Physiology and Biochemistry

Nutrition and Respiration of Basidiospores and Mycelium of *Pisolithus tinctorius*

Willard A. Taber and Ruth A. Taber

Professor and research scientist, respectively, Department of Biology and Department of Plant Sciences, Texas Agricultural Experiment Station, Texas A&M University, College Station 77843.

We thank David Lewis and Alan Hunter for locating fresh collections of puffballs from Texas.

Accepted for publication 22 June 1981.

ABSTRACT

Taber, W. A., and Taber, R. A. 1982. Nutrition and respiration of basidiospores and mycelium of *Pisolithus tinctorius*. Phytopathology 72:316-322.

Basidiospores of the mycorrhizal puffball, *Pisolithus tinctorius*, germinate rarely if at all in vitro, and this may be due at least in part to an inability to respire significantly until certain specific conditions are met. A low rate of respiration was detected by gas exchange and production of ^{14}C-CO_2 when approximately 15 mg of spores wetted with Tween-80 solution and suspended in phosphate buffer were exposed to ^{14}C-glucose for extended periods of time in the Warburg respirometer. Respiration was not stimulated by the presence of B vitamins, trace elements (including citrated iron), a mixture of vitamins and trace elements, or yeast extract. Respiration of exogenous glucose was low and was not accompanied by a marked increase in rate of respiration over endogenous respiration. Respiration was partially inhibited by a mixture of sodium azide and iodoacetate. To test the possibility that *P. tinctorius* mycelium might possess either the exotic growth requirements or limited carbohydrate metabolism of some other basidiomycetous mycorrhizal fungi, a study of nutritional requirements and growth patterns of mycelial cultures was undertaken. Mycelium respired glucose, mannose, mannitol, cellobiose, and trehalose, but grew well only on glucose and cellobiose of the several carbon sources examined. Growth occurred in cultures with nonionized succinic acid as the sole carbon source, but not on either ionized or nonionized acetic acid. Spores and mycelium both contained lipids and a similar array of nonreducing polyhydroxy compounds; the latter probably consisted of polyols and a disaccharide. One or more of these constituents may be the substrates for endogenous respiration.

Additional key words: ectomycorrhizae.

Pisolithus tinctorius (Pers.) Coker and Couch is a widely distributed puffball that forms mycorrhizal relationships with roots of a number of species of trees (11,26). Mycorrhizae enhance the uptake of certain nutrients from soil and also increase resistance to invasion by certain pathogens (19,21,29,35,49). The protection afforded by the mycorrhizal association in tree feeder roots against penetration by pathogenic fungi has been documented by numerous workers(5,6,9,12,15,16,20,22–24,28,33,34,41,48,49). The role of ectomycorrhizae as biological deterrents to infections by *Phytophthora*, *Pythium*, *Fusarium*, *Cylindrocladium*, and *Rhizoctonia* has been related to production of antibiotics by the fungal symbionts, physical presence of the hyphal mantle formed by the fungus, chemical inhibitors released by the host, and exudations produced as a result of the association (19). Some species of trees show little or no growth without a mycorrhizal association, even though other soil microorganisms also may play a role in this growth stimulation (3,45). *P. tinctorius* is a more effective mycorrhizal symbiont on certain trees than other naturally occurring mycorrhizal basidiomycetes (26); therefore, introducing this fungus into soils might result in the eventual increase in production of fuel and fiber (35). For example, incorporation of *P. tinctorius* into mine spoils is followed by the establishment of trees (35), and addition of spores (25) or cultured mycelium to fumigated nursery soils leads to the establishment of the mycorrhizal relationship with seedlings (27,29,30). The empirical addition of the fungus to soil can eventually lead to symbiotic growth; however, little is known of the biology of the fungus itself (46). Colonization may not always occur and it has been hypothesized that *P. tinctorius* sometimes colonizes the rhizoplane with difficulty because of the activities of other microorganisms (3). Other factors such as nutrition, physiology, spore germination, and formation of antibiotics (21,28), however,

