Glyceollin Production Associated with Control of Phytophthora Rot of Soybeans by the Systemic Fungicide, Metalaxyl

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ABSTRACT

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Hypocotyls of soybean seedlings (cultivar Altona) inoculated with the compatible race 6 of *Phytophthora megasperma* var. *sojae* (*Pms*) developed typical spreading watersoaked lesions. When the roots of seedlings were supplied with the systemic fungicide *N*-(2,6-dimethylphenyl)-*N*-(methoxyacetyl) alanine methyl ester (metalaxyl), the lesion development was reduced. At metalaxyl concentrations of 20 μg/ml lesions became restricted, brown, necrotic, and indistinguishable from lesions in untreated hypocotyls inoculated with the incompatible race 4 of *Pms*. Glyceollin production in the first 12 hr following inoculation of hypocotyls of metalaxyl-treated seedlings with *Pms* race 6 was similar to that in the race 4 incompatible interaction. This rate was not maintained over longer incubation periods, and maximum glyceollin levels, which were reached after 24–36 hr, usually were about two thirds as great as those present in the incompatible interaction. Metalaxyl was taken up rapidly by roots and transported to the infection site. In metalaxyl-treated seedlings, race 6 gave rise to an incompatible response even when treatment was delayed until 3 hr after inoculation.

Reilly and Klarman (15) reported that several fungicides stimulated the production of the phytoalexin, glyceollin, in soybean (*Glycine max* L., Merr.) hypocotyl tissues. They suggested that possibly phytoalexins might contribute to the effectiveness of fungicides in the field, especially if this exceeds expectations from in vitro activity. There have been several reports of compounds with low in vitro fungitoxity that are effective fungicides in the plant (6,8,12,13) and Cartwright et al (2) have presented evidence that control of rice blast by dichlorocyclopropanes is accompanied by phytoalexin production.

Recently, in tests with several systemic fungicides for control of Phytophthora rot of soybean (14), it was observed that limited hypersensitive necrotic lesions developed when control was successful. These lesions were similar to those which develop in typical imcompatible interactions with the causal fungus, *Phytophthora megasperma* Drechs. var. *sojae* Hildeb. (*Pms*) and in which inhibitory levels of glyceollin accumulate (18).

In this paper we report that glyceollin is produced in inoculated hypocotyls of soybean seedlings treated with the systemic fungicide metalaxyl (*N*-[2,6-dimethylphenyl]-*N*-[methoxyacetyl] alanine methyl ester) and compare production of it under these conditions with that in a typical incompatible interaction.

MATERIALS AND METHODS

Details of growth of soybean seedlings and *Pms* races, and of inoculation procedures have been given in detail previously (14,18). Etiolated 5-day-old soybean (cultivar Altona) seedlings were placed horizontally in racks in glass trays and the roots were covered with cellucotton soaked with deionized water or metalaxyl solution. After incubation for 24 hr, or other periods as indicated, at 25 C in the dark, the hypocotyls were inoculated with four 10-μl droplets of a *Pms* zoospore suspension (1×10^5 zoospores per milliliter) spaced approximately 0.5 cm apart starting 1.5 cm below the cotyledons. The trays were closed with plastic film and incubated for 24 hr in the dark at 25 C. Races 4 and 6 of *Pms* were used, these are incompatible and compatible, respectively, on soybean cultivar Altona.

Metalaxyl solutions were prepared in deionized water with an analytical standard of the compound.

