Selective Isolation and Enumeration of *Laetisaria arvalis* from Soil

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ABSTRACT

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Three MBC fungicides (benomyl, thiabendazole, thiophanate-methyl) at 25 μg a.i./ml of potato-dextrose agar completely inhibited linear growth of *Rhizoctonia solani* but not that of *Laetisaria arvalis* (*Corticium sensu lato*), an antagonist of *R. solani* and *Pythium ultimum*. Using this information, a semiselective medium was developed for the direct isolation and enumeration of *L. arvalis* from soil. The medium, designated as WAA-HC+TBZ, contained, per liter: agar, 15 g; streptomycin SO_4, chlorotetracycline HCl, and thiabendazole, 100, 50, and 25 mg, respectively; and Hunt and Cobb solution, 5 ml. The average percentage recovery of the antagonist from artificially infested soil with a multiple pellet soil-sampler (MPSS) was 66%. The table beet seed colonization method of isolation was more sensitive than the MPSS technique in recovering *L. arvalis* from soil but did not yield quantitative data on numbers of propagules per gram of soil. Isolation of *L. arvalis* was also obtained on the WAA-HC+TBZ medium by the dilution-plate method, but the numbers recovered were smaller than those obtained with the MPSS. Numbers of propagules of *L. arvalis* in seven field soils ranged from 0 to 7.0 per gram of soil. No significant difference of population density of *L. arvalis* was observed on the medium from soils infested or uninfested with *R. solani*.

A basidiomycetous fungus with distinct clamp connections was isolated by M. G. Boosalis from sugar beet residue in soil of western Nebraska (8). This fungus was referred to as *Corticium sensu lato* (4,9) or as *Corticium* sp. (3,5). In 1980, Burdsall et al (1) placed this species in the genus *Laetisaria* and named the fungus *L. arvalis* Burdsall sp. nov.

Although *L. arvalis* was found to be a good antagonist against *Rhizoctonia solani* Kühn (8,9) and *Pythium ultimum* Trow (3), providing substantial biological control of these pathogens in the field (3,7,8), no quantitative studies of the ecology and survival of the antagonist in soil have been reported. The scarcity of information on the survival of *L. arvalis* in soil is due, almost exclusively, to lack of culture media and precise techniques for its isolation and enumeration. Techniques that employ beet seed colonization to detect *R. solani* in soil (10) are not suitable for accumulation of quantitative data for the antagonist. This further complicates the possibility of recovering *L. arvalis* from soil, especially in the presence of *R. solani*.

The present study was undertaken to formulate selective media for isolation of *L. arvalis* from soil and to adapt these media for use with selective techniques to recover *L. arvalis* from soil without interference from *R. solani*, the gross morphology of which is similar to that of the antagonist. A preliminary report of this work was presented (11).

MATERIALS AND METHODS

Isolates of *L. arvalis*. Ten isolates of *L. arvalis* were used in this study. H. H. Burdsall, Jr., Forest Products Laboratory, Madison, WI 53705, provided isolates ZH-1, ZH-2, ZH-3, ZH-4, ZH-5, and ZH-6. M. G. Boosalis provided the original isolate subsequently designated by us as LA-1. Isolates LA-2, LA-3, and LA-4 were obtained by the senior author from Beltsville soils. Stock cultures of *L. arvalis* were maintained on Difco potato-dextrose agar (PDA) slants at 5 C.

Production of sclerotia. All isolates of *L. arvalis* were grown on autoclaved oats (100 g of oat seed, 100 ml of tap water) for 4 wk. Sclerotia were harvested by wet-sieving the colonized oat seed through a 1.41-mm sieve in tandem with a 0.25-mm sieve. The sclerotia collected on the 0.25-mm sieve were washed with tap water and air-dried. Because the sclerotia develop aggregate masses when dried, they were ground in a Wiley mill with a 0.425-mm screen to separate them as much as possible. The grinding had no effect on viability of sclerotia. Weighed samples of each isolate were examined with a dissecting microscope to determine the number of sclerotia per gram of dry weight.