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

0031-949X/82/03031607/$03.00/0
©1982 The American Phytopathological Society

might also be involved. Trappe (41) stressed the importance of careful selection of inoculum for ectomycorrhizal inoculation of nursery stock and discussed the need for developing the relatively simple and inexpensive inoculation techniques because of their great potential for improving plant growth and survival. Puffballs contain enormous numbers of basidiospores, but no in vitro method has been devised to determine spore viability. Spores germinate rarely, if ever, in vitro and a single report records a germination rate of only one in a thousand (4). A preliminary survey (*unpublished*) employing various enzymes, seedlings, and other factors verified the recalcitrant dormancy of *P. tinctorius* spores. Since the mycorrhizal relationship can be established from mycelial cultures, it is important to understand the nutrition and physiology of mycelia. Mycelial cultures derived from sporocarpic tissues have been grown on complex organic media containing thiamin (13,21,27,40), but the fungus has not been cultured on synthetic media and its nutritional requirements have not been defined. Many basidiomycetous mycorrhizal fungi can utilize only a few carbon sources for growth (32).

This research was conducted to determine: whether ungerminated basidiospores of *P. tinctorius* respire (10,46), if respiration can be used as an index of spore lot viability, if spores respond to exogenous nutrients or are constitutionally dormant, and some aspects of the nutritional pattern and requirements for mycelial growth.

MATERIALS AND METHODS

Spores and cultures. Two collections of basidiospores were used in this investigation: those from a puffball collected near College Station, TX, in 1978, and those from a puffball collected on the Lamar University Campus, Beaumont, TX, in January 1980. After the peridium was removed, spores were sifted through several layers of sterile cheesecloth and collected in a sterile petri dish. Spores were stored at 8 C (25). Several 20-mg samples of each spore lot were plated on glucose-peptone-yeast extract agar to determine the number of contaminants present.

Two mycelial isolates of *P. tinctorius* were also studied: Georgia isolate 246 was kindly supplied by D. H. Marx, Institute for Mycorrhizal Research, U.S. Forest Service, Southwest Forest Experiment Station, Athens, GA, and the Texas isolate, ATCC 42409, was isolated by the authors from sporocarp tissue of the puffball collected at Beaumont, TX. Cultures were maintained on Difco potato-dextrose agar enriched with 0.1 mg thiamine per liter. Even though the cultures were maintained under sterile mineral oil, transfers were necessary approximately every 4 mo to maintain viability.

Respiration. The consumption of oxygen and evolution of ^{14}C-CO_2 by basidiospores and mycelium were measured at 30 C in a Warburg respirometer (43) with air as the gas phase. Carbon dioxide evolution was measured by the direct method (43). Results were expressed as μl O_2 consumed, μl CO_2 evolved, Q_{O_2}, and as respiratory quotient (RQ). An aliquot of each radioactive glucose solution was combusted and the radioactivity was measured (37); the μmoles of glucose respired were calculated from the radioactivity in the respired CO_2. Respired CO_2 in the alkali-containing center well was collected, mixed CO_3, NH_4Cl, and $BaCl_2$. The $BaCO_3$ precipitate was collected on Tared S&S 589 Blue Ribbon filter paper (Schleicher and Schuell, Inc., Keene, NH 03431) and washed sequentially with water, acetone, and diethyl ether. After the precipitate was dried, radioactivity was counted as described previously (36,37) allowing for background, coincidence, self-absorption, and variation in machine performance. Radioactivity was counted in a Model 1152 Spectro/shield low-background gas-flow counter (Nuclear Chicago Corp., Des Plains, IL 60018). The final concentrations of sodium azide and iodoacetate were 10^{-2} and 10^{-3} M, respectively. The number of spores in each spore suspension was determined with the Petroff-Hausser bacteria counter, and the dry weight equivalent of spores was measured by drying aliquots of a given suspension at 100 C for 48 hr and weighing after the spores had cooled in a desiccator. Heat-killed *P. tinctorius* spores were added to some Warburg vessels of several earlier experiments to determine if the observed very low gas exchange values were artifacts due to systematic error in correcting for barometric pressure changes. The vessels also received ^{14}C-glucose.

