Temperature Effects on Basidiospore Germination and on Infection of Slash Pine Seedlings by *Cronartium quercuum* f. sp. *fusiforme*

E. George Kuhlman and William D. Pepper

Research plant pathologist and station biometrician, respectively, Southeastern Forest Experiment Station, U.S. Department of Agriculture, Forest Service, 320 Green Street, Athens, GA 30602.
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ABSTRACT

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In vitro germination of basidiospores of 10 isolates of *Cronartium quercuum* f. sp. *fusiforme* occurred within 24 h at temperatures of 8–32 C. At 32 C, only very short germ tubes developed. Germination was frequent at temperatures of 12–28 C, and 90% of the maximum germination occurred at 14.6–29.6 C. On the basis of second-degree polynomial regressions, the optimum temperatures during 24 h of moist incubation for the infection of pines were 17.7 and 19 C in two experiments. Moist incubation for 24 h at 11.6–23.4 C resulted in ≥90% of the maximum infection rate for susceptible slash pine seedlings. Basidiospores were able to initiate infections over a broad temperature range of 8–28 C during a 24-h period of moist incubation. The shape and height of curves for germination of each rust isolate or infection of each pine family did not differ significantly, indicating neither factor interacted with temperature.

Additional keywords: fusiform rust, *Pinus elliottii*.

The fungus *Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme* causes fusiform rust disease of slash (*Pinus elliottii* Engelm. var. *elliottii*) and loblolly (*P. taeda* L.) pines in the southern United States. In the spring, aeciospores produced on perennial galls on pines infect immature oak leaves. There, telia produce haploid basidiospores, which are capable of infecting new pine tissue. Although the environmental conditions enabling the basidiospores to germinate and infect the pines are critical elements in disease development, only a few pathologists have studied these conditions. Previously, Kuhlman (3) reported that varying incubation temperatures after a uniform 20 C during 24 h of moist incubation had no effect on relative susceptibility. Siggers's (8) detailed 1947 study of the temperature requirements for germination of all spore stages of *C. q. fusiforme* has been the accepted standard. He tested five rust isolates from Mississippi and one from North Carolina in temperature-maintaining equipment that had fluctuating temperatures. Siggers (8) reported that basidiospores germinated at temperatures of 13–29 C and that no germination occurred at 9 or 11 C. The largest mean germination rate was 78% at 23 C, and the range among tests was 46–93%. At 25 C, the mean rate dropped to 44%, but the range was 13–82%.

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Siggers suggested the optimum temperature for germination was 22 C. Bega (1) reported that basidiospores of the related species *C. ribicola* J. C. Fisch. germinated well over a broad temperature range of 0.5–24 C in 24 h; but in 6 h, maximum germination occurred at 16 C. Basidiospores of another member of the genus, *C. commandrae* Peck, germinated at temperatures of 8–28 C; the most rapid germination occurred at 18 C (2).

A high relative humidity is also required for telial and basidiospore germination. Snow (9) reported that some infection of inoculated slash pine seedlings occurred after moist incubation for 4 h at 16 C, but more infection occurred on seedlings after 6 or 16 h of moist incubation. Siggers (8) suggested that a minimum of 18 h with temperatures of 16–26 C and humidity close to the moisture-saturation point were needed for abundant pine infection by *C. q. fusiforme*. On the basis of infection of pine seedlings exposed to natural inoculum in Mississippi, Snow et al (10) postulated that infection of pine occurred when relative humidity was ≥97% and temperature was above 16 C for at least 9 h. When methods for artificial inoculation of pine were developed, a temperature of 20 C for moist incubation seems to have been selected arbitrarily (4,5,11). This temperature was deemed adequate because 80–100% of susceptible seedlings developed galls (5,11). However, occasionally the infection rate dropped to 50–65% with no apparent change in conditions. The lack of temperature boundaries for infection of pine led to the study described here. The objectives were to determine the effect of a range of temperatures on basidiospore germination and of a range of temperatures during moist incubation on infection of susceptible slash pine seedlings and to develop regression curves to show the effects of deviations from the optimum temperatures on germination and infection.

