Variation in Sensitivity Among Anastomosis Groups of *Rhizoctonia solani* to the Antibiotic Gliotoxin

R. W. JONES, Former Research Assistant, and R. E. PETTIT, Associate Professor, Department of Plant Pathology and Microbiology, Texas A&M University, Texas Agricultural Experiment Station, College Station 77843

ABSTRACT

Jones, R. W., and Pettit, R. E. 1987. Variation in sensitivity among anastomosis groups of *Rhizoctonia solani* to the antibiotic gliotoxin. Plant Disease 71:34-36.

Considerable variation in sensitivity to gliotoxin was found among anastomosis groups (AG) of *Rhizoctonia solani*. In broth culture, gliotoxin at 2 or 4 ppm was fungistatic to fungicidal toward the mycelium disks of AG-1 (sasakii), AG-2-1, AG-2-2, and AG-3. Isolates of AG-1 (microsclerotial [ms]), AG-4, and AG-5 were much less sensitive, requiring gliotoxin concentrations of 8 ppm for complete inhibition. Sclerotia of AG-1 (ms), AG-1 (sasakii), and AG-2-2 showed reduced viability after submersion in gliotoxin solutions of 20 and 40 ppm. Sclerotia of AG-3, AG-4, and AG-5 were much less sensitive. Linear growth of *Rhizoctonia* mycelium toward gliotoxin in wells cut in agar plates did not differ greatly among anastomosis groups, but growth was generally reduced compared with check treatments.

The role of antibiosis in suppressing *Rhizoctonia solani* Kühn by *Gliocladium virens* Miller, Giddens, & Foster was first studied by Weindling and Fawcett (11). Suppression has been reported to be caused by a "lethal principle" that occurs in culture filtrates of *Trichoderma lignorum* (reidentified as *G. virens* by Webster and Lomas [9]). The lethal principle has subsequently been determined to be gliotoxin, a diketopiperazine antibiotic with a reactive sulfur linkage (10). Numerous reports of its effects on *R. solani* have been published, ranging from no observable effect to fungicidal action (1,2,4–6,10). This study was done to investigate the basis for the previously conflicting reports on the activity of gliotoxin toward *R. solani*. The concentrations of gliotoxin and the treatment methods followed those of previous studies.

MATERIALS AND METHODS

Isolates of *R. solani*. Isolates representing each anastomosis group (AG) according to Ogoshi (8) were maintained on defined agar media.

Media preparation. A defined medium was prepared consisting of (per liter) D-glucose, 8 g; $NaNO_3$, 2 g; DL-asparagine, 1 g; KCl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KH_2PO_4, 0.25 g; $FeSO_4$, 5 mg; and thiamine-HCl, 3 mg. After autoclaving, the medium was buffered with 0.1 M sodium phosphate (pH 5.8), then 20-ml aliquots were pipetted into sterile 125-ml flasks for liquid cultural studies. For the agar plate assays, agar was added to a concentration of 0.75%.

Assay procedure. Gliotoxin, obtained in purified crystalline form from E. R. Squibb Co., was dissolved in warm absolute ethanol at 1 mg/ml. Aliquots of the gliotoxin solution were micropipetted into flasks containing 20 ml of liquid medium to provide concentrations of 0, 2, 4, 6, and 8 ppm. Solvent concentrations were equalized throughout these experiments by adding appropriate volumes to the controls and lower gliotoxin treatments.

The liquid medium was then seeded with 3-mm-diameter mycelial disks of *R. solani* growing on the defined agar medium. The flasks were then incubated 7 days in the dark at 25 C. Mycelial mats were placed on preweighed filter paper disks and dried at 85 C to constant weight.

Mycelial disks that showed little or no linear growth of *R. solani* biotypes was sterile, distilled water, split in half, and plated on potato-dextrose agar (PDA) and water agar. Presence or absence of growth was noted 48 hr later.

The ability of gliotoxin to inhibit the linear growth of *R. solani* biotypes was determined on the defined agar medium. Three 5-mm-diameter wells were cut in the agar near the edge of each plate. Ethanol solutions containing 40 and 80 μg gliotoxin were micropipetted into the wells; absolute ethanol was added to the third well of each plate. Each plate was seeded from the same cultures used for the liquid media tests by placing an inverted mycelial disk in the center of each plate. Linear growth toward each well was measured daily for 5 days.