Soils. Rumford sandy loam (RSL, pH 6.0) was brought to the laboratory from a Beltsville field, passed through a 3-mm screen, and used immediately. In one experiment, in addition to RSL, five field soils from Maryland and one from Washington state were assayed for *L. arvalis* populations.

Effect of fungicides on radial growth of *L. arvalis* and *R. solani*. The following fungicides were used: pentachloronitrobenzene (PCNB, 75% WP, Olin Corp., Agriculture Division, Little Rock, AR 72203), benomyl (50% WP, E. I. du Pont de Nemours & Co., Wilmington, DE 19898); chloroneb (65% WP, E. I. du Pont de Nemours & Co.); chlorothalonil (75% WP, Diamond Shamrock Corp., Painesville, OH 44077); iprodione (50% WP, Rhône-Poulenc, Lyon, France); nystatin (Mycostatin, 4,960 units per milligram, Calbiochem-Behring Corp., La Jolla, CA 92307); carboxin (Vitavax 34% FF, Uniroyal Chemical, Bethany, CT 06525); thiabendazole (42.28% F, Merck & Co., Chemical Division, Rahway, NJ 07065); captan (50% WP, Chevron Chemical Co., Richmond, CA 94804); and thiophanate-methyl (70% WP, Pennwalt Corp., Oak, IL 60521). The fungicides were suspended in sterile distilled water and added at 25 μg a.i./ml to the autoclaved PDA before it was dispensed into petri dishes (20 ml per dish).

The 10 isolates of *L. arvalis*, isolate R-35 of *R. solani* (from the senior author's collection), and cultures of the four standard anastomosis groups of *R. solani* (AG-1, AG-2, AG-3, AG-4) were transferred to PDA containing fungicide at 0 or 25 μg a.i./ml. Colony radii were measured after 3, 5, and 7 days of incubation.

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Isolation media. Two basal media were used for isolation: water agar (1.5% agar) containing streptomycin SO_4 and chlorotetracycline HCl at 100 and 50 μg/ml, respectively (WAA) (10), and WAA containing 5 ml/L of a solution used by Hunt and Cobb (6) for the isolation of wood-rotting basidiomycetes. This solution contained the following: 95% ethanol and distilled water, 50 ml each; phenol, 1 g; benomyl, 0.32 g; and 2,6-dichloro-4-nitroaniline (DCNA, Botran®, 75% WP, TUCO, Division of the Upjohn Co.; Kalamazoo, MI 49001), 0.21 g. The second basal medium is hereafter referred to as WAA-HC.

Nystatin, benomyl, and thiabendazole (TBZ) were added singly to WAA and WAA-HC media at 25 μg a.i./ml. The various media were evaluated for isolation of *L. arvalis* from soil in the presence or absence of *R. solani*.

Isolation methods. *L. arvalis* was recovered from soil using the multiple pellet soil-sampler (MPSS) described by Henis et al (2). The table beet seed (*Beta vulgaris* L.) colonization method, hereafter referred to as colonization method (10), and the dilution-plate method were also used in some experiments for comparison. Soil dilutions were made by suspending the equivalent of 1 g of air-dry soil in 9 ml of sterile tap water and shaking the suspensions by hand for 1 min. One-milliliter aliquots were removed from the containers while the liquid was agitated by a magnetic stirrer and were spread on the media (six plates per replication). The plates were incubated in the dark at 25 C for 3–4 days and then washed with a cotton swab and a small stream of tap water to carefully remove all the soil and bacteria from the agar surface. Colonies of *L. arvalis* were best seen with an oblique fluorescent light while the agar surface was still wet. Colonies were examined microscopically for the presence of clamp connections on the mycelium.

