

Determining Inoculum Potentials of *Cylindrocladium floridanum* in Cropped and Chemically-Treated Soils by a Quantitative Assay

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ABSTRACT

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A quantitative assay was developed for determining inoculum potentials of *Cylindrocladium floridanum* in soil. Inoculum potentials were measured by determining the percentage of soil samples in a total of 100 1-g samples that produced signs of *C. floridanum* disease on alfalfa seedlings. Inoculum potentials of *C. floridanum* were directly correlated with percentages of diseased black spruce seedlings in four soils tested. *Cylindrocladium floridanum* survived for one growing season in field plots to which it was introduced regardless of soil type, cover crops, or chemical treatments. Three nonhost cover crops (oats, rye, and wheat) significantly increased the inoculum potential of *C.*

floridanum during one growing season, whereas corn, soybeans, and buckwheat did not detectably alter inoculum potentials when compared to nontreated plots. No cover crop measurably altered the germination of microsclerotia of *C. floridanum* or numbers of soil microorganisms. Trichlorodinitrobenzene applied prior to soil infestation with *C. floridanum* reduced microsclerotial germination and inoculum potentials of *C. floridanum*. Methyl bromide, applied prior to infestation, resulted in increased inoculum potentials in a sandy soil and decreased inoculum potentials in a silt loam compared to inoculum potentials in nontreated plots.

Additional key words: methyl bromide, trichlorodinitrobenzene, cover crops, microsclerotia, soil microbiology.

In Minnesota, *Cylindrocladium floridanum* Sob. & Seymour causes an important root rot of conifers that formerly was attributed to *C. scoparium* Morgan (21). It has destroyed up to 80% of the seedlings in some nursery beds, and has caused losses amounting to more than \$40,000 in a single nursery during one year (14, 17). At one location, damage was so severe that the nursery was abandoned. Although much effort has been directed toward controlling *Cylindrocladium* root rot and clarifying its effect upon host plants, few attempts have been made to measure populations or to elucidate factors that influence survival of this fungus in soils.

The purpose of this investigation was to develop a method for measuring the inoculum potential (5) of *C. floridanum* under nursery conditions. Since cover crops and chemical treatments are frequently employed in forest nurseries, an attempt was made to determine what effects these factors have on the survival of *C. floridanum* in soil throughout one growing season.

MATERIALS AND METHODS

Quantitative assay.—Experiments were performed using four Minnesota soils: Knife River sand from the Knife River Nursery, Two Harbors, which had a pH of 5.9 and a maximum water-holding capacity of 41%; Omega sand from the University of Minnesota Forest Research Center, Cloquet, which had a pH of 5.4 and a maximum water-holding capacity of 45%; and Waukegan silt loam from the University of Minnesota Agricultural Experiment Station, St. Paul, which had a pH of 5.9 and a maximum water-holding capacity of 83%. The fourth soil consisted of a mixture of Waukegan silt loam and sand

(3:1, v/v) with a combined pH of 6.9.

The Knife River sand was naturally infested with *C. floridanum*. The Omega sand and the Waukegan silt loam were infested with *C. floridanum* in the field by mixing 38.6 kg of Knife River sand with the upper 10 cm of soil in 2 m square plots. The soil mixture was steamed for 1 hour at 121 C, and mixed with artificially prepared inoculum of *C. floridanum* at a ratio of 20 parts soil to one part inoculum. The artificial inoculum consisted of 5% cornmeal and 95% sandy loam (approximate moisture content 11%) which had been autoclaved for 1 hour, infested with *C. floridanum*, and incubated for 90 days.

