Decline of *Phellodendron amurense* in Tokyo: Associated Fungi and Pathogenicity of Associated *Cylindrocladium* spp.

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ABSTRACT

Watanabe, T., Hagiwara, S., and Narita, I. 1995. Decline of *Phellodendron amurense* in Tokyo: Associated fungi and pathogenicity of associated *Cylindrocladium* spp. Plant Dis. 79:1161-1164.

Three *Cylindrocladium* species were isolated from *Phellodendron amurense* seedlings together with fungi belonging to 24 genera, and 11 genera from the tree roots. The three species were tested for pathogenicity on leaflets and seedlings of *P. amurense*. *Cylindrocladium colhounii* caused leaf blight and subsequent abscission of leaflets of seedlings, saplings, and trees, and damping-off of potted seedlings, but *C. camellea* and *C. tenue* were not pathogenic. The significance of *Cylindrocladium* species in relation to decline of *P. amurense* trees in Tokyo is discussed.

Phellodendron amurense Rupr. is one component of the natural flora of a 20-ha garden of The Institute for Nature Study, National Science Museum in Tokyo, Japan. Among a total of 155 mature trees (9 to 76 cm, D.B.H.) in the garden, 36 trees were dead and nearly 30 trees were diseased by the end of December 1993. Diseased trees were characterized by loss of turgidity and delayed new leaflet development in the spring. Leaflets that developed on diseased trees were smaller than those on healthy ones. Canopies of the diseased trees were less dense than those of healthy trees because of less numerous and smaller leaflets (Fig. 1A). As disease progressed, new leaflets did not develop in the spring, and dieback of twigs occurred, resulting in the decline of the entire tree. Diseased trees had damaged root systems. The number of lateral roots under 5 mm in diameter was reduced, and there were numerous discolored roots. The root epidermis did not appear healthy, was readily sloughed off, water-soaked, and became dark yellow. Abundant seedlings emerged near mature trees but died within a year. Diseased seedlings were often brown, had smaller roots and basal stems, and the root epidermis was disintegrated or sloughed-off. Diseased leaflets had slightly sunken, brown, necrotic lesions with indistinct margins.

In 1992, three *Cylindrocladium* species were identified from diseased seedlings: *C. camelliae* Venkataramani & Venkata Ram and *C. colhounii* Peerally (anamorph of *Calonectria colhounii* Peerally) (= *C. colhounii* var. macroconidialis Crous, Wingfield & Alfenas) (2) and *C. tenue* (Bugnicourt) T. Watanabe. The morphology, temperature responses, and taxonomy of these fungi have been described by Watanabe (8).

In this report, the pathogenicity of these three *Cylindrocladium* spp. to *P. amurense* was studied, and the significance of *C. colhounii* is discussed in relation to the decline of *P. amurense* trees.

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Accepted for publication 22 May 1995.

MATERIALS AND METHODS

Isolation of fungi. Fungi were isolated from roots selected at random from three diseased and two healthy trees, and 41 diseased and 10 healthy seedlings. Roots were washed and segments (27 mm^3) were plated on water agar (WA) and maintained at 25°C. Pure cultures were obtained by removing hyphal tips and transferring them to potato-dextrose agar (PDA).

Pathogenicity tests and isolates tested. In preliminary inoculation studies conducted by placing 4-mm agar culture disks of test fungi onto leaflets of *P. amurense* (one disk/leaflet) in a moist condition, *C. colhounii* isolates were the most virulent among 26 fungal isolates belonging to 10 genera selected from dominant fungi known as plant pathogens; others were very weakly pathogenic or nonpathogenic. In addition, during isolation processes, *Cylindrocladium* spp. often sporulated on diseased leaflets and roots of seedlings in plates. Therefore, further pathogenicity tests were conducted using four isolates of three *Cylindrocladium* spp. including *C. camelliae* (isolate 92-202), *C. tenue* (isolate 92-246), and *C. colhounii* (two isolates 92-211; -260). All isolates were obtained from the roots of *P. amurense* seedlings, except *C. colhounii* isolate 92-211, which was isolated from leaflets of *P. amurense* seedlings. Morphology, temperature responses, and taxonomy of these fungi were described previously (8,9).

