Morphology, Cultural Characteristics, and Pathogenicity of *Rhizosphaera kalkhoffii* on *Picea* spp. in Northern Minnesota and Wisconsin

JENNIFER JUZWIK, Research Plant Pathologist, USDA Forest Service, North Central Forest Experiment Station, 1992 Folwell Avenue, St. Paul, MN 55108

ABSTRACT

Juzwik, J. 1993. Morphology, cultural characteristics, and pathogenicity of *Rhizosphaera kalkhoffii* on *Picea* spp. in northern Minnesota and Wisconsin. Plant Dis. 77:630-634.

Morphology, cultural characteristics, and pathogenicity of *Rhizosphaera kalkhoffii* from spruce (*Picea* spp.) showing premature needle loss in northern Minnesota and Wisconsin were investigated. Pycnidiospores from needles, conidia of the *Hormonema*-like synanamorph, and pycnidiospores produced in culture were similar for Colorado blue, Engelmann, Norway, and white spruce collections and isolates. Pycnidiospores from black spruce needles were smaller than those from Colorado blue or white spruce ($P < 0.05$). Colony diameters at 14 days were similar on four different spruce needle extract agars for isolates from Colorado blue, Engelmann, and white spruce. Growth profiles of the fungus from Colorado blue, Engelmann, and white spruce on three agar media at five temperatures, and in three liquid media at three temperatures, were also similar to each other, with optimum growth occurring at either 20 or 25 C. Needle cast symptoms and *R. kalkhoffii* pycnidia developed within 12 mo on black, Colorado blue, Norway, and white spruce after inoculation with pycnidiospores of an isolate of the fungus from either Colorado blue or white spruce.

Rhizosphaera kalkhoffii Bubák has been considered the causal agent of a needle cast disease on spruce (*Picea* spp.) (11), although pathogenicity of *R. kalkhoffii* has not previously been demonstrated by artificial inoculation (11,18). According to Nicholls et al (10), the fungus has caused serious damage to Colorado blue spruce (*P. pungens* Engelm.) in Christmas tree plantations in Wisconsin, Minnesota, Indiana, and Michigan and has also been reported in ornamental nurseries and in ornamental landscape plantings of blue spruce (12).

The fungus has been found on various spruce species in forest plantations and natural stands (2,6,18,19) but was not considered a serious defoliator in these situations. In forested areas of Japan, *R. kalkhoffii* is considered a weak pathogen of *Pinus densiflora* Siebold & Zucc., particularly those subjected to drought and SO_2 (1,15). It is also not considered the primary cause of "top-dying" of Norway spruce (*P. abies* (L.) H. Karst.) in Europe (2), a disease primarily due to water shortage in the crown (11). The fungus, however, was associated with total defoliation of large white spruce (*P. glauca* (Moench) Voss) in the University of Wisconsin Arboretum in the mid-1950s (5).

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Accepted for publication 16 February 1993.

It is not known whether *R. kalkhoffii* is the cause of, or only a secondary contributor to, the premature needle loss and branch mortality that have been occurring in forest plantations of spruce in northern Minnesota and Wisconsin since the early 1980s (17). Pycnidia of the fungus were common on needles of white spruce in 40-yr-old plantations on the Hayward Ranger District, Chequamegon National Forest, Wisconsin, in 1983 (memorandum on file, USDA Forest Service Northeastern Area State and Private Forestry, St. Paul, MN). Premature needle loss and lower branch mortality were observed in these plantations. Pycnidia of the fungus were observed on needles of white, black (*P. mariana* (Mill.) B.S.P.), and Norway spruce in 15- to 60-yr-old plantations and natural stands on the Glidden, Hayward, and Park Falls Ranger districts of the Chequamegon National Forest and on 20- to 60-yr-old white, Colorado blue, and Engelmann (*P. engelmannii* Parry ex Engelm.) spruce at the Cloquet Forestry Center, University of Minnesota, Cloquet. Similar observations have been made by me and others in ornamental plantings and windbreaks of white and Colorado blue spruce in the Cloquet and Hayward areas. Studies have been initiated to elucidate the role of *R. kalkhoffii* in the development of premature needle loss and branch mortality on spruce in forested areas. The objective of this particular study was to compare the morphology, cultural characteristics, and pathogenicity of *R. kalkhoffii* from spruce species in forest plantations and windbreaks affected by premature needle loss and lower branch mortality in northern areas of Minnesota and Wisconsin.