Alfalfa plants (*Medicago sativa* L. 'Vernal') were used as a bioassay indicator (3, 15, 22). Twenty-five 2 × 9 cm soil cores were taken at random from each soil. Each core was stored in a plastic bag at 4 C until assayed. The samples were mixed while in the bags, and four 1-2 g subsamples per bag were transferred to glass vials 1 cm high and 1.75 cm in diameter. Two to four drops of deionized water were added and four or five seeds of alfalfa were pressed into the soil in each vial. Vials were incubated in storage dishes at 22-25 C and 100% relative humidity. The seedlings were examined for conidia or microsclerotia of *C. floridanum* after 8 and 14 days. The presence of either fungus structure on one or more seedlings in a vial was counted as a positive reaction. Alfalfa seedlings without signs of *C. floridanum* were immersed in 1% aqueous sodium hypochlorite solution for 15 seconds, rinsed in sterile distilled water, and placed on 1% extract medium containing 30 µg/ml (30 ppm) of aureomycin. Since *C. floridanum* was isolated from only 2 of 250 seedlings, we assumed that the presence of conidia or microsclerotia was sufficient to identify

infected plants. The number of positive reactions per 100 vials per plot was used to express the inoculum potential in percent. Three replications of this procedure were performed for each soil.

To correlate *C. floridanum* inoculum potentials with losses of conifer seedlings, each of the four soils was collected in 25 13-cm pots, and two fall-lifted 2-year-old black spruce (*Picea mariana* Mill.) seedlings, which had been stored at 4 C for 3 months, were planted in each pot. After 12 weeks in a greenhouse at 18-26 C, seedlings with foliar symptoms of root rot were removed and the roots were sectioned and examined for microsclerotia of *C. floridanum*. Seedlings with both foliar symptoms and microsclerotia in the roots were considered to have root rot caused by *C. floridanum*.

Field plots.—The influence of cover crops and chemical treatments on inoculum potential of *C. floridanum* were evaluated in field plots on the soils at St. Paul and Cloquet, Minnesota. Twelve plots were established at each location. At each location three chemical treatments and four cover crop treatments were applied in factorial design. Each plot was 2 m square and separated from other plots by strips 1 m wide. Four plots were treated with trichlorodinitrobenzene (Chemagro 2635, 70% wettable powder), equivalent to 22.4 kg/hectare (ha) (15.7 kg/ha active ingredient). This fungicide is active against some root-rotting organisms, but in initial tests it did not prevent *Cylindrocladium* root rot. Four plots were covered with 0.102-mm (4-mil) plastic film and fumigated with Dowfume MC-2 (98% methyl bromide and 2% chloropicrin) at the rate of 0.91 kg/4m². The plastic was removed 5 days after treatment. Four plots received no chemical treatment.

After the chemical treatments, all 12 plots at each location were infested with *C. floridanum*. The cover crops planted on the Waukegan silt loam were wheat (*Triticum aestivum* L.), sweet corn (*Zea mays* L.), and soybeans [*Glycine max* (L.) Merr.]. Cover crops planted on the Omega sand were buckwheat (*Fagopyrum esculentum* Moench.), rye (*Secale cereale* L.), and oats (*Avena sativa* L.). Three plots at each location were left fallow.

Chemical treatments and crops were arranged in rows which were randomly located. All plots were hand-weeded periodically. Plots on the Waukegan silt loam were treated with methyl bromide on 21 June and with trichlorodinitrobenzene on 23 June 1967. They were infested with *C. floridanum* and planted with cover crops on 27 June. On the Omega sand both chemicals were applied on 24 May 1968 and infested with *C. floridanum* and planted with cover crops on 29 May. A methyl bromide-treated plot, a trichlorodinitrobenzene-treated plot, and a control plot, all without cover crops were established on the Waukegan silt loam on 21 May 1968, for comparison with the non-cover-cropped Omega sand.

Soil samples for assay of inoculum potentials of *C. floridanum* were collected on six dates from July to October in 1967, but only during August in 1968 at St. Paul; and on seven dates from June to October in 1968 at Cloquet.

Other measurements.—Soil fungistasis in field plots was estimated by the method of Morrison (15). Microsclerotia of *C. floridanum* were produced, treated, and stored by the methods of Morrison (15). The soil

from Cloquet was adjusted to 30-40% of maximum water-holding capacity; those from St. Paul to 40-50% of maximum water-holding capacity as determined by the method of Keen and Raczkowski (10). A sample of the soil was placed in a 5-cm diameter petri dish and the surface was smoothed. Microsclerotia in aqueous suspension were placed on the soil and incubated 4 days at 28 C. Microsclerotia that produced at least one germ tube were considered to have germinated.

