

## Relationship Between Localized Glyceollin Accumulation and Metalaxyl Treatment in the Control of Phytophthora Rot in Soybean Hypocotyls

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### ABSTRACT

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Soybean seedlings (cultivar Altona) were treated with the systemic fungicide metalaxyl by immersing the roots in solutions of 0.5, 1.0, 1.5, or 2.0  $\mu\text{g/ml}$  for 18 hr. Controls were immersed in water only. After treatment, hypocotyls were inoculated with drops of zoospore suspensions of *Phytophthora megasperma* f. sp. *glycinea* (*Pmg*), race 4 (incompatible) or race 6 (compatible). Water drops were used on uninoculated controls. After incubation for 24–72 hr, the reaction type (resistant or susceptible) was recorded and the tissues at the inoculation site were excised and analyzed for metalaxyl and for glyceollin (a phytoalexin) by bioautography, gas-liquid chromatography, and spectrophotometry. Plants inoculated with the incompatible race developed limited necrotic lesions regardless of metalaxyl treatment. Untreated plants inoculated with the compatible race 6 developed spreading water-soaked lesions. However, in metalaxyl-treated plants the compatible race produced limited necrotic lesions by 24 hr, similar to those produced with the incompatible race. These lesions

remained restricted throughout 72 hr incubation, except in plants treated with 0.5  $\mu\text{g}$  metalaxyl per milliliter. In the latter, lesions had spread by 48 hr and hypocotyls were extensively colonized by 72 hr. Generally, fungicide concentrations were greater in infected lesions than at similar sites that received water droplets instead of inoculum. Lesions in plants protected from the compatible race contained metalaxyl concentrations in excess of the in vitro  $\text{EC}_{90}$  for *Pmg* (0.8  $\mu\text{g/ml}$ ). This concentration was not reached in lesions in plants treated with only 0.5  $\mu\text{g}$  metalaxyl per milliliter. Except in untreated plants inoculated with the compatible race, glyceollin concentrations exceeded the in vitro  $\text{EC}_{90}$  in all lesions examined. In spite of this, in plants only partially protected by 0.5  $\mu\text{g}$  metalaxyl per milliliter, spread of the fungus was not restricted more than would be expected from the metalaxyl concentration alone. The results suggest that glyceollin does not contribute to inhibition of spread of the fungus under these conditions.

Van der Kerk (20) drew attention to several early examples of the protection of plants from disease by systemic compounds that interfered with the normal biochemical relationship between plants and pathogens. More recently Cartwright et al (2) and Langcake and Wickins (8,9) provided evidence that phytoalexin production may be involved in the control of blast in rice plants treated with dichlorocyclopropanes. Reilly and Klarman (16) also reported that several fungicides stimulated production of the phytoalexin, glyceollin, in soybeans (*Glycine max* L. Merr.). In tests with several systemic fungicides (12), we observed that control of *Phytophthora megasperma* f. sp. *glycinea* (*Pmg*) in soybean hypocotyls resulted in hypersensitivity typical of a resistant response to incompatible races of *Pmg*. Subsequently it was shown (21) that, in hypocotyls treated with metalaxyl [DL-methyl *N*-(2,6-dimethylphenyl)-*N*-(2-methoxyacetyl) alaninate], glyceollin accumulation was associated with this type of response. In incompatible interactions, glyceollin accumulation has been considered to be the main event leading to restriction of fungal growth (1,5,6,25). Although metalaxyl alone is strongly inhibitory to *Pmg*, we suggested previously (21) that, in tissues with less than fully inhibitory concentrations of metalaxyl, localized accumulation of glyceollin might play a supplementary role in control of the pathogen. The present study attempts to resolve the significance of glyceollin accumulation in such instances by determining the concentration of metalaxyl and glyceollin at the lesion site in the natural hypersensitive response to the incompatible race 4 and in the fungicide-mediated hypersensitive response to the compatible race 6 of *Pmg*.

### MATERIALS AND METHODS

The procedures used for growing soybean seedlings and races of *Pmg* and for producing zoospores have been reported previously

(12,21–23). Metalaxyl solutions were prepared in deionized water from an analytical standard of the compound (kindly supplied by Ciba Geigy Canada Ltd., Mississauga, Ontario).