MATERIALS AND METHODS

Comparison of pycnidia on needles. Branches with needles colonized by *R. kalkhoffii* were collected from the lower 2.5 m of the crown of several spruce species in northern Minnesota and Wisconsin (Fig. 1). Each fungus collection was marked with a collection location, stand location, and tree number, i.e., one collection consisted of colonized needles obtained from one tree. Needles with *R. kalkhoffii* were removed from each collection, placed in screw-top vials with formalin-propionic acid (7), and aspirated under vacuum. The ages of needles removed from each collection were: Colorado blue spruce, 3 yr; white spruce, 4 yr; black spruce, 3 yr; and Norway spruce, 4 yr. Thin cross sections of needles were made with a razor blade and mounted in lactophenol for microscopic observation of pycnidia. All observations were made with bright-field microscopy at 200×. Pycnidiospores were obtained from swollen, globose, brown pycnidia with spores exuding on black, Colorado blue, and white spruce needles of branch pieces placed in moist chambers for 24–48 hr. Spores were mounted in glycerin, and dimensions (length and width) of 25 spores from each collection were measured to the nearest 0.1 μm at 1,000× under oil.

Isolates. Pycnidiospores obtained from swollen, globose, brown pycnidia on needles of Colorado blue, Engelmann, Norway, and white spruce kept in moist chambers for 24–48 hr were transferred to acidified potato-dextrose agar (four drops of 25% lactic acid per 100 ml of medium) and incubated at 25 C in the dark. Polysporic isolates were maintained at 5 C on potato-dextrose agar (PDA) slants. Fourteen isolates obtained from nine collection locations were used in experiments as noted.

Cultural comparisons. Dishes of PDA and Leonian agar (LOA) (16) were inoculated with a 6-mm-diameter plug of one of 12–14 isolates of *R. kalkhoffii*. Following incubation at 25 C in the dark for 2 wk on PDA, conidia of the *Hormonema*-like synanamorph were removed and mounted in glycerin on microscope slides. Dimensions of 100 conidia per isolate (14 isolates total) were measured at 1,000× under oil. Pycnidia were produced by isolates on LOA after 3–4 wk of incubation at 25 C in the dark. Dimensions of 100 pycnidiospores per isolate (12 isolates total) were similarly

measured at 1,000×.

The cultural appearances of nine *R. kalkhoffii* isolates (four from Colorado blue, four from white, and one from Engelmann spruce) grown on PDA, LOA, and Colorado blue spruce needle extract agar (NEA) for 2 wk at 25 C in the dark were compared. Four dishes were used per isolate per medium. NEA was prepared by grinding 150 g of ≥2-yr-old needles in a Waring blender for 2–3 min, simmering them in 500 ml of distilled water for 1 hr, then straining them through Miracloth (Calbiochem Corp., La Jolla, CA). To obtain 1 L of medium, 10 g of dextrose, 20 g of agar, and 500 ml of distilled water were added to 500 ml of needle extract, and the solution was autoclaved at 1 kg/cm^2 (15 psi) for 20 min at 121 C. Four dishes per isolate per medium were incubated for 3 wk at 25 C in the dark for comparison of sporulation. Each isolate on each medium was observed microscopically for sporulation, using slide mounts in glycerin and water.

