Carpogenesis and basidiosporogenesis by
Flammulina velutipes, Schizophyllum commune,
and Trametes versicolor in vitro ¹

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K e y w o r d s: Basidiospore, oidia, hymenium, basidiome, carpophore, carpogenesis, basidiosporogenesis

1. Introduction

Wood-decay basidiomycetes produce fruiting bodies that generate enormous quantities of basidiospores. These fungi are the primary source of infection of wood used above ground. The basidiospores are readily dispersed by wind, rain, or insects. They are capable of germinating to form vegetative hyphae, which cause wood biodeterioration. In most cases, basidiospores are not affected by unfavourable atmospheric conditions.


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It is often very difficult to induce cultures to produce fruiting structures and basidiospores in vitro. Consequently, there is very little information on basidiospore production in vitro or on regulatory factors. Therefore, most investigators have studied mycelial growth of wood-decay fungi rather than basidiospore germination, even though wood is primarily infected by the basidiospores.

A primary objective of the wood preservative industry is to find preservatives that inhibit basidiospore germination and thus prevent decay at its inception. Studies in this area require a large supply of viable basidiospores. Since basidiospores can be very difficult to obtain from nature, a method is needed for obtaining a large supply of viable basidiospores in vitro.

The purpose of our study was to gain a better understanding of the factors that regulate and control basidiospore production. The study focused on two white-rot fungi, *Schizophyllum commune* and *Trametes versicolor* frequently found on wood exposed above ground, and one edible mushroom that produces the white-rot fungus *Flammulina velutipes*.

### 2. Materials and methods

#### 2.1 Basidiomycetes

Dikaryotic isolates of the white-rot basidiomycetes *Schizophyllum commune* Fr. [MAD619] and *Trametes versicolor* (L. ex Fr.) Pilat. (= *Coriolus versicolor*) [MAD-697] were obtained from the Center for Forest Mycology Research at the Forest Products Laboratory in Madison, Wisconsin. *Flammulina velutipes* (Fr.) was obtained from the Department of Applied Mycology of the Agricultural Sciences National Institute in Suweon, Korea. The cultures, which were maintained on 2% malt extract agar (MEA) or potato dextrose agar (PDA), were kept in the dark at 27°C and 70% relative humidity.

#### 2.2 Media

The media used for formation of fruiting bodies and production of basidiospores by *S. commune* and *T. versicolor* were complete plus yeast extract medium (CYM), which has been used extensively for fruiting of *S. commune* Fr. (R. B. STEVENS, 1974), PDA (Difco, Detroit, MI), MEA, and a chemically defined medium containing various concentrations of glucose (0.5%, 1%, 2%, 4%, or 8%, w/v) or Walseth cellulose and ammonium tartrate (2, 5, 10, 20, 50, or 100 mM). The Walseth cellulose was prepared from Whatman fibrous cellulose CF-11 powder (C. S. WALSETH, 1952): 20 g of cellulose powder was swollen in 85% phosphoric acid and regenerated repeatedly by washing with cold distilled water followed by 1% Na2CO3 and

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washed again until neutral (C. S. Walsh, 1952). An additional 25 mM KH₂PO₄, (J. Bjurman, 1984) was added to the basal medium (BIII) (T. K. Kirk et al., 1986) for the chemically defined medium. The final pH of the chemically defined medium was 5.2-5.5.

The BIII medium contained, per liter, 1.47 × 10⁻² mol KH₂PO₄, 2.03 × 10⁻³ mol MgSO₄·7H₂O, 6.8 × 10⁻⁴ mol CaCl₂·2H₂O, 5.93 × 10⁻⁶ mol thiamine·HCl, and 10 ml of a trace element solution. The basal trace element solution contained, per liter, 7.8 × 10⁻³ mol nitriloacetic acid, 1.2 × 10⁻² mol MgSO₄·7H₂O, 2.9 × 10⁻³ mol MnSO₄·H₂O, 1.7 × 10⁻¹ mol NaCl, 3.59 × 10⁻¹ mol FeSO₄·7H₂O, 7.75 × 10⁻⁴ mol CuSO₄·5H₂O, 2.1 × 10⁻⁴ mol Al₃K(SO₄)₂·12H₂O, 9.0 × 10⁻⁵ mol CaCl₂, 3.48 × 10⁻¹ mol ZnSO₄·7H₂O, 4 × 10⁻¹ mol Na₂CO₃, 2.1 × 10⁻¹ mol Al₃K(SO₄)₂·12H₂O, and 4.1 × 10⁻⁵ mol Na₂MoO₄·2H₂O.