Etiolated 5-day-old cultivar Altona soybean seedlings were carefully uprooted, and groups of 60 seedlings were placed in 400-ml plastic beakers containing 200 ml of metalaxyl solutions or water to cover the roots. The plants were allowed to take up the fungicide in the dark for 18 hr, then they were washed thoroughly under running tap water to remove excess fungicide from the surface and placed horizontally in racks in glass trays as described previously (12,21). For any one treatment, 20 plants were inoculated at the top of the hypocotyl with four 10- $\mu\text{l}$  drops of a race 4 (incompatible) or race 6 (compatible) zoospore suspension ( $10^5$  zoospores per milliliter) or with drops of distilled water. The trays were covered with plastic film and incubated at 25 C for 24 hr. The diffusate drops were then removed, and tissue samples 0.5–1.0 mm thick were excised from the area directly underneath the droplets. In some experiments the diffusates and excised tissues were combined for analysis, whereas in others the diffusate drops were removed 5 hr after inoculation and discarded. Neither glyceollin nor metalaxyl was detectable in the diffusates after this period of incubation. The excised tissues were extracted in 4 ml of 95% ethanol for 2–4 hr. The ethanol was collected and replaced by 4 ml of fresh ethanol, and the samples were boiled in a water bath for 2 min. The ethanol was collected and the tissues were extracted further in the same volume of fresh ethanol at 4 C overnight. The tissues received a final rinse with three 1.0-ml volumes of ethanol, and the combined ethanol extracts were reduced to dryness in a rotary evaporator. The dry residue was then extracted with four 0.5-ml volumes of ethyl acetate. The pooled extracts were transferred to vials and reduced to dryness at 40 C under  $\text{N}_2$  gas.

**Glyceollin determinations.** The residue in the vials was redissolved in 100 or 200  $\mu\text{l}$  of ethyl acetate, and either the entire sample or a fraction of it (based on equivalent dry weight of extracted tissues) was applied to two or three channels on a Whatmann LK6DF thin-layer chromatography (tlc) plate. Chromatograms were developed in either ethyl acetate:tertiary

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butanol (95:5, v/v), or acetone:chloroform:ammonium hydroxide (50:50:1, v/v). Glyceollin was located on chromatograms by quenching of fluorescence in ultraviolet (UV) light and reference to a glyceollin standard. Silica gel containing the glyceollin was scraped from the plates, placed in small glass columns, and eluted with three 1.0-ml volumes of absolute ethanol. The UV spectrum of each eluate was recorded from 230 to 310 nm by using a scanning spectrophotometer. The glyceollin concentration was calculated from the maximum absorbance at 285 nm and the extinction coefficient,  $\epsilon = 10,300$ .

**Metalaxyl determinations. Thin-layer bioautography.** Samples of the ethyl acetate extract from 3.5 mg of tissue (dry weight basis) and glyceollin and metalaxyl standards were spotted on precoated 20 × 20-cm silica gel plates (Polygram SIL NHR, Machery Nagel & Co., Postfach 307, 5160 Düren, West Germany) and developed in ethyl acetate:tertiary butanol (95:5). The plates were dried and sprayed with encysted zoospores of *Pmg* in V-8 agar as described elsewhere (10). The chromatograms were incubated for 24 hr at 25 C in closed Pyrex trays lined with moist paper. Zones of growth inhibition were demonstrated by immersing chromatograms in a suspension of charcoal in water (3–4%, w/v). The radii of the inhibition zones were measured and metalaxyl was estimated by reference to a standard curve prepared from measurements of inhibition zones obtained with a series of metalaxyl standards (10).

**Gas-liquid chromatography (glc).** The entire ethyl acetate-soluble fraction of the ethanol extract of lesions, obtained from 40 plants per treatment, was chromatographed as described previously. Metalaxyl was located on the plates by the spray procedure of Singh and Tripathi (17). The fungicide was eluted from the silica gel with four 0.5-ml volumes of ethyl acetate and the eluate was concentrated under N<sub>2</sub> gas and transferred to a small conical glc vial. Ten microliters of methyl meristate, as an internal standard, was added, and the sample was rinsed to the bottom of the vial with ethyl acetate. The solvent was evaporated under N<sub>2</sub> gas and the final residue was dissolved in 10  $\mu$ l of ethyl acetate. Three or four 0.5–2.0- $\mu$ l aliquots from each ethyl acetate solution were injected into a Hewlett-Packard gas-liquid chromatograph (flame ionization detector; column 180 cm × 1.5 mm packed with 3% SE 30 on Gas Chrom Q 80-100; initial column temperature 200 C; N<sub>2</sub> flow rate 10 ml/min). Retention time for metalaxyl was 6.7 min. After analysis, the column was purged of less volatile material at 250 C for 16 min. A metalaxyl standard was injected periodically to monitor column performance.

