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

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ABSTRACT


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 ED50 for inhibition of mycelial growth of Pmg race 4 was about 100 µg of glyceollin per milliliter; with race 6, the ED50 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 ED50 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 sporopollen (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.

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silica gel 60 F 254 TLC plates (0.25 mm) with chloroform-ethanol (100:3, v/v), 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. ED50 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 in 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 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 1a-hydroxyphaseollin. 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 AgNO3 (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 PX5, 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.

**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 ED50 for inhibition of mycelial growth for race 4

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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

<table>
<thead>
<tr>
<th>Fungal race</th>
<th>Glyceollin (μg/ml) during adaptation period (8 hr)</th>
<th>Final glyceollin conc. (μg/ml)</th>
<th>Germ tube length (μm) after:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>8 hr</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td>1,135</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>35</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>35</td>
<td>364 w</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>40</td>
<td>464 w</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>45</td>
<td>378 w</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>50</td>
<td>315 w</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
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<td>243 y</td>
</tr>
<tr>
<td></td>
<td>10</td>
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<td>414 z</td>
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<td>60</td>
<td>469 z</td>
</tr>
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<td></td>
<td>10</td>
<td>65</td>
<td>468 z</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>70</td>
<td>423 z</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>75</td>
<td>328 y</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>80</td>
<td>320 y</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>90</td>
<td>265 y</td>
</tr>
</tbody>
</table>

*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).
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

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**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 races 1 and 2 on V-8 juice agar.

At the end of the 9-hr adaptation period, phytoalexins were adjusted to the values in parenthesis indicate the quantity of glyceollin per milliliter for 9 hr. Subsequently, glyceollin was adjusted to 200–230 μg of glyceollin per milliliter after 2 days of incubation, growth of *Pmg* races 4 and 6 on absorptive pads was impregnated with nutrient medium containing 200 μg glyceollin with HPLC. The proportions of isomers I, II, and III races 1 and 2 on V-8 juice agar.

**DISCUSSION**

The ED₉₀ values of glyceollin for inhibition of mycelial growth of *Pmg* races 4 and 6 on absorptive 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 ED₉₀ 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 absorptive pads impregnated with nutrient medium containing 200 μg glyceollin was measurable after 4–6 days only (Fig. 1). Since the use of liquid medium and absorptive 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 ED₉₀ 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 ED₉₀ 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 nondegradable 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 *Pmg*.

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.

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

<table>
<thead>
<tr>
<th>Fungus</th>
<th>Glyceollin added (μg)</th>
<th>Incubation period (days)</th>
<th>Glyceollin extracted (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>race 1, mycelium</td>
<td>100 (20)</td>
<td>1</td>
<td>75.3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>79.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 (control)</td>
<td>78.1</td>
<td></td>
</tr>
<tr>
<td>race 4, cysts</td>
<td>140 (35)</td>
<td>6</td>
<td>96.6</td>
</tr>
<tr>
<td>adapted</td>
<td>96.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>not adapted</td>
<td>97.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>autoclaved</td>
<td>91.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>race 4, mycelium</td>
<td>175 (35)</td>
<td>1</td>
<td>118.5</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>121.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 (control)</td>
<td>125.4</td>
<td></td>
</tr>
<tr>
<td>race 6, mycelium</td>
<td>125 (25)</td>
<td>1</td>
<td>93.3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>89.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 (control)</td>
<td>92.6</td>
<td></td>
</tr>
</tbody>
</table>

* All samples were incubated at room temperature.

* For details see Materials and Methods.

* Glyceollin was added to 3-day-old cultures in 5 ml of bean broth.

* Values in parenthesis indicate the quantity of glyceollin per milliliter.

* Means are calculated from two experiments.

* Cultures were autoclaved prior to the addition of glyceollin.

* 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.

* 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.

LITERATURE CITED


