A Selective Medium for the Enumeration and Isolation of Phoma betae from Soil and Seed

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ABSTRACT

A selective medium developed for the enumeration and isolation of *Phoma betae* from soil and seed consisted of: 4 g K_2HPO_4; 1.5 g KH_2PO_4; 25 ml soil extract; 200 mg boric acid; 100 mg each of streptomycin sulfate, chlorotetracycline, and benomyl; 10 g sucrose; and 17 g agar in 1,000 ml distilled water, adjusted to pH 7.0 with HCl before autoclaving. Sucrose and the antibiotics were added to the molten agar (50 C) after autoclaving. *Phoma betae* was characterized by the production of small, dark hyphal masses resembling microsclerotia on the underside of the culture in contact with the petri dish. The medium was used to enumerate *P. betae* in field soils that had been cropped to sugar beets for five consecutive years, in soil from commercial root storage sites, and in assaying seed samples.

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Phoma betae Frank is a common pathogen of sugar beet seedlings and stored roots (2). The fungus is seedborne and has been reported from England as infecting an average of 39% of seeds during a 12-year period (1). Information is limited concerning the survival of this fungus in the soil. Pool and McKay (5) reported that *P. betae* survived for 3 months when infected sugar beet leaves were stored in soil in boxes and exposed to outdoor conditions. The fungus was not viable after 5 to 8 months when infected leaves were buried in the ground.

At the North Dakota Agricultural Experiment Station, *P. betae* pycnidia were isolated from overwintered sugar beet debris (W. M. Bugbee, *unpublished*). The presence of *P. betae* in soil from this field could be detected by placing soil particles directly on water agar and examining the underside of the plate for characteristic growth conformations as described by Mangan (4).

A selective medium was necessary to quantitatively measure dispersal and factors affecting the survival of *P. betae* in field soils. A selective medium used to enumerate *Collectotrichum coccodes* (3) was used as the starting point.

MATERIALS AND METHODS.–Inoculum of *P. betae* was produced by growing the fungus on sterile, moist barley grain. Infected kernels were air-dried and ground in a mill to pass a 60-mesh screen. Inoculum was added to sterile or nonsterile Fargo silty clay soil. Soil to be assayed was air-dried and passed through a 2-mm screen before dilution.

Thirty-g soil samples were suspended in 300 ml of 0.1% sterile water agar. The suspensions were mixed for 2 min in a food blender, and 1-ml aliquots were dispensed in petri plates. Molten agar (50 C) was poured into the plates. The plates were swirled to disperse the soil suspension.

The base medium consisted of 4 g K_2HPO_4; 1.5 g KH_2PO_4; 25 ml soil extract; and 1,000 ml distilled water, adjusted to pH 7.0 with HCl before autoclaving. Soil extract was prepared by suspending 1 kg of soil in 1 liter of water and steaming the mixture at 104-110 C for 30 min in the autoclave.

The carbon sources and antibiotics were added to the molten agar at 50 C. Different carbon sources were tested in amounts calculated to give approximately similar quantities of carbon/liter: 10 g each of polygalacturonic acid (PGA), glucose, fructose, and galactose; 5 g sucrose; and 3.3 g raffinose. Antibiotics added (0.1 mg/ml) were cycloheximide, streptomycin sulfate, and chlorotetracycline. A pentachloronitrobenzene (PCNB) suspension was prepared according to Farley (3), and added to the medium at .002, .005, .025, or .05 mg/ml. Benomyl [methyl-1-(butylcarbamoyl)-2-benzimidazolecarbamate] was suspended in distilled water and 0.05% alkyl phenoxypolyethoxyethanol and added to the medium at 0.02, 0.1, or 0.2 mg/ml.

Growth was determined by measuring colony diam. Agar plugs, 3 mm in diam were taken from the edge of young colonies; two plugs were transferred to each plate containing agar medium with various amendments. Growth values were averaged from five plates (10 colonies).

Seed were washed for 30 min in running tapwater to remove the water-soluble fungicide Dexon [*p*-(dimethylamino) benzenediazo sodium sulfonate]. The seeds were rinsed in distilled water and plated on selective agar at 10 seeds/plate and 50 seeds/cultivar.

