



## Induced distal defence potentiation against *Phytophthora sojae* in soybean

D-S. PARK\*, S. LANDINI, M. Y. GRAHAM and T. L. GRAHAM†

Department of Plant Pathology, The Ohio State University, Columbus, OH 43210, U.S.A.

(Accepted for publication 4 April 2002)

Soybean phenylpropanoid defence responses to the wall glucan elicitor (WGE) from *Phytophthora sojae* include the accumulation of phenolic polymers and glyceollin in cells immediately proximal to the point of treatment and the accumulation of conjugates of the isoflavones, daidzein and genistein, in distal cells. Since daidzein is a glyceollin precursor and genistein is both toxic to *P. sojae* and implicated in local potentiation of competency for the glyceollin response, it has been hypothesized that the distal cell response might raise the defence potential of cells distant from the infection court. In this paper, it is demonstrated that the WGE is indeed highly effective in protecting cells distal to the point of treatment from infection by *P. sojae*. Mycolaminaran, jasmonic acid (JA), methyl jasmonate and the ethylene precursor, 1-amino-cyclopropane carboxylic acid (ACC), are also effective, while salicylic acid (SA) is not. Methyl jasmonate, WGE and mycolaminaran are most effective, resulting in nearly complete protection against the pathogen even in the universally susceptible line, Williams. Dose response data revealed two distinct types of protection shared by all treatments. The first contains the pathogen to the point of inoculation, while the second is lesion limiting. Lesion limiting protection correlates very well with pre-infectional levels of genistein conjugates and accumulation of a specific peroxidase isozyme, P 2-4, and with post-infectional accumulation of glyceollin, suggesting that this type of protection may be the result of a distal potentiation of glyceollin elicitation competency. The mechanism underlying local containment protection is unknown, but it does not correlate well with any of the established phenylpropanoid responses.

© 2002 Elsevier Science Ltd. All rights reserved.

**Keywords:** Soybean; Glycine max; wound signals; *P. sojae* wall glucan elicitor; mycolaminaran; jasmonic acid; ethylene; salicylic acid.

### INTRODUCTION

The chemical or genetic enhancement of induced disease resistance in plants is a very attractive strategy for disease control. Several forms of systemically induced disease resistance, commonly referred to as systemic acquired resistance (SAR), [24, 28, 36, 38] and induced systemic resistance (ISR), [45] are particularly attractive and have been the subject of many investigations. The signaling and signal transduction mechanisms involved in both SAR and ISR have been investigated extensively. Salicylic acid (SA) appears to be a central signaling component in SAR [8, 29] and jasmonic acid (JA) and ethylene appear to be

important to ISR [10, 45]. In addition, chemical mimics or agonists of SA have been developed and even employed commercially [23, 27, 43]. SAR and ISR appear to involve, at least in part, the systemic induction of defence-related proteins including several classes of pathogenesis-related (PR) proteins [39, 44], although the specific proteins induced in these two responses differ. Finally, recent work in parsley and cucumber suggests that some of the resistance responses induced by SA, JA and their mimics may involve, in part, the potentiation of local resistance responses [25, 26, 37, 41; reviewed in Refs. 20, 21].

However, the development of effective systemic resistance in some plants has been somewhat more difficult. In legumes, although systemic induced resistance has been demonstrated in *Phaseolus vulgaris*, including induction by the SA mimic dichloroisonicotinic acid, its potential activation in other legumes has not been as convincingly demonstrated [for a review, see Ref. 9]. In soybean (*Glycine max* (L.) Merrill), several short reports or preliminary studies have described the induction of localized disease resistance to *Phytophthora sojae* (Kauf. and Gerde.). The resistance-inducing agents have included non-host or

\* Current address: Department of Life Science, Center for Plant Intracellular Trafficking, Pohang University of Science and Technology, Pohang, 790-784, Korea

† To whom all correspondence should be addressed. E-mail: [graham.1@osu.edu](mailto:graham.1@osu.edu)

Abbreviations used in the text: ACC, 1-amino cyclopropane carboxylic acid; HR, hypersensitive response; ISR, induced systemic resistance; JA, jasmonic acid; JAME, jasmonic acid methyl ester; MGG; malonyl glucosyl genistein; PR, pathogenesis related; SA, salicylic acid; SAR, systemic acquired resistance; WGE, wall glucan elicitor

incompatible Phytophthora strains [32, 40, 46], cell wall and other glucan elicitors [2, 5] and ethylene pretreatment [49]. The induction of short-range or weakly systemic resistance in soybean has also been reported. For instance, injection of soybean cotyledons with conidial suspensions of *Colletotrichum truncatum* or *C. lagenarium* or heat-killed conidial suspensions of *C. lagenarium* provided resistance against subsequent inoculation of epicotyls with *C. truncatum* [47]. Truly systemic induced resistance in soybean has been more elusive. For instance, although early reports from industry suggested that a systemic "long term protection response" could be induced in soybeans by the cell wall glucan elicitor from *P. sojae* (WGE) and the ethylene precursor, 1-amino-cyclopropane carboxylic acid (ACC), protection was only partial and was not effective in the field [6, T. L. Graham, unpublished]. Also, despite many investigations with SA in the authors' and other labs, protective effects or induced resistance responses to SA in soybean have not yet been uncovered. While it is possible that certain plants, such as soybeans, put more emphasis on local responses and/or on secondary product responses, it is also possible that the critical signals and conditions for effective systemic responses and/or the induction of PR proteins simply have not been identified.

At the biochemical level, multiple phenylpropanoid defence responses [15] appear to be involved in local defence of soybean tissues against incompatible isolates of *P. sojae*. In cells immediately proximal to the hypersensitive response to infection, conjugates of the isoflavones daidzein and genistein are hydrolysed [22]. Genistein is directly antibiotic to *P. sojae* [35], while daidzein is the precursor of the phytoalexin glyceollin [11], which subsequently accumulates in the same proximal cell zone [22]. In addition, phenolic polymers are also deposited [30], possibly providing a barrier against infection. The glucan elicitor from the cell wall of *P. sojae* (WGE) is also capable of inducing the phenolic polymer and glyceollin responses in cells proximal to wounded or HR dying cells [17]. However, in addition, the glucan elicitor induces a large net accumulation of conjugates of genistein and daidzein in cells distal (up to several hundred cells) from the point of elicitor treatment [18]. It was hypothesized that this distal accumulation of isoflavones could raise the defence potential of these cells distant from the infection court. In this paper, this hypothesis has been tested and some of the signal molecules which might be involved in mediating the response have been examined. The authors reasoned that the demonstration of such a protection response and its biochemical characterization might provide valuable insights towards the development of truly systemic responses in soybean.

## MATERIALS AND METHODS

### Chemicals

The intact WGE was prepared from the cell walls of race 1 of *P. sojae* (Kauf. and Gerde.) according to Ayers *et al.* [2] and as described previously [18]. Before use, the unfractionated and insoluble wall glucan preparation was sonicated and then autoclaved for 3 h in deionized double distilled water [2].

Glutathione, coumestrol, SA, ACC, and (–) JA were obtained from Sigma Chemical Company, St. Louis, MO, U.S.A. Jasmonic acid methyl ester (JAME) was obtained from Bedoukian Research Inc. (Danbury, CT, U.S.A.).

### Growth of soybean seedlings and Phytophthora cultures

Soybean seeds [*Glycine max* L. (Merr.) cvs. Williams and Williams 79] were kindly provided by Dr A. F. Schmitthenner (OARDC, Wooster, OH, U.S.A.). Seedlings were grown as described previously [18] with slight modifications. Instead of vermiculite, seedlings were grown in Metromix 360 (Sierra Grace, Milpitas, GA, U.S.A.) at 26°C with 500  $\mu\text{E m}^{-2} \text{s}^{-1}$  of light and a 14-hour photoperiod. The flats were immediately watered very thoroughly for germination. After 3 days, the plants were watered every other day from the top. Plants were not fertilized. A field isolate of *P. sojae* race 1 was also obtained from Dr A. F. Schmitthenner and was maintained on clarified lima bean agar.

The cultivars used in this study included Williams, a universally susceptible line, and Williams 79 and Harosoy, which carry the *Rps1c* and *Rps7* genes, respectively, for race-specific resistance to *P. sojae*. Williams 79 is resistant to *P. sojae* race 1, while Harosoy is susceptible. Williams was chosen as the main cultivar in the study because it is universally susceptible. Thus, the presence of (and potential effects of) race-specific *Rps* genes would not interfere with the expression of protection, which we hypothesized would be a general resistance phenomenon. An analysis of the effects of several *Rps* genes on isoflavone defence responses in soybean is reported elsewhere [1]. Williams was also chosen because it is an inherently incompetent line for glyceollin production. That is, WGE does not induce glyceollin in Williams unless elicitation competency has been pre-induced either by application of competency factors or by close proximity of the treated cells to wounded or hypersensitively dying cells [17]. Williams 79 was chosen as a near-isogenic line of Williams with race-specific resistance to *P. sojae* race 1. It allowed us to compare induced protection in Williams to the incompatible response of Williams 79 to the same pathogen isolate. It also allowed us to examine if additional protection could be induced in a line already showing

race-specific resistance. Harosoy was chosen because it is susceptible to race 1, but it is inherently elicitation competent [1]; that is, Harosoy does not require pre-induction of competency for the expression of the glyceollin response to WGE.

#### *Cotyledon infection and elicitor assays*

Cotyledons from eight-day-old seedlings, unless otherwise noted, were harvested in small batches and used immediately. Cotyledons were cut with a razor blade to form a small, shallow oval wound (approximately 4 mm diameter) at the non-petiole end and this wound was treated with water, elicitor or other effector in 20  $\mu$ l. Ten replicate cotyledons were used per treatment and placed inside an inverted petri plate containing a wet filter paper to retain humidity during the assay. Separate plates were set up in parallel to monitor infection and for harvest for HPLC and phenolic polymer analyses. Initial incubation was in constant light at 200  $\mu$ E m<sup>-2</sup> S<sup>-1</sup>. For the infection plates, at various times after treatment, the cotyledon tissue approximately 7–8 mm away from the edge of the wound and toward the petiole end was inoculated with a small square agar plug (1.5  $\times$  1.5 mm) of *P. sojae* race 1 mycelia grown on clarified lima bean agar (20 g lima beans per liter). The cotyledon surface was pricked lightly with a dissection needle to just break the epidermis before placing the agar plug on the surface. Although breaking the epidermis was not necessary for infection, it led to more synchronous infections. Mock inoculations were performed using lima bean agar plugs. After inoculation, plates were moved to lower light intensity (100  $\mu$ E m<sup>-2</sup> S<sup>-1</sup>) to facilitate infection. At various times after inoculation, disease was rated on a scale consisting of: 0, no spread of symptoms from the point of inoculation; 1, 1–10%; 2, 10–25%; 3, 25–50%; 4, 50–75%; 5, 75–100% spread of symptoms over the total area of the cotyledon. After 96 h, a typical resistant response is characterized by a pink to red response at the point of inoculation, sometimes with a limited dark brown to black colored necrotic area spreading usually only in the surface cells but sometimes slightly into internal tissues as well (rating 1 or 2), while a typical susceptible response leads to a grayish-green, spreading, water-soaked lesion with extensive internal tissue maceration and a rating of 3–5.

At various times before and after infection, tissues were harvested from separate plates for HPLC and phenolic polymer analyses. Please note that in the descriptions that follow we use “proximal” and “distal” to denote the distance in relation to the point of treatment, not the point of inoculation. In fact, as described above, the point of inoculation was at a distal cell zone. Although treatments and inoculations were made and infections were followed in the same manner in all experiments, two

protocols were used to harvest tissues for biochemical analysis depending on the experiment. In all cases replicate tissues were pooled for biochemical analysis.