Effect of temperature on growth. Petri dishes containing 20 ml of PDA, LOA, or NEA were inoculated with a 6-mm-diameter plug of one of nine isolates (four from Colorado blue, four from white, and one from Engelmann spruce) from actively growing cultures on PDA. Four dishes of each isolate were enclosed in plastic bags, inverted, and incubated in the dark at 10, 15, 20, 25, or 30 C. After 14 days, two diameter measurements, 90° apart, were made of each colony. The experiment was performed two times.

Erlenmeyer flasks (250 ml) containing 50 ml of liquid media of potato extract (Difco), Leonian medium (LOA without agar), or Colorado blue spruce needle extract were inoculated with four replications of the same isolates and incubated in the dark at 20, 25, or 30 C to compare colony biomass growth in each. After 14 days, all replicates were harvested by vacuum filtration, oven-dried (40 C) for 24 hr, and weighed. The experiment was performed three times.

Effect of spruce needle extract on growth and pycnidial production. Needle extract agars using ≥2-yr-old needles from black, Colorado blue, Norway, and white spruce were prepared as described previously. Each petri dish, containing 20 ml of one of the spruce agars, was inoculated with a 6-mm-diameter plug of one of the nine isolates, actively growing on PDA, used in the temperature and culture appearance studies. Four culture dishes of each isolate were enclosed in plastic bags, inverted, and incubated in the dark at 25 C. Two diameter measurements, 90° apart, were made after 14 days for each colony. The experiment was performed two times.

Pathogenicity. Two different spruce stock types were used for the pathogenicity experiments. In the first type, bare root 2+0 (2 yr in seedbed and 0 yr in transplant bed) black, Colorado blue, Norway, and white spruce seedlings were lifted in October 1989 at three state forest nurseries in Minnesota and Wisconsin, transplanted into 15-cm-diameter pots with peat:vermiculite (2:1), and overwintered outdoors in a cold frame prior to inoculation in early June 1990. The second stock type, used in the second inoculation experiment in August 1990, was spruce seedlings grown in five cavity root trainers (Spencer Lemaire Co., Edmonton, Alta.) with peat:vermiculite for 3 mo, transplanted into 15-cm-diameter pots with peat:vermiculite, and grown for 6 mo in the greenhouse.

One Colorado blue spruce isolate from Hayward and one white spruce isolate from Cloquet were grown in still culture of liquid Leonian medium for 3 wk in the dark at 25 C for production of pycnidia. The first isolate was from a windbreak tree growing within 2 km of affected white spruce in forest plantations. The other isolate was from an affected white spruce in a 30-yr-old plantation. Vigorous stirring of fungal growth with a sterile glass rod for 30 sec was followed by magnetic stirring for 2 min on a stir plate to free pycnidiospores. The liquid cultures were then passed through a Miracloth lining in a Büchner funnel by means of a vacuum. The filtrate was placed in a centrifuge (1,700 rpm) for 4 min. The supernatant was decanted, and the pelleted pycnidiospores were suspended in sterile sodium acetate buffer (pH 4.7–4.8). The concentration of spores was estimated with a hemacytometer and adjusted, when necessary, by dilution with sterile buffer. Viability of inoculum was tested on dishes of PDA. Sterile buffer was used for the control treatment.

A Power Pak (Sigma, St. Louis, MO) aerosol spray was used to deliver a fine mist of the spore suspension (4.1×10^6 spores per milliliter in June, 2.5×10^7 in August) to each potted seedling placed in small inoculation chambers designated for one isolate or control suspension only. The inoculum was sprayed to the drip point, vertically down the foliage on each side of each seedling. The seedlings were immediately placed in a chamber for 48 hr, with frequent mistings. Two inoculation experiments were conducted, one in early June and one in mid-August 1990. Twenty-five trees of each spruce species were inoculated with each isolate on both inoculation dates. All inoculated trees were moved to a cold frame outdoors and held for 12 mo, at which time they were assessed.

