCEPTIBILITY OF SOYBEANS TO *Phytophthora megasperma* f.sp. *glycinea* : PHENYLALANINE AMMONIA-LYASE AND GLYCEOLLIN IN THE HOST; GROWTH AND GLYCEOLLIN I SENSITIVITY OF THE PATHOGEN.

9.1 Summary

In soybean cultivars differring in the effect of temperature on the development of resistance, phenylalanine ammonia-lyase (PAL) activity was determined in hypocotyls at 25 or 33°, following infection with *Phytophthora megasperma* f.sp. *glycinea* or treatment with the abiotic elicitor, $AgNO_3$. PAL activity was less at 33°C than at 25°C in each of six cultivars examined but was lowest in two cultivars previously shown to develop susceptibility at 33°C. Glyceollin accumulation was determined in response to $AgNO_3$-treatment and was higher at 33°C than at 25°C in four cultivars tested. The increase was marginal with two temperature-sensitive cultivars but more than 50 percent in two cultivars that remained resistant at 33°C. There were significant differences among 18 races of the pathogen for growth rates *in vitro*, the effect of temperature (25 or 33°C) on growth, sensitivity to glyceollin I and the interaction of temperature and glyceollin I sensitivity. Growth of some races (eg. races 2,7) was strongly inhibited at 33°C, that of others was similar at both temperatures, and that of one (race 19) was faster at 33°C than at 25°C. Minimal restriction of growth at 33°C and relative tolerance to glyceollin I in race 4 combined with a major suppression of PAL activity and little increase in glyceollin accumulation at 33°C in cv. Altona was consistent with temperature-induced susceptibility in this race-cultivar interaction. The possibility that combinations of physiological variables

in host and pathogen, rather than, or in addition to, putatitive recognition systems, may define reaction-types is discussed.

9.2 Introduction

The influence of temperature on the development of resistance and susceptibility in the interaction of soybeans with Phytophthora megasperma f.sp. glycinea has been the subject of several previous reports [Chamberlain and Gerdemann, 1966; Classen and Ward, 1985; Keeling, 1985; Rose et al., 1982; Ward and Buzzell, 1983; Ward and Lazarovits, 1982]. Similar observations have been made for other diseases [e.g., Bailey et al., 1980; Daly, 1972; Elliston et al.,1977; Gousseau et al., 1985] and Vanderplank [1978] has proposed that temperature-induced susceptibility may be due to copolymerization of host and pathogen proteins following changes in their tertiary structure caused by increasing temperatures. However, there do not appear to be any essential reasons why temperature-induced changes in susceptibility should be related only to putatitive host-pathogen recognition phenomena. In the soybean-Phytophthora megasperma f.sp. glycinea interaction, for example, no links were found between temperature-induced susceptibility and the production and activity of elicitors of the phytoalexin, glyceollin [Classen and Ward, 1985; Murch and Paxton,1980].

In addition to recognition phenomena that may lead to the establishment of compatibility or incompatibility and the triggering of defence reactions, many other biochemical attributes of host and pathogen presumably contribute to the development of resistant or susceptible reaction types. It was reported previously that the interaction of some combinations of Phytophthora megasperma f.sp. glycinea races and soybean cultivars were temperature sensitive (incompatible at 25°C, compatible at 32.5°C) while others were not

(incompatible at both temperatures) [Keeling, 1985; Ward and Buzzell, 1983; Ward and Lazarovits, 1982]. If defense related processes are affected more by temperature changes in some cultivars than in others and, similarly, temperature-related differences for 'pathogenicity' factors exist between races, then, temperature-induced susceptibility would be expected to develop most readily between cultivars most affected and races least affected by elevated temperatures. Although the nature of such factors remains to be defined, two aspects of host and pathogen physiology that may be involved were examined in this chapter. In the host, the effect of temperature on phenylalanine ammonia-lyase(PAL) activity (Fig. 7.7) and production of the phytoalexin, glyceollin were measured in several soybean cultivars differing in temperature sensitivity. PAL activity and mRNA transcription have been reported to increase specifically in incompatible interactions and not in compatible interactions [Bonhoff et al., 1986; Esnault et al., 1987; Chapter 8]. In the pathogen, the influence of temperature on growth and sensitivity to glyceollin I of 18 races of Phytophthora megasperma f.sp. glycinea was determined. The results indicate that temperature-induced susceptibility in some race-cultivar combinations may be related to quantitative changes in these aspects of host and pathogen physiology.

9.3 Materials and Methods

9.3.1 Host

Seeds of soybean cultivars/lines Altona, Harosoy, Harosoy 63, PRX8122-1, Corsoy-79, L-70-6494 were grown in trays of vermiculite for 6 days in the dark as described earlier to obtain etiolated seedlings [Chapter 2.1.1].

9.3.2 Pathogen

Phytophthora megasperma f.sp. glycinea race 4 and race

6 [Ward and Lazarovits,1982] were grown routinely on V8 juice agar at 25° C. Zoospores were produced as described previously [Chapter 2.2.2.2]. A 10 µl drop of zoospore suspension (10^5/ml)was used as inoculum for both races. Etiolated hypocotyls of Altona($\underline{Rps_6}$) are resistant to race 4 and susceptible to race 6 at 25°C and susceptible to both races at 33°C. Races 1 to 15 and 17-19 were maintained similarly and used for in vitro bioassays described below.

9.3.3 Abiotic Elicitor

A freshly prepared solution of $AgNO_3$ (10^{-3} M) was used as an abiotic elicitor [Chapter 2.3]. It was applied to etiolated hypocotyls in 10 µl drops in the same way as zoospore suspensions.

9.3.4 Inoculation and Incubation

Etiolated hypocotyls were arranged horizontally in glass trays as described previously [Chapter 2.1.2]. Hypocotyls were inoculated by placing two drops of zoospore suspension or $AgNO_3$ solution, approximately 1 cm apart, 2 cm below the cotyledons. After inoculation with Phytophthora megasperma f.sp. glycinea race 4 or 6 (cv.Altona) or treatment with $AgNO_3$ (all cultivars) seedlings were incubated in the dark at 25° C or 33° C and 100 % RH.

9.3.5 Determination of PAL Activity

Hypocotyl sections approximately 1.75 cm long containing the inoculated sites were excised, weighed and immediately frozen in liquid nitrogen and stored at -70°C. The enzyme activity was determined following the methods described in chapter 7.3.10 and expressed as n moles cinnamic acid produced in one minute per g fresh weight of tissue.

9.3.6 Determination of Reducing Sugars

Reducing sugars in the supernatant used in the determination of PAL activity were determined following the method of Nelson [1944] and expressed as mg per g fresh weight of tissue.

9.3.7 Determination of Glyceollin

Tissues from lesions developed following inoculation with zoospores or $AgNO_3$ treatment were excised and extracted in ethanol (95%) and glyceollin was separated by thin layer chromatography as described in chapter 2.7. Glyceollin concentrations are expressed in µg per 10 treated sites.

9.3.8 Preparation of Glyceollin I

Seven-day old etiolated hypocotyls of cv. Altona were arranged horizontally in glass trays (40 seedlings/tray) and sprayed with $AgNO_3$ (10^{-3} M) solution. After incubation for 48 h at 25°C, $AgNO_3$-treated tissues were boiled in 95 % ethanol for 15 minutes and glyceollin I was extracted and purified following the methods described previously [Chapter 2.5].

9.3.9 Bioassays

Races 1 to 15 and 17-19 of Phytophthora megasperma f.sp. glycinea were grown in Petri dishes (90 mm diameter) containing 6 ml clarified 10% V8 juice agar (agar 1.5%) at 25°C in the dark. Plugs (5 mm diameter) were cut from the advancing margin of the colony and transferred to Petri dishes (35 mm diameter) containing the assay media prepared as follows.

An ethanol solution of glyceollin I was mixed with molten 10% V8 juice in a flask in a water bath (50°C) to give concentrations of 10 and 75 µg/ml. Ethanol concentra-

tions were adjusted to 2% in all cases including controls without glyceollin I. The media were dispensed immediately into Petri dishes (35 mm diameter, 0.8 ml/dish) on a slide warmer to allow formation of a uniform thin layer before setting . The Petri dishes were transferred briefly to the bench top for the agar to solidify and then to glass trays containing wet paper towels and sealed, except during inoculation, with plastic film. Prior to dispensing of medium, the Petri dishes were serially numbered (1-36) and randomly inoculated with the 18 races (1 treatment, 2 Petri dishes for each race). The Petri dishes of each pair were separated randomly into two groups, one group was incubated at 25°C the other at 33°C. A second replicate was set up in the same way. Eighteen Petri dishes containing one glyceollin I level and each of the 18 races were incubated in one glass tray and constituted one replicate.

Growth was measured first at 36 h following inoculation and thereafter at 24 h intervals. This was achieved by taking Polaroid photographs of groups of plates in the same sequence on successive days. The negatives were projected on to a graphic tablet of an Apple IIe computer at a magnification of 3.2 x using a photographic enlarger. The area of the colony was computed from a series of points on the periphery of the image and from this a value for mean colony-diameter was derived. Because of the differences in growth rates, data are based on the 24 h period 36 to 60 h for 0 and 10 µg/ml glyceollin I and from 60 to 84 h for 75 µg/ml. The data are reported as radial growth in mm/day and analysed following a split-plot design [Little and Hills, 1978] with temperature in the main plots, levels of glyceollin I in sub-plots and races of Phytophthora megasperma f.sp. glycinea in sub-sub-plots.

9.4 Results

9.4.1 Phenylalanine Ammonia-lyase Activity Following Inoculation with Phytophthora megasperma f.sp. glycinea

In the resistant response of cv. Altona to Phytophthora megasperma f.sp. glycinea race 4 at 25°C, PAL activity was significantly greater than in controls 3 h following inoculation, it reached a peak at 12 h and, thereafter, declined (Fig. 9.1). In the susceptible response of cv. Altona to race 6 at 25°C, PAL activity although higher than in uninoculated controls did not increase appreciably during the first 7 h, but, thereafter, it increased throughout the 22 h period studied. The pattern of response changed at 33°C. There was a significant increase in PAL activity by 7 h following inoculation with race 6 but activity declined after 12 h. In the temperature-induced susceptibile reaction of cv. Altona to race 4 at 33°C PAL activity was much lower than at 25°C for the first 12 h. It was comparable to that in the cv. Altona-race 6 interaction at 33°C until 7 h but thereafter the pattern of activity was similar to, although higher than that of the normal susceptible reaction to race 6 at 25°C. No temperature-related differences in PAL activity were detected in the water treated controls.