In the first set of experiments (used for the data in Fig. 7 and 9), two samples were harvested with time. The first sample was a section harvested using a number one cork borer (inside diameter, 4 mm) to obtain a cylinder of cells from the point of treatment. The uppermost 0.5 mm section of this column of cells, representing the treated surface, was sliced and saved as the proximal cell sample. The remainder of the column was discarded. The second sample was a transverse section 1.5 mm thick, spanning the entire cross section of the cotyledon at the inoculation site (distal cell sample). It was sampled before inoculation or 24 and 48 h after inoculation regardless of the extent of infection (or qualitative lesion type) after removal of the 1.5 mm square *P. sojae* or control agar plug. This harvest procedure is shown as an inset to Fig. 7(a).

In the second set of experiments (used for the data in Fig. 8), the treated (proximal) cell population was harvested in the same manner as above, but the distal, infected tissues were harvested with a modified protocol. In these experiments, lesions were classified into two qualitatively different types. These different lesion types were harvested separately for analysis. In those cases where the lesion did not spread radially from the point of inoculation (but may have spread very slightly downward below the point of inoculation), the same 1.5 mm transverse distal cell section described above was first taken, but it was further cut to remove outlying tissues, so that only a 1.5 mm square column of tissues immediately below the point of inoculation was sampled. This column was further cut cross-wise into infected and uninfected zones. Only the upper, infected zone was analysed. This protocol is shown in the inset in Fig. 8(a). In those cases where infection had spread radially from the point of inoculation, the infected tissue was harvested by cutting a section to the shape of the entire infected area. Uninfected tissues were carefully trimmed from the bottom of the lesion if the lesion had not spread to the entire depth of the cotyledon. This protocol is shown in the inset to Fig. 8(b). For the sake of clarity, the sections actually taken are described again in each experiment as it is discussed in the Results section below.

#### *HPLC and phenolic polymer analyses*

HPLC analysis was performed as described previously [14]. Replicate sections per treatment were pooled together and either extracted and analysed immediately for soluble metabolites by HPLC or stored intact at  $-20^{\circ}$ C for later use. Individual peaks were confirmed by running standards and analysing the UV spectra.

Glyceollin concentrations reported are the total of the peaks for all glyceollin isomers [14].

Phenolic polymers and other insoluble phenolics were measured in the pellet from the HPLC extraction. The pellet was drained and resuspended in 1 ml 95 % ethanol and allowed to soak overnight at room temperature. A second 95 % ethanol wash (1 ml) was then carried out for at least 30 min and the pellet collected by centrifugation. The pellet was then finely suspended in 1 ml 1.25 M sodium hydroxide. This mixture was vortexed 3–4 times over the next few hours and then left for a total of 20 h for extraction at room temperature. The sodium hydroxide saponifies esterified phenolics and also extracts high molecular weight phenolic polymers. At 20 h, samples were vortexed and centrifuged at 13,000 g for 5 min. The absorbance (320 nm) of each extract was taken by adding 100  $\mu$ l of extract to 900  $\mu$ l 1.25 M sodium hydroxide. A standard curve was made using ferulic acid in 1.25 M sodium hydroxide and values were expressed as mg ferulic acid equivalents per g fresh weight tissue.

#### *Electrophoresis and peroxidase activity staining*

Peroxidases were extracted from 10 pooled distal cell sections using 50 mM calcium chloride as described previously. Polyacrylamide gel electrophoresis and peroxidase activity staining with 3-amino-9-ethylcarbazole was also as described previously [16]. Stained gels were photographed and digitally scanned, and the images quantified using ImageQuant software (Version 3.30, Molecular Dynamics). Specifically, plotted values for peroxidase activity represent the sum of the total pixel values in each band minus background using volume integration in ImageQuant.

## RESULTS

#### *Distal protection of soybean tissues with the glucan elicitor*

We first examined the effects of treatment with WGE on infection by *P. sojae* at sites distal to the point of elicitor application. Disease progress curves (24–96 h) for infection of Williams soybeans are shown in Fig. 1, including those for unwounded controls, wounded water-treated controls and tissues treated immediately after wounding with 10–300  $\mu$ g ml<sup>-1</sup> WGE. Half-maximal elicitation of glyceollin occurs at 30  $\mu$ g ml<sup>-1</sup> WGE in the classical cut cotyledon assay [18]. Effective protection is observed following proximal treatment with 100 and 300  $\mu$ g ml<sup>-1</sup> WGE when the challenge inoculation at the distal site is 48 h after treatment with the glucan [Fig. 1(a)]. Similar results are obtained if the challenge inoculation is made 96 h after elicitor treatment (data not shown). If the

cotyledons are incubated in the dark immediately following inoculation, protection is substantially reduced with a 48-h challenge inoculation [Fig. 1(b)] or eliminated if the challenge inoculation is delayed until 96 h (data not shown). As shown in Fig. 2 similar results are obtained with Harosoy, except that some protection is evident even at 30  $\mu$ g ml<sup>-1</sup> WGE and is equally effective in infections proceeding in the light [Fig. 2(a)] and dark [Fig. 2(b)]. However, protection is not evident in Harosoy tissues if the challenge inoculation is delayed until 96 h (data not shown). In both Williams and Harosoy, the protection afforded by the WGE results in symptoms generally similar to incompatible lesions seen with the same isolate of *P. sojae* on Williams 79 (a red color at the point of inoculation by 24 h and/or a very slightly spreading dark necrotic surface lesion with a disease rating of 1–2 over the period 48–96 h). Protection experiments were also performed on Williams 79. Even with this cultivar, which normally shows an incompatible lesion to race 1, proximal WGE treatment provided enhanced distal lesion containment characterized by somewhat slower lesion development (data not shown).

The capacity for maximal proximal defence responses to WGE in the cut cotyledon assay is an induced and transient phenomenon and is related to the establishment of wound-induced elicitation competency [17]. Maximum responsiveness in Williams cotyledons to WGE occurs at about 4 h after wounding, after which responsiveness declines [17]. We thus examined the effect on protection of delayed application of WGE to the wounded proximal cotyledon tissues. As shown in Fig. 3(a), with delayed application of elicitor, even 30  $\mu$ g ml<sup>-1</sup> glucan provided protection if elicitor was applied 4–8 h after wounding [Fig. 3(a)]. At 100  $\mu$ g ml<sup>-1</sup> WGE [Fig. 3(b)], protection was effective over a wider range, representing 0–8 h delay in elicitor application. In both cases, protection was not established if the elicitor was applied at 16 h or later. If anything, the establishment of disease was actually more rapid with treatment delays over 16 h.

Mycolaminaran is a lower molecular weight  $\beta$ -1,3/ $\beta$ -1,6 storage glucan found in the cytoplasm of *P. sojae* mycelia [3]. Although it is a relatively weak glyceollin elicitor [48, T. L. Graham, unpublished], we decided to test its capacity for induction of distal protection with a 4 h delayed application after wounding. At 333  $\mu$ g ml<sup>-1</sup> mycolaminaran provided protection (final disease rating at 96 h, 1.2) similar to that seen with 100  $\mu$ g ml<sup>-1</sup> WGE. However, mycolaminaran was still protective at 37  $\mu$ g ml<sup>-1</sup> (final disease rating at 96 h, 1.7). The relative effectiveness of WGE and mycolaminaran in providing protection closely reflected their relative capacity for glyceollin induction in the treated proximal cells ( $r^2 = 0.8043$ , data not shown), suggesting that the

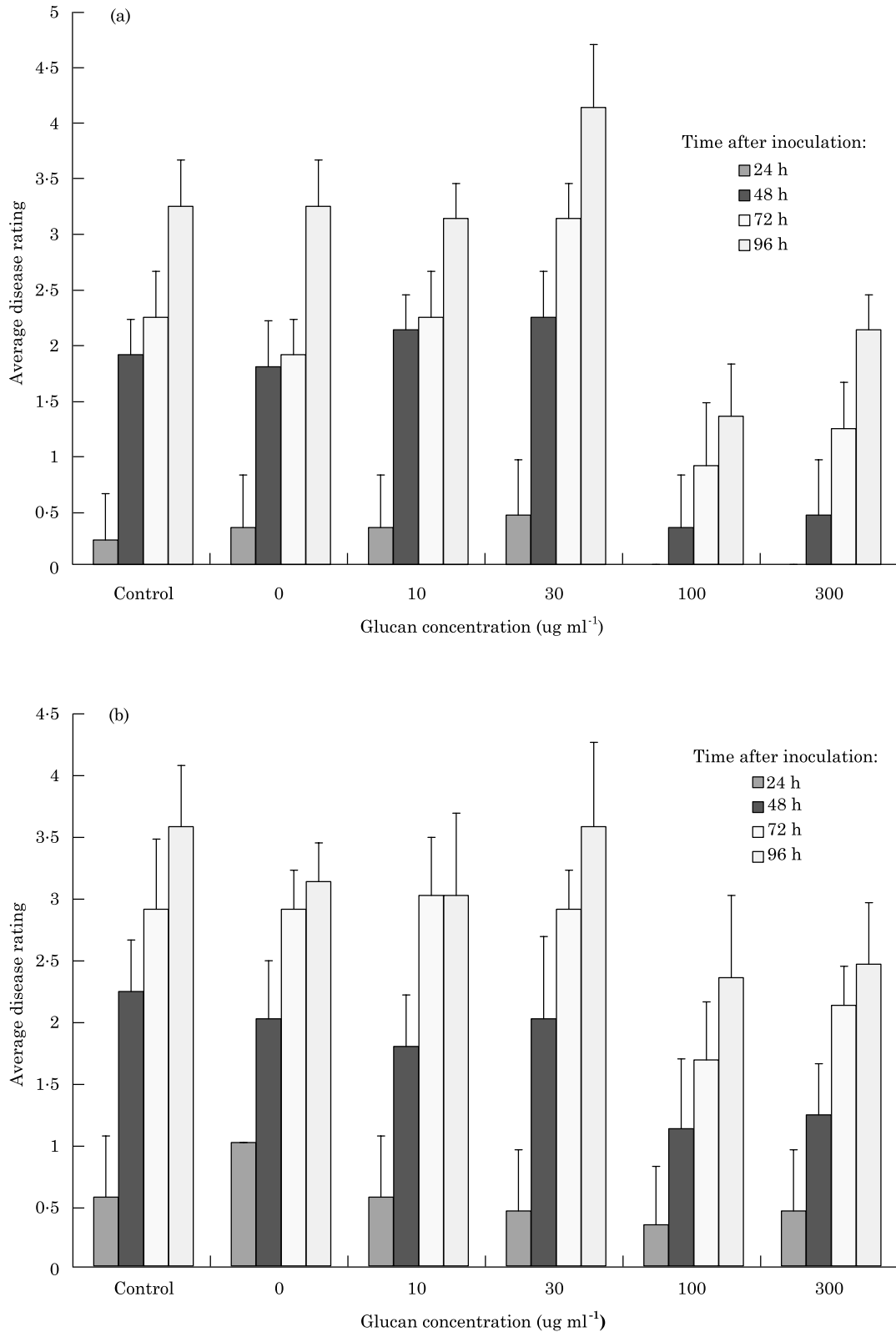


FIG. 1. Disease progress curves for Williams cotyledons treated with glucan elicitor and subsequently inoculated with *P. sojae*. Glucan elicitor was applied at one end of the cotyledon immediately after wounding at 0, 10, 30, 100 and 300  $\mu\text{g ml}^{-1}$ . The control is an untreated, non-wounded cotyledon. Cotyledons were inoculated 7–8 mm from the edge of the glucan-treated wound with *P. sojae* 48 h after treatment and then incubated in the light at  $100 \mu\text{E m}^{-2} \text{s}^{-1}$  (a) or in the dark (b). Disease ratings were taken every 24 h.