Soils to be assayed with the MPSS were adjusted to 12–15% moisture content, and about 30 g quantity was placed in a petri plate. The surface of the soil was pressed lightly and smoothed with a bent spatula. Soil samples were taken with the MPSS as described by Henis et al (2), and these were delivered on the surface of the media (four plates per replication, 15 pellets each). Additional pellet samples were dried at 30–32 C for 24 hr to determine the average dry weight of the pellet samples. The plates were incubated at room temperature and examined for the presence of *L. arvalis* and *R. solani* hyphae after 1, 2, and 3 days of incubation. The number of propagules per gram of soil was estimated from the percentage of pellets colonized by applying the first order of the Poisson distribution, as was done by Henis et al (2) for *R. solani*:

$$\log_e \frac{1}{1 - X}$$

where X is the proportion of pellets colonized. This transformation depends on the assumption that the propagules are distributed randomly in soil (12).

Except where otherwise stated, five replications were used throughout and all experiments were done twice.

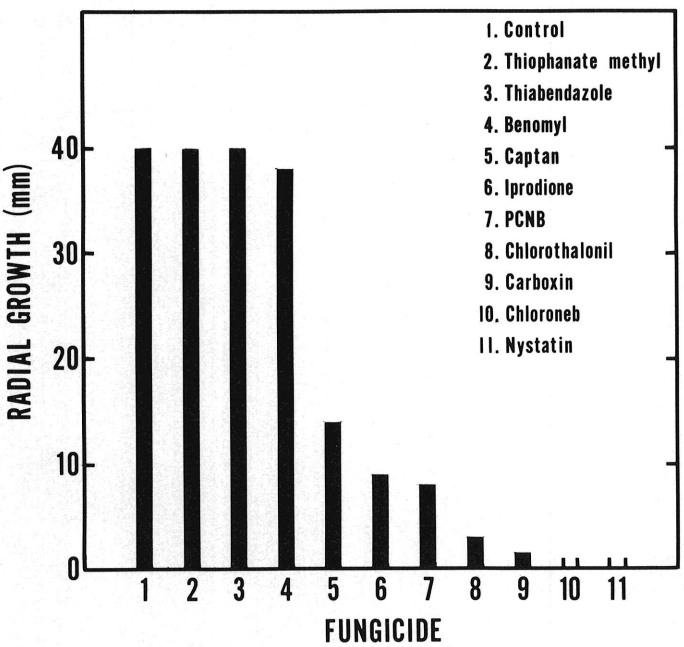

Fig. 1. Effect of 10 fungicides and a fungistatic antibiotic (nystatin) added to potato-dextrose agar at 25 μg a.i./ml on the linear growth of *Laetisaria arvalis* (isolate ZH-5). Radii were measured after 5 days of incubation.

TABLE 1. Recovery of *Laetisaria arvalis* (isolate ZH-5) and *Rhizoctonia solani* from soil with a pellet sampler as affected by the medium of isolation

Basal medium and additives[a]	Propagules per gram of soil	
	R. solani	*L. arvalis*
Water agar plus antibiotics (WAA)[b]		
Nystatin	0	0
Benomyl	0	2.1 a[c]
None	1.5	5.9 b
Thiabendazole	0	10.3 c
WAA + Hunt & Cobb solution[d]		
Nystatin	0	0
Benomyl	0	5.8 b
None	0	10.5 c
Thiabendazole	0	13.4 d

[a] All added at 25 μg a.i./ml of medium.
[b] Water agar (1.5%) containing streptomycin sulfate and chlorotetracycline HCl at 100 and 50 μg/ml, respectively.
[c] Values followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.
[d] WAA fortified with 5 ml/L of a solution used by Hunt and Cobb (6) for the isolation of wood-rotting basidiomycetes.

RESULTS

Tolerance of *L. arvalis* to fungicides. The three MBC fungicides, benomyl, TBZ, and thiophanate-methyl, did not inhibit the linear growth of *L. arvalis* (isolate ZH-5) at 25 g a.i./ml of PDA (Fig. 1). Captan, iprodione, and PCNB reduced linear growth to one-fourth of that obtained on fungicide-free PDA.

Chlorothalonil, carboxin, and especially chloroneb and nystatin were toxic to *L. arvalis*. Six additional isolates of *L. arvalis* tested in a separate experiment behaved the same way as did isolate ZH-5 on PDA amended with the fungicides (Fig. 1).

