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WORKSHOP: Organisms and Methods for Biological Control of Nematodes

Methods for the Study of *Verticillium chlamydosporium* in the Rhizosphere

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Abstract: Methods for screening isolates of the nematophagous fungus, *V. chlamydosporium*, for their ability to colonize the surface of plant roots are described. Significant differences in the extent of colonization were observed in sterile conditions and in soil; plant species and cultivars also differed in their ability to support a selected isolate of the fungus. Although fungal density could be estimated using a semi-selective medium, it was not possible to separate differences in vegetative growth from differences in sporulation. There was a weak positive correlation between estimates of fungal density on the roots of plants grown in sterile conditions and the extent of hyphal growth measured by direct observation.

Key words: biological control, nematode, plant host, rhizosphere colonization, root-knot nematode, screening method, selective media, *Verticillium chlamydosporium.*

Colonization of the rhizosphere by the nematophagous fungus, *Verticillium chlamydosporium*, is essential for the control of *Meloidogyne* spp. by this agent. The fungus does not cause lesions or affect root growth but appears to proliferate in the rhizoplane and rhizosphere (11). However, isolates of the fungus differ markedly in their ability to spread on roots (11) and the extent of colonization differs significantly between plant species (10). Thus, there is a need to identify *V. chlamydosporium* isolates capable of extensive growth in the rhizosphere and plants able to support the fungus. Egg masses of root-knot nematodes within large galls are physically isolated from infection because the fungus is confined to the surface of roots. Hence, control of these nematodes is most effective when egg masses on small galls are exposed in the rhizosphere. Gall size is most affected by host plant susceptibility and nematode density.

Selective media have been developed for monitoring changes in the abundance of *V. chlamydosporium* as estimated from the numbers of colony-forming units (cfu) on dilution plates (4,9). These media have been used to demonstrate the importance of the rhizosphere for the survival of the fungus in soil (7) and the effects of soil texture, application rate, and temperature on the density of the fungus in the rhizosphere. However, selective media do not allow differentiation among colonies derived from hyphal fragments, conidia, or chlamydospores. Hence, changes in vegetative growth or in the extent of sporulation cannot be distinguished with these techniques. In this paper we describe some methods developed for studying the colonization of the root surface by *V. chlamydosporium* on different plant species.

Materials and Methods

Isolates of *V. chlamydosporium* were collected from a wide range of soils infested with *Heterodera* and *Meloidogyne* spp. from suspensions of eggs or chlamydospores plated on 0.8% water agar (8). Pure cultures of each isolate were made on 1.7% corn meal agar, and mixtures of hyphae and spores were stored at 4 C on silica gel (14) until required for testing. More than 110 isolates have been collected and stored since 1980; all were tested for their ability to colonize plant rhizospheres within 2

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years of their date of collection and most within 12 months.

Screening for ability to grow in the rhizosphere: Conidia of each isolate were produced in shaken cultures of Czapek Dox Broth with additional salts (5) at 15 C. After 14 days, the spores were separated from mycelium on a 10-μm-pore sieve and suspended in 1% manucol, and the volume adjusted to provide 10^6 conidia/ml. Ten barley (*Hordeum vulgare* cv. Triumph) seeds were surface disinfested in 7% calcium hypochlorite for 45 minutes on a wrist action shaker and washed five times with sterile distilled water. The seeds were dipped in the spore suspension, for an infestation level of ca. 10^5 conidia/seed, and air-dried overnight in a sterile cabinet. The seeds were planted in sterile vermiculite in glass tubes (15 cm × 2.5-cm-d) stoppered with cotton and grown for 10 days at 19 C. Each isolate was tested in triplicate. Controls were untreated seeds dipped in the manucol solution only. Before planting, the presence of viable inoculum of each isolate was checked by plating three treated seeds on 0.7% water agar and incubating for 7 days at 19 C; colonies of the fungus developed from all seeds. Two roots from the seedling grown in each tube were cut into 1-cm segments and assessed for colonization after incubation on 0.7% water agar plates for 7 days at 19 C. The proportion of segments colonized and their distance from the initial inoculum on the seed was recorded.

