Techniques

Isolation of *Ceratocystis wageneri* from Forest Soil with a Selective Medium

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ABSTRACT

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A selective medium containing 800 μg/ml cycloheximide was developed for the isolation of *Ceratocystis wageneri* from soil. Fifty-nine soil samples were collected from around roots of infected ponderosa pines and assayed. In 11 cases, *C. wageneri* was isolated in quantities of up to 12,000 propagules per gram of soil and, in one case, was found 4–6 cm from the infected root. Most propagules apparently were conidia but several bits of mycelium removed from soil also proved to be *C. wageneri.* Mycelial growth through soil for short distances may be an important means of spread of the pathogen. Other species of *Ceratocystis* were isolated from soil and were tolerant of high concentrations of cycloheximide.

Additional key words: dispersal, *Pinus ponderosa, Verticicladiella wagenerii.*

Ceratocystis wageneri Goheen and Cobb (*Verticicladiella wagenerii* Kendrick) is a root-infecting fungus that causes a disease of several conifer species in western North America. The most common hosts are ponderosa pine (*Pinus ponderosa* Laws.) and Jeffrey pine (*P. jeffreyi* Grev. and Balf.) in the Sierra Nevada; Douglas fir (*Pseudotsuga menzesii* [Mirb.] Franco) in the California coast ranges, Oregon, Washington, and British Columbia; pinyon pine (*P. edulis* Engelm.) in Colorado, Utah, New Mexico, and Arizona; and singleleaf pinyon pine (*P. monophylla* Torr.) in southern California (3,6). While apparently not a serious

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problem in undisturbed stands, the pathogen has induced damage in some disturbed forests, particularly where stands are composed of nearly pure ponderosa pine or Douglas fir (1,5).

The fungus enters the host through roots and invades xylem tracheids (15), spreading through the root system and lower stem of the tree. Although the pathogen alone is capable of killing the host, trees infected with *C. wageneri* often are attacked and killed by bark beetles (Coleoptera:Scolytidae), which preferentially infest the weakened, infected trees (3,4).

How *C. wageneri* is transmitted from tree to tree is not well understood. However, most infected pines in the Sierra Nevada occur in infection centers or foci ranging in size from small areas containing several trees to 10 or more hectares with hundreds of trees (1). This pattern of distribution, coupled with evidence obtained by examination of insect galleries in roots (5), suggests that there are at least two different modes of transmission: one by which new infection centers are initiated, probably involving a bark beetle vector (4); and a second by which the pathogen spreads to adjacent trees at the margins of expanding infection centers.

Several investigators have suggested (12,15) that cross-over via root grafts is responsible for the latter type of spread. Moreover, Landis and Helburg (10) excavated the roots of pinyon pine in an infection center in western Colorado and traced the dark stain (a distinct symptom of the disease) from tree to tree through root contacts and grafts. On the other hand, in California most infection in ponderosa pine occurs through small rootlets within 15 cm of, but not in actual contact with, infected roots (4). This implies that the pathogen moves through soil either by mycelial growth, by vector, or as water-borne spores.

Attempts to isolate *C. wageneri* from soil or insects with standard nutrient agar media were unsuccessful. Other fast-growing microorganisms invariably colonized the medium, completely obscuring or inhibiting any *C. wageneri* that may have been present. Preliminary work toward a selective medium indicated that *C. wageneri* was relatively tolerant to cycloheximide and several other fungicides. Cycloheximide has been used in selective media for *C. ulmi* (11) and other *Ceratocystis* spp. (14).

Thus, there were two objectives of the research reported here: to develop a selective medium for *C. wageneri*; and to determine, through soil isolations, whether the pathogen occurs in soil, and if so, whether it is occurring as mycelium, spores, or in association with a vector.

MATERIALS AND METHODS

Selective medium. Compounds initially screened for use in a selective medium were: 10–200 μg/ml of cycloheximide, 0.35–3.5 μg/ml of phenyl mercury ammonium acetate, and 2–50 μg/ml of the following: cadmium succinate, pentachloronitrobenzene (PCNB), copper (as $CuSO_4$), dicloran, mancozeb, zineb, dodine, anilazine, thiram, benomyl, thiabendazole, and thiophanate-methyl.

