Spore Production and Artificial Inoculation Techniques for *Gremmeniella abietina*

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ABSTRACT

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Viable conidia of *Brunchorstia pinea* (teleomorph = *Gremmeniella abietina*) were produced in flasks containing 20% V-8 broth incubated at 16–18 C under a 16-hr photoperiod with a light intensity of approximately 100 μE m^{-2} sec^{-1}. More than 6×10^7 spores per flask were usually produced in 21 days under these conditions. Red pine seedlings were inoculated with a suspension of conidia that had been produced in this way, held at 18 C at high relative humidity for 3 days, and transferred to 4 C. Disease symptoms of Scleroderris shoot blight, as indicated by loose fascicles, appeared after as few as 56 days in the dark at 4 C.

Additional keywords: Ascocalyx abietina, Pinus resinosa, Scleroderris lagerbergii.

Scleroderris shoot blight, caused by *Gremmeniella abietina* (Lagerb.) Morelet (= *Ascocalyx abietina* (Naumov) Schlaepfer-Bernhard, anamorph = *Brunchorstia pinea* (Karst.) Hohn.), occurs on members of the Pinaceae in the northern Lake States, northeastern regions of the United States, eastern Canada, Europe, and Japan. Dorworth and Krywienczyk (6) defined at least three serotypes of *G. abietina*—North American (NA), European (EU), and Asian—based on immunologic comparisons. This grouping is supported somewhat by differences in gross morphology of cultures (5).

Both the NA and the EU serotypes occur in North America. Where the NA serotype is predominant, the disease affects primarily *Pinus* spp. Small plantation trees and lower branches of larger trees are affected, to about 2 m above ground level (4). Trees smaller than about 2 m can be killed by the disease; taller trees will typically survive the infection (3,7). Disease associated with the NA serotype occurs north of about 45° N latitude. The EU serotype, first discovered in North America in 1975 (7), affects many members of the Pinaceae. Entire crowns of large trees may be affected, and such trees may be killed. Disease caused by this serotype occurs as far south as 43° N latitude.

The differences in symptom expression associated with the two serotypes may be due to differences in pathogenicity and host range, to environmental differences where the two serotypes predominate, or to some combination of the two. The basis for differences in symptom expression has not been clarified, for two principal reasons. First, there has been no reliable laboratory method to conduct inoculation studies. Thus, the necessary comparisons in pathogenicity and host range have not been made. Second, key aspects of the interaction of the physical environment with disease development have not been understood.

Obstacles to development of reasonably rapid and reliable methods for study of this disease in the laboratory have included the year-long period from initial penetration to symptom development in nature (9) and lack of reliability of and the long incubation times required for existing methods of producing spores in the laboratory (1,8,10). For field inoculations, the variable and often low viability and the short shelf-life of laboratory-grown spores were additional obstacles.

The objectives of the study described in this manuscript were: 1) to develop a reliable, rapid method to produce viable conidia in quantities sufficient to conduct statistically meaningful inoculation studies, and 2) to develop a reliable technique for reproducing disease symptoms under artificial conditions in a time span shorter than the full year necessary for the natural disease cycle.

MATERIALS AND METHODS

The isolate of *G. abietina* used in this study, ASL 4-b-3, was derived from a single ascospore collected in 1982 from an infected red pine tree in Bayfield County, WI. Serological testing confirmed the isolate to be the NA serotype (C. N. Davis, *personal communication*). Stock cultures of the fungus were maintained in the dark on V-8 agar slants at 4 or 8 C. Active cultures were maintained on V-8 agar plates at 16 or 18 C, with or without light, and transferred at monthly intervals. Flasks for spore production were inoculated with a 3-mm-diameter plug cut from the outer edge of an actively growing colony.

Incubators used to determine light and temperature requirements for sporulation were Sherer Controlled Environment Chambers (Marshall, MN) Model CEL 25-7HL and Model 4-4, an Environmental Growth Chamber (Chagrin Falls, OH) Model M-3, or a General Electric Model 8A 11TC refrigerator. All incubators maintained temperatures within ±2 C. Light in the incubators was provided by cool-white fluorescent bulbs. Relative humidity was not controlled. Light intensity was measured with a Li-cor (Lincoln, NE) LI-188 integrating quantum meter. Temperatures were monitored periodically with thermistors attached to a CR-21 data logger (Campbell Scientific, Logan, UT) or Hampshire Controls Corp. (Exeter, NH) Sentry-Q digital readout, or with copper-constantan thermocouples attached to a chart recorder.