Assessing growth in the rhizosphere: After selection of isolates in the in vitro assay, the isolates were tested for their ability to colonize the rhizosphere in nonsterilized soil and to control nematode multiplication. A procedure has been developed in which chlamydospores of the fungus are mixed with soil and added to pots infested with root-knot nematodes in which tomato (*Lycopersicon esculentum* cv. Pixie) plants are grown. Here, only the methods for assessing growth of the fungus in the rhizosphere are discussed. Two tests were conducted in which four different isolates were compared for their ability to grow on the roots of tomato plants, and different plant species were screened for their ability to support a selected isolate. Isolates of the fungus were cultured on a 1:1 mixture (v/v) of sand and milled barley grain for 21 days at 25 C. The colonized medium was washed on a 50-μm-pore sieve, and chlamydospores and some hyphal fragments were collected on a sieve with 10-μm openings. The chlamydospores were added to sterile sand and stored at 4 C until required. Before addition to soil, the spores from a known weight of sand were suspended in water and their number estimated in a hemocytometer. The inoculum in sand was added to nonsterilized soil at a rate of 5,000 chlamydospores/g soil and thoroughly mixed. The treated soil was dispensed in 750-g aliquots into 12-cm-d pots and planted with one of a range of plants. After 2 weeks, the soil was inoculated with 2,500 juveniles of *M. incognita.*

After ca. 6 weeks, an assessment of rhizosphere competence in nonsterilized soils was obtained with a semi-selective medium (9) to estimate the proliferation of *V. chlamydosporium* on the root surface. Root systems were washed, blotted dry, weighed, cut into segments (0.5–1 cm long), and mixed, and a 1-g subsample was taken. The sample was crushed in 9 ml of 0.05% agar solution with a sterile pestle and mortar and a dilution range prepared (10^{-1} to 10^{-3}) (9). Two 0.2-ml subsamples of each dilution were incubated at 25 C on the semi-selective medium for 2–3 weeks, at which time colonies were counted. The intersect method was used to measure root length (15) and with a measurement of average root segment diameter, used to estimate root surface area for calculation of cfu/cm^2 root. Data were subjected to analysis of variance.

Measurement of rhizosphere colonization: The relationship between hyphal length and cfu on a range of plant species and cultivars grown in sterile conditions was investigated. As described above, test tubes (15 × 2.5-cm-d) were half-filled with vermiculite, dampened, plugged with cotton, and autoclaved. Seeds were surface disinfested in 7% CaOCl for 45 minutes on a shaker, rinsed, and placed aseptically on seed-germinating medium (10 g glucose, 0.1 g each of yeast extract and peptone, 12 g agar/liter distilled water) at 25 C to check for the development of contaminants; plates were examined after 24–36 hours when the seeds had germinated. Plugs of *V. chlamydosporium* (4 mm × 2-mm-d) were removed from the edges of colonies on corn meal agar and pushed below the surface of the vermiculite and a sterile germinated seed was transferred to the tube. Six tubes for each plant species or cultivar were incubated at 25 C.

After 3 weeks, the plants in half the tubes were removed, shaken free of vermiculite, and the roots stored in water with a few drops of formaldehyde (40% w/v). A length of root (5 cm) was stained with aniline blue (6) and hyphal density estimated by a modification of the line intersect method (13). The remaining plants were used to assess growth in the rhizosphere, as estimated from the numbers of cfu, by the method described above. Data were subjected to correlation analysis.

Results

Screening for the ability to grow in the rhizosphere: In sterile conditions, few (<5%) isolates of *V. chlamydosporium* failed to spread on the surface of barley roots (Table 1). Most roots were >10 cm in length, and ca. 45% of the isolates colonized >80% of the

TABLE 1. Extent of rhizosphere colonization of barley cv. Triumph seedlings inoculated with different isolates of *Verticillium chlamydosporium*.

Category (% colonization)	No. of isolates	Colonization (%) Mean†	Colonization (%) SE
0–20	11	6	2.3
21–40	10	28	1.6
41–60	15	50	1.7
61–80	22	72	1.3
81–100	42	94	0.9

† Mean proportion of root segments colonized by the fungus within each category; estimated from two roots from each plant and three replicate plants treated with each fungal isolate.

TABLE 2. Differences in the proliferation of four isolates of *Verticillium chlamydosporium* on roots of tomato plants in soil.

Fungal isolate	Root colonization Cfu × 10^3	Root colonization Log_{10}
10	46.6	1.66
33	7.4	0.90
40	43.0	1.61
98	15.0	1.07
Untreated control	0	0.00
	SED = 0.13	

Data are means of five replicates.

root surface. However, the test detected only the presence or absence of the fungus and was not able to distinguish differences in the degree of colonization of different fungal isolates.