Sclerotia were produced from one isolate of each anastomosis group by

Table 1. Growth inhibition of *Rhizoctonia solani* mycelium exposed to gliotoxin in liquid culture for 7 days

Anastomosis group	Area of origin	Host	Isolate	Gliotoxin (ppm) 2	4	6	8
1 (microsclerotial)	Florida	Radish	1101	83[a]	67	32	0
	Quebec, Canada	Pine	1102	95	67	36	0
1 (sasakii)	Texas	Rice	1201	0	0	–	–
	Texas	Rice	1202	0	0	–	–
	Texas	Rice	1203	0	0	–	–
2 (type 1)	Australia	Crucifer	2101	0	0	–	–
	Hokkaido, Japan	Sugar beet	2102	50	0	–	–
2 (type 2)	Minnesota	Carrot	2201	0	0	–	–
	Georgia	Corn	2202	0	0	–	–
	Colorado	Sugar beet	2203	40	0	–	–
	Colorado	Sugar beet	2204	20	0	–	–
	Tokyo, Japan	Soil	2205	18	0	–	–
3	Texas	Potato	3001	80	68	24	–
	California	Potato	3002	0	0	–	–
	Colorado	Potato	3003	0	0	–	–
4	Texas	Peanut	4001	83	74	40	0
	Minnesota	Carrot	4002	93	70	37	0
5	Minnesota	Soil	5001	91	74	40	0

[a] Expressed as the percentage of mycelial dry weight relative to the control. Values represent the average of three trials with four replicates each.

Present address of first author: Department of Plant Pathology, University of California, Berkeley 94720.

Journal Series Paper 21333 of the Texas Agricultural Experiment Station.

Accepted for publication 5 September 1986 (submitted for electronic processing).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

culturing *R. solani* on 200 g of moistened, autoclaved oat seed in 500-ml flasks incubated at 20 C in the dark. After 3 wk, the sclerotia were aseptically removed from the surface of the oat seed. Mature sclerotia of uniform size, produced by each biotype, were saved to determine their sensitivity to gliotoxin.

Gliotoxin solution was pipetted into sterile test tubes and the volume brought to 10 ml with sterile water to provide final concentrations of 0, 20, and 40 ppm gliotoxin. Twenty freshly harvested sclerotia from oat seed cultures of each *R. solani* anastomosis group were placed in individual tubes at each concentration and shaken to ensure submersion of the sclerotia. After 4 hr, the sclerotia were rinsed three times in 10 ml of sterile, distilled water, blotted to remove excess moisture, and plated on water agar. The sclerotia were incubated 48 hr and observed to determine if they were 1) viable with normal hyphal growth, 2) viable with reduced or distorted growth, or 3) nonviable.

RESULTS

Considerable variation was observed in the response of *R. solani* mycelium to low levels of gliotoxin (Table 1). Where no growth was observed, the mycelium often proved nonviable. The apparent viability of the mycelium differed within a treatment and depended on whether it was plated onto PDA or water agar.

In general, members of AG-1 (sasakii), AG-2-1, AG-2-2, and AG-3 were completely inhibited at 4 ppm gliotoxin; AG-1 (sasakii) was also consistently inhibited at 2 ppm.

The variation in gliotoxin sensitivity was less in the linear growth assay than in the broth culture assay, although inhibition was still observed (Fig. 1).

In the limited tests performed on sclerotia, a pattern of inhibition different from that of the mycelium was observed (Table 2). Sclerotia from AG-1 (microsclerotial [ms]) were much more sensitive than the mycelium, whereas sclerotia of AG-2-1, AG-2-2, and AG-3 were less sensitive to gliotoxin than the mycelium.

DISCUSSION

Three assay techniques were employed to determine their effects on observed gliotoxin sensitivity. Broth culture assays could be considered comparable to colonization of a substrate occupied by a gliotoxin-producing organism. Isolates of *R. solani* unable to grow in broth culture containing low levels of gliotoxin may be unable to colonize organic matter in soil occupied by *G. virens*.

Reduced linear growth toward wells of gliotoxin could be seen as analogous to the growth of *R. solani* hyphae toward a substrate occupied by *G. virens*, such as a seed coat or biocontrol carrier granule. The reduced growth may delay infection and allow for increased parasitism of *R. solani* hyphae by *G. virens* or other microorganisms. Brewer et al (3) proposed a similar example where the increased lag time in growth of *Bacillus subtilus* in the presence of gliotoxin could allow for increased phagocytosis by other microbes. The inhibition produced by gliotoxin may be nutrient-dependent as seen by the enhanced viability of mycelium disks plated on PDA, compared with water agar, after gliotoxin exposure.