Conidia produced in flasks were suspended by using a rubber policeman to dislodge spore tendrils from the colony, adding additional sterile distilled demineralized water if necessary. The entire spore suspension was pipetted into a sterile glass vial or test tube and mixed with a Vortex mixer. The number of conidia per flask was estimated from the average of two hemacytometer counts.

To estimate spore viability, 0.25 ml of a conidial suspension was spread onto a 9-cm-diameter water-agar plate and plates were incubated at 20 C without light for 3 days. Spores were then stained with lactophenol-cotton blue and examined microscopically. A spore was considered germinated if it had produced a germ tube equal to or greater than the width of the spore.

Inoculation and symptom assessment. Spore suspensions used as inoculum were prepared from V-8 broth flask cultures as described below and contained approximately 1.0×10^6 conidia/ml. Conidia were not more than 2 wk old when used, and their viability was 90% or greater. Inoculum was applied to the current-year growth of the red pine seedlings after candles had begun to flush, but before needle elongation, by brushing in an upward motion with a No. 1 artist's paint brush (1). Between 1×10^5 and 2×10^5 spores per seedling were applied to the stems and bract surfaces of each seedling in this manner. Seedlings were assessed for symptoms by means of the "pull test" technique (9). All loose fascicles were plated on V-8 agar supplemented with either 100 mg/L of streptomycin or 1 drop of 80% lactic acid per plate. Basal sections of needles from each fasicle were placed on two plates, one of which was incubated at 4 C and the other at 18 C. Suspect colonies of *G. abietina* were transferred to V-8 agar slants and incubated at 18 C under a 16-hr photoperiod at 100 μE m^{-2} sec^{-1} light intensity to favor sporulation. Only trees with loose fasicles from which spore-producing colonies of *G. abietina* were recovered were considered infected.

Effect of temperature, photoperiod, and light intensity on spore production. To determine an optimum temperature for spore production, 14 V-8 broth flasks were inoculated and placed under an 8-hr photoperiod with a light intensity of about 100 μE m^{-2} sec^{-1} at either 12, 14, 16, or 18 C. Total spore production was determined after 21 days. To investigate the effect of photoperiod and light intensity on spore production, 14 V-8 broth flasks were inoculated and placed at 18 C under a 2-, 8-, 16-, or 24-hr photoperiod at a light intensity of about 100 μE m^{-2} sec^{-1}. Half of the flasks in each treatment were covered with two layers of cheesecloth; this reduced the intensity of light reaching these flasks to about 40 μE m^{-2} sec^{-1}. Total spore production was determined after 22 days.

Effect of medium and time and concentration in storage on spore viability. The viability of spores produced by the methods of Blenis et al (2) and Hudler et al (8) was compared to that of spores produced on V-8 agar plates inoculated with a conidial suspension or in V-8 broth flasks as described above. All media were incubated at 18 C under a 16-hr photoperiod with a light intensity of approximately 100 μE m^{-2} sec^{-1}. To assess the effect of time in suspension on spore viability, 10 replicate suspensions of V-8 broth-grown conidia (4.68×10^5 conidia/ml) were prepared in dilute V-8 broth; 10 additional suspensions were prepared in sterile distilled demineralized water. Germination was assessed after storage at 8 C for 0, 4, 8, 24, 48, and 96 hr, at 4-day intervals through the 28th day, and again at 49 days. To test the effect of concentration on viability of V-8 broth-grown spores over time, aliquots of a 1.15×10^7 conidia/ml suspension were diluted after 4–10 days at 8 C to 1.15×10^6 conidia/ml; all suspensions were then stored for an additional 1–12 days, for a maximum total time in suspension of 16 days. Viability of spores in both the concentrated and dilute suspensions was tested at 24-hr intervals. The concentrated suspension was diluted to 1.15×10^6 conidia/ml immediately before plating.

Artificial inoculation-ambient penetration-controlled overwintering experiment. Six 8-mo-old containerized seedlings were planted in each of eight 23.5- × 19.0- × 19.0-cm wooden flats and buried to ground level at a site near Butternut, WI, an area where the disease is known to occur naturally. The seedlings were inoculated with a spore suspension in the late afternoon of 8 June 1984 and misted with water immediately thereafter. The seedlings were left at the Butternut site until 10 December to ensure seedling hardiness. One flat was then transferred to and reburied at a site near Madison, and one was left at the site of inoculation. One of the remaining flats was transferred to controlled temperature rooms or walk-in cold storage rooms at each of the six conditions listed in Table 1. After 105 days, those seedlings at −30 C and +4 C were transferred to 16 C, 8-hr photoperiod, to induce bud break and allow any further disease development. Seedlings in the field were assessed for symptom development after 121 (Butternut) or 147 (Madison) days. Seedlings in all growth rooms were assessed daily after 105 days.

