A Medium to Enhance Recovery of *Aphanomyces* from Infected Plant Tissue

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ABSTRACT

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This report describes a selective medium (MBV) that permits growth of *Aphanomyces* while inhibiting or restricting growth of several root-associated fungi that commonly obscure *Aphanomyces* on isolation plates. MBV contains the following ingredients per liter of water: 10 g of Difco-Bacto agar, 10 g of Difco cornmeal agar, 30 mg of metalaxyl, 5 mg of benomyl, and 200 mg of Vancocin. MBV inhibits or greatly restricts growth of *Fusarium, Rhizoctonia, Phytophthora,* and several *Pythium* species. Some isolates of *Pythium* were able to grow in the presence of 30 mg of metalaxyl but at rates lower than for *Aphanomyces*. If *Alternaria, Mucor,* or *Rhizopus* interfere with isolation, 0.5 mg of amphotericin B can be used in the medium.

Aphanomyces causes root rot of several economically important plants (6). Crops affected in Wisconsin include peas, beans, and alfalfa. Accurate diagnosis of Aphanomyces root rot is often hindered because of difficulty in isolating this fungus from infected tissue. The difficulty arises because *Aphanomyces* generally is associated with other root-invading fungi, and some of these fungi, especially *Pythium* and *Rhizoctonia*, may obscure *Aphanomyces* on isolation plates. Since 1925, when *Aphanomyces* was first isolated from pea roots (3), various methods have been used to enhance recovery of this fungus from diseased tissue. Most techniques have depended on repeated or prolonged washing of tissue before isolation (4). These methods, later modified by the use of streptomycin, were employed to reduce bacterial contamination, but as Jones and Drechsler (3) noted, there was no satisfactory way to separate *Pythium* from *Aphanomyces* on isolation plates. Eckert and Tsao (2) identified antibiotics that permitted growth of *Pythium* while inhibiting *Aphanomyces*, but until recently, there has not been a selective agent that inhibits *Pythium* while permitting growth of *Aphanomyces*. We recently observed that metalaxyl, a systemic fungicide developed for control of diseases caused by *Pythium* and *Phytophthora* (7), has little in vitro activity against *Aphanomyces* at concentrations that inhibit Pythiacious fungi (5). On the basis of this observation, we have developed a medium that improves efficiency in isolating *Aphanomyces* from infected plant tissue.

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MATERIALS AND METHODS

Various fungicides and antibiotics were tested for their ability to permit growth of *Aphanomyces* while inhibiting growth of several other fungi commonly associated with diseased roots. For these tests, the materials were incorporated into agar medium and plates of the media were seeded with single plugs of the test fungi taken from actively growing colonies on suitable media. Plates were incubated at 22 ± 2 C and increase in colony diameter was recorded over a period of 2–7 days. The following materials were used in the final medium: metalaxyl, as an emulsifiable concentrate dissolved in 95% ethanol at 10 mg a.i./ml; benomyl, as a wettable powder added directly to the medium; Vancocin, added as a sterile water solution; and amphotericin B, also added as a sterile water solution. The agar base of the medium was made from Difco-Bacto agar and Difco cornmeal agar, and the antimicrobials were added after autoclaving. The fungi tested were isolates from diseased plants or from culture collections at the Department of Plant Pathology, University of Wisconsin at Madison.

From these results, a medium that encouraged growth of *Aphanomyces* while inhibiting other root fungi was formulated. This medium was tested for efficacy in isolating *Aphanomyces* from infected roots collected in the field. Plant roots, dug from plots known to be infested with *Aphanomyces* and other root-infecting fungi, were washed thoroughly, immersed in 0.5% NaOCl for 2 min, rinsed, blotted dry, and cut into 2-cm segments. Alternate segments from each root were placed on cornmeal agar and the selective medium. The plates were then incubated in the laboratory (22 ± 2 C) and observed daily for 5 days. Fungal colonies growing from the root segments were counted and the fungi classified as *Aphanomyces* or other on the basis of microscopic observation of hyphae or other structures. The proportion of colonies classified as *Aphanomyces* was recorded for each trial. About 10% of the total fungal colonies were transferred to pure culture for more rigorous identification. In all cases, the initial classification as *Aphanomyces* or non-*Aphanomyces* was confirmed.

