# Germination of Basidiospores of Fomes applanatus

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ABSTRACT

Up to 78% of the basidiospores of *Fomes applanatus* collected from sporophores in nature germinated within 1 week on artificial media containing glucose, but only in the presence of certain bacteria, fungi, yeasts, or volatiles from colonies of *Ceratocystis fagacearum*. Culture filtrates of certain yeasts stimulated spore germination. Germination rarely occurred under other conditions, and never was greater than 1%. Propyl, ethyl, or sodium acetates did not induce germination, nor did ethanol, any of 14 vitamins tested, kinetin, indoleacetic acid, $CO_2$, isovaleric acid, acetaldehyde, or isoamyl alcohol.

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Heartrot causes more loss of timber than any other disease of living trees in the United States (3). In spite of the importance of this disease, very little is known about the process of infection. It is assumed that infection results from wind-disseminated basidiospores. However, basidiospores of many Hymenomycetes that cause heartrot do not germinate on artificial media or in distilled water (6). One such fungus is *Fomes applanatus* (Pers. ex Wallr.) Gill.

There are only two reports of successful germination of basidiospores of *F. applanatus*. White (10) found that even though spores from most sporophores did not germinate at all, spores from a few did so; however, germination was always low, generally only a fraction of 1%. He concluded that the viability of the spores varied but was inherently low. However, Aoshima (1) found that up to 30% of the basidiospores of *F. applanatus* germinated after incubation periods of up to 1 year. Germination rarely occurred during the first 3 to 4 months of incubation. He concluded that the spores possessed an internal dormancy factor.

*Fomes applanatus* produces basidiospores from early spring until late fall (10). From a teleological viewpoint it seems disadvantageous to the fungus for its spores to lie dormant for 3 to 12 months before being able to germinate and infect. In view of other studies of germination of basidiospores of hymenomycetous fungi (6), it seems probable that basidiospores of *F. applanatus* germinate shortly after deposition upon a suitable substrate.

The following studies were done to determine conditions conducive to germination of basidiospores of *F. applanatus*. Such information would help elucidate the process of infection of standing trees.

MATERIALS AND METHODS.—Basidiospores were collected on glass microscope slides suspended 5 to 10 mm below *F. applanatus* sporophores in nature. When the spores were to be used promptly, the slides were stored in plastic boxes at 5 C; otherwise they were stored at −25 C.

Two standard media were used: 2% malt extract agar (MA = 20 g Difco malt extract, 20 g agar/liter of distilled water); and glucose-salts-thiamine agar [GSTA = 20 g glucose, 20 g agar, mineral salts (4, p. 427), 10 μg thiamine HCl, and 300 μg streptomycin/liter of distilled water].

Basidiospores were suspended in sterile distilled water and pipetted onto the desired medium in petri dishes. In the culture filtrate studies, the spores were suspended in sterile GST broth (GSTA without streptomycin or agar) as well as in the culture filtrates, and resulting suspensions were pipetted onto GSTA.

The spores were incubated at 21 and/or 27 C in diffuse light or dark for 7 days. Percentage of germination was based on a minimum of 200 spores observed at ×450. In all studies there were at least three replications per treatment; all studies were done with at least two different spore collections.

*Amendment of the culture medium.*—Sodium, ethyl, or propyl acetate was added to MA in concentrations of 1, 10, 100, 1,000, 10,000, and 40,000 ppm. The medium was cooled nearly to the point of solidification before the test material was added. Ethyl and propyl acetates were dissolved in 95% ethanol to insure mixing in the medium. An equal amount of ethanol without acetate was added to other MA.

Ethanol was added to GSTA in concentrations of 1, 10, 100, 1,000, 10,000, and 100,000 ppm. Kinetin, indoleacetic acid (IAA), or both were added to GST at 5, 10, 20, and 25 ppm. Glucose was added to GSTA without streptomycin in concentrations of 1, 2, 4, and 5% (w/v); and to GSTA, at concentrations of 0, 2.5, 5.0, 7.5, and 10.0% (w/v).

Discs of Whatman No. 1 filter paper were dipped into solutions (1 mg compound/1 ml of glass-distilled water or 30% ethanol) of kinetin, IAA, (or both), vitamin A acetate, ascorbic acid, biotin, calciferol, choline chloride, cyanocobalamine, myo-inositol, niacinamide, paraaminobenzoic acid, pyridoxine, *d*-panthothenate, riboflavin, thiamine-HCl, or DL-α-tocopherol, and then placed on GSTA upon which spores had previously been spread.