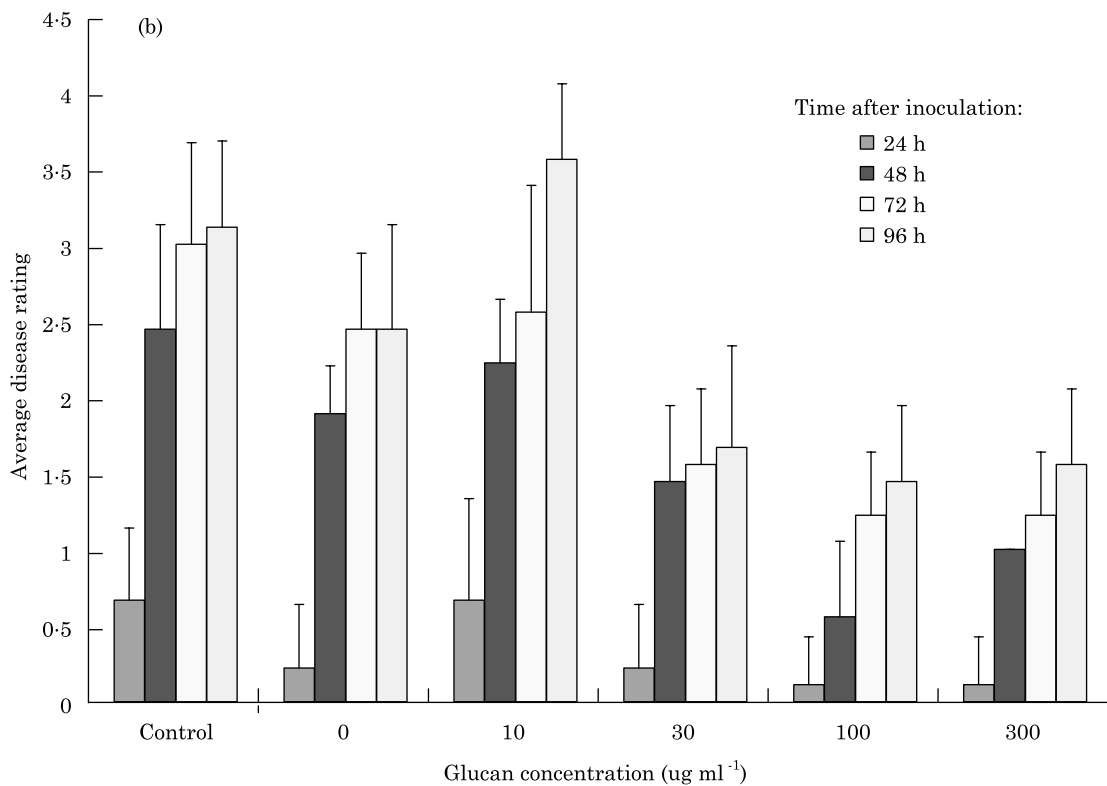
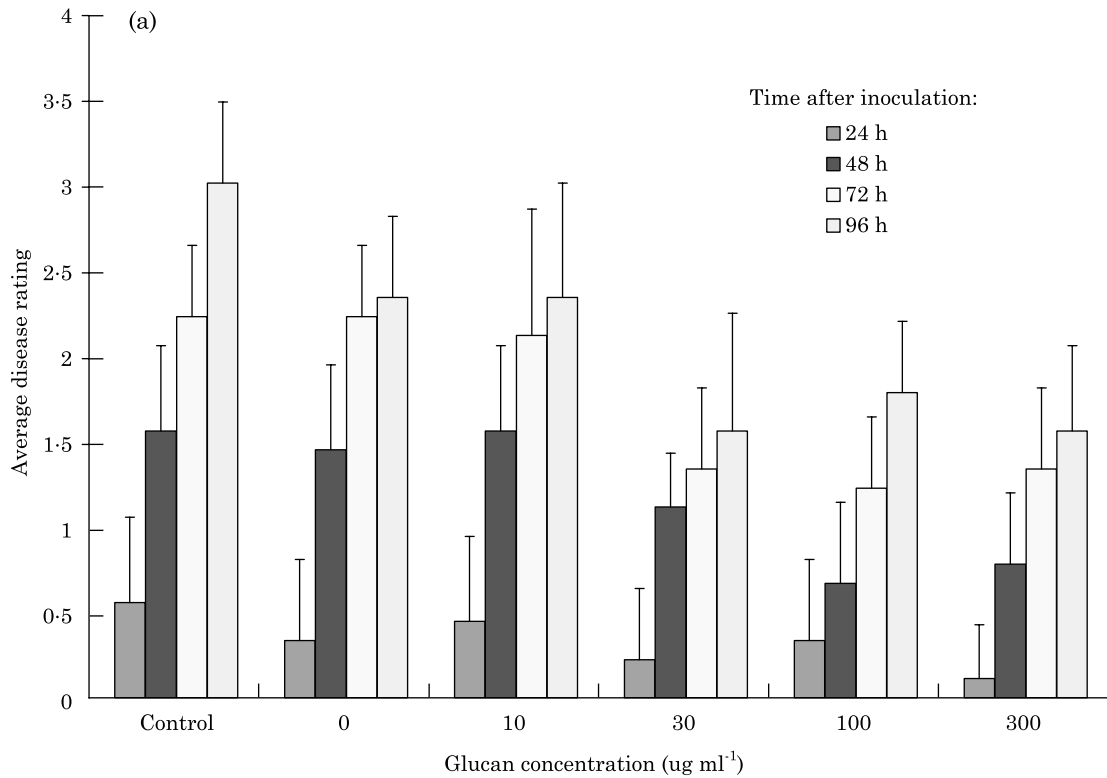


FIG. 2. Disease progress curves for Harosoy cotyledons treated with glucan elicitor and subsequently inoculated with *P. sojae*. Glucan elicitor was applied at one end of the cotyledon immediately after wounding at 0, 10, 30, 100 and 300  $\mu\text{g ml}^{-1}$ . The control is an untreated, non-wounded cotyledon. Cotyledons were inoculated 7–8 mm from the edge of the glucan-treated wound with *P. sojae* 48 h after treatment and then incubated in the light at 100  $\mu\text{E m}^{-2} \text{s}^{-1}$  (a) or in the dark (b). Disease ratings were taken every 24 h.

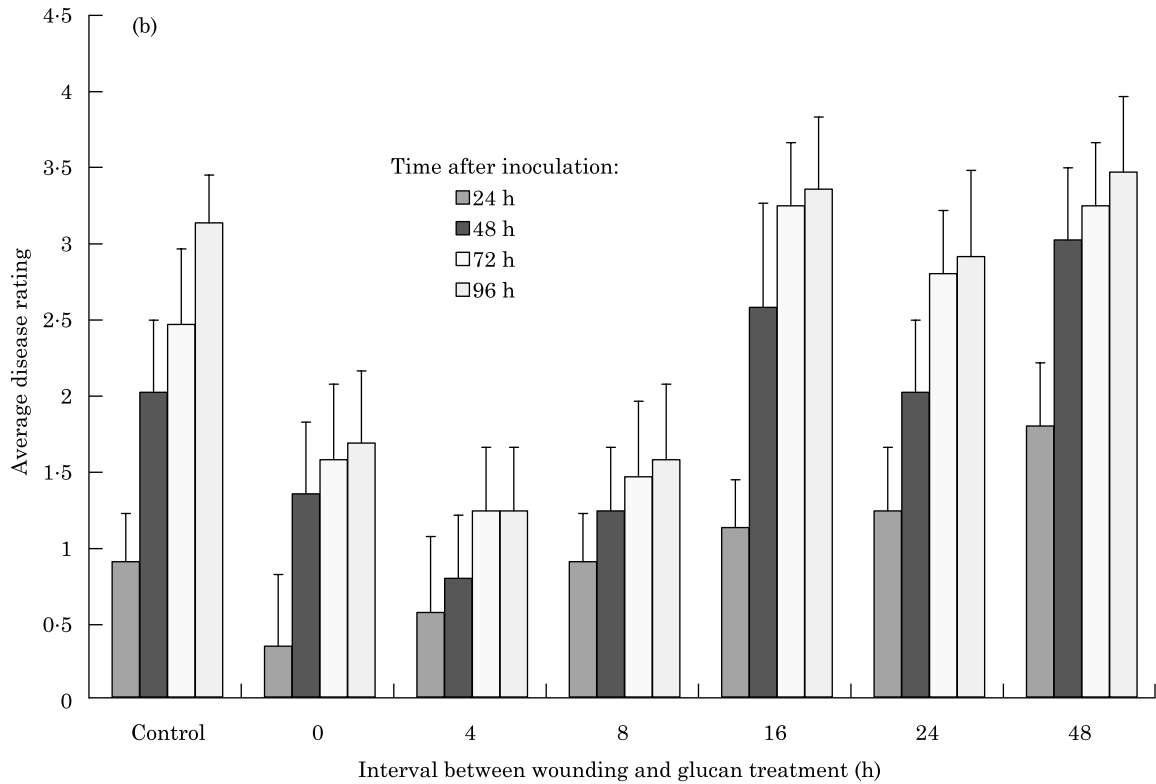
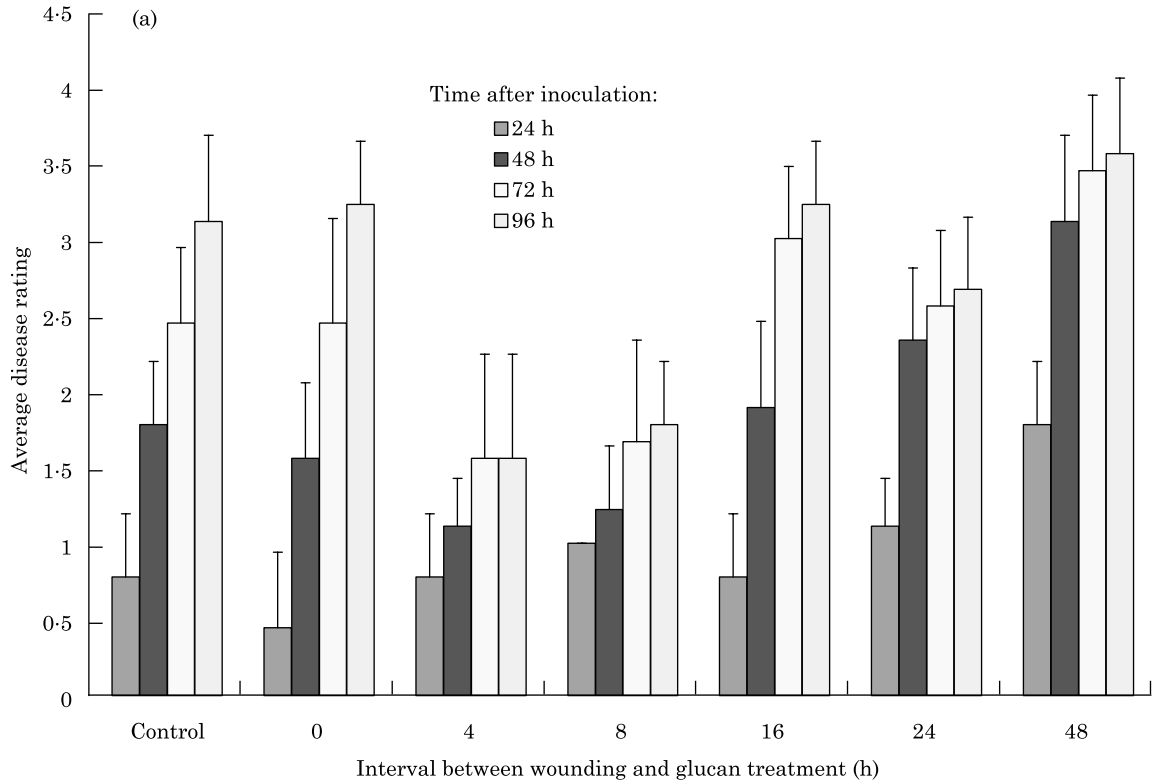


FIG. 3. Effect on induced disease protection of delaying application of glucan elicitor for various times after wounding. Williams cotyledons were wounded at 0 h and then glucan applied immediately to the wounded surface ( $t = 0$ ) or after 4, 8, 16, 24 and 48 h. Elicitor concentrations were  $30 \mu\text{g ml}^{-1}$  (a) or  $100 \mu\text{g ml}^{-1}$  (b). The control was wounded and treated with water at 0 h. Cotyledons were inoculated with *P. sojae* 48 h after treatment at a point 7–8 mm from the edge of the glucan-treated wound.

level of elicitation in the proximal cells correlated to the distal protection response.

