Temperature-Induced Changes in Specificity in the Interaction of Soybeans with *Phytophthora megasperma* f. sp. *glycinea*

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ABSTRACT

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The optimum temperature for growth of *Phytophthora megasperma* f. sp. *glycinea* races 4 and 6 in vitro was 27.5 C; there was little growth at 35 C and none at 37.5 C. Growth of cultures preincubated at 25 C was slowed or arrested by exposure to 37.5 C but resumed at 25 C after a lag, except when the period of exposure was extended to 48 hr. Etiolated soybean hypocotyls (cultivar Altona) inoculated with zoospores of race 4 (incompatible) or race 6 (compatible) were incubated at temperatures ranging from 15 to 37.5 C. At temperatures above 27.5 C, seedlings became increasingly susceptible to race 4 and at 32.5 C the responses (lesion size, necrosis, and glyceollin production) were indistinguishable from the responses to the compatible race 6. Susceptibility to race 4 could be induced by temperature elevation following initial periods of incubation at 25 C for up to 8 hr, but not 12 hr, after inoculation. A period of 8–12 hr at the elevated temperature was required for the induction of susceptibility. With continuous incubation above 35 C, hypocotyls appeared resistant to both races and developed restricted necrotic lesions with only light brown flecking at 37.5 C. Inoculated seedlings incubated first for 2–8 hr at 25 C and then exposed to 37.5 C for 4–12 hr developed intense brown necrotic lesions and high glyceollin levels in response to both races. After returning to 25 C, plants inoculated with race 6 became susceptible, despite theoretically inhibitory glyceollin concentrations, and developed spreading lesions. Plants inoculated with race 4 remained resistant. The results indicate that even high glyceollin concentrations may not always be inhibitory in vivo and suggest that further study is required to determine the significance of glyceollin in disease resistance.

Phythophthora rot of soybeans has been the subject of extensive physiological and genetic studies and has been used as a model system for the study of host-pathogen interactions and the role of phytoalexins in disease resistance (eg, 1,9,13,14,18,25,28,29). Little attention, however, has been given to the effect of temperature on these processes. Most studies have been made under greenhouse conditions and temperature fluctuations frequently have been wide (eg, 15,20). More precise temperature control has been reported for laboratory studies, although choice of temperature, usually between 20 and 25 C, seems to have been arbitrary (10,14,28,29). Eye et al (8) reported that root infection was optimal between 20 and 30 C; and Chamberlain (4) and Chamberlain and Gerdemann (5) demonstrated that heat treatment (>40 C) of plants prior to inoculation could induce temporary susceptibility, but no other systematic study of the effect of incubation temperature on disease development has been published. In several other plant diseases, incubation temperatures have been found not only to influence disease development, but also the expression of resistance and susceptibility (3,6,7,11,17). Such temperature-sensitive systems have proved to be useful tools in the study of host-pathogen interactions.

We report here that a similar system exists in the interaction between soybean and *Phytophthora megasperma* f. sp. *glycinea*.

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MATERIALS AND METHODS

Isolates of races 4 and 6 of *Phytophthora megasperma* Drechs. f. sp. *glycinea* (Hildeb.) Kuan and Erwin (*Pmg*) were grown routinely on V-8 juice agar at 25 C in the dark. Procedures for zoospore production have been described in detail (26). Zoospores in suspensions were adjusted to 1×10^5/ml with sterile distilled water for inoculation purposes.

Soybeans were of the cultivar Altona, which is susceptible to race 6 and resistant to race 4 at 25 C. Seeds were supplied by R.I. Buzzell (Research Station, Agriculture Canada, Harrow, Ontario) and etiolated seedlings were grown as described previously (26).

Six-day-old seedlings were washed in running tap water, blotted dry, and placed in glass trays as described previously (26). The intact hypocotyls were inoculated by placing a 10-μl droplet of zoospore suspension on the hypocotyl surface, about 2 cm below the cotyledons. Inoculated plants were incubated in the dark for the times and at the temperatures indicated in the results. Incubators were calibrated to the indicated temperatures. However, in experiments involving transfers to and from incubators at various temperatures, uncontrolled fluctuations occurred due to the frequent opening of the incubators, and the elevated temperatures (32.5–37.5 C) should be regarded as nominal only.