The media mainly used for carpogenesis of F. velutipes were PDA, MEA, and sawdust medium. The sawdust medium consisted of 80% white oak sawdust, 10% wheat bran, and 10% millet, with water added for a final moisture content of 60%. Fifty, 75, or 100 g of sawdust medium were added to each storage dish and then pressed tightly before autoclaving.

2.3 Fruiting body formation

Plastic petri plates (90-mm-diameter) were used for 20 ml of complex medium or chemically defined medium, and Pyrex storage plates (Corning no. 3250) were used for 50 ml of the same media. Plates with chemically defined medium were inoculated at the center with a 6 mm mycelial disk from a fresh culture.

Preincubation was carried out in the dark (plates covered with aluminum foil) at 27 °C and 70% relative humidity except for F. velutipes, which was incubated at 14 °C to 15 °C. After the colonies reached a diameter of 25 to 35 mm, the plates were uncovered, inverted, and incubated at 14 °C to 15 °C under black light (Spectroline ENF-24, 115 V, 60 H3, 24 amp; ultraviolet light with emission maximum of 355 nm) or fluorescent light (General Electric, 2-15 W, standard cool white). A 12-h-light and 12-h-dark cycle was used for the entire incubation. To aerate the cultures, the lids of inverted plates were opened aseptically once a week.

The structures that produced basidiospores were regarded as the fruiting bodies, although their morphology was sometimes different from that in nature. The lids of the petri plates and storage plates were covered with precut, colored, sterile weighing paper for collection of basidiospores.

2.4 Basidiospore production

The technique for in vitro carpogenesis and basidiosporogenesis was that used by S. C. Croan and T. L. Highley (1991). At the first sign of spore production, basidiospores were collected from inverted plates of fruiting cultures by transferring the weighing paper in the plate lids to a sterile plastic petri plate. The collected basidiospores were immediately lyophilized and then stored at -20 °C in the dark. Basidiospore production was initially determined by microscopic examination of spore prints deposited under the fruiting cultures. The production of basidiospores was usually visible to the naked eye because the prints were creamy white against the colored weighing paper. Basidiospores on the weighing paper were suspended
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in sterile 0.005% Tween 20 solution and tested for viability on MEA plates after being counted with a haemocytometer.

2.5 Oidium production

Plates cultured with PDA or MEA were inoculated with the dikaryotic strain of *F. velutipes* and incubated at 14 °C-15 °C for 2-3 weeks or until mycelial growth covered the whole surface of the plates. An oidial suspension was obtained by adding 5 to 10 ml of sterile 0.005% Tween 20 solution to the plates with mycelial growth and scraping off the surface mycelial growth with a sterile glass rod, bent to resemble a hockey stick. The oidial suspension was filtered through two to three layers of glass wool in a funnel / flask to separate the oidia from hyphal fragments. The number of oidia in suspension was counted using a haemocytometer.

3. Results

Light was required for carpogenesis and basidiosporogenesis by *F. velutipes*, *S. commune*, and *T. versicolor*. When these basidiomycetes were grown in the dark, normal vegetative growth was obtained but no fruiting bodies were produced. The basidiospores were produced under the hymenial structures (fruiting bodies) on various media exposed to black light or cool white fluorescent light. The hymenial layer was produced in the center of the plate around the inoculum plug, in the middle of the plate, or at the margin of colonial growth; mycelia sometimes grew continuously beyond the hymenial layer. For *S. commune*, and *T. versicolor*, fruiting bodies were formed 2 to 5 weeks later under black light than under cool white fluorescent light. The light source did not make any difference for normal carpogenesis and basidiosporogenesis by *F. velutipes*.

