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Nuclear behavior during basidiospore germination in *Cronartium quercuum* f. sp. *fusiforme*

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Abstract: Nuclear behavior during basidiospore germination in *Cronartium quercuum* f. sp. *fusiforme* was examined on glass slides and host seedlings using 4, 6-diamidino-2-phenylindole staining. Mononucleate basidiospores of *Cronartium quercuum* f. sp. *fusiforme* normally were produced following meiosis in the teliospore. However, a subsequent mitotic division often occurred within each basidiospore resulting in a short-lived binucleate condition. Within 1 h after spores were released from the basidium, one of the two nuclei in most basidiospores began to degenerate. Ninety-five percent of basidiospores directly cast from telia onto seedlings of *Pinus taeda* germinated directly forming thin germ tubes. More than 93% of germ tubes were mononucleate. On glass slides, however, 79% of basidiospores germinated indirectly, forming a secondary basidiospore. During indirect germination, degeneration of the second nucleus occurred quickly without exception. No differences in nuclear behavior were found between direct and indirect germination on host plants or on glass slides. However, nuclear movement from basidiospores into germ tubes was faster on the pine seedlings than on glass slides.

Key Words: direct germination, indirect germination, nuclear degeneration, *Pinus taeda, Quercus rubra,* rust fungus

INTRODUCTION

Fusiform rust disease is a major disease in pine plantations of the southeastern United States. Understanding the basic biology of the pathogen, *Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme,* is essential to understanding the disease cycle and the development of successful biological control methods. In this paper, we report on a subsequent mitotic division in *C. quercuum* f. sp. *fusiforme* basidiospores that occurred following meiosis. This additional mitotic division was not found to be associated with different types of basidiospore germination.

Previous studies of nuclear behavior in basidiospores of other rust fungi have reported a binucleate stage occurring after meiosis and before spore release from sterigmata (Anikster, 1983; Kaneko, 1975). Kaneko (1975) examined the nuclear behavior of teliospores and sporidia in *Coleosporium petasitis* Cooke during indirect and direct germination, and found 97% of basidiospores were binucleate 0–2 h after release from sterigmata. Similar nuclear behavior also was observed in *C. helianthi* and *C. vernoniae* by Olive (1942) and in *C. sidae* by Sanwal (1953). Bauer (1986) and Bauer and Oberwinkler (1988) found basidiospores of several rust species, including *Cronartium asclepiadeum,* (Wilde) Fr., *Gymnosporangium clavariiforme* (Pers.) DC., *Puccinia malvacearum* Bert., *Phragmidium violaceum* (C. F. Shultz) Wint., and *Coleosporium tussilaginis* (Pers.) Lev., were binucleate until they formed a hypha, appressorium, or secondary basidiospore, at which time one nucleus degenerated. This condition also was observed in *Gymnosporangium juniperi-virginianae* Schwein (Mims and Richardson 1989, 1990).

In a previous study of *Cronartium quercuum* f. sp. *fusiforme,* Spaine and Kaneko (1993) found that more than 95% of washed basidiospores cast onto water agar germinated directly, forming thin germ tubes. Unwashed basidiospores germinated indirectly, forming secondary basidiospores. The objective of this study was to determine whether germination type, direct or indirect, influences or relates to nuclear behavior.

MATERIALS AND METHODS

Leaves of *Quercus rubra* L. were inoculated with aeciospores (mixed gall collection, Clarke County, Georgia) of *Cronartium quercuum* f. sp. *fusiforme.* Leaves bearing telia were collected 3 wk after inoculation. To stimulate both germination types, directly cast and washed basidio-spores were used (Spaine and Kaneko, 1993).

In direct-cast spore treatments, oak leaves with telia

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were hydrated for 12 h in darkness at 20 C in a petri dish containing moist filter paper. This procedure initiated the formation of basidiospores. Leaves were then suspended for 1 h directly over either glass slides sprayed with distilled H_2O or needles and stems of 3-mo-old *Pinus taeda* seedlings that had been cut off at the stem collar. The 0^a hour incubation represented spores harvested after a 30-min casting period. All other incubation periods followed a 1-h casting period. Slides and seedlings were incubated in a petri dish with moistened filter paper for 0, 1, 2, 4, or 6 h.