Each potted seedling was examined for general condition (live vs. dead), needle symptoms, and pycnidia development in stomata. Reisolation of *R. kalkhoffii* was attempted from symptomatic attached needles showing globose pycnidia in the stomata. Five such needles were taken from each symptomatic seedling for isolation. The isolation technique used was similar to that used to obtain original cultures.

Data analyses. Analyses of variance (ANOVA) were performed on spore dimension data sets, and treatment comparisons were made with standard t tests when the F statistic was significant (Fischer's LSD) (13). Spore dimensions (length and width) were first analyzed within each host species. If no differences ($P > 0.10$) were found in length or width among collection locations when the collections were nested within location, then means were pooled according to host source of collection or isolate and

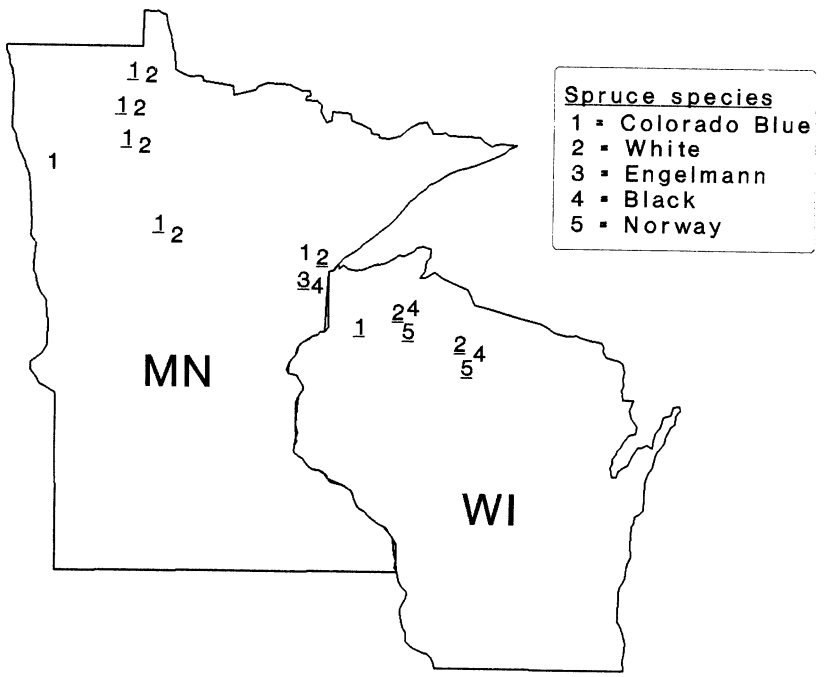

Fig. 1. Locations of *Rhizosphaera kalkhoffii* collections in Minnesota and Wisconsin; isolates were obtained from collections indicated by underlined numbers.

ANOVA for host effect was performed. Response for an isolate in the experiments involving growth at five temperatures was defined as the mean diameter growth of eight dishes used for the isolate on each medium in the two experiments. Means were pooled according to host source of isolate for each of the three media. Response of an isolate's growth in liquid media at three temperatures was defined as the dry weight of mycelial growth from 12 flasks used for the isolate in the three experiments. ANOVA was performed on both growth data sets, and treatment comparisons were made with standard t tests when the F statistic was significant.

The growth data for isolates on different spruce needle extract agar were also analyzed by ANOVA and standard t tests. Response for an isolate was defined as the mean diameter growth on eight dishes of the two experiments. Data were analyzed first for isolates grouped according to host within each extract medium, then for pooled means for all media groups according to host.

RESULTS AND DISCUSSION

Comparison of pycnidia on needles. The pycnidia observed in needle cross sections of black, Colorado blue, Norway, and white spruce were similar to published descriptions of *R. kalkhoffii* (6,14,18,19). No morphological differences were observed in pycnidia among the four spruce species. Width of the globose pycnidia on each of the four spruce species ranged from 48 to 62 μm. Pycnidiospores from all spruce species ranged from 4.4 to 9.1 μm in length and from 2.4 to 5.0 μm in width (Table 1). Pycnidiospore dimensions of isolates from each spruce species were similar to those in published descriptions of *R. kalkhoffii* (3,14). No differences were found in length or width among collection districts ($P > 0.10$). Significant differences were found between white or Colorado blue spruce collections and black spruce collections ($P < 0.05$), but not between white and Colorado blue spruce collections ($P = 0.20$) (Table 1).

