Phytotoxicity to Crop Plants and Herbicidal Effects on Weeds of Viridiol Produced by *Gliocladium virens*

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ABSTRACT

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When the biological control agent, *Gliocladium virens*, was grown on an autoclaved rice medium, it produced a phytotoxin that caused necrosis of cotton seedling radicles. The phytotoxin was identified as viridiol, a compound previously reported from *G. virens*. Viridiol, a close relative of the antifungal compound viridin, had little antibiotic activity against a variety of fungi and bacteria, but it was herbicidal to germinating pigweed seed when used in vitro. Because of its instability, viridiol was not an effective herbicide when it was introduced into field soil. However, when a dried and ground preparation of *G. virens* cultured on rice was worked into pigweed-infested soil above planted cotton seed, viridiol apparently was produced in sufficient quantity and duration to prevent pigweed emergence without apparent harm to emerging cotton seedlings. The longevity of *G. virens* in the dried rice preparation and the ease with which it is stored and applied, suggest its possible use as a biological control agent to suppress pigweed in cotton plantings.

Additional key words: *Amaranthus retroflexus*, carbon-13 NMR.

Gliocladium virens is a destructive mycoparasite of *Sclerotinia sclerotiorum* (9) and a biological control agent for Rhizoctonia root rot of white beans (10) and Rhizoctonia and Pythium damping-off of cotton seedlings (4). Although it is an active parasite of *Rhizoctonia solani*, *G. virens* does not parasitize *Pythium ultimum* (4). Biocontrol of *P. ultimum* is due to the production of the antifungal antibiotic, gliovirin, by the mycoparasite (5). Strains of *G. virens* also produce the antifungal and antibacterial compound, gliotoxin (1), the antibacterial compound heptelidic acid (6), and the antifungal compound viridin (2). Viridiol, a compound with a molecular structure closely related to that of viridin, has also been reported from *G. virens* (8).

In the course of studies on optimum inoculum production and application of *G. virens* to suppress cotton seedling disease, we found that treatment with *G. virens* preparations grown on rice prevented cotton seed from emerging after a 2-wk incubation period. Examination of seeds excavated from the soil showed that, other than death of the radicle tip, the partially germinated seeds appeared healthy. This paper describes a study we made to determine the cause of this phenomenon and our efforts to use this phytotoxic effect for weed control. Since pigweed (*Amaranthus retroflexus* L.) is an important weed pest in cotton fields, the effect of this phytotoxin on the germination and subsequent growth of the weed was examined.

The objectives of the experiments reported here were: to isolate and identify the phytotoxic compound, to determine the herbicidal activity of the compound to pigweed in vitro, to establish the herbicidal effectiveness of *G. virens* cultured on rice in nonsterile soil, to ascertain whether herbicidal activity in soil was due to the stabilizing effect of the rice medium on a compound already present or due to subsequent production of phytotoxin by the fungus acting on the rice substrate, and to determine the antifungal and antibacterial activity of the phytotoxin.

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MATERIALS AND METHODS

Strain GV-P of *Gliocladium virens* Miller et al was grown for 6 days on a medium consisting of 140 g of long-grain rice and 250 ml of H_2O that was autoclaved for 15 min at 121 C. The cultures were then air-dried, ground to pass through a 20-mesh screen, and stored at 25 C until used.

Ten-gram lots of dried and ground GV-P rice culture, uninoculated rice, GV-P Peat moss-Czapek broth (PMCZB) culture (4), and 100 g of GV-P potato-dextrose agar (PDA) culture were extracted with two 100-ml volumes each of 80% aqueous acetone and the acetone was removed in vacuo. The aqueous residues were extracted with two equal volumes of chloroform and the chloroform was removed in vacuo. The extract residues were then dissolved in 1-ml aliquots of acetone.

Ten cottonseeds (*Gossypium hirsutum* 'Stoneville 213') were rolled into chromatography paper cylinders (15 × 10 cm) and test solutions consisting of 0.125 ml of each acetone extract adjusted to 5 ml with H_2O were pipetted over the seeds. The germination cylinders were covered with wax paper, except for the ends, and incubated at 30 C for 3 days in the dark. The seeds were then examined for signs of germination and damage to the radicle.