Mycelium to be used in the respirometer was grown on the synthetic medium described below supplemented with 0.1 mg of thiamin HCl per liter. The glucose stock solution was autoclaved separately and 33 ml of the basal medium was dispensed to each cotton-plugged 125-ml Erlenmeyer flask. The cultures were incubated stationary at 28 C and briefly homogenized in a Waring Blendor the day before use.

The effects of vitamins, selected salts, and yeast extract on respiration were measured. The vitamins were used at the concentrations given below under the heading "Mycelial Growth." The salts were added at the following concentrations: $FeSO_4 \cdot 7H_2O$, 35 μM (with 35 μM citrate); $MgSO_4 \cdot 7H_2O$, 1.2 mM; $MnSO_4 \cdot H_2O$, 18 μM; $ZnSO_4 \cdot 7H_2O$, 15 μM; $CaCl_2 \cdot 2H_2O$, 41 μM; and $CuCl_2 \cdot 2H_2O$, 2.0 μM. Yeast extract was added at a rate of 30 mg per vessel.

For comparison, respiration of spores of the common fungus *Penicillium frequentans* was measured. Spores were treated the same as the *P. tinctorius* spores.

Mycelial growth. The ability of vegetative mycelium grown in synthetic medium to use various carbon and nitrogen sources for growth and to grow without the presence of added B vitamins was determined.

Vegetative mycelia to be used as inoculum sources were grown either on the synthetic medium described below or on 1% glucose-0.3% yeast extract broth. Mycelia were briefly homogenized in a Waring Blendor and washed with sterile distilled water by centrifugation. Approximately 1-mg of mycelial fragments was added to each cotton-plugged 125-ml Erlenmeyer flask, which contained 33 ml of liquid medium.

The basal medium contained the following constituents (grams per liter of water): NH_4NO_3, 1.0; KH_2PO_4, 2.5; K_2HPO_4, 2.0; $MgSO_4 \cdot 7H_2O$, 0.3; $FeSO_4 \cdot 7H_2O$, 0.005; $ZnSO_4 \cdot 7H_2O$, 0.0044; $MnSO_4 \cdot H_2O$, 0.003; $CaCl_2 \cdot 2H_2O$, 0.006; $CuCl_2 \cdot 2H_2O$, 0.00003; $CoCl \cdot 2H_2O$, 0.00002; NaCl, 0.0025; and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.0018. All carbohydrates except cellulose and starch were filter-sterilized and added to the basal medium to give 4 gram equivalents of carbon per liter of medium. Starch and cellulose (washed Whatman #1 filter paper) were autoclaved dry in 125-ml Erlenmeyer flasks to which sterile basal medium was added. The noncarbohydrate carbon sources, acetic acid (adjusted to pH 4) and acetate (pH 7), were filter-sterilized. Succinic acid (adjusted to pH 4.0) and potassium succinate (pH 6.8) were autoclaved.

D-glucose and sucrose (Fisher Scientific, Pittsburgh, PA 15219), D-mannose (Matheson, Coleman & Bell, Norwood, OH 45212), and trehalose (Sigma Chemical Co., St. Louis, MO 63178) were used without further treatment. Some other carbohydrate carbon sources received additional treatment for purification. Excess amounts of D-galactose and D-xylose (Sigma), mannitol (Matheson, Coleman & Bell), lactose and maltose (Difco Laboratories, Detroit, MI 48232), and cellobiose (both Difco and Sigma), were first dissolved in hot water and then either crystallized or precipitated in the cold (4–6 C) with or without the addition of ethanol. In addition, maltose and D-xylose solutions were decolorized with a minimum amount of charcoal before recrystallization. All contaminating pigments in maltose and xylose were removed with charcoal. The maltose remaining in solution after charcoal treatment was completely decolorized, but contained traces of two polyhydroxy compounds whose migration in paper chromatography (developed with the solvent system ethyl acetate:acetic acid:water [6:2:2, v/v]) suggested they might be a trisaccharide and a slightly larger oligosaccharide. The authenticity of cellobiose was further checked by paper chromatography and by using a Fisher-Johns melting-point apparatus. Some flasks of nonglucose medium also contained 0.5 g of supplemental glucose per liter of medium to allow sufficient growth to induce the utilization of other carbon sources, should that be genetically possible.