9.4.2 Phenylalanine Ammonia-lyase Activity and Glyceollin Production Following $AgNO_3$ Treatment

The effect of temperature on PAL activity in the host without the interaction with the pathogen was determined using the abiotic elicitor, $AgNO_3$. This elicits glyceollin accumulation in etiolated hypocotyls without wounding [Stossel, 1982; Chapter 3.4.4.1]. At 25°C the pattern of PAL activity in $AgNO_3$-treated hypocotyls (Fig. 9.2) was comparable to that in the resistant response to the pathogen at 25°C (Fig. 9.1). However, at 33°C, although PAL activity was significantly higher than at 25°C by 3 h, it was less than

Fig. 9.1
Phenylalanine ammonia-lyase activity in hypocotyls of soybean cv. Altona inoculated with Phytophthora megasperma f.sp. glycinea race 4 or race 6 and incubated at 25°C or 33°C. Soybean cv. Altona is resistant to race 4 at 25°C but susceptible at 33°C, it is susceptible to race 6 at both temperatures. Race 4 at 25°C (○——○) and 33°C (○---○), race 6 at 25°C (●——●) and 33°C(●--●) or water treatment at 25°C(Δ——Δ) and 33°C(Δ--Δ).

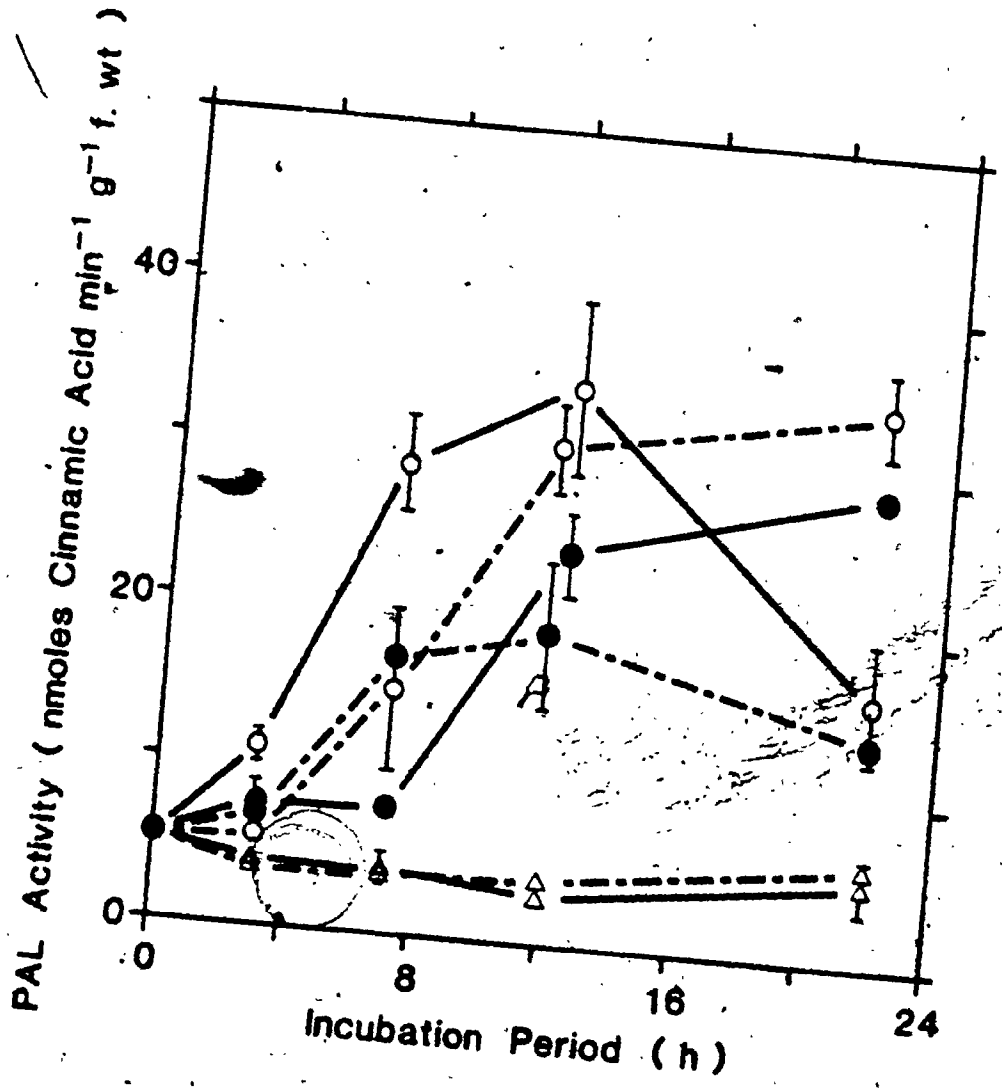
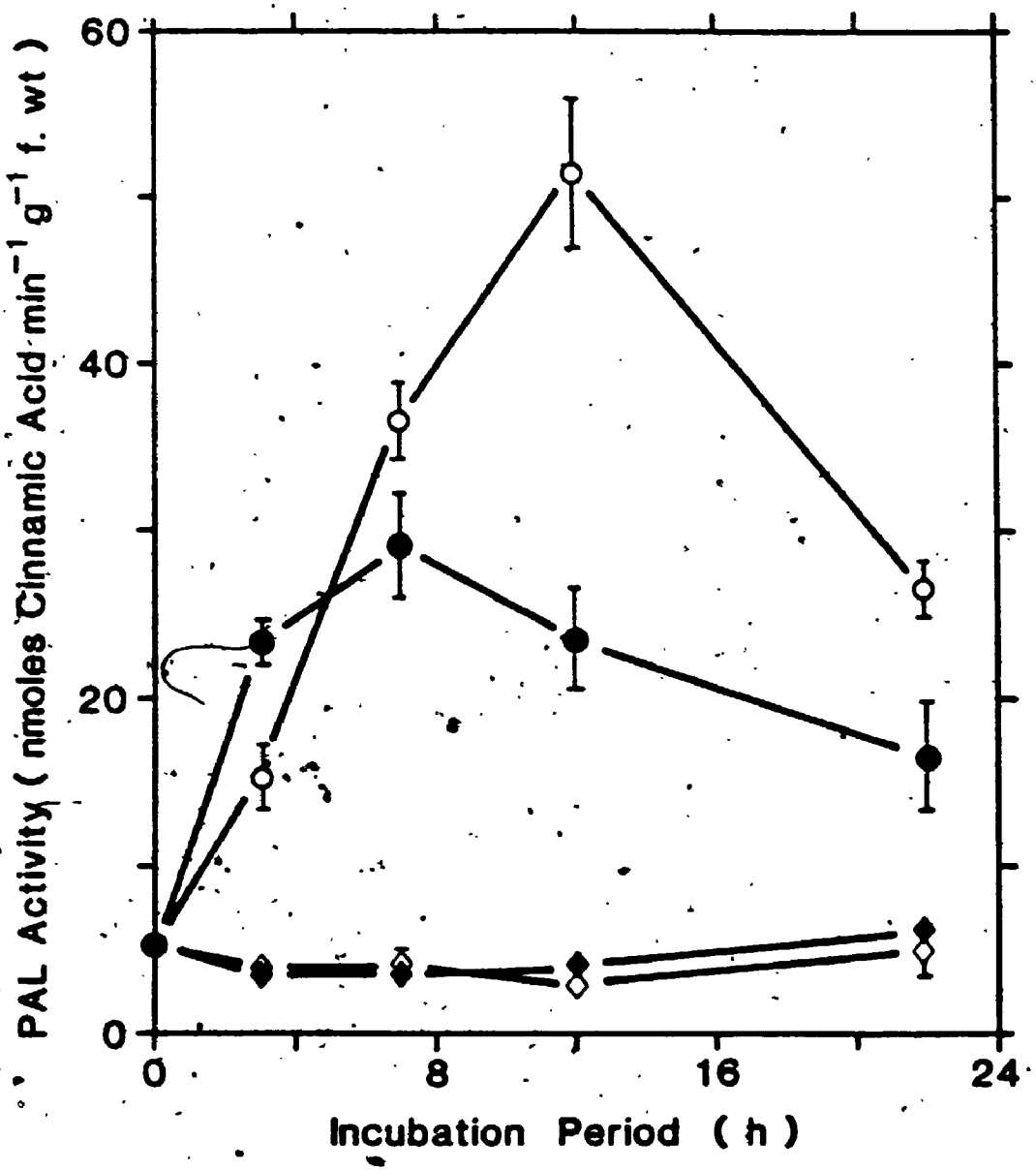

PAL Activity (nmoles Cinnamic Acid min^{-1} g^{-1} f. wt)
0
20
40
0
8
16
24
Incubation Period (h)
A

Fig. 9.2

Phenylalanine ammonia-lyase activity in hypocotyls of soybean cv. Altona following treatment with $AgNO_3$ and incubation at 25°C(○——○) and 33°C(●——●) or water at 25C(◇——◇) and 33°C(◆——◆).


60
40
20
0
0
8
16
24
PAL Activity (nmoles Cinnamic Acid min^{-1} g^{-1} f. wt)
Incubation Period (h)

at 25°C by 7 h and continued to decline subsequently. At 12 h PAL activity at 33°C was only about half that at 25°C. Comparisions with two other cultivars after incubation for 12 h indicated that they differed widely in the effect of temperature on PAL activity (Table 9.1). Thus while activity in Altona was reduced 50 per cent at 33°C that in Harosoy 63 was reduced less than 30 per cent and even then was comparable to that in Altona at 25°C. There were small differences between the three cultivars in the levels of reducing sugars, but no indication that reduced PAL activity at 33°C was due to a depletion of the general precursor pool due to enhanced respiration or other demands (Table 9.1). In Harosoy there was an increase in reducing sugars at the higher temperature.