Exposure to light stimulated carpogenesis only for specific ages and sizes of colonies. Different stages of mycelial growth were examined by exposing the dark-grown cultures to light at various distances. The dark-grown colonies were 25 to 35 mm in diameter when they were exposed to light at a distance of 30 to 40 cm. After 8 weeks, no fruiting had occurred in dark grown colonies > 10 or < 70 mm in diameter. When exposed to light at a distance of 60 to 80 cm under the same conditions, hymenial structures were not formed during the 8-week period, but the cultures showed signs of fruiting, indicated by fluffy mounds of mycelial growth (T. versicolor, Fig. 1) or various sizes of abnormal fruiting bodies (S. commune, Fig. 2 a/b), without basidiosporogenesis. The 12-h-light / 12-h-dark cycle induced more spores than did continuous light. Basidiospores were produced only rarely under continuous light.

For *F. velutipes*, fruiting bodies were formed on the colony surface or on 22- to 80-mm stems above the surface. Fruiting bodies formed 9- to
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40-mm-diameter caps on PDA or sawdust (Fig. 3); the number and size of fruiting bodies was proportional to the amount of sawdust medium used. The best carpogenesis was induced by 100 g of medium. F. velutipes grew and formed fruiting bodies very easily at 15 °C on PDA or sawdust medium in 4 to 6 weeks, by which time the whole area of medium was colonized by mycelia, which produced enormous quantities of basidiospores. The basidiospore prints on the side and bottom of the storage dish indicated great quantities of basidiospores on PDA (Fig. 4). Oidia were easily produced by gentle maceration of dikaryotic mycelial growth on MEA or PDA petri plates. Aerial hyphae germinated from the oidia did not have clamp connections.

Figure 1: Abnormal fruiting bodies and fluffy mounds of mycelial growth of Trametes versicolor

Fruiting bodies of S. commune (Fig. 5) and T. versicolor (Fig. 6) were formed on MEA, PDA, CYM, and chemically defined medium. For S. commune, fruiting bodies were formed at the margin of the colony, 5 to 15 mm above the surface of the agar plate. Mycelial growth sometimes extended beyond the fruiting zone.
The colonial growth of S. commune and T. versicolor differed, depending on the concentration of nitrogen or carbon. Colony growth was restricted at the higher concentration of ammonium tartrate or glucose, but fruiting was never observed.

Carpogenesis and basidiosporogenesis by S. commune usually depended on the concentration of carbon and nitrogen in the medium. Carbon or nitrogen depletion stimulated carpogenesis and basidiosporogenesis (Table 1). Carpogenesis occurred in glucose-starved (0.5% -1% glucose) cultures with 2 to 50 mM nitrogen. With 2% glucose, the formation of fruiting bodies was slowed by 2 to 4 weeks. More than 4% glucose suppressed fruiting and stopped carpogenesis. Walseth cellulose (2%) was found to be the best carbon source for inducing fruiting body.
formation and subsequent basidiosporenogenesis at any nitrogen concentration (Table 1). Walseth cellulose apparently overcame nitrogen repression and also induced good fruiting (Table 1, Fig. 5). However, the addition of glucose to Walseth cellulose suppressed carpogenesis, indicating the strong catabolic repression of fruiting body formation (Table 1). Fruiting bodies were formed after about 3 to 6 weeks and basidiospores were produced in approximately 3 to 12 weeks. Peak production was reached in about 4 weeks.

For T. versicolor, carpogenesis occurred in about 4 to 8 weeks and basidiospores were produced in about 4 to 16 weeks. Nitrogen depletion (2-5 mM) triggered the formation of carpophores and basidiospores as a secondary metabolic phenomenon, nitrogen depletion stimulated the onset of carpogenesis and basidiosporogenesis at 0.5%, 1%, 2%, and 4% glucose (Table 2, Fig. 6). The ammonium tartrate concentration (10 to 100 mM nitrogen) was found to suppress fruiting (Table 2).
Figure 3: Normal fruiting bodies of Flammulina velutipes on (1) PDA and (2) various amounts of sawdust medium: (a) 50 g, (b) 75 and (c) 100 g.