The isomers of glyceollin do not separate in the systems used here and are treated as a single compound. For glyceollin determination, diffusate droplets and the inoculated sections (2 cm) removed from 10 hypocotyls were transferred to 6 ml of 95% ethanol in test tubes and steeped for 72 hr. They were then heated at 100 C for 2 min by immersion of the tubes in a boiling water bath as described by Yoshikawa et al (20). The ethanol was removed and the hypocotyl sections were rinsed twice with 3 ml aliquots of absolute ethanol. The combined ethanol extracts were evaporated to dryness and the residue was applied quantitatively in ethyl acetate to two channels of a silica gel LK6DF (Whatman) thin-layer chromatography plate and chromatographed in benzene-methanol (95:8). Glyceollin was located on the chromatogram by fluorescence quenching in ultraviolet light and reference to a glyceollin standard. Silica gel in the glyceollin bands was scraped from the plates and eluted (in a small column) with 3 ml of absolute ethanol. Glyceollin was measured directly in the eluate using a scanning spectrophotometer by its absorbance at 285 nm and calculated by using the extinction coefficient, 10,300. After extraction, infected tissue was dissected from the hypocotyl tissues, dried and weighed. Glyceollin is reported in micrograms per milligram dry weight of infected tissue.

RESULTS

Lesions on hypocotyls inoculated with race 4 (incompatible) were restricted to the area of the inoculum droplet and surface cells displayed a typical hypersensitive response with limited brown necrosis. This response was not influenced by metalaxyl treatment, and the dry weight of infected tissue remained constant over a range of concentrations (Fig. 1A). In contrast, race 6 (compatible), in untreated hypocotyls, spread rapidly and caused extending watersoaked rotting lesions without browning. With increasing metalaxyl concentrations, the size of the lesions decreased, as indicated by the dry weight of infected tissue (Fig. 1A). They changed from watersoaked to intensely brown and necrotic and at the highest concentrations they were restricted and similar in appearance to the hypersensitive lesions produced by race 4.

Largest quantities of glyceollin were produced in tissues inoculated with race 4 and these were not influenced by metalaxyl treatment (Fig. 1B). Hypocotyls inoculated with race 6 produced only small amounts of glyceollin, but the amounts increased extensively with increasing amounts of metalaxyl, up to about 60%

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of those in the incompatible interaction. The time-course of glyceollin production (Fig. 2) indicates that production stimulated by metalaxyl never reaches that in the incompatible reaction. Rates of production in the first 12 hr following inoculation were similar, but subsequently the rate due to metalaxyl treatment tended to fall below that in the incompatible interaction. Maximum glyceollin levels were reached at similar times and the decline followed similar patterns in both the incompatible interaction and the metalaxyl-treated race 6 interaction. Metalaxyl treatment alone without inoculation did not stimulate glyceollin accumulation.

The timing of metalaxyl treatment did not affect the outcome of the interaction or glyceollin production in hypocotyls inoculated with race 6 unless it was delayed beyond 3 hr after inoculation (Table 1). When metalaxyl treatment was delayed until 6 hr after inoculation there was considerable spread of the fungus into the tissue surrounding the inoculation site, and glyceollin did not accumulate to as high a concentration. These results indicate that metalaxyl is rapidly taken up by the seedlings and transported to the hypocotyl region.

DISCUSSION

Control of *Pms* infection of soybean hypocotyls by metalaxyl causes a hypersensitive type of response accompanied by glyceollin production that appears to duplicate a typical race-cultivar incompatible interaction. Even if metalaxyl reached concentrations at the infection site that were less than fully inhibitory, it seems quite possible that the glyceollin accumulating there could play a critical role in preventing disease development. Several other investigators have reported hypersensitive symptoms during control of fungal diseases by systemic fungicides (3,4,7,9,13,14, 16,17), and in one case the associated production of phytoalexinlike compounds was described (2). These reports include quite different fungicides and host pathogen combinations, so it is possible that association of hypersensitivity with control by

TABLE 1. Influence of the time of addition of metalaxyl relative to the time of inoculation on glyceollin production in soybean (cultivar Altona) hypocotyls inoculated with *Phytophthora megasperma* var. *sojae* (race 6, compatible)

Time of metalaxyl addition (hr)[a]	Incubation[b] period	Glyceollin µg/mg of dry tissue	
		Metalaxyl	Control
−24	24	14.5	...
0	24	12.0	2.3
+ 3	27	12.0	3.2
+ 6	30	9.7	3.1
+12	36	5.6	3.5

[a] Metalaxyl (20 µg/ml) added to roots of 5-day-old etiolated Altona soybean hypocotyls (cultivar Altona) at the times indicated relative to the time of inoculation, 0 hr. Hypocotyls were inoculated with four droplets (10 µl) of a zoospore suspension (1×10^5 zoospores per milliliter) of *Phytophthora megasperma* var. *sojae* race 6.