MATERIALS AND METHODS

Basidiospore germination. Rust isolates were collected from loblolly pines in Georgia and South Carolina. Aeciospores were from composite gall collections: M-85 from Clarke and Greene counties, GA; Hous. from Houston County, GA; and Mill. from Baldwin County, GA. Urediniospores were from single-aeciospore isolates: SC-20-9, SC-20-12, SC-20-21, SC-35-2, and SC-35-5 from Colleton County, SC; and LHNC-2-31 and LHNC-2-40 from Halifax County, NC. Basidiospores were produced by the methods of Matthews and Rowan (5) from telia on northern red oak (*Quercus rubra* L.) seedlings planted in Fafard Mix No. 2 (Conrad Fafard, Inc., Agauam, MA) in 10-cm pots. After collection in acid water (pH 2.0), the basidiospores were washed several times with cold deionized water and stored at 4 C for 1–14 days prior to use.

Before inoculation, plates to be incubated at temperatures below ambient were stored for 24 h at 4 C, while those to be incubated at or above ambient were stored at room temperature. Suspensions of 50,000 basidiospores per milliliter were prepared, and 0.2 ml of suspension was pipetted onto the surface of 1.5% Agar-Agar (Fisher Scientific, Fair Lawn, NJ) in a 60-mm petri dish. The spores were spread evenly on the agar surface with a glass rod. Plates were incubated unstacked at the designated temperature for 24 h in the dark and then placed at 4 C until spore germination was tallied.

Incubators were assigned temperatures for each of the three or four replications from a random numbers table. Experiment 1 included 11 temperature treatments (10–30 C at intervals of 2 C), four single-aeciospore isolates (S-20-21, SC-35-5, LHNC-2-31, and LHNC-2-40), and four replications. Experiment 2 had seven temperatures (8–32 C at intervals of 4 C), seven isolates (four single-aeciospore isolates from South Carolina and the three composite isolates), and three replications. At least 100 spores were observed in each plate. A spore was considered to have germinated if its germ tube was at least as long as the spore diameter.

Pine inoculation. Slash pine seeds from two rust-susceptible sources, half-sib family 3051-3 and multitree source SSC, were floated in water overnight at 20 C and germinated in vermiculite. The germlings were transferred to Fafard Mix No. 2 in planting tubes and grown 15 seedlings per tray in a greenhouse for 6 wk. For the first pine inoculation experiment, each of the four replications had two trays of each source for each of the 11 temperatures (10–30 C at intervals of 2 C). In the second inoculation experiment, each of four replications included two trays of source SSC for each of the seven temperatures (8–32 C at intervals of 4 C). In the second experiment, seedlings were brought to the approximate treatment temperature prior to inoculation. Trays of seedlings to be exposed to moist incubation at temperatures below 20 C were incubated for 24 h at 4 C below their incubation temperature. Seedlings to be incubated at or above 20 C were placed at the incubation temperature for 24 h before inoculation. In both experiments, the trays of seedlings were sprayed with 50,000 basidiospores per milliliter generated from isolate M-85 in the automated concentrated basidiospore spray system (5). Individual trays were immediately placed in plastic bags that had been sprayed inside with water. Each bagged tray of seedlings was sprayed with a fine mist of water for 10 s before the bag was sealed. The trays of seedlings in each temperature treatment were inoculated, bagged, and placed in an incubator with no lights before another temperature treatment was started. Incubators were randomly assigned temperatures for each of the four replicates in each of the experiments. After 24 h of moist incubation, the trays of seedlings were removed from the bags, and all seedlings were placed in a greenhouse for 6 mo with temperatures fluctuating from 22 to 28 C. Seedlings were classed as infected if they had one or more galls 6 mo after inoculation.

Statistical analysis. Plots of raw percentages and the arcsines of their square roots suggested that both percent germination and percent infection have a quadratic relationship with incubation temperature. This relationship makes biological sense: percentages are near zero at both temperature extremes and rise to a maximum at intermediate temperatures. Data from each experiment were used to fit second-degree polynomials of the form

$$ASP = B_0 + B_1 T + B_2 T^2,$$

where ASP = predicted value on the arcsine-square root scale; B_0, B_1, and B_2 are least squares estimates of regression coefficients; and T = temperature. When more than one pine family or more than one rust isolate were included in an experiment, multiple covariance analyses were performed with SAS procedure GLM (7). These analyses were needed to determine whether a single quadratic model could represent all families or isolates or whether separate models were needed.

