Selective Medium for *Ceratocystis ulmi*

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ABSTRACT

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A medium containing linoleic acid, cycloheximide, dicloran, triphenyltin hydroxide, chloramphenicol, and streptomycin sulfate in potato-dextrose agar was developed for selective isolation of *Ceratocystis ulmi*. The medium facilitated the rapid isolation of *C. ulmi* from elm tissue and bark beetles. An aggressive isolate of *C. ulmi* retained pathogenicity after being cultured on the medium, and all isolates of the fungus tested grew on the medium. A strain of *C. montia*, causal agent of blue stain of conifers, also grew on the selective medium. *C. montia*, like *C. ulmi*, forms conidia exogenously; *C. fimbriata* and *C. fagacearum*, two species that form conidia endogenously, did not grow on the medium.

Selective media for isolating *Ceratocystis ulmi* (Buisman) C. Moreau, based on the pathogen's high tolerance to cycloheximide, have been described by Holmes (3), Holmes and Mannett (4), and Schneider (9). These media incorporate cycloheximide and penicillin or streptomycin in a potato-dextrose agar base (4,9). Schneider's medium, which incorporates 200 μg/ml cycloheximide and 10 μg/ml streptomycin sulfate, has been used successfully to isolate *C. ulmi* and species of *Ceratocystis* associated with blue stain of conifers (9,12). However, as many as 12% of the plates contained contaminating organisms (9). We developed a more highly selective medium for *C. ulmi*.

MATERIALS AND METHODS

Organisms and inoculation procedures. All experiments were conducted with an aggressive strain of *C. ulmi* (designated Cu5F) obtained from N. Van Alfen, Utah State University, Logan. Other isolates of *C. ulmi* were obtained from L. R. Schreiber at the USDA Shade Tree and Ornamental Plants Laboratory, Delaware, Ohio. *C. montia* Rumbold, a causal agent of blue stain in conifers, was isolated from a locally infected lodgepole pine and identified by D. E. Mathre, Montana State University. *C. fagacearum* (Bretz) Hunt was obtained from D. Hindal, University of West Virginia. A culture of *C. fimbriata* (ATCC 13323) was obtained from the American Type Culture Collection. European elm bark beetles, *Scolytus multistriatus* (Marsham), were supplied by J. Lanier, Syracuse University.

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All isolates were maintained on potato-dextrose agar (PDA) (Difco) and were inoculated onto stock plates or test medium by placing an agar mycelial plug 6 mm in diameter onto the fresh medium. Inoculated plates were maintained at 26 C. Unused plates were stored in plastic bags at 4 C.

Seedlings were inoculated by a modification of the technique described by Schreiber and Stipes (10). Greenhouse cultivated elm seedlings (1–1.5 m tall) were inoculated 10 cm above the soil surface. A wound made by pulling back a 0.5 × 2.0 cm patch of bark was flooded with an aqueous suspension of spores and mycelia from *C. ulmi* selective medium (CuSM) or PDA plates. The inoculation site was taped, and trees were examined for vascular discoloration 2 mo later.

Isolation of contaminating fungi. At least seven genera of contaminating fungi were isolated from elm wood samples placed on plates of Schneider's medium (9). These fungi, including *Chaetomium* sp., *Fusarium* sp., *F. roseum* Lk., *Penicillium* sp., *Trichoderma* sp., *Rhizopus* sp., *Alternaria* sp., and *Gliocladium penicilloides* Corda, were used as test organisms on selective media.

Preparing the selective medium. CuSM was prepared as follows: 39 g of dehydrated PDA was added to 850 ml of distilled water, autoclaved at 121 C for 20 min, and cooled to 55 C. Additional components were prepared as follows: 500 mg of sodium salt of linoleic acid (Sigma Chemical Co., St. Louis, MO), 1 mg of 75% wettable powder dicloran (2,6-dichloro-4-nitroaniline) (Upjohn Co., Kalamazoo, MI), 10 mg of 47.5% wettable powder triphenyltin hydroxide (Thompson-Hayward Chemical Co., Kansas City, KS), 200 mg of cycloheximide (Sigma), 30 mg of chloramphenicol (Parke, Davis & Co., Detroit, MI), and 100 mg of streptomycin sulfate (Sigma) were added to 150 ml of sterile, distilled water. The suspension was agitated on a shaker for 30 min and added to the PDA. Further sterilization was not required to prevent contamination when the laboratory plates were poured and inoculated under a laminar flow hood.

RESULTS AND DISCUSSION

Schneider's medium supported the growth of numerous contaminating fungi and bacteria. To enhance the selectivity of Schneider's medium with additional microbial inhibiting agents, we tested 20 fungicides. Only three—dicloran, triphenyltin hydroxide, and thiram—were found to be effective against species of *Rhizopus*, the most troublesome fungal contaminant.