Appearance and sporulation in culture. Dimensions (length and width) of conidia produced on PDA by the *Hormonema*-like synanamorph of 13 isolates from four spruce species are given in Table 2, and dimensions of pycnidiospores produced on LOA by 12 isolates from four spruce species are given in Table 3. Mean dimensions of conidia ranged from 7.5 to 8.6 μm in length and from 4.5 to 5.3 μm in width (Table 2). The lengths were similar to, but the widths were greater than, those previously reported for the *Hormonema*-like synanamorph (4). Mean dimensions of pycnidiospores produced on LOA ranged from 6.4 to 7.9 μm in length and from 3.8 to 5.2 μm in width. These dimensions were similar to previously published values (4,12). No differences were found in length or width among isolate collection districts ($P > 0.10$) for both data sets. No differences were detected ($P > 0.05$) when pooled means of spore measurements were analyzed across hosts (with more than one isolate per host).

Table 1. Dimensions of pycnidiospores of *Rhizosphaera kalkhoffii* from needles of three *Picea* species collected in Minnesota and Wisconsin[y]

Spruce host	No. of collections	Length (μm)		Width (μm)	
		Mean	Range of means	Mean	Range of means
Colorado blue	13	7.5 a[z]	5.5–8.4	4.2 a	2.9–4.8
White	11	6.9 a	4.9–9.1	3.9 a	2.5–5.0
Black	5	5.6 b	4.4–6.5	2.9 b	2.4–3.5

[y] Based on means for 25 spores per collection.
[z] Means followed by the same letters are not significantly different ($P \geq 0.05$).

Table 2. Dimensions of condidia produced on potato-dextrose agar by *Hormonema*-like synanamorph of isolates of *Rhizosphaera kalkhoffii* from four *Picea* species[z]

Spruce host	No. of collections	Length (μm)		Width (μm)	
		Mean	Range of means	Mean	Range of means
Colorado blue	6	8.6	8.1–9.1	5.3	5.0–5.9
White	4	8.2	8.0–8.3	5.0	4.8–5.2
Norway	2	7.5	4.9–13.0	4.5	3.2–6.9
Engelmann	1	8.5	6.9–12.1	5.3	4.0–6.9

[z] Based on means for 100 spores per isolate. No statistically significant differences ($P > 0.05$) were found among hosts with more than one isolate per host.

Table 3. Dimensions of pycnidiospores produced on Leonian agar by isolates of *Rhizosphaera kalkhoffii* from four *Picea* species[z]

Spruce host	No. of collections	Length (μm)		Width (μm)	
		Mean	Range of means	Mean	Range of means
Colorado blue	6	7.9	7.5–8.5	5.2	4.8–5.9
White	4	7.3	6.6–7.8	4.6	4.0–5.0
Norway	1	6.4	4.9–8.9	3.8	3.2–5.3
Engelmann	1	7.9	6.1–10.1	4.9	4.0–5.7

[z] Based on means for 100 spores per isolate. No statistically significant differences ($P > 0.05$) were found among hosts with more than one isolate per host.