Symptoms were examined 24, 48, and 72 hr after inoculation and were categorized as follows. Typical resistant-type lesions (R) were restricted to the area covered by the inoculum droplet and were uniformly brown and necrotic. Susceptible-type lesions (S) spread rapidly and the hypocotyl became water-soaked above and below the inoculated sites without brown discoloration. Some of the temperature treatments altered these responses, so that resistant lesions spread (Rs) and susceptible lesions developed varying degrees of brown necrosis (Sn). Lesions were measured along the length of the hypocotyl above and below the inoculated site by using dividers and a millimeter rule. In compatible-type interactions the limit of water-soaking was assumed to be the boundary of the lesion. Values are based on measurements of 10 or 20 lesions per treatment.

Glyceollin in tissues was determined as described in detail previously (28) except that infected tissues at the sites of inoculation were excised from 10 or 20 hypocotyls prior to glyceollin extraction. Glyceollin is expressed in micrograms per gram fresh weight of tissue and is corrected for background levels obtained for uninoculated tissues.

RESULTS

The optimum temperature for growth in vitro of both races of *Pmg* was about 27.5 C (Fig. 1). Growth decreased sharply at temperatures above the optimum and did not occur at 37.5 C. Race 6 tended to grow more slowly (10–15%) than race 4 at all temperatures. The effect of exposure to temperatures above the optimum for growth also was determined by transferring growing colonies from 25 C to 30–37.5 C for various periods and then returning them to 25 C (Fig. 2). Transfer to 30 C caused no detectable change in growth rate. Exposure to 32.5 C for 48 hr reduced the subsequent growth rate slightly, but exposure for 24 hr had little effect. More significant changes in growth rate were caused by transfer to 35 or 37.5 C. Growth was halted at 37.5 C. It did not recover from a 48-hr exposure, and there was a lag of 24 hr before it recommenced after a 24-hr exposure. Shorter lags occurred after shorter exposures. The responses of both races to the temperature treatments were similar.

Uninoculated soybean seedlings remained healthy and continued to grow, without observable ill effects, for at least 72 hr at all incubation temperatures (15–37.5 C).

In inoculated soybean seedlings at 25 C, race 4 (incompatible) caused restricted brown necrotic lesions with high glyceollin levels, whereas race 6 (compatible) caused spreading water-soaked lesions with low glyceollin levels (Table 1). Similar results were obtained at lower temperatures. At higher incubation temperatures the interaction with race 4 became increasingly compatible, and at 32.5 C it was indistinguishable from that with race 6. Glyceollin levels were very low, lesions were similar in size and water-soaked with some diffuse browning. At 35 C, plants were resistant to both races. Lesions closely resembled incompatible-type lesions at 25 C, but glyceollin levels were low.

When susceptibility to race 4 was induced by incubation at 32.5 C, plants remained susceptible after returning to 25 C (Table 2). Lesion lengths and glyceollin levels resembled those in plants maintained at 32.5 C throughout.

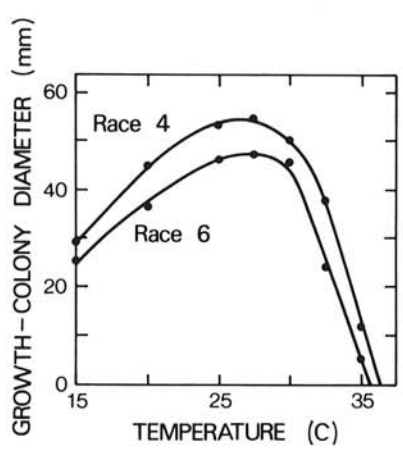

Fig. 1. Influence of temperature on growth of races 4 and 6 of *Phytophthora megasperma* f. sp. *glycinea* on V-8 juice agar (incubation period 96 hr).