Each of these fungicides was added singly to 2% potato dextrose agar (PDA) acidified to pH 4 by the addition of three drops of concentrated sulfuric acid per 200 ml of agar. Preliminary tests indicated that this pH was optimum for *C. wageneri* growth in culture. Streptomycin sulfate at 200 μg/ml was added to all media to suppress bacterial growth (13). Several of the compounds used were heat labile and thus were added to the PDA after it had been autoclaved. These were streptomycin, cycloheximide, PCNB, mancozeb, and dicloran. The other compounds were added before autoclaving.

To compare growth of *C. wageneri* on the different media, a 1-mm^2 piece of agar and mycelium from the edge of an actively growing colony on PDA was transferred to the center of a 90-mm-diameter petri plate containing approximately 10 ml of medium. Five plates (replicates) of each medium were incubated for 2 wk at 18 C (the temperature generally considered optimum [12] for *C. wageneri* growth). Colony diameters were measured and recorded as the mean of two measurements taken at right angles to one another.

C. wageneri was tolerant to several fungicides. Several of these were tested for suppression of common soil inhabiting fungi. A 1/1,000 dilution of a soil sample from Blodgett Research Forest at 1,300 m elevation in the central Sierra Nevada was prepared by using the procedure of Johnson and Curl (9). One milliliter of this dilution was mixed with 10 ml of cooled (45 C) medium and incubated at 18 C. Five replicates were prepared for each medium tested. After 1 wk, a qualitative description of the suppressiveness was recorded.

On the basis of results from the above tests, a concentration series of cycloheximide media, ranging from 50 to 1,000 μg/ml, also was tested for effects on growth of *C. wageneri* and for effectiveness in isolating the fungus in 1/100 soil dilutions. For the latter, *C. wageneri* conidia were added to raw forest soil in a concentration that would yield a final dilution of 20 viable spores per plate. Numbers of colonies of *C. wageneri* and of other fungi were recorded after 2 wk of incubation.

Soil isolations. Soil samples for isolation were collected from around infected roots of ponderosa pines in the Blodgett Research Forest. Most of the trees were heavily infected and either visibly weakened or recently killed by *C. wageneri* alone or in conjunction with bark beetles. Where possible, soil was collected near larger, heavily infected roots that were still at least partly alive.

Two different sampling procedures were used to collect samples seven times during a 10-mo period. The first procedure, used from February through July, involved one composite soil sample collected from around each selected root and included soil from as far as 5–10 cm from the root. In the second procedure, used in October and November, four incremental soil samples were collected at different distances from each selected root so that the distance from the root at which viable propagules of the pathogen existed could be determined.

In the incremental sampling, one side only of a diseased root was exposed, leaving the soil face at right angles to the root undisturbed. Soil samples were carefully removed from four 2-cm-wide increments paralleling the root at distances ranging from 0 to 8 cm from the root. The increment farthest from the root was sampled first, followed progressively by nearer increments. Care was taken to avoid mixing of increments or including root or other organic material in the sample. Samples were placed in polyethylene bags and stored at 3 C until isolations were performed. In addition, the roots were sampled to determine whether they contained viable *C. wageneri*. Soil temperatures and soil water matric potential (ψ_m) were measured at each sampling site at 20 cm depth. Matric potentials greater than −0.6 bar were determined in the field with a tensiometer. ψ_m was calculated for drier soils from moisture content determinations and moisture characteristics.

Ninety-one root and associated soil samples were collected during the seven sampling dates. Isolations indicated that the fungus was not viable in 56 roots, and isolations were not attempted from the soil samples associated with 32 of them. The remaining 59 soil samples taken immediately adjacent to roots were tested for presence of the pathogen. Soil increments other than those immediately adjacent to the root were tested only when *C. wageneri* was shown to be present in the nearest soil increment. Isolations from roots were made by aseptically transferring small chips of stained xylem to petri plates containing the selective medium described in the Results section.