In a further comparison, the effect of temperature on PAL activity and glyceollin accumulation following $AgNO_3$ -treatment was determined for two pairs of soybean cultivars that ef[illegible]ther did (cvs. Altona, PRX8-122-1) or did not (cvs. Corsoy 79, L-70-6494) exhibit temperature-induced susceptibility in an earlier study [Ward and Buzzell, 1983] (Table 9.2). In all four cultivars PAL activity was less at 33°C than at 25°C but the decrease in activity was much greater in the cultivars with temperature-sensitive interactions than those without. In the non-temperature sensitive cultivar L-70-6494 the difference between the activities at the two temperatures was within the standard errors. Similarly, although all four cultivars produced more glyceollin at 33°C than at 25°C, increases were relatively minor in the temperature-sensitive cultivars but extensive in the other two cultivars. Changes in PAL activity and glyceollin accumulation were correlated. Thus, cvs. Corsoy 79 and L-70-6494 that had the smallest decreases in PAL activity at 33°C had the greatest increases in glyceollin accumulation.

Table 9.1

Phenylalanine ammonia-lyase activity and reducing sugar levels in soybean cultivars following $AgNO_3$-treatment and incubation at 25°C or 33°C.

Cultivar	Temperature		% decrease[a]
	25°C	33°C	
(a) PAL-activity (n mole cinnamic acid min^{-1}/g f. wt)[b]			
Harosoy	54.7±0.9[c]	35.9±0.7	34.4
Harosoy 63	58.9±0.1	41.9±1.4	28.9
Altona	46.1±4.9	24.0±2.8	47.9
(b) Reducing sugars (mg/g f. wt)			
Harosoy	11.6±0.2	14.5±0.9	----
Harosoy 63	10.3±0.2	10.6±0.0	----
Altona	9.6±0.1	9.8±0.6	----

[a] Percent decrease of PAL activity at 33°C from that at 25°C.

[b] PAL activity and reducing sugar levels were determined 12 h following $AgNO_3$ treatment.

[c] Data are mean and S.E. from two replications.

Table 9.2

Comparison of phenylalanine ammonia-lyase activity and accumulation of glyceollin at 25°C and 33°C following $AgNO_3$ treatment in soybean cultivars that differ in temperature-induced susceptibility to *Phytophthora megasperma* f.sp. *glycinea*

Cultivar	Temperature		% change[a]
	25°C	33°C	
(a) PAL-activity (n mole cinnamic acid min^{-1}/g f. wt)[2]			
PRX8-122-1	45.8±1.9[d]	17.1±1.6	-62.7
Altona	42.3±2.5	23.6±1.1	-44.3
Corsoy 79	52.4±1.4	38.8±4.1	-25.9
L-70-6494	46.4±4.9	41.1±2.9	-11.5
(b) Glyceollin (µg/10 treated sites):			
PRX8-122-1	72.0±4.5	80.8±7.0	+12.2
Altona	45.3±1.1	51.9±0.1	+14.6
Corsoy 79	105.9±5.2	159.5±4.5	+50.6
L-70-6494	101.8±7.1	178.3±5.4	+75.1

[a] Percent decrease of PAL activity at 33°C was calculated over that at 25°C, while percent increase of glyceollin accumulation at 33°C was calculated over that at 25°C.

[b] PAL activity was determined 12 h and glyceollin 20 h following $AgNO_3$ treatment.

[c] Cultivars Altona and PRX8-122-1 displayed temperature-induced susceptibility in a previous study [Ward and Buzzell, 1983] Corsoy 79 and L-70-6494 did not.

[d] Data are mean and S.E. from two replications.

9.4.3 Influence of Temperature and Glyceollin I on Growth of Phytophthora megasperma f.sp. glycinea in vitro

There were major differences among the 18 races of Phytophthora megasperma f.sp. glycinea in their growth rates and responses to temperature and glyceollin I (Fig. 9.3, Table 9.3). Analyses of the data of Table 9.3 demonstrated that there were significant differences for the effects of temperature and levels of glyceollin I; for the interaction effects of temperature and glyceollin I levels; among the races and their interaction with temperature or glyceollin I levels, or with temperature and glyceollin I levels (Table 9.4). The overall effect of the 2 temperatures or 3 glyceollin I levels on the 18 races differed significantly from each other (Table 9.5). However, at the highest glyceollin I level (75 µg/ml) there was no overall significant effect of temperature on growth.

Fifteen of the 18 races grew more slowly in the control medium at 33°C than at 25°C (Fig. 9.3, Table 9.3) but the extent of the reduction varied widely. Thus growth of race 7 at 33°C was only 16% of that at 25°C, growth of races 2 and 13 was about 40% and growth of the majority was between 70 and 80% that at 25°C. However, two races (4 and 18) grew as rapidly at 33°C as at 25°C and one race (19) grew most rapidly at 33°C.

Sensitivity to glyceollin I differed appreciably among races (Fig. 9.3, Table 9.3). At 25°C growth with 10 µg/ml glyceollin I ranged from 69% (race 11) to 104% (races 13 and 15) of corresponding controls. With 75 µg/ml the range was from 7 to 44%. At 33C there were further differences. Some races (7,9,12,13,17) grew better with 10 µg/ml glyceollin I than without it, others were unaffected (4,15) while others were inhibited (1,2,6,8). For some races (4,7,9) glyceollin I at one or both of the concentrations was relatively less inhibitory at 33°C than at 25°C. The actual

Fig. 9.3

Effect of temperature and glyceollin I on growth of Phytophthora megasperma f.sp. glycinea, races 1-15 and 17-19. Temperature, levels of glyceollin I and incubation periods were as follows: A) 25°C, 0 µg/ml, 84 h; B) 25°C, 10 µg/ml, 84 h; C) 25°C, 75 µg/ml, 168 h; D) 33°C, 0 µg/ml, 84 h; E) 33°C, 10 µg/ml, 84 h; F) 33°C, 75 µg ml^{-1}, 168 h. In each group races were arranged as follows: from left to right, top row, races 1,2,3,4,5,6; middle row, races 7,8,9,10,11,12, and bottom row, races 13,14,15,17,18,19.

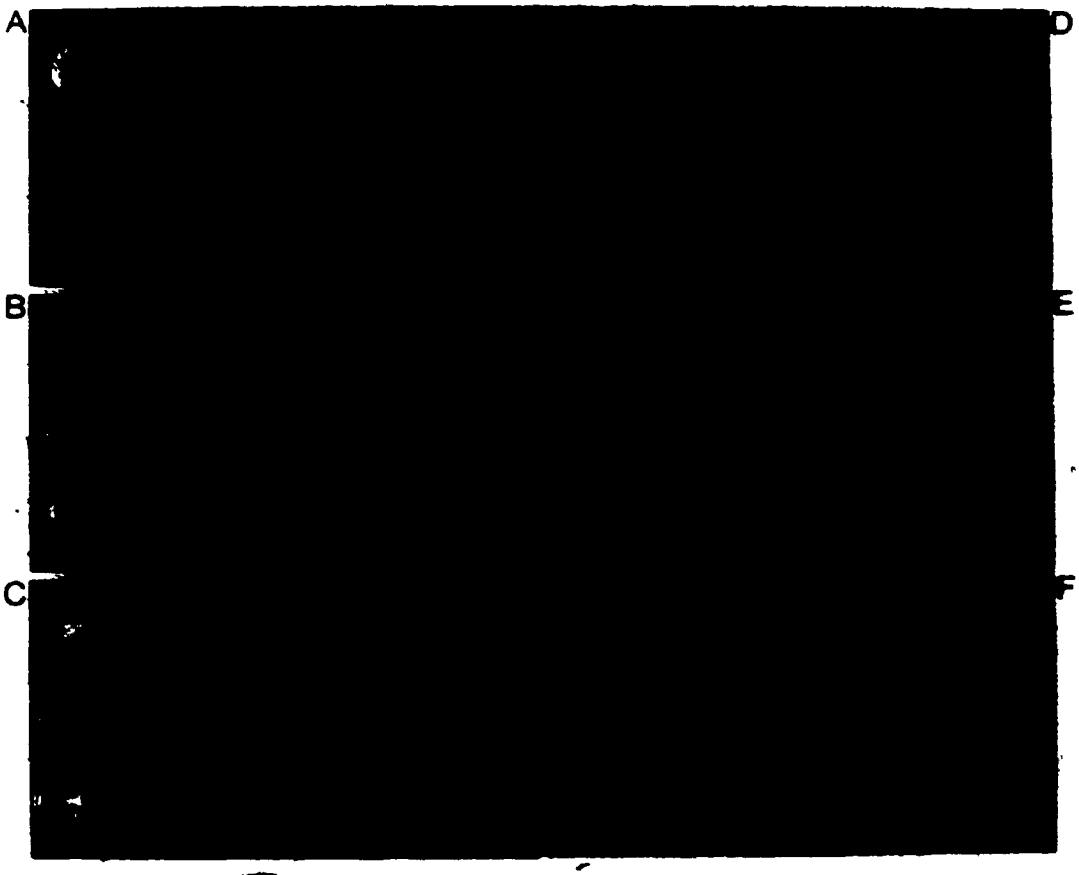

A
D
B
E
C
F

Table 9.3

Effect of temperature and glyceollin I levels on radial growth (mm/day) of 18 races of Phytophthora megasperma f.sp. glycinea.