#### *Protection of cotyledons with other potential signal molecules*

We were interested in examining other signal molecules for protection and comparing their effects to those of the glucan elicitors. The effects of the ethylene precursor, ACC, and JA on protection with a 4 h delayed application to proximal cells after wounding are shown in Fig. 4. Although protection was seen at all levels of ACC, the lowest concentration (11  $\mu\text{M}$ ) was the most effective. Dilution of ACC to 3.7  $\mu\text{M}$  led to diminished protection (data not shown). Although 10  $\mu\text{M}$  JA was ineffective, 20 and 40  $\mu\text{M}$  were effective. Unlike protection by the glucan elicitor, protection by ACC and JA seemed to break down somewhat with time, resulting in higher disease ratings at 72 h compared to the glucan-induced protection. However, it should be noted that the increase in disease ratings for ACC and JA after 48 h were due to the spread of non-water soaked (i.e. not typical susceptible) lesions, as discussed further below. Similar results to those in Fig. 4 were seen in three additional experiments.

Fig. 5 shows protection induced by mixtures of ACC and JA and by JAME. Protection by the mixtures of ACC and JA was somewhat more effective, uniform and stable than either treatment alone (compare to Fig. 4). JAME provided protection at all concentrations tested, but was again most effective at the lowest concentration (6.6  $\mu\text{M}$ ) in these experiments. Dilution to 2.2  $\mu\text{M}$  led to diminished protection (data not shown). The protection at 6.6  $\mu\text{M}$  was as effective as the highest levels of WGE. Similar results were seen in two additional experiments. SA (50  $\mu\text{M}$ –2 mM) was ineffective in inducing protection, while reduced glutathione (0.5–1 mM) led to statistically significant, but weak protection at both 0.5 and 1 mM (data not shown).

#### *Lesion symptoms associated with susceptibility and protection*

A discussion of the different types of lesions seen is useful at this point, since as we will see below, the biochemical responses of the different types of lesions differed significantly. Photographs of typical *P. sojae*-infected Williams cotyledons (72 h post inoculation) inoculated 48 h after proximal treatment with water or with WGE at 100  $\mu\text{g ml}^{-1}$  (with a 4 h delay in application) are shown

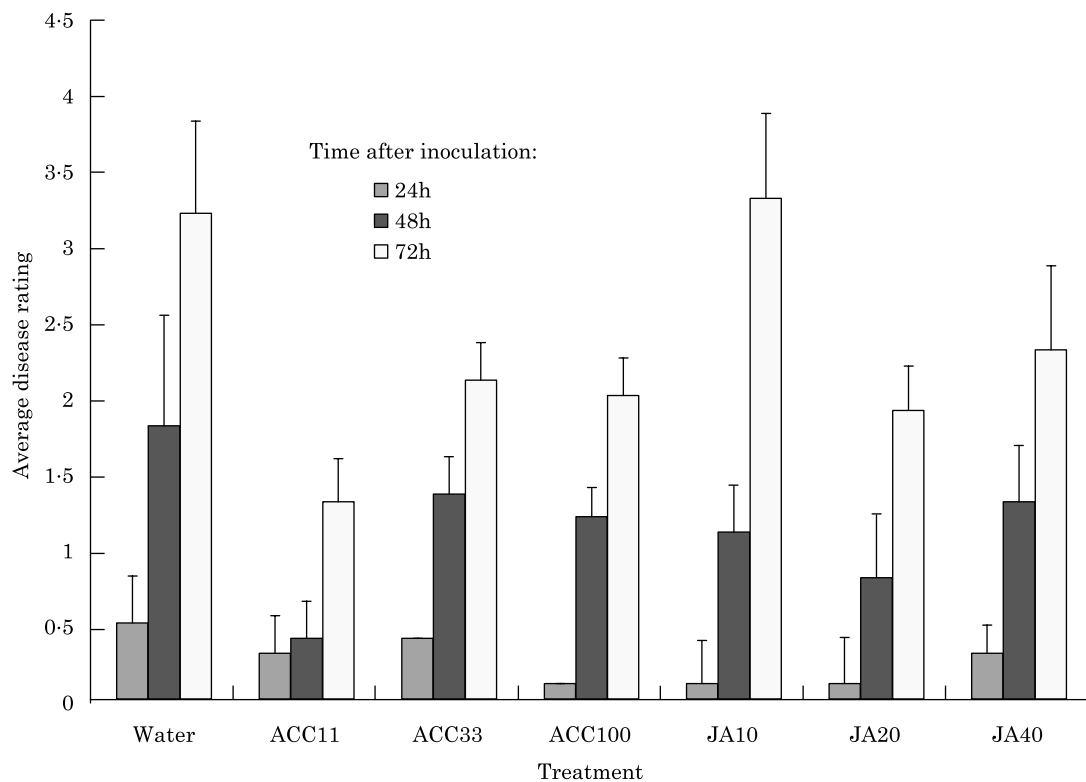


FIG. 4. Disease progress curves of Williams soybeans treated with ACC or JA and subsequently inoculated with *P. sojae*. ACC or JA at the various concentrations ( $\mu\text{M}$ ) indicated were applied 4 h after wounding. After incubation for 48 h at 200  $\mu\text{E}$ , cotyledons were inoculated with *P. sojae* at a point 7–8 mm from the edge of the treated wound and incubated at 100  $\mu\text{E m}^{-2} \text{s}^{-1}$ . Disease ratings were taken every 24 h.

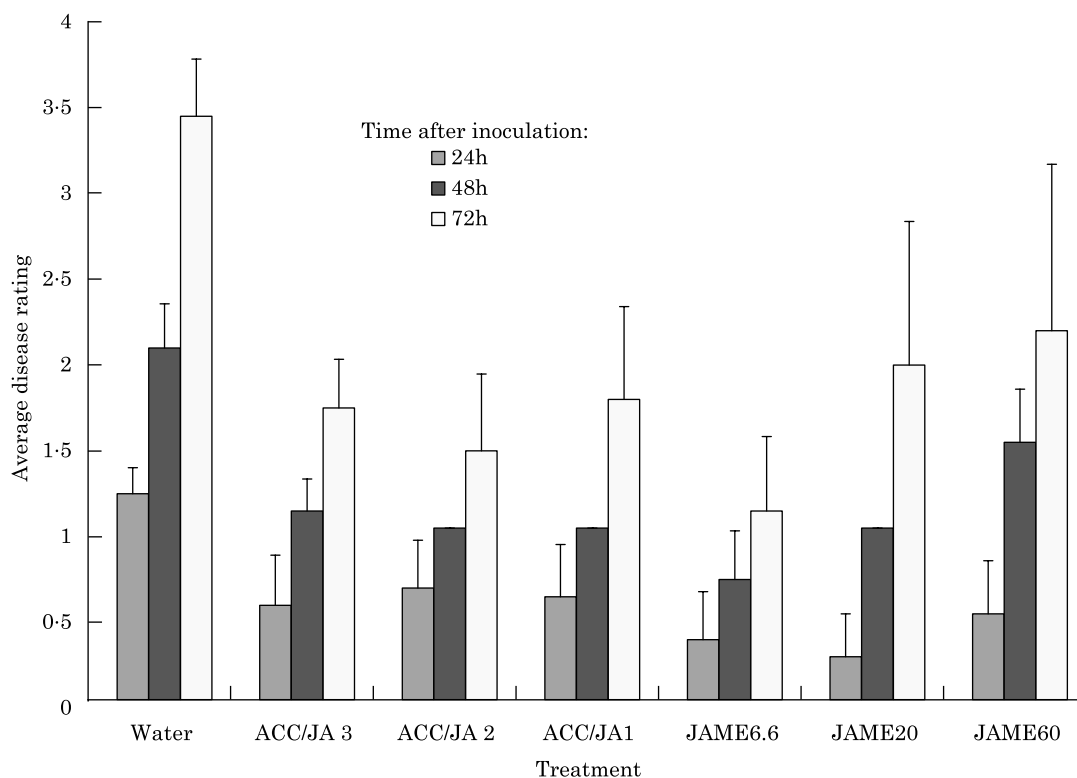


FIG. 5. Disease progress curves of Williams soybeans treated with a combination of ACC and JA or with JAME and subsequently inoculated with *P. sojae*. ACC and JA were kept at a constant ratio with serial 1:3 dilutions of ACC/JA. ACC/JA 1 = 100/60  $\mu\text{M}$ , ACC/JA 2 = 33/20  $\mu\text{M}$  and ACC/JA 3 = 11/6.7  $\mu\text{M}$ . These mixtures or JAME at the various concentrations ( $\mu\text{M}$ ) indicated was applied 4 h after wounding. After incubation for 48 h at 200  $\mu\text{E m}^{-2} \text{s}^{-1}$ , cotyledons were inoculated with *P. sojae* at a point 7–8 mm from the edge of the treated wound and incubated at 100  $\mu\text{E m}^{-2} \text{s}^{-1}$ . Disease ratings were taken every 24 h.

in Fig. 6(a). The agar block for the water control has dried on the surface. The red color of the agar block in the WGE-treated cotyledon is due to spontaneously formed derivatives of trihydroxypterocarpan precursors of glyceollin and is a typical response to infection or elicitor [19, 50]. As illustrated, this level of glucan usually led to nearly complete containment of the infection at the point of inoculation. Dissection of these tissues showed that although infection occurred, it was localized to points immediately (usually 1–2 mm or less) below the point of inoculation and characterized by tight, slightly brownish to red-brown lesions. With certain cotyledons, especially at the lower levels of WGE, the tissues showed only partial protection, characterized by slightly spreading necrotic lesions (ratings 1–2) usually restricted to the cotyledon surface (not shown). These first two types of lesions seen in protected tissues are similar to those seen in incompatible (race-specific resistant) responses. In contrast, the grayish-green color of the infected control reflects complete maceration and water-soaking. As shown in Fig. 6(b), upper panel, when protection was even less effective, lesions sometimes developed with time as spreading patches of sunken necrotic tissues, but there

was still no tissue maceration or rotting. Lesions for mycolaminaran- and ACC-induced protection were similar to those for WGE. Protection afforded by JA and JAME usually also either resulted in nearly complete containment or in partial protection with spreading lesions. Similar to other treatments, partial protection was sometimes seen as a spreading necrotic lesion. The further breakdown of protection from JA treatment, however, sometimes resulted in lesions as shown in Fig. 6(b) (lower panel). While the pathogen did not spread uniformly through all of the tissue and did not cause maceration, it apparently partially colonized underlying tissues and eventually emerged again at the surface, causing scattered bright red spots typical of areas of glyceollin accumulation [Fig. 6(b)]. Indeed, the presence of high levels of glyceollin in these red areas was confirmed by HPLC.