Washed spores were prepared by a method similar to the one described by Miller (1970). Oak leaves were hydrated as described above. Leaves were then hung for 1 h over a petri dish containing H_2O adjusted to pH 2.2 with HCl. Discharged basidiospores were collected on an Advantec[1] millipore filter (3.0 μm) (Toyo Roshi, Ltd., Japan), and washed with distilled H_2O. Suspensions of collected basidiospores in distilled H_2O were sprayed onto seedlings of *Pinus taeda* or glass slides in petri dishes containing moistened filter paper.

All petri dishes of slides and seedlings were kept at 20 C in darkness for 0, 1, 2, 4, and 6 h. Five slides and five seedlings were prepared for washed and unwashed spores for each incubation period. Immediately after removal from the petri dishes, slides were air-dried at 50 C. To observe germination of basidiospores on seedlings, the needles and stem of a seedling were placed on a glass slide previously sprayed with distilled H_2O, and the basidiospores were washed with distilled H_2O from the seedlings. These slides also were air-dried at 50 C. Five slides were prepared for unwashed basidiospores, and five for washed basidiospores. To determine the nuclear behavior within basidiospores, the nuclear conditions in both germination types were examined by 4, 6-diamidino-2-phenylindole (DAPI) staining methods.

Before DAPI staining, all dried slides were fixed in a solution of absolute ethanol and acetic acid (3:1) for 10 min, washed in distilled H_2O for 10 min five times, and air-dried. Slides were stained in DAPI following the method described by Kuroiwa and Suzuki (1980), but changing the DAPI concentration to 0.5 μg/mL. Observations were made with an Olympus BH-2 epifluorescence microscope. Spores (50 on each of five slides, 250 total) were examined for each of the 25 treatments. Nuclear conditions in basidia developed from teliospores were examined by staining germinating telia with DAPI.

[1] The use of trade or firm names in this publication is for reader information and does not imply endorsement by the U.S. Department of Agriculture of any product or service.