Race	Glyceollin I (µg/ml)								
	0			10			75		
	25°C	33°C	%[1]	25°C	33°C	%	25°C	33°C	%
1	5.73[a][2]	4.20[b]	73	4.91[a]	2.81[b]	57	1.87[a]	1.13[b]	60
2	4.97[a]	1.96[b]	39	4.33[a]	0.60[b]	14	1.29[a]	0.28[b]	22
3	5.19[a]	2.90[b]	56	4.12[a]	2.18[b]	53	1.37	1.20	88
4	4.91	4.70	96	4.07	4.54	112	1.47[b]	2.04[a]	139
5	5.65[a]	4.03[b]	71	4.61[a]	3.40[b]	74	2.27	1.91	84
6	4.89[a]	3.65[b]	75	3.94[a]	2.50[b]	64	2.17	1.99	92
7	5.29[a]	0.86[b]	16	4.50[a]	0.99[b]	22	0.38	0.49	129
8	4.67[a]	4.02[b]	86	4.35[a]	3.10[b]	71	1.48	1.15	78
9	5.61[a]	3.94[b]	70	4.86[a]	4.36[b]	90	0.64	0.77	120
10	5.40[a]	3.72[b]	69	4.65[a]	3.07[b]	66	1.00	1.01	101
11	6.21[a]	3.89[b]	63	4.28[a]	3.40[b]	79	1.36	1.34	99
12	6.06[a]	5.27[b]	87	5.39	5.56	103	1.05	0.91	87
13	5.91[a]	2.27[b]	38	6.16[a]	2.74[b]	45	1.24	0.94	76
14	4.67[a]	3.27[b]	70	4.71[a]	3.18[b]	68	1.88	1.52	81
15	3.92[a]	2.97[b]	76	4.09[a]	3.05[b]	75	0.93	0.85	91
17	4.97[a]	2.84[b]	57	4.49	4.29	96	0.66	0.61	92
18	5.28	5.27	100	4.98	4.61	93	0.97	1.06	109
19	5.81[b]	6.75[a]	116	5.54	5.20	94	0.81	0.88	109

[1] Radial growth at 33°C as a percentage of that at 25°C.
[2] Radial growth (mm day^{-1}). LSD=0.49 for temperatures within a single glyceollin I level for each race. Races with significant differences in growth at the two temperatures are identified with superscripts a and b.
LSD=0.69 (within a column) for all 18 races at a single temperature and glyceollin I level.

Table 9.4

Mean sum of squares for the effect of different levels of temperature and glyceollin I on the radial growth of 18 races of Phytophthora megasperma f.sp. glycinea[a]

Source	df	Mean sum of squares
Replication	1	0.07
Temperature(T)	1	57.60*
Error(a)	1	0.02
Glyceollin I level(L)	2	227.30**
TxL	2	10.73**
Error(b)	4	0.23
Race(R)	17	3.58**
RxT	17	2.19**
RxL	34	1.30**
RxLxT	34	0.55**
Error(c)	102	0.12

* Significant at P=0.05 ** Significant at P=0.01

[a] Radial growth was determined for 18 races of Phytophthora megasperma f.sp. glycinea at two temperatures (25 and 33°C) and three glyceollin I levels (0,10 and 75 µg/ml). Refer to data of Table 9.3.

Table 9.5

The effect of temperature and glyceollin I levels on the radial growth (mm/day) of 18 races of *Phytophthora megasperma* f.sp. *glycinea*.

Temperature (°C)	Glyceollin I level(μg ml^{-1}) 0	10	75	Mean
25	5.29^{A}_{a}	4.66^{B}_{b}	1.27^{E}_{c}	3.74^{a}
33	3.69^{C}_{a}	3.31^{D}_{b}	1.12^{E}_{c}	2.71^{b}
Mean	4.49^{a}	3.99^{b}	1.19^{c}	

Values not accompanied by the same letter in each series of superscripts and subscripts differed significantly at the P=0.05 level as follows:
A,B,C,D,E superscripts for groups among different temperature and glyceollin I level combinations.
a,b,c subscripts for different glyceollin I levels at each temperature.
a,b,c superscripts for either over all effect of temperature or glyceollin I level. Analysis based on data of Table 9.3:

growth rates of races 4,5 and 6 with 75 µg/ml glyceollin I at 33°C were appreciably higher than any of the other races.

9.5 Discussion

Resistance and susceptibility of soybeans to Phytophthora megasperma f.sp. glycinea are mediated by a series of major genes in the host that condition specific interactions with a complementary series of races of the pathogen. By analogy with other examples in which the genetics of the pathogen is understood, it has been assumed that there is a gene-for-gene relationship [e.g. Ellingboe, 1983]. Several authors have postulated that in such relationships host and pathogen genes code for a recognition process probably involving molecules at host pathogen interfaces [Daly, 1984]. On these assumptions the induction of a susceptible phenotype in soybeans by temperature change is equivalent to loosing (or in reverse, gaining) a major gene for resistance and, consequently, a change in the recognition process. Daly [1984] has pointed out, however, that one of the difficulties with recognition models, as generally envisaged in gene-for-gene host-pathogen interactions, is that they are "yes or no" systems, whereas even in well defined interactions of hosts with biotrophic fungi this is seldom the case. In the interaction of soybean cv. Altona with race 4 of Phytophthora megasperma f.sp. glycinea [Ward and Lazarovits, 1982] there is a gradual change from resistance at 25°C through a series of intermediate reaction-types at increasing temperatures to complete susceptibility at 32-33°C. This suggests that if a recognition phenomenon is involved, either it must have a quantitative dimension, which is perhaps difficult to reconcile with a "yes or no" system, or it is overridden by other components of the interaction for which there must be a genetic basis also. It might be suggested that concepts of resistance and suscep-

tibility in soybeans and identity of genes controlling the process would be rather different if the incubation temperature generally used by investigators had been a few °C higher.

Phenylalanine ammonia-lyase activity in soybeans is enhanced in the first few hours following inoculation in the resistant but not in the susceptible response of hypocotyls [(Fig. 9.1) and Chapter 8.4.1.2] and roots [Bonhoff et al., 1986] to Phytophthora megasperma f.sp. glycinea. Phenylalanine ammonia-lyase is an important enzyme in the biosynthesis not only of glyceollin but also of lignins, phenolic compounds in general and melanins, all of which have been associated with resistant responses in various host plants [Ward, 1986]. Activity of PAL, is therefore, a useful indicator of the activation of defense-related responses. Consistent with this, was the demonstration in the present study that the induction of susceptibility in cv. Altona to Phytophthora megasperma f.sp. glycinea race 4 at 33°C was associated with a suppression of PAL activity from that in the resistance response at 25°C to a level initially similar to that in the susceptible response to race 6 (Fig. 9.1). At later stages (12 and 22 h) PAL activity continued to increase in both the susceptible responses to race 4 at 33°C and to race 6 at 25°C. These later increases evidently do not correlate with the development of resistance (or with the accumulation of glyceollin [Ward and Lazarovits, 1982]).

The suppresssion of PAL activity at 33°C appears to be primarily a property of the host and not of the host-pathogen interaction, since activity in response to $AgNO_3$ also was much lower at 33°C than at 25°C in several cultivars examined (Tables 9.1 and 9.2). There was no comparable decrease in the availability of reducing sugars that serve as sources of energy as well as of precursors for

biosynthetic pathways including that of the phenylpropanoids. Evidence that changes in PAL activity and glyceollin accumulation may be related directly to temperature-induced susceptibility was provided also by analysis of $AgNO_3$-treated hypocotyls. In two cultivars for which temperature-induced susceptibility has been reported [Ward and Buzzell, 1983], increasing the incubation temperature from 25 to 33°C caused a much greater decrease in PAL activity and a much smaller increase in glyceollin accumulation than in two cultivars in which resistance is not affected by temperature.

There were appreciable differences in growth rate among the 18 races of Phytophthora megasperma f.sp. glycinea at 25°C and even wider differences at 33°C. The races differed also in their sensitivity to glyceollin I and in the extent to which this was influenced by incubation temperature. The data provide further evidence of the wide range of variability that appears to be characteristic of species of the genus Phytophthora [Erwin, 1983]. They are based on cultures maintained by mass mycelial transfer that may provide a better picture of the overall variability within a race than random, and possibly atypical, single zoospore isolates [see Chapter 6]. The data emphasize that there are major differences between the races in addition to the genes for avirulence that they are presumed to carry. In comparisions of host interactions with race differing in virulence it may not be wise to assume that all differences between interactions are related only to the presence or absence of avirulence genes in the pathogen. It is possible that relative genetic differences between races are greater than between near-isogenic lines of the host. The data may serve also to supplement information about races obtained by cultivar reactions. Thus, races 6 and 7 are sometimes difficult to distinguish by their reactions on the differential

cultivars [Moots et al., 1983], however, they differ distinctly in sensitivity to temperature and to glyceollin I.

Without comparing rates of tissue colonization in universally susceptible cultivars it cannot be assumed that in vitro growth rates bear a direct relationship to those in infected plants. Nevertheless, the degree of suppression of growth at 33°C probably provides a measure of temperature-sensitivity that may apply to behaviour in the plant. On this basis, temperature-induced susceptibility would be more probable with some races than with others. Among races 1-6 tested in the previous studies [Ward and Buzzell, 1983; Ward and Lazarovits, 1982] growth of race 2 in vitro was strongly suppressed at 33°C and no breakdown in resistance to this race was reported, growth of race 4 was only slightly suppressed and two cultivars were reported to be susceptible to this race at elevated temperatures. Race 2 was also one of the most sensitive races to glyceollin I especially at 33°C whereas race 4 was one of the least sensitive. There were, however, inconsistencies also. Thus, growth of race 3 at 33°C was only 56% of that at 25°C and yet susceptibility to race 3 was induced at elevated temperatures in two cultivars [Ward and Buzzell, 1983]. However, this may have been offset, at least in one of the cultivars (PRX8-122-1), by greatly reduced PAL activity at 33°C with only marginally increased levels of glyceollin (Table 9.2). Keeling [1985] reported several additional examples of temperature-induced susceptibility including cultivars inoculated with race 2. However, in that study seedlings were inoculated by insertion of mycelium into hypocotyl wounds and it was shown previously that wounding caused breakdown of resistance of several cultivars at elevated temperatures [Ward and Buzzell, 1983].