Populations of soil fungi, bacteria, and actinomycetes at Cloquet were estimated using serial dilution plates. The soil was adjusted to 30-40% of maximum water-holding capacity and stored at 4 C prior to assay. Isolation media (9) were Allen's soil extract agar for bacteria and actinomycetes, and peptone-dextrose agar plus rose bengal and aureomycin for fungi.

Soil moisture content on an oven-dry basis was calculated for all plots at each sampling date. Soil temperature, air temperature, and precipitation data were obtained from weather stations about 200 and 400 m from the plots at St. Paul and Cloquet, respectively.

RESULTS

Quantitative assay.—Inoculum potentials of *C. floridanum* ranged from 8 to 100% in the four soils tested and were positively correlated with percentages of diseased black spruce seedlings found in the same soils (Fig. 1).

Inoculum potentials of *Cylindrocladium floridanum* in the field.—*Cylindrocladium floridanum* survived in all plots on two soil types regardless of soil treatments or cover crops for at least one growing season (Fig. 2 and 3). The quantitative alfalfa assay detected *C. floridanum* at every sample date from every plot. Inoculum potentials fluctuated during the growing season irrespective of soil treatments, cover crops, or soil types (Fig. 2 and 3). At both locations the lowest average seasonal infection potentials occurred during August or early September. The average inoculum potential of *C. floridanum* in the

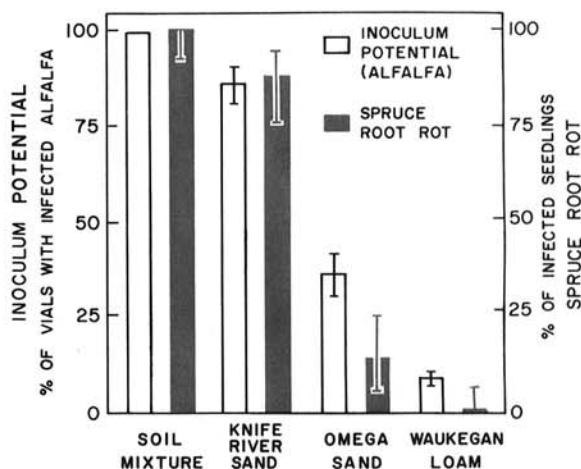


Fig. 1. Infection potentials of *Cylindrocladium floridanum* determined by the alfalfa bioassay, and percentages of black spruce seedlings infected with *C. floridanum* in four soils. Confidence intervals at $P = 0.05$.

Waukegan silt loam was greater ($P=0.05$) at the end of the growing season in October than at any other time during the growing season (Fig. 2). In the Omega sand, inoculum potentials were significantly higher ($P=0.05$) in June, July, early August, and September than they were in late August (Fig. 3).

Average seasonal inoculum potentials of *C. floridanum* were greater in plots cropped with all grasses except corn than they were in fallow plots. Average seasonal inoculum potentials in the Waukegan silt loam in plots containing soybeans and corn were not different from

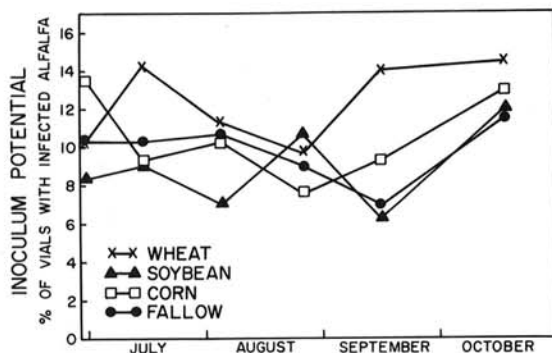


Fig. 2. The effect of cover crops on inoculum potentials of *Cylindrocladium floridanum*, determined by the quantitative alfalfa assay, in Waukegan silt loam. Data are averages from methyl bromide, trichlorodinitrobenzene, and nontreated plots. Plots were infested with *C. floridanum* by the addition of naturally infested soil on 27 June 1967.

those of fallow plots (Table 1), but the inoculum potentials in plots of wheat were 22% greater than in fallow soil. In the Omega sand, average seasonal inoculum potentials in plots with oats and rye were 65% and 56% greater, respectively, than those in fallow plots, but the potentials in the buckwheat plots were not significantly different from those in fallow plots (Table 2). Interacting effects of crops and chemical treatments were present in both soils.