Of all permutations of these three fungicides at 0, 1, 10, and 100 μg/ml, the most effective combination that inhibited *Rhizopus* sp. and *Aspergillus* sp. with the least effect on *C. ulmi* was 1 μg/ml dicloran and 10 μg/ml triphenyltin

Table 1. Isolation of *Ceratocystis ulmi* and contaminating fungi from naturally infected or healthy elm trees

Characteristic	Medium		
	Acid PDA	Schneider's	CuSM[x]
No. of elm samples	16	16	16
No. of elm samples with *C. ulmi*[y]	0	8	8
Fungal contaminants[z]	3.4 ± 1.3 a	2.4 ± 1.7 b	1.3 ± 1.2 c

[x] *C. ulmi* selective medium.
[y] *C. ulmi* not detected either because contaminating fungi overgrew plates or because the organism was absent.
[z] Mean number per plate and standard error. Means followed by dissimilar letters are significantly different ($P < 0.05$) according to least significant difference test.

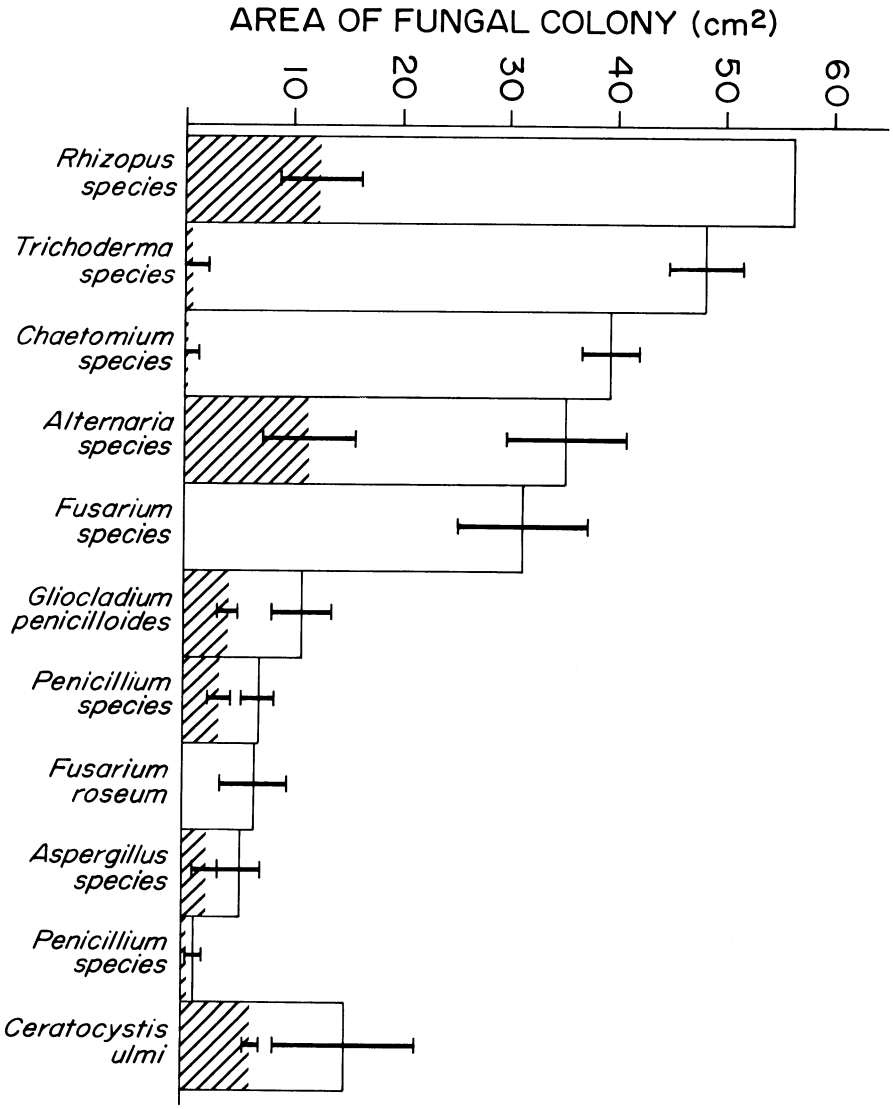

Fig. 1. Means and standard errors of colony areas of *Ceratocystis ulmi* (Cu5F) and contaminant fungi on potato-dextrose agar (unshaded) and on *C. ulmi* selective medium (shaded).

hydroxide. An unsuccessful attempt to induce coremia based on the work of Hindal and MacDonald (2) showed that adding linoleic acid greatly facilitated growth of *C. ulmi*.

