In Vitro Effects of Glyceollin on *Phytophthora megasperma* f. sp. *glycinea*

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ABSTRACT

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Inhibition of *Phytophthora megasperma* f. sp. *glycinea* (*Pmg*) races 1, 4, and 6 by glyceollin was examined in several bioassays. On absorbent pads impregnated with bean broth or soybean broth, the ED_{50} for inhibition of mycelial growth of *Pmg* race 4 was about 100 μg of glyceollin per milliliter; with race 6, the ED_{50} was 70 μg/ml using pads impregnated with bean broth and 100 μg/ml using pads impregnated with a sucrose-asparagine medium. For inhibition of germ tube growth in liquid media, however, the ED_{50} was approximately 15 μg of glyceollin per milliliter for race 4 and 25 μg/ml for races 1 and 6. Germ tube growth of cysts and sporelings (cysts allowed to germinate for 8 hr prior to exposure to glyceollin) was equally affected by glyceollin. Pre-exposure of cysts to noninhibitory concentrations of glyceollin, phaseollin or kievitone induced some tolerance to glyceollin. There was no evidence for glyceollin metabolism.

Additional key words: adaptation to glyceollin, *Glycine max*.

The role of phytoalexins in host resistance is still not clear. Although numerous studies support their primary role in the defense against fungal invaders (eg, 8,15,26), some authors raised doubts (3,4,17,23).

In vitro bioassays provide estimates for inhibitory concentrations of phytoalexins. The significance of these estimates may be limited, for such bioassays do not reflect in vivo conditions. Thus, to better imitate these conditions, an in vitro study should include experiments on (i) adaptation of a fungus to phytoalexin(s) by pre-exposure to noninhibitory phytoalexin concentrations, a situation that likely occurs following host penetration, and (ii) the possible change in sensitivity to phytoalexins with increasing mycelial age.

Studies on Fungi Imperfecti and Ascomycetes demonstrated adaptation to phytoalexins (6,11,19) and tolerance based either on phytoalexin detoxification (eg, 5, 11, 20) or on nondegradative mechanisms (6,11). Oomycetes were investigated less extensively. *Phytophthora capsici* so far is the only oomycete shown to convert a phytoalexin (25). *Phytophthora megasperma* f. sp. *glycinea* (*Pmg*) failed to degrade glyceollin during a 24-hr period (27).

The lack of information on adaptation of *Pmg* to glyceollin, and the discrepancy of the ED_{50} values reported for the inhibition of mycelial growth by glyceollin (10,26) prompted me to investigate some effects of glyceollin on *Pmg*. Liquid cultures were chosen, for they appear to provide more consistent and reliable data (18) and may better reflect in vivo conditions than cultures on solid media.

MATERIALS AND METHODS

Organisms and culture media. *Phytophthora megasperma* Drechs. f. sp. *glycinea* Kuan and Erwin (*Pmg*) race 1 was obtained from J. Ebel, University of Freiburg (FRG), and *Pmg* races 4 and 6 were from E. W. B. Ward, Agriculture Canada, London, Ontario. Stock cultures were grown on bean or soybean broth agar or on the medium described by Keen (9) modified as follows: $NaMoO_4 \cdot 2 H_2O$ and $MnCl_2 \cdot 2 H_2O$ were replaced by 0.02 mg of MoO_3 and $MnSO_4 \cdot H_2O$, respectively per liter. Bean broth was prepared by gently simmering 30 g of beans in 1 L of distilled water for 90 min. After filtration through cheesecloth, the volume of the filtrate was adjusted to 1 L with distilled water; agar, 15 g/L, was added if desired. Soybean broth was prepared similarly. Four-day-old cultures of *Pmg* races 4 and 6, and 3-day-old cultures of *Pmg* race 1, on bean broth agar were used for zoospore and cyst production with sterile distilled water according to the method described elsewhere (12). Suspensions contained 10^4–5×10^4 spores per milliliter.