TABLE 1. Effect of incubation temperature on germination of basidiospores of *Cronartium quercuum* f. sp. *fusiforme*[a]

Temperature (C)	Germination (%)			
	Experiment 1		Experiment 2	
	Average	Range	Average	Range
8	...[b]	...	9	2–23
10	29	24–33	...	...
12	41	32–50	69	54–91
14	54	40–67	...	...
16	56	44–75	81	67–95
18	60	42–83	...	...
20	63	49–91	85	71–95
22	59	45–86	...	...
24	60	44–84	82	69–95
26	55	38–75	...	...
28	52	36–72	81	70–96
30	49	35–67	...	...
32	...	...	55	43–69

[a] Experiment 1 included four single-aeciospore isolates and four replicates. Experiment 2 had four single-aeciospore isolates, three composite isolates, and three replicates.
[b] No data collected.

For each fitted curve, we determined the maximum temperature and the maximum predicted value for germination or infection. The fitted model was then equated to 90% of this arcsine value, and roots of the resulting equation were determined. Provided they are real, these two roots are inverse estimates of threshold values of temperatures corresponding to 90% of the maximum predicted value of *ASP*.

The graphical analog to this analytic procedure shows the curve of the second-degree polynomial with a horizontal line intersecting the vertical axis at 90% of maximum predicted value. This line will intersect the curve at two points. Vertical perpendiculars dropped from these two points will intersect the horizontal axis at points T_1 and T_2, which define the lower and upper threshold temperatures, respectively, corresponding to 90% of maximum predicted *ASP*.

The final step in the analysis was the determination of simultaneous 95% confidence limits for estimates of threshold temperature values. We used the Working-Hotelling-Scheffe method presented by Miller (6) to construct simultaneous 95% confidence bands for second-degree polynomials. Unlike the nonsimultaneous method, this procedure ensures the application of the 95% confidence coefficient at all values of *T*. By converting Miller's (6) formula 5a into an equation and squaring both sides, we defined a fourth-degree polynomial and attempted to find four roots: R1, R2, R3, and R4, where R1 is the smallest in value and R4 is the largest. Provided they are real, these roots are the 95% confidence limits for the two temperature threshold values. For practical purposes, we are interested only in the smallest and largest roots. Then we can state with 95% confidence that both true threshold values are contained in the interval (R1, R4).

RESULTS

Basidiospore germination. Germination of basidiospores occurred over the entire range of temperatures (8–32 C) used in these experiments (Table 1). In the second experiment, germination averaged 69–85% when the incubation temperatures were 12–28 C. In the first basidiospore germination experiment, almost half (49%) of the spores germinated normally at 30 C. In the second experiment, more than half of the basidiospores germinated at 32 C, but the germ tubes were never more than twice as long as the diameter of the spore. A very small number of spores (9%) germinated at 8 C, and the lengths of germ tubes were similar to those at other temperatures after 24 h of moist incubation.

Covariance analyses generally indicated that one second-degree polynomial provided adequate fit to the data when experiments involved more than one pine family or more than one rust isolate. *F* tests performed in covariance analyses for three of the four experiments indicated that regression coefficients, including intercepts, did not differ significantly ($P > 0.05$). This result implies that curves for each family or isolate had the same height and shape. The homogeneous shape of curves implies that neither the family factor nor the isolate factor interacted with temperature.

In the second basidiospore germination experiment, one curve was not adequate for the seven isolates. The seven curves had homogeneous shapes but failed the *F* test of equal intercepts ($P > 0.05$). Further analyses of the data indicated that two second-degree polynomials were appropriate, one for isolates M-85, SC-20-12, and SC-35-2 and a second for isolates SC-20-9, SC-20-21, Hous., and Mill.