RESULTS AND DISCUSSION

On unamended cornmeal agar, the growth rate of commonly encountered *Pythium* species was about two to three times that of the *Aphanomyces* isolates. Metalaxyl, at a concentration of 25 ppm in the medium, almost completely inhibited radial growth of *Pythium ultimum* and *P. irregulare* while reducing growth of *Aphanomyces* isolates only about 25% (Table 1). Growth of two unidentified *Pythium* isolates with filamentous sporangia was reduced about 70% at 25 ppm of metalaxyl. These changes resulted in *Pythium* growth rates substantially less than, or in one case equal to, *Aphanomyces* growth rates. At metalaxyl concentrations of 50 or 100 ppm, the growth rate of one *Aphanomyces* isolate was reduced to or below that of the filamentous-sporangia *Pythium* isolates.

Preliminary tests showed that several other amendments were useful in improving selectivity of the medium. Benomyl (5 ppm) greatly restricted growth of *Rhizoctonia, Fusarium, Verticillium,* and *Thielaviopsis*. Vancocin (200 ppm) was effective in reducing bacterial growth. Occasionally, *Alternaria* or *Mucor* interfered with isolation of *Aphanomyces*; amphotericin B (0.5 ppm) effectively inhibited these organisms but also somewhat reduced growth of *Aphanomyces*. The inhibitory effect of the fungicides on target organisms was

enhanced by halving the nutrient level customarily used in cornmeal agar; the reduced nutrient level also stimulated *Aphanomyces* to grow as a larger, less dense colony than it does on full-strength cornmeal agar. The final formulation of the medium (MBV) contains the following ingredients: 10 g of Difco-Bacto agar, 10 g of Difco cornmeal agar, 30 mg of metalaxyl, 5 mg of benomyl, 200 mg of Vancocin, and 1 L of water; 0.5 mg of amphotericin B should be included if *Alternaria* or *Rhizopus* are common contaminants. In pure-culture radial growth tests, MBV reduced the growth of most common root colonizers below that of *Aphanomyces* isolates (Table 2). Some isolates of *Pythium* present potential problems because their growth rates, although reduced by the metalaxyl, remain in the same range as those of some *Aphanomyces* isolates. These *Pythium* isolates were generally of the filamentous-sporangial type, although one spherical-sporangial isolate was able to make significant growth on MBV. The occurrence of metalaxyl-tolerant *Pythium* isolates from field soils is consistent with similar observations by Cook et al (1) in Washington. The isolates we recovered, although able to grow in the presence of 30 ppm of metalaxyl, were sufficiently inhibited by it to permit recovery of *Aphanomyces* in the presence of these *Pythium* isolates. Other fungi that could interfere with recovery of *Aphanomyces* on MBV are *Alternaria, Mucor,* and *Rhizopus*, but these were seldom encountered in isolations from field material. Furthermore, they can be largely inhibited by inclusion of amphotericin B in the medium if they are a problem (Table 2). Because this material also reduces growth of *Aphanomyces*, we recommend its use only where it is found necessary.

Performance of the medium was assessed by isolating from pea, bean, and alfalfa roots collected from fields known to harbor *Aphanomyces* and other soilborne pathogenic fungi. Compared with cornmeal agar, the MBV medium increased the efficiency of recovering *Aphanomyces* from infected root tissue (Table 3).

Use of purified agar (Difco-Bacto agar and Difco cornmeal agar) in the medium permits macroscopic and microscopic observation of fungal growth without interference from agar impurities. On the basis of morphological characteristics, *Aphanomyces* is easily distinguished among fungi that grow on this medium. It forms a sparse, arachnoid, wandering growth on and within the medium; this distinguishes it from most *Pythium* isolates, whose growth is more strongly directional. *Aphanomyces* may also be recognized by its characteristic hyphal morphology. It has large-diameter hyphae with granular cytoplasm, side branches are often short with a pointed apex, and the main hyphae commonly branch in a Y-shaped junction (Fig. 1). *Pythium* isolates, in contrast, usually have a less granular cytoplasm, longer, unpointed side branches, and lack the characteristic Y-shaped junctions in the main hyphae (Fig. 1). *Rhizopus* is the only other fungus we have seen on isolation plates that resembles *Aphanomyces* in morphology. It too has large-diameter hyphae with granular cytoplasm. As noted before, however, it is rarely encountered and is easily distinguishable by the black aerial sporangia it produces after a few days' growth.