#### *HPLC and phenolic polymer analyses of tissues before and after infection*

To gain some knowledge of the biochemical status of protected tissues both before and after infection, we

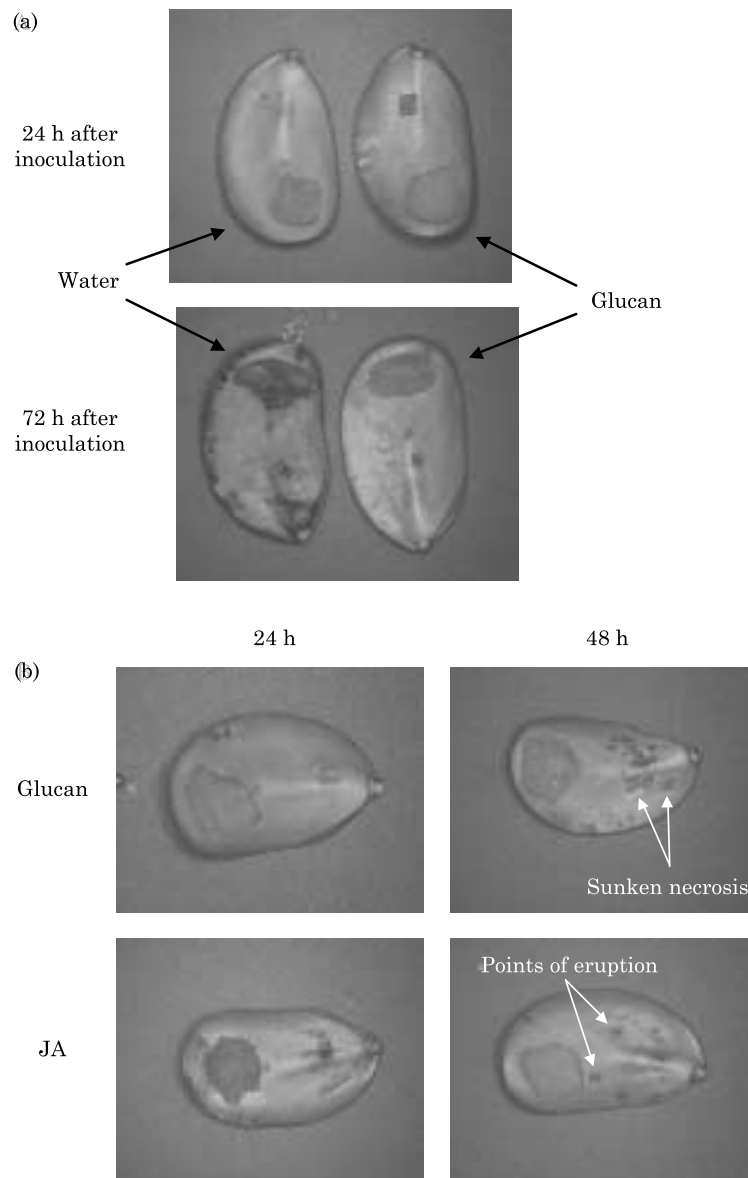


FIG. 6. Typical symptoms associated with protection of soybean cotyledon tissues. (a), Williams cotyledon tissues treated with water or  $100 \mu\text{g ml}^{-1}$  WGE and infected 48 h post-treatment with Race 1 of *P. sojae*. Pictures are at 24 and 72 h after inoculation. (b), Spreading lesions seen in some cotyledons showing partial protection. Upper row were glucan treated  $30 \mu\text{g ml}^{-1}$  and lower were JA treated  $20 \mu\text{M}$ . Pictures were taken at 24 and 48 h after inoculation. Please note the sunken necrosis seen in partial protection by the glucan and the eruptions of red-colored lesions seen in partial protection with JA.

examined the various well-established phenylpropanoid defence responses by HPLC and phenolic polymer analyses [15]. As a result of the different lesion types observed and in order to gain different perspectives on the protection responses, somewhat different sections were harvested from the infected, distal tissues for analysis depending on the experiment. In all experiments, the proximal cell layers at the point of treatment were analysed at the time of inoculation of distal cells. Moreover, sections at the point of inoculation (distal from the point of treatment) were analysed both before inoculation and at various times after inoculation. Such

analyses were carried out for many replicate experiments for all of the various treatments described above. Thus, very large amounts of data were collected and analysed. In each case, however, the general trends were similar and we thus present here only some overall observations and the data for a few representative experiments.

In all cases where susceptible lesions were analysed, both the infected water-soaked lesion and a 1.5 mm section of healthy tissues ahead of the lesion (infection front) were harvested. Observations were very similar to those reported previously [22], so we only describe the overall results here for comparative purposes. In the

water-soaked, macerated tissues, there was extensive hydrolysis of the isoflavone conjugates of daidzein and genistein, but very little to no net accumulation of glyceollin. In tissues just ahead of the visible infection front, isoflavone conjugate hydrolysis had begun, but again there was little net accumulation of glyceollin.

Protected tissues usually consisted of two general types of lesions, those contained nearly completely to the point of inoculation (no radial spread) and those with a resistant, but radially spreading necrotic phenotype. Analysis of these tissues differed somewhat with different sets of experiments. Although described in detail in Materials and Methods, we reiterate some aspects here to facilitate the description of results. In the experiments for which the disease results are described in Fig. 3, we harvested a 1.5 mm transverse cross section at the point of inoculation before (0 h) or at various times (24 and 48 h) after inoculation for HPLC analysis. This includes just the point of inoculation (a 1.5 mm square plug of agar was used) and some peripheral tissues. When these sections were analysed (data shown in Fig. 7), the accumulations of the phenylpropanoid molecules normally associated with local defence in soybean showed no clear relationships with protection [compare the results in Fig. 7 with disease ratings shown in Fig. 3(b)]. Although the malonyl-glucosyl conjugates of both genistein [Fig. 7(a)] and daidzein (data not shown) showed an overall induction by the glucan in distal cells at the point of inoculation prior to infection, neither the levels in these tissues before (0 h) or after (4–48 h) inoculation showed any clear correlation with protection. Moreover, glyceollin accumulation was relatively low {compared to a typical incompatible response [22]} and showed an apparent positive correlation to disease rather than protection [compare Fig. 7(b) to Fig. 3(b)]. Scatter plots correlating the 48 h data for glyceollin accumulation with the 48 h and 96 h data for average disease rating for all treatments yielded a positive correlation with  $r^2$  values of 0.78 and 0.75, respectively. Using equivalent data from the experiment in which  $30 \mu\text{g ml}^{-1}$  glucan was used,  $r^2$  values of 0.59 and 0.61 were obtained. Thus, in these sections of tissue, glyceollin accumulation showed a strong correlation with disease instead of protection. Though not quite as striking, phenolic polymer accumulation also showed an apparent inverse correlation with protection [compare Fig. 7(c) to Fig. 3(b)].

The protection experiment shown in Fig. 3(b), and analysed biochemically in Fig. 7, was repeated twice with very similar overall results and the same conclusions. Although we obtain independent disease ratings for each of the ten replicate cotyledons within a treatment, leading to the error bars for standard deviation within an experiment shown in Fig. 3, due to the minimal amount of tissue required and the time consuming nature of

each biochemical analysis by HPLC profiling, we pool all replicate cotyledon samples from each treatment for HPLC analysis. This gives an average biochemical value for the pooled tissues. A discussion and statistical analysis of this approach has been presented earlier [14]. In most cases, we are then able to present the biochemical data as an average of several experiments, allowing the presentation of standard errors in figures such as Fig. 7. However, for these very complex experiments we found that the precise disease progress curves and biochemical analyses shifted somewhat in magnitude and timing from one experiment to another, such that averaging the biochemical data tended to obscure important trends. Thus, while very similar trends were seen in all three experiments, we chose to present the data from one representative experiment throughout.

To further confirm and extend these results, a series of three follow-up experiments were carried out in which tissues were harvested in a more discrete manner to determine if the qualitatively different lesion types (contained versus spreading necrotic) were possibly associated with different biochemical responses. In these experiments, after treatment of proximal cells with various agents (including various dose responses with WGE, mycolaminaran, JAME and combinations of JA and ACC), we again harvested distal tissues at the time of inoculation (48 h after treatment, 0 h after inoculation) and at various times (24 and 48 h) after inoculation. However, the different lesion types were harvested and analysed separately. In those cases where the lesion had not spread radially beyond the point of inoculation, a small (approximately 1.5 mm square) column of cells was harvested. It was then examined with a dissecting scope for the extent of actual infection (brownish red lesion) and the infected portion of the column was collected for analysis [inset, Fig. 8(a)]. The infected portions of these lesions generally showed only 1–2 mm spread directly below the point of inoculation. In the case of lesions showing radially spreading necrosis or patchy necrosis, the infected areas were carefully cut out to the exact shape of the lesion and saved as a different fraction for analysis [inset, Fig. 8(b)]. These lesions spread radially forming a roughly circular lesion of diameter from 2 mm up to 3–4 mm for necrotic (resistant) lesions and up to 6–8 mm for water-soaked (susceptible) lesions. While necrotic lesions were normally limited to upper layers of cotyledon tissue, water-soaked lesions usually spanned the depth of the cotyledon.

Analysis of the locally highly contained lesions below the point of inoculation and the larger, radially spreading necrotic lesions showed very different trends. While glyceollin accumulated in both types of lesions to varying levels, the correlation of glyceollin to the spread of the pathogen was opposite in the two types of lesions. For the lesions largely contained at the point of inoculation, we

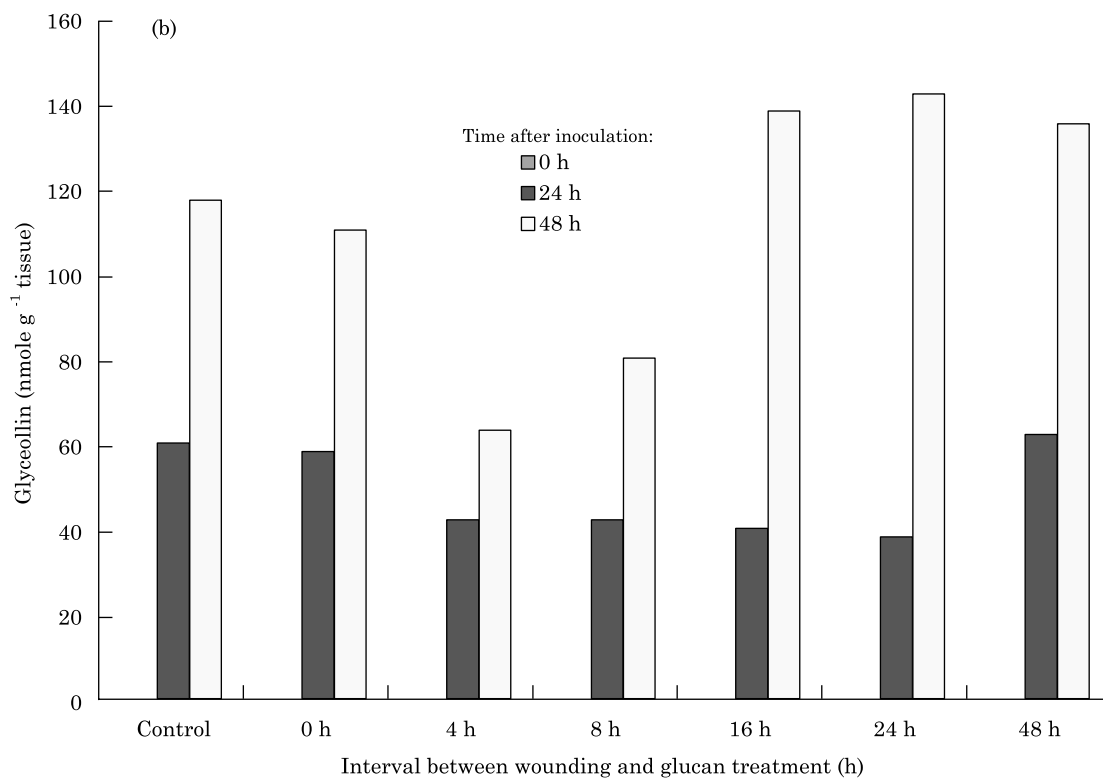
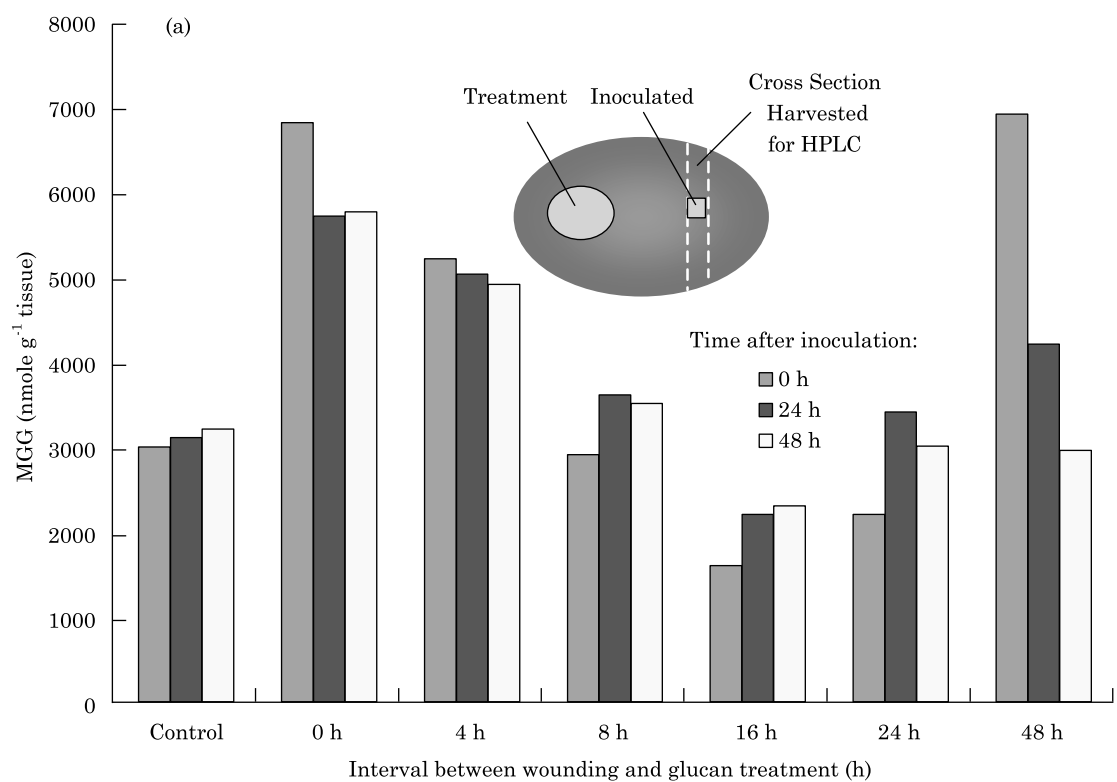