In a separate experiment, benomyl and TBZ were added to PDA at 25 g a.i./ml and the media were inoculated with five isolates of *L. arvalis*, isolate R-35 of *R. solani*, and the four anastomosis group standards (AG-1, AG-2, AG-3, AG-4). Neither fungicide had an appreciable effect on linear growth of the five isolates of *L. arvalis*. Benomyl and TBZ, on the other hand, inhibited growth of *R. solani* completely. Because of their inability to inhibit growth of *L. arvalis* at 25 g a.i./ml, benomyl and TBZ were selected for further tests to isolate the antagonist from soil.

Isolation media. *L. arvalis* inoculum (isolate ZH-5, grown on sand-corn meal for 4 wk) was added to RSL (naturally infested with *R. solani*) at 0.1% (w/w, dry weight) and mixed thoroughly. In this test, the antagonist inoculum contained both sclerotia and mycelia. Benomyl, nystatin, and TBZ were added to WAA and WAA-HC singly at 25 g a.i./ml, and the media were used to isolate *L. arvalis* from soil, in the presence of *R. solani*, with the MPSS. Nystatin was used because it was found not to affect greatly the linear growth of *R. solani* at 25 g a.i./ml (G. C. Papavizas, *unpublished*). The best recovery of *L. arvalis* with the MPSS was obtained on the WAA-HC medium containing TBZ (WAA-HC+TBZ) and on WAA+TBZ (Table 1). Benomyl added to both basal media did not improve recovery of *L. arvalis* from soil. *R. solani* failed to grow except from some pellets placed on WAA (1.5 propagules per gram of soil). Nystatin and benomyl (at 25 g a.i./ml), therefore, were not included in subsequent tests.

Utilizing the same soil naturally infested with *R. solani* and freshly fortified with *L. arvalis*, a second test was performed to compare recovery of *L. arvalis* with the three methods. Quantitative recovery was obtained only with the MPSS (Table 2). Comparable isolation was obtained on the basal media with TBZ, and on WAA-HC without TBZ. *L. arvalis* was also recovered with the dilution-plate method, but only about one-fifth of the number of propagules per gram of soil were recovered compared to the number obtained with the MPSS. All table beet seeds plated out on the four media were colonized by *L. arvalis*. No interference was observed from *R. solani*.

Evaluation of the WAA-HC+TBZ medium with the MPSS. Sclerotia of *L. arvalis* (isolate ZH-5), obtained from oat cultures and counted with the aid of a stereoscopic microscope, were added to soil at a rate of 0, 5, 10, 15, 20, 25, and 30 sclerotia per gram of air-dry soil. These amended soils were assayed with the MPSS and the WAA-HC+TBZ medium. The number of propagules recovered from the seven amended soils, as estimated with the Poisson distribution formula, was 0, 3.5, 6.4, 9.9, 12, 16, and 21, respectively, corresponding to 0, 70, 64, 66, 60, 64, and 70% recovery, respectively (Fig. 2). Eighty percent of table beet seed was colonized by *L. arvalis* in soil infested with five sclerotia per gram and 100% was colonized at all other concentrations of sclerotia.

Recovery of various isolates of *L. arvalis* from soil. Sclerotia of eight isolates of *L. arvalis* were added to soil at 15 sclerotia per gram of air-dry soil. Recovery from soil was attempted with the MPSS on the WAA-HC+TBZ medium 1 hr after soil infestation. Recovery ranged from 37% with isolate ZH-1 to 162% with isolate ZH-4 (Table 3). The percentage recovery of ZH-5 was 73%. Recoveries of more than 100% were obtained with isolates LA-1, ZH-2, and ZH-4.