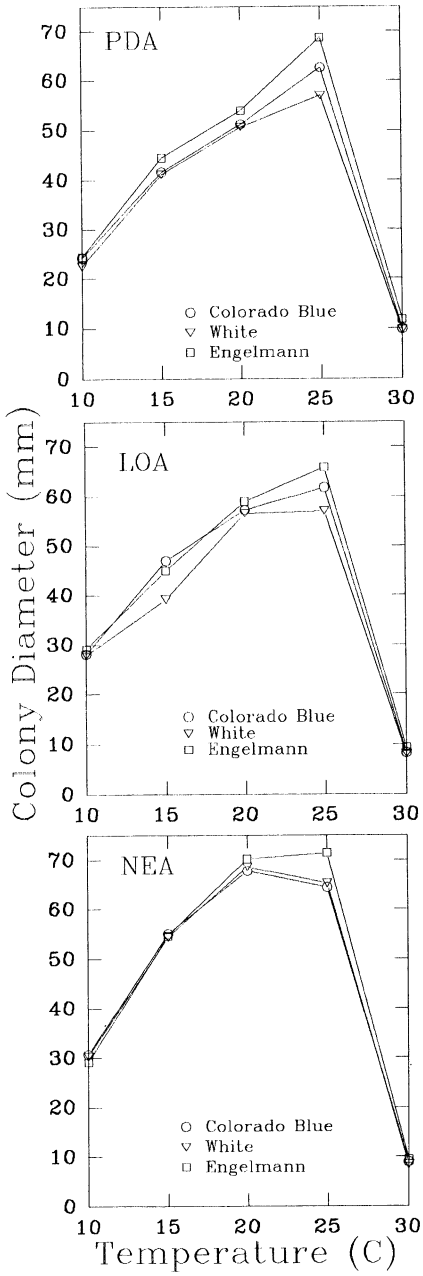

Fig. 2. Mean diameter of mycelial growth of *Rhizosphaera kalkhoffii* isolates from Colorado blue (four isolates), white (four isolates), and Engelmann (one isolate) spruce on potato-dextrose agar (PDA), Leonian agar (LOA), and Colorado blue spruce needle extract agar (NEA) after 14 days at five temperatures. Values represent average of eight colonies resulting from replicated experiments of four plates each, including a 6-mm-diameter inoculum plug.

Appearance of colony growth of isolates of *R. kalkhoffii* from Colorado blue, Engelmann, and white spruce was similar on each of three growing media and similar to published descriptions (3,18).

Sporulation of nine *R. kalkhoffii* isolates (four from white, four from Colorado blue, and one from Engelman spruce) was observed 3 wk after dishes of agar were inoculated. Conidia of the *Hormonema*-like synanamorph were produced in a manner similar to that of *R. oudemansii* Maubl. (9) and were common on PDA and LOA for all isolates, whereas conidia were infrequent on NEA for all isolates. When present, conidia were produced on mono- and polyphialides from intercalary or terminal hyphal cells. Rudimentary (immature) pycnidia were also common on PDA and LOA. Well-formed (mature) pycnidia were observed on the margins of all isolates on LOA and NEA. In the experiment involving different spruce agars, pycnidial production was most rapid on NEA from white spruce and slowest on NEA from black spruce.