The sensitivity of sclerotia is particularly important for isolates that depend on these as the main survival structure. Sensitivity of sclerotia was tested at higher concentrations of gliotoxin because a colonized sclerotium would provide a considerable food base for toxin production. Sclerotial morphology seemed to play a role in the sensitivity to gliotoxin. Isolates of AG-1 (ms) and AG-1 (sasakii), both the most sensitive to gliotoxin, have a similar structural morphology consisting of an outer rind encompassing melanized sclerotial cells. The outer rind may enhance permeability to gliotoxin. Size of the sclerotium was not considered important because the biomass of the AG-1 (sasakii) is quite high relative to the others, yet it was highly sensitive.

The differential reactions of some isolates, when tested as mycelium in broth culture, as sclerotia, or as mycelium growing toward gliotoxin, exemplify the need for multiple assay procedures in determining the sensitivity of *R. solani* to antibiotics or fungicides.

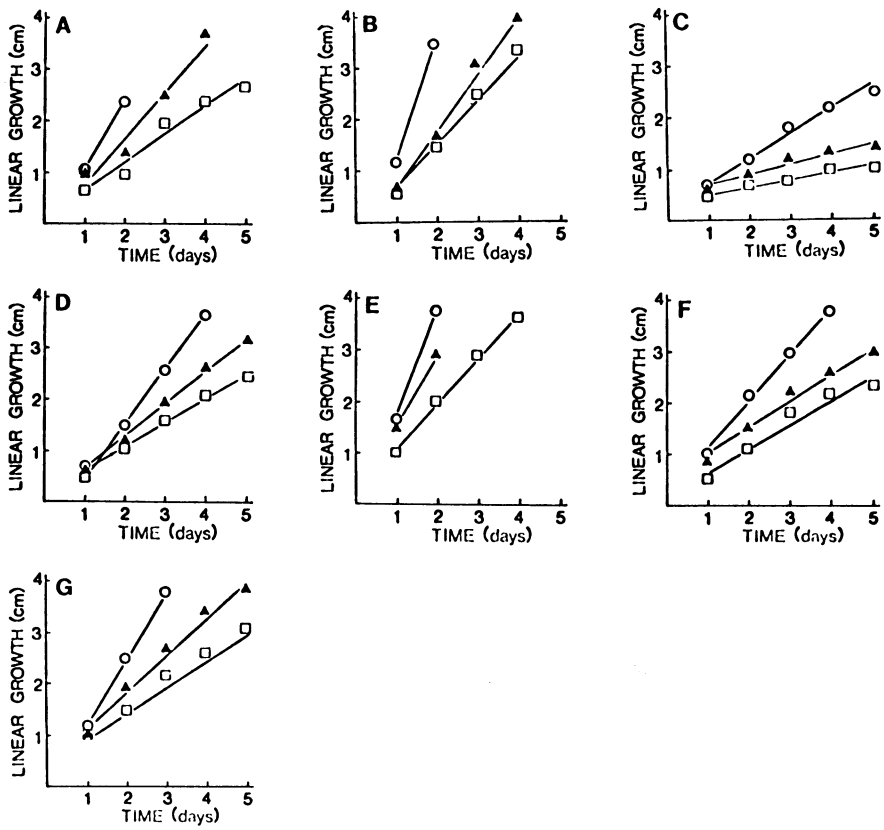

Fig. 1. Linear growth of different *Rhizoctonia solani* anastomosis groups toward gliotoxin: **(A)** AG-1 (microsclerotial), **(B)** AG-1 (sasakii), **(C)** AG-2 type 1, **(D)** AG-2 type 2, **(E)** AG-3, **(F)** AG-4, and **(G)** AG-5. Coefficients of determination ranged from 0.82 to 0.99. o—o = Control, ▲—▲ = 40 μg gliotoxin, and □—□ = 80 μg gliotoxin.

Table 2. Viability of *Rhizoctonia solani* sclerotia after 4 hr of submersion in gliotoxin solution

Isolate	Anastomosis group	Gliotoxin (ppm) 20 N[y]	20 R	20 D	40 N	40 R	40 D
1101	1	9 a[z]	8 a	3 b	5 a	5 a	10 b
1201	1	14 a	4 b	2 b	3 a	4 a	13 b
2101	2	19 a	0 b	1 b	17 a	2 b	1 b
2201	2	17 a	2 b	1 b	12 a	2 b	6 b
3001	3	20 a	0 b	0 b	20 a	0 b	0 b
4001	4	20 a	0 b	0 b	20 a	0 b	0 b
5001	5	20 a	0 b	0 b	20 a	0 b	0 b

[y] N = normal germination and growth, R = restricted growth, and D = dead.