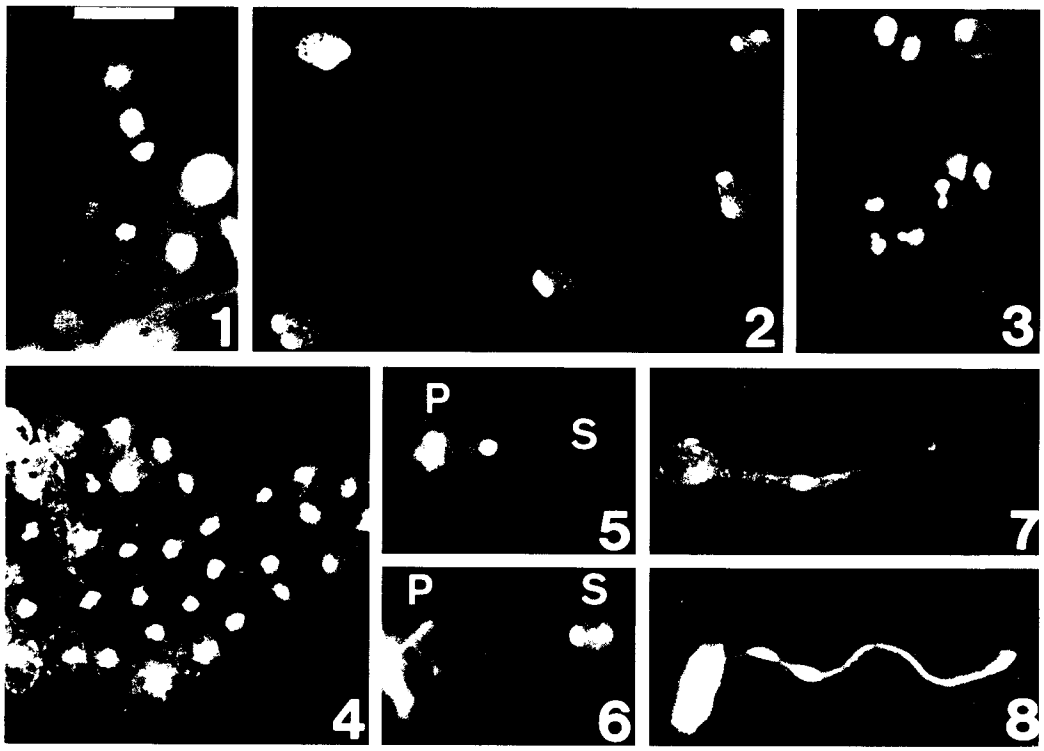
RESULTS

Each cell of a basidium contained one nucleus (FIG. 1), but mitosis was observed in immature basidiospores still attached to sterigmata. The binucleate condition was short-lived, with most spores becoming mono-nucleate before germination. Washing spores apparently hastened this process. Nuclear behavior was similar during both types of germination, direct, i.e., formation of a thin germ tube, or indirect, i.e., by repeated secondary basidiospore formation.

FIGURES 1–8 illustrate the nuclear conditions observed in the basidium and basidiospores during indirect and direct germination. Each cell of the basidium was mononucleate (FIG. 1). When basidiospores were first cast from the basidium, the majority of spores were binucleate (FIG. 2). However, after a 1–2 h period, one of the two nuclei was degenerating and fading (FIG. 3). When degeneration of the second nucleus was complete, basidiospores remained mononucleate (FIG. 4). In basidiospores that germinated indirectly, the nucleus often was seen migrating into the sterigma (FIG. 5). The nucleus then migrated into the secondary basidiospore where it underwent a second mitotic division (FIG. 6). The binucleate condition in the secondary basidiospore also was short-lived, and one nucleus degenerated quickly leaving a mononucleate spore. During direct germination, typically a single nucleus migrated into the germ tube (FIG. 7). However, occasionally a binucleate condition was observed in the germ tube (FIG. 8).

Effects of substrate and of basidiospore washing on the frequency of nuclear conditions during basidiospore germination of *C. quercuum* f. sp. *fusiforme* are summarized in FIG. 9. When basidiospores were cast directly onto glass slides, after 30 min in the casting chamber and 0-h further incubation, 85% of the spores had two nuclei of equal size (FIG. 9, a). However, after 1-h in the casting chamber with 0-h further incubation, only 19% of the spores were binucleate (FIG. 9, a), 76% contained one normal-sized nucleus, and one smaller nucleus (FIG. 9, b); and 5% were mononucleate (FIG. 9, c). Mononucleate basidiospores increased to 73% after a 1 h incubation period (FIG. 9, c).

Basidiospores directly cast onto glass slides began to develop germlings after a 1-h incubation period. However, 65% of the spores germinated indirectly, producing secondary basidiospores within a 6-h incubation (FIG. 9, i–q) period. Only one nucleus was observed (FIG. 5) in 98% of primary spores or secondary sterigmata from the 1- to 6-h incubation period (FIG. 9, l–o). This suggests nuclear degeneration occurred rapidly during indirect germination. After



FIGS. 1–8. Fluorescence micrographs of *Cronartium quercuum* f. sp. *fusiforme* illustrating different nuclear conditions observed in the basidium and basidiospores during indirect and direct germination. P = primary basidiospore; S = secondary basidiospore. Bar = 20 μm for all figures. 1. Basidium with one nucleus in each cell. 2. Binucleate basidiospores. 3. Binucleate basidiospores, with one nucleus degenerating. 4. Degeneration of second nucleus nearly complete. 5. Indirect germination with one nucleus in secondary sterigma. 6. Indirect germination, with mitotic division in secondary spore. 7. Direct germination, with one nucleus in germ tube. 8. Direct germination showing two nuclei in germ tube.

secondary spores were produced, mitotic division occurred in these spores (FIG. 6; FIG. 9, p–q). At the 6-h incubation period, the number of secondary spores with unequal-sized nuclei (FIG. 9, q) increased, suggesting nuclear degeneration in the secondary spores.

