Isolation of Cylindrocladium from Soil or Infected Azalea Stems with Azalea Leaf Traps

R. G. Linderman

Plant Pathologist, Plant Science Research Division, ARS, USDA, Beltsville, Maryland 20705.

The author acknowledges the technical assistance of Anita L. Schmidt.

Accepted for publication 13 February 1972.

ABSTRACT

Healthy, detached azalea leaves, inserted into infested soil or into infected azalea stems, were used to trap and identify several species of *Cylindrocladium*. Using this method, *C. scoparium* and *C. theae* were recovered from infected azalea stems, often when direct plating methods failed. These two species, plus *C. floridanum, C. ilicicola, C. crotalariae,* and *C. quinqueseptatum* were successfully trapped from artificially infested field soil or greenhouse (GH) soil mix, but *C. parvum* and an undescribed *Cylindrocladium* sp. were not. *Phytophthora cinnamomi* was not recovered either from soil or infected azaleas by this method.

By means of the leaf trap method, *C. scoparium* was found to persist in artificially infested vermiculite or perlite for 2 months, and in field soil or GH soil mix for at least 5 months (the latter being infested with as low as 9 conidia/g soil). *Cylindrocladium floridanum* was trapped from three naturally infested field soils and one artificially infested field soil diluted with uninfested soil from 13 down to ca. 3 microsclerotia/g soil.

Phytopathology 62:736-739.

Additional key words: soil assay, azalea diseases, survival in soil, species identification.

The symptoms of certain root and stem diseases of azalea are often similar, even though the causal organism is different. Either *Phytophthora cinnamomi* Rands or species of *Cylindrocladium* may cause wilt and dieback of the entire plant or portions of it. Stem rot, girdling, root discoloration, root rot, and yellowing of the foliage are also common symptoms. Thus, it is often not possible to determine the cause of the malady based on symptoms alone. Isolations of pathogenic organisms are often unsuccessful because secondary organisms invade the tissue killed by the primary pathogen. Therefore, a consistent and reliable method for isolating the causal organism from infected azaleas would significantly aid in disease diagnosis and control.

Several methods have been reported for assaying soil for *Cylindrocladium* (3, 6, 7, 8, 9), but these methods are time-consuming. This paper reports a new technique for isolating *Cylindrocladium* from natural soil and artificial mixes, as well as from infected azaleas, which is superior in many ways to those previously reported.

MATERIALS AND METHODS.—Leaves of azalea [*Rhododendron obtusum* (Lindl.) Planch. 'Red McCaw' or 'Kingfisher'] were used as bait to trap species of *Cylindrocladium* from infested soil or infected woody azalea tissue. Numerous other cultivars tested were equally effective.

Isolation from soil.—Mature, healthy azalea leaves were removed from the plant and inserted halfway into moistened soil, petiole down, in plastic moist chambers (Fig. 1). After 3-7 days, the leaves were removed and those with dark brown-to-black lesions were surface-sterilized in 0.5% sodium hypochlorite for 5 min. Lesions were cut out with a sterile cork borer and plated out on acidified potato-dextrose agar (APDA) (1 drop 25% lactic acid/plate with 20 ml medium).

Isolation from diseased plants.—Azalea plants naturally or artificially infected with several known fungus pathogens were used. Plants were either symptomless or were expressing symptoms ranging from twig dieback to total wilt. Plants were pruned to within 1 inch of the soil line, and the remaining stems were split longitudinally with pruning shears or a knife. An azalea trap leaf was inserted into each split stem (Fig. 2), and the pot was covered with a polyethylene bag for 1 week and maintained either in the greenhouse or on the laboratory bench. Afterward, the leaf was removed and examined under the dissecting microscope for brown lesions and sporulation. Sporulation was often visible on the lesions caused by *Cylindrocladium* spp., and plating was unnecessary. Lesions without sporulation were surface-sterilized with sodium hypochlorite (0.5% for 5 min) and plated on APDA. For comparative purposes the remaining stem tissue was also plated on APDA after 10- to 15-min surface sterilization in 0.5% sodium hypochlorite.

Infestation of field soil, vermiculite, perlite, and GH soil mix.—Fungi used were grown either on agar or on vermiculite moistened with Czapek-Dox broth. Conidia were produced on an agar medium consisting of malt extract, yeast extract, dextrose, and agar (5:2:2:20 g/liter) (MYDA) from which they were removed by washing, and were added to GH soil mix (equal parts of loamy soil, peat moss, and perlite), vermiculite, or perlite. Vermiculite cultures containing sclerotia, chlamydospores, hyphal fragments, and conidia were mixed into either field soil or GH soil mix.