Isolations from soil were made by using a modification of the soil dilution method of Johnson and Curl (9). Each soil sample was thoroughly mixed, and a representative 10-g subsample (at field moisture content) was taken. All visible root fragments were removed from this subsample, and the sample was then diluted twice in 90 ml of sterile water for a final dilution of 1/100. One milliliter of this final suspension was pipetted into each of six petri dishes, followed by addition of 10 ml of selective medium (cooled to approximately 45 C). Plates were then swirled to distribute the sample. Plates were incubated at 18 C and examined twice (at 2 and 4 wk) for the presence of *C. wageneri*. Numbers of *C. wageneri* colonies on each plate were recorded, and the presence of other *Ceratocystis*-type fungi also was noted.

To investigate the origins (spores, mycelia, or organic matter) of colonies appearing on the plates, a soil sample collected in October

and shown to contain viable *C. wageneri* was "fractionated" according to a modified Warcup procedure (16). One gram of soil was shaken with 100 ml of water in a 250-ml graduated cylinder, and 1 ml of this suspension was immediately pipetted into each of eight plates. The suspension was allowed to settle for 60 sec, and 1 ml of the supernatant was pipetted into each of eight more plates. The remaining supernatant was poured through a 0.1 mm sieve, and 1 ml of this filtrate also was transferred to each of eight plates. Finally, the coarse material remaining in the bottom of the cylinder was washed several times with a distilled water jet, and divided among three petri plates. Thus, the plated fractions were: the whole suspension; the supernatant after large soil particles (>0.05 mm) had settled out; the filtrate from which all particles (including organic matter) larger than 0.1 mm in diameter had been removed; and the coarse soil fraction. About 10 ml of selective medium was added to all plates, which were incubated at 18 C and examined after 2 wk for the presence of *C. wageneri* colonies.

In addition, a second soil sample (also collected in October) containing *C. wageneri* was wet-sieved in the above manner and the material trapped on the sieve was washed under running water. About 25 pieces of dark hyphae or mycelial strands were removed from the 0.1 mm sieve, plated, and incubated. In addition, several roots were examined under low magnification for signs of the pathogen on root surfaces. Dark, coarse mycelial strands were observed in a number of cases, and these were transferred to petri plates containing the selective medium.

In another test, several hundred nematodes were collected by the Baermann funnel method (7) from 300 g of a soil sample (collected in March) containing *C. wageneri*. The nematodes were washed in three changes of distilled water. They were then divided among eight plates containing selective medium which were incubated at 15 C and checked for *C. wageneri* colonies after 2 and 4 wk.

RESULTS

Selective medium. *C. wageneri* was tolerant of even the highest concentration of cycloheximide tested. Compared to the PDA controls, growth rate was significantly ($P = 0.01$) reduced, only on the medium with 1,000 μg/ml cycloheximide, and in this case growth was reduced by only 21% (41 mm diameter/2 wk compared to 52 mm in the check). Colony diameters in media with 100, 200, and 500 μg/ml cycloheximide were 46, 48, and 45 mm, respectively. Furthermore, high concentrations of cycloheximide in soil dilution plates suppressed growth of most soil fungi. Other fungicides were less effective and were not tested further either because they inhibited growth of *C. wageneri* at low concentrations (dicloran, mancozeb, zineb, anilazine, thiram, benomyl, thiabendazole, thiophanate-methyl, and phenyl mercury ammonium acetate, or because they were relatively ineffective in suppressing soil microorganisms on dilution plates at moderate concentrations (cadmium succinate, PCNB, copper, and dodine).

When conidia of *C. wageneri* were added to soil and isolations were attempted by soil dilution onto the cycloheximide medium, recovery of the fungus was good. For example, when cycloheximide was present at 200 to 1,000 μg/ml, about 90% of the viable spores germinated and formed visible colonies (Fig. 1). In comparison, the control (no cycloheximide) plates were rapidly overrun by other fungi, and no *C. wageneri* colonies were seen.