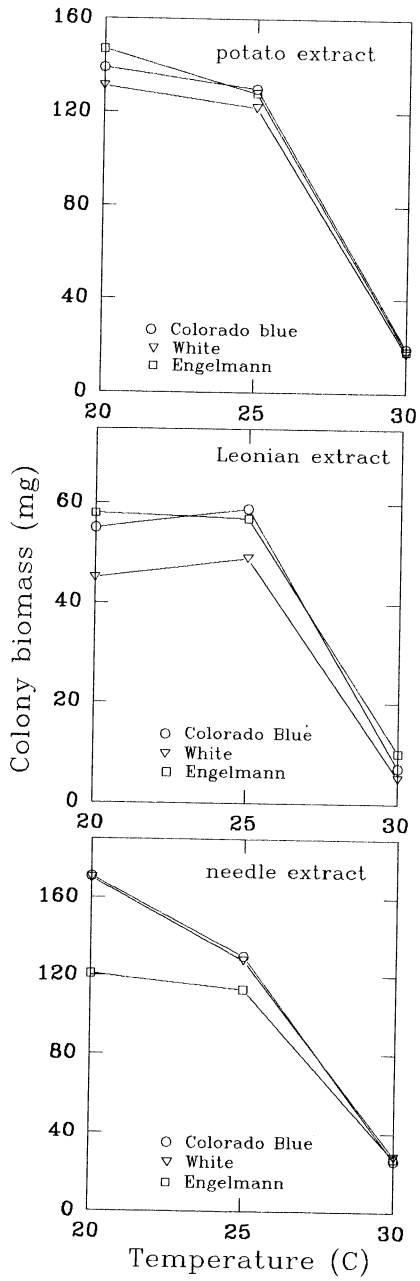

Fig. 3. Mean production of biomass of *Rhizosphaera kalkhoffii* isolates from Colorado blue (four isolates), white (four isolates), and Engelmann (one isolate) spruce in potato extract, Leonian extract, and Colorado blue spruce needle extract liquid media after 14 days at three temperatures. Values represent average of eight colonies resulting from replicated experiments of four flasks each, including a 6-mm-diameter inoculum plug.

Growth in culture. *Colony diameter on three solid media at five temperatures.* No consistent or significant differences in diameter of isolates from each host species (Colorado blue, Engelmann, and white spruce) were found at 10, 15, 20, 25, and 30 C on PDA (Fig. 2). The diameter of each group of isolates was greatest at 25 C, and significant differences occurred when means of all isolates were combined ($P < 0.05$). The diameter of Colorado blue and Engelmann spruce isolates was greatest at 25 C on LOA; the white spruce isolates grew similarly at 20 and 25 C, but when means for all isolates were combined, colony diameter was significantly greater at 25 C (Fig. 2). The colony diameter of the isolates on NEA was similar except for a slight difference for one Engelmann spruce isolate at 25 C (Fig. 2). Mean values and ranking of colony diameter of isolates from each host were consistent between experiments for the five temperatures with each of the three media. The optimum temperature for mycelial growth on all three media (25 C) was similar to previously published data (8,15), and growth was good from 15 to 25 C on all three media. The sharp decline in growth between 25 and 30 C on all media also had been observed previously (8).

Biomass production in three liquid media at three temperatures. R. kalkhoffii isolates produced more than twice the biomass at 20 and 25 C in potato extract and Colorado blue spruce needle extract than in Leonian liquid medium (Fig. 3). No significant differences in biomass production were found among isolates from each host in all media and at all temperatures. Biomass production by *R. kalkhoffii* from *Pinus densiflora* Siebold & Zucc. (Japanese red pine) on potato decoction solution at 25 C for 8 days as reported by Tanaka and Chiba (15) was greater than that found in this study.

Diameter growth on four needle extract agars. All isolates grew well on extract agar prepared from black, Colorado blue, Norway, and white spruce needles. There were no significant differences in colony diameters among the isolates from each host within the different extract media or across the different media. Average combined colony diameters for the nine isolates on each extract medium were: black spruce agar, 62.6 cm; Colorado blue spruce agar, 61.1 cm; Norway spruce agar, 63.2 cm; and white spruce agar, 68.7 cm. In contrast, Kumi and Lang (8) reported that growth of seven isolates of *R. kalkhoffii* from seven spruce species was greatest on Colorado blue spruce needle extract agar and slowest on Norway spruce needle extract agar.

Pathogenicity. Both the Colorado blue spruce and white spruce isolates of *R. kalkhoffii* used in the pathogenicity trials

Table 4. Pathogenicity of *Rhizosphaera kalkhoffii* on four *Picea* species inoculated in June and August 1990