Inoculation of leaflets. Inoculum was placed directly onto leaflets of seedlings, saplings, and mature trees. In Tokyo, leaflets were inoculated on 5-month-old potted seedlings and 2-year-old saplings in a natural stand (Fig. 1B) and in plastic containers (50 × 30 × 8 cm). Five seedlings or saplings were used in each case. In Tsukuba, leaflets were inoculated on 5-month-old potted seedlings, 2-year-old potted saplings, and 3-year-old field-grown saplings, using 5 plants for each age. Leaflets on at least 10 branchlets were also inoculated on three trees (>20 years old) in the garden of Forestry and Forest Products Research Institute (FFPRI) at Tsukuba. Agar culture disks (4 mm diameter) removed from 6-day-old PDA and WA cultures were used as inocula. One disk was placed in the center of each leaflet. Uninoculated PDA and WA disks were used as controls. Inocula were covered with pieces of wetted cotton (1.5 cm^2 per piece) and taped on the leaflets to maintain moisture (Fig. 1C). Inoculated leaflets were kept moist by covering with plastic bags for at least 2 days. Leaflets were scored for infection after 7 days.

Experiments were conducted using a total of 12 or 18 leaflets (two or three leaflets/isolate or control) in each experiment and repeating one to three times separately outdoors or in the greenhouses in June or September 1993, and September 1994 in both Tokyo and Tsukuba.

Inoculation of seedlings by soil infestation. Three-month-old potted seedlings (three per pot) were inoculated by burying inocula 1 to 2 cm deep near the stems of seedlings. Inocula were harvested from 10-day-old potato-dextrose broth cultures in 9-cm plastic petri dishes. The contents of a single culture were added to each 10-cm-diameter polyethylene pot with 500 ml of soil. As controls, uninoculated potato-dextrose broth was used. Seedlings were observed for disease occurrence 10 days after inoculation. This experiment was conducted using a total of six seedlings for each isolate or control, and repeated once under greenhouse conditions, separately in