Production of phytoalexins. Soybean seedlings, *Glycine max* (L.) Merr. 'Altona,' were grown in vermiculite (previously soaked in water overnight) in the dark at 25 C and 90% RH for 5 days. Subsequently, hypocotyls were arranged as described by Ward et al (24), and glyceollin accumulation was induced abiotically by placing 10-μl droplets of 1 mM $AgNO_3$ on the hypocotyls (21). Treated seedlings were incubated in the dark at 25 C and 90% RH for 48 hr. Glyceollin was extracted with 95% ethanol (21). The extract was dried, and the residue was partitioned with distilled water-ethyl acetate (1:1, v/v) and chromatographed on Whatman PLK5F preparative silica gel plates with toluene-methanol (95:8, v/v). Glyceollin (ie, glyceollin isomers I–III [13,22]) was detected by reference to a standard (kindly provided by E. W. B. Ward, Agriculture Canada, London, Ontario) and its fluorescence quenching at 254 nm. Silica gel containing glyceollin was scraped from the plates, eluted with ethanol, concentrated in vacuo at 40 C, and further purified on Sephadex LH-20 (columns 23 × 1.5 or 40 × 2.5 cm) eluted with 95% ethanol. Glyceollin concentration was calculated from the absorbance at 285 nm and the extinction coefficient (1).

Seeds of *Phaseolus vulgaris* were imbibed in distilled water for 2 hr. The water was drained off, and the beans were imbibed in 1 mM $AgNO_3$ for 2 hr. Subsequently, they were rinsed in distilled water and incubated in the dark at 25 C and 90% RH for 5 days. Phytoalexins were extracted according to the procedure used for the extraction of glyceollin. Chromatography was done on Whatman PLK5F silica gel plates with chloroform-methanol (25:1, v/v) (7). Phaseollin and kievitone were localized by fluorescence quenching in UV (254 nm) and their R_f-values (7). Silica gel containing phaseollin and kievitone, respectively, was scraped from the plates and eluted with ethanol, and the compounds were identified by their absorbance spectra in UV (2,7). Phaseollin was further purified on Sephadex LH-20 (column 23 × 1.5 cm) eluted with 95% ethanol. Kievitone was rechromatographed on Merck

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silica gel 60 F_{254} TLC plates (0.25 mm) with chloroform-ethanol (100:3, v/v) (2,7), eluted with ethanol and rechromatographed using toluene-ethyl acetate-methanol (25:8:4, v/v). Concentrations were calculated from the absorbance at 280 nm (phaseollin) and 293 nm (kievitone) and the extinction coefficients (2).

Bioassays. All assays were done at room temperature except the mycelial growth assay (25 C). Phytoalexins were dissolved in ethanol and added with Hamilton precision syringes. The final concentration of ethanol in the cultures, including controls, was 2% (0.5–1.0% during periods of adaptation to inhibitory glyceollin concentrations). Cyst germination, germ tube growth, and mycelial growth were not affected by 2% ethanol. For the determination of the length of germ tubes, only germinated cysts were considered. ED_{50} values were estimated from dosage response curves.

Mycelial growth assay. For assays of inhibition of mycelial growth, 2 ml of bean or soybean broth or 2 ml of the sucrose-asparagine medium (9) were dispensed into polystyrene petri dishes (Millipore, 49 × 9 mm). Subsequently, glyceollin was added and thoroughly mixed with the medium. This was covered with a sterile, pure cellulose absorbent pad (Millipore, 47-mm diameter), and an inoculum plug, 5 mm in diameter, from a culture on the corresponding solid medium was placed at the center. Colony diameters (minus the diameter of the inoculum plug) were measured after incubation for 2–7 days at 25 C. Data represent means obtained from duplicate experiments.

To determine the adsorption of glyceollin on the absorbent pads, 2 ml of bean broth and 40 µl of ethanol containing 400 µg glyceollin were pipetted into petri dishes, and covered with an absorbent pad or left uncovered (controls). After 1 hr and 7 days, respectively, glyceollin in the pads was extracted by agitating each pad in 100 ml 95% ethanol for 30 min, squeezing it out, and rinsing it with 95% ethanol; petri dishes were dipped into the ethanol and rinsed, and control dishes were rinsed similarly. The solvent was evaporated, and the residues were chromatographed on Whatman LK6DF silica gel plates with toluene-methanol (95:8, v/v). Glyceollin quantities were determined as described above.

Germ tube growth assay. For the determination of the inhibition of germ tube growth, 0.9 ml of suspension of either cysts or sporelings (the cyst suspension harvested from the medium was incubated at room temperature for 8 hr prior to use) were added to sterile tubes containing 0.1 ml of bean broth or 0.1 ml of sterile distilled water with 20% sucrose and 0.05% potassium citrate (14). Subsequently, glyceollin was added. Germ tubes were measured after 8 and 24 hr.