In October, 50 samples from roots of the cover crop from each plot were washed in a 1% solution of sodium

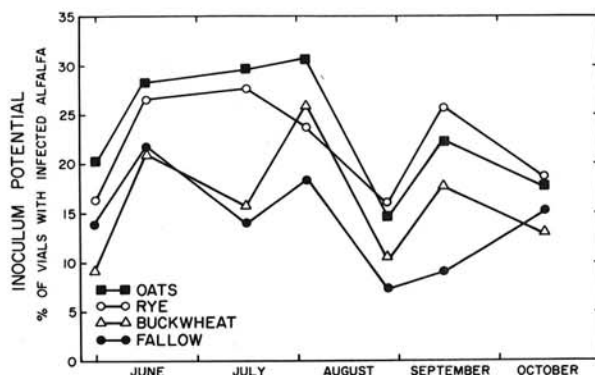


Fig. 3. The effect of cover crops on inoculum potentials of *Cylindrocladium floridanum*, determined by the quantitative alfalfa assay, in Omega sand nursery soil. Data are averages from methyl bromide, trichlorodinitrobenzene, and nontreated plots. Plots were infested with *C. floridanum* by addition of naturally infested soil on 29 May 1968.

TABLE 1. Inoculum potentials of *Cylindrocladium floridanum* in Waukegan silt loam as affected by cover crops and chemical treatments applied prior to infestation with *C. floridanum*

Chemical soil treatment	Inoculum potential ^a (%)				Mean of chemical treatment ^b
	Wheat	Soybeans	Corn	Fallow	
Trichlorodinitrobenzene	4.8	5.0	6.6	7.0	5.9
Methyl bromide	13.9	10.6	9.7	8.8	10.7
No treatment	9.8	6.5	8.3	7.6	8.0
Mean of cover crops ^c	9.5	7.3	8.3	7.8	

^aPercentage of 100 subsamples of soil in which alfalfa seedlings became infected with *C. floridanum*. Values are averages from six sampling dates from June to October of one season. Interactions of soil treatment and cover crop, $LSD_{0.05} = 2.3$.

^b $LSD_{0.05} = 1.2$.

^c $LSD_{0.05} = 1.2$.

TABLE 2. Inoculum potential of *Cylindrocladium floridanum* in Omega sand as affected by cover crops and by chemical treatments applied prior to infestation with *C. floridanum*

Chemical treatment	Inoculum potential ^a (%)				Mean of chemical treatments ^b
	Buckwheat	Oats	Rye	Fallow	
Trichlorodinitrobenzene	19.0	18.7	17.1	14.1	17.2
Methyl bromide	11.0	24.1	20.7	14.1	17.5
No treatment	18.1	27.3	28.4	14.4	22.0
Mean of cover crops ^c	16.0	23.4	22.1	14.2	

^aPercentage of 100 subsamples of soil in which alfalfa seedlings became infected with *C. floridanum*. Values are averages from six sampling dates from June to October of one season. For interactions of soil treatment and cover crops, $LSD_{0.05} = 6.5$.

^b $LSD_{0.05} = 3.3$.

^c $LSD_{0.05} = 3.8$.

hypochlorite, rinsed in sterile distilled water, placed on 1% malt agar containing 30 $\mu\text{g}/\text{ml}$ (30 ppm) aureomycin, and incubated at 23-25 C. *Cylindrocladium floridanum* was isolated from two samples of soybean roots from a methyl bromide-treated plot in the Waukegan silt loam. In the Omega sand nursery soil, *C. floridanum* was recovered from approximately 40% of the buckwheat plants in all three chemical treatments. *Cylindrocladium floridanum* was not recovered from roots of any other cover crop.