The nitrogen sources were added to nitrogen-free basal medium

at 0.7 g nitrogen per liter of medium. Urea was filter-sterilized and, where necessary, the final pH of the medium was adjusted to pH 6.5 with KOH. The nitrogen sources were NH_4NO_3, $NaNO_3$, urea, L-glutamic acid, and (Difco Bacto) peptone. The final medium contained glucose as the carbon source and the complete supplement of vitamins.

The cultures were incubated stationary at 28 C (14) in a PsycroTherm (New Brunswick Scientific Co., Edison, NJ 08817).

Growth responses to externally supplied B vitamins were measured by using the prototype medium containing glucose and ammonium nitrate. Both the Georgia and Texas isolates were examined. Selected data from several experiments are reported. Detection of an absolute thiamine requirement required two serial transfers through vitamin-free media. Possibly, thiamine was concentrated from the initial complete medium and was not readily washed from the mycelium. All vitamin stock solutions were sterilized by filtration through an ultrafine fritted-glass filter. Inositol was added to a final concentration of 1,000 μg/L and biotin and cobalamin to a concentration 10 μg/L each. Thiamine, pyridoxal, nicotinamide, folic acid, calcium pantothenate, and riboflavin each were added to final concentrations of 100 μg/L medium. Growth was expressed as dry weight of mycelium collected on tared Whatman #4 filter paper and dried at 97 C in an oven for 24 hr. [U-^{14}C]-Glucose was purchased from New England Nuclear, Boston, MA 02118. All other chemicals were of the highest grades available.

Primary shunt metabolites. The low rate of respiration of basidiospores that was observed in this study could be due to the lack of respirable primary shunt metabolites (38,39,44) such as lipids, polyols, or trehalose. It is postulated (7) that exogenous carbon sources are not utilized until first converted to endogenous nutrients. If that is true in this instance, low respiration in the presence of glucose might be due to poor conversion of exogenous glucose to one or more of these metabolites. Extraction procedures, assays, and paper chromatography were employed to reveal whether or not basidiospores and mycelium grown in vitro contained primary shunt metabolites. Various procedures were used, but only one is described below. Spores (500 mg) were ground in a mortar and pestle with 5 g of 200-μm glass beads and then extracted with petroleum ether (30 C boiling) followed by diethyl ether. The amount of lipid was measured gravimetrically. The defatted spores were treated with hot water. The aqueous extract was assayed for total polyols by using the periodate-chromotropic acid procedure and for carbohydrates by using the anthrone procedure (31). The extract was also examined by paper chromatography using Whatman #1 paper and the solvent system previously described herein. One sheet was sprayed with alkaline silver nitrate (42) and a duplicate sheet with aniline phthalate, which detects reducing sugars (2). The water extract was deionized and decolorized by treatment with Dowex 1 × 8 (Cl^-) and Dowex 50 × 8 (H^+) resins and polyols and oligosaccharides adsorbed to 6 g of charcoal. The compounds were eluted from the charcoal with 20% aqueous ethanol. Evaporation yielded crystals that were dissolved in a minimal amount of water and chromatographed as described above. Mycelium of the Georgia isolate grown in the glucose-ammonium nitrate synthetic medium was freeze-dried, oven-dried, ground briefly in a mortar and pestle (but without added glass beads), and treated as above.