[b] Hypocotyls were harvested and glyceollin was extracted after 24 hr of incubation following the addition of metalaxyl except in the −24 hr treatment which was harvested 24 hr following inoculation.

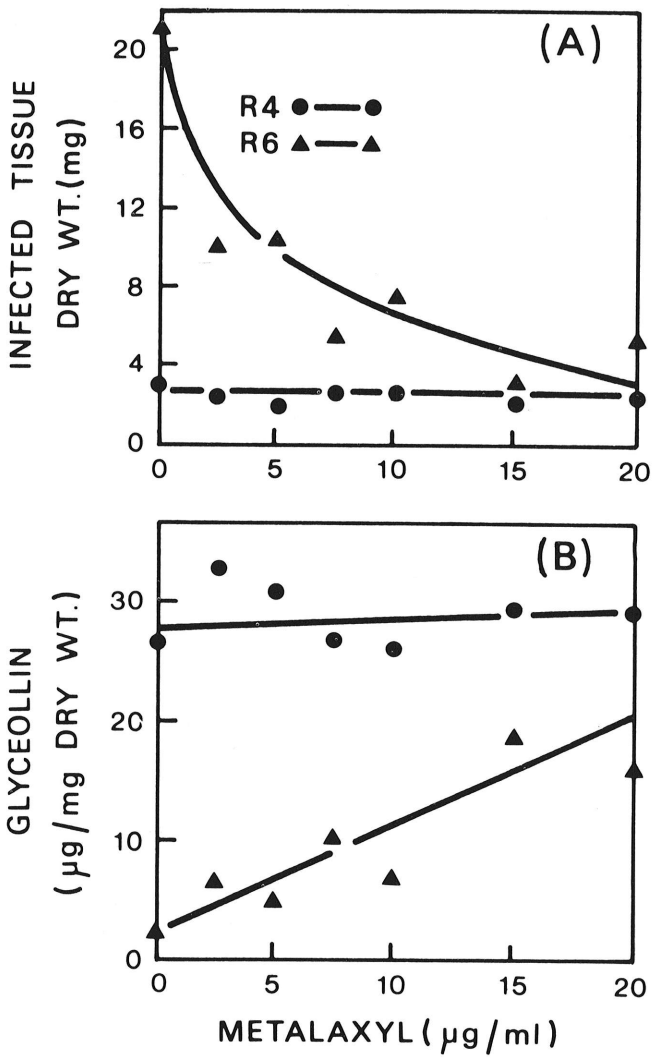

Fig. 1. Influence of metalaxyl applied to roots, on infection (dry weight of infected tissue, Fig. 1A) and glyceollin production (Fig. 1B) in soybean (cultivar Altona) hypocotyls inoculated with zoospores of *Phytophthora megasperma* var. *sojae* race 4 (incompatible, •——•) and race 6 (compatible, ▲——▲) and incubated for 24 hr.