Adaptation to inhibitory doses of phytoalexins. To test adaptation to glyceollin, 0.9 ml cyst suspension was incubated for 8 hr (unless indicated otherwise) with 0.1 ml of bean broth and 7.5 and 10 µg of glyceollin for *Pmg* races 4 and 6, respectively. Subsequently, the glyceollin concentration was adjusted to growth-inhibitory concentrations as indicated in Table 1, ranging from 35–90 µg/ml. Cysts in controls were exposed to the inhibitory concentration only. Germ tubes were measured after 24 hr, including the 8-hr incubation period.

The effect of pre-exposure to low concentrations (2.5 or 5 µg/ml) of either phaseollin or kievitone on the sensitivity of *Pmg* races 4 and 6 to glyceollin was studied similarly. After 9 hr, 32.5 and 45 µg glyceollin were added to tubes with cysts of *Pmg* race 4 and 6, respectively, and germ tubes were measured 13 hr later. Data are means obtained from 25 germ tubes of duplicate experiments.

Glyceollin metabolism studies. Tests for glyceollin metabolism were done with cysts of *Pmg* race 4 and with 3-day-old mycelia of all three races.

To sterile 25-ml Erlenmeyer flasks containing 3.6 ml of a suspension of cysts and 0.4 ml of bean broth, 30 µg glyceollin was added. After 9 hr, glyceollin concentration was adjusted to 35 µg/ml, and incubation proceeded for 6 days. The two kinds of controls consisted of (i) similar preparations with 35 µg glyceollin per milliliter (inhibitory concentration) but without pre-exposure to 7.5 µg glyceollin per milliliter, and (ii) preparations that received 35 µg glyceollin per milliliter on the fourth day, following autoclaving and prior to further incubation for 6 days.

For tests with mycelium, 5 ml of bean broth in sterile 25-ml Erlenmeyer flasks were inoculated with a mycelial plug from bean broth agar, 5 mm in diameter, and incubated for 3 days. Glyceollin, 20, 25, and 30 µg/ml medium for *Pmg* races 1, 6, and 4, respectively, was then added, and incubation proceeded for 1–4 days. Controls were prepared similarly, but the cultures were killed by autoclaving prior to the addition of glyceollin.

Following incubation, the 4-ml samples with cysts and the 5-ml samples with mycelium were transferred to tubes. Methanol (10 ml) and chloroform (5 ml) were added, and glyceollin was extracted according to the method described by Kistler and VanEtten (11) for the extraction of phaseollin and la-hydroxyphaseollone. After a second addition of chloroform (5 ml) and of 4 ml of distilled water, 8 ml of the chloroform phase was removed with a pipette and evaporated to dryness in a Savant Speed Vac Concentrator. Residues were resuspended in ethyl acetate and chromatographed on Whatman LK6DF plates using toluene-methanol (95:8, v/v). Glyceollin was eluted and determined as described above. Data were corrected for the total volume of the chloroform extract (10 ml).

To determine whether *Pmg* caused a change in the isomeric proportion of glyceollin, 175 µg glyceollin were added to 5-day-old cultures of *Pmg* race 4 in 5 ml of bean broth, and extracted after 8 days according to the method of Kistler and VanEtten (11) and chromatographed as above. Glyceollin, induced with $AgNO_3$ (see 'Production of phytoalexins' section) and isolated from hypocotyls of soybean seedlings cultivar Altona, served as control. Glyceollin isomers were separated on a 25 × 0.46-cm column of Partisil (Whatman PXS, 5-µm particle size) eluted with a mixture of hexane and propane-2-ol with a flow rate of 0.8 ml/min, and monitored at 288 nm (22). Glyceollin isomers I, II, and III, the proportions of which are cultivar and age-specific (22), were identified by their absorbance spectra in UV (13). Data were obtained through an integrator (Shimadzu C-R1A Chromatopac) and are means from duplicate samples.