RESULTS

Respiration. *Spores.* Basidiospores collected from puffballs contained a few contaminant microorganisms and it was necessary to determine if the numbers present might be sufficient to be a source of measurable respiration. The arithmetic mean and standard deviation of the number of contaminants in the College Station and Beaumont spore collections (based on platings) were 169 ± 64 and 173 ± 105 cells per milligram of *P. tinctorius* spores (~10^8 spores), respectively. Three of the most common contaminants were isolated, grown on a glucose-peptone-yeast extract medium, and examined in the respirometer for detectable respiration at concentrations equivalent to the total number of contaminant cells in the spore lots of *P. tinctorius*. That is, a washed suspension of cells from pure culture of each strain was prepared and an amount of each suspension giving a number of cells equivalent to that present in the *P. tinctorius* suspension was added to designated Warburg vessels. No respiration was detected,

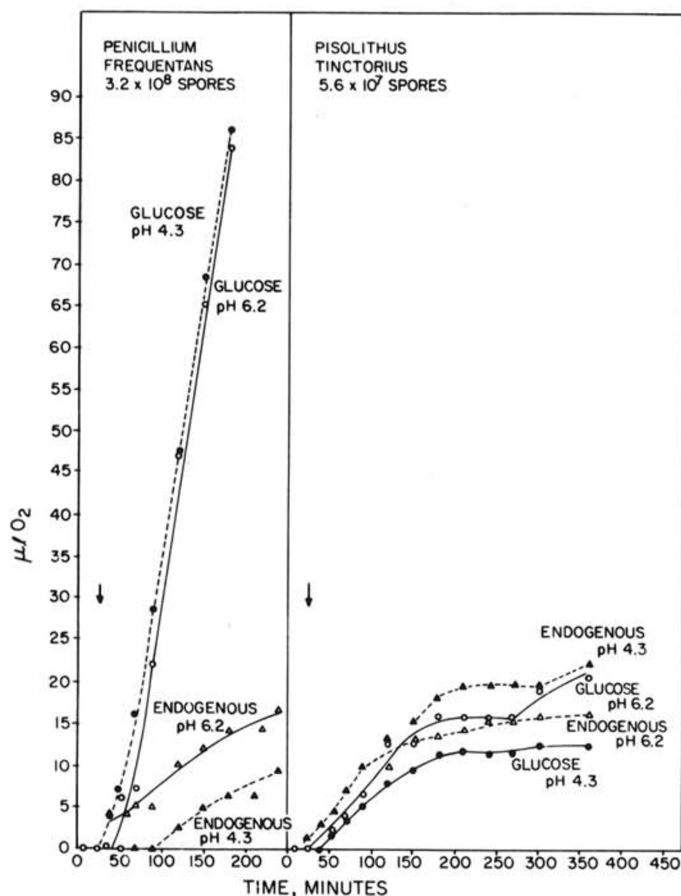

Fig. 1. Oxygen consumption by spores of *Pisolithus tinctorius* and *Penicillium frequentans* in the presence and absence of exogenous glucose. Each Warburg vessel received 7 mg (dry weight equivalent) of spores of *P. tinctorius* (from College Station, TX) or 4.4 mg (dry weight equivalent) of *Penicillium frequentans* spores.

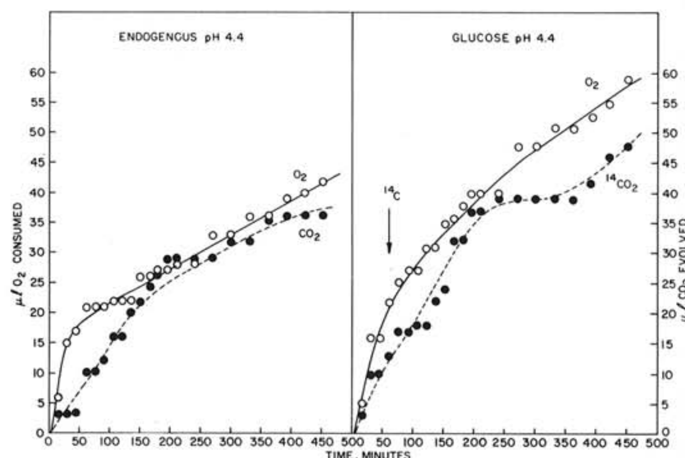

Fig. 2. Consumption O_2 and evolution of CO_2 in the presence and absence of exogenous glucose by spores of *Pisolithus tinctorius*. Arrow indicates time that glucose was introduced into the main compartment. Gas exchange attributed to respiration of added glucose is calculated by substracting the volume of gas in the left hand square from the values in the right hand square. Each flask received 16 mg (dry weight equivalent) of spores from College Station, TX.

even after 5 hr of incubation and no $^{14}C-CO_2$ evolution was detected, indicating that these few contaminants were not the source of the respiration observed when incubating *P. tinctorius* spores in the respirometer. For an additional assessment of detectability of respiration by small numbers of cells, respiration of spores of the common fungus, *Penicillium frequentans*, was measured (Fig. 1). Extrapolation from this respiration rate downward to a concentration of spores 100 times that of all contaminants in the puffball spore lots revealed that the value (0.11 μl O_2) would be below that detectable by the respirometer.

