Germination and Infection by Basidiospores of *Athelia* (*Sclerotium*) *rolfsii*

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ABSTRACT

Punja, Z. K., and Grogan, R. G. 1983. Germination and infection by basidiospores of *Athelia* (*Sclerotium*) *rolfsii*. Plant Disease 67:875-878.

Two isolates of *Sclerotium rolfsii* from golf greens of bentgrass and annual bluegrass in Sacramento, CA, formed the basidial state (*Athelia rolfsii*) on potato-dextrose agar containing 2% activated charcoal. Basidiospore deposits were obtained by suspending pieces of agar with hymenium over petri dishes containing 1% Noble water agar that was adjusted to various pHs or that contained osmotica. Highest germination (85–94%) of the basidiospores was observed at 24–30 C, at a solute potential (ψ_s) of −2.5 to −5 bars, and at pH 2.0–4.0. No basidiospores germinated below 15 C, at ψ_s less than −40 bars, and at pH above 7.0. Bentgrass-annual bluegrass plugs from uninfected golf greens and bentgrass (Penncross) started in the greenhouse from seed were inoculated either with an aqueous suspension of basidiospores or by direct spore deposits from fruiting cultures; disease symptoms developed after incubation for 7 days at 100% relative humidity and 28 C. Extensive hyphae developed from germinating basidiospores and appressoria were formed on the leaf surface. Isolations from diseased leaves and crowns yielded typical colonies of *S. rolfsii*. This is the first report of infection by basidiospores of *S. rolfsii*; however, the epidemiological significance of the sexual state has not been established.

Additional key words: southern blight, turf

Sclerotia of *Sclerotium rolfsii* represent the resistant structures by which the fungus survives in soil and which can initiate infection of susceptible hosts (8). With the exception of mycelium growing in soil or on organic matter, sclerotia probably serve as the main source of inoculum for initiation of disease. In published descriptions of the disease cycle of *S. rolfsii*, the basidial state (*Athelia rolfsii*) is generally omitted or considered to have a minor role in the initiation of disease (2). This is mainly because the teleomorph has seldom been observed in nature and occurs infrequently on laboratory media.

Since 1975, *S. rolfsii* blight, or southern blight, has become a disease of increasing importance on golf greens in California. The disease may be spread from one golf green to another by sclerotia and mycelium carried with turf clippings by mowers and other machinery and possibly by golfers. Symptoms and control of this disease are described elsewhere (11). Numerous isolates of *S. rolfsii* obtained from naturally infected golf greens in California from 1975 to 1981 have been induced to form the basidial state on culture media (10).

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Accepted for publication 14 January 1983.

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Because of the consistency with which these particular isolates sporulated in culture compared with isolates of *S. rolfsii* from other hosts or areas, the possibility existed that basidiospores produced by these turf isolates could play a role in the spread of the disease. Their ability to infect susceptible host tissue has, however, never been demonstrated. In this paper, we describe the effect of temperature, solute (osmotic) potential (ψ_s), and pH on basidiospore germination and demonstrate that basidiospores are capable of initiating disease on turf under optimal conditions of temperature and moisture. The process of infection of bentgrass leaves by hyphae from germinating basidiospores is described briefly. Preliminary results from this study have been published (7).

MATERIALS AND METHODS

Basidiospore production and germination. Isolates 1126 and 2672 of *S. rolfsii* that originated from bentgrass (*Agrostis tenuis* Sibth.) and annual bluegrass (*Poa annua* L.) golf greens in Sacramento, CA, were used in this study. Both isolates formed the sexual state (*Athelia rolfsii*) consistently on potato-dextrose agar (PDA) containing 2% activated charcoal. The conditions promoting formation of the teleomorph are described in detail elsewhere (10).