Assessing growth in the rhizosphere: Isolates differed ($P < 0.001$) in their ability to proliferate in the rhizosphere of tomato plants grown in soil infested with root-knot nematodes (Table 2). Isolates 10, 40, and 98 colonized >80% of root segments in the in vitro test, but isolate 98 failed to proliferate in the rhizosphere in nonsterile soil. Isolate 10 increased most ($P < 0.001$) in the rhizosphere of tomato plants and least in that of soybean (Table 3).

Measurement of rhizosphere colonization: In the absence of nematodes, plants also differed ($P < 0.001$) in their ability to support the proliferation of isolate 10 (Table 4). In the sterile conditions of this test, the density of the fungus in the rhizosphere was much greater than in the test in soil. Different cultivars of the same plant species also differed in their ability to support the fungus. There was a weak positive correlation ($r = 0.55$; $P < 0.05$) between estimates of colonization based on the numbers of cfu and those based on hyphal length.

Discussion

The methods described above facilitated screening of many different isolates of *V. chlamydosporium* for their ability to colonize plant rhizospheres and provided information on factors that influence the relative

TABLE 3. Differences in the proliferation of *Verticillium chlamydosporium* isolate 10 on the roots of different plant species infested with *Meloidogyne incognita*.

Host species	Root colonization	
	Cfu × 10^2	Log_{10} cfu
Tomato *Lycopersicon esculentum*	236.9	2.37
Cabbage *Brassica oleracea*	189.9	2.18
Potato *Solanum tuberosum*	87.8	1.93
Tobacco *Nicotiana tabacum*	42.6	1.62
Pigeon pea *Cajanus cajan*	34.8	1.40
Pepper *Capsicum annuum*	29.3	1.33
Cotton *Gossypium hirsutum*	22.2	1.13
Sorghum *Sorghum bicolor*	19.0	1.24
Wheat *Triticum vulgare*	15.6	1.18
Soybean *Glycine max*	13.8	1.15
SED ($P < 0.001$)		0.16

Data are means of three replicates.

abundance of the fungus on roots. In practice, isolates that colonized <80% of the root segment are not effective colonizers of the rhizosphere in tests in field soil (Kerry, unpubl.). Thus, by incorporating a screen for rhizosphere colonization into the selection procedure of potential biological control agents, it would be possible to reject almost 50% of the isolates on this criterion alone. Clearly, the plant has a significant effect on the colonization of the rhizosphere in both sterile and nonsterile conditions. This fungus is probably a weak competitor in the rhizosphere and was much more abundant on the surface of sterile roots than on those grown in soil. However, direct comparisons between different tests can be misleading because fungal colonization is affected by factors influencing plant growth such as light intensity and the presence of nematodes in roots (J. M. Bourne, unpubl.). The fungus is more abundant on roots infected with root-knot nematodes (12), which affect

TABLE 4. Relationship between estimates of the numbers of colony-forming units (cfu) and hyphal length of *Verticillium chlamydosporium* on the roots of different plants after 3 weeks growth in sterile conditions in the absence of nematodes.

Plant	Root colonization	
	Cfu × 10^3/cm^2	Hyphal length cm/cm^2
Sunn hemp *Crotalaria* spp.	155.5	35.5
Kale *Brassica oleracea*	37.3	31.5
Eggplant *Solanum melongena*	33.7	2.6
Tomato *Lycopersicon esculentum* CV. M82-1-8-VF	17.5	11.0
Onion *Allium cepa*	11.2	25.5
Cabbage *Brassica oleracea*	8.9	22.5
Maize *Zea mays* (cv. Phormia)	7.9	11.0
Tomato *Lycopersicon esculentum* (cv. Pixie)	3.7	7.0
Sorghum *Sorghum bicolor*	3.6	3.0
Melon *Citrullus lanatus*	2.6	5.5
Millet *Pennisetum americanum*	1.4	10.0
Pigeon pea *Cajanus cajan*	1.4	0.1
Soybean *Glycine max*	0.4	3.1
Maize *Zea mays* (cv. Katumani)	0.3	11.0
Bean *Phaseolus vulgaris*	0.2	1.0
Okra *Abelmoschus esculentus*	0.1	1.0

Data are means of two replicates.