An additional 100 g of GV-P rice culture was extracted and concentrated as described previously, and the extract was fractionated according to the scheme in Fig. 1. The active compound resulting from the isolation procedure was subjected to proton NMR and UV and mass spectrographic analyses, and its melting point was obtained. These data were compared with those from a compound previously isolated from this fungus (8). The ^{13}C NMR spectrum of the active compound was compared to that reported for demethoxyviridin (3).

Determination of herbicidal activity. Pigweed seeds were placed on filter paper in petri dishes moistened with an aqueous solution of the compound. After 5 days of incubation at 30 C, the seeds were examined for germination and damage.

Samples of the extracts from GV-P rice, GV-P PMCZB, GV-P PDA, and the rice control were spotted on Baker TLC plates (J. T.

Baker Chemical Company, Jackson, TN) along with the phytotoxin from *G. virens* and developed two-dimensionally in chloroform-acetone (90:10) and ethyl acetate. Chromatograms were observed under UV light to detect by absorbance the presence of the phytotoxic compound.

Efficacy of GV-P rice culture as an herbicide in natural soil. Cotton seed were planted in nonsterilized soil from a cotton field at a depth of 3.5 cm and the furrow was closed. The top 1.5-cm of soil was removed from the surface and 0.5 g (approximately 100 seeds) of pigweed seeds was mixed into it. The 1.5 cm of soil was then replaced, and the surface was uniformly covered with a 10-cm-wide band of 0, 6, 12, 18, 24, 30, 36, 42, or 48 g of GV-P rice culture per meter of row. The treatment was worked into the soil to a depth of 1.5 cm, and the flats were incubated in a growth chamber at 26 C during the day and 18 C at night. The photoperiod was 14 hr. After 14 days, the numbers of emerged and surviving pigweed seedlings were counted. The cotton seedlings were examined for evidence of phytotoxicity and weighed.

Preliminary experiments with adding the phytotoxin (viridiol) to soil and a report in the literature (8) indicated that the purified compound was too unstable to be useful as an herbicide in field soil. Therefore, the question emerged as to whether herbicidal activity by the GV-P rice mixture in soil was due to the presence of already elaborated phytotoxin stabilized by the other culture ingredients, or to continued production of phytotoxin by the fungus acting on the substrate. To resolve this question, we added the following treatments to the surface of nonsterile soil in petri dishes: GV-P rice, GV-P rice extracted thoroughly with water to remove phytotoxin, dry ground rice granules as a control, and heat-killed GV-P rice. Moist filter paper was laid over the treated soils and pigweed seed was sprinkled on the filter paper. The plates were incubated at 25 C for 5 days and then examined to determine the effect of these treatments on pigweed seed germination and subsequent growth. The water extract of GV-P rice and an 80% acetone extract of heat-killed GV-P rice were assayed by the germination cylinder method to confirm the presence of phytotoxin in the former and to determine the effect of heat on phytotoxin in the latter.

Antibiotic spectrum of the herbicide. A sample of the purified phytotoxin was diluted to 1,000, 500, and 100 μg/ml in 50% methanol, and 75-μl aliquots were placed in wells cut into agar immediately after inoculum from fungi or bacteria was spread over the surface. PDA plates were inoculated with *Rhizoctonia solani* Kühn, *Pythium ultimum* Trow, *Saccharomyces cerevisiae* Meyen, and *Verticillium dahliae* Kleb. *Bacillus thuringiensis* Berliner, *Corynebacterium tritici* (Hutchinson) Burkholder, *Escherichia coli* (Migula) Castellani and Chalmers, and *Pseudomonas cepacia* Burkholder were assayed on medium 523 (7). The plates were incubated at 25 C and observed after 24 and 48 hr for signs of growth inhibition near the wells.

Phytotoxicity spectrum of GV-P rice granules. Moist nonsterile soil in petri dishes was sprinkled with GV-P-infested rice granules, or nontreated, and the soil was covered with moistened filter paper. Seeds of the crop plants, soybean (*Glycine max* (L.) Merr.), wheat (*Triticum aestivum* L.), grain sorghum (*Sorghum vulgare* Pers.), and the seeds or propagules of the weeds, nutsedge (*Cyperus esculentus* L.), morning glory (*Ipomoea purpurea* (L.) Lam.), and Johnson grass (*Sorghum halepense* (L.) Pers.) were placed on the filter paper. The closed dishes were incubated in the dark for 5 days, then the seedlings were examined for symptoms of phytotoxicity.