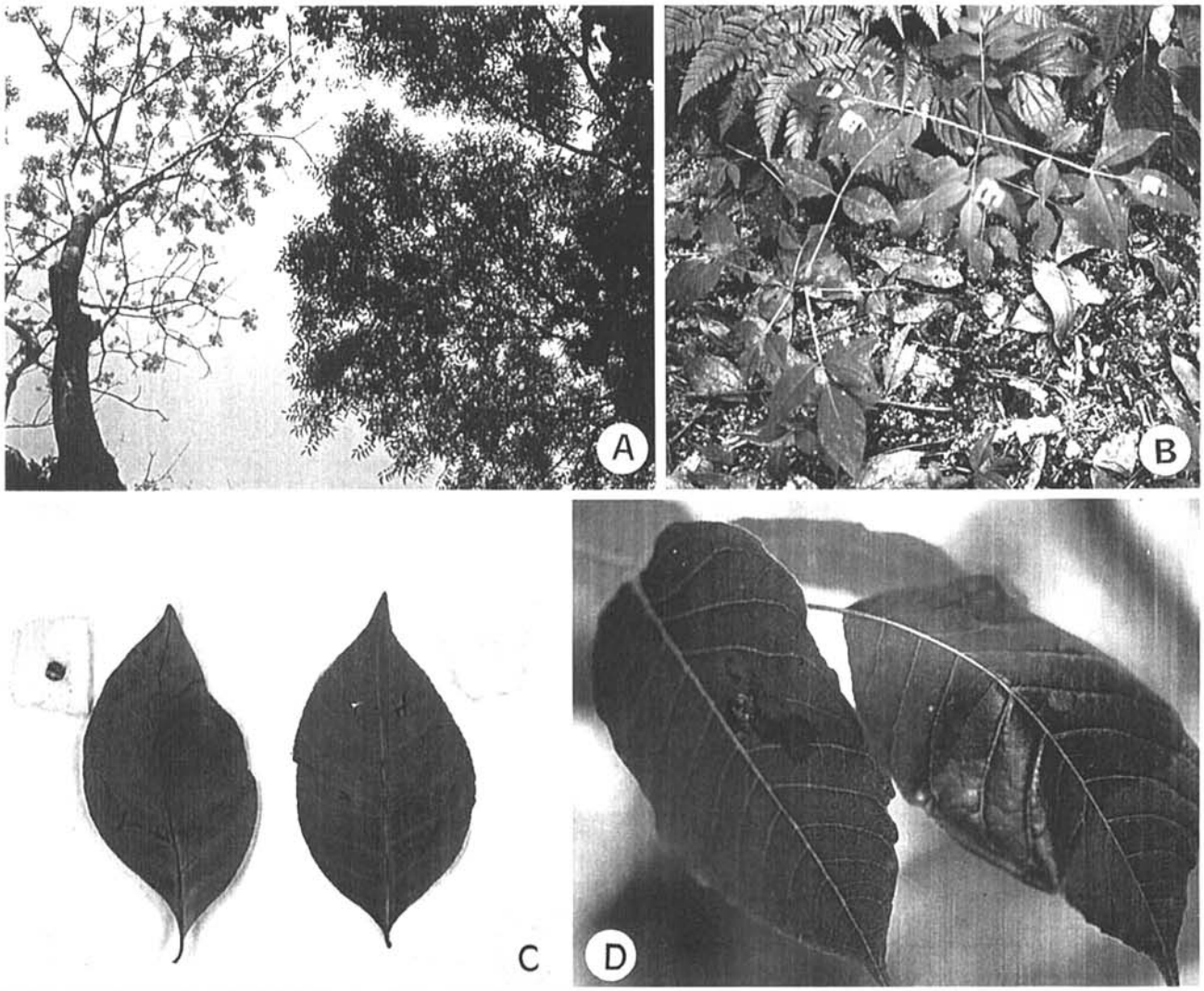

Fig. 1. Declining *Phellodendron amurense* tree and pathogenicity of three *Cylindrocladium* species: **(A)** Declining *Phellodendron amurense* tree (left) in comparison with healthy tree (right) at The Institute for Nature Study, National Science Museum in Tokyo; **(B)** inoculated leaflets of 2-year-old *P. amurense* sapling in a natural stand in Tokyo; **(C)** blighted leaflet (left) inoculated with *C. colhounii* (isolate 92-211), and healthy leaflet used as control (right) from natural sapling in Tokyo together with inocula (agar disks) on pieces of cotton cover used to keep inocula moist; **(D)** symptoms on a leaflet of mature tree (left) 4 days after inoculation with *C. colhounii* (isolate 92-260), and healthy leaflet (right) with potato-dextrose agar disk as control following removal of wetting material.

both Tokyo and Tsukuba. Outdoor temperatures in Tokyo and Tsukuba during the experiments ranged between 10.9 and 32.7°C (mean 23.9°C) and 14.3 and 31.9°C (mean 25°C), respectively. Temperatures in the greenhouses ranged between 18 and 30°C.

RESULTS

Fungi isolated. A total of 293 fungal isolates were obtained from 140 root segments of five trees and 137 root segments of 51 seedlings. A total of 141 isolates from incubated samples of tree roots on WA belonged to at least 11 genera (species name, if known, and isolate number for representative isolates are given in parentheses): *Acremonium* Link:Fr. (92-251), *Cylindrocarpon* Wollenweb. (*C. destructans* (Zinssmeister) Scholten, 92-219; -234), *Fusarium* Link:Fr. (*F. solani* (Mart.) Sacc. emend. W. C. Snyder & H. N. Hans., 92-218), *Gliocladium* Corda (*G. catenulatum* Gilman & E. Abbott, 92-227), *Humicola* Traaen (92-250), *Mortierella* Coem. (*M. elongata* Linnemann, 92-228), *Pythium* Pringsh. (92-221; -224), *Rhizoctonia* DC. (92-253; -254), *Stilbum* Tode (92-223; -238), *Verticillium* Nees, and unknown fungi with clamped hyphae (92-213; -214). Among these fungi, *Stilbum*, and basidiomycetous fungi with clamped hyphae were isolated only from the diseased tree roots.