**Extraction efficiency.** For each experiment, a duplicated series of standard samples of glyceollin and metalaxyl was added to tubes containing 1 g (fresh weight) of untreated soybean hypocotyl tissue. These samples were processed the same as experimental material, and the results obtained were used to calculate the efficiency of recovery (glyceollin, 50–60%; metalaxyl, 40–50%). In addition, in all experiments, standard samples were tested to check that conditions used to prepare standard curves had been maintained. Data have been corrected for loss during extraction.

**Determination of tissue weights.** After tissues were extracted, they were transferred to preweighed aluminum foil and dried overnight in an oven at 80 C. Metalaxyl or glyceollin concentrations are expressed as micrograms per gram fresh weight of tissue on the basis that 1 mg dry weight of extracted tissue weighed 30.02 mg prior to extraction.

**Determinations of in vitro toxicity.** The inhibitory concentrations for metalaxyl and glyceollin in vitro were determined by radial growth bioassays. One hundred microliters of a 50% ethanol solution of a metalaxyl or glyceollin standard was injected into 2.4 ml of sterile water which was immediately mixed with 2.5 ml of hot V-8 juice agar (2%). Approximately 1.5 ml of this agar was then poured into each of three petri plates (3.5 cm in diameter). Agar plates that received ethanol only were prepared similarly. When they had cooled, the plates were inoculated with a plug of mycelium from the periphery of a *Pmg* colony growing on V-8 juice agar. Radii of colonies were measured after 3 days of incubation at 24 C. A standard curve relating percent inhibition to the logarithm of concentration was prepared to determine the relationship between the concentration of metalaxyl or glyceollin

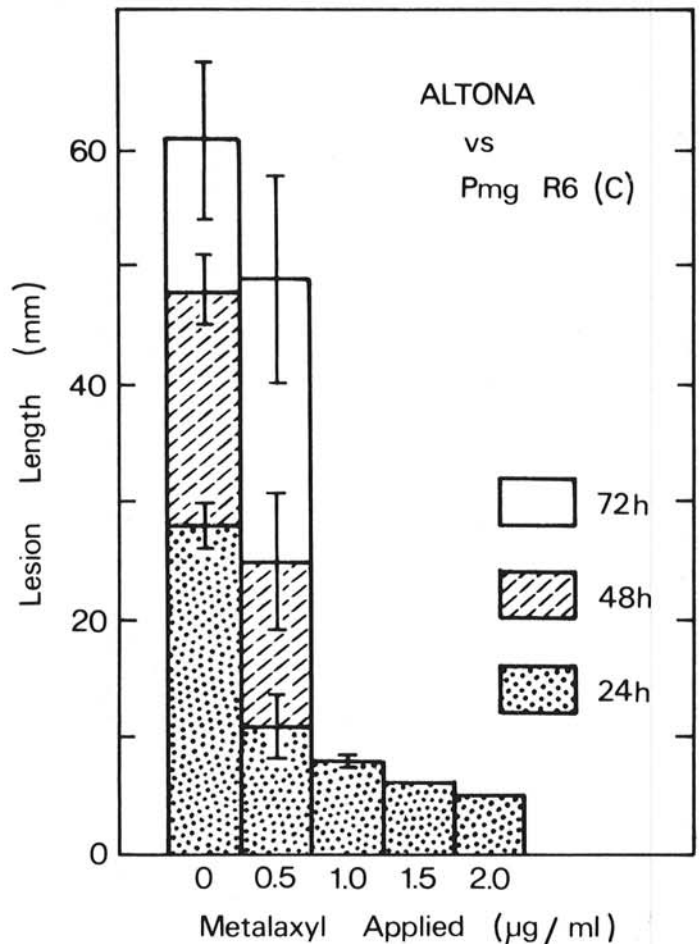
and growth inhibition.