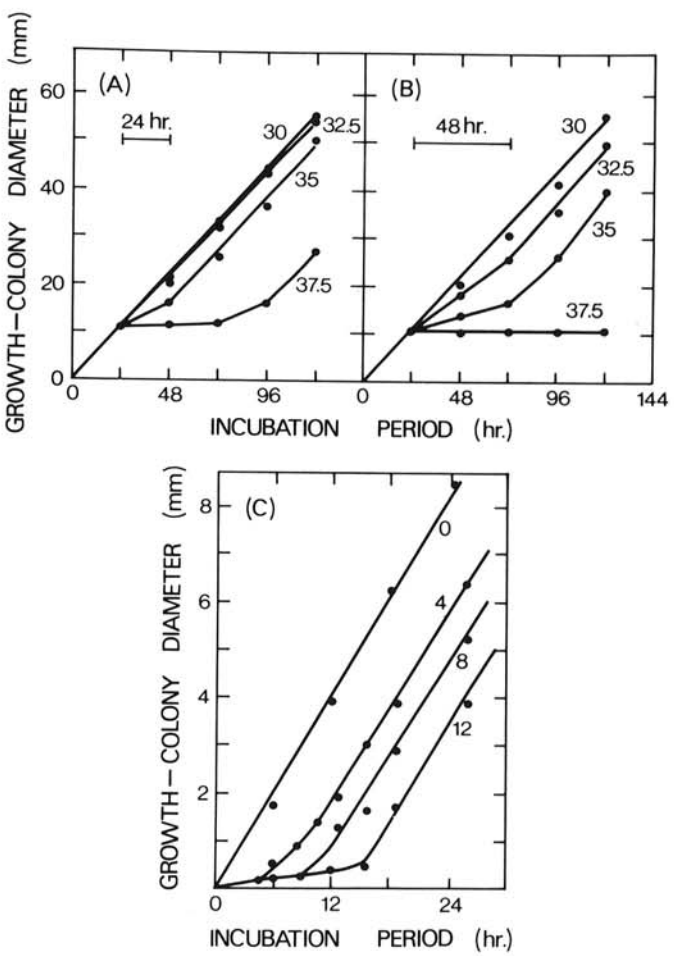

Fig. 2. Effect of periods of exposure to above optimum temperature on growth of races 4 and 6 of *Phytophthora megasperma* f. sp. *glycinea* on V-8 juice agar. **A,** 24 Hr exposure to 30, 32.5, 35 and 37.5 C. **B,** 48 Hr exposure to the same temperatures, **C,** exposure to 37.5 C for 0, 4, 8, and 12 hr. All cultures were preincubated for 24 hr at 25 C (not shown in C) before transfer to the higher temperature and returned to 25 C after the periods indicated.

A series of experiments was performed to determine the effects of timing and duration of elevated temperatures on interactions with both races of *Pmg*. These followed the format indicated in Table 3. Inoculated plants were incubated at 25 C for 0–12 hr, transferred to a higher temperature for 2–12 hr, and then returned to 25 C. These time periods were chosen on the basis of a preliminary observation, in which removal of inoculum droplets from hypocotyls at various times after inoculation showed that infection was established within 2 hr, and after microscope studies indicated extensive tissue colonization by the compatible race within 12 hr (23). The experiments were carried out at various elevated temperatures between 32.5 and 37.5 C. Induction of susceptibility increased with the temperature elevation up to 35 C; this is illustrated here with results from 34 C (Table 3). Generally, 8 or 12 hr exposure to the elevated temperature was required to initiate lesion spread and the tendency towards compatibility. These changes in response were not influenced by preincubation at 25 C for periods of up to 8 hr, but were much reduced when preincubation was extended to 12 hr. The interaction with the compatible race 6 was also influenced by temperature elevation. The lesions developed a brown discoloration in contrast to the typical pale, water-soaked appearance of compatible-type lesions at 25 C. There was no effect on lesion size. As with the incompatible interaction, 8–12 hr of exposure to the elevated temperature was required to induce the changes. However, unlike the incompatible interaction, these changes did not occur if preincubation at 25 C was >4 hr.

Quite different results were obtained when 37.5 C was used as the higher temperature (Table 4). This experiment was carried out with both race 4 and race 6. Typical race 4 results are illustrated here by one example in which 8 hr of exposure to 37.5 C followed 4 hr of incubation at 25 C. Timing and duration of the 37.5 C exposure had little effect on response to race 4. Dark, necrotic, resistant-type lesions developed in all cases. Glyceollin levels were somewhat less than in the 25 C race 4 controls but were still typical of an incompatible response. At 24 hr, the response to race 6 in most treatments was indistinguishable from that to race 4. Lesions were restricted and necrosis and glyceollin levels were high; in fact, higher than those produced following infection by race 4. In two treatments, both following 4 hr of exposure to 37.5 C, susceptible lesions with some browning developed and less glyceollin accumulated. Evidently, after 4 or 8 hr of incubation at 25 C, 4 hr of exposure to 37.5 C was insufficient to induce a fully resistant-type response. Generally, the longer the exposure to 37.5 C (i.e. 4, 8 or 12 hr) the more resistant the response; by 48 hr after incubation, however, all lesions had begun to spread and continued to do so in the subsequent 24 hr. Lesions became intensely brown at and around the inoculated site within 24 hr following inoculation and the browning increased at 48 and 72 hr. Glyceollin levels, except in the initially susceptible interactions, remained high in tissue excised from the inoculated region. Where spreading occurred, glyceollin levels at the periphery of lesions were comparable to those measured in susceptible controls.