Roots and leaflets of most seedlings were lightly or severely diseased. A total of 152 isolates from seedlings belonged to 28 genera: *Acremonium* (93-339), *Alternaria* Nees (*A. alternata* (Fr.:Fr.) Keissl., 93-308), *Aphanomyces* de Bary (*A. cladogamus* Drechs., 92-207), *Arthrinium* Kunze:Fr. (93-401), *Cladosporium* Link: Fr. (*C. cladosporioides* (Fresen.) G. A. De Vries, 93-306), *Colletotrichum* Corda in Sturm. (92-249; 93-337), *Cylindrocarpon* (*C. destructans*, 92-210), *Cylindrocladium* Morg., *Dactylaria* Sacc., *Fusarium* (*F. moniliforme* J. Sheld., 92-267, *F. roseum* (Link:Fr.), 93-303), *Gliocladium* (92-241), *Helicomyces* Link (*H. roseus* Link, 93-408), *Humicola* (*H. grisea* Traaen, 92-209), *Monacrosporium* Oudem. (92-273; 93-404), *Mortierella* (*M. alpina* Peyronel, 93-318, *M. humilis* Linnemann: W. Gams, 93-331), *Mucor* P. Mich.: Fr. (*M. meguroense* T. Watanabe, 92-268, *Mucor* spp., 93-328; -329), *Myrothecium* Tode:Fr., *Nectria* (Fr.) Fr. (92-201), *Penicillium* Link:Fr. (93-332; -335), *Pestalotia* De Not. (92-212; -265), *Phoma* Sacc. (92-208; -262), *Phytophthora* de Bary, *Pyrenochaeta* De Not. (93-345), *Pythium* (*P. sylvaticum* W. A. Campbell & J. W. Hendrix, 93-310; -311, *P. torulosum* Coker & F. Patterson, 92-244, *Pythium* spp., 92-243; 93-330), *Rhizoctonia* (92-263; 93-344), *Trichoderma* Pers.:Fr. (93-327), *Verticillium*, and unknown fungi including Sphaeropsidales (93-334; -405). In addition, *Botrytis, Epi-*

Table 1. Pathogenicity of *Cylindrocladium camelliae* (isolate 92-202), *C. colhounii* (isolates 92-211; -260), and *C. tenue* (isolate 92-246) on leaflets of 2-, and 3-year-old saplings of *Phellodendron amurense* in natural and artificial stands tested separately in Tokyo and Tsukuba[y]

	Tokyo						Tsukuba					
	2-year-old sapling						2-year-old sapling			3-year-old sapling		
	(Potted)			(Natural stand)			(Potted)			(Transplanted)		
	Blighted		Abscissed	Blighted		Abscissed	Blighted		Abscissed	Blighted		Abscissed
Inocula	%	% area	%	%	% area	%	%	% area	%	%	% area	%
Control	0	0 a[z]	0	0	0 a	0	0	0 a	0	0	0 a	0
C. camelliae	0	0 a	0	0	0 a	0	0	0 a	0	0	0 a	0
C. colhounii												
92-211	100	62 b	100	100	13 b	100	100	23 c	100	100	16 b	100
92-260	100	84 b	100	100	17 b	100	100	16 b	100	100	24 b	100
C. tenue	0	0 a	0	0	0 a	0	0	0 a	0	0	0 a	0

[y] Percentage of leaflets blighted or abscised and average percentage of blighted area 7 days after inoculation (one 4-mm-diameter potato-dextrose agar [PDA] culture disk placed/leaflet) at temperatures of 10.9 to 32.7°C. Each value is a mean of one or two replicates using a total of three to six leaflets per isolate or control. Sterile PDA disks served as control.

[z] Values with the same letter within a column do not differ significantly ($P = 0.01$) according to the least significant difference test.

coccum, Pithomyces, and *Torula* that were not obtained from roots were just observed only from seedling leaflets without isolation. Several fungi among these isolates, including *Helicomyces roseus* and *Mucor meguroense,* were described and illustrated elsewhere (9,10). Most of these fungal isolates are deposited in the Gene Bank, National Institute of Biological Resources, Ministry of Agriculture, Forestry and Fisheries (MAFF) in Tsukuba.

Pathogenicity tests. Brown lesions developed around the point of inoculation as early as 2 days after inoculation with *C. colhounii* on leaflets of 5-month-old potted seedlings in several tests, and on potted, container- and field-grown, 2- to 3-year-old saplings (Table 1; Fig. 1C) and mature trees (Table 2; Fig. ID). Diseased leaflets abscised as early as 4 days after inoculation, whereas uninoculated control leaflets, and leaflets inoculated with *C. tenue* and *C. camelliae,* remained healthy (Tables 1 and 2; Fig. 1C, D).