Oxygen uptake by *P. tinctorius* spores over a period of 5.5 hr (Fig. 1) was very low; the $Q_{O_2} = 0.3$. Unlike the response of a typical fungus, *P. frequentans* ($Q_{O_2} = 7$), the rate of respiration did not change following introduction of the exogenous substrate. The observed low level of oxygen consumption was not an artifact attributable to systematic error in correcting for barometric changes in the absence of respiration, however, because several experiments included flasks charged with heat-killed spores and ^{14}C-glucose, and no gas exchange or formation of $^{14}C-CO_2$ was recorded for these flasks. If the slight manometric changes interpreted as O_2 uptake were not artifacts, then CO_2 evolution should also be measurable. Evolution of CO_2 was detected (Fig. 2) and the RQ for endogenous respiration (left panel) approximated 1, which is characteristic of carbohydrate respiration. Carbon dioxide respired in the presence of ^{14}C-glucose was radioactive (right panel). The presence of $^{14}C-CO_2$ in the alkaline solution of the center well of flasks for various experiments receiving unheated spores established beyond doubt that respiration had occurred. Respiration was not very sensitive to pH; respiration rates were similar at pH 4.4 and 6.5. After 480 min of incubation in glucose at pH 4.4 and 6.5, spores had consumed 48 and 43 μl O_2, respectively. In some of the Warburg flasks, ^{14}C-glucose was introduced after 420 min of incubation to determine if longer soaking in aerated buffer would predispose spores to increased rate of uptake and/or respiration of glucose. No increase in rate of O_2 uptake occurred within 60 min of this late introduction of glucose. Based on radioactivity of respired CO_2 at both pH values, 20 mg of spores respired 0.015 of 36 μmoles of added glucose in 7.5 hr.

In a separate experiment *P. tinctorius* spores took up 37 μl O_2 during 420 min in the presence of glucose and 16 μl in the presence of glucose and a mixture of sodium azide and iodoacetate. Inhibition was not absolute; however, spores respiring endogenously consumed 18 μl O_2. The inhibitors may have blocked exogenous respiration, but not endogenous respiration.

Respiration was not stimulated by the presence of: (a) added B vitamins, (b) a mixture of trace elements, (c) a mixture of (a) and (b), or (d) added yeast extract (Fig. 3).

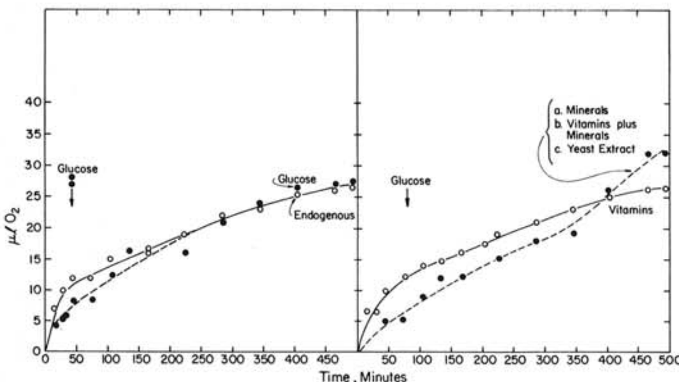

Fig. 3. Oxygen uptake of spores of *Pisolithus tinctorius* in the presence and absence of the indicated supplements. The vitamin supplement contained the seven vitamins listed under Materials and Methods. The mineral supplement gave final concentrations as follows: $FeSO_4$, 35 μM (with 35 μM sodium citrate); $MgSO_4$, 1.2 mM; $MnSO_4$, 18 μM; $ZnSO_4$, 15 μM; $CaCl_2$, 41 μM; and $CuCl_2$, 2 μM. Yeast extract was added at a rate to give final concentration equivalent to 10 g per liter of fluid. Only the flasks designed to measure endogenous respiration did not receive glucose at the time indicated by the arrow. Each flask received 10 mg (dry weight equivalent) of spores from College Station, TX.