TABLE 1. Tolerance of *Phytophthora megasperma* f. sp. *glycinea* races 4 and 6 to inhibitory doses of glyceollin induced by pretreatment with low concentrations of glyceollin

Fungal race	Glyceollin (µg/ml) during adaptation period (8 hr)[a]	Final glyceollin conc. (µg/ml)[b]	Germ tube length (µm) after:[c] 8 hr	24 hr
4	0	0		1,135
	35	35	0	0
	7.5	35	364 w	660 x
	7.5	40		464 w
	7.5	45		378 w
	7.5	50		315 w
6	0	0		948
	50	50	0	0
	10	50	243 y	435 z
	10	55		414 z
	10	60		469 z
	10	65		468 z
	10	70		423 z
	10	75		328 y
	10	80		320 y
	10	90		265 y

[a] Cysts were incubated at room temperature in one-tenth strength bean broth containing no, 7.5 or 10 µg glyceollin per milliliter.

[b] At the end of the adaptation period, glyceollin was adjusted to a concentration between 35 and 90 µg/ml. The final concentration of ethanol was 2%.

[c] Means followed by a different letter are significantly different ($P<0.025$).

RESULTS

Glyceollin, 200 µg/ml, repressed mycelial growth of *Pmg* races 4 and 6 for 4–6 days (Fig. 1). Subsequently, some growth was measurable. The ED_{50} for inhibition of mycelial growth for race 4

on absorbent pads impregnated with either bean or soybean broth was about 100 μg glyceollin per milliliter. For race 6, an ED_{50} of approximately 70 μg and 100 μg glyceollin per milliliter was measured using absorbent pads impregnated with bean broth or the sucrose-asparagine medium, respectively (Fig. 1). To determine whether the phytoalexin was adsorbed on the pad or stayed in solution, 400 μg of glyceollin were added to petri dishes with impregnated pad and subsequently extracted. After 1 hr and 7 days, respectively, 92.6 and 97.5% of the glyceollin extracted from controls (petri dishes without absorbent pad) were recovered.

Germ tube growth was more sensitive to glyceollin than mycelial growth. In bean broth (Fig. 2B, D, and G) or 2% sucrose with 0.005% potassium citrate (Fig. 2A and F), the ED_{50} for inhibition of germ tube growth was about 20 and 15 μg glyceollin per milliliter for cysts of *Pmg* races 1 and 6 and *Pmg* race 4, respectively. The ED_{50} for inhibition of germ tube growth of race 4 determined after 8 hr (Fig. 2C) and after 24 hr (Fig. 2D) were identical. The ED_{50} for inhibition of germ tube elongation of sporelings of races 4 and 6 (Fig. 2E and H) were the same as those determined for inhibition of germ tube growth with cysts (Fig. 2D and G).

To test for adaptation to glyceollin, cysts of *Pmg* race 4 were incubated for 4–8 hr with a sub-inhibitory concentration of glyceollin (7.5 μg/ml) prior to exposure to growth inhibitory concentrations. Following adaptation periods of 4–6 hr and subsequent exposure to 35 μg glyceollin per milliliter, germination was still suppressed. After 8 hr of adaptation, germination rate was 50% compared to 77% in untreated controls. Mean length of germ tubes at the end of the adaptation period was significantly less than the mean length determined after 24 hr, 13 hr of which was with 35 μg of glyceollin per milliliter (Table 1). This difference, however, was not significant if the initial adaptation period was followed by exposure to 40 μg of glyceollin per milliliter or more (Table 1). Germ tubes of race 6, following adaptation with 10 μg of glyceollin per milliliter for 8 hr, were significantly shorter at the end of the adaptation period than after the additional exposure to glyceollin in concentrations ranging from 50 μg/ml (inhibitory concentration) to 70 μg/ml for 13 hr (Table 1).

To determine the specificity of the adaptation of *Pmg* to growth inhibitory concentrations of glyceollin, similar experiments were done with phaseollin and kievitone. Incubation of cysts of race 4 with 2.5 μg/ml of phaseollin or kievitone for 9 hr prior to the addition of 32.5 μg of glyceollin per milliliter allowed germ tube elongation (Table 2). Glyceollin, 45 μg/ml, added to cysts of *Pmg* race 6 previously exposed to either 2.5 or 5 μg/ml of phaseollin or kievitone for 9 hr, did not stop germ tube growth (Table 2). In contrast, growth was suppressed in controls lacking pre-exposure to noninhibitory concentrations of phaseollin or kievitone (Table 2).