Twenty-one percent of spores on glass slides germinated directly (FIG. 7). For these spores, 96% of the germ tubes contained one nucleus (FIG. 9, g), and 4% of the germ tubes contained two nuclei (FIG. 9, h). This suggests that nuclear degeneration also occurred during direct germination within the first 6-h incubation period.

Germination appeared to occur earlier in spores directly cast onto seedlings than in those cast onto glass slides. After a 1-h casting period, with 0-h further incubation, 30% of the basidiospores already had germinated. As time passed, the ratio of ungerminated mononucleate spores (FIG. 9, c) to binucleate spores increased. This was the result of one of the two nuclei degenerating. On seedlings, 95% of the spores germinated directly. This was compared to 21% of the spores on glass slides. Of these, 97% of the germ tubes were mononucleate (FIG. 9, g), and 3% were binucleate (FIG. 9, h). These results show that nuclear degeneration occurred in the course of direct germination.

DISCUSSION

Our results indicated that nuclear behavior was independent of germination type in *C. quercuum* f. sp. *fusiforme* basidiospores. Most washed basidio-spores germinated directly (96%), regardless of substrate (FIG. 9). This observation supported previous findings (Spaine and Kaneko 1993). Comparison of the 30 min/1 h casting period with no further incubation period demonstrates how rapidly nuclear degeneration occurred. At 30 min casting period, 19% of the ungerminated washed basidiospores on glass were mononucleate. After 1 h casting time and 1 h incubation time, this had increased to 60%. After the 2 h incubation period, 66% of ungerminated spores were mononucleate. The frequency of ungerminated mononucleate basidiospores increased in the longer incubation periods on both glass slides and seedlings. This indicates that nuclear degeneration also occurred in the washed basidiospore treatment, usually

Treatment	Incubation period (hr)	Ungerminated Spores (a) 2 N	(b) 1 N and 1 DN	(c) 1 N	Direct Germination (d) 2 N in PB	(e) 1 N and 1 DN in PB	(f) 1 N in PB	(g) 1 N in GT	(h) 2 N in GT	Indirect Germination (i) 2 N in PB	(j) 1 N and 1 DN in PB	(k) 1 N and 1 DN in SS	(l) 1 N in PB	(m) 1 N in SS	(n) 1 N in SB and 1 DN in SS	(o) 1 N in SB	(p) 2 N in SB	(q) 1 N and 1 DN in SS	% Total
Direct cast on glass slides	0[a]	84.8	12.8	2.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100
	0	18.8	76.0	5.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100
	1	2.4	22.4	73.2	0	0	0	0.4	0	0	0	1.6	0	0	0	0	0	0	100
	2	0.8	10.8	64.8	0	0.4	4.8	0.4	0	0	0.8	17.2	0	0	0	0	0	0	100
	4	0.4	7.2	14.8	1.2	1.6	13.6	3.2	0	0.4	0.8	33.2	0	4.0	1.2	2.0	11.2	5.2	100
	6	0.8	5.2	18.0	0.4	0	5.2	5.2	0.4	0	0.4	20.4	0	19.2	0.4	9.2	6.0	9.2	100
Direct cast on seedlings	0	6.8	19.6	44.0	2.4	1.6	19.2	1.6	0	0	0	4.8	0	0	0	0	0	0	100
	1	5.6	8.4	40.0	1.6	2.4	30.0	9.2	0	0	0	2.0	0	0	0	0	0.4	0.4	100
	2	0.4	0.8	22.0	0	0.4	37.2	36.0	1.6	0	0	1.6	0	0	0	0	0	0	100
	4	0.4	0	6.0	0.4	0.8	39.6	52.8	0.4	0	0	0	0	0	0	0	0	0	100
	6	0	1.6	10.8	0	0.4	4.8	72.8	3.6	0	0	1.2	0.4	3.2	0	0.8	0.4	0	100
Washed on glass slides	0[a]	11.2	69.6	19.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100
	0	25.6	60.0	14.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100
	1	12.8	27.6	58.8	0	0	0.8	0	0	0	0	0	0	0	0	0	0	0	100
	2	5.2	16.4	65.6	0	1.2	9.6	0	0	0	0	2.0	0	0	0	0	0	0	100
	4	1.2	7.2	22.0	0.4	4.0	48.8	12.8	0.8	0	0.4	1.6	0	0	0	0	0	0.8	100
	6	1.6	7.2	22.0	0	1.2	33.2	29.6	2.0	0	0	2.0	0	0.8	0	0.4	0	0	100
Washed on seedlings	1	6.0	20.8	60.4	3.6	0	8.0	0	0	0	0.4	0.8	0	0	0	0	0	0	100
	2	2.8	11.2	34.4	2.8	4.4	28.8	12.4	0.8	0	0	1.2	0	0	0	0.4	0	0.4	100
	4	1.6	6.0	21.2	2.0	6.0	30.0	28.4	2.8	0	0	1.6	0	0	0	0.4	0	0.4	100
	6	0	0	7.2	0	0.8	3.2	84.4	3.2	0	0	0.4	0	0.4	0.4	0	0	0	100