Determination of spore concentrations of C. scoparium.—Concentrations of conidial suspensions of *C. scoparium* Morgan to be added to soil were determined by plating 1 ml/plate of each suspension on duplicate MYDA plates to which 3 ppm

Endomycin (Upjohn Co., Kalamazoo, Mich.) and 0.75% pentachloronitrobenzene (PCNB) had been added. The PCNB restricted colony growth, and Endomycin stimulated sporulation on the colonies (5). The plate colonies could be counted after 48 hr, but were still distinct for several weeks.

RESULTS.—*Isolation from diseased plants.*—Numerous attempts were made using the leaf trap technique to isolate *Cylindrocladium* from naturally or artificially infected dead or dying azaleas. The age, cultivar, and growing conditions of the plants varied. The results in Table 1 show the per cent recovery of *Cylindrocladium* with the leaf trap technique.

Whenever leaf traps were placed in azaleas known to be infected with *Phytophthora cinnamomi* (based on prior isolations), *P. cinnamomi* was never recovered. On one occasion, however, a saprophytic isolate of *Cylindrocladium* having a hastate vesicle was recovered from a plant killed by *P. cinnamomi.*

Since the leaf trap technique readily permitted recovery of *Cylindrocladium* but not *Phytophthora* from infected azaleas, I inoculated 20 rooted azalea cuttings (cultivar Kingfisher) with vermiculite cultures of both *C. scoparium* and *P. cinnamomi.* As these plants died, reisolation of the pathogens was attempted by means of the azalea leaf trap technique. Leaf lesions and basal stem tissue were plated on APDA after 1 week. *Cylindrocladium scoparium* was recovered in every case where it was added as inoculum. *Phytophthora cinnamomi* was not isolated by the leaf trap technique, even when recovered by direct isolation from the basal stem tissue of inoculated plants.

I compared direct plating on APDA with the leaf trap technique on 2-year-old plants inoculated with *C. scoparium* the previous year (cultivar Kingfisher), or naturally infected with *C. scoparium* (cultivar Hershey's Red). Many of these plants had been dead several months, but were maintained on the greenhouse bench. In both of these experiments (2 and 3), *Cylindrocladium* was more readily recovered by the leaf trap method than by direct plating of the basal stem tissue (Table 1). In addition, *C. scoparium* grew into the plating medium from the leaf trap lesions several days earlier than it did from directly plated infected stem tissue, and *C. scoparium*

TABLE 1. Recovery of *Cylindrocladium scoparium* from infected azalea plants using the azalea leaf trap method except where comparison was made with direct plating of infected stem tissue after the leaf trap was removed

Experiment no.	Plant treatment[a]	No. attempts	% Recovery
1	Noninoculated controls	25	0
	Inoculated-symptomless	25	35
	Inoculated-dead	19	32
2	Inoculated-dead		
	Leaf trap method	18	55
	Direct plating method[b]	18	11
3	Wilt or dieback symptoms		
	Leaf trap method	17	29
	Direct plating method[b]	17	23
4	Noninoculated controls	5	0
	Inoculated-symptomless	11	45
	Inoculated-dead	10	60

[a] Experiments 1 and 2 involved 2-year-old artificially infected Kingfisher azaleas; experiment 3 involved 2-year-old naturally infected Hershey's Red azaleas; experiment 4 involved 3-month-old artificially infected Kingfisher azaleas.

[b] Comparisons between leaf trap method and direct plating of stem tissue after the leaf trap was removed.