Average seasonal inoculum potentials of *C. floridanum* were significantly lower in plots treated with trichlorodinitrobenzene than in nontreated plots on both the Omega sand and the Waukegan silt loam (Tables 1 and 2). The effect of methyl bromide upon the average seasonal inoculum potentials of *C. floridanum* varied with soil type. Average seasonal inoculum potentials were 34% greater in methyl bromide-treated plots than in nontreated plots on the Waukegan silt loam (Table 1), but on the Omega sand, methyl bromide reduced the average seasonal inoculum potentials 20% below inoculum potentials in the nontreated soil (Table 2).

Germination of microsclerotia in field plot soil.—Germination of microsclerotia fluctuated between 10 and 80% on Omega sand collected at different dates during the growing season. Greatest germination (between 40 and 80%) occurred on soil collected in July; germination decreased throughout the remainder of the summer and autumn. Cover crops exerted no significant influence upon percentage of germinating microsclerotia in the Omega sand. The only chemical that affected germination of microsclerotia was trichlorodinitrobenzene. It reduced ($P = 0.05$) the average seasonal germination of microsclerotia by 44%.

Soil fungi, bacteria, and actinomycete populations in field plots.—Bacteria and actinomycete populations were not affected by cover crops or chemical treatments in a way that could be correlated with *C. floridanum* infection potentials. Average seasonal populations of fungi in the Omega sand were significantly affected ($P=0.05$) only by methyl bromide, not by trichlorodinitrobenzene or cover crops. Average seasonal numbers of fungal colonies were increased by 93% in the methyl bromide-treated plots compared to untreated plots.

Moisture and temperature in field plots.—Inoculum potentials of *C. floridanum* were not correlated with soil moisture content, air temperatures, or soil temperature.

Inoculum potential related to soil type.—Average

inoculum potentials in the Waukegan silt loam in August 1968, were 42% lower and germination of microsclerotia was 48% less, whereas fungal colonies were 83% more numerous when compared to the Omega sand (Table 3). However, significantly more fungal colonies ($P = 0.05$) were found in the methyl bromide-treated Omega sand (150,000/g soil) than in the methyl bromide-treated Waukegan silt loam (68,000/g soil).

DISCUSSION

Cylindrocladium floridanum survived for one growing season in all plots to which it had been introduced, regardless of seasonal fluctuations, soil types, cover crops, or chemical treatments. There was no evidence that populations of *C. floridanum* were decreasing (Fig. 2 and 3). These results confirm evidence for the ability of *C. floridanum* to survive long periods under a variety of soil conditions and to withstand cultural and chemical control measures (17, 21).

The quantitative alfalfa assay method could be used to test nurseries for hazard of *Cylindrocladium* root rot prior to planting. Care must be taken, however, when correlating the inoculum potentials detected through the quantitative alfalfa bioassay with actual losses in the field. The spruce used in this study were grown in the greenhouse and losses obtained under such conditions will differ from losses in the field. Also black spruce are generally thought to be more sensitive to *C. floridanum* than most other coniferous seedlings. It may be assumed, based on Figure 1, that losses due to *C. floridanum* will begin when inoculum potentials rise above 12%. Similarly, *C. floridanum* inoculum potentials above 80% will result in almost total loss of coniferous seedlings over a 2- or 3-year period.

The seasonal fluctuation of *C. floridanum* inoculum potentials is similar to variation described for other soil fungi (4, 6) in that minimum populations often occur during midsummer or early fall. The soil environment may be more inhibitory to root infection by *C. floridanum* during the summer or early fall than at other times during the growing season.