A selective medium capable of supporting growth of *C. wageneri* and of suppressing other fungi was chosen for use in subsequent studies. This medium was prepared as follows:

> A stock solution of 2% cycloheximide (Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178) and 0.5% streptomycin sulfate (Sigma Chemical Co.) was prepared by first dissolving 2 g of cycloheximide in 100 ml of sterile water. Solution was hastened by heating to about 90 C. After the solution was cooled to room temperature, 0.5 g of streptomycin sulfate was added. In preparing the agar, three drops of H_2SO_4 were added to 200 ml of melted PDA to acidify it to pH 4. When the agar was cooled to below 60 C, 8 ml of the stock cycloheximide-streptomycin solution were added to 200 ml of medium. The final medium contained 800 μg/ml cycloheximide and 200 μg/ml streptomycin sulfate (187 μg/ml streptomycin).

Results of a subsequent study indicated that growth of *C. wageneri* was stimulated by the addition of up to 1,000 μg/ml Mn (8); thus, it is recommended that 100 μg/ml Mn (as $MnCl_2$) be added to the above medium for more rapid growth of the fungus.

Field isolations. *C. wageneri* was isolated from 11 of 59 soil samples tested (Table 1). Identification was based on morphology of the fungus grown on PDA, and pathogenicity was confirmed by inoculating ponderosa pine seedlings in the greenhouse. Numbers of colonies on dilution plates ranged from one colony per six plates (~20 propagules per gram of soil at field moisture content) to about 120 colonies per plate (~12,000 propagules per gram). In one case (October sample), *C. wageneri* was found in soil increments 2–4 cm (12,000 propagules per gram) and 4–6 cm (80 propagules per gram) from the root as well as in the sample taken from nearest the root (6,500 propagules per gram).

C. wageneri was found in the soil in February, March, April, October, and November, but not in samples taken in late May or

TABLE 1. Incidence of viable *Ceratocystis wageneri* isolated from soil during a 10-mo period

Month	Samples tested (no.)	Samples with viable *C. wageneri* (no.) root	soil[a]	Propagules (no./gram)
February	9	5	1	500
March	4	1	2	20, 6,500
April	7	3	1	100
May	10	6	0	...
July	4	2	0	...
October	14	11	5	30, 30, 3,000, 6,500[b], 12,000
November	11	7	2	30, 80
Totals	59	35	11	

[a]Taken from immediately adjacent to root (0–2 cm).
[b]*C. wageneri* also was isolated from soil samples 2–4 cm (12,000 propagules per gram) and 4–6 cm from root (80 propagules per gram).

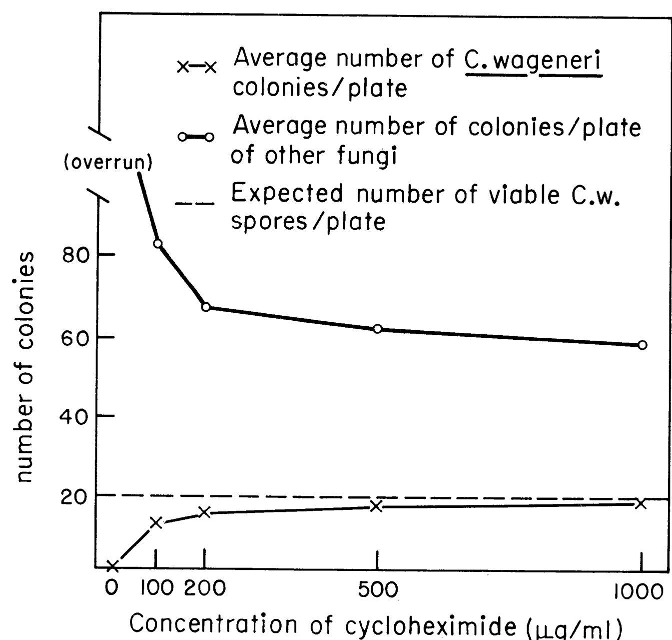

Fig. 1. Effect of concentration of cycloheximide in potato dextrose agar medium on recovery of *Ceratocystis wageneri* from soil into which a known concentration of spores had been introduced. A 1/100 soil dilution was employed.