A thick layer of agar was poured into petri dishes.

A 20-mm-diam well was cut into the agar in each dish, and the bottom of the well was sealed with melted agar. One ml of 10% glucose or 1% lactose solution was pipetted into these wells, producing a short-term concentration gradient of these compounds across the agar. Spores were then spread onto the agar surface.

The pH of the MA was adjusted, after autoclaving, with lactic acid or ammonium hydroxide to vary from 3 to 8 by units. The pH of GSTA was similarly adjusted from 2 to 9 by units.

*Amendment of the atmosphere.*—These experiments were of two types: In the first, 14-day-old petri dish cultures of *Ceratocystis fagacearum* (Bretz) Hunt were inverted over dishes of MA with *F. applanatus* spores sown on the agar surface (7). The dishes were taped together and incubated 4 days. Subsequently, gases from 19 colonies of *C. fagacearum* grown on the agar-coated sides of 1-gal jugs were bubbled for 3 months through 50 ml of cold 95% ethanol, which then was incorporated into GST at 2% concentration (v/v).

In the second, air in desiccators was replaced with $CO_2$ so that the atmosphere in the desiccators contained 0.1, 0.5, 1, 5, 10, 20, or 30% $CO_2$. Spores on MA and GSTA, with or without streptomycin, were incubated in these atmospheres. Similar tests were made exposing spores that had been stored for 1 to 5 months or 24 hr to normal or 30% $CO_2$ atmospheres. In addition, spores were incubated on GSTA in atmospheres containing 1, 10, and 100 ppm of vapors of isovaleric acid, isoamyl alcohol, or acetaldehyde.

*Effects of microorganisms.*—During the above studies it was noted that certain microorganisms introduced with the spores stimulated the basidiospores to germinate. Therefore, sterile pieces of dialysis membrane were laid across many of these colonies on MA. *Fomes applanatus* basidiospores were spread onto the membrane. One-half of these dishes was incubated in desiccators containing normal atmospheres; the other half was incubated in 30% $CO_2$ to determine whether the stimulation was due to $CO_2$ liberated by the organisms. Controls consisted of spores spread onto MA with or without the membrane under the spores. One-half of the control dishes in each category was incubated in 30% $CO_2$.

Three techniques were used in attempts to separate the *F. applanatus* basidiospores from associated microorganisms collected with the basidiospores. First, surface sterilization of the basidiospores was attempted with sodium hypochlorite at concentrations as low as 0.05% and ethanol at concentrations as low as 5% for periods as short as 15 sec. Second, spores were suspended in sterile distilled water and the suspension was filtered through a 3-$\mu$m Millipore filter (Millipore Corp., Bedford, Mass.). Spores caught on the filter were washed repeatedly with sterile distilled water. Third, suspensions of spores were centrifuged at various gravities to precipitate the basidiospores, but not the bacteria or yeasts.

Some of the stimulatory microorganisms were grown in 1% malt broth (10 g malt extract/liter of distilled water) or in GST broth. The cultures were filtered by suction or pressure through a Seitz filter or a 0.45-$\mu$m Millipore filter. Spores of *F. applanatus* were suspended in the filtrates and pipetted onto GSTA.

RESULTS.—During these studies, it was noticed that basidiospores adjacent to colonies of unidentified bacteria and fungi, including species of *Hormodendrum, Pullularia, Rhodotorula,* and *Candida*, germinated freely. Maximum germination reached 78% in 72 hr in small areas next to these colonies. Occasionally germination was highest adjacent to the colony, but in most cases the germination percentage increased for a short distance from the colony, then decreased sharply with increasing distance from the colony (Fig. 1). This stimulation by microorganisms was observed both in atmospheres containing high levels of $CO_2$ and in normal atmospheres. Therefore, it appears that $CO_2$ is not the compound which stimulates germination, at least of fresh spores. On the other hand, in some cases high concentrations of $CO_2$ seemed to increase germination of spores stored longer than 1 month. In studies during the winter months, germination of stored spores was higher in atmospheres containing high percentages of $CO_2$. For example, in the study previously described involving dialysis membrane, 1% germination occurred on the MA controls incubated in normal atmosphere, whereas 17% of the spores incubated in 30% $CO_2$ germinated. These spores had been stored over 5 months in the freezer.