Recovery from natural soils. Seven field soils were assayed with the MPSS and the table beet seed colonization method on the WAA-HC+TBZ medium. No propagules were recovered with the MPSS from three soils and a very small number from three other soils (Table 4). Only a silty clay soil from Beltsville, MD, yielded 7 propagules per gram of air-dry soil. This soil sample came from a cucumber field that had been infested with *L. arvalis* (isolate LA-1) for the control of Rhizoctonia fruit rot 3 mo before the assay. The percent colonization of table beet seed ranged from 0 to 80% in the soil from the cucumber field. Isolations made from colonized table beet seed on PDA slants from the natural soils yielded, in addition to *L. arvalis*, a fungus, orange in color, with small (0.1 mm in diameter), orange sclerotia but without clamp connections. The recovery of such a fungus, unidentified, complicated the recovery of *L. arvalis* from natural soils.

DISCUSSION

The new medium, WAA-HC+TBZ, allows development of *L. arvalis* hyphae from soil without interference from *R. solani*, another basidiomycete whose hyphae resemble those of the antagonist when growing out of the colonized seed or soil pellets. *R. solani* is completely inhibited by TBZ at 25 μg a.i./ml or by benomyl at 8 μg a.i./ml added to the medium containing the solution used by Hunt and Cobb (6) to isolate wood-rotting basidiomycetes. The medium also allows formation of clamp connections, a morphologic character that distinguishes *L. arvalis* from soil saprophytes and even from other basidiomycetes such as *R. solani* that do not form clamp connections. The WAA-HC+TBZ medium is a good substrate for hyphal development, which can be distinguished by examining the bottoms of petri plates against a fluorescent light within 1–3 days of incubation. An estimation of the number of propagules of *L. arvalis* per gram of soil can then be made with the Poisson distribution formula in a fashion similar to that used by Henis et al (2) for *R. solani*.

The new *L. arvalis*-selective medium (WAA-HC+TBZ) described in this paper gave satisfactory results in this research and may be used in studies to determine population dynamics of *L. arvalis* in soil as affected by various environmental factors. The medium, however, used with the colonization method or with the MPSS to recover *L. arvalis* from natural soils has at least one disadvantage. Although it excludes *R. solani*, the medium allows

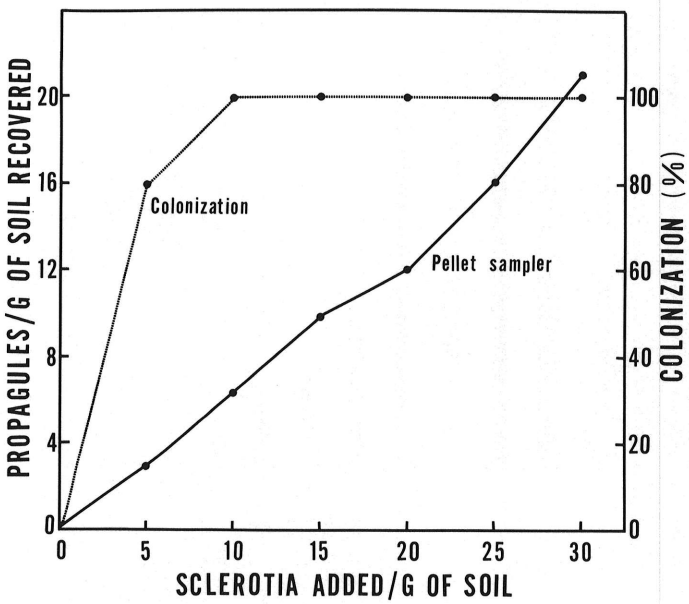

Fig. 2. Recovery of *Laetisaria arvalis* (isolate ZH-5) from soil infested with sclerotia of the antagonist by the table beet seed method (in percent colonization) and the multiple pellet soil-sampler technique (in propagules per gram of soil).

TABLE 2. Recovery of *Laetisaria arvalis* (isolate ZH-5) from soil with the dilution plate method, a pellet sampler, and with the table beet seed colonization method as affected by the medium of isolation

Basal medium with or without thiabendazole (TBZ)	Propagules per gram of soil		Table beet seed colonization (%)
	Dilution plate	Pellet sampler	
Water agar plus antibiotics (WAA)[b]	3 a[a]	3 a	100
WAA+TBZ	6 b	30 b	100
WAA + Hunt & Cobb (HC) solution[c]	7 b	36 b	100
WAA–HC+TBZ	9 c	35 b	100

[a] In each column, values followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.
[b] Water agar (1.5%) containing streptomycin sulfate and chlorotetracycline HCl at 100 and 25 μg/ml, respectively.
[c] WAA fortified with 5 ml/L of a solution used by Hunt and Cobb (6) for the isolation of wood-rotting basidiomycetes.