FIG. 7. Caption on next page.

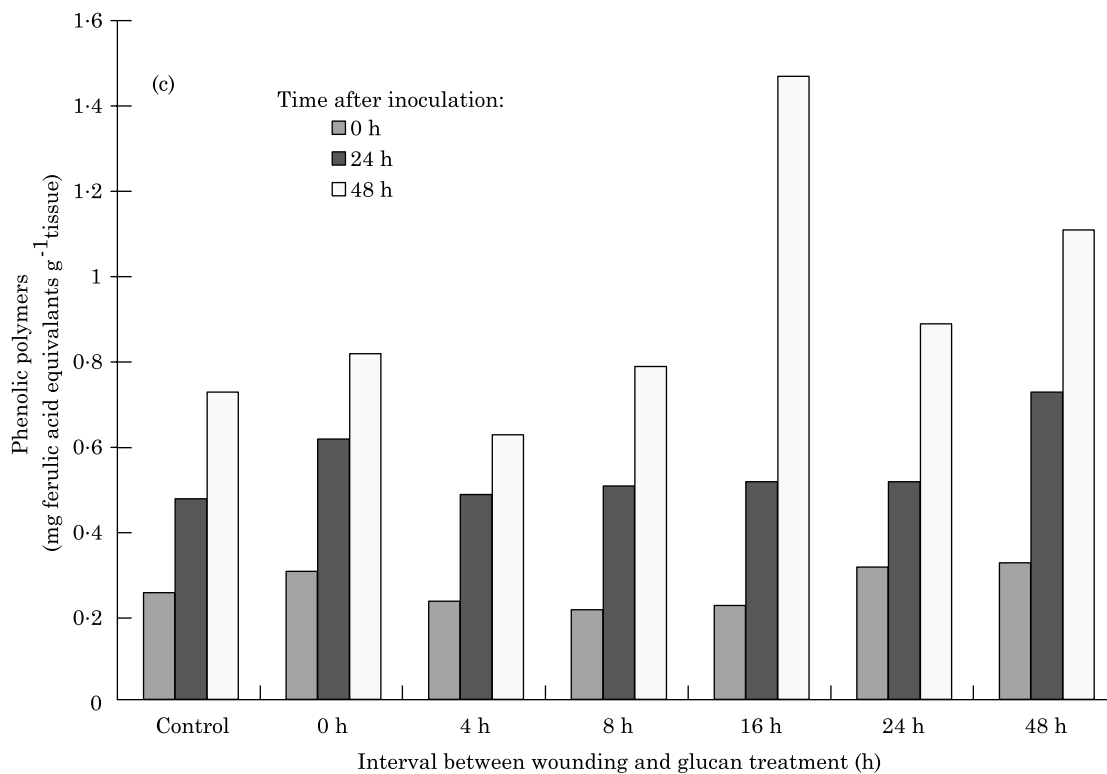


FIG. 7. Net accumulations of defense-related phenylpropanoids in the glucan protection experiment in Fig. 3(b). Data are shown for tissues harvested just before inoculation (0 h) and 24 and 48 h after inoculation for the various delayed times of elicitor application after wounding (0–48 h). (a), malonyl glucosyl genistein (MGG); (b), glyceollin; (c), phenolic polymers. The same sampling procedure shown in the insert for Fig. 7(a) was used for Fig. 7(b) and 7(c).

plotted glyceollin levels (48 h after inoculation) for all treatments in all three experiments (a combination of over 30 data points) on a tissue weight basis against the average weight (and thus the average amount of disease) of the harvested lesions [Fig. 8(a)]. In these samples, glyceollin accumulation showed a very strong positive correlation to the amount of disease ( $r^2$  value of 0.83). Thus, these results were qualitatively similar to those obtained with the somewhat less discrete tissue sectioning described for the experiments in Fig. 3. Quantitatively, the glyceollin levels and the regression coefficients were somewhat higher with the more discrete sectioning procedure. Taken together, the experiments suggest that glyceollin accumulation in narrow cross sections or columns of cells at the point of inoculation of protected tissues does not correlate to protection.

In contrast, in those cases where analyses were conducted on the radially spreading lesions (i.e. the pathogen was not stopped immediately at or just below the point of inoculation) biochemical analyses gave markedly different results. In Fig. 8(b), we have plotted on the  $y$  axis the glyceollin levels (on a weight basis) in such tissues 48 h after inoculation for a single representa-

tive experiment including treatments with water, WGE (3.3, 11, 33, 100  $\mu\text{g ml}^{-1}$ ), mycolaminaran (37, 111, 333, 1000  $\mu\text{g ml}^{-1}$ ), JAME (7, 20, 60  $\mu\text{M}$ ) and various combinations of JA and ACC. We have also included on these plots the glyceollin levels for susceptible lesions. Similar results were obtained in two other experiments. Plotted on the  $x$  axis is the average disease rating for each of the treatments at 48 h. At first glance, the data fit an exponential curve. However, further analysis showed that the data clearly grouped into two subsets, those which had typical susceptible water-soaked spreading lesions 96 h after inoculation (e.g. water controls) and those which had resistant, spreading necrotic lesions as described above. As can be seen, in all cases where a susceptible lesion was seen, the glyceollin levels were below 300–400  $\text{nmol gm}^{-1}$  tissue and the correlation of glyceollin to disease rating was very low ( $r^2 = 0.04$ ). Above this threshold of glyceollin, increasing protection was seen with increasing glyceollin accumulations ( $r^2 = 0.88$ ). Thus, in spreading necrotic lesions, a very strong correlation of protection to glyceollin levels was seen. In these same tissues, a relatively strong correlation of protection to MGG levels prior to infection was also

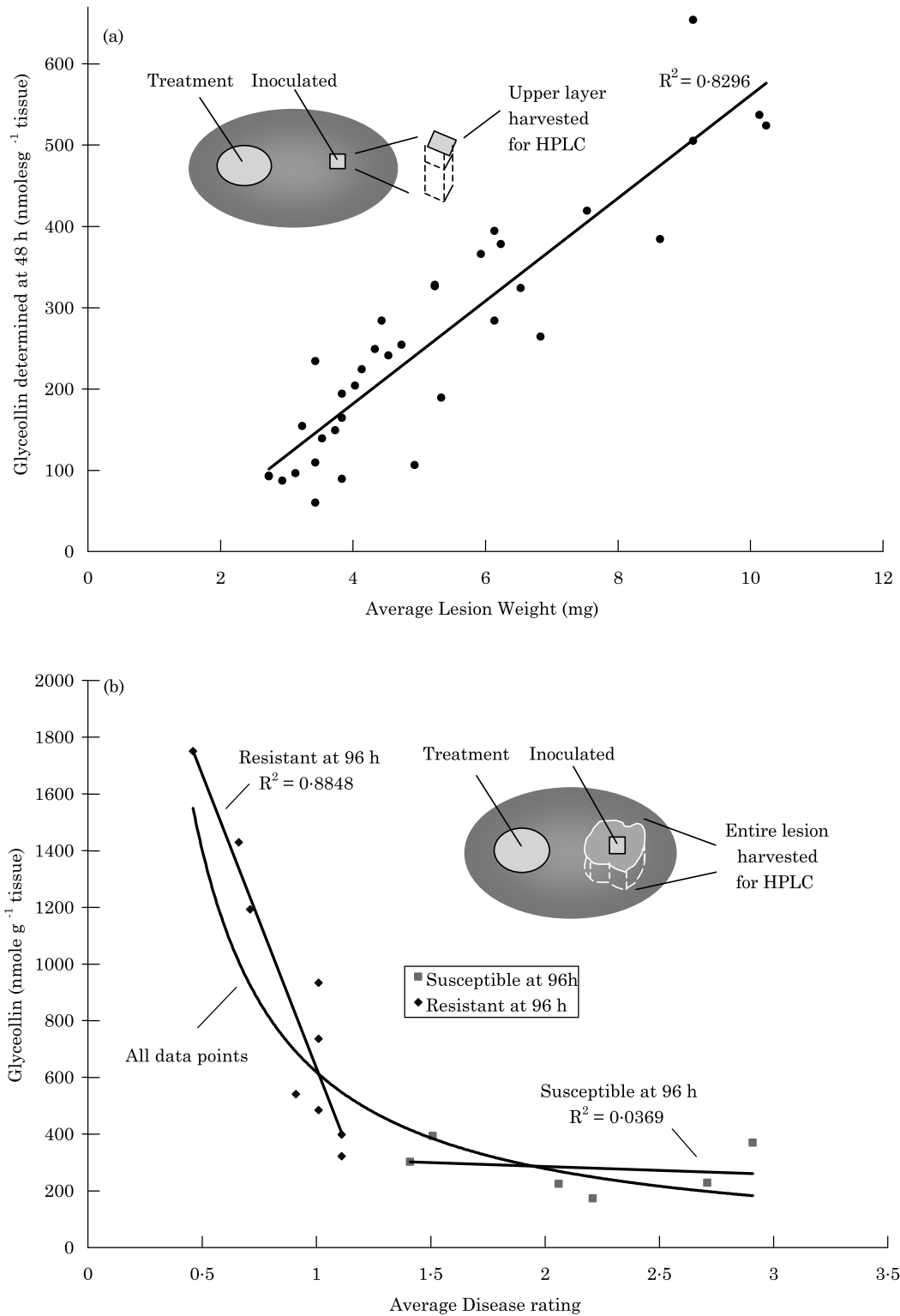


FIG. 8. Scatter plots of glyceollin levels accumulating after infection in infected tissues. (a) Glyceollin levels 48 h after infection in protected lesions contained to the point of inoculation (see insert for sampling procedure) are plotted against the average weight of the harvested lesion. Treatments included WGE, mycolaminaran, JAME, and ACC/JA from three separate experiments. (b) Glyceollin levels 48 h after infection in tissues with spreading lesions (see insert for sampling procedure) are plotted against the corresponding average disease ratings at 48 h. Treatments included water, WGE, mycolaminaran, JAME and ACC/JA treatments from a single representative experiment. For regression analysis, the data was treated as one group (exponential curve) or as two separate groups (linear curves), representing treatments that showed resistant ( $r^2 = 0.8848$ ) or susceptible ( $r^2 = 0.0369$ ) lesions at 96 h.