Mycelium. Respiration was demonstrated in mycelium grown in synthetic medium and was higher on a weight basis than that of spores. Oxygen uptake was stimulated by the presence of glucose, trehalose, mannose, mannitol, and cellobiose, but not by galactose and sucrose (Fig. 4). These data suggest that the first five carbon sources were respirable by mycelium of *P. tinctorius*. Based on radioactivity in respired CO_2 and in glucose, 0.415 μmole of glucose was respired by 6 mg (dry weight equivalent) of mycelium after 4 hr of incubation.

Growth. *Vitamin requirements*. In one experiment, the effect on growth of adding B vitamins singly and in various combinations was measured using as the inoculum mycelium that had been grown in YD broth (1% glucose and 0.3% yeast extract) and subsequently washed four times with sterile distilled water after brief homogenization in a Waring Blendor. Only limited growth occurred in the absence of thiamine (Table 1). In subsequent experiments, the inoculum source was grown on the complete medium described under "Materials and Methods." When the mycelium to be used as the inoculum source was washed with water the day before the experiment, stored overnight in water at 8 C, and washed four times again the next day, 1.05 g (dry weight equivalent) of mycelium was produced per liter of medium in the presence of thiamine and 0.2 g in its absence. A third such experiment produced similar results, but when fresh vitamin-free media and thiamine-containing media were inoculated with fragments of mycelium from the above negative control, growth occurred only in the presence of thiamine. Thus, it appears that an absolute requirement for exogenous thiamine exists, and that when it is present from previous culture conditions it is not readily washed out of the mycelium.

Carbon sources. Of the carbon sources examined, only glucose

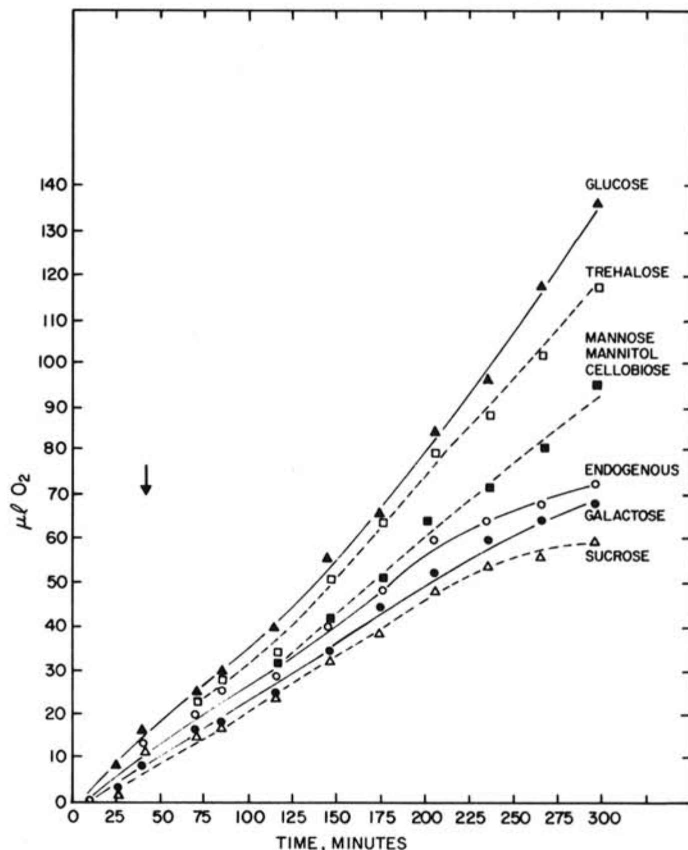

Fig. 4. Oxygen uptake in the presence of various sugars and mannitol by mycelium of *Pisolithus tinctorius*. Uptake of mannose, mannitol, and cellobiose is represented by one line. Each flask received the equivalent of 5.6 mg dry weight of mycelium. Radioactivity was present only in glucose. Carbon sources were introduced at the time indicated by the arrow. Georgia isolate.