In experiment 1, we produced only a single curve for isolate LHNC-2-40, with the maximum germination at 21.7 C (Fig. 1A). In experiment 2, the temperature for maximum germination was 21.4 C for a group of three isolates (M-85, SC-20-12, and SC-35-2) (Fig. 1B) and 22.0 C for a group of four isolates (SC-20-9, SC-20-21, Hous., and Mill) (Fig. 1C). Maximum predicted germination for these three models were 87.5, 95, and 89%, respectively. From these models, we estimate that ≥90% of maximum germination occurs where the temperature of the environment is between 16.4 and 27.4 C. Confidence limits presented on the graphs enable us to state with 95% confidence that ≥90%

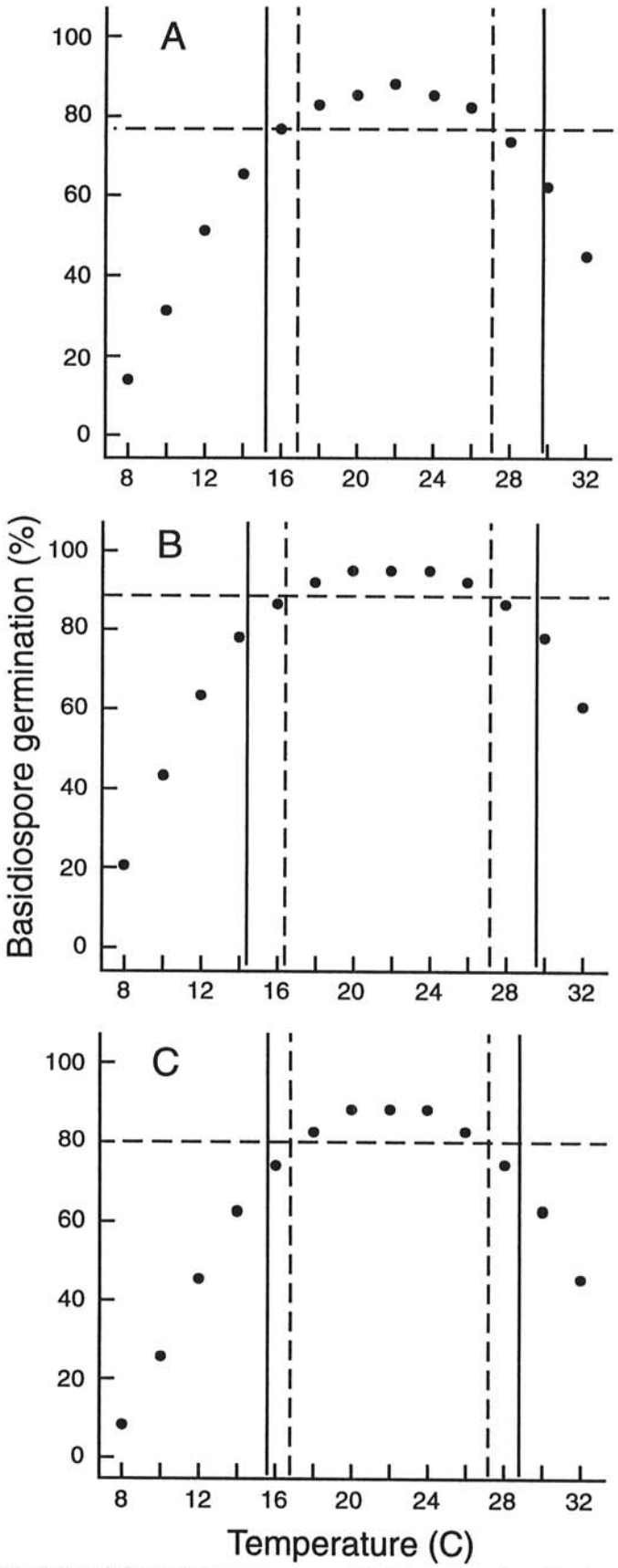

Fig. 1. Plot of effect of temperature on predicted frequency of germination of basidiospores of *Cronartium quercuum* f. sp. *fusiforme*. The horizontal lines and the inner vertical lines correspond to 90% of the maximum predicted value, and the outside vertical lines correspond to the 95% confidence limits. **A,** Single aeciospore isolate LHNC-2-40; **B,** combined data for isolates M-85, SC-20-12, and SC-35-2; and **C,** combined data for isolates SC-20-9, SC-20-21, Hous., and Mill.

of maximum predicted germination will occur when the temperature is between these limits. For Figure 1A, the confidence interval is 14.9–29.5 C.