[z] Means in rows of individual treatments followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test. Means represent the average of three trials. All sclerotia in the ethanol-treated control exhibited complete germination and normal growth.

Equally important is the use of members from each anastomosis group. Most reports on the inhibition of *R. solani* by gliotoxin were published before formalization of the anastomosis group concept.

A positive determination of the anastomosis groups to which previously tested *R. solani* isolates belonged cannot be made without the original cultures. Data from this study suggest that isolates of *R. solani* previously reported as sensitive to gliotoxin belonged to AG-2 (type 1 and 2) and AG-3. The isolate employed by Allen and Haenseler (1) released a dark brown pigment into the Czapek broth. We have found this characteristic only of isolates of AG-2 when grown in Czapek broth plus thiamine. Those reports where *R. solani* isolates were obtained from potato suggest they were members of AG-3 (2,5).

Although purified gliotoxin was generally used in previous reports, some data were based on crude culture filtrates, which would raise the possibility of a response to other antibiotics produced by *G. virens*, namely viridin and gliovirin (7,9). When tested at the levels reported for gliotoxin in these studies, viridin (either alone or in combination with gliotoxin) failed to inhibit *R. solani* (R. W. Jones, *unpublished*). Gliovirin, though not tested, might be expected to possess a spectrum of activity similar to gliotoxin because they are both diketopiperazines (3). Also, an AG-4 isolate has been reported to be insensitive to gliovirin (7).

The successful application of biological control will depend on a more complete characterization of the target system than that currently practiced. This paper provides an example of the variation in sensitivity to antibiosis exhibited not only by different anastomosis groups within a fungal species but by different morphological structures within an isolate.

LITERATURE CITED

1. Allen, M. C., and Haensler, C. M. 1935. Antagonistic action of *Trichoderma* on *Rhizoctonia* and other soil fungi. Phytopathology 25:244-252.
2. Aluko, M. O., and Hering, T. F. 1970. The mechanisms associated with the antagonistic relationship between *Corticium solani* and *Gliocladium virens*. Trans. Br. Mycol. Soc. 55:173-179.
3. Brewer, D., Hannah, D. E., and Taylor, A. 1966. The biological properties of 3,6-epidithiadiketopiperazines. Inhibition of growth of *Bacillus subtilis* by gliotoxins, sporidesmins, and chetomin. Can. J. Microbiol. 12:1187-1195.
4. Dennis, C., and Webster, J. 1971. Antagonistic properties of species-groups of *Trichoderma*. I. Production of nonvolatile antibiotics. Trans. Br. Mycol. Soc. 57:25-39.
5. Gulliver, K. 1946. The inhibitory action of antibiotics on plant pathogenic bacteria and fungi. Ann. Bot. (n.s.) 10:271-282.
6. Howell, C. R. 1982. Effect of *Gliocladium virens* on *Pythium ultimum, Rhizoctonia solani,* and damping-off of cotton seedlings. Phytopathology 72:496-498.
7. Howell, C. R., and Stipanovic, R. D. 1983. Gliovirin, a new antibiotic from *Gliocladium virens*, and its role in the biological control of *Pythium ultimum*. Can. J. Microbiol. 29:321-324.
8. Ogoshi, A. 1975. Grouping of *Rhizoctonia solani* Kühn and their perfect stages. Rev. Plant Prot. Res. 8:93-103.
9. Webster, J., and Lomas, N. 1964. Does *Trichoderma viride* produce gliotoxin and viridin? Trans. Br. Mycol. Soc. 47:535-540.
10. Weindling, R. 1941. Experimental consideration of the mold toxins of *Gliocladium* and *Trichoderma*. Phytopathology 31:991-1003.
11. Weindling, R., and Fawcett, H. S. 1936. Experiments in the control of Rhizoctonia damping-off of citrus seedlings. Hilgardia 10:1-16.
12. Wright, J. M. 1956. The production of antibiotics in soil III. Production of gliotoxin in wheat straw buried in soil. Ann. Appl. Biol. 44:461-466.