Following adaptation of cysts of *Pmg* race 4 to 35 μg glyceollin per milliliter by pre-exposure to 7.5 μg of glyceollin per milliliter, appreciable hyphal growth developed during 6 days of incubation. To study the metabolism of glyceollin, this was extracted and

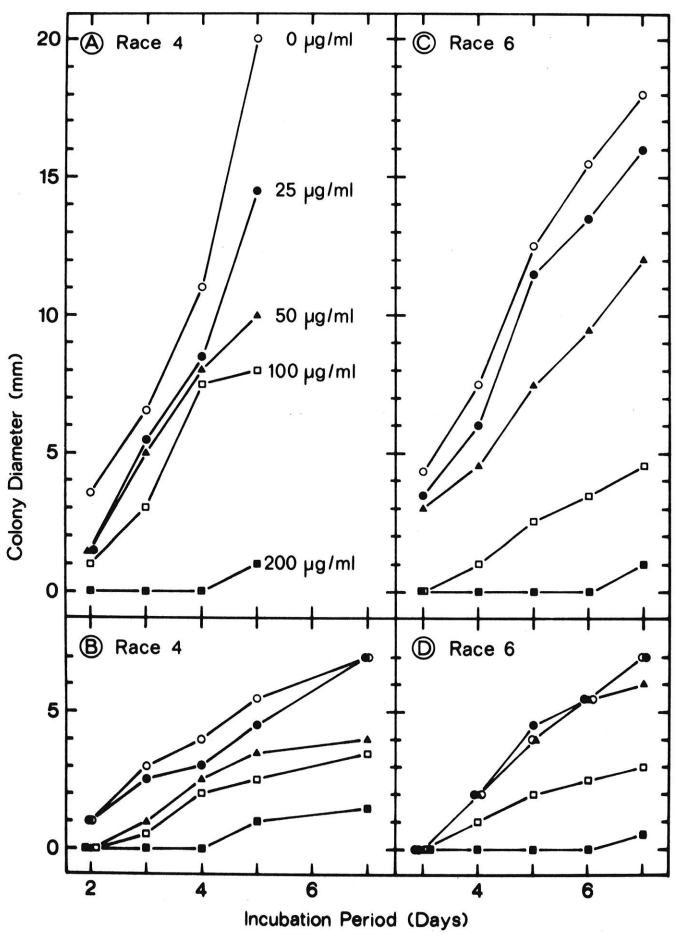

Fig. 1. Growth of *Phytophthora megasperma* f. sp. *glycinea* races 4 and 6 on absorbent pads impregnated with **A and C,** bean broth; **B,** soybean broth; and **D,** a sucrose-asparagine medium, respectively, amended with glyceollin.