To obtain basidiospore deposits, pieces of agar (about 0.5 cm^2) with hymenium were attached to the lids of plastic petri dishes (60 × 15 mm) with double-sided transparent tape and inverted over dishes containing 1% Difco Noble agar (Difco Laboratories, Detroit, MI 48232) that was either unadjusted or adjusted to different pH or that contained various osmotica. The dishes were incubated at 27 C and in the dark. Basidiospores were usually discharged within 24 hr; at this time, the lids with hymenia were removed and replaced with sterile lids. All dishes then were placed in plastic bags and incubated for 48 hr at 27 ± 2 C. Percent germination was determined from counts of about 100 randomly selected basidiospores in each of five replicate dishes that were viewed directly under the low power (×125) of a compound microscope. All experiments were repeated at least once. The data presented are the means of five replicates; percentage germination values in each replicate were not significantly different ($P = 0.05$).

Effect of temperature. Dishes with basidiospores deposited onto 1% Noble water agar were incubated at temperatures ranging from 6 to 33 C, at 3 C increments.

Effect of solute (osmotic) potential. The basal medium employed was 1% Noble water agar (pH 5.6 after autoclaving). The ψ_s was adjusted to values between −2.5 and −58 bars by adding appropriate amounts of either NaCl or $CaCl_2 \cdot 2H_2O$ as outlined by Robinson and Stokes (14). After autoclaving at 121 C and 1.05 kg/cm^2 pressure, sterile distilled water was added to the flasks to compensate for losses in volume during autoclaving. About 8 ml of osmotically adjusted agar was poured into each petri dish.

Effect of pH. Noble agar was added to 1% to mixtures of 0.15 M each of citric acid and tris buffers (9) and autoclaved. The pH of cooled agar was readjusted, when necessary, to the final desired pH value (in the range of 2.0–9.0) before pouring. The values reported represent the final pH of agar at the end of the experiment and were determined using a flat-surface combination electrode (A. H. Thomas Co., Philadelphia, PA 19106).

Initiation of disease by basidiospores. Bentgrass-annual bluegrass plugs (10 × 6 cm) from uninfected golf greens and 4-wk-old bentgrass (Penncross) started from seed were inoculated with basidiospores using two methods: Fruiting cultures on charcoal-PDA were either inverted 8 cm above the pots containing the turf for 18 hr or 30 ml of an aqueous suspension of basidiospores (about 50 spores per milliliter) was added to each pot. The pots were covered with plastic bags and incubated at 28 ± 2 C for 7 days. The experiment was repeated twice and

four replicate pots were included for each isolate and inoculation method. Control pots either had nonsporulating cultures of *S. rolfsii* growing on charcoal-PDA suspended over them or received sterile distilled water. From diseased areas that developed in inoculated pots, infected leaves and crowns were plated onto PDA and incubated at room temperature for 4 days.

Infection process. To establish the method of penetration by hyphae from germinating basidiospores, light and scanning electron microscopy were employed. For light microscopy, individual leaves of bentgrass taken from pots 48 hr after inoculation were fixed and cleared in acetic alcohol (1 part glacial acetic acid:1 part 95% ethanol) for 2 days, then transferred into 85% lactic acid where they were stored for 4 days (16). The leaves were subsequently mounted in 0.1% cotton blue in lactophenol on glass slides and examined in the light microscope. For scanning electron microscopy, individual leaves were exposed to vapors of OsO_4 (1% in 0.01 M phosphate buffer, pH 7.2) for 12 hr. Subsequently, they were dehydrated in an ethanol series (from 30 to 100%, 45 min in each), then transferred into amyl acetate where they were stored for 5–7 days. The specimens were critical-point dried, attached to 12-mm Al stubs with double-sided transparent tape, sputter-coated with gold (using argon as the inert gas), and examined with a Cambridge scanning electron microscope (SEM).