RESULTS.–*Phoma betae* did not grow when PCNB was used at a concn. of only 0.002 mg/ml. Average diam of 6-day-old colonies on agar

containing 0.02, 0.1, or 0.2 mg/ml of benomyl were 18, 10, and 9 mm, respectively.

PGA or sucrose was used as the carbon source in combination with certain antibiotics to observe the effect on growth and cultural characteristics of *P. betae* (Table 1). The cultures were incubated at 20 or 25 C. Cycloheximide at 0.1 mg/ml inhibited the growth of *P. betae*. Growth was more rapid with PGA as the carbon source, than with sucrose at 25 C in the presence of streptomycin sulfate or chlorotetracycline. Although the fungus grew most rapidly on PGA or raffinose, the hexose sugars and sucrose were of greater diagnostic value because of the production of the characteristic dark hyphal holdfast-like masses (Fig. 1). Average colony diam after 14 days at 22 C with glucose, fructose, sucrose, galactose, PGA, and raffinose as carbon sources were 5.2, 5.4, 5.8, 6.4, 9.2, and 10.0 mm, respectively [LSD ($P = 0.05$) = 0.7 mm].

Boron is stimulatory to *P. betae* (6). Results in Table 2 show an insignificant increase in the recovery of propagules per gram of soil, and a significant increase in growth when boric acid was used with soil extract.

The final medium used in subsequent work consisted of: 4 g K_2HPO_4; 1.5 g KH_2PO_4; 25 ml soil extract; 200 mg boric acid; 100 mg each of streptomycin sulfate, chlorotetracycline, and benomyl; 10 g sucrose; and 17 g agar in 1,000 ml of distilled water, adjusted to pH 7.0 with HCl before autoclaving. Sucrose and the antibiotics were added to the molten agar (50 C) after autoclaving.

Recovery of *P. betae* from soil artifically inoculated with a known number of conidia was attempted; however, conidia failed to germinate on soil dilution plates. In another attempt to measure recovery, sterile and nonsterile field soil was amended with barley-grain inoculum in the amounts of 1, 5, 10, and 20% (w/w). Based on colony counts, the number of propagules/g sterile soil from each of the concns was 18, 84, 144, and 274, respectively; and number of propagules/g nonsterile soil was 12, 76, 130, and 216, respectively. Differences between the two soil types was not statistically significant.

Recovery from naturally infested soil.—A plot at Fargo had been continuously cropped to sugar beet for 5 yr. The prevalence of Phoma leaf spot in 1972 indicated a moderate amount of infection. An assay of soil samples taken from four sites at this plot on April 24, 1973, revealed a *Phoma* sp. population of 6, 64, 714, and 734 propagules/g soil. *Phoma betae* was isolated from the soil-dilution plates by aseptically transferring pieces of dark hyphal masses from the underside of the agar sheets to new agar plates. One isolate was tested for pathogenicity and was virulent on sugar beet storage roots. Soil collected on 8 May 1973, from three sites in Moorhead, Minn., where roots had been stored during the 1972-73 processing season, showed a *Phoma* population of 6, 170, and 200 propagules/g soil.

Assay of seed samples.—Seed samples of eight sugar beet cultivars used in the 1973 growing season were obtained from four seed companies. When seeds were plated on the selective medium, dark hyphal masses indicative of *P. betae* developed on the glass surface below the seeds. Occasionally, a ×10 hand lens was used to observe these hyphal masses when they were obscured by other fungi. The percentage of seed with *P. betae* ranged from 0 to 82%. The mean for all seed samples was 32%.