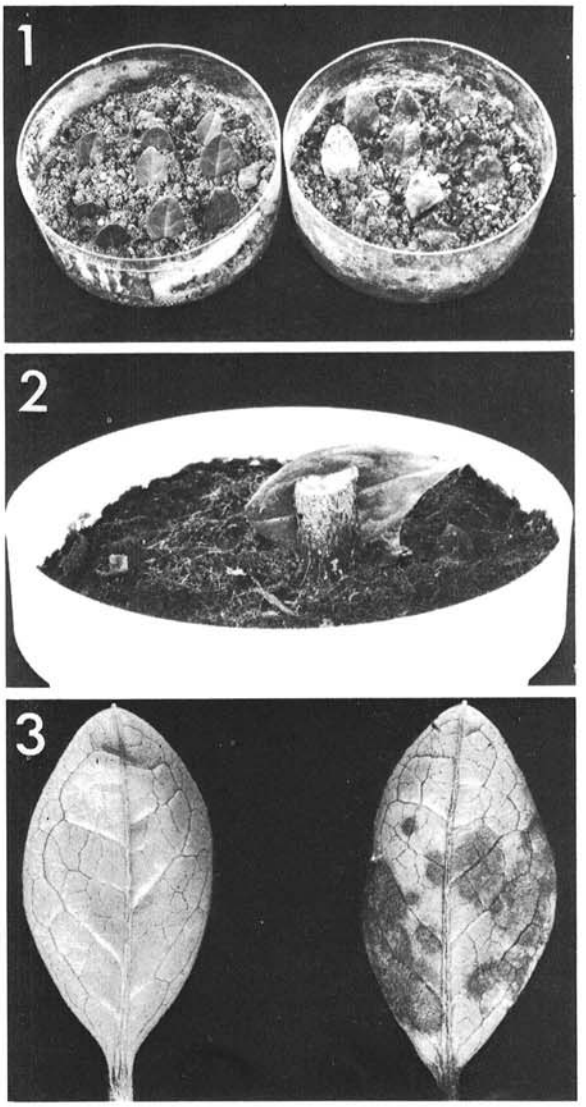

Fig. 1-3. 1) Assay of greenhouse soil mix infested with conidia of *Cylindrocladium scoparium*, using azalea leaves as traps. Container on the left is uninfested control. Note abundant sporulation on trap leaves 7 days after being inserted into infested soil. **2)** Azalea leaf inserted into split azalea stem which has trapped the pathogen, *Cylindrocladium scoparium.* **3)** Appearance of azalea leaves inserted into uninfested soil (left) and soil infested with *Cylindrocladium scoparium* (right) after 72 hr.

was usually the only organism present. Many fungi generally grew from the woody tissue pieces into the plating medium, but *C. scoparium* was detectable because of the presence of brown pigmented sclerotia and chlamydospores.

Cylindrocladium theae (Petch) Alfieri et al. (2) and an undescribed *Cylindrocladium* sp. with a hastate vesicle were also recovered in leaf traps, as were species of *Colletotrichum, Rhizoctonia, Pestalotia,* and *Gloeosporium* and, less frequently, *Phomopsis* sp. and *Fusarium oxysporum* Schlecht. *Cylindrocladium theae* is a recently reported pathogen on azalea (1, 2), but the role of the others as primary pathogens is presently under investigation.

Recovery of Cylindrocladium from soil.—The persistence of *C. scoparium* in GH soil mix, perlite, and vermiculite infested with conidia was assayed periodically. The concentrations of conidial suspensions were determined on MYDA plus Endomycin and PCNB prior to adding them to the GH soil mix. The pathogen was recovered in leaf traps from perlite and vermiculite 2 months after infestation. It was recovered from GH soil mix 5 months after infestation with only 9 conidia/g of soil, but not at 2.5 conidia/g of soil even at the beginning of the experiment.

The effect of soil moisture was studied in a sandy loam field soil infested with vermiculite cultures of *C. scoparium*. Abundant conidia, sclerotia, and chlamydospores were observed on the pieces of vermiculite prior to their incorporation into the soil. Although the population after infestation was unknown, the leaf trap assay indicated that *C. scoparium* was still viable after the soil was stored for 5 months in the laboratory. Water was added periodically to maintain the original moisture level (14% by weight). Subsamples of the soil were taken and their moisture levels adjusted to 0, 7, and 21% by adding water or drying. The azalea leaf assay indicated *C. scoparium* was still viable at 7, 14, and 21% moisture after 2 months, but was not viable in the air-dry soil even at the beginning of the test. The fungus was not recovered even after remoistening the dry soil.

Cylindrocladium floridanum Sobers & Seymour was readily recovered from three naturally infested field soils assayed with azalea leaves. These soils were from a peach nursery in Pennsylvania, a mature peach orchard in Maryland, and a blueberry field in Maryland. Another peach orchard soil (supplied and assayed by D. J. Weaver, ARS, USDA, Byron, Ga.) artificially infested 1 year before with microsclerotia, was diluted with uninfested field soil. *C. floridanum* was recovered from the soil even when the concentration of the fungus was reduced from 13.3 down to ca. 3 microsclerotia/g.