RESULTS

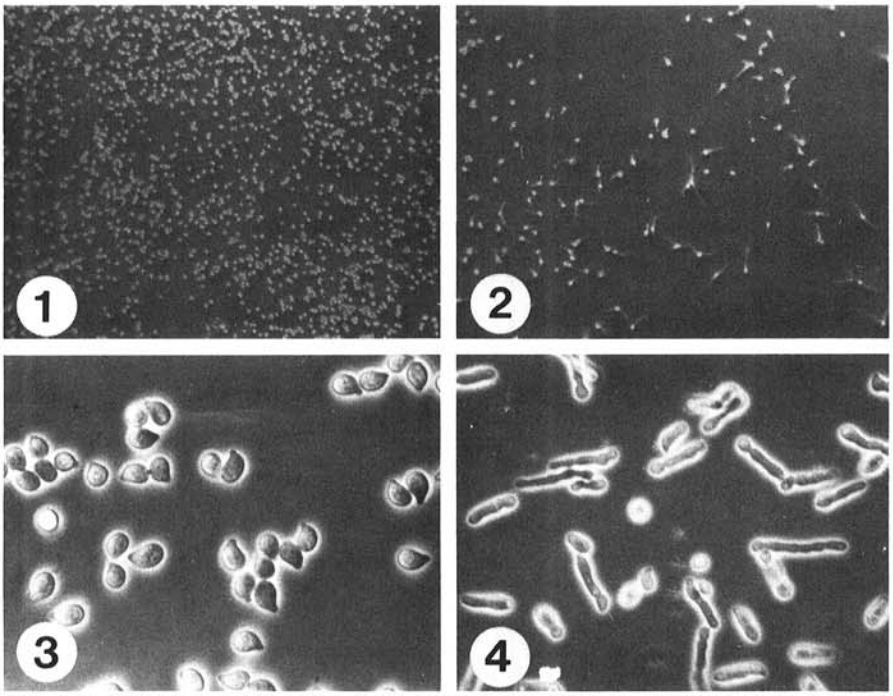
Basidiospore production and germination. Both isolates of *S. rolfsii* were induced to fruit consistently on charcoal-PDA. Large numbers of basidiospores were deposited on water agar after 18–24 hr of incubation in the dark (Figs. 1 and 2). Each germinating basidiospore usually had produced one or two germ tubes after 48 hr of incubation (Figs. 3 and 4). An extensive mycelium developed after incubation for an additional 48 hr at 27 ± 2 C.

Effect of temperature. Highest (about 94%) germination of basidiospores on 1% Noble water agar was observed at 27 C; below 15 C and above 33 C, percent germination was significantly reduced (Fig. 5). The temperature range optimal for basidiospore germination was 24–30 C.

Effect of solute (osmotic) potential. The response of basidiospores to NaCl was slightly different than to $CaCl_2 \cdot 2H_2O$ (Fig. 6). In general, however, germination was highest at ψ_s of about −2.5 bars, and as the ψ_s was lowered, germination decreased. Percent germination was zero at ψ_s of −30 bars using $CaCl_2 \cdot 2H_2O$, and −43 bars using NaCl (Fig. 6).

Effect of pH. The basidiospores germinated best on water agar adjusted to low pH (from 2.0 to 4.0); as the pH was increased, percent germination was reduced and no basidiospores germinated at pH 7.0 or above (Fig. 7).

Initiation of disease by basidiospores. Turf plugs taken from uninfected golf



Figs. 1–4. Basidiospore deposits of *Athelia* (*Sclerotium*) *rolfsii* on 1% Noble water agar. **(1)** Nongerminated basidiospores (×85). **(2)** Germinating basidiospores (×100). **(3)** Nongerminated basidiospores (×850). **(4)** Germinating basidiospores (×550). Note extensive germ tube growth.