With the possible exception of glucose, none of the chemicals added to the media stimulated the spores to germinate. In studies with 1, 2, 4, and 5% glucose in GSTA with streptomycin, the average germination was 42, 28, 42, and 49%, respectively. Even in these, no germination was noted except in the presence of a bacterium that grew sparsely over

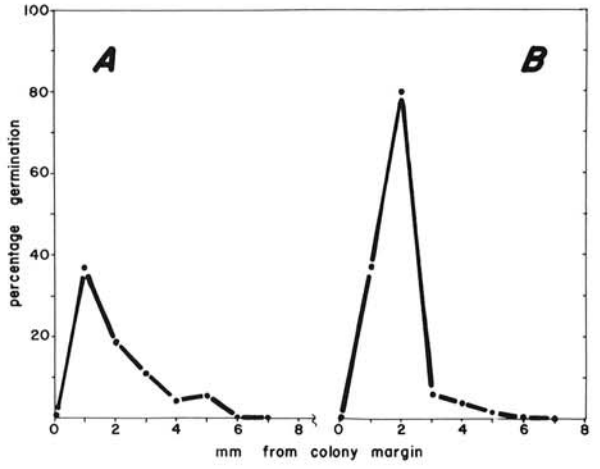


**Fig. 1.** Germination of basidiospores of *Fomes applanatus* at various distances from colonies of **A)** *Rhodotorula* sp.; and **B)** *Pullularia* sp.

the surface of the agar. Germination never occurred on media without glucose. No germination was observed at any concentration of glucose unless one or more of the associated microorganisms was present.

No germination occurred on media with adjusted pH except around colonies of stimulatory microorganisms on media of pH 5.0 and 6.0. However, equal percentages of germination occurred around colonies of yeasts in control dishes of GSTA. Thus, variation of pH indirectly affected germination by controlling growth of stimulatory organisms.

Isovaleric acid, isoamyl alcohol, and acetaldehyde at the concentrations used did not affect germination.

Spores incubated in the presence of *C. fagacearum* germinated freely; 41% germinated in the first trial; and 42% in the second. When ethanol through which volatile compounds from *C. fagacearum* colonies had passed was incorporated into GST, no stimulation occurred. Other studies had shown 2% ethanol to be nontoxic. Because the material is volatile, the concentration in GST gradually would have decreased to nil, passing a concentration favorable for germination had inhibitory amounts been added. Therefore, the stimulatory agent was not present, at least not in sufficient concentration to stimulate spore germination.

Attempts to surface-sterilize the basidiospores were unsuccessful. The basidiospores appeared to be more sensitive to the sterilants than were the spores of the associated microorganisms.

Attempts to germinate spores on dialysis membranes placed over colonies of stimulatory microorganisms failed with one exception. Twelve percent of the spores pipetted onto a membrane laid on a Rhodotorula colony germinated when incubated in 30% $CO_2$. Seventeen percent of the spores of the same suspension germinated in the presence of associated microorganisms without membrane when pipetted onto MA, but none germinated when on the membrane. The use of dialysis membrane did not separate the spores from the other organisms, which was the original purpose. Growth of the other organisms was profuse on the surface of the membrane.

When spores were suspended in culture filtrates of stimulatory organisms, occasionally germination in all areas of the petri dish approximately equaled the germination adjacent to colonies of the stimulatory microorganisms. Therefore, the stimulatory substance was present in these culture filtrates. Stimulation was rarely evident when the cultures had been suction-filtered. One stimulatory yeast was isolated from a sporulating *F. applanatus* basidiocarp in nature. Nineteen percent of the spores suspended in the suction-filtered culture filtrate of this yeast germinated when streaked on GSTA; 16% of the spores suspended in noninoculated and suction-filtered GST broth germinated when similarly treated. In both cases, germination was restricted to areas adjacent to colonies of associated organisms. However, 46% of the spores suspended in the pressure-filtered culture filtrate germinated; germination was not related to distance from colonies of associated microorganisms.

DISCUSSION.—The collection of spores from nature for use in these studies was necessary because *F. applanatus* does not produce basidiospores in culture. However, this led to several difficulties. Foremost was the variable amounts and kinds of microorganisms collected with the spores. These led to wide disparities between replicates in an experiment and between repetitions of an experiment. For example, in one experiment germination in the presence of isoamyl alcohol was sometimes higher than the controls. The general pattern of germination over the three concentrations used, however, could not be attributed to the changing concentrations of the alcohol. In subsequent trials, no germination occurred. The germination observed in the first trial was attributed to the differences in microflora on the various dishes.