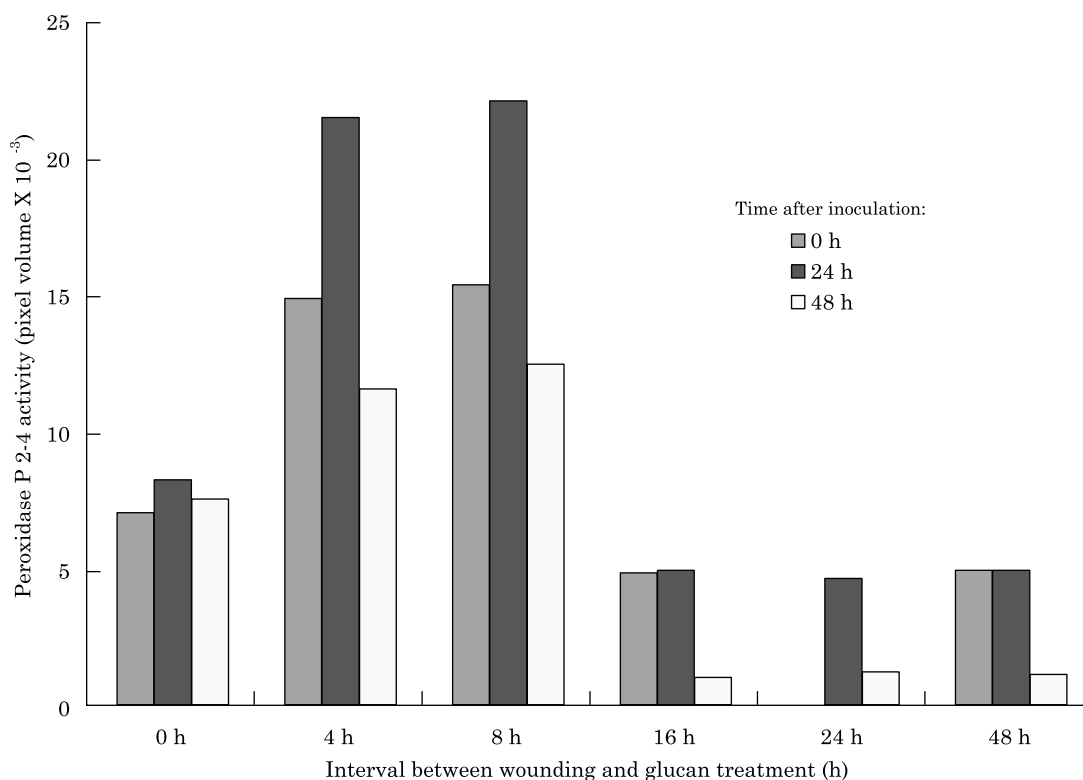


FIG. 9. Induction of peroxidase isozyme P 2-4 in the WGE protection experiment in Fig. 3(b). Data are shown for tissues harvested just before inoculation (0 h) and 24 and 48 h after inoculation for the various delayed times of elicitor application after wounding (0–48 h). Levels of P 2-4 activity in the corresponding water-treated controls have been subtracted from the plotted values. The sampling procedure was the same as that shown in the insert for Fig. 7(a).

apparent ( $r^2 = 0.77$ , data not shown), while the MGD levels prior to infection showed a poor correlation ( $r^2 = 0.034$ ).

#### *Peroxidase induction in tissues before and after infection*

One protein defence that has been studied in some detail in soybean is the accumulation of peroxidase isozymes (now classified as PR-9) in response to WGE [16]. In the WGE response work, three groups of peroxidase isozymes were identified based on their mobility on electrophoresis, only one of which (Group 2) was induced in response to glucan. The induction of several Groups 2 isozymes correlated well with phenolic polymer deposition in proximal cell layers. The lowermost isozyme in Group 2, P2-4, on the other hand, was induced more strongly in distal cells where phenolic polymers did not accumulate in response to elicitor [16]. All four isozymes of Group 2 were also induced in response to infection in all experiments reported here. However, only P2-4 was induced in distal cells by WGE treatment *prior to infection*. Moreover, while induction of the upper isozymes of Group 2 (P2-1 through P2-3) showed a good correlation with phenolic polymer deposition in all experiments (ranging from

$r^2 = 0.53$  to  $r^2 = 0.71$ ), the induction of P2-4 showed a very poor correlation to phenolic polymer deposition ( $r^2 = 0.03$ ). As shown in Fig. 9, the induction of P2-4 was maximal when application of glucan elicitor was delayed 4–8 h after wounding of proximal cells and shows a good overall correlation with protection of these tissues as shown in Fig. 3(b). These experiments, though repeated several times in the format of the experiment in Fig. 3, have not yet been carried out with the different lesion types as described above.

## DISCUSSION

The results presented here clearly demonstrate that treatment of soybean tissues with the WGE from *P. sojae* leads to the protection of tissues distal to the point of treatment with elicitor. Protection can also be induced by the related cytoplasmic glucan, mycolaminaran, and by ACC (the ethylene precursor), JA and its methyl ester. The classical systemic acquired resistance (SAR) signal molecule, SA, was inactive in these experiments. The results reported here strongly confirm and significantly extend previous experiments suggesting that local and/or

weakly systemic potentiation of soybean defence responses can occur [2, 5, 32, 40, 47, 49]. In particular, as discussed below, we have carried out exhaustive analyses of the correlations of protection to phenylpropanoid responses of treated and challenged tissues, a process which has led to the description of two possibly very distinct types of disease protection, and we have begun to characterize some of the signaling components potentially involved.

The distal protection against *P. sojae* induced by the various treatments examined here is characterized by lesions that are symptomatically somewhat similar to those seen in incompatible infections. That is, rather than being water-soaked and spreading, the lesion is often contained at the point of inoculation or dark colored and necrotic in those cases where there is a limited radial spread. Moreover, when spreading is evident, the lesion in protected tissues usually occurs only on the surface or slightly below the surface and only a short distance from the point of inoculation. At lower levels of protecting treatments, lesions with eruptions of necrosis at a further distance are sometimes seen which indicate some internal colonization by *P. sojae*. The data presented here suggest that there may be two distinct mechanisms involved in the overall protection of these tissues. In those cases where the pathogen was nearly completely contained at the point of inoculation (i.e. there was some very limited infection below, but there was no visible spread radially beyond the initial point of inoculation), glyceollin levels showed a strong positive correlation to the extent of *disease progression* below the point of inoculation. From our experience, the most likely interpretation of this data is that the glyceollin accumulating is simply the result of the greater amount of host-pathogen contact in these tissues and is thus a consequence of infection and not a determinant of resistance. While the data cannot rule out a contribution of glyceollin accumulation to protection, the results suggest that uncharacterized, non-phenylpropanoid defence responses may be operating in this highly effective local containment type of protection. Since JA and ACC also induce this response, perhaps it is related to PR protein accumulations, which will be examined in future work. It is very important to point out that, although the lesions seen are symptomatically similar, the induced local containment of the compatible pathogen reported here appears to be fundamentally different from the local containment associated with an actual incompatible infection, where glyceollin accumulation has been shown to occur at high levels and correlate to resistance in these same tissues [see, e.g. 22].

In contrast, in those cases where the lesion had visibly spread slightly or occurred in necrotic patches, but was still typical of a resistant response (necrotic, with no water soaking or tissue maceration) the levels of accumulation of glyceollin after infection showed a strong correlation to protection. In these same tissues, genistein conjugate

levels prior to infection also showed a strong correlation to protection, but daidzein conjugate levels did not. While daidzein is a precursor of glyceollin, genistein has been implicated in the establishment of glyceollin elicitation competency [20, 21]. Thus, it would appear that the increased capacity for glyceollin accumulation in protected tissues may not be due directly to increased pools of the glyceollin precursor. Instead, the correlation to increased genistein levels may suggest that protection correlates to increased elicitation competency for glyceollin synthesis of these tissues.

Taken together, the results of our studies suggest that the distal defence potentiation cannot be explained completely by a simple priming of previously characterized local phenylpropanoid defence responses. The results suggest that the possible involvement of two distinct mechanisms of protection, a non-phenylpropanoid response which leads to a nearly total restriction of the pathogen to the point of inoculation (local containment) and a phenylpropanoid related lesion-limiting resistance that apparently becomes involved if there is limited necrotic spread of the lesion.

Other than secondary product defences, the most studied are those involving the pathogenesis related (PR) proteins. Compared with many other plant species, the PR proteins have been comparatively little studied in soybean. Of the proteinaceous defences in soybean, one studied in some detail is the accumulation of peroxidases (PR-9) in response to glucan elicitor [16]. We report here the induction of peroxidase isozymes following infection in soybean. As previously described for response to the glucan elicitor [16], only Group 2 peroxidases were induced in response to infection. The upper isozymes of Group 2 (P2-1 through P2-3) were induced only in infected tissues and correlated well with phenolic polymer deposition, but not with protection. On the other hand, the activity of isozyme P 2-4, previously shown to be induced by elicitor in distal cells [16], was induced prior to infection in distal cells in the current studies as well and correlated well with protection. Isozyme P2-4, then, is induced in distal cells by glucan elicitor and may function in a protection mechanism other than phenolic polymer deposition. Peroxidases can have many functions in overall redox regulation. Indeed, a role for peroxidases in hydrogen peroxide generation in plant defence has been proposed by several labs [e.g. 4, 20, 21]. A specific peroxidase isozyme (Nox II), which is activated by genistein, is thought to play a role in establishment of competency (local defence potentiation) for the glyceollin response in soybean [20, 21]. How isozyme P 2-4 relates to Nox II is under investigation.

The activities of ACC, JA and JAME and the lack of activity of SA suggest that the distal cell protection response in soybeans may share some attributes in common with non-SA mediated ISR (or ISR-like)

responses [10, 45]. JA and/or ACC induce specific classes of PR proteins in a number of plants, including the proteinase inhibitors [13], defensins and thionins [12, 33]. Ethylene causes a relatively weak further induction of the constitutively expressed elicitor-releasing glucanase (a PR-2 protein) in soybean [31, 42] and PR-1 mRNA is abundantly expressed in *P. sojae* - infected tissues [34]. A number of other PR gene homologs are present in the soybean EST database and we are currently examining the expression of these genes in the various distal protection responses described here.

An interesting aspect of the distal protection responses described here is that protection is maximal under conditions where proximal elicitation competency is maximal. Thus, although the two types of protection described here do not both correlate to enhanced glyceollin accumulation in the infected tissues, the establishment of both types of protection does correlate to at least some parameters associated with establishment of the glyceollin elicitation competency of the signal-generating proximal tissues. This suggests that the processes underlying establishment of proximal glyceollin elicitation competency may be related to the processes underlying the generation of possible signals for both forms of distal cell protection. Indeed, with the glucan elicitors (WGE and mycolaminaran) there is a very good correlation between proximal elicitation of glyceollin and all aspects of distal protection.

The protection studies described here used predominantly the universally susceptible soybean cultivar, Williams. Protection was also seen with the cultivar Harosoy and enhanced resistance was even seen in Williams 79, which has race-specific resistance to the *P. sojae* race used in these studies. These various observations suggest that the protection phenomenon is probably associated with non-race specific or general resistance (sometimes called horizontal resistance).

Although protection in the cotyledon model system will allow further characterization at a molecular level in this well defined system, we are also interested in extending these results to the whole plant level and to truly systemic protection. Recently, the herbicides lactofen [7] and norflurazon [30] have been shown to induce resistance in soybean, but little is known about the systemic nature of the protection. It will be interesting to determine how these chemically induced forms of resistance relate to the glucan/JA/ACC distal cell protection reported here.