RESULTS

Cotton seeds treated with extracts of rice or *G. virens* grown on PMCZB or PDA germinated and produced healthy seedlings with 5-cm-long white radicles. Seeds treated with extract from GV-P rice culture produced necrotic radicles from 0.5 to 1.0 cm in length.

On TLC plates, active zones were found at R_f 0.12 and R_f 0.27 after development with chloroform-acetone (90:10) and ethyl acetate, respectively. Phytotoxic activity was also found in the precipitates formed by adding water or cyclohexane to methanol extracts (Fig. 1).

Comparison of the chromatographic behavior; UV, proton NMR, and mass spectra; and the melting point of the purified phytotoxin with those of a compound previously described from this fungus (8) showed that the phytotoxic component in the GV-P rice culture was viridiol (Fig. 2). The ^{13}C resonances (Fig. 2) were assigned by comparison of spectral data with that of demethoxyviridin (3) and by off-resonance and selected decoupling experiments.

Experiments with viridiol-treated moist filter paper and pigweed seeds showed that viridiol was very toxic to the emerging pigweed radicle. Seeds that germinated later in the experiment, however, were much less severely affected than those that had germinated initially.

Two-dimensional TLC of acetone extracts from GV-P rice, GV-P PMCZB, GV-P PDA, and purified viridiol, confirmed that the toxic effects of GV-P rice were due to viridiol. Spots corresponding to viridiol were very prominent in extract from GV-P rice, but they were absent or present in only trace amounts in the PMCZB and PDA extracts.

GV-P rice incorporated into the top 1.5-cm of cotton field soil planted with cotton and pigweed seeds strongly suppressed pigweed emergence (Fig. 3). Cotton emergence and seedling weight were not affected, and examination of the root system showed no apparent damage, even at the highest treatment levels. A minimum concentration of 24 g of GV-P rice per meter of row was required in order to effectively control pigweed emergence in cotton plantings.

Pigweed seeds placed on moist filter paper over nonsterile soil treated with GV-P rice, rice granule control, GV-P rice extracted

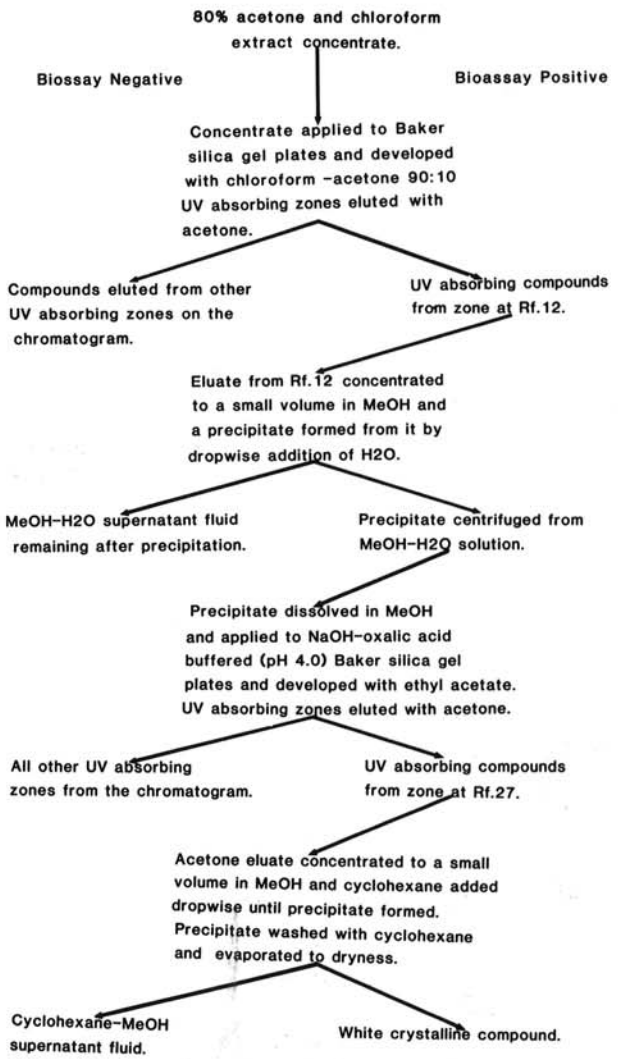

Fig. 1. Isolation procedure used to obtain pure viridiol from *Gliocladium virens* cultured on rice.