All spore collections used in these studies contained associated microorganisms. However, even with other microorganisms present on the culture media, the effects of agents that might induce germination could be easily studied. In the controls, germination occurred only adjacent to colonies of certain organisms (Fig. 1); in dishes in which stimulatory agents were present, such as volatiles produced by colonies of *C. fagacearum*, germination occurred evenly over the surface of the medium regardless of the presence of other microorganisms.

Often spores germinated abundantly adjacent to colonies of associated microorganisms in an experiment in which very little, if any, germination occurred elsewhere. It seemed highly unlikely that such a concentration of viable spores would be found grouped together in a suspension of otherwise nonviable ones. Therefore, White's (10) conclusion that the inherent viability of these spores is low is unacceptable. Viability of these spores is high—we observed 78% germination. Further, Aoshima's conclusion (1) that spores of *F. applanatus* have an internal dormancy which requires them to lie dormant for several months before germination also is unacceptable; up to 78% of freshly collected spores germinated in 72 hr, but only in the presence of stimulatory microorganisms.

One compound which may be necessary for germination is glucose; germination was nil without it. However, scanty growth of other microflora on media low or lacking in glucose, and the fact the germination did not always occur when glucose was present, suggest that the effect of glucose may, in part, be due to the effect that it has on promoting growth of yeasts and other stimulatory organisms. If an external supply of glucose is necessary, it alone is not sufficient for germination. Therefore, the germination requirements of *F. applanatus* differ from those of *F. igniarius* reported by Manion & French (5). However, since Manion & French used basidiospores collected from nature, and since we have always observed associated microorganisms in such spore collections, it is possible that Manion &

French actually were observing the same phenomenon as we.

*C. fagacearum* emits some substance(s) that stimulates basidiospores of *F. applanatus* to germinate. We have been unable to isolate the stimulatory compound or even to detect it by gas chromatography. Acetates, isoamyl alcohol, isovaleric acid, and acetaldehyde, reported to affect germination of other basidiomycetes (6), did not stimulate germination.

The role of $CO_2$ in the germination of spores of *F. applanatus* is not clear. It was not necessary for the germination of fresh spores. Although higher percentages of aged spores often germinated in 30% $CO_2$ than in normal atmospheres, even under these conditions the stimulation by microorganisms was still quite apparent. Therefore, the stimulatory substance is not $CO_2$ or carbonate ion. Furthermore, since variation of pH did not affect germination, $CO_2$ probably is not active through alteration of pH.

Since none of the growth regulators or 14 common vitamins was stimulatory, it seems that they are not active in stimulation of germination at the rates applied. We do not know whether thiamine is required, since it was present in all of the media used.

Since not all filtrates from a stimulatory organism were stimulatory, it appears that these organisms do not produce this substance continuously, or that the substance is quite unstable. Furthermore, since pressure filtration of the cultures greatly increased the frequency with which stimulation occurred, the active agent probably is volatile. We have been unable to isolate the stimulatory compound. Our results are similar to those of others who have applied the "red yeast" technique of Fries (2) to certain Gasteromycetes and mycorrhizal fungi. Although technique is useful in obtaining low levels of germination—usually less than 1%—to the best of our knowledge no one has been able to identify the stimulatory substance(s).

Abundant data indicate that bacteria, yeasts, and non-Hymenomycetes precede decay fungi in living trees (9). However, previous studies have not related this metabiotic sequence to the infection process. Our studies show that microorganisms collected with the basidiospores of *F. applanatus* in nature strongly stimulate them to germinate on artificial media. It appears highly probable that the same phenomenon occurs in nature; Paine (8) has shown that basidiospores of several decay fungi are stimulated to germinate on dead branch stubs colonized by blue-staining fungi and other microorganisms. Especially intriguing is the fact that culture filtrates of a yeast isolated from the hymenium of a living sporophore of *F. applanatus* from nature were quite stimulatory.

In summary, we have found that spores of *F. applanatus* are able to germinate as soon as cast, if they are placed in the presence of certain bacteria, yeasts, Fungi Imperfecti, or *C. fagacearum*. Although the material that stimulates germination probably is volatile, we have been unable to isolate and identify it.

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