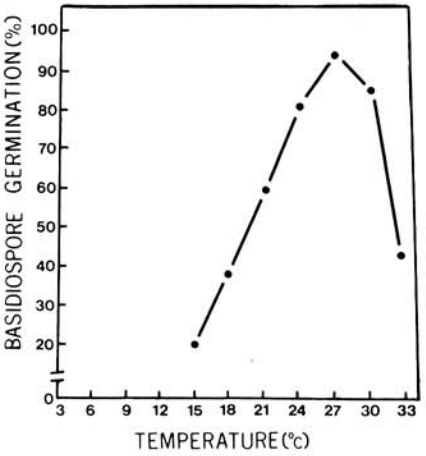


Fig. 5. Effect of temperature on germination of basidiospores of *Athelia* (*Sclerotium*) *rolfsii* on 1% Noble water agar. Points represent the mean germination percentage of about 100 basidiospores in each of five replicate dishes. Germination was rated after 48 hr of incubation.

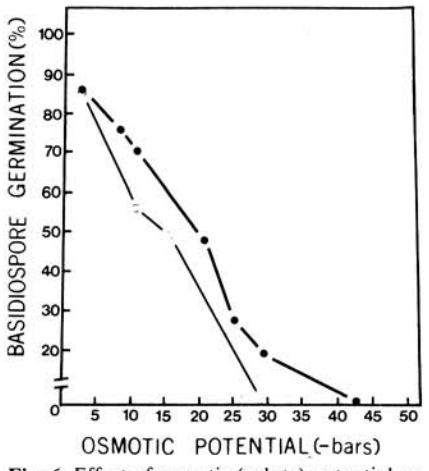


Fig. 6. Effect of osmotic (solute) potential on germination of basidiospores of *Athelia* (*Sclerotium*) *rolfsii*. The osmotica used were NaCl (•——•) and $CaCl_2 \cdot 2H_2O$ (o——o). Points represent the mean germination percentage of about 100 basidiospores in each of five replicate dishes. Germination was rated after 48 hr of incubation.

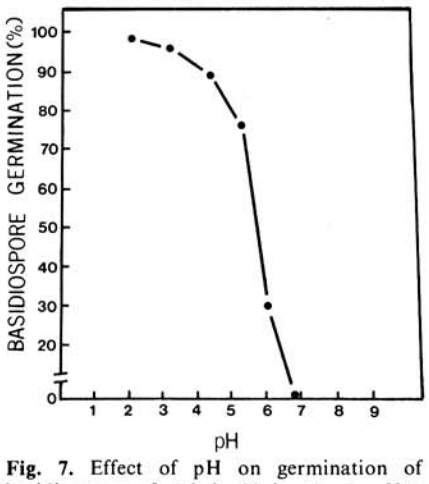


Fig. 7. Effect of pH on germination of basidiospores of *Athelia* (*Sclerotium*) *rolfsii*. The pH of 1% Noble water agar was adjusted with citric acid-tris buffer. Points represent the mean germination percentage of about 100 basidiospores in each of five replicate dishes. The pH and percent germination were determined after 48 hr of incubation.

greens and 4-wk-old bentgrass started from seed developed disease symptoms in the greenhouse within 7 days of inoculation with basidiospores of *A. rolfsii*. Both isolates were pathogenic to turf, and inoculation either by direct deposition of basidiospores or with an aqueous suspension resulted in disease. Extensive necrosis and death of the turf was observed. Isolations from diseased leaves and crowns yielded characteristic colonies of *S. rolfsii*. Control pots over which nonsporulating cultures of *S. rolfsii* were inverted did not develop disease symptoms.

Infection process. Basidiospores germinated on the surfaces of bentgrass leaves to produce an extensive mycelium. Numerous hyphal tips in contact with the host epidermal cells developed swollen appressorial-like structures (Figs. 8–10). Penetration through the epidermis by hyphae produced from these appressoria and growth of hyphae in the epidermal cells were observed (Fig. 9). Extensive mycelial growth and appressorial formation were observed also with the SEM on the surfaces of bentgrass leaves inoculated with basidiospores (Figs. 11 and 12).