Cover crops may influence soil fungi, whether they are hosts or not. Root-infecting fungi can maintain populations by growth in the rhizospheres of nonsusceptible plants, using exudate as their substrate (1, 8, 13, 19, 20). Some nutrients in the rhizosphere may be absorbed by resting structures of fungi and utilized for

TABLE 3. A comparison of inoculum potentials, germination of *Cylindrocladium floridanum* and microbial populations in two soils. Values are averages from single methyl bromide-treated, trichlorodinitrobenzene-treated, and nontreated plots from each soil type in August 1968

Soil type	Inoculum potential ^a (%)	Germination of microsclerotia ^b (%)	Colonies per gram of dry soil	
			Bacteria and actinomycetes ^c ($\times 10^6$)	Fungi ^c ($\times 10^3$)
Omega sand	18.3	34.7	13.7	73.3
Waukegan silt loam	10.7	18.0	17.0	134.3
LSD _{0.05}	5.4	9.3	N.S.	55.2

^aPercentage of 100 vials with alfalfa infected with *C. floridanum*.

^bValues are averages of three subsamples per plot.

^cValues are averages of two subsamples per plot.

survival without subsequent germination and growth (7). Such activities may have sustained *C. floridanum*, which was able to survive and even increase its inoculum potential under nonhost grass cover crops.

Although buckwheat and soybeans were infected by *C. floridanum*, they did not increase inoculum potentials over the growing season, as did oats, wheat, and rye. No satisfactory explanation is available for this anomaly. It is known that some leguminous host plants are not killed by *C. floridanum* in the field. The fungus attacks the roots of these often symptomless carriers and invades the cortex, producing abundant microsclerotia. During estimation of inoculum potentials of *C. floridanum* on plots with infected plants, microsclerotia may have been concentrated in host tissue. This would result in consistently low inoculum potential readings, even though propagule numbers were large. In the grass plots no infection of roots was observed, but *C. floridanum* may have been growing in the rhizosphere, thus occupying a larger soil volume which was reflected in the sampling technique. Grasses in general possess a far greater root surface area than do legumes, and this alone may have been responsible for increases *C. floridanum* inoculum potentials observed in this study.

Regardless of the mechanism involved, forest nursery personnel should avoid planting these grass cover crops if possible. Attempting to "starve out" *C. floridanum* under so-called nonsusceptible hosts could actually double inoculum potentials during the first growing season. Corn may be a suitable substitute for wheat, rye, or oats, since it did not affect inoculum potentials.

Trichlorodinitrobenzene increased the fungistasis of the soil and decreased the inoculum potentials of *C. floridanum*. It was not known if this fungicide inhibited *C. floridanum* directly, or whether the inhibition was mediated by other organisms.

The variable effects of methyl bromide-treated soil upon the inoculum potentials of *C. floridanum* in the two soils in this study (Tables 1 and 2) could be explained by reinvasion of the fumigated soil by antagonistic microorganisms (2, 11, 12, 17, 18). More fungi were present in methyl bromide-fumigated Omega sand than in similarly treated Waukegan silt loam, although in samples from trichlorodinitrobenzene-treated and nontreated plots more fungi were present in the latter soil (Table 3). These results indicate that methyl bromide-treated Omega sand was reinvaded far more rapidly by fungi than was the Waukegan silt loam. Since soil fungi may reestablish soil fungistasis (16) the high populations of fungi in methyl bromide-treated Omega sand may be responsible for reducing the inoculum potentials of *C. floridanum* in these plots below that in nontreated plots (Table 2). In methyl bromide-treated Waukegan silt loam in which populations of fungi were lower than in corresponding nontreated plots, the lack of antagonistic fungi may have resulted in greater inoculum potentials of *C. floridanum* than were present in the nontreated plots (Table 1).

Greater inoculum potentials of *C. floridanum* existed in the Omega sand nursery soil, in which *Cylindrocladium* root rot is known to become severe, than in the Waukegan silt loam. Soil fungistasis may have been responsible for these results. Fewer microsclerotia of *C. floridanum* germinated on the silt loam than in the

Omega sand nursery soil (Table 3). More soil fungi were found in the silt loam than in the corresponding Omega sand (Table 3), except in the methyl bromide-treated plots. A greater variety of potential antagonists and competitors were probably present in the silt loam.

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