Artificial inoculation-controlled penetration-controlled overwintering experiment. On 22 May 1985 the new growth of 12 seedlings held at 26 C/21 C during the previous experiment was inoculated in the laboratory. After inoculation, seedlings were placed in the dark in a dew chamber at 18 C and 100% relative humidity for 3 days to allow for initial penetration, then transferred to 4 C in the dark. All seedlings were misted and covered with plastic bags to maintain a high relative humidity during the incubation period. One flat of six seedlings was transferred to 16 C, 8-hr photoperiod after 28 days, the second remained at 4 C. Symptom development was assessed after 56 days.

TABLE 1. Infection on seedlings overwintered under artificial conditions or in the field[a]

Treatment	Time (days)	Infection (%)
Artificial conditions		
−30 C[b]	105	0
−20 C, +4 C[b]	45, 60	100
16 C[c]	105+	0
20 C[c]	105+	0
24 C[c]	105+	0
26 C/21 C[d]	105+	0
Field conditions		
Madison	121	100
Butternut	147	100

[a]Seedlings were inoculated in June 1984 at the Butternut site where they remained until transfer to the various treatments on 10 December.
[b]Dark.
[c]8-hr photoperiod.
[d]12-hr photoperiod.

RESULTS AND DISCUSSION

Effect of temperature, photoperiod, and light intensity on spore production. Preliminary experiments revealed that conidia could be produced within 21 days in large numbers in 250-ml Ehrlenmeyer flasks containing 25 ml of 20% V-8 broth. Spore production in those with V-8 broth seemed to be quite dependent on conditions of incubation; therefore, the effects of temperature, photoperiod, and light intensity on fungal growth and sporulation were investigated.

Flasks incubated at 18 C produced a significantly greater number of spores ($P = 0.01$) than did those at 12 or 14 C. After 22 days, a significantly larger number of spores ($P = 0.05$) was produced under the 16- and 24-hr photoperiods (9.17×10^7 and 7.72×10^7, respectively) than under the 2- or 8-hr photoperiods (4.66×10^7 and 5.18×10^7, respectively). When all treatments were analyzed separately, the production of spores at a 16-hr photoperiod and a light intensity of about 100 μE m^{-2} sec^{-1} was significantly greater ($P = 0.05$) than in any other treatment (Table 2). More spores were produced at the higher light intensity, but this difference was not significant when data from all photoperiods were combined (as determined by Duncan's multiple range test).

When light intensities were maintained at approximately 100 μE $m^{-2}sec^{-1}$, V-8 broth flasks incubated at 16–18 C with a 16-hr photoperiod produced an average of $6.21 \pm 3.34 \times 10^7$ conidia per flask ($n = 68$) within 21 days. Spore production was consistent and reliable in over 500 flasks inoculated over the course of 4 yr. The technique was successful with an isolate of the EU serotype (SF-4, collected from *Larix sibirica* Ledeb. in Finland [6]) as well as with NA isolate ASL 4-b-3.

Although the results of our light intensity experiment (Table 2) indicate only a slight effect of the difference between light intensities of 40 and 100 μE $m^{-2}sec^{-1}$ on sporulation, in repeated trials, an observable decrease in spore production occurred as fluorescent lights aged and light intensity decreased from 100 to about 80 μE $m^{-2}sec^{-1}$. This suggests that some other factor, presumably the change in light quality as the lights aged, in addition to the decrease in light intensity, is critical for spore production. This possibility is additionally supported by the observations that decreased spore production was observed when flasks were incubated under incandescent as well as fluorescent bulbs, or in an incubator containing a light barrier that cut out infrared radiation. In another experiment, sporulation occurred only after 6 wk when the light intensity was decreased to 40 μE $m^{-2}sec^{-1}$ by decreasing the number of fluorescent bulbs. Thus, it is clear that adequate light is necessary, but that we do not understand fully the interaction between light quality, light intensity, and sporulation.

Effect of medium and time and concentration in storage on spore viability. To determine if the spores produced in our standard conditions could survive storage and transport to the field, the effect of conditions of production, dilution, and storage on viability of conidia was examined. After 21 days, no spores had been produced on Hudler's medium; thus, comparison of viability of spores produced under different growth conditions could not be done until cultures were 30 days old. Spores produced in V-8 broth flasks and on V-8 agar plates had higher and less variable germination than did those produced on media of either Blenis et al (2) or Hudler et al (8) (Table 3).