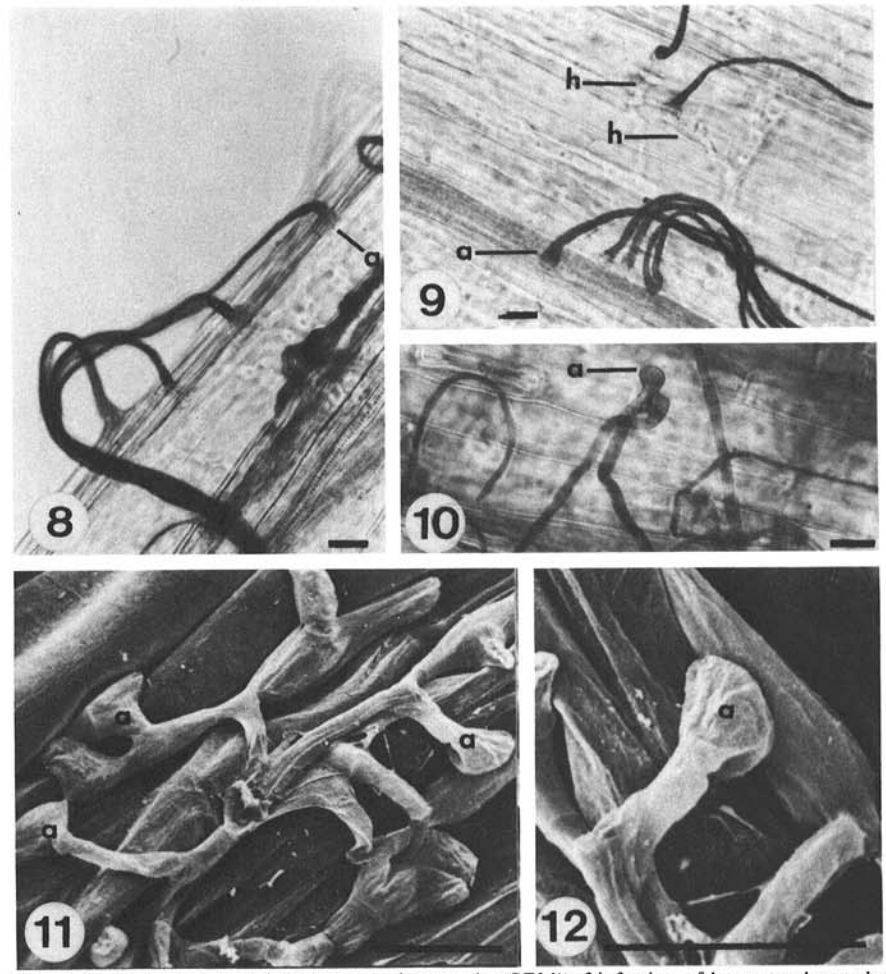
DISCUSSION

With the exception of the earlier work of Goto (3,4), there are no previous reports on factors influencing the germination of basidiospores of *S. rolfsii*. Goto reported that optimal germination of basidiospores occurred at temperatures between 25 and 31 C and between pH 4.2 and 4.6; the upper and lower limits of temperature and pH for germination were 37 and 16 C and pH 5.2 and 2.2, respectively. Each germinating basidiospore usually produced one or two germ tubes. The results obtained in this study on the effect of temperature and pH on basidiospore germination substantiate those reported earlier by Goto (3,4), except we observed germination of basidiospores up to a pH of about 6.0. At pH 7.0, germination of basidiospores and of sclerotia (9) was inhibited. The normal microenvironmental conditions in the thatch layer of golf greens (high relative humidity, temperature of 28–30 C, pH of about 5.8, and presence of an organic substrate for growth) would be conducive to the germination of basidiospores. Under these conditions, growth of mycelium and production of oxalic acid by the fungus would lower the pH of an unbuffered substrate to levels optimal for continued growth (9).