The MBV medium has been useful in diagnosis of pea and bean root rot specimens to determine whether *Aphanomyces* is contributing to the

Table 1. Radial growth rate (mm/24 hr) of *Aphanomyces* and *Pythium* isolates on cornmeal agar[a] amended with metalaxyl

Isolate	Metalaxyl concentration			
	0 ppm	25 ppm	50 ppm	100 ppm
A. euteiches f. sp. *pisi*	8	6	5	3
A. euteiches f. sp. *phaseoli*	8	6	5	3
Aphanomyces sp.[b]	6	5	3	2
P. ultimum	24	0	0	0
P. ultimum	25	0	0	0
P. irregulare	22	<1	<1	<1
P. irregulare	24	<1	<1	<1
Pythium sp.[c]	16	5	3	3
Pythium sp.[c]	17	3	3	2

[a] Cornmeal agar contained 10 g of Difco-Bacto agar + 10 g of Difco cornmeal agar per liter.
[b] *Aphanomyces* isolate obtained from diseased alfalfa seedling.
[c] Unidentified *Pythium* isolates with filamentous sporangia.

Table 2. Radial growth rate (mm/24 hr) of selected soil fungi on nonselective and selective media

Fungus	Number of isolates	Medium[a]		
		CMA	MBV	MBVA
Aphanomyces euteiches f. sp. *pisi*	3	8–9[b]	7–8	5–6
A. euteiches f. sp. *phaseoli*	3	7–8	5–6	5–6
Aphanomyces sp.[c]	3	5–8	4–5	4–5
Pythium ultimum	1	24	<1	0
P. irregulare	3	18–22	<1	<1
P. paroecandrum	1	26	0	0
P. mamillatum	1	16	0	0
Pythium sp.[d]	2	15–24	0–5[e]	0–5[e]
P. aphanidermatum	1	21	1	<1
P. torulosum[e]	1	10	3	3
Pythium sp.[f]	2	16–17	3–4	3–4
Phytophthora spp.[g]	3	3–5	0	0
Rhizoctonia sp.	4	9–12	0–1	0–1
Fusarium solani[h]	3	2–4	0–1	0–1
F. roseum	1	6	0	0
F. oxysporum f. sp. *phaseoli*	1	6	0	0
Verticillium albo-atrum	2	1–2	0	0
Thielaviopsis sp.	1	2	0	0
Alternaria sp.	1	4	3	<1
Mucor sp.	1	9	7	1
Mortierella sp.	1	1	1	<1
Rhizopus sp.	1	18	17	5

[a] CMA = 10 g of Difco-Bacto agar + 10 g of Difco cornmeal agar per liter; MBV = CMA with 30 ppm of metalaxyl, 5 ppm of benomyl, and 200 ppm of vancomycin; MBVA = MBV with 0.5 ppm of amphotericin B added.
[b] Range of growth rates observed among the isolates of each fungus tested. Growth rate of each isolate is the average of two trials, with two plates per trial.
[c] Isolated from alfalfa seedling roots.
[d] Unidentified *Pythium* with spherical sporangia and no oospores.
[e] Isolates recovered from tissue plated on MBV medium.
[f] Unidentified *Pythium* isolates with filamentous sporangia.
[g] One isolate each of *P. megasperma, P. cactorum,* and *P. cinnamomi.*
[h] Two isolates of *F. solani* f. sp. *phaseoli* and one of *F. solani* f. sp. *pisi.*

Table 3. Efficiencies of nonselective and selective media in isolation of *Aphanomyces* from diseased root tissue[a]

Host	*Aphanomyces* colonies (percentage of total fungal colonies appearing on medium)	
	Cornmeal agar	MBV medium
Pea	27	86
Bean	7	51
Alfalfa	3	26

[a] Isolations attempted from roots of 50 field-grown plants per host. Each root was cut into segments and alternate segments were placed on each medium.

