Selective Medium for Recovering *Verticicladiella procera* from Soils and Symptomatic White Pines

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ABSTRACT

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A selective medium was developed for recovering *Verticicladiella procera* from infested soils and symptomatic white pines. The medium, with the pH adjusted to 4.5–5.0 before autoclaving, contained glucose, microelements, chlorotetracycline hydrochloride, cycloheximide, and streptomycin sulfate. The pathogen was recovered regularly when wood samples from cankers or soils from the rhizosphere of symptomatic trees were incubated on this medium for 7–15 days at 20 C.

Verticicladiella procera Kendrick causes a root decline in eastern white pine, *Pinus strobus* L. Diseased trees exhibit delayed bud break, crooking of new shoots, chlorotic drooping needles, and retention of dead needles for a year or more after tree death. Diffuse cankers with resin-soaked, irregular zones of brown discoloration are also present on the stem near the ground line and on the roots of infected trees (1,5,6,8). The fungus has been reported to cause root cankers and occasional death of *Pinus resinosa* Ait. (red pine) (6) and has been isolated from the roots of several other conifers (3,4).

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Significant losses are attributed to this pathogen in several Christmas tree and landscape white pine plantings in West Virginia. The actual involvement of *V. procera* in these losses has not been confirmed satisfactorily because the fungus is difficult to isolate from infected wood and suspect soils (1,5). This report describes a defined, selective medium that can be used to isolate *V. procera* from suspect soils and infected wood.

MATERIALS AND METHODS

Initially, we tested five antimicrobial agents—chlorotetracycline hydrochloride, cycloheximide, pentachloronitrobenzene, pimaricin, and streptomycin sulfate—to determine their effect on the in vitro growth and sporulation of *V. procera*. Test agents were incorporated into a defined glucose and asparagine agar (GA) and a Difco potato-dextrose agar (PDA). The GA contained 10.0 g of glucose, 2.0 g of L-asparagine, 1.0 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.2 mg of Fe^{++}, 0.2 mg of Zn^{++}, 0.1 mg of Mn^{++}, 0.1 mg of thiamine, 0.1 mg of pyridoxine, 5.0 μg of biotin, 15 g of Difco Bacto agar, and 1 L of distilled water. When an antimicrobial agent was included in the test media, it was suspended in 10 ml of sterile distilled water and added to 1 L of the sterile cooled media before being dispensed into sterile glass or plastic petri plates.

After these initial tests, GA and PDA containing various combinations of chlorotetracycline hydrochloride, cycloheximide, and streptomycin sulfate were tested for recovery of *V. procera* from suspect soils and symptomatic wood. Results indicated that the media required further modification to improve the reliability of pathogen recovery. We modified the GA medium by reducing the concentration of carbon and nitrogen, changing the carbon and nitrogen sources, and omitting macroelements and vitamins. All test media contained chlorotetracycline hydrochloride, cycloheximide, and streptomycin sulfate at 50 mg/L each. In vitro growth and ability to recover the pathogen from suspect soils were evaluated on these media. The medium that was developed seemed to be suitable for isolating the pathogen from suspect soils.

After the medium was developed, additional soils were sampled and assayed for *V. procera*. A total of 281 soil samples were collected from the rhizosphere of white pines in Christmas tree and landscape plantings with and without symptoms of white pine root decline. Different numbers of samples were collected on each date, and the rhizosphere of each tree was usually sampled only once. The assay was conducted by sieving soils through 2-mm mesh and suspending a 1-g sample in 9 ml of distilled water in 250-ml Erlenmeyer flasks. This suspension was shaken on a rotary shaker (100 rpm) for 15 min and diluted to concentrations

of 10^{-2}, 10^{-3}, and 10^{-4}. A 1-ml aliquot of each dilution from each soil sample was spread evenly on the surface of the test medium and incubated at 20 C. Observations for *V. procera* were made after 7–15 days of incubation.

We also conducted tests to determine the suitability of this medium for isolation of *V. procera* from symptomatic wood. Discolored, resin-soaked wood samples were collected from stem and root cankers on symptomatic Christmas or landscape white pines. Wood pieces were also collected from white pine seedlings that had been artificially inoculated in stems and roots with *V. procera*. Small chips, 1–2 cm long, were removed from the wood pieces, surface sterilized in 10% sodium hypochlorite for 2 min, rinsed four times in sterile distilled water, placed on the test medium, and incubated at 20 C for 7–15 days.

RESULTS

Chlorotetracycline hydrochloride, cycloheximide, and streptomycin sulfate at 50 mg/L did not inhibit the in vitro growth or sporulation of *V. procera* on GA or PDA. Limited growth occurred when these media contained pentachloronitrobenzene at 50 mg/L, but sporulation was reduced and the morphology of the conidiophores was altered. No growth occurred on media containing pimaricin at 50 mg/L.