The efficacy of the azalea leaf assay for detection of species of *Cylindrocladium* other than *C. scoparium* and *C. floridanum* in soil was determined. Vermiculite cultures of several other species were incorporated into GH soil mix and a field soil. These soils were assayed initially, and again after 2 and 4 weeks' incubation in the laboratory. The known species added included *C. scoparium* (isolates 1, 21, 42, 30, 32), *C. floridanum* (isolates 7, 31, 24), *C. theae* (isolates 27, 50), *C. crotalariae* (Loos) Bell & Sobers (isolate 36), *C. ilicicola* (Hawley) Boedijn & Reitsma (isolate 25-ATCC 16162), *C. quinqueseptatum* Boedijn & Reitsma as identified by E. K. Sobers (isolate 26-ATCC 16550 labeled *C. pteridis* Wolf), and *C. parvum* Anderson (isolate 23-ATCC 16315). In addition, unknown isolate 33 (possible *Calonectria hederae* Arnaud) and an undescribed *Cylindrocladium* sp. having a hastate vesicle (isolates 43, 44) were also added. Only *C. parvum* (isolate 23) and the undescribed *Cylindrocladium* sp. (isolates 43, 44) were not recovered by means of the leaf trap method, presumably because they are not pathogenic on azaleas.

DISCUSSION.—Selective recovery of *Cylindrocladium* spp. from infected azaleas after secondary invasion by saprophytic organisms has not been reported. In this regard, the leaf trap method is decidedly more effective than direct isolation by plating. Still other points in its favor are speed and selectivity. When leaf lesions were plated out as compared to woody stem tissue, *Cylindrocladium* grew from the leaf lesions faster than from the wood tissue. Furthermore, sporulation of all the isolates tested was stimulated around the plated azalea leaf tissue disc, even those isolates which typically sporulate very little. These features greatly simplify purification and identification. Moreover, *Phytophthora cinnamomi*, which induces symptoms most like those of *Cylindrocladium*, does not invade azalea leaves. Thus, if no lesions are present on the assay leaves, one might suspect that *P. cinnamomi* is the pathogen.

The existing indirect assays of soil for *Cylindrocladium* (3, 4, 7) may require several weeks to perform, and the direct methods (6, 8, 9) involve laboratory manipulations which may reduce the actual propagule numbers assayed. If the population is already low, *Cylindrocladium* may not be detected. While the method I have presented is not quantitative, it does involve whole, unmanipulated soil, and its potential as a sensitive qualitative indexing procedure for nurseries and greenhouse growers is great. Based on results reported here, populations as low as 3 microsclerotia/g soil can be detected. Furthermore, the leaf trap method can be used to index symptomless plants during various stages of production. The potential of the technique is good; it needs only to be tested under a variety of conditions.

LITERATURE CITED

1. ALFIERI, S. A., JR., R. G. LINDERMAN, R. H. MORRISON, & E. K. SOBERS. 1971. Pathogenicity of Cylindrocladium theae and C. scoparium to roots and leaves of azalea. Phytopathology 61:883 (Abstr.).
2. ALFIERI, S. A., JR., R. G. LINDERMAN, R. H. MORRISON, & E. K. SOBERS. 1972. Comparative pathogenicity of Calonectria theae and

Cylindrocladium scoparium to leaves and roots of azalea. Phytopathology 62:647-650.
3. BUGBEE, W. M. 1962. Host range and bioassay of field soil for Cylindrocladium scoparium. Phytopathology 52:726 (Abstr.).
4. BUGBEE, W. M., & N. A. ANDERSON. 1963. Infection of spruce seedlings by Cylindrocladium scoparium. Phytopathology 53:1267-1270.
5. HORST, R. K., & H. A. J. HOITINK. 1968. Occurrence of Cylindrocladium blights on nursery crops and control with fungicide 1991 on azalea. Plant Dis. Reptr. 52:615-617.
6. MORRISON, R. H., & D. W. FRENCH. 1969. Direct isolation of Cylindrocladium floridanum from soil. Plant Dis. Reptr. 53:367-369.
7. THIES, W. G., & R. F. PATTON. 1966. Spot-plate technique for the bioassay of Cylindrocladium scoparium. Phytopathology 56:1116-1117.
8. THIES, W. G., & R. F. PATTON. 1970. An evaluation of propagules of Cylindrocladium scoparium in soil by direct isolation. Phytopathology 60:599-601.
9. THIES, W. G., & R. F. PATTON. 1970. The biology of Cylindrocladium scoparium in Wisconsin forest tree nurseries. Phytopathology 60:1662-1668.