Germination of spores held in suspension (4.68×10^5 conidia/ml, in either water or V-8 broth) at 8 C was high ($98.1 \pm 7.1\%$, $n = 298$) throughout the 49-day experimental period. No measurable decline in germinability with time was detected nor was there a difference between viability of spores suspended in water and those suspended in V-8 broth. Microconidia were observed after 8 days. Short, abnormal germ tubes were produced after 12 days in suspension. Germination of conidia initially suspended at 1.15×10^7 conidia/ml and diluted to 1.15×10^6 conidia/ml at different times was greater than 95% after 16 days for all treatments. Germ tubes in all cases were long and normal appearing, and no microconidia were observed.

Hudler et al (8) concluded from their spore viability studies that spore suspensions for field inoculations should not be prepared in advance and carried to the field. In contrast, the spores produced by our technique, kept cool during transit, provided healthy inoculum for extended inoculation trips. We may have improved maintenance of spore viability during transportation to the field by preparing a concentrated spore suspension in the laboratory and diluting it with sterile water to the desired concentration at the inoculation site.

Artificial inoculation-ambient penetration-controlled overwintering experiment. At the Butternut and Madison sites, all seedlings were covered with snow throughout the winter of 1984-1985, and all developed symptoms. Of those placed under controlled conditions, all seedlings held at −20 C for 45 days followed by +4 C for 60 days developed symptoms. No symptoms were observed on seedlings held at any other controlled temperature (Table 1).

Artificial inoculation-controlled penetration-controlled overwintering experiment. All seedlings inoculated under laboratory conditions in May 1985 were apparently healthy after 28 days at 4 C. After 56 days, all seedlings exposed to 4 C for 28 days followed by 16 C for 28 days were free of symptoms. Of the seedlings exposed to 4 C for 56 days, loose fascicles were found on the current-year growth of all six seedlings and on 1-yr-old needles of one seedling. Longitudinal sectioning revealed that the infections on all seedlings had been initiated in the current-year growth and were progressing downward toward the 1-yr-old growth. Thus, infection most likely arose from the inoculation immediately preceding the 4 C exposure, not the inoculation in the field a year earlier. Lesions were comparable to those observed in seedlings infected naturally in the field.

Significance. The spore production technique developed in this study satisfactorily provided a source of viable conidia for use in our inoculation experiments. The results of our inoculation experiments demonstrate that a lengthy summer-fall incubation period after inoculation is not necessary for Scleroderris shoot blight to develop, and that the disease cycle of Scleroderris shoot blight can be reproduced, from inoculation to symptom expression, under entirely artificial conditions, by exposing

TABLE 2. Number of spores produced in V-8 broth flasks incubated at 18 C for 22 days under different light regimes

Photoperiod (hr)	Light intensity (μE $m^{-2}sec^{-1}$)	Spores/Flask $\times 10^7$ [a,b]
2	40[c]	3.55 ± 2.04 a
2	101	5.87 ± 1.75 ab
8	42[c]	5.32 ± 1.63 ab
8	105	6.02 ± 1.45 b
16	39[c]	8.83 ± 0.92 c
16	98	9.56 ± 2.86 d
24	38[c]	7.08 ± 2.44 bc
24	94	8.44 ± 2.59 c

[a] Values represent means ± standard deviation of seven replicates.
[b] Values followed by different letters are significantly different ($P = 0.05$), as determined by Duncan's multiple range test.
[c] Light intensities in these flasks were modified by covering the flasks with three layers of cheesecloth, a procedure that was estimated to attenuate light intensity by about 60%.

TABLE 3. Number and percent germination of spores produced by various methods, incubated at 18 C under a 16-hr photoperiod of approximately 100 μE m^{-2} sec^{-1} light intensity for 30 days

Method	Number of spores[a]	Percent germination[a]
Hudler et al (8)	$1.37 \pm 1.85 \times 10^8$ a[b]	52.4 ± 42.7 a
Blenis et al (2)	$3.65 \pm 1.49 \times 10^8$ b	71.1 ± 17.8 ab
V-8 agar plates	$6.60 \pm 5.85 \times 10^6$ a	98.9 ± 1.1 b
V-8 broth flasks	$7.52 \pm 1.72 \times 10^7$ a	97.6 ± 1.3 b

[a] Values represent means ± standard deviations of five replicates.
[b] Numbers followed by different letters are significantly different ($P = 0.05$), as determined by Duncan's multiple range test.

inoculated seedlings to +4 C for as few as 56 days. The development of reliable techniques to produce inoculum and produce disease symptoms under artificial conditions will allow controlled experiments involving all serotypes of *G. abietina*, and will greatly facilitate further study of all aspects of the disease caused by this pathogen. Particularly, these methods will allow those experiments to be done that will resolve differences in pathogenicity and response to the physical environment of the NA and the EU serotype of *G. abietina*.

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