In the soil-infestation study, 14 out of a total of 24 3-month-old seedlings inoculated with *C. colhounii* shriveled and collapsed within 10 days after inoculation. None of 24 seedlings in pots infested with *C. camelliae* and *C. tenue*, nor 12 noninfested controls, were diseased. The fungus was readily recovered from diseased leaflets and seedlings throughout the experiments.

Table 2. Pathogenicity of *Cylindrocladium camelliae* (isolate 92-202), *C. colhounii* (isolates 92-211; -260), and *C. tenue* (isolate 92-246) on leaflets of *Phellodendron amurense* trees at Tsukuba, using potato-dextrose agar (PDA) and water agar (WA) cultures as inocula[y]

	Blighted		Abscised	
Inocula	PDA	WA	PDA	WA
Control	0 a[z]	0 A	0 A	0 A
C. camelliae	0 a	0 A	0 A	0 A
C. colhounii				
92-211	75 b	78 B	50 B	78 B
92-260	83 b	78 B	58 B	78 B
C. tenue	0 a	0 A	0 A	0 A

[y] Percentage of leaflets blighted or abscised 7 days after inoculation (one 4-mm-diameter PDA or WA culture disk placed/leaflet) at temperatures of 14.3 to 31.9°C. Each value is a mean of three or four replicates using a total of nine or 12 leaflets per isolate or control. Sterile PDA or WA disks served as control.

[z] Values with the same letter within a column do not differ significantly ($P = 0.01$ for lowercase letters, $P = 0.05$ for uppercase letters) according to the least significant difference test.

DISCUSSION

Cylindrocladium colhounii, isolated from seedlings of *P. amurense*, caused severe leaf blight and subsequent abscission following inoculation of leaflets, and damping-off of 3-month-old potted seedlings following soil inoculation. Subsequently, pathogenicity has been demonstrated by spraying leaflets with conidial suspensions (T. Watanabe, *unpublished*). Thus, pathogenicity was demonstrated in a number of different plant ages, and experimental sites and conditions.

Cylindrocladium colhounii was described first by Peerally (5) in 1973 from Mauritius as a minor but common pathogen of tea (*Thea sinensis* L.). On *Eucalyptus robusta* Sm. and *Callistemon citrinus* (Curtis) Skeels (= *C. lanceolatus* (Sm.) DC.) it caused leaf spots (6). In pathogenicity tests on tea and peanut (*Arachis hypogaea* L. cv. Cabri) seedlings, inoculated leaves turned black or dark brown, and finally abscised (5). In Queensland, Australia, considerable damage to leaves and fruit of *Annona squamosa* L. caused by *C. colhounii* was also reported (3).

According to Linderman (4), ethylene, produced by the interaction between *Cylindrocladium* and azalea tissue, was most likely involved in leaf abscission that occurs with the disease. In an additional study, Axelrood-McCarthy and Linderman (1) demonstrated that ethylene was produced by *C. floridanum* Sob. & Seymour and *C. scoparium* Morg. in culture. Thus, abscission of blighted leaflets by *C. colhounii* in this study may also be due to ethylene production.

In the natural habitat, seedlings were numerous early in the season but most gradually died, probably due to the brown leaf blight disease named in this study. Although *Cylindrocladium* species were not successfully isolated from mature tree roots, or aerial apical parts of mature trees, they may be present in apical parts of defoliated branches.

Sinclair et al. (7) stated that significant damage by *Cylindrocladium* to woody plants outside nurseries and greenhouses is unusual, but *C. scoparium* has been found associated with root rot and decline of peach trees in orchards, of tulip trees in a plantation, and of sweet gum in a natural forest stand.

Cylindrocladium colhounii may be involved in initiating the decline of *P. amurense* in Tokyo by causing leaf blight and subsequent abscission, thus weakening the trees.

In pathogenicity tests, *C. colhounii* was pathogenic and is believed to be involved in the decline of *P. amurense*. However, some of the other fungi isolated may also be involved; further work on the pathogenicity of these fungi is in progress.

ACKNOWLEDGMENTS

We are grateful to E. George Kuhlman, Plant Disease Forestry Sciences Laboratory, Turner B. Sutton, North Carolina State University, and anonymous reviewers for critical review of the manuscript and editorial advice.

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