root exudation and the supply of nutrients in the rhizosphere (3). Although the selective medium enabled the isolation of the fungus from soil, there was a relatively poor correlation between estimates of hyphal growth measured by direct staining and the numbers of cfu counted on dilution plates, presumably because there were considerable differences in sporulation in the rhizosphere of different plants species or cultivars. To elucidate the relationship between root exudation and the abundance of the fungus in the rhizosphere in soil, there is a need for specific techniques to differentiate sporulation from vegetative growth in situ. Estimates of the biomass of *V. chlamydosporium* are not readily related to the numbers of cfu even in sterile conditions (9), and the use of polyclonal antibodies to study colonization has met with limited success (1). Reporter genes such as GUS (*Escherichia coli* β-D-glucuronidase gene) have been used successfully to study colonization of rhizosphere bacteria (2) and would allow transformed isolates of the fungus to be visualized on the surface of roots in nonsterile conditions.

Literature Cited

1. Clyde, J. M., and H. J. Atkinson. 1992. An immunological approach to studies on the colonisation of the rhizosphere by *Verticillium chlamydosporium* and its effect on the multiplication of *Heterodera schachtii*. Proceedings of the ESN 21st International Nematology Symposium, Albuferia, Portugal, April 1992. Nematologica 38:404.

2. Broek, A. V., J. Michiels, A. V. Gool, and J. Vanderleyden. 1993. Spatial-temporal colonization patterns of *Azospirillium brasiliense* on the wheat root surface and expression of the bacterial nifH gene during association. Molecular Plant-Microbe Interactions 6:592–600.

3. Dourhout, R., F. J. Gommers, and C. Kolloffel. 1993. Phloem transport of carboxyyfluorescein through tomato roots infected with *Meloidogyne incognita*. Physiological and Molecular Plant Pathology 43: 1–10.

4. Gaspard, J. T., B. A. Jaffee, and H. Ferris. 1990. Association of *Verticillium chlamydosporium* and *Paecilomyces lilacinus* with root-knot nematode infested soil. Journal of Nematology 22:07–213.

5. Irving, F., and B. R. Kerry. 1986. Variation between strains of the nematophagous fungus, *Verticillium chlamydosporium* Goddard. II. Factors affecting parasitism of cyst nematode eggs. Nematologica 32: 474–485.

6. Jones, P. C. T., and J. E. Mollison. 1948. A technique for the qualitative estimation of soil microorganisms. Journal of Canadian Microbiology 2:54–69.

7. Kerry, B. R. 1988. Two microorganisms for the biological control of plant parasitic nematodes. Proceedings of the Brighton Crop Protection Conference—Pests and Diseases (1988). 603–607.

8. Kerry, B. R., and D. H. Crump. 1977. Observations on fungal parasites of females and eggs of the cereal cyst nematode, *Heterodera avenae*, and other cyst nematodes. Nematologica 23:193–201.

9. Kerry, B. R., I. A. Kirkwood, F. A. A. M. de Leij, J. Barba, M. B. Leijdens, and P. C. Brookes. 1993. Growth and survival of *Verticillium chlamydosporium* Goddard, a parasite of nematodes, in soil. Biocontrol Science and Technology 3:355–365.

10. Kerry, B. R., and F. A. A. M. de Leij. 1992. Key factors in the development of fungal agents for the control of cyst and root-knot nematodes. Pp. 139–144 *in* E. C. Tjamos, G. C. Papavizas, and R. J. Cook, eds. Biological control of plant diseases. New York: Plenum Press.

11. Leij, de F. A. A. M., and B. R. Kerry. 1991. The nematophagous fungus, *Verticillium chlamydosporium* Goddard, as a potential biological control agent for *Meloidogyne arenaria* (Neal) Chitwood. Revue de Nématologie 14:157–164.

12. Leij, de F. A. A. M., B. R. Kerry, and J. A. Dennehy. 1992. The effect of fungal application rate and nematode density on the effectiveness of *Verticillium chlamydosporium* as a biological control agent for *Meloidogyne incognita*. Nematologica 38:12–122.

13. Rovira, A. D., E. I. Newman, J. H. Bowen, and R. Campbell. 1974. Qualitative assessment of the rhizosphere microflora by direct microscopy. Soil Biology and Biochemistry 6:211–216.

14. Smith, D., and A. H. S. Onions. 1983. A comparison of some preservation techniques for fungi. Transactions of the British Mycological Society 81: 535–540.

15. Tennant, D. 1975. A test of a modified line intersect method of estimating root length. Journal of Ecology 63:995–1001.