After storage at -20 °C for 10 to 14 months, T. versicolor basidiospores showed 60% to 85% viability and S. commune basidiospores 20% to 60% viability.
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Figure 4: Abundant basidiospore production by Flammulina velutipes.

Table 1: Effect of carbon and nitrogen concentration on carpogenesis by Schizophyllum commune

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a Nitrogen source, ammonium tartrate.
b No basidiospores, +- abnormal fruiting without basidiospores, + up to 25% fruiting bodies and basidiospores on colony margin, ++ 25%-50%, +++ 50%-75%, ++++ more than 75%.
b Gl is Glucose; WC is Walseth cellulose (2%).
Figure 5: Normal fruiting bodies of Schizophyllum commune.
F i g u r e 6: Pores on surface of Trametes versicolor fruiting bodies.
4. Discussion

In work with the white-rot basidiomycete *Pycnoporus cinnabarinus* (Jacq. Fr.) Karst., carpogenesis was repressed by ≥ 100 mM nitrogen with Walseth cellulose under black light (J. Bjurman, 1988). Our results showed that Walseth cellulose was not only the best carbon source for inducing fruiting bodies but could also reverse nitrogen repression of *S. commune*. When glucose was added to Walseth cellulose, no fruiting bodies were synthesized, indicating that fruiting body formation was under strong glucose repression. When glucose was used as the sole carbon source, *S. commune* carpogenesis was regulated by both nitrogen and glucose starvation. The low concentration of glucose (0.5% to 1%) or nitrogen (2 to 20 mM) triggered earlier onset of carpogenesis by 2 to 3 weeks. At a concentration of ≤ 2% glucose, nitrogen repression was observed. Higher concentrations of glucose (≥ 8%) at any concentration of nitrogen repressed carpogenesis and basidiosporogenesis. Concentrations of 5% to 7% glucose were not examined.

For the basidiomycetes *Lentinula edodes* (G. F. Leatham and M. A. Stahmann, 1987), *S. commune* (Fr.) (D. J. Niederprem, 1963), and *Gloeophyllum trabeum* (Pers.: FY) (S. C. Croan and T. L. Highley, 1991), light was found to be essential for carpogenesis and subsequent basidiospore production. In our study, carpogenesis of white-rot basidiomycetes was photoactivated; cool, white fluorescent light induced carpogenesis 3 to 5 weeks earlier than did black light.

Carpogenesis and basidiosporogenesis by the white-rot basidiomycetes *Phanerochaete chrysosporium* Burds. (M. H. Gold and T. M. Chang, 1979) and *Pycnoporus cinnabarinus* (Jacq.: Fr.) Karst. (J. Bjurman, 1988)

**Table 2: Effect of carbon and nitrogen concentration on carpogenesis by *Trametes versicolor*[^a^]**

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[^a^]: Carbon source, glucose; nitrogen source, ammonium tartrate.

— No basidiospores, + – abnormal fruiting without basidiospores, + up to 25% fruiting bodies and basidiospores on colony margin, ++ 25%-50% fruiting bodies and basidiospores.
and the brown-rot basidiomycete Gloeophyllum sepiarium (Wulf. Fr.) Karst. 163-BS-1 (J. B. JURMAN, 1984) were reported to be controlled by glucose and nitrogen limitation. Similarly, we found that carbon and nitrogen limitation controlled S. commune carpogenesis, which suggests that the regulatory mechanisms of S. commune are similar to those of these white-and brown-rot basidiomycetes.

Carpogenesis by T. versicolor is evidently governed by a different mechanism because it was controlled by nitrogen metabolic repression alone. At \( < 4\% \) glucose, nitrogen repression was not observed at the 2- to 5-mM nitrogen concentration. Higher concentrations of glucose (\( \geq 8\% \)) at any concentration of nitrogen repressed carpogenesis. Glucose concentrations of 5\% to 7\% were not examined. The effect of nitrogen repression on fruiting body formation and basidiospore production was the same in T. versicolor and S. commune.