TABLE 1. The effect of polygalacturonic acid (PGA), sucrose, temp, and antibiotics on the growth of *Phoma betae* after 14 days at 22 C[a]

Antibiotics, (0.1 mg/ml)	Temp, (C)	Colony diam (mm)[b] PGA (5 g/liter)	Sucrose (10 g/liter)
None	20	38	32
	25	35	34
Cycloheximide	20	0	0
	25	0	0
Streptomycin sulfate	20	31	30
	25	38	26
Chlorotetracycline	20	29	28
	25	36	25
Benomyl	20	18	16
	25	14	12
LSD ($P = 0.05$)		8.5	

[a]Basal medium: 4 g K_2HPO_4; 1.5 g KH_2PO_4; 25 ml soil extract; 17 g agar; and 1,000 ml distilled water, adjusted to pH 7.0 with HCl before the addition of agar. Antibiotics, sucrose, and PGA were added to molten agar (50 C) after autoclaving.

[b]Growth values were averaged from five plates, two colonies/plate.

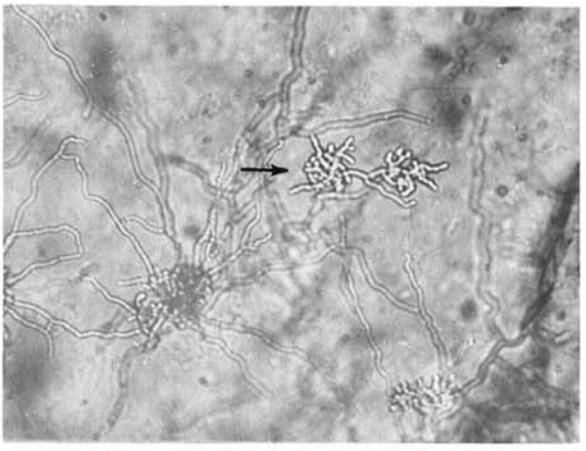

Fig. 1. Initial behavior of hyphae of *Phoma betae* when contacting glass surface as seen from underside of plate culture. (×145).

DISCUSSION.—The formation by *P. betae* of holdfastlike structures on a glass surface, aids in early identification by microscope. The medium described here is useful, not only because it is selective for the fungus, but also because it induces the holdfasts or hyphal knots to turn dark brown to black, thus

TABLE 2. The effect of boric acid and soil extract on the growth and recovery of *Phoma betae* from soil[a]

Boric acid (mg/ml)	Propagules/g soil		Colony diameter, mm[b]	
	soil extract		soil extract	
	+	-	+	-
0	16	12	1.7	1.5
0.005	15	12	1.8	1.6
0.05	14	11	1.7	1.1
0.1	12	12	2.1	1.7
0.2	17	9	2.5	1.2
LSD ($P = 0.05$)	N.S.		0.3	

[a]Basal medium: 4 g K_2HPO_4; 1.5 g KH_2PO_4; 25 ml soil extract; 100 ppm each of streptomycin sulfate, chlorotetracycline, and benomyl; 10 g sucrose; 17 g agar; 1,000 ml distilled water, adjusted to pH 7.0 before autoclaving. Antibiotics and sucrose were added to the molten agar (50 C) after autoclaving.

[b]Colony diam is an average of 10 random colonies from each of five plates after 4 days of incubation at 22 C.

facilitating the observation of colonies with the naked eye.

The observation of holdfasts during these experiments agreed with those of Mangan (4). The holdfasts do not appear to be pycnidial initials. Rather, fully developed holdfasts resemble microsclerotia. Further investigations are necessary to determine the occurrence of these structures in nature and the role they might play in survival of the fungus in the soil.

Farley (3) stated that soil extract was required to obtain the desired development of sclerotia of *Colletotrichum coccodes*. Our results show that the stimulatory effect of boric acid was most evident in the presence of the soil extract. Warren (7) also has shown that a mixture of hexose sugars and boric acid mimics the stimulatory effect of pollen on lesion development by *P. betae* on sugarbeet leaves.

The selective medium will aid in studying factors that affect the survival of *P. betae*. We know that the fungus can overwinter in the Red River Valley of North Dakota and Minnesota. Use of the selective medium will enable us to determine if *P. betae* inhabits the roots of nonhost plants as a means of survival in the summer. Also, the soil at storage sites should be assayed in the fall when the storage begins. Conceivably, *P. betae* will still be present, because large amounts of root debris remain in this soil after the stored beets have been removed and processed.

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