In the interaction of Phytophthora megasperma f.sp. glycinea race 4 with cv. Altona the combination of the

pathogen's ability to grow well at 33°C and relatively low sensitivity to glyceollin I together with a major suppression of PAL activity and little increase in glyceollin accumulation in the host appear to be consistent with temperature-induced susceptibility. If this interpretation is correct, it implies that glyceollin plays a significant role in resistance. Testing of a wider range of race-cultivar combinations is necessary before any firm conclusions can be reached about the general importance of temperature effects on these host and pathogen characteristics in resistance and susceptibility. Presumably many additional physiological attributes of host and pathogen contribute to the development of a host-pathogen interaction. The results reported here serve to indicate that combinations of physiological variables in host and pathogen rather than, or in addition to, recognition systems involving hypothetical cell-surface components may define reaction-types in some interaction of hosts and pathogens.

CHAPTER 10

GENERAL DISCUSSION

10.1 Discussion

Phytophthora rot of soybeans [Glycine max (L.) Merr.] caused by Phytophthora megasperma Drechs. f.sp. glycinea (Hildeb.) Kuan and Erwin has been reported to develop on all parts of soybean plants [Sinclair, 1982]. Resistance to Phytophthora megasperma f.sp. glycinea is governed by single genes (Rps) that occur in different allelomorphs and also in different loci [Schmitthenner, 1985]. Although the genetics of the pathogen is not known the expression of cultivar specific or host resistance of soybeans to a series of physiological races of the pathogen follows the classical "gene-for-gene" system put forward by Flor [1956]. The expression of Rps genes takes place in almost all soybean organs studied [Hahn et al., 1985; Keeling et al., Keen and Horsch, 1972; 1976; Lockwood and Cohen, 1978; Morrison and Thorne, 1978; Chapter 3]. Expression of Rps genes, however, is supplemented by age-related or organ-specific resistance in this host-pathogen interaction [Lazarovits et al., 1981; Paxton and Chamberlain, 1969; Stossel et al., 1981; Ward et al., 1981; Chapter 3 and 4]. As a result, Rps genes play an exclusive role in the expression of resistance only for short periods of time during the development of soybean organs. In older soybean organs age-related resistance duplicates the effect of Rps genes. From light and electron microscope studies, Stossel and co-workers [1980, 1981] concluded that at the cellular level the expression of age-related resistance of soybeans to a virulent race of Phytophthora megasperma f.sp. glycinea is basically similar to that of a cultivar-specific resistant reaction to an avirulent race. The effect of the Rps gene is duplicated

also in susceptible soybean hypocotyls that have been pre-exposed to light before inoculation with a virulent race [Ward and Buzzell, 1983]. In such green hypocotyls, however, if the epidermis and a few cortical cell layers were removed before inoculation with a virulent race, resistance was decreased. When the epidermis and a few cortical cell layers were removed from etiolated hypocotyls of a resistant cultivar, although resistance persisted, there was some spreading from the inoculated wounded sites [Chapter 7].

Ward [1984a] concluded that although the interactions between sbybeans and Phytophthora megasperma f.sp. glycinea are classified into genetically controlled classes viz. compatible and incompatible interactions, at the cellular level the differences are much less clearly defined. Together with the observations from physiological studies he concluded that resistance and susceptibilility are not two absolute alternatives but are the extreme expressions of quantitative differences. The expression of both resistant and susceptible responses on the same trifoliate of 28 day-old-plants of the susceptible cv. Harosoy (Fig. 4.2) and 12 day-old-plants of the resistant cv. Harosoy 63 [Chapter 4] also indicates that a delicate balance exists between resistance and susceptibility. Decline in phenylalanine ammonia-lyase activities in hypocotyls of resistant cultivars and increase in sensitivities of avirulent races of Phytophthora megasperma f.sp. glycinea to glyceollin I following temperature increases from 25 to 33°C were correlated with temperature-induced susceptibility [Chapter 9]. This indicates that a delicate balance between resistance and susceptibility exists in etiolated hypocotyls. Therefore, it is possible that, although the expression of cultivar- or race-specific resistance leads to two phenotypic classes viz. resistant and susceptible, at the biochemical or molecular level the presumed recognition between gene products of the Rps genes

of soybean cvs. and the products of genes for virulence or avirulence of Phytophthora megasperma f.sp. glycinea races may take place in a quantitative fashion. Presumably each Rps gene codes for a different receptor with specific structural sites that recognize the products of genes for virulence or avirulence.

Ellingboe [1976, 1981] considered that specificity in a "gene-for-gene" system [Flor; 1956] resides in the incompatibility that is conditioned by the interaction between genes for resistance (R) and avirulence (A). All other allelic combinations viz. R/a, r/A and r/a result in compatibility. If, as discussed, age-related and cultivar-specific resistance of soybeans are basically similar at ultrastructural, physiological and biochemical levels [Stossel et al., 1980, 1981; Ward et al., 1981], then according to Ellingboe's arguments, for a susceptible cultivar to express age-related resistance it must acquire in the course of ageing or maturation the equivalent of an Rps gene, complementary to an avirulence gene already present in the race in question.

Vanderplank [1978, 1982, 1984], on the other hand, argued that specificity resides in the interaction between the host alleles for resistance (R) and pathogen alleles for virulence (a). Compatibility is the outcome of all the combinations where there are no unmatched 'a' alleles, while, incompatibility results in all cases where this is not the case. According to this hypothesis, the R/a combination will result in the most compatible interaction, while r/A, r/a or R/A are progressively less compatible interactions. This hypothesis is generally consistent also with obsevations with soybeans of age-related resistance and of enhanced resistance in light-exposed hypocotyls. Thus, the Rps gene products may be assumed to be receptors, for example on the host plasmamembrane, that trigger plant defence responses

(mechanisms will be discussed later). It the number of receptors per unit area of plasmamembrane increases with cell-age of maturity and if, as proposed by Vanderplank, compatibility results from the interaction of R gene products with those of virulence genes, a, then in mature cells there may be many more receptors than a gene products. Such surplus receptors would be free to activate the plant defence genes that code for enzymes of pathways involved in synthesis of defence-related products. Conversly, in immature trifoliates of the resistant cultivar, Harosoy 63, there may be too few receptors to tip the reaction towards resistance. This may be the reason that in trifoliates treated with the abiotic elicitor, $AgNO_3$, the amount of glyceollin in trifoliates of 12-day-old plants was only one fifth of that in trifoliates of 14-day-old plants [Chapter 4]. A similar line of evidence comes from the work of Ward et al., [1981]. They also observed that much more glyceollin accumulated in older than in younger parts of etiolated hypocotyls following biotic elicitor treatment. Possibly this is a reflection of a greater number of receptors in the older tissue.

Crute and Norwood [1986] put forward another hypothesis basically similar to that of Ellingboe [1976, 1981] and concluded that Vanderplank's interpretation of the "gene-for-gene" hypothesis does not explain the data they obtained for inheritance in the lettuce-Bremia lactucae interaction. They observed gene dosage effects or incomplete dominance especially for the genes for resistance of lettuce to the pathogen. According to Crute and Norwood [1986] the interaction takes place between alleles for susceptibility (r) and avirulence (A). In the abscence of 'r', allele 'A' provokes resistance. This model, however, does not help to explain the basis of age-related resistance.

Circumstantial evidence for receptors in the plasma-

membrane that mediate compatibility or incompatibility was provided by the demonstration of incompatibility of 'pea pod endocarp' tissues to cells of diverse plant and animal origin [Teasdale et al., 1974]. Such incompatibility between species of the same or two different kingdoms disappeared when protoplasts were fused, that is when they were not separated by their plasmamembranes [Bushnell, 1985]. However, there were also exceptions to this view and Bushnell [1985] has argued that plants do not have a universal ability to recognise 'non-self' but are stimulated by products of other organisms. However, from the accumulated evidence for stimulation of plant-defence-genes by wounding, biotic or abiotic elicitors, and infection [Bailey, 1982; Dixon, 1986; Ward, 1986; Yoshikawa, 1983; Yoshikawa et al.,1979; Chapter 3, 7, 8, 9], it may be proposed that plants recognise not virulent or avirulent races but an array of stimuli that may all cause varying degree of stress. Hargreaves and Bailey [1978] and later Hahn et al., [1981] demonstrated the presence of water-soluble elicitors in extracts of plant tissues. These were first termed constitutive elicitors but now are generally referred to as endogenous elicitors. Thus, following infection, or treatment with biotic or abiotic elicitors or simply wounding or exposing the plant tissues to environmental stress e.g. freezing, UV-light, endogenous elicitors are released from plant tissues, most probably from cell walls [Dixon, 1986]. As such endogenous elicitors trigger plant defence responses, it is proposed here that they do so by interacting with the receptors coded for by genes for resistance (R genes).

Possibly, the release of endogenous elicitors is basically a general response to wounding. In other words, all treatments with biotic or abiotic agents or infection with microorganisms may cause wounds to varying degrees

thereby releasing endogenous elicitors and activating plant-defence-genes [Ward, 1986]. Recent evidence to support this possibility has been provided by Liang et al. [1987]. They reported that in beans there are 4 genes that code for phenylalanine ammonia-lyase (PAL), the first enzyme of the phenylpropanoid pathway that leads to many products of intermediary metabolism in addition to defence related compounds e.g. phytoalexins, lignins etc. Significantly, of these four genes, one coded for PAL both following infection and wounding.

Differences in the extent of activation of plant-defence-genes following mechanical wounding, elicitor treatments or infection should be related to the degree and duration of wounding or irritation caused by these different agents. Dixon et al. [1981] demonstrated that continued stimulation is essential for phytoalexin accumulation in cell-suspension cultures. Due to their phytotoxic nature abiotic and also probably biotic, elicitors cause wounds that are locally more extensive than mechanical wounds [Ward, 1986] and, therefore, higher concentrations of phytoalexins accumulate. In microbial infection, while the initial contact may create a small wound, the irritation caused by infection is persistent. As the pathogen attempts to spread the stimulation of the host is likely to continue until the pathogen is sealed off (resistant) or the host can no longer successfully respond (susceptible). Rapid glyceollin I metabolism with a very short half life [Chapter 7] in both infected tissues and tissues wounded only indicates that phytoalexin accumulation is dependent upon the rate of biosynthesis, and this in turn will depend on the amount of irritation or wounding produced. The time courses of accumulation of glyceollin I or III [Chapter 3] or PAL activity [Chapter 9], especially in the resistant response is, therefore, also the time course of irritation caused by the

fungus. When growth of the fungus in the lesion decreases, the amount of irritation is reduced and so is the rate of biosynthesis [Chapter 7] and accumulation [Chapter 3] of glyceollin I or PAL activity [Chapter 9]. Because of the rapid metabolism of glyceollin I, the accumulation is finely controlled by the plant. This presumably prevents harmful effects on the plant, for glyceollin I is phytotoxic and an inhibitor of electron transport in soybean mitochondria [Boydston et al., 1983]. Therefore, possibly the levels of glyceollin I correlate with the amounts of irritation or endogenous elicitor released.