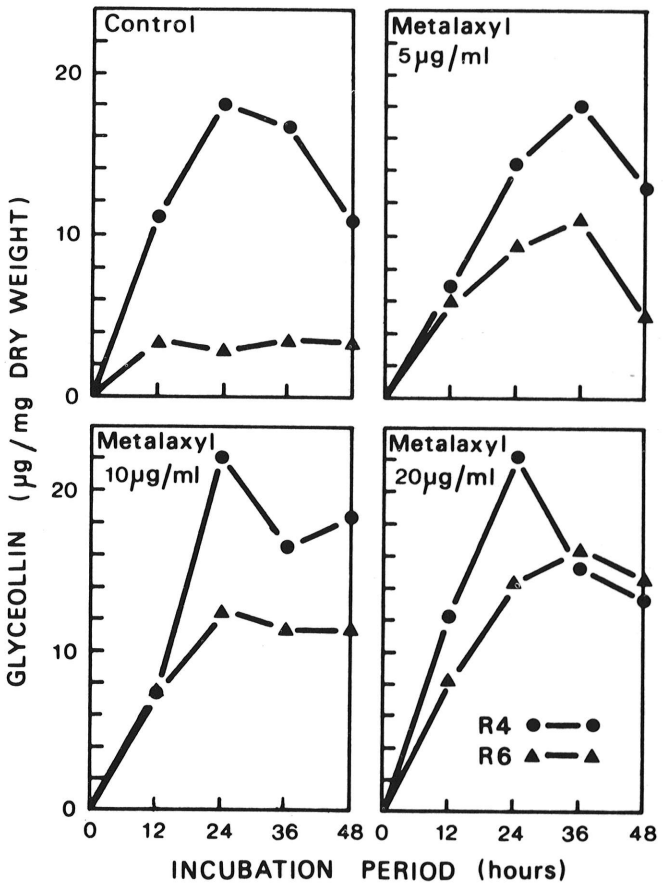

Fig. 2. Time course of glyceollin production in soybean hypocotyls (cultivar Altona) inoculated with zoospores of *Phytophthora megasperma* var. *sojae* after the application of metalaxyl to roots of etiolated soybean seedlings, race 4 (incompatible, •——•), race 6 (compatible, ▲——▲).

systemic fungicides is of more general occurrence than the small number of examples suggests.

These results may have implications both for the development of systemic fungicides and the explanation of resistance mechanisms. Inhibition of the fungus at the infection site appears to provide the conditions necessary for initiation of a hypersensitive response. Various authors have suggested that a compatible interaction may be transformed into an incompatible one by inhibiting the growth rate of the pathogen, giving sufficient time for host defense mechanisms to come into play before the pathogen spreads to fresh tissue (eg, 1,19). Certainly, the time-course of glyceollin production in the first 12 hr after inoculation (Fig. 2) suggests that the race 6 compatible interaction has been rendered very similar to the race 4 incompatible interaction by metalaxyl administration.

Probably more pertinent to the results are the observations of Király and coworkers (5,11) that application of fungitoxic antibiotics to infection sites changed compatible interactions into incompatible ones. They proposed that the fungus, killed by antibiotics, released 'endotoxins' which caused the host cells to react hypersensitively. More recent evidence suggests that fungal cell wall polymers, which elicit phytoalexin accumulation (1,10), could be the functional equivalent of such 'endotoxins'. If this is true, the ability to cause the release of such elicitors from fungal cell walls could provide a basis for the development of successful systemic chemicals for disease control. Quite different conclusions were drawn by Cartwright et al (2) from similar responses in the control of rice blast by dichlorcyclopropanes. Because the in vitro fungitoxicity of these compounds was low, Cartwright et al (2) suggested that they sensitized the plant so that it responded in a resistant manner to the invading fungus. However, the compounds were fungitoxic under some conditions in vitro, so the possibility that they are also toxic in vivo, or, for example, affect cell wall integrity in a manner not evident in growth assays, should not be ruled out.