**Measurements of lesion size.** Ten plants for each concentration of fungicide tested were inoculated with one 10- $\mu$ l drop of zoospores from the compatible race. These plants were incubated as described above together with the correspondingly treated plant used for glyceollin and metalaxyl determinations. Lesion lengths were measured at 24, 48, and 72 hr.

## RESULTS

Lesions on hypocotyls inoculated with race 4 (incompatible) were restricted to the area of the inoculum droplet, and surface cells displayed a typical, necrotic hypersensitive response in both untreated and metalaxyl-treated plants. Untreated hypocotyls inoculated with race 6 (compatible) developed rapidly expanding water-soaked lesions, whereas all metalaxyl-treated plants inoculated with race 6 developed restricted lesions with considerable necrosis within 24 hr after inoculation (Fig. 1). These lesions remained restricted except in plants treated with only 0.5  $\mu$ g metalaxyl per milliliter. In these plants, lesions had spread within 48 hr and hypocotyls were extensively rotted within 72 hr (Fig. 1). At the highest metalaxyl concentration, necrosis tended to be less extensive with both races than in the typical hypersensitive response with race 4 in untreated plants.

Thin-layer bioautographs of extracts from metalaxyl-treated plants inoculated with water droplets displayed only one inhibitory spot; this co-chromatographed with the metalaxyl standard (Fig. 2A). The size of the inhibition zone was proportional to the



**Fig. 1.** The relationship between lesion length and metalaxyl treatments on seedlings of soybean cultivar Altona at 1, 2, and 3 days after inoculation with the compatible (C) race 6 of *Phytophthora megasperma* f. sp. *glycinea*. Vertical lines indicate the deviation from the mean obtained from measurements of 10 plants.

metalaxyl concentration administered. Extracts from plants that did not receive metalaxyl had no inhibitory activity (Fig. 2A). No zones of inhibition were detected on bioautographs of extracts from untreated plants inoculated with race 6 (compatible) (Fig. 2B). However, two inhibitory spots were detected on bioautographs of extracts from metalaxyl-treated plants inoculated with this race. A large one that corresponded to metalaxyl was found in all extracts, and a smaller one that corresponded to glyceollin was found in all extracts except those from plants that received 0.5  $\mu\text{g}$  metalaxyl per milliliter. In these latter plants, the level of glyceollin was below the limit of detection by this technique. The inhibition zone corresponding to glyceollin was detected also on bioautographs of extracts from metalaxyl-treated and untreated plants inoculated with race 4 (incompatible) (Fig. 2C).

The concentrations of metalaxyl (Table 1), estimated from thin-layer bioautographs, in the tissue of lesions from *Pmg*-inoculated plants were generally higher than the concentrations in comparable tissue from uninoculated plants.

In subsequent experiments metalaxyl was determined by glc (Table 1). The inoculum drops were removed after 5 hr of incubation as a precaution against loss of metalaxyl and glyceollin into the diffusate and to ensure that the values obtained represented tissue concentrations only. Although the values obtained by glc tended to be higher, they generally confirmed the trends indicated by thin-layer bioautography. Except for plants treated with 0.5  $\mu\text{g}$  metalaxyl per milliliter, metalaxyl levels in tissues of lesions from treated plants greatly exceeded the  $\text{EC}_{90}$  for growth inhibition in vitro (Table 2) and were several times greater than those in uninfected tissue. These plants were completely protected and the