TABLE 3. Recovery of various isolates of *Laetisaria arvalis* from soil with a pellet sampler on the WAA–HC+TBZ medium[a]

Isolate added[b]	Propagules per gram of soil	Recovery (%)
ZH-1	5.5 a[c]	37
LA-2	8.2 b	55
ZH-5	11.0 c	73
ZH-6	12.0 c	80
ZH-3	15.0 d	100
LA-1	16.8 d	112
ZH-2	17.3 d	115
ZH-4	24.3 e	162

[a] Water agar (1.5%) containing streptomycin sulfate and chlorotetracycline HCl at 100 and 25 μg/ml, respectively; 5 ml/L of Hunt & Cobb solution (6); and 25 μg/ml of thiabendazole.
[b] Fifteen propagules were added per gram of soil.
[c] Values followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.

TABLE 4. Isolation of *Laetisaria arvalis* from various soils, using the pellet sampler and the table beet seed colonization methods on the WAA−HC+TBZ[a] medium

Soil and origin	Table beet seed colonized (%)	Propagules per gram of soil as determined with the pellet sampler
Loamy sand, Maryland	0	0
Sandy loam, Maryland	3.3 a[b]	0
Sandy loam, Washington	1.3 a	0
Silty clay, Maryland	6.0 a	0.3 a
Garden soil, Maryland	25.3 b	0.7 b
Sandy loam, Maryland	6.5 a	0.9 c
Silty clay, Maryland[c]	80.0 c	7.0 d

[a] Water agar (1.5%) containing streptomycin sulfate and chlorotetracycline HCl at 100 and 25 μg/ml, respectively; 5 ml/L of Hunt and Cobb solution (6); and 25 μg/ml of thiabendazole.

[b] In each column, values followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.

[c] Soil from a plot that was infested with *L. arvalis* in June 1981. Assay was performed 3 mo after infestation.

recovery of at least one unidentified, nonsporulating fungus, orange in color, that looks very much like *L. arvalis* but that does not develop clamp connections on its mycelium. When in doubt, transfers can be made to PDA to ascertain that colonies developing from seed or soil pellets are actually *L. arvalis*. Another apparent disadvantage is that a recovery of more than 100% was observed when a known number of sclerotia of certain isolates was added to soil (Fig. 2). This may appear to be the result of an error in counting the sclerotial inoculum with the microscope or enumerating the antagonist from soil. We believe that this phenomenon may have been caused by the sclerotia of some isolates breaking in soil or carrying dry mycelium easily separated from sclerotia during mixing, thus increasing the actual numbers of propagules added at the outset.

Comparison of the data from the table beet seed colonization method for *L. arvalis* with those obtained with the MPSS shows that the former method is more sensitive than the MPSS technique (Tables 3 and 4). For instance, 80% of the table beet seed was colonized by *L. arvalis* when the seed was incubated for 2 days in a silty clay soil from Beltsville, MD (Table 4). The MPSS technique with the same soil yielded seven propagules per gram of air-dry soil. In another experiment, even five sclerotia per gram of soil resulted in 80% seed colonization (Fig. 2), but the method could not distinguish concentrations of 10 sclerotia or more per gram of soil. The great sensitivity, but apparent lack of quantitative ability, of the seed colonization method may be due to the fact that *L. arvalis* is a dynamic organism capable of progressively colonizing organic matter in soil. With the MPSS, the soil pellets are removed from soil and immediately placed on the selective medium. Because of this discrepancy between the two methods, we suggest that the table beet seed colonization method be used to isolate *L. arvalis* from soils where populations may be very low and the MPSS technique be used for both isolation and quantitative assays of *L. arvalis*.

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