This research was supported by grants from USDA-NRI, the Ohio Soybean Council and the Ohio Plant Biotechnology Consortium. Salaries and research support were also provided by State and Federal Funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University, U.S.A.

## REFERENCES

1. **Abassi PA, Graham MY, Graham TL.** 2001. Effects of soybean genotype on the glyceollin elicitation competency of cotyledon tissues to *Phytophthora sojae* glucan elicitors. *Physiological and Molecular Plant Pathology* **59**: 95–105.
2. **Ayers AR, Ebel J, Valent BS, Albersheim P.** 1976. Host-pathogen interactions. X. Fractionation and biological activity of an elicitor isolated from the mycelial walls of *Phytophthora megasperma* var. *sojae*. *Plant Physiology* **57**: 760–765.
3. **Bartnicki-Garcia S, Wang MC.** 1983. Biochemical aspects of morphogenesis in *Phytophthora*. *Phytophthora: Its Biology, Taxonomy, Ecology and Pathology* In: Erwin DC, Bartnicki-Garcia S, Tsao PH, eds, pp. 129. St. Paul, MN: American Phytopathological Society Press.
4. **Bolwell GP, Davies DR, Gerrish C, Auh C-K, Murphy TM.** 1998. Comparative biochemistry of the oxidative burst produced by rose and French bean cells reveals two distinct mechanisms. *Plant Physiology* **116**: 1379–1385.
5. **Bonhoff A, Grisebach H.** 1988. Elicitor-induced accumulation of glyceollin and callose in soybean roots and localized resistance against *Phytophthora megasperma* f. sp. *glycinea*. *Archives of Biochemistry and Biophysics* **246**: 149–154.
6. **Castanho B, Bass JT, Lundry DR, Graham TL.** 1981. The protection of soybean plants against *Phytophthora megasperma* var. *sojae* (PMS) by phytoalexin elicitors. *Phytopathology* **71**: 866.
7. **Dann EK, Diers BW, Hammerschmidt R.** 1999. Suppression of Sclerotinia stem rot of soybean by lactofen herbicide treatment. *Phytopathology* **89**: 598–602.
8. **Dempsey DA, Shah J, Klessig DE.** 1999. Salicylic acid and disease resistance in plants. *Critical Reviews in Plant Sciences* **18**: 547–575.
9. **Deverall BJ, Dann EK.** 1995. Induced resistance in legumes. *Induced Resistance to Disease in Plants* In: Hammerschmidt R, Kuc J, eds, Boston, MA, USA: Kluwer Academic Publishers 1–30.
10. **Dong X.** 1998. SA, JA, ethylene, and disease resistance in plants. *Current Opinion Plant Biology* **1**: 316–323.
11. **Ebel J.** 1986. Phytoalexin synthesis: the biochemical analysis of the induction process. *Annual Reviews of Phytopathology* **24**: 235–264.
12. **Eppe P, Apel K, Bohlmann H.** 1995. An Arabidopsis thaliana thionin gene is inducible via a signal transduction pathway different from that for pathogenesis-related proteins. *Plant Physiology* **109**: 813–820.
13. **Farmer EE, Ryan CA.** 1992. Octadecanoid-derived signals in plants. *Trends in Cell Biology* **2**: 236–241.
14. **Graham TL.** 1991. A rapid, high resolution HPLC profiling procedure for plant and microbial aromatic secondary metabolites. *Plant Physiology* **95**: 584–593.
15. **Graham TL.** 1994. Cellular biochemistry of phenylpropanoid responses of soybean to infection by *Phytophthora sojae*. *Handbook of Phytoalexin Metabolism and Action* In: Daniel M, Purkayastha RP, eds. New York: Marcel Dekker, Inc, 85–116.
16. **Graham MY, Graham TL.** 1991. Rapid accumulation of anionic peroxidases and phenolic polymers in soybean cotyledon tissues following treatment with *Phytophthora megasperma* f. sp. *glycinea* wall glucan. *Plant Physiology* **97**: 1445–1455.
17. **Graham MY, Graham TL.** 1994. Wound-associated competency factors are required for the proximal cell responses of soybean to the *Phytophthora sojae* wall glucan elicitor. *Plant Physiology* **105**: 571–578.

18. **Graham TL, Graham MY.** 1991. Glyceollin elicitors induced major but distinctly different shifts in isoflavonoid metabolism in proximal and distal soybean cell populations. *Molecular Plant-Microbe Interactions* **4**: 60–68.
19. **Graham TL, Graham MY.** 1996. Signaling in soybean phenylpropanoid responses: dissection of primary, secondary, and conditioning effects of light, wounding, and elicitor treatments. *Plant Physiology* **110**: 1123–1133.
20. **Graham TL, Graham MY.** 1999. Role of hypersensitive cell death in conditioning elicitation competency and defense potentiation. *Physiological and Molecular Plant Pathology* **55**: 13–20.
21. **Graham TL, Graham MY.** 2000. Defense potentiation and elicitation competency: redox conditioning effects of salicylic acid and genistein. In: Stacey G, Keen NT, eds. *Plant Microbe Interactions*. St. Paul, MN: APS Press, 181–220.
22. **Graham TL, Kim JE, Graham MY.** 1990. Role of constitutive isoflavone conjugates in the accumulation of glyceollin in soybean infected with *Phytophthora megasperma*. *Molecular Plant-Microbe Interactions* **3**: 157–166.
23. **Gullino ML, Leroux P, Smith CM.** 2000. Uses and challenges of novel compounds for plant disease control. *Crop Protection* **19**: 1–11.
24. **Hunt MD, Neuenschwander UH, Delaney TP, Weymann KB, Friedrich LB, Lawton KA, Steiner H-Y, Ryals JA.** 1996. Recent advances in systemic acquired resistance research: A review. *Gene* **179**: 89–95.
25. **Kauss H, Krause K, Jeblick W.** 1992. Methyl jasmonate conditions parsley suspension cells for increased elicitation of phenylpropanoid defense responses. *Biochemical and Biophysical Research Communications* **189**: 304–308.
26. **Kauss H, Theisinger-Hinkel E, Mindermann R, Conrath U.** 1992. Dichloroisonicotinic and salicylic acid, inducers of systemic acquired resistance, enhance fungal elicitor responses in parsley cells. *The Plant Journal* **2**: 655–660.
27. **Kessmann H, Staub T, Hofmann C, Maetzke T, Herzog J, Ward E, Uknes S, Ryals J, Cook RJ.** 1994. Induction of systemic acquired disease resistance in plants by chemicals. *Annual Reviews of Phytopathology* **32**: 439–459.
28. **Kuc J.** 1995. Systemic acquired resistance. *Aspects of Applied Biology* **42**: 235–242.
29. **Mauch-Mani B, Metraux JP.** 1998. Salicylic acid and systemic acquired resistance to pathogen attack. *Annals of Botany* **82**: 535–540.
30. **Mohr PG, Cahill DM.** 2001. Relative roles of glyceollin, lignin and the hypersensitive response and the influence of ABA in compatible and incompatible interactions with *Phytophthora sojae*. *Physiological and Molecular Plant Pathology* **58**: 31–41.
31. **Park R-D, Kim K-S, Cho M-J.** 1992. Purification and characterization of ethylene-induced beta-1,3-glucanase from soybean *Glycine max* (L.) leaves. *Korean Biochemical Journal* **25**: 597–603.
32. **Paxton JD, Chamberlain DW.** 1967. Acquired local resistance of soybean plants to *Phytophthora* spp. *Phytopathology* **57**: 351–353.
33. **Penninckx IAMA, Eggermont K, Terras FRG, Thomma BPHJ, De Samblanx GW, Buchala A, Metraux J-P, Manners JM, Broekaert WE.** 1996. Pathogen-induced systemic activation of a plant defensin gene in arabidopsis follows a salicylic acid-independent pathway. *Plant Cell* **8**: 2309–2323.
34. **Qutob D, Hrabec PT, Sobral BWS, Gijzen M.** 2000. Comparative analysis of expressed sequences in *Phytophthora sojae*. *Plant Physiology* **123**: 243–253.
35. **Rivera-Vargas LI, Schmitthenner AF, Graham TL.** 1993. Soybean flavonoid effects on and metabolism by *Phytophthora sojae*. *Phytochemistry* **32**: 851–857.
36. **Ryals JA, Neuenschwander KH, Willits MG, Molina A, Steiner H-Y, Hunt MD.** 1996. Systemic acquired resistance. *Plant Cell* **8**: 1809–1819.
37. **Siegrist J, Jeblick W, Kauss H.** 1994. Defense responses in infected and elicited cucumber (*Cucumis sativus* L.) hypocotyl segments exhibiting acquired resistance. *Plant Physiology* **105**: 1365–1374.
38. **Sticher L, Mauch-Mani B, Metraux J-P.** 1997. Systemic acquired Resistance. *Annual Reviews of Phytopathology* **35**: 235–270.
39. **Stintzi A, Heitz T, Prasad V, Wiedemann-Merdinoghi S, Kauffmann S, Geoffroy P, Legrand M, Fritig B.** 1993. Plant pathogenesis related proteins and their role in defense against pathogens. *Biochimie* **75**: 687–706.
40. **Svoboda WE, Paxton JD.** 1972. Phytoalexin production in locally cross-protected Harosoy and Harosoy-63 soybeans. *Phytopathology* **62**: 1457–1460.
41. **Thulke O, Conrath U.** 1998. Salicylic acid has a dual role at the activation of defense-related genes in parsley. *Plant Journal* **14**: 35–42.
42. **Tkeuchi Y, Yoshikawa M, Takeba G, Tanaka K, Shibata D, Horino O.** 1990. Molecular cloning and ethylene induction of messenger RNA encoding a phytoalexin elicitor-releasing factor, beta-1,3-endoglucanase, in soybean. *Plant Physiology* **93**: 673–682.
43. **Uknes S, Vernooij B, Morris S, Chandler D, Steiner H-Y, Specker N, Hunt M, Neuenschwander U, Lawton K, Starrett M, Friedrich L, Weymann K, Negrotto D, Grolach J, Lanahan M, Salmeron J, Ward E, Kessmann H, Ryals J.** 1996. Reduction of risk for growers: Methods for the development of disease-resistant crops. *New Phytologist* **133**: 3–10.
44. **van Loon LC.** 1997. Induced resistance in plants and the role of pathogenesis-related proteins. *European Journal of Plant Pathology* **103**: 753–765.
45. **van Loon LC, Bakker PAHM, Pieterse CMJ.** 1998. Systemic Resistance Induced by Rhizosphere Bacteria. *Annual Reviews of Phytopathology* **36**: 453–483.
46. **Ward EWB.** 1983. Effect of mixed and consecutive inoculations on the interactions of soybeans with races of *Phytophthora megasperma* f. sp. *glycinea*. *Physiological Plant Pathology* **23**: 281–294.
47. **Wrather JA, Elrod JM.** 1990. Apparent systemic effect of *Colletotrichum truncatum* and *C. lagenarium* on the interaction between soybean and *C. truncatum*. *Phytopathology* **80**: 472–474.
48. **Yoshikawa M, Keen NT, Wang M-C.** 1983. A receptor on soybean *Glycine max* cultivar Harosoy membranes for a fungal elicitor of phytoalexin accumulation. *Plant Physiology* **73**: 497–506.
49. **Yoshikawa M, Takeuchi Y, Horino O.** 1990. A mechanism for ethylene-induced disease resistance in soybean: enhanced synthesis of an elicitor-releasing factor,  $\beta$ -1,3-endoglucanase. *Physiological and Molecular Plant Pathology* **37**: 367–376.
50. **Zahringer U, Shaller E, Griesebach H.** 1981. Induction of phytoalexin synthesis in soybean. Structure and reactions of naturally occurring and enzymatically prepared prenylated pterocarpanes from elicitor-treated cotyledons and cell cultures of soybean. *Zeitschrift fuer Naturforsch* **36**: 234–341.