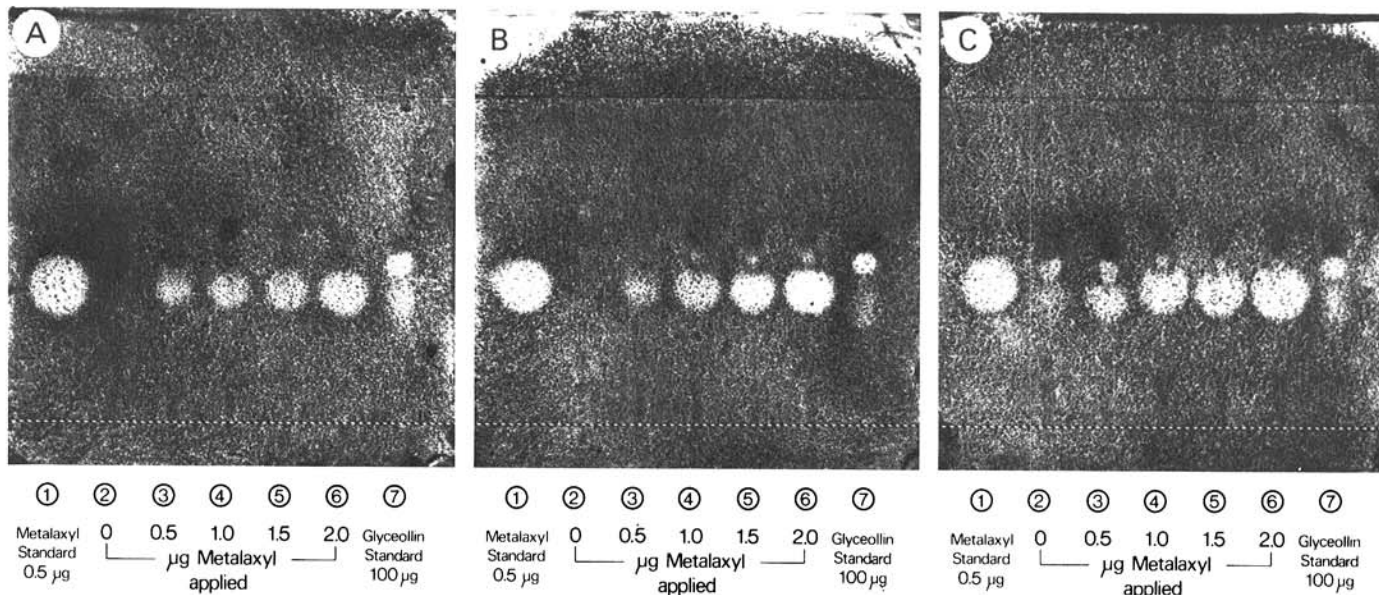


Fig. 2. Thin-layer bioautographs of extracts from lesions of metalaxyl-treated seedlings of soybean cultivar Altona inoculated with A, drops of distilled water, or suspensions containing zoospores of B, race 6 (compatible) or C, race 4 (incompatible) of *Phytophthora megasperma* f. sp. *glycinea*.

TABLE 1. Concentrations of metalaxyl and glyceollin in tissues at sites on soybean hypocotyls inoculated with race 4 (incompatible) or race 6 (compatible) of *Phytophthora megasperma* f. sp. *glycinea* following treatment of the roots of soybean seedlings with metalaxyl

Experiments <sup>a</sup>	Inoculum <sup>b</sup>	Metalaxyl supplied to roots <sup>c</sup> ( $\mu\text{g}/\text{ml}$ ):				
		0	0.5	1.0	1.5	2.0
Thin-layer bioautography Residual metalaxyl <sup>d</sup> ( $\mu\text{g}/\text{g}$ fr. wt.)	Water	0	0.11 $\pm$ 0.06	0.25 $\pm$ 0.26	0.43 $\pm$ 0.37	1.18 $\pm$ 0.54
	Race 4	0	0.43 $\pm$ 0.01	1.22 $\pm$ 0.52	2.19 $\pm$ 1.13	4.94 $\pm$ 2.13
	Race 6	0	0.32 $\pm$ 0.22	0.89 $\pm$ 0.59	1.65 $\pm$ 0.73	2.50 $\pm$ 1.36
Glyceollin ( $\mu\text{g}/\text{g}$ fr. wt.)	Race 4	540 $\pm$ 206	1,185 $\pm$ 133	569 $\pm$ 143	813 $\pm$ 266	629 $\pm$ 83
	Race 6	10 $\pm$ 14	220 $\pm$ 24	473 $\pm$ 203	363 $\pm$ 127	243 $\pm$ 90
Gas-liquid chromatography Residual metalaxyl ( $\mu\text{g}/\text{g}$ fr. wt.)	Water	0	0.13 $\pm$ 0.01	0.42 $\pm$ 0.04	0.33 $\pm$ 0.17	0.90 $\pm$ 0.40
	Race 4	0	1.23 $\pm$ 0.35	2.06 $\pm$ 0.08	4.24 $\pm$ 0.61	4.88 $\pm$ 1.16
	Race 6	0	0.81 $\pm$ 0.25	1.75 $\pm$ 0.35	3.13 $\pm$ 1.17	4.05 $\pm$ 0.04
Glyceollin ( $\mu\text{g}/\text{g}$ fr. wt.)	Race 4	1,200 $\pm$ 643	851 $\pm$ 436	1,334 $\pm$ 709	987 $\pm$ 418	1,361 $\pm$ 721
	Race 6	63 $\pm$ 9	706 $\pm$ 127	949 $\pm$ 495	1,143 $\pm$ 469	709 $\pm$ 311