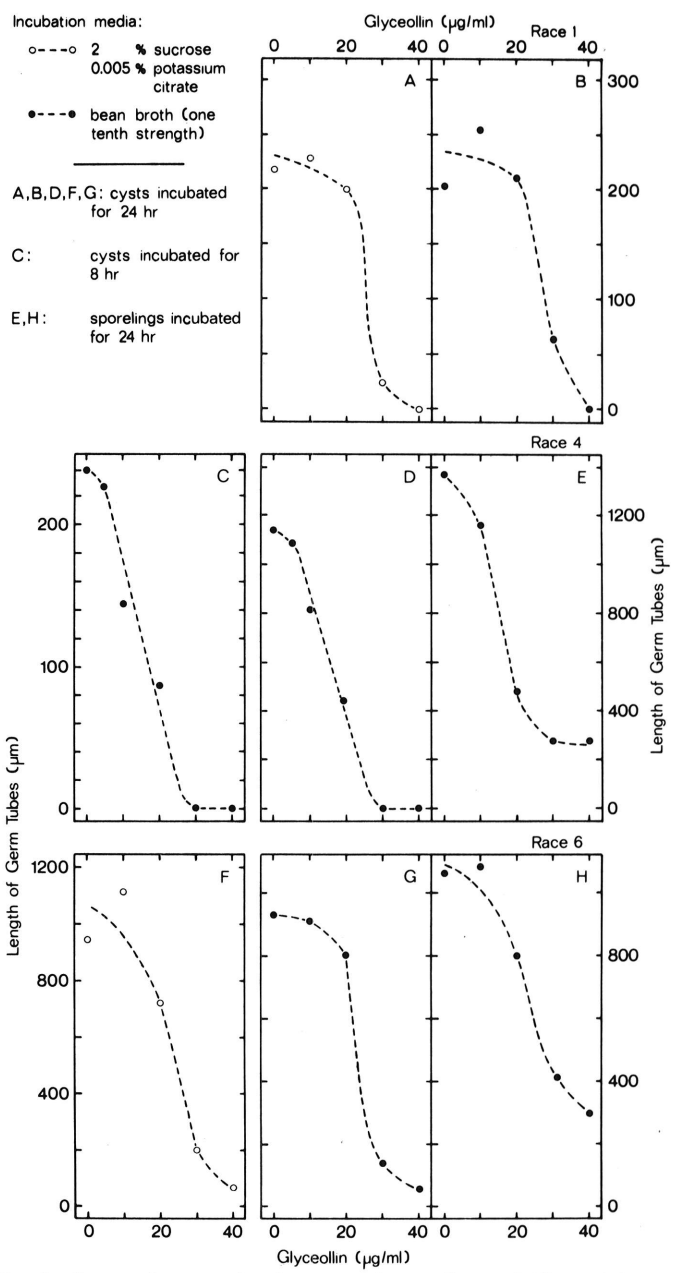

Fig. 2. Germ tube growth of cysts and sporelings of *Phytophthora megasperma* f. sp. *glycinea* races 1, 4, and 6 exposed to glyceollin.

compared with similar extracts from controls with unadapted cysts (no growth), and from autoclaved controls. No difference in pattern of fluorescence quenching at 254 nm on TLC plates was detected, and all extracts contained similar quantities of glyceollin (Table 3). In experiments with glyceollin in noninhibitory concentrations given to 3-day-old mycelia of *Pmg* races 1, 4, and 6, the amount of glyceollin extracted after 1 and 4 days was similar to that in autoclaved controls (Table 3). The influence of *Pmg* on the isomeric proportion of glyceollin was studied by analyzing glyceollin with HPLC. The proportions of isomers I, II, and III were 22.4:1.6:1 and 22.5:1.5:1, respectively, for glyceollin prior and subsequent to contact for 4 days with *Pmg* race 4.

DISCUSSION

The ED_{50} values of glyceollin for inhibition of mycelial growth of *Pmg* races 4 and 6 on absorbent pads were in the range of those reported by Yoshikawa et al (26) for *Pmg* race 1 on various solid media. While these authors determined an ED_{90} for inhibition of mycelial growth of 200–230 μg of glyceollin per milliliter after 2 days of incubation, growth of *Pmg* races 4 and 6 on absorbent pads impregnated with nutrient medium containing 200 μg glyceollin per milliliter was measurable after 4–6 days only (Fig. 1). Since the use of liquid medium and absorbent pads assured that about 95% of the glyceollin added stayed in solution, this method may be more effective than incorporation of glyceollin into agar (26); this could explain the slight difference between the results reported by Yoshikawa et al (26) and those described here, but not the discrepancy between these two studies and that of Keen et al (10), which produced an ED_{50} of 7×10^{-5} M (23.7 μg/ml) glyceollin (6a-hydroxyphaseollin) for inhibition of mycelial growth of *Pmg* races 1 and 2 on V-8 juice agar.

Cyst wall formation, germination and germ tube elongation were inhibited at a significantly lower glyceollin concentration than inhibition of mycelial growth. Germ tube formation was inhibited at about half the concentration of glyceollin found in diffusates of compatible soybean hypocotyls 24 hr after inoculation with zoospores of *Pmg* (12). The use of liquid media in all assays of the present work permits comparison of the results. It appears, thus, that the sensitivity of *Pmg* to glyceollin decreased with increasing age.

Pre-exposure of cysts to a noninhibitory concentration of glyceollin induced tolerance to a glyceollin concentration slightly (*Pmg* race 4) or significantly (*Pmg* race 6) above the ED_{100} for inhibition of germ tube growth. Apparently, the ability to adapt to glyceollin varies between races. Adaptation to glyceollin also was indicated by the lag phase of 4–6 days for mycelial growth with 200 μg of glyceollin per milliliter (Fig. 1). Since *Pmg* races 1, 4, and 6 did not alter glyceollin over a period of 6 days, adaptation to glyceollin must be based on nondegradative mechanisms.

Tolerance was not specifically induced by glyceollin, for pre-exposure to low concentrations of either phaseollin or kievitone had the same effect (Table 2). Tolerance induced by structurally different phytoalexins already has been shown for *Colletotrichum lindemuthianum* (19) and *Nectria haematococca* (6), but has not been reported for oomycetes.