with water, or heat-killed GV-P rice, showed the following responses: seedlings over the rice control and heat-killed GV-P rice treatments produced normal radicles with no stunting or lesion development, radicles produced by seedlings over GV-P rice or water-extracted GV-P rice were stunted, with necrotic radicle tips and lesion development progressing toward the seeds. Both the water extract of GV-P rice and the 80% acetone extract of heat-killed GV-P rice were strongly phytotoxic to cotton seedlings.

Bioassays of viridiol against representive fungi and bacteria showed that viridiol was not active even at 1,000 μg/ml against any of the fungi and was weakly inhibitory only to *B. thuringiensis* among the bacteria.

Phytotoxicity assay of the GV-P rice granules against seeds of representative crops and weeds showed that the fungus culture was phytotoxic to all of the seedlings. Seedling radicles from nontreated controls were normal in appearance, whereas those treated with GV-P rice were stunted, and the tips were necrotic.

DISCUSSION

Viridiol is a dihydro-derivative of viridin (8), an antifungal compound produced by *G. virens* (11). In contrast to viridin which is active against a wide range of fungi (2), viridiol showed no activity against any of the representative fungi tested. Both viridin and gliotoxin have been shown to inhibit seedling root growth of wheat, white mustard, and red clover (12). However, the root necrosis associated with viridiol treatment was not observed for those compounds. The differences between viridin and viridiol with respect to their relative toxicities to higher plants and fungi may be related to the difference in groups on the carbon resonating 61.60 δ (Fig. 2). Viridin has a ketone group on this carbon while in viridiol the ketone has been reduced to a hydroxyl.

The fungal strain used in this study, although producing large amounts of the antibiotic gliovirin (5), apparently did not produce phytotoxic levels of either gliotoxin or viridin, since viridiol was the only phytotoxin found in the culture extracts. The production of viridiol appears to be governed by the nature of the substrate on which the fungus is grown. Strain GV-P will produce substantial amounts of viridiol in rice cultures but only trace amounts in PMCZB or on PDA. Thus, the formulation medium is an important determinant of the phytotoxic potential of *G. virens*.

Viridiol has been reported to be unstable in aqueous alkaline solutions (8) and this tendency was confirmed by our observations on the loss of phytotoxic activity to late-germinating pigweed seeds in neutral assay solution. The purified compound also appears to have little potential for use as an herbicide placed directly into soil. However, when a dry granular preparation of the fungus culture is worked into the soil, the phytotoxin subsequently produced by the fungus remains active long enough to prevent emergence of pigweed seedlings.

Placement of the GV-P rice preparation relative to seeds is important. If added to the furrow with cotton seed, it will be extremely phytotoxic to the cotton seedlings. If, however, the GV-P rice treatment is worked into soil above the seed after furrow closing, it will prevent pigweed seedling emergence without adversely affecting cotton seedlings. The optimum treatment level with GV-P rice appears to be 24 g per meter of row.

The phytotoxicity spectrum of the GV-P rice culture appears to be rather broad, since the seedling radicles of all the crops and weeds treated with the preparation showed symptoms of stunting and necrosis.

From the effects of the water leaching and heat killing of GV-P rice on pigweed germination, it is apparent that inhibition of pigweed emergence does not stem from a stabilizing effect of the rice fungus mixture on viridiol. Extraction of already elaborated viridiol from the GV-P rice preparation did not reduce the herbicidal effect of the preparation in soil. Inhibition apparently comes from the subsequent production of viridiol by *G. virens* acting on the rice substrate mixed into the soil, since heat killing of the fungus eliminated herbicidal activity without destroying existing viridiol.