TABLE 1. Influence of temperature on resistance and susceptibility, lesion length and glyceollin production in soybean hypocotyls (cultivar Altona) inoculated with race 4 (incompatible) or race 6 (compatible) of *Phytophthora megasperma* f. sp. *glycinea*

Temp (C)	Race	Reaction[a]	Lesion[b] (mm)	Glyceollin[c] (μg/g fr. wt.)
15	4	R	7	644
	6	S	18 ± 2.3	241
20	4	R	7	1,059
	6	S	36 ± 1.9	44
25	4	R	7	2,578
	6	S	53 ± 3.2	24
27.5	4	Rs	25 ± 16.7	1,559
	6	S	56 ± 4.6	27
30	4	Sn	44 ± 17.8	529
	6	S	52 ± 3.4	73
32.5	4	Sn	52 ± 4.3	180
	6	Sn	54 ± 6.1	116
35	4	R	6	172
	6	R	6	203

[a] R = resistant, Rs = resistant with spread, S = susceptible, Sn = susceptible with browning. See text for complete details. Observations were made 48 hr after inoculation.

[b] Lesions were measured along the length of the hypocotyl, values are means and standard deviations from 20 hypocotyls.

[c] Glyceollin was determined in tissue excised from the site of inoculation and calculated as micrograms per gram of tissue fresh weight.

DISCUSSION

The results indicate that elevated incubation temperatures can have opposite effects on the interaction between soybeans (cultivar Altona) and race 4 (incompatible) and race 6 (compatible) of *Pmg*. From 27.5 to 32.5 C, seedlings became increasingly susceptible to race 4; at and above 35 C seedlings were resistant to both races.

Induction of susceptibility by temperature elevation is similar to that described for a number of other temperature-sensitive systems (3,6,11,17) and should be a useful experimental tool for manipulating the soybean-*Pmg* interaction. It did not appear to be associated with any loss of vigor in the seedlings or with increased vigor in the pathogen. The optimum temperature for growth of the latter (in vitro) was 27.5 C, whereas greatest susceptibility occurred at 32.5 C, a temperature at which in vitro pathogen growth was reduced appreciably. Furthermore, according to Murch and Paxton (16), the ability to synthesize glyceollin, at least in cultivar Harosoy 63, is still close to maximum at 32.5 C. Thus, although clearly associated with lower glyceollin levels, temperature-induced susceptibility probably cannot be ascribed to a lack of potential for glyceollin production.

An induction period of 4–8 hr at elevated temperatures was required for compatibility. That the induction period was similar, even after preincubation for 8–12 hr at 25 C, indicates that the incompatible interaction can be prevented late in its development without influencing temperature-mediated processes leading to susceptibility. In incompatible interactions at 25 C, light necrotic flecking of the hypocotyl surface becomes visible to the unaided eye 8–12 hr following inoculation. Temperature-induced susceptibility appeared to override resistance after plants were returned to 25 C. Microscopy has shown that, within lesions, individual hypha-host

TABLE 2. Reaction type, lesion length, and glyceollin production following temperature-induced susceptibility of soybean (cultivar Altona) hypocotyls to race 4 (incompatible) of *Phytophthora megasperma* f. sp. *glycinea*

Temp (C)[a]		Incubation period					
		24 hr			48 hr		
0–24 hr	24–48 hr	Reaction[b]	Lesion (mm)	Glyceollin (μg/g fr. wt.)	Reaction	Lesion (mm)	Glyceollin (μg/g fr. wt.)
25	25	R	6	1,634	R	6	1,350
32.5	32.5	Rs	26.0 ± 1.6	156	Sn	47.5 ± 3.0	45
32.5	25	Rs	25.1 ± 4.5		Sn	48.2 ± 2.3	91

[a] Inoculated seedlings were incubated at 25 or 32.5 C for 48 hr, or for 24 hr at 32.5 C, followed by 24 hr at 25 C. Data were obtained at 24 hr and 48 hr.