Recognition of endogenous elicitors by receptors coded for by R genes, while consistent with age-related and quantitative resistance effects, does not provide the specificity required by "gene-for-gene" host-pathogen interactions. However, it may be proposed that each of the receptors coded by R genes has one general site for recognition of endogenous elicitors and a specific site that is recognized by the gene products of a corresponding gene for virulence. Possibly both sites overlap or, alternatively, binding to the specific site of the receptor by the product of a gene for virulence results in changes in the configuration of the general site so that the endogenous elicitor subsequently cannot bind.

The role of defence-related compounds elaborated through the phenylpropanoid pathway from the common precursor phenylalanine in disease resistance is evident from the work presented in chapter 9. Elevated temperature causes a decrease in PAL-activity in temperature-sensitive cultivars. On the other hand reduced sensitivity of some Phytophthora megasperma f.sp. glycinea races to glyceollin I correlates with temperature-induced susceptibility. Therefore, a critical balance, for expression of reaction type, occurs between the amounts of defence-related substances synthesized and

sensitivity of the pathogens to those substances. Tegtmeier and VanEtten [1982] in genetical studies also observed that ascospore progeny of Nectria haematococca, which were not able to demethylate pisatin, were low in virulence and sensitive to pisatin; whereas, all isolates that were highly or moderately virulent on pea could demethylate pisatin and were tolerant of pisatin. There is also much circumstantial evidence for the association of phytoalexins with disease resistance [e.g. Bailey et al., 1980; Bailey, 1982; Hahn et al., 1985; Mayama et al., 1982; Yoshikawa et al., 1978].

Ward and Lazarovits [1982] observed that when growth of a virulent race was stopped at high temperature, a high concentration of glyceollin accumulated, comparable to that in a resistant response. However, when the infected plants were returned subsequently to a normal temperature, compatibility was restored. This may not have been due to uneven concentrations of glyceollin at the cellular level and the escape of a few hyphae from regions with low glyceollin concentrations, as suggested by Keen [1986]. In the light of evidence in chapter 7 a more probable explanation is that it is due to rapid metabolism of the glyceollin and removal of the stimulus for glyceollin biosynthesis. At the higher temperature, growth of the fungus is inhibited; therefore, the gene product from the gene for virulence may not be synthesized and endogenous elicitors would have access to receptor sites, turn on plant-defence-genes and the development of a resistant response would follow. After returning to the lower temperature the virulence gene would become active again and its product would compete for receptor sites with the endogenous elicitors. Rapid metabolism by the host would remove glyceollin and the virulence gene product would eliminate further stimulation of biosynthesis. A separate study using the systemic fungicide, metalaxyl [N-(2, 6-dimethylphenyl) -N-(methyl-acetyl) alanine methyl

ester] an inhibitor of RNA and protein synthesis in Phytophthora spp., by Ward [1984b] indicated that RNA and protein synthesis by Phytophthora megasperma f.sp. glycinea is essential for compatibility or, in terms of the proposal made here, suppression of resistance mechanisms. It was shown also that a virulent race could survive and spread from lesions produced from predominantly avirulent inoculum [Ward, 1983]. These studies are consistent with the view that virulence gene products play a role in inhibiting the expression of plant-defence-genes. They are presumably actively released, whereas in plants the receptors are presumably constitutive. Possibly, virulence gene products are rapidly produced and liberated into intercellular spaces of the host so that they can pre-occupy receptor sites in advance of the release of endogenous elicitors. Evidence that fungal components may spread into host tissues surrounding invading hyphae has been provided by the detection of antigens from fungal fimbriae by immuno-electron microscopy [Svircev et al., 1986].

The proposed model for the expression of compatible or incompatible interactions is apparently similar to the "non-specific elicitor and specific suppressor" model discussed recently by Crute et al., [1985] and is consistent with the view expressed by Ward and Stoessl [1976] that specificity should be associated with compatibility. It is also in part consistent with models proposed individually by Bushnell and Rowell [1981] and Heath [1981]. However, the proposed model attempts to accommodate quantitative, age-related and non-host aspects of resistance with cultivar-specific resistance that follows the gene-for-gene system of Flor [1956]. Further, in the proposed model the non-specific elicitor is of endogenous origin, arising from the plant itself and not from the invading microorganism as suggested by others. It, therefore, covers a wide range of microorganisms

and of other stresses that cause the accumulation of defence-related compounds. The "non-specific elicitor and specific suppressor" model arose from studies by Doke et al. [1979, 1980]; Doke and Tomiyama [1980]; Garas et al. [1979]; Ziegler and Pontzen [1982]. In this and also in the model proposed here, specificity is accounted for by the interaction of the gene products of genes for resistance (R) and genes for virulence (a). In the proposal put forward by Vanderplank [1978, 1982] the R/a combination was considered to be the most compatible because compatibility is due to the specific interaction between products of genes for resistance (R) and virulence (a). Other combinations were considered less compatible, however, there was no evidence for this in a recent study by Crute and Norwood [1986]. In the present proposal compatibility is observed in all but the R/A combination and should be similar in each combination for the following reasons: 1) in the R/a combination the virulence gene product occupies the receptors coded by R, endogenous elicitors are not recognized and plant defence genes are not turned on, 2) in the r/a and r/A combinations there are no receptors and, therefore, similarly there is no activation of plant defence genes. The R/A combination is incompatible, because the product of gene A fails to preoccupy the receptors coded by the R gene and hence endogenous elicitors turn on the plant-defence-genes. Evidence that the gene product of R is essential for a resistant response comes from the work of Loegering and Sears [1981]. They observed that deletion of a chromosomal arm carrying a dominant resistance gene resulted in a susceptible response. On the other hand Flor [1960] observed that when avirulence genes were removed from homozygotes, presumably through deletion mutation, a compatible interaction instead of an incompatible interaction developed. This apparently indicates that there is no requirement for a product of the gene

for virulence to establish compatibility in the presence of an R gene. According to this the model proposed here is not correct. However, Flor's [1960] study may also indicate that genes for virulence and avirulence are regulatory instead of structural genes. The avirulence gene product would then be a repressor protein for the structural gene in the pathogen that codes for the product specific for the receptor coded by the complementary R gene in the host. When the avirulence gene was removed by deletion in Flor's study the necessary structural gene was derepressed and susceptibility was the result [Fig. 10.1].

A similar phenomenon may have occurred in the study by Staskawicz et al. [1984] who cloned an avirulence gene from race 6 of Pseudomonas syringae pv. glycinea. When the gene was mobilized into the virulent race 5 through transconjugation, race 5 became avirulent to an otherwise susceptible cultivar of soybean. The merodiploid (race 5 + avirulence gene from race 6) thus carried both virulence and avirulence genes. The introduced avirulence gene may have suppressed the expression of the structural gene for virulence the product of which possessed specificity (suppressor) for the receptors coded by the corresponding R gene in the host [Fig. 10.1]. Tepper and Anderson [1984] also emphasized that "major genetic changes conditioning specificity may be regulatory loci rather than in structural genes". They discussed the importance of mutation of regulatory rather than structural loci for evolution at the organismal level.

In the foregoing discussion a model has been proposed to accomodate the biochemical data from cultivar-specific resistance with the requirements of the classical "gene-for-gene" hypothesis, considering only one pair of loci in the host and pathogen. However, in nature the final outcome of an interaction is likely to be the result of the interplay

Fig. 10.1
Hypothetical model for the control mechanisms for expression of a gene for virulence in the pathogen.

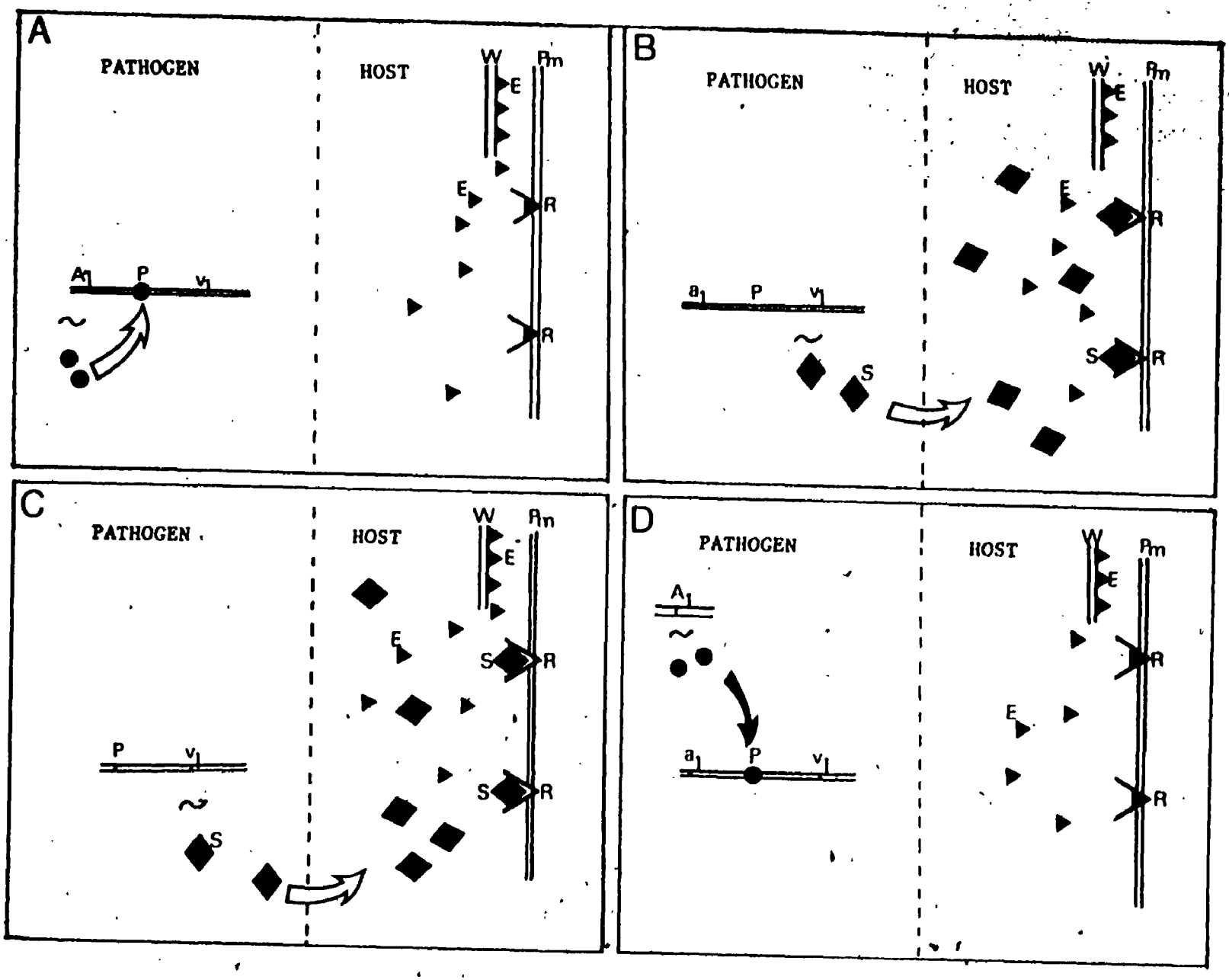
A_1 : gene for avirulence in Flor's hypothesis
v_1 : structural gene, the products [S] of which specifically bind the receptors [R] in the host plasmamembrane coded by R genes for resistance
P : promoter region for gene v_1
E : Endogenous elicitor
Pm : Plasmamembrane
W : Cell Wall