The data show that races of *Pmg* may differ in their sensitivity to glyceollin, that this sensitivity significantly decreases with mycelial age, and that some tolerance to glyceollin may be induced by low concentrations of glyceollin or other isoflavonoid phytoalexins. At present, the evolution of glyceollin accumulation in vivo on a cellular level and the sensitivity of the penetrating hyphae of *Pmg*—depending not only on fungal race but also on hyphal age and induced glyceollin tolerance—are not known. Therefore, the importance of glyceollin in resistance to *Pmg* remains unclear. New

TABLE 2. Tolerance of *Phytophthora megasperma* f. sp. *glycinea* races 4 and 6 to inhibitory concentrations of phaseollin (P) and glyceollin (G) or kievitone (K) and glyceollin (G), following pre-exposure of cysts to low concentrations of phaseollin and kievitone, respectively

Fungal race	Concentration of phytoalexins (μg/ml) during the 9-hr adaptation period[a]	Final phytoalexin conc. (μg/ml)[b]	Mean length of germ tubes (μm) after:[d] 9 hr	24 hr
4	0	0	...[c]	1,075
	2.5 (P)	2.5 (P) 32.5 (G)	172 w	346 x
	2.5 (P) 32.5 (G)	2.5 (P) 32.5 (G)	0	0
	2.5 (K)	2.5 (K) 32.5 (G)	146 w	350 x
	2.5 (K) 32.5 (G)	2.5 (K) 32.5 (G)	0	0
6	0	0	214 w	955 x
	2.5 (P)	2.5 (P) 45.0 (G)	186 w	289 x
	5.0 (P)	5.0 (P) 45.0 (G)	229 y	414 z
	2.5 (P) 45.0 (G)	2.5 (P) 45.0 (G)	0	0
	2.5 (K)	2.5 (K) 45.0 (G)	280 w	383 x
	5.0 (K)	5.0 (K) 45.0 (G)	246 y	401 z
	2.5 (K) 45.0 (G)	2.5 (K) 45.0 (G)	0	0

[a] Cysts were incubated at room temperature in 1 ml of one-tenth strength bean broth with or without phaseollin (P), kievitone (K), and glyceollin (G).
[b] At the end of the 9-hr adaptation period, phytoalexins were adjusted to the concentrations indicated. The final concentration of ethanol was 2%.
[c] Not determined.
[d] Mean germ tube length at the end of the adaptation period is significantly shorter than that determined after 24 hr of incubation (w and x, $P<0.005$; y and z, $P<0.025$).

TABLE 3. In vitro degradation of glyceollin by races 1, 4, and 6 of *Phytophthora megasperma* f. sp. *glycinea*

Fungus	Glyceollin added (μg)	Incubation period (days)[a]	Glyceollin extracted (μg)[b]
race 1, mycelium[c]	100 (20)[d]	1	75.3[e]
		4	79.0[e]
		4 (control)[f]	78.1[e]
race 4, cysts	140 (35)[d]		
adapted[g]		6	96.6[e]
not adapted		6 (control)	97.7[e]
autoclaved[h]		6 (control)	91.2[e]
race 4, mycelium[c]	175 (35)[d]	1	118.5
		4	121.9
		4 (control)[f]	125.4
race 6, mycelium[c]	125 (25)[d]	1	93.3[e]
		4	89.2[e]
		4 (control)[f]	92.6[e]

[a] All samples were incubated at room temperature.
[b] For details see Materials and Methods.
[c] Glyceollin was added to 3-day-old cultures in 5 ml of bean broth.
[d] Values in parenthesis indicate the quantity of glyceollin per milliliter.
[e] Means are calculated from two experiments.
[f] Cultures were autoclaved prior to the addition of glyceollin.
[g] Tolerance to glyceollin was induced by pre-exposure of cysts to 7.5 μg of glyceollin per milliliter for 9 hr. Subsequently, glyceollin was adjusted to 35 μg/ml.
[h] Cysts were incubated for 4 days, autoclaved prior to the addition of glyceollin, and subsequently incubated for 6 days.

techniques (eg, 16) and further studies are needed to conclusively demonstrate the role of glyceollin in restricting *Pmg* in soybean.

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