[b] R = resistant, Rs = resistant with spread, S = susceptible, Sn = susceptible with browning. Lesions were measured along the length of the hypocotyl, values are means and standard deviations for 20 lesions. Glyceollin was determined in tissue excised from the site of inoculation and calculated as micrograms per gram fresh weight of tissue.

TABLE 3. Effect of timing and duration of temperature elevation on resistance and susceptibility of soybean hypocotyls (cultivar Altona) to race 4 (incompatible) and race 6 (compatible) of *Phytophthora megasperma* f. sp. *glycinea*

Incubation[a] (hr) at:		Race 4		Race 6	
25 C	34 C	Reaction	Lesions/mm[b]	Reaction	Lesions/mm[b]
0	2	R	7 ± 0.5	S	49 ± 3.6
0	4	R	6 ± 1.2	Sn	40 ± 5.5
0	8	Rs	12 ± 3.6	Sn	48 ± 2.4
0	12	Rs	30 ± 10.6	Sn	42 ± 2.6
2	2	R	7 ± 0.8	S	49 ± 4.8
2	4	Rs	16 ± 10.9	S	47 ± 4.7
2	8	Sn	31 ± 11.8	Sn	46 ± 3.2
2	12	Sn	29 ± 9.6	Sn	41 ± 3.8
4	2	R	8 ± 2.1	S	45 ± 1.9
4	4	R	7 ± 0.4	S	46 ± 1.8
4	8	Rs	25 ± 14.4	Sn	40 ± 2.6
4	12	Sn	29 ± 8.2	Sn	42 ± 4.4
8	2	R	8 ± 1.8	S	47 ± 1.0
8	4	Rs	21 ± 9.2	S	43 ± 2.4
8	8	Sn	32 ± 11.9	S	43. ± 0.6
8	12	Sn	31 ± 9.1	S	47 ± 4.0
12	2	R	7 ± 1.7	S	47 ± 3.2
12	4	R	8 ± 1.8	S	46 ± 2.4
12	8	Rs	16 ± 13.0	S	46 ± 2.2
12	12	Rs	9 ± 1.6	S	32 ± 4.1
72	0	R	7 ± 0.5	S	44 ± 3.1
0	72	Rs	23 ± 6.9	Rs	20 ± 8.9

[a] Inoculated plants were incubated sequentially at 25 and 34 C for the periods indicated and then returned to 25 C for the remainder of the 48 hr period, except for the 34 C control which remained at 34 C throughout.
[b] R = resistant, Rs = resistance with spread, S = susceptible, Sn = susceptible with browning. Lesions were measured along the length of the hypocotyl, values are means and standard deviations for 10 lesions per treatment.

cell interactions are seldom either all incompatible or all compatible (21,22). Elevated temperatures below 35 C presumably increase the proportion of compatible combinations sufficiently to permit the pathogen to spread through the host tissue. Higher temperatures, at which the growth rate of the pathogen is greatly reduced, may permit the balance to shift in the opposite direction and incompatibility is the result.

Lesions produced after exposure to 37.5 C appeared to be identical to incompatible lesions at 25 C. This could be due to a reversion of temperature sensitivity at the higher temperature or to a direct effect of temperature on the pathogen. The second of these seems the most probable. Growth of the fungus in vitro was halted at 37.5 C and did not resume until after a lag. This suggests that the fungus suffers appreciable damage at 37.5 C, especially as it is killed by long exposures to that temperature. Thus, it is probable that growth in the host also is stopped while at 37.5 C and does not resume immediately after the infected plant is returned to 25 C. Necrosis may be a reaction of the host cells to materials liberated from damaged hyphae. The development of a hypersensitive type of response following exposure to elevated temperature has been described for other diseases (3,12,19). It has also been demonstrated where infecting fungi have been inhibited by antibiotics (16) or systemic fungicides (27). Jones and Deverall (12) postulated that a specific toxin was released by *Puccinia recondita* in wheat at high temperature, and Király et al (16) considered hypersensitivity following antibiotic treatments to be a response to toxic material released from infecting fungi killed by the antibiotics. Both Rahe (19) and Bailey et al (3) demonstrated that the temperature-induced hypersensitive response of *Phaseolus vulgaris* to *Colletotrichum lindemuthianum* was accompanied by the production of the phytoalexin, phaseollin. Both authors concluded that their results were consistent with the view that phytoalexins restrict the growth of *C. lindemuthianum* in resistant-type reactions in *P. vulgaris*. In general, the results of these authors are similar to those reported here for soybeans and *Pmg*. Our results, however, differ in that in spite of the development of lesions with all the manifestations of a resistant response, including theoretically inhibitory levels of glyceollin (ED_{90} 230 μg/ml in vitro [29]), the compatible race spread from the initial lesion site after returning inoculated plants to 25 C.