Pine inoculation. Significant numbers of susceptible slash pine seedlings had galls 6 mo after a 24-h moist incubation at 10–28 C. In the first inoculation experiment, the statistical analysis indicated that the pine sources did not differ significantly. Gall development peaked at 82% after moist incubation at 18 C in the first experiment and at 90% at 20 C in the second experiment (Table 2). Only 12% of the seedlings developed galls after moist incubation at 8 C. The treatment at 32 C produced no galls, and only 3% of the seedlings had galls after moist incubation at 30 C. The second-degree polynomials of the two experiments indicate that optimum temperatures for infection of susceptible slash pine seedlings were 17.7 and 19.2 C. These conditions produced galls on 86 and 94% of seedlings, respectively (Fig. 2A and B). The polynomials suggest that at least 90% of the maximum infection should occur over a temperature range of 11.6–23.4 C in experiment 1 and 13.6–25.0 C in experiment 2.

DISCUSSION

The temperatures of 21.4–22.0 C predicted as optimum for basidiospore germination agree with those of Siggers (8). However, basidiospores germinated at a higher frequency at 12–28 C in our experiments than in his. Germination occurred at higher and lower temperatures than those Siggers reported. He indicated less than 3% germination at 13 C and no germination at 11 C. In six tests, Siggers found only 5% of the spores germinating at 29 C, while 18% germinated at 27 C. In our tests, basidiospores germinated from 8 to 30 C. In addition to using his self-described unsophisticated methods of temperature control, Siggers followed germination in water under coverslips on glass slides. Excess free water depressed basidiospore germination in our preliminary trials, so we opted to observe germination of the spore suspension on the agar surface. Most of Siggers's dikaryon sources were from southeastern Mississippi, and these may be less tolerant of cold than the more northerly sources we used. Basidiospore viability appears to be affected by many factors that we do not understand. When spores are freshly collected from vigorous telia, germination can be expected at the frequency shown in Figure 1. Three of the isolates in the first experiment had poor germination and were considered atypical. Siggers (8) reported that attempts to store sporidia were unsuccessful. The technique of Matthews and Rowan (5) usually enables basidiospore storage for several weeks in a refrigerator. Spaine and Kaneko (12) reported that basidiospores cast in acid water (pH 2.2 or 5.5) and washed in distilled water had a high frequency of direct germination compared with the low frequency of direct germination of basidiospores cast directly on 0.05–3% agar. However, bacterial contamination sometimes reduces germination. If spores are stored inadequately or telia are less vigorous, germination can be reduced, as happened with three isolates in experiment 1.

Seedlings for the second inoculation experiment and all agar plates were incubated for 24 h at temperatures at or below the treatment temperature to minimize the time the plants or plates took to reach the treatment temperature. The frequency of gall formation at 12–28 C in the two seedling inoculation experiments is not different (Table 2), which indicates there was neither a change in the host susceptibility with the pretreatment nor an opportunity for the pathogen to initiate penetration during the transition from ambient to incubation temperature in the first experiment.

Relatively high levels of infection of susceptible slash pines occurred during moist incubation over a broad temperature range. Resistance in some families may be expressed only at temperatures other than 20 C. Rather than having only the optimum temperature for germination and infection, we wanted to develop

TABLE 2. Effect of temperature during moist incubation on infection of two susceptible slash pine sources, 3051-3 and SSC, by basidiospores of *Cronartium quercuum* f. sp. *fusiforme*

Temperature (C)	Seedlings with galls (%)			
	Experiment 1			Experiment 2
	3051-3	SSC	Average	SSC
8	...[a]	...	...	12
10	69	60	64	...
12	56	63	59	64
14	80	80	80	...
16	73	86	79	87
18	90	74	82	...
20	64	80	72	90
22	82	74	78	...
24	84	73	79	76
26	50	65	57	...
28	26	28	27	38
30	2	4	3	...
32	...	...	...	0

[a] No data collected.