When plated on the various media, an aqueous spore-mycelial suspension of *C. ulmi* contained 9×10^6 propagative units on PDA acidified to pH 4.5 with lactic acid, 9×10^6 units on Schneider's medium, and 3×10^6 units on CuSM. That is, about 70% of the propagative units lost viability on the selective medium compared with acid PDA or Schneider's medium. This reduction may be acceptable, considering the high selectivity of CuSM.

When cultivated on CuSM, only species of *Rhizopus* and *Alternaria* outgrew the Cu5F strain of *C. ulmi* (Fig. 1). Colony development of other contaminating fungi was dramatically reduced. Except for the *Alternaria* sp. encountered only once, the *Rhizopus* sp. was the only contaminant that overgrew selective medium plates. This occurred in about 15% of the attempts to isolate *C. ulmi*. However, overgrowth occurred fewer times on CuSM than on Schneider's medium.

Isolation of *C. ulmi* on CuSM required 5–7 days; the fungus was identified by colony morphology and conidial characteristics. All eight isolates of *C. ulmi* tested grew on CuSM; colony area was reduced 11–60% compared with colonies obtained on PDA.

We compared the effectiveness of acid PDA, Schneider's medium, and CuSM in eliminating contaminants and in isolating *C. ulmi* from elm wood (Table 1). The mean number of contaminants per plate was significantly lower ($P < 0.05$) on Schneider's medium than on acid PDA plates, and a further significant decrease in number of contaminants was obtained on CuSM.

C. ulmi was isolated from eight of 16 elm samples on Schneider's medium, and the same trees yielded the fungus on CuSM (Table 1). *C. ulmi* could not be identified on acid PDA plates because of contaminating microorganisms.

C. ulmi was isolated on CuSM from 23% of European bark beetles collected from galleries in trees with Dutch elm disease. The fungus was isolated with the same efficiency after beetles were surface disinfested with 0.5% sodium hypochlorite solution for 2 min, indicating that the fungus was retained internally.

Previous investigators (5,7,8) reported that 4–76% of the beetle population carried *C. ulmi*. However, Parker et al (7) reported that only 18% of the carriers retained the fungus internally. Use of the selective medium could resolve this apparent discrepancy and help to clarify interrelationships among vector, fungus, and host. The beetle population assayed in this study may have lost externally borne conidia or mycelia in transit from New York to Montana. Alternatively, surface treatment may not have eliminated all externally borne conidia, although this seems unlikely.

C. ulmi (Cu5F) grown on CuSM retained phytopathogenicity, as measured by its ability to cause vascular discoloration when introduced into elm seedlings in the greenhouse. *C. ulmi* could be recovered up to 100 cm above the inoculation site from trees inoculated with fungus obtained from both CuSM and PDA plates.

Clinton and McCormick (1), as early as 1936, indicated that *C. ulmi* rarely forms coremia in vitro. When formed on artificial media, coremia often appear as white or yellow staghorn-like growths (1). Occasionally, coremia more similar to those seen in situ, typified by a brown stalk with a tuft of conidia, are observed. Among compounds incorporated into CuSM to induce coremia formation, catechin [trans-2-(3,4-Dihydroxyphenyl)-3,4-dihydro-2H-1-benzopyran-3,5,7-triol] induced coremia with strain Cu5F at concentrations of 10–50 m*M*. Taylor et al (11) reported that this compound induced coremia in *C. ulmi*.

Coremia were most abundant in cultures on CuSM with catechin maintained in continuous darkness. They appeared approximately 10 days after the fungus was transferred and were most abundant near the mycelial plug.

CuSM may also help in isolating other species of *Ceratocystis*. For instance, *C. montia* was readily isolated from samples of lodgepole pine infected with blue stain. *C. fagacearum*, which has been shown to be inhibited by cycloheximide (6), and *C. fimbriata* did not grow on the selective medium. These latter species form conidia endogenously, whereas *C. ulmi* and *C. montia* form conidia exogenously. Although few species were tested, B. C. Hemming, Montana State University, has suggested that medium selectivity may correlate with the mechanism of conidial formation, a useful differential taxonomic characteristic.

Although dicloran reduced colony growth of *Rhizopus* about 80%, *Rhizopus* still overgrew colonies of *C. ulmi*. Although plating recovery of *C. ulmi* propagules was only 30% with the

selective medium, all eight isolates of *C. ulmi* tested grew on the medium. Virulence was not lost, and this recovery rate is often sufficient for isolation from tissue and epidemiological studies.

Selectivity of CuSM with other wilt organisms, such as species of *Verticillium* or *Cephalosporium*, was not tested. The medium was highly selective against contaminating organisms, as shown by the significant decrease in extraneous organisms when isolations were made from elm wood samples. CuSM appeared to retain high selectivity for more than 6 mo when maintained at 4 C in plastic petri dish bags.

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