A] Normal incompatible interaction: 'A_1' suppresses the expression of 'v_1', endogenous elicitors switch on the plant-defence-genes.

B] Normal compatible interaction: 'v_1' expresses constitutively and suppressor 'S' inhibits the expression of the plant-defence-genes.

C] Compatible interaction as observed by Flor [1960] due to deletion of A_1 gene that resulted in constitutive expression of v_1 gene.

D] In merodiploid obtained by Staskawicz et al. [1984], gene products from A_1 gene suppressed the expression of v_1 and, hence, incompatibility resulted.


A
PATHOGEN
HOST
W
Pm
E
E
R
R
P
B
PATHOGEN
HOST
W
Pm
E
E
R
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R
P
C
PATHOGEN
HOST
W
Pm
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R
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PATHOGEN
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of more than one locus or at least more than one allele in the host and many loci in the pathogen. According to the 'gene-for-gene' hypothesis, an interaction is incompatible if in both the host and pathogen a corresponding pair of dominant resistance and avirulence genes are present at least in one locus. However, it does not mean that other loci are not playing any role. Take for example, an interaction between a host with the genotype R_1 R_2 r_3 R_4 and a pathogen with the genotype a_1 a_2 A_3 A_4 (race 1; Fig. 10.1). The subscripts indicate corresponding loci or gene pairs. The interaction of this combination will result in incompatibility according to Flor's 'gene-for-gene' system (because of R_4/A_4) and also according to the model proposed here (receptor of R_4 will be bound by the endogenous elicitors). At locus 1 (R_1/a_1) and at locus 2 (R_2/a_2) the outcome is compatibility, because gene products from a_1 and a_2 will pre-occupy receptors coded by R_1 and R_2 respectively. At the locus 3, r_3 does not code for a receptor, therefore, endogenous elicitors cannot elicit the plant-defence-genes and there is no interaction. Since receptors coded by R_4 cannot be blocked by the pathogen, endogenous elicitors are recognized by these receptors and they trigger a resistant reaction, although in the loci 1 and 2 interactions are for compatibility. The interactions among alleles can be outlined as in Fig. 10.2.

According to the proposed model a universally susceptible cultivar should not develop age-related resistance because it will not have any genes coding for receptors. The cv. Wayne is considered to be a universal susceptible cultivar of soybean to Phytophthora megasperma f.sp. glycinea. However, etiolated hypocotyls of this cultivar expressed age-related resistance when inoculated with a virulent race [Bhattacharyya and Ward, unpublished]. Therefore, it must have at least an unidentified Rps gene for resistance which

Fig. 10.2
Types of interaction at different comlementary loci of a host with two pathogen races, and the final phenotype of such interactions at four complementary loci in both host and pathogen on the basis of the proposed model.

is responsible for the expression of age-related resistance. Furthermore, Rutherford et al. [1985] presented evidence for switching on of plant-defence-genes in this cultivar in response to a single zoospore isolate obtained from a virulent race, and an unidentified Rps gene must be inferred. A similar observation was recorded for this cultivar following inoculation with single zoospore isolates obtained from Isolate I.1 [Chapter 6]. In Vanderplank's scheme [1978, 1982] also a "universally susceptible" host genotype does not exist. According to the model proposed here cultivars of soybeans will differ in numbers of

	Host (R_1R_1 R_2R_2 r_3r_3 R_4R_4)				Phenotype
	Locus	R_1R_1	R_2R_2	r_3r_3	R_4R_4	
Pathogen (race$_1$) ($a_1a_1a_2a_2A_3A_3A_4A_4$)	a_1a_1	C				Incompatibile interaction
	a_2a_2		C			
	A_3A_3			-		
	A_4A_4				I	
Pathogen (race$_2$) ($a_1a_1a_2a_2A_3A_3a_4a_4$)	a_1a_1	C				Compatible interaction
	a_2a_2		C			
	A_3A_3			-		
	a_4a_4				C	

C = Compatible interaction
I = Incompatible interaction
- = no interaction but leads to compatible interaction due to absence of receptors coded by R_3 gene

receptors coded by Rps genes they carry. Those with many Rps genes should have more receptors than those with fewer Rps genes. This may possibly give rise to differences in the degree of compatibility among the cultivars to a virulent race. For example, in the present study, cultivar Harosoy although susceptible to Phytophthora megasperma f.sp. glycinea race 1 (formerly regarded as universally susceptible) carries an unidentified Rps gene(s) conferring resistance to races 12, 16, 18 and 19 [Keeling, 1982] while none have been reported for cultivar Wayne. This may be the reason that cv. Harosoy was less susceptible than cv. Wayne to a number of single zoospore isolates obtained from isolate 1.1. [Chapter 6]. Such residual effects of genes for resistance have been described by Martin and Ellingboe [1976]. However, more detailed studies are required to confirm this possibility. If true, it may explain quantitative differences in the balance between compatible and incompatible interactions.

Evidence that cultivar- or race- specific host genes may exist but have not been reported due to unavailability of appropriate avirulent races is provided by the work of Staskawicz cited by Day [1986]. He observed that when cloned fragments of DNA from the tomato bacterial pathogen Pseudomonas syringae pv. tomato were introduced into P. syringae pv. glycinea, some transformants showed some new cultivar-specific avirulence in soybeans. This report has at least two important implications; e.g. i) In addition to reported genes for resistance there may be many more genes for resistance that are nullified by the corresponding virulence genes of different races of a pathogen and hence are hidden. In other words, "universal susceptibility" may be a concept that is impossible to prove. ii) P. syringae pvs. tomato and glycinea, presumably, descended from a common ancestor. The basis of their classification into two pathovars is that of

host range. Presumably, in the course of evolution they gained virulence genes or lost avirulence genes that were complementary to the resistance genes of either tomato or soybeans. Loss of avirulence genes may be the more probable alternative [in support of Flor [1960] also]. In that case, Staskawicz may have restored an avirulence gene from pv. tomato to pv. glycinea that the latter had lost during the course of its evolution. The virulence gene of pv. glycinea in the transformant evidently failed to nullify the receptors coded by the corresponding hidden resistance genes in the host. Similar results can be expected from transformation of pv. tomato by the cloned DNA fragments from pv. glycinea. In this context, a pathogen can be defined as the one which lost all the avirulence genes complementary to all resistance genes in the host in due course of evolution. Also it can be concluded that the same gene for resistance plays a role not only against races of a pathogen but also against all non-pathogens that can penetrate the first line of (non-specific) defence e.g., cuticle [Kolattukudy and Koller, 1983]. If this assumption is correct then resistance of plants was the primary situation and the development of compatibility with the pathogen was secondary. Heath [1985] also discussed the possibility of achieving basic compatibility by a successful parasite on some members of a plant species as an alternative to the generally accepted possibility of superimposing cultivar-specific resistance on already established basic compatibility. She termed the non-host resistance that is overcome by the successful parasite "residual resistance". She speculated that such residual non-host resistance could be the reason for cultivar-specific resistance as long as resistance involves active plant responses, controlled by a single gene.

10.2 Conclusions

1) Although the expression of cultivar-specific resistance in soybeans leads to two phenotypic classes viz. resistant and susceptible, in most of the cases following infection with Phytophthora megasperma f.sp. glycinea races, at the biochemical or molecular level recognition between the gene products of the Rps genes of soybean cultivars and the gene products of the genes for virulence or avirulence of the pathogen races takes place in a quantitative fashion.

2) 'Recognition' is a continuous event and it takes place in freshly invaded tissues to switch on the plant-defence-genes in this host-pathogen interaction. Rapid metabolism of glyceollin I indicates that the level of accumulation depends upon the rate of biosynthesis and biosynthesis in turn depends upon the switching on of plant-defence-genes through the 'recognition' process.

3) It is hypothesized that active responses of plants to any biotic or abiotic stimuli take place through receptors coded by the Rps genes of soybeans. Endogenous elicitors or active components, released from plant cell walls following wounding caused by such stimuli, are recognized by the receptors. Gene products of the virulence genes can nullify the receptors so that the recognition does not take place. Genes for avirulence or virulence may have regulatory functions for each of the operons coding gene products specific to the receptors of complementary Rps genes.