<sup>a</sup>Two series of experiments were performed. In the first series, metalaxyl was estimated from the diameter of zones of growth inhibition on thin-layer chromatograms sprayed with encysted zoospores of *Pmg*. In the second series, metalaxyl was determined by gas-liquid chromatography. In both series, glyceollin was determined spectrophotometrically by its absorbance at 285 nm, after partial purification by thin-layer chromatography. Data are the means and standard errors from two separate experiments in each series.

<sup>b</sup>Hypocotyls were inoculated with four 10- $\mu\text{l}$  drop of either water or zoospore suspension ( $10^5$  zoospores per milliliter) of race 4 (incompatible) or race 6 (compatible) of *Pmg*.

<sup>c</sup>Roots of 5-day-old etiolated seedlings of soybean cultivar Altona were immersed in metalaxyl solutions in distilled water for 18 hr prior to inoculation.

<sup>d</sup>Infected tissue immediately beneath the inoculum drop, or a similar amount of tissue in water controls, was excised and extracted in ethanol. Metalaxyl and glyceollin were determined in the extracts following chromatography as described.

compatible race 6 did not spread from the inoculated site. Concentrations in tissues after treatment of plants with 0.5  $\mu\text{g}$  metalaxyl per milliliter were well below the  $\text{EC}_{90}$  level according to the thin-layer bioautography results but were at (race 6) or above (race 4) the  $\text{EC}_{90}$  level according to the glc data (Table 1). In addition to differences between the values obtained by the two techniques, there were large differences between the values obtained in separate experiments. Regardless of this variation, however, the level of metalaxyl in these plants was insufficient to prevent spread of race 6 lesions.

Glyceollin accumulation in the tissue from lesions of metalaxyl-treated plants was similar to that reported previously (21). High concentrations were detected in tissues inoculated with race 4 (incompatible), and these did not appear to be affected significantly by metalaxyl treatment (Table 1). Infected tissues from untreated hypocotyls inoculated with race 6 (compatible) contained only small amounts of glyceollin, but very much larger amounts accumulated in lesions in metalaxyl-treated plants. Glyceollin levels usually were lower than in lesions from plants inoculated with race 4 although there was considerable variation between experiments. There was no evidence of a consistent relationship between glyceollin accumulation and metalaxyl concentration. However, glyceollin levels were equal to or higher than the in vitro  $\text{EC}_{90}$  (Table 2) in tissues from lesions of all inoculated plants that had been treated with metalaxyl.

## DISCUSSION

Hypersensitivity and phytoalexin production have been considered to be expressions of monogenic soybean resistance to *Pmg* (1,5,6). However, it is evident from the results described here as well as from other studies (3,7,11,14,15,21,23) that these responses can occur in compatible interactions, in which resistance genes are not expressed, if infected plants are exposed to chemical or physical conditions that are unfavorable to the pathogen. The results reported here indicate also that in metalaxyl-treated plants these responses are insufficient to confer resistance.

Treatment of soybean seedlings with metalaxyl at concentrations of 1  $\mu\text{g}/\text{ml}$  or higher completely prevented spread of race 6 (compatible) lesions. The concentration of metalaxyl in the tissues of these lesions always exceeded the  $\text{EC}_{90}$  for metalaxyl in vitro. Spread of race 6 lesions, however, was not prevented by treatments with 0.5  $\mu\text{g}$  metalaxyl per milliliter although the rate of spread in the first 24 hr was reduced 46–73% compared with that in untreated plants (Fig. 1). Somewhat higher inhibition would have been expected if the in vivo activity of metalaxyl exactly duplicated its in vitro activity. Thus, 0.32  $\mu\text{g}$  metalaxyl per milliliter determined by bioautography and 0.81  $\mu\text{g}/\text{ml}$  determined by glc (Table 1) should have caused 80–90% inhibition (Table 2). It appears, therefore, that the metalaxyl concentration in tissue infected with race 6 was sufficient to account for all the inhibition of lesion spread that was observed. The glyceollin that accumulated in these lesions, although it exceeded the in vitro  $\text{EC}_{90}$  or  $\text{EC}_{100}$  according to the bioautographic or glc data, respectively (Tables 1 and 2), evidently did not limit the spread of the fungus.