F. velutipes easily formed fruiting bodies and produced an enormous quantity of basidiospores on MEA, PDA, or sawdust medium in 4 to 6 weeks, by which time the whole area of medium was colonized by mycelia. The occasional exposure of dark-grown cultures to light during daily monitoring was enough to induce fruiting bodies, but their caps were closed. The 12 h-light/12 h-dark cycle induced the formation of fruiting bodies with open caps and subsequently produced basidiospores. However, oidia could easily be produced from log-phase dikaryotic mycelia. C. T. INGOLD (1980) and R. F. O. KAMP (1980) found that aerial hyphae germinating from oidia that formed from dikaryotic mycelia of F. velutipes were homokaryons.

5. Summary

Basidiospores of wood-attacking basidiomycetes are a primary source of infection that causes wood deterioration. Most studies on the evaluation of wood preservatives have used mycelia or basidiospores obtained from wild mushrooms. The objective of this study was to develop in vitro methods that promote carpogenesis and basidiosporogenesis by the white-rot basidiomycetes Flammulina velutipes, Schizophyllum commune, and Trametes versicolor. After preincubation in the dark at 27 °C for 3 to 5 days, basidiospores were produced in 4 to 16 weeks in basidiomes exposed to light at 15 °C. Adequate light exposure, aeration, and low temperature treatment after preincubation were essential for fruiting body formation. For S. commune, carpogenesis and basidiosporogenesis were controlled by nitrogen and carbon limitation; for T. versicolor, carpogenesis was induced by nitrogen limitation. The best carbon source for carpogenesis and subsequent basidiosporogenesis was Walseth cellulose. Optimal conditions were established for carpogenesis and basidiosporogenesis of S. commune, T. versicolor, and F. velutipes. Methods were developed for producing a large number of basidiospores by these basidiomycetes in vitro and for obtaining homokaryotic oidia from the dikaryotic hyphae of F. velu-
tipes. The results demonstrate the feasibility of using oidia or basidiospores rather than mycelia to evaluate potential wood preservatives.

Zusammenfassung

Carpogonese und Basidiosporogenese in Flammulina velutipes,
Schizophyllum commune und Trametes versicolor in vitro


Résumé

Carpogénèse et basidiosporogénèse in vitro de Flammulina velutipes,
Schizophyllum commune et Trametes versicolor

Les basidiospores des basidiomycètes attaquant le bois sont une source primaire d'infection causant la détérioration du bois. La plupart des études sur l'évaluation des produits de préservation du bois utilisent le mycélium ou les basidiospores des champignons sauvages. L'objectif de cette étude était de développer des méthodes in vitro qui induisent la carpogénèse et la basidiosporogénèse chez les pourritures blanches Flammulina velutipes, Schizophyllum commune et Trametes versicolor. Après une pré-incubation dans le noir à 27 °C pendant 3 à 5 jours, les basidiospores ont été produites en 4 à 16 semaines dans les basides exposées à la lumière à 15 °C. Une exposition adéquate à la lumière, l'aération, et un traitement à basse température après pré-incubation ont été essentiels pour la formation des structures reproductives. Pour S. commune, la carpogénèse et la basidiosporogénèse ont été contrôlées par une source d'azote limitée. La meilleure source de carbone pour la carpogénèse et la subseqüente basidiosporogénèse a été la cellulose Walseth. Les conditions optimales ont été établies pour la carpogénèse et la basidiosporogénèse de S. commune, T. versicolor et F. velutipes. Les méthodes ont été développées pour pro-
duire un grand nombre de basidiospores in vitro et pour obtenir des oidies homokaryotiques à partir d’hyphes dicaryotiques de *F. velutipes*. Les résultats démontrent la possibilité d’utiliser des oidies ou des basidiospores au lieu du mycélium pour évaluer le potentiel des produits de préservation du bois.

References


2 Material und Organismen 31/1


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