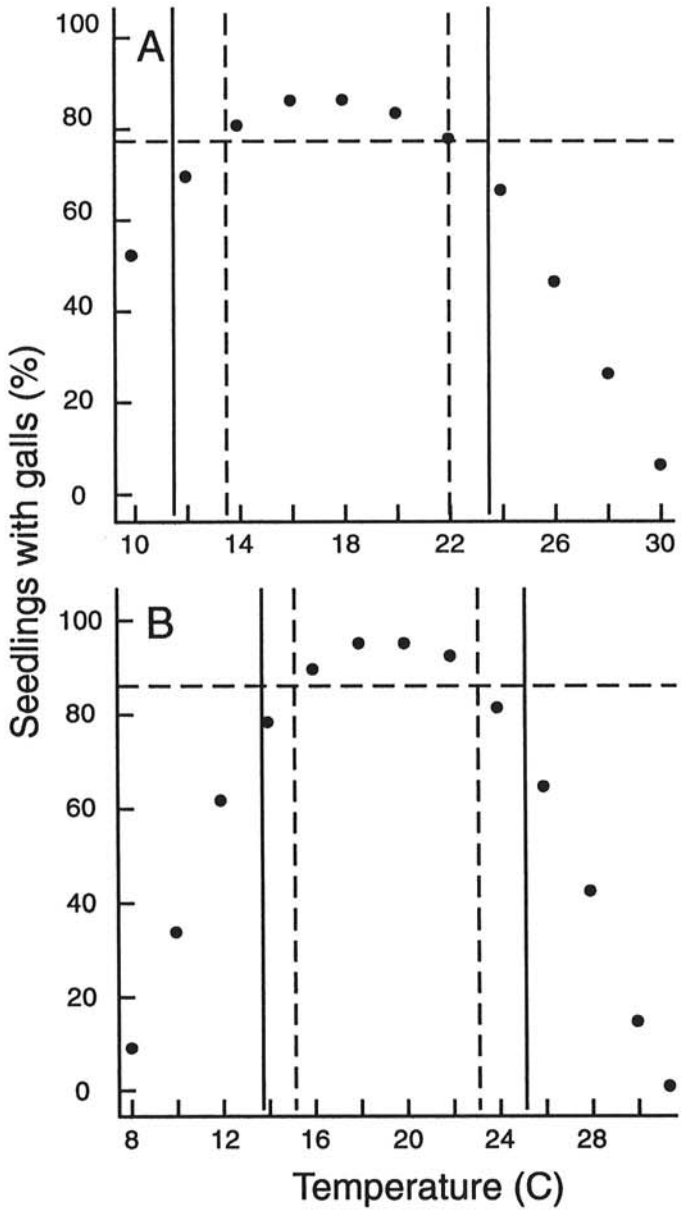

Fig. 2. Plot of predicted frequency of fusiform rust galls on slash pine seedlings 6 mo after inoculation with basidiospores of *Cronartium quercuum* f. sp. *fusiforme* and moist incubation at various temperatures. The horizontal lines and the inner vertical lines correspond to 90% of the maximum predicted value, and the outside vertical lines correspond to the 95% confidence limits. **A,** Combined data for inoculations of family 3051-3 and source SSC; and **B,** data for second inoculation of source SSC.

statistical methods for providing a confidence interval for the temperature range for these attributes within a percentage of the maximum (optimum). Initially, we thought 70% of the maximum would be useful, but we chose 90% when it became obvious that the slopes near the maximum were gentle and that germination and infection could occur readily over a broad range. For basidiospores, ≥90% of the maximum germination occurred over temperature ranges of 14.9–29.5 C (Fig. 1A), 14.6–29.6 C (Fig. 1B), and 15.4–28.7 C (Fig. 1C), according to estimates of 95% confidence intervals. Pine infection occurred at slightly lower temperatures and in a narrower temperature range than basidiospore germination did, but ≥90% of the maximum infection occurred over the ranges of 11.6–23.4 C (Fig. 2A) and 13.6–25 C (Fig. 2B). Basidiospores of the fusiforme rust fungus have the capacity to germinate and infect susceptible slash pine seedlings over a broad temperature range. Krebill (2) and Bega (1) have shown that other species of *Cronartium* germinate over broad temperature ranges in vitro. This, however, is the first demonstration that a *Cronartium* rust can infect its host over broad temperature ranges. Successful inoculations resulting in a high frequency of infection can be expected when moist incubation occurs at temperatures between 12 and 25 C.

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