When chlorotetracycline hydrochloride, cycloheximide, and streptomycin sulfate were each included at 50 mg/L in GA or PDA, *V. procera* was occasionally isolated from suspect soils. However, many other, faster-growing fungi also grew on these media, making reliable recovery of the pathogen impossible.

When suspect soils were assayed for *V. procera* on modified GA containing the three antibiotics, recovery of the pathogen was improved and growth of competing fungi often reduced. The omission of glucose reduced growth of the contaminants and the pathogen; however, glucose at 2.0 g/L allowed sufficient growth of *V. procera* for identification and also restricted the growth of contaminants. Recovery of the pathogen was not improved by the substitution of glucose with cellobiose, fructose, sorbose or trehalose at 2.0 g/l, carbon sources that favored in vitro growth of *V. procera* (7).

The omission of L-asparagine also reduced contaminant growth but allowed sufficient growth of the pathogen for identification. When proline, a nitrogen source favorable for the in vitro growth of the fungus, or phenylalanine, a poor nitrogen source for in vitro growth (7), was substituted for asparagine, excessive growth of contaminants resulted in reduced recovery of *V. procera*. When the microelements and vitamins were omitted, growth of contaminants was reduced but recovery and identification of the pathogen were not affected.

Based on these results, we used a defined medium containing 2.0 g of glucose; 0.2 mg of Fe^{++}; 0.2 mg of Zn^{++}; 0.1 mg of Mn^{++}; 50 mg each of chlorotetracycline hydrochloride, cycloheximide, and streptomycin sulfate; 1 L of distilled water; and 15 g of Difco Bacto agar to test for recovery of *V. procera* from suspect soils and symptomatic wood. This medium will be referred to as *Verticicladiella procera* isolation medium (VPIM). Further tests indicated that pathogen recovery was improved when the pH of VPIM was adjusted from 4.5 to 5.0 before autoclaving; however, recovery was not improved when the concentration of the three antibiotics was increased to 100 mg/L.

About 72% of all soil samples collected from the rhizosphere of symptomatic trees were positive for *V. procera* using VPIM. The pathogen was also recovered from about 4% of the samples collected in the rhizosphere of asymptomatic trees (Table 1). In addition, the fungus was recovered from an average of 53% of the wood samples taken from cankered areas on symptomatic trees using this medium. When stem or root samples from white pine seedlings that had been artificially inoculated with the pathogen were incubated on VPIM, 74% of the samples yielded *V. procera* (7).

Table 1. Recovery of *Verticicladiella procera* on a selective medium from soils collected in 1979 in the rhizosphere of white pines with and without symptoms of white pine root decline

Month sampled	Trees symptomatic			Trees asymptomatic		
	Number tested	Number positive	Percentage positive	Number tested	Number positive	Percentage positive
March	5	4	80	...[a]	...	...
April	101	101	100	5	1	20
May	7	0	0	5	0	0
June	5	0	0	25	0	0
July	28	5	18	15	0	0
September	26	16	62	6	0	0
October	7	7	100	5	1	20
November	21	11	52	5	1	20
December	...	...	...	15	0	0
Total	200	144	72	81	3	4

[a] None tested.

DISCUSSION

V. procera was recovered regularly from symptomatic wood using VPIM. The rate of recovery was equal to or better than that reported by other workers who have developed means for isolating the fungus from diseased trees (1,5). These data provide good evidence for involvement of *V. procera* in white pine root decline. The use of VPIM also provided a means for recovering the pathogen from a high percentage of the soils collected from the rhizosphere of symptomatic white pines (Table 1). The rate of recovery was better than that reported by other workers (8,9). Occasionally, the pathogen was recovered from soils collected from the rhizosphere of asymptomatic trees. It is not known whether these trees were infected and not yet showing symptoms, whether they died after the soil samples were collected, or whether the fungus was part of the natural flora of these soils and not associated with disease development.

There is little information on the biology of *V. procera*, and nothing is known of the natural distribution of this organism in soil. The pathogen is reported to be widespread in New York State and to be causing losses in white pine grown in poorly drained sites (6). Even though many soils in West Virginia are heavy and poorly drained, disease occurrence and the ability to recover the pathogen there were not always associated with poor drainage. Diseased trees were observed on steep hillsides, and the fungus was often recovered from the rhizosphere of those trees. The fungus also seemed to be most difficult to recover from suspect soils in the warmer months (Table 1). It is not known whether this reduction reflected a seasonal fluctuation of the pathogen in soil.

Hicks et al (2) suggested that *Ceratocystis* (*Verticicladiella*) *wageneri* Goheen and Cobb, a root-infecting organism that causes a disease similar to eastern white pine root decline in several western conifer species, may be quite widespread in forest soils. With the development of a selective medium that is suitable for recovering *V. procera* from soil, investigations on the distribution, dissemination, and survival of this pathogen can be conducted. These studies are needed to aid in the formulation of appropriate control measures.

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