Much evidence of a circumstantial nature has been presented to

TABLE 4. Reaction type, lesion length, and glyceollin production following temperature-induced resistance in soybean hypocotyls (cultivar Altona) to *Phytopthora megasperma* f. sp. *glycinea*

Race no. and incubation (hr)[a] at		Total incubation period								
		24 hr			48 hr			72 hr		
25 C	37.5 C	Reaction[b]	Lesion/mm[c]	Glyceollin[d]	Reaction	Lesion/mm	Glyceollin	Reaction	Lesion/mm	Glyceollin
Race 6										
2	4	R	7.4 ± 1.3	1,716	Sn	35.4 ± 3.2	1,056	Sn	50.2 ± 3.1	383
2	8	R	5.5	1,452	Rs	25.4 ± 5.4	1,881	Sn	44.7 ± 6.3	1,124
2	12	R	6.0	1,372	Rs	19.7 ± 4.3	2,442	Sn	39.9 ± 12.9	1,092
4	4	Sn	12.5 ± 1.3	689	S	45.1 ± 6.9	401	S	62.5 ± 3.5	129
4	8	R	7.1 ± 0.9	1,163	Rs	28.4 ± 6.2	1,202	Sn	50.2 ± 3.8	382
4	12	R	5.0	2,541	Rs	7.3 ± 1.4	2,284	Rs	25.7 ± 15.1	1,837
8	4	Sn	13.7 ± 2.8	315	S	43.0 ± 4.8	314	S	59.6 ± 3.5	178
8	8	R	6.6 ± 0.7	1,486	Rs	21.1 ± 3.5	1,665	Rs	37.5 ± 8.9	1,318
8	12	R	6.7 ± 0.8	1,155	Rs	12.5 ± 2.8	1,188	Rs	39.9 ± 2.3	1,419
72	0	S	25.8 ± 2.6	132	S	50.3 ± 2.3	182	S	64.8 ± 3.7	185
0	72	R	5.0	254	R	5.0	215	R	5.0	198
Race 4										
4	8	R	5.0	617	R	6.5	1,091	R	6.5	...
72	0	R	6.0	961	R	6.0	1,551	R	6.0	990
0	72	R	6.0	132	R	6.0	188	R	6.0	174

[a] Inoculated plants were incubated sequentially at 25 and 37.5 C for the periods indicated and then returned to 25 C until 24, 48, or 72 hr following inoculation.
[b] R = resistant, Rs = resistant with spread, S = susceptible, and Sn = susceptible with browning.
[c] Means and standard deviations of lesion lengths from 10 hypocotyls.
[d] Glyceollin micrograms per gram (fr. wt.) of excised hypocotyl tissue from the site of inoculation.

support a role for glyceollin in disease resistance (eg, 13,14,29). The present results appear to provide equally strong circumstantial evidence that it does not. Neither glyceollin nor necrosis conditioned resistance in these experiments. Obviously, problems of timing and localization, which beset most studies of the role of phytoalexins, also prevail here. The ability of *Pmg* to survive theoretically toxic concentrations of glyceollin is difficult to explain, unless toxicity in vivo differs from that in vitro due to solubility or to some form of compartmentalization in this basically compatible interaction, that may enable some hyphae to escape. From results to be published elsewhere we have concluded that partial inhibition of disease development by the systemic fungicide, metalaxyl, caused the production of glyceollin levels that would be inhibitory in vitro, but similarly these did not halt the progress of the fungus. Bailey and Rowell (2) also demonstrated that viable hyphae of *C. lindemuthianum* could be isolated from lesions on *P. vulgaris* despite high phytoalexin levels.

Vanderplank (24) has argued that the most supportable role for phytoalexins is as agents of defense against secondary invaders. Clearly, further critical studies must be made to determine if they have additional significance in the resistance of soybeans to *Pmg*.

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