July (Table 1). However, numbers of samples were small and statistically significant differences among months were not detected. Soil temperature ranged from 3 C in March to 13 C in October. Matric potential ranged from −15 bars in July to −0.1 bar in March. No significant relationship was observed between the occurrence of the fungus in soil and either soil temperature or matric potential.

The fractionated soil sample yielded 42 colonies of *C. wageneri* per plate (4,200 propagules per gram) from the whole suspension. In comparison, the numbers of colonies on plates made from the supernatant and the 0.1-mm sieved filtrate were both significantly higher, 69 and 62 colonies, respectively. Since most mycelial fragments are assumed to be in association with coarse soil particles (16) the fact that filtering did not reduce the number of *C. wageneri* colonies isolated suggests that most of the colonies originated from spores. The number of colonies of other fungi was higher on the plates made from the whole suspension, possibly accounting for the smaller number of *C. wageneri* colonies that developed on these plates. The coarse soil particle fraction yielded only 45 propagules of *C. wageneri* per gram.

Two of the 25 pieces of dark mycelium from the second wet-sieved soil sample yielded *C. wageneri* colonies. The other pieces were either nonviable or gave rise to unidentified fungi.

Several blue-stain-type fungi were found on dilution plates throughout the study. These were unidentified *Graphium* species in 10 samples and *Leptographium* or *Verticicladiella* species in 15 soil samples. Mycelial strands removed from the surfaces of infected roots were, in several cases, also identified as *Leptographium, Verticicladiella,* or *Graphium* sp after being plated on selective medium. None of these coarse mycelial strands yielded *C. wageneri* colonies. No *C. wageneri* was isolated from nematodes.

DISCUSSION

By using the selective medium developed for isolation of *C. wageneri,* the fungus was found in forest soils. Most propagules were probably spores, since removal of large mineral and organic particles (to which much of the mycelia in soil is expected to be attached [16]) did not decrease the number of colonies appearing on plates. However, fragments of *C. wageneri* mycelia also were observed in soil samples, suggesting that the organism may grow through soil as hyphae or mycelial strands. Furthermore, in a laboratory study (8) *C. wageneri* was shown to readily grow for several centimeters from colonized wood blocks through nonsterile forest soil. So far the fungus has been found no farther than 4–6 cm from inoculum sources (ie, infected roots), but this could be far enough to account for at least some of the infection observed by Goheen (4).

Goheen (4) found that most root infection in ponderosa pine in the central Sierra Nevada occurred through small rootlets less than 5 mm in diameter. Contrary to previous reports (10,12,15), few of these rootlets were in contact with other infected roots but were almost invariably within 15 cm of them. Earlier, it was hypothesized that insects or other arthropods might be responsible for this short distance spread. While insect vectoring cannot be dismissed entirely, negative results of isolation from nematodes and insects (authors' *unpublished*) and the absence of evidence of feeding on rootlets by larger insects (4), together with results of this investigation which indicate growth through soil, make such a process less probable.

Use of the selective medium resulted in isolation of several unidentified *Graphium* and *Verticicladiella* or *Leptographium* species, as well as *C. wageneri,* from soil. Thus, the medium should aid in studies of the biology of these fungi. Very little is known about the occurrence of these fungi in forest soils, and insect vectors sometimes are suggested as the means of spread with no supporting data. Quite possibly, more of these fungi are capable of growing through soil than heretofore suspected. For example, root graft transmission or above-ground insect transmission cannot explain the incidence of *C. fagecearum* infection in chestnut oaks adjacent to previously infected red or black oaks (2). Conceivably, this pathogen also may be growing through soil short distances from infected red oak roots to infect roots of adjacent oaks.

The suggestion that this group of fungi might in general be more widespread inhabitants of soil deserves further attention.

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