Several authors have concluded that the accumulation of glyceollin concentrations similar to those demonstrated here following metalaxyl treatment accounts for the restriction of the

fungus that occurs in lesions in incompatible interactions (1,5,6,25). It is difficult to explain, therefore, why, in apparently similar lesions in plants treated with 0.5  $\mu\text{g}$  of metalaxyl per milliliter, glyceollin did not restrict the spread of the compatible race. Possibly, glyceollin does not accumulate uniformly throughout the lesion and may not reach inhibitory concentrations in the vicinity of the growing hyphal tips. There is evidence that, even in incompatible interactions, glyceollin concentrations in freshly invaded tissues are lower than in tissues in older parts of the lesion (25). There is also the possibility that glyceollin is compartmentalized in metalaxyl-restricted lesions and not in race-specific incompatible interactions. The responses of the host cells in both situations, however, appeared to be very similar in histological and ultrastructural studies (18). It was concluded from those studies that metalaxyl acted primarily on the fungus and that the manifestations of incompatibility that developed were secondary effects, possibly in response to materials liberated from damaged hyphae and to reduction in the rate of spread of the fungus. Although a causal relationship between glyceollin production and resistance does not appear to hold for metalaxyl-treated plants, the association between glyceollin production and necrosis remains consistent and supports previous conclusions that glyceollin production is associated more closely with necrosis than with resistance (11,23).

Although the evidence suggests that metalaxyl alone is responsible for restricting the growth of *Pmg* in treated plants, the possibility that host responses other than glyceollin production may complement the effect of the fungicide have not been ruled out. Hickey and Coffey (4) found that *Peronospora pisi* mycelium in metalaxyl-treated pea leaves retained a normal ultrastructural appearance for several weeks. Fungal death was attributed to starvation of the fungus brought about by encasements formed by the host cells around the haustoria. Such host responses, however, were not observed in metalaxyl-treated soybean plants 24 hr after infection with a compatible *Pmg* race (18). On the contrary, mature hyphae were observed to contain damaged plasmalemma and mitochondrial membranes. Such damage could cause leakage of phytotoxic metabolites from the injured or killed fungus and induce a hypersensitive reaction, as was postulated by Érsek et al (3) and Király et al (7).

The accumulation of metalaxyl at the lesion site, consistently observed here, has been related in other studies to increased transpiration due to injury to the plant surface (13,15,19,24). In this study, metalaxyl accumulated even when diffusates were removed from the lesions, and hence increased concentrations cannot be explained simply by diffusion of fungicide from the surrounding tissues into the diffusates. It was observed that, when diffusates were removed, droplets frequently reformed on the surface of the lesion. This suggests that, under the influence of root pressure in the apoplast, water with fungicide in solution moves freely through the necrotic tissues to the surface. Tissues in lesions probably are thus more rapidly suffused with fungicide than those in uninfected parts of the hypocotyl. Evaporation from the surface also would result in further concentration of the fungicide. In seedlings treated with 0.5  $\mu\text{g}$  of metalaxyl, this process of fungicide accumulation resulted in the development of concentrations in lesions in excess of the in vitro  $\text{EC}_{90}$ , whereas concentrations in uninfected tissues remained well below the  $\text{EC}_{75}$  (Tables 1 and 2). The enhancement of localized fungicide concentrations by infection may contribute significantly to the protective and curative properties of metalaxyl.

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TABLE 2. Effective concentrations (EC) for the inhibition of growth of *Phytophthora megasperma* f. sp. *glycinea* by glyceollin or metalaxyl<sup>a</sup>

	Glyceollin ( $\mu\text{g}/\text{ml}$ )	Metalaxyl ( $\mu\text{g}/\text{ml}$ )
$\text{EC}_{50}$	56	0.07
$\text{EC}_{75}$	112	0.23
$\text{EC}_{90}$	200	0.80
$\text{EC}_{95}$	316	2.0†

<sup>a</sup> Values were derived from a probit plot of percentage inhibition of radial growth of *Pmg* race 4 against a range of concentrations of glyceollin or metalaxyl. The value marked with a dagger symbol was obtained by extrapolation.

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