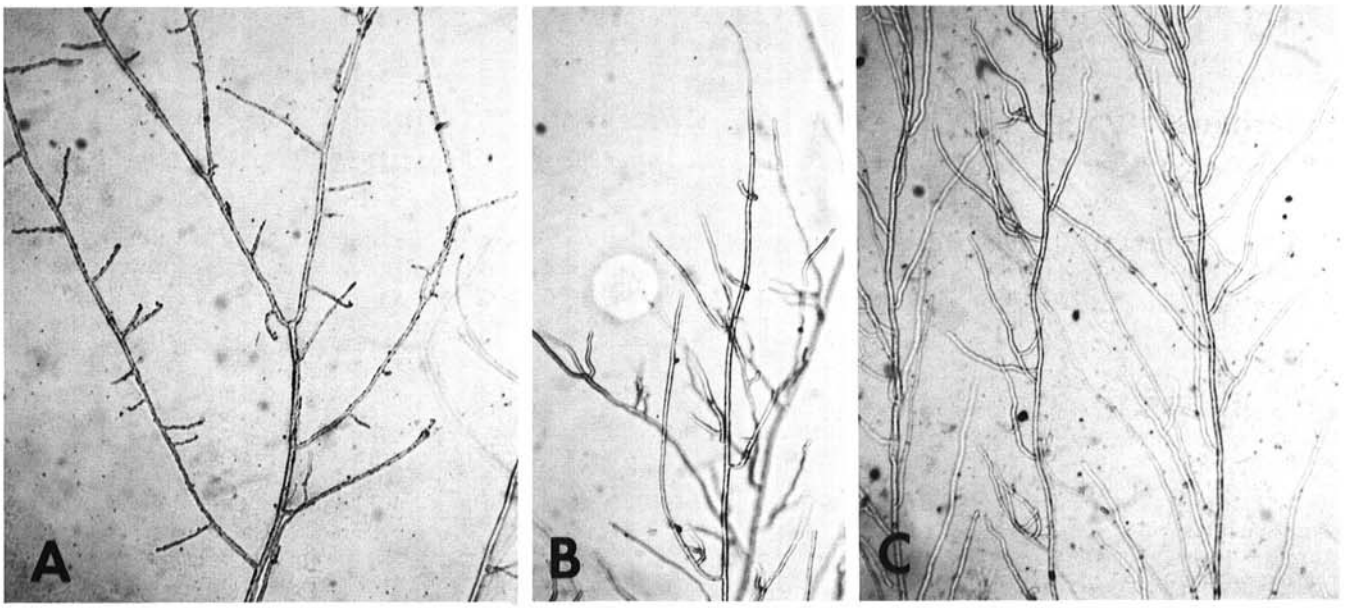

Fig. 1. Comparison of the hyphal morphology of *Aphanomyces* and *Pythium*. **(A)** Appearance of *Aphanomyces* hyphae on MBV medium. (Appearance on cornmeal agar is identical.) Note short, tapered side branches, granular cytoplasm, and Y-shaped junctions. **(B)** *Pythium* sp. on MBV medium. **(C)** *Pythium ultimum* on cornmeal agar (×75.6).

disease in a given situation. It has also aided in the study of a stand-establishment problem in some Wisconsin alfalfa fields, where it appears that *Aphanomyces* may be important (E. B. Holub, P. A. Delwiche, and C. R. Grau, *unpublished*). Although the medium is effective in recovering actively growing *Aphanomyces* from plant tissue, we have not found it useful in direct assessment of the soil population of this fungus. In dilution plating of either naturally infested field soil or pasteurized soil artificially reinfested with oospores of *Aphanomyces*, no colonies of the pathogen were observed on this medium, perhaps because requirements for oospore germination were not met on the plates.

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