LITERATURE CITED

1. AYERS, A. R., B. VALENT, J. EBEL, and P. ALBERSHEIM. 1976. Host-pathogen interactions. XI. Composition and structure of wall-released elicitor fractions. Plant Physiol. 57:766-774.
2. CARTWRIGHT, D., P. LANGCAKE, R. J. PRYCE, and D. P. LEWORTHY. 1977. Chemical activation of host defence mechanisms as a basis for crop protection. Nature 267:511-513.
3. CRUTE, I. R., S. A. WOLFMAN, and A. A. DAVIS. 1977. A laboratory method for screening fungicides for systemic activity against *Bremia lactucae*. Ann. Appl. Biol. 85:147-152.
4. DEKKER, J., and R. G. VAN DER HOEK-SCHEUER. 1964. A microscopic study of the wheat-powdery mildew relationship after application of the systemic compounds procaine, griseofulvin and 6-azouracil. Neth. J. Plant Pathol. 70:142-148.
5. ÉRSEK, T., B. BARNA, and Z. KIRALY. 1973. Hypersensitivity and resistance of potato tuber tissues to *Phytophthora infestans*. Acta Phytopathol., Acad. Sci. Hung. 8:3-12.
6. ERWIN, D. C., S. D. TSAI, and R. A. KHAN. 1976. Reduction of the severity of Verticillium wilt of cotton by the growth retardant, tributyl[(5-chloro-2-thienyl)methyl]phosphonium chloride. Phytopathology 66:106-110.
7. FAHIM, M. M. 1967. The effect of systemic griseofulvin pretreatment on invasion of bracken prothalli by *Botrytis cinerea*. Ann. Bot. 31:173-177.
8. HARDISON, J. R. 1971. Systemic fungistatic activity of 1,1,1-tri chloro-3-nitro-2-propanol against smut fungi in grasses. Phytopathology 61:936-939.
9. HOCH, H. C., and M. SZKOLNIK. 1979. Viability of *Venturia inaequalis* in chlorotic flecks resulting from fungicide application to infected *Malus* leaves. Phytopathology 69:456-462.
10. KEEN, N . T. 1975. Specific elicitors of plant phytoalexin production: determinants of race specificity in pathogens? Science 187:74-75.
11. KIRÁLY, Z., B. BARNA, and T. ÉRSEK. 1972. Hypersensitivity as a consequence, not the cause, of plant resistance to infection. Nature 234:456-457.
12. LANGCAKE, P., and S. G. A. WICKINS. 1975. Studies on the mode of action of the dichlorocyclopropane fungicides: effects of 2,2-di chloro-3,3-dimethylcyclopropane on the growth of *Piricularia oryzae* Cav. J. Gen Microbiol. 88:295-306.
13. LANGCAKE, P., and S. G. A. WICKINS. 1975. Studies on the action of the dichlorocyclopropanes on the host-parasite relationship in the rice blast disease. Physiol. Plant Pathol. 7:113-116.
14. LAZAROVITS, G., C. H. UNWIN, and E. W. B. WARD. 1980. Rapid assay for systemic fungicides against Phytophthora rot of soybeans. Plant Dis. 64:163-165.
15. REILLY, J. J., and W. L. KLARMAN. 1972. The soybean phytoalexin, hydroxyphaseollin, induced by fungicides. Phytopathology 62:1113-1115.
16. SZKOLNIK, M. 1974. Unusual post-infection activity of a piperazine derivative fungicide for the control of cherry leaf spot. Plant Dis. Rep. 58:326-329.
17. SZKOLNIK, M. 1974. Unique post-infection control of cedar-apple rust on apple with triforine. Plant Dis. Rep. 58:587-590.
18. WARD, E. W. B., G. LAZAROVITS, C. H. UNWIN, and R. I. BUZZELL, 1979. Hypocotyl reactions and glyceollin production of soybean cultivars inoculated with zoospores of races of *Phytophthora megasperma* var. *sojae*. Phytopathology 69:951-955.
19. WOOD, R. K. S. 1972. Introduction: disease resistance in plants. Proc. R. Soc. Lond., B Biol. Sci. 181:213-232.
20. YOSHIKAWA, M., U. YAMAUCHI, and H. MASAGO. 1978. Glyceollin: its role in restricting fungal growth in resistant soybean hypocotyls infected with *Phytophthora megasperma* var. *sojae*. Physiol. Plant Pathol. 12:73-82.