Since GV-P has been reported to parasitize the sclerotia of several soilborne pathogens (9,10), incorporation of this treatment into the soil may have the beneficial effect of reducing pathogen inoculum in addition to its herbicidal activity.

The results of this study indicate that similar problems of phytotoxin production on various substrates may be encountered with other biological control agents. The effects may not be as striking as in this case, but significant yield loss may still result. If so, it may be necessary to genetically alter the biocontrol agent to reduce production of the phytotoxin, or a simple change in substrate may be all that is necessary.

The characteristics of *G. virens* that allow storage for long periods and application in a dry granular form (4) make this fungus an excellent candidate as a biological herbicide. At present, however, the amount of material necessary to achieve complete control, and the expense of the substrate, do not make it economically feasible to use this treatment on a field basis.

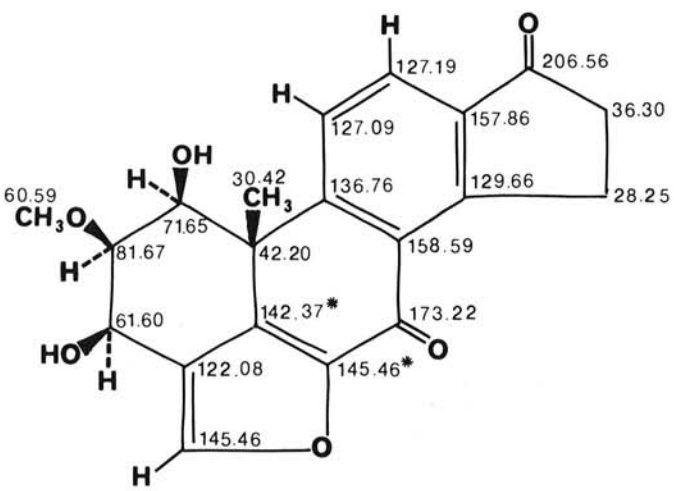

Fig. 2. Molecular structure of the herbicidal steroid, viridiol (8). Numbers indicate the ^{13}C chemical shifts (δ) at the designated positions. Numbers followed by an asterisk indicate carbon assignments that may be interchanged.

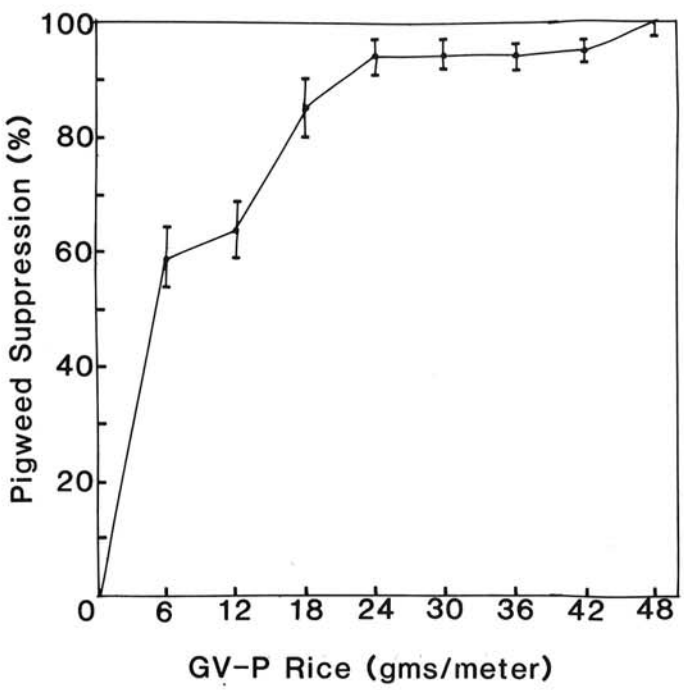

Fig. 3. Effect of GV-P rice treatments on suppression of pigweed seedling emergence in cotton plantings. Granules were applied in a 10-cm-wide band over the closed furrow and worked into the soil. Bars represent the standard deviation computed from five replications.

However, through mutation and screening techniques, we may obtain more efficient viridiol-producing strains of *G. virens*, and thus reduce the level of inoculum required to achieve complete weed control. Alternative substrates that are cheaper than rice may also be found to stimulate viridiol production in *G. virens*. Research to achieve these goals is currently under way.

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