In repeated attempts, Goto (3,4) could not obtain infection of various host plants by inoculating with basidiospores and attributed this to the inhibition of basidiospore germination by the fairly alkaline pH (greater than 5.2) of the leaf surfaces. In contrast, basidiospores inoculated to pots of turf in this study germinated and produced an extensive mycelium on the leaf surface and appressorial-like structures were formed upon contact of numerous hyphal tips with the leaf surface. Other workers (5,6,15) have also observed formation of appressorial-like structures on terminal portions of infecting hyphae of *S. rolfsii* on various hosts inoculated either with sclerotia or mycelial fragments. These structures apparently facilitated the attachment to and penetration by the hyphae into susceptible host tissues.

Previous failures to obtain infection with basidiospores may have been due to the method of inoculation used. Goto placed basidiospore suspensions in distilled water (pH 6.1) directly onto leaves of various plant species and incubated them in a moist chamber. The absence of infection was attributed to the failure of basidiospores to germinate (4). In some of our preliminary studies in which basidiospores were similarly inoculated directly onto sugar beet leaves and carrot roots in petri dishes, we also did not obtain infection (*unpublished*). In this study, however, when the basidiospores were inoculated to pots of turf that contained living and dead plant material, infection was obtained. The basidiospores conceivably could have germinated to produce mycelium that colonized the organic matter before initiating infection of living tissue. Basidiospores inoculated onto dead plant tissues can establish vigorous mycelial growth (*unpublished*). A relationship between a food base of nonliving organic matter or a nutrient source and initiation of infection by basidiospores and ascospores of some plant pathogens has been noted by others (1,12,13). In fact, Goto (4) observed a dramatic increase in germination of basidiospores after adding various organic and inorganic acids and salts to distilled water agar, indicating these materials either served as a nutrient source or reduced the pH to a level optimal for basidiospore germination.

Basidiospores of *S. rolfsii* and ascospores of *Sclerotinia sclerotiorum* (12) can germinate in distilled water, but for appressorial formation and infection of host tissues by *S. sclerotiorum* to proceed, an exogenous nutrient source must be present (1,12). Although it appears likely, we have not determined



Figs. 8–12. Light and scanning electron micrographs (SEM) of infection of bentgrass leaves by germinating basidiospores of *Athelia* (*Sclerotium*) *rolfsii*. All light micrographs show hyphae stained with 0.1% cotton blue 48 hr after inoculation. **(8)** Attachment of hyphae to leaf surface by appressoria (a). **(9)** Appressoria (a) and hyphal growth (h) in the epidermal tissues. **(10)** Appressoria formed at tips of infecting hyphae. **(11)** Appressorial formation on leaf surface (SEM). **(12)** Close-up of an appressorium seen in Figure 11 (SEM). Scale bars = 20 μm.

whether basidiospores of *S. rolfsii* have a similar requirement for an exogenous nutrient source.

Because all isolates of *S. rolfsii* from turf in California fruit consistently, formation of the sexual state may have achieved some selective advantage in the life cycle. These field isolates are presumed to be heterokaryons, and all are pathogenic to turfgrass; similarly, many single-basidiospore strains are pathogenic in vivo (7). Mycelium from germinating basidiospores of *S. rolfsii* could establish homokaryotic colonies, which through hyphal anastomoses could become heterokaryotic; both heterokaryons and homokaryons can initiate disease (*unpublished*). We have no evidence, however, to implicate the involvement of basidiospores in initiation or spread of disease on golf greens under field conditions. Also, factors influencing survival and spread of basidiospore inoculum in nature have not been determined. The extent of variability and genetic relatedness among field isolates of *S. rolfsii* from turf in California indicate that the involvement of basidiospores in spread of the disease may be minimal (*unpublished*). Further epidemiological studies are, however, required to clarify the role of the basidial state in the disease cycle of *S. rolfsii*.

ACKNOWLEDGMENTS

We thank J. Hall for preparing the illustrations and T. Unruh and C. Pratt for providing numerous turf samples for inoculation studies.

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