10.3 Proposals for future work

Rapid metabolism of glyceollin I observed in infected and wounded tissues [Chapter 7] indicates that it would be profitable to look for enzymes that are involved in the metabolism of glyceollin I. Identification of such an enzyme(s), and its purification, and production of antibodies

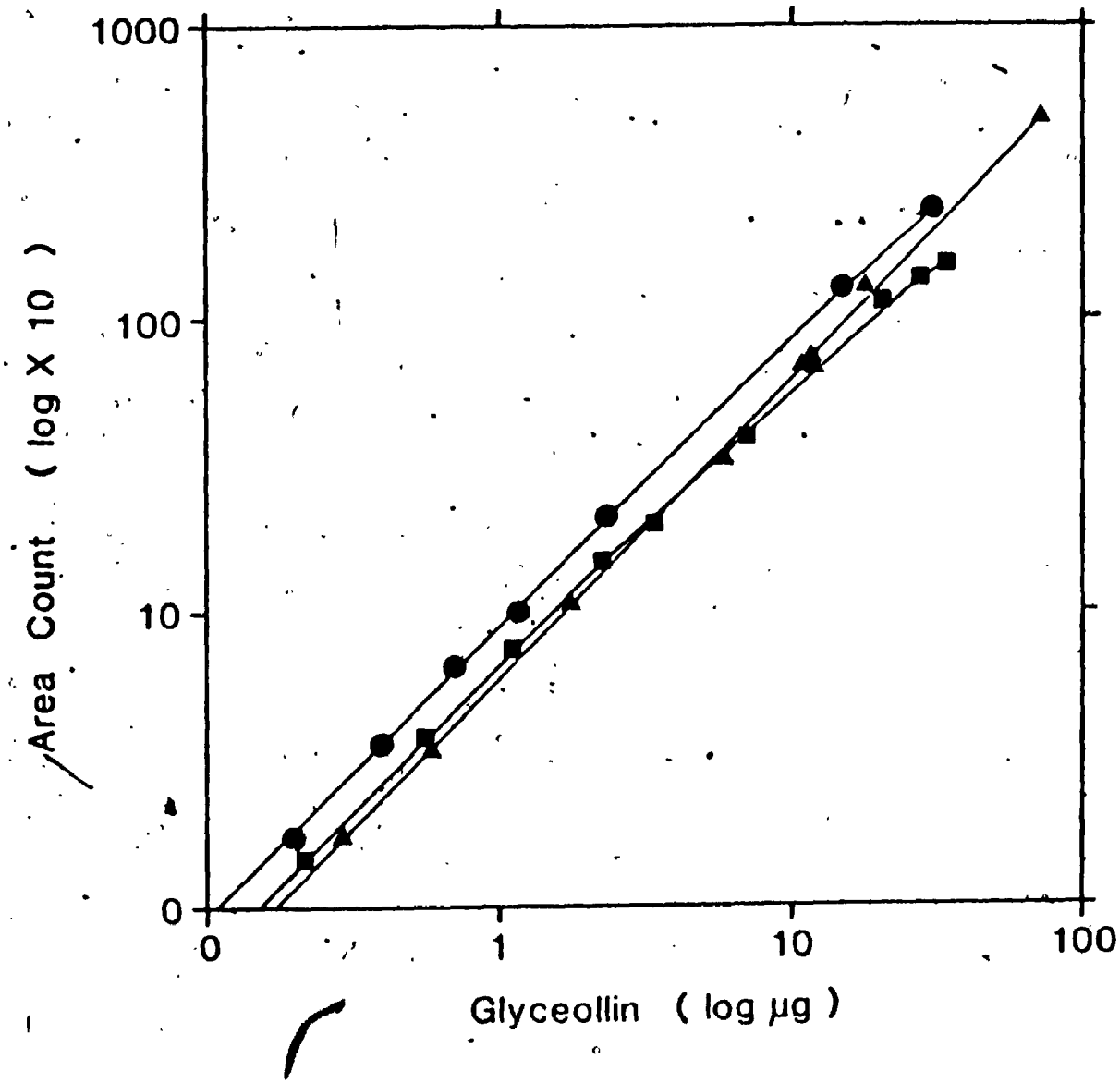
against it will enable us in the near future to localize the cellular-sites for glyceollin I metabolism by immuno-electron microscopy. Site of synthesis could be identified similarly , if the enzyme that elaborates glyceollin I from glycinol or glyceollidin I can be identified, on the same preparation for sites of metabolism following double immuno-cytochemical labeling technique [Bendayan, 1982]. Such studies will possibly reveal invaluable information regarding the role of glyceollin I in soybean-Phytophthora megasperma f.sp. glycinea interaction in addition to the mechanisms of its accumulation.

Studies can be carried out also to understand the molecular mechanisms of generation of variability in successive single-zoospore generations of Phytophthora megasperma f.sp. glycinea race 1 or Isolate 1.1 [Chapter 6]. In such studies isolation and characterization of the mitochondrial genome from two extreme types among the single-zoospore isolates may be useful.

If the susceptible response of trifoliates of 12 day-old plants of the resistant cv. Harosoy 63 to Phytophthora megasperma f.sp. glycinea race 1 [Chapter 4 and 8] is considereed to be due to non-expression of its Rps_1 gene, a molecular biological study can be undertaken to isolate the Rps_1 gene as follows: 1) A cDNA library constructed for trifoliates of 14 day-old plants of cv. Harosoy 63 (gene expressed) can be screened by hybridizing it with the mRNAs isolated from trifoliates of 12 day-old plants of the same cultivar. 2) The cDNAs that were not hybridized by mRNAs could be mobilized into a suseptible cv. Harosoy (rps_1) using a suitable vector and a methology e.g., electroporation [Fromm et al., 1985]. 3) Transformants can be screened using a drug resistant marker and then evaluated for the expression of Rps_1 gene.

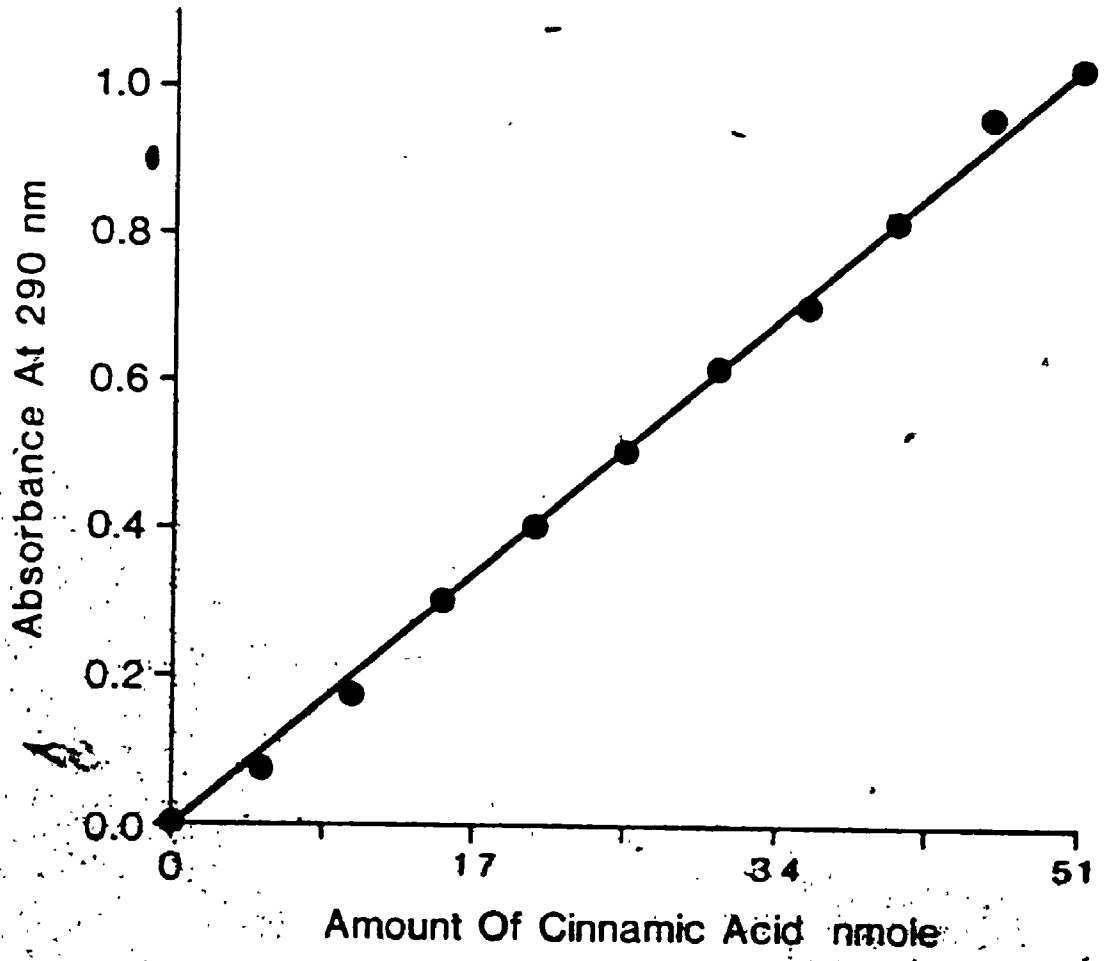
Appendix I

Standard graphs for the quantification of glyceollin isomers following HPLC analysis. Glyceollin I (○), glyceollin II (□) and glyceollin III (△). The graphs were fitted by injecting known concentrations of each of the isomers and monitoring the absorbances at 285 nm.


1000
100
10
0
Area Count (log X 10)
0
1
10
100
Glyceollin (log μg)

Appendix II.

Standard graphs for the determination of trans-cinnamic acid produced (n moles) as a measure for phenylalanine ammonia-lyase activity [see Chapters 7, 8 and 9]. The graph was fitted by measuring absorbances of known amounts of cinnamic acid at 290 nm.


Absorbance At 290 nm
1.0
0.8
0.6
0.4
0.2
0.0
0
17
34
51
Amount Of Cinnamic Acid nmole

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