FIG. 9. Effects of substrate and basidiospore washing on the percenage frequency of nuclear conditions during various phases of basidiospore germination of *Cronartium quercuum* f. sp. *fusiforme*. 0[a] = spores harvested after a 30-min casting period; all other incubation periods followed a 1-hr casting period; N = normal nucleus; DN = degenerating nucleus; PB = primary basidiospore; GT = germ tube; SS = secondary sterigmata; and SB = secondary basidiospore.

before development of germ tubes. When germ tubes developed but nuclei remained in the primary spores, 93% were mononucleate condition (FIG. 9, d–f) on glass slides and 78% were mononucleate seedlings (FIG. 9, d–f).

When nuclei migrated into the developed germ tube, 94% were in the mononucleate condition (FIG. 9, g) on glass slides, and 95% on seedlings. This frequency is similar to that observed in the direct germination of direct-cast spores and strongly suggests that nuclear degeneration occurred regardless of spore washing.

In *Coleosporium petasitis,* the smaller of the two nuclei in basidiospore germlings was thought to be degenerating (Kaneko, 1975). Using transmission electron microscopy, Bauer and Oberwinkler (1988) and Mims and Richardson (1989) clearly demonstrated the smaller nuclei were degenerating in *Cronartium asclepiadeum* and in *Gymnosporangium juniperi-virginianae,* respectively. Similar behavior also occurred in *Cronartium quercuum* f. sp. *fusiforme* basidiospores. When cast onto moist glass slides, one of the two nuclei degenerated before germ tube development.

This study showed that fundamental nuclear behavior was similar in direct and indirect germination on slides (non-host) and seedlings (host). However, the percentage direct germination was much higher in direct-cast spores on host surfaces and in washed spores on both host and non-host surfaces. The only difference found between direct and indirect germination involved a few instances during direct germination in which nuclear degeneration failed to occur before germ tube development. No such instances were observed during indirect germination. These results differ from observations in other rust species (Bauer, 1986; Kaneko, 1975; Mims and Richardson, 1989). These earlier studies suggested no nuclear degeneration occurred during direct germination.

Spaine and Kaneko (1993) stated that basidiospore exudates and other external factors affect germination type of *C. quercuum* f. sp. *fusiforme* basidiospores. This follow-up study shows that germination type is not controlled by differences in nuclear behavior. However, nuclear degeneration advanced more quickly on host plants than on artificial surfaces.

Nuclear degeneration during direct germination may establish the way for the haploid condition needed before spermatization. Mycelia and haustoria of *Cronartium* species in pine tissues have been reported to be primarily mononucleate (Jewell et al., 1962; Jewell and Walker, 1965).

The significance of nuclear degeneration during indirect germination is unclear. Mims and Richardson (1989) suggested that it might prevent secondary spores from becoming tetranucleate because mitosis seems to be a common event during maturation of secondary spores. In this study, only one tetranucleate secondary basidiospore was observed. Therefore, nuclear degeneration in the process of indirect germination of basidiospores may be a necessary event for rust fungi that have binucleate basidiospores.

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