	Inoculated trees			No. of trees at assessment[y]		
Isolate source	Month	Spruce species	No.	Live	Symptoms + pycnidia	Fungus recovered
Colorado blue spruce	June	Colorado blue	25	24	11	6
	August	Colorado blue	25	25	17	1
	June	White	25	24	18	16
	August	White	25	25	17	10
	June	Norway	25	24	3	3
	August	Norway	25	25	8	2
	August	Black	25	25	7	4
White spruce	June	Colorado blue	25	25	5	3
	August	Colorado blue	25	25	3	1
	June	White	25	25	14	5
	August	White	25	25	4	4
	June	Norway	25	25	5	4
	August	Norway	25	25	2	1
	August	Black	25	25	6	5
Control[z]	June	Colorado blue	25	25	0	...
	August	Colorado blue	25	25	0	...
	June	White	25	24	0	...
	August	White	25	25	0	...
	June	Norway	25	25	0	...
	August	Norway	25	25	0	...
	August	Black	25	25	0	...

[y] Trees were assessed 12 mo after inoculation.
[z] Inoculated with sterile buffer.

resulted in symptom development and pycnidia formation on varying numbers of four spruce species inoculated (Table 4). From 22 to 70% of the spruce inoculated with the Colorado blue spruce isolate yielded symptomatic foliage and pycnidia, and *R. kalkhoffii* was recovered from 10–52% of the trees. Similarly, 14–56% of the spruce inoculated with the white spruce isolate developed characteristic needle symptoms and pycnidia; *R. kalkhoffii* was successfully reisolated from 8–20% of the trees. Symptomatic foliage accompanied by pycnidia formation was not observed on the control trees. Foliar symptoms observed on black, Colorado blue, Norway, and white spruce 12 mo after inoculation included yellow-brown and brown 2- to 4-yr-old needles attached to the main stem or the oldest needles attached to lateral branches and cast brown needles lodged in lateral branches. Fresh pycnidia were observed on brown 3- and 4-yr-old needles attached to lateral branches and the main stem of all four species of bareroot origin and on the older brown needles attached to the lateral branches and main stem of all four species of greenhouse origin. In general, no differences were apparent in symptom development, pycnidial development, and reisolation of the fungus between the two inoculation experiments. Additional isolates should be tested to determine whether virulence differences exist between isolates from Colorado blue spruce and isolates from white spruce.

Koch's postulates have now been fulfilled for *R. kalkhoffii* on *Picea* species. Both a Colorado blue spruce isolate and a white spruce isolate were shown to be pathogenic on four different species of spruce. These results, however, are contrary to those reported for Norway spruce in Europe. Following inoculation experiments with *R. kalkhoffii* on Norway spruce, Dotzler (4) concluded that the fungus is a saprophyte living as an epiphyte on the needle surfaces. Diamandis (2) also reported that *R. kalkhoffii* did not have a primary involvement in the development of foliar symptoms in "top-dying" of Norway spruce by attacking needles of the last 2 yr's growth. In contrast, Tanaka and Chiba (15) concluded that *R. kalkhoffii* is a weak pathogen on current season's needles of *Pinus densiflora* growing under normal conditions in Japan.

Overall, results of mophological and cultural comparisons of *R. kalkhoffii* collections and isolates from Colorado blue, Engelmann, and white spruce in northern Minnesota and Wisconsin show a high level of similarity. Further investigation of black and Norway spruce isolates, however, would be of interest to explore the possible existence of a different strain of the fungus. *R. kalkhoffii* on Norway spruce in Britain is considered to be a different strain of the fungus observed on other spruce species, because of the low optimum temperature for mycelial growth and saprophytic growth of the fungus on Norway spruce (2,3).

The results of these morphological, cultural, and pathogenicity studies suggest that the fungus on white spruce is no different from that on Colorado blue spruce and, presumably, could spread from one host species to the other and infect needles from either species. Whether *R. kalkhoffii* from black or Norway spruce could infect Colorado blue and white spruce needles, and vice versa, is unknown and requires further study.

ACKNOWLEDGMENTS

I thank D. Rugg, mathematical statistician, for advice and guidance concerning statistical analyses; J. Walla, J. O'Brien, D. W. French, C. Ash, and four anonymous reviewers for critical review of the manuscript; and K. Stolhammer and M. Warn for technical assistance.

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