and, unexpectedly, cellobiose favored abundant growth (Table 2). Growth on glucose was similar whether glucose was autoclaved separately from the remainder of the medium or sterilized by filtration through an ultrafine fritted-glass filter (data from an earlier harvest period). Abundant growth on crystallized cellobiose (two sources) but not on cellulose, starch, maltose, sucrose, or trehalose would appear to be an anomaly, but both strains of *P. tinctorius* reacted similarly on repetitions of the test. Paper chromatography of cellobiose did not reveal any contaminating carbohydrates. The trace amounts of contaminating carbohydrates in decolorized and recrystallized maltose previously described probably would not support the trace of growth observed on the maltose carbon source (Table 2); therefore, it is concluded that maltose is used, although poorly. Under the conditions of the experiment, the small concentrations of glucose added to allow sufficient protein synthesis to induce the enzymes required for the utilization of the other carbon sources did not lead to the eventual utilization of these other carbon sources (Table 2). Growth occurred on nonionized succinic acid, but not on ionized succinic acid or on either nonionized or ionized acetic acid (Table 2).

TABLE 1. Effect of vitamins on growth of Georgia isolate (#246) of *Pisolithus tinctorius* mycelium after 9 days

Vitamin added	Dry wt (g/L)[a]	Final pH
All[b]	2.2 ± 0.1	8.1
None[c]	0.8 ± 0.1	6.0
Biotin	0.9 ± 0.9	6.1
Thiamine	2.2 ± 0.1	7.8
Biotin + thiamine	2.2 ± 0.01	7.9
Biotin + thiamine + citrate	2.3 ± 0.01	8.1
Biotin + thiamine + pyridoxal + nicotinamide + riboflavin + B_{12}	2.2 ± 0.1	7.9
Pyridoxal	0.9 ± 0.01	6.2
Nicotinamide	1.0 ± 0.04	6.2
Riboflavin	1.0 ± 0.06	6.1

[a] Growth expressed as arithmetic mean and standard deviation of dry weight of mycelium from three flask cultures.
[b] Biotin, thiamin, pyridoxal, nicotinamide, folic acid, pantothenate, riboflavin, B_{12}, inositol, and citrate chelator. See text for concentration.
[c] In a separate experiment, limited growth also occurred in the medium to which no vitamins had been added, but when vitamin-free medium was inoculated with washed mycelium from these cultures, no growth occurred.

TABLE 2. Growth of Georgia isolate (#246)[a] of *Pisolithus tinctorius* mycelium on various carbon sources for 25 days

Carbon source	Dry wt (g/L)	Final pH
Glucose	4.6 ± 0.1	4.3
Mannitol	0.6 ± 0.02	5.6
Mannitol + glucose	1.2 ± 0.02	5.4
Trehalose	0.04	6.1
Trehalose + glucose	Trace	6.0
Sucrose	Nil	6.0
Sucrose + glucose	Trace	6.0
Maltose	0.09 ± 0.01	6.1
Maltose + glucose	1.2 ± 0.1	4.8
Cellobiose	3.02 ± 0.1	3.0
Cellobiose + glucose	3.2 ± 0.1	3.0
Lactose	Nil	6.1
Lactose + glucose	0.3	5.8
Starch	Trace	6.0
Starch + glucose	Trace	5.8
Cellulose	Nil	6.1
Cellulose + glucose	Trace	6.0
Galactose	Nil	6.1
Galactose + glucose	Trace	6.0
Xylose	Nil	6.1
Xylose + glucose	Trace	6.0
Succinic acid	1.3 ± 0.01	3.5
Succinate	Nil	7.2
Acetic acid	Nil	4.2
Acetate	Nil	6.7

[a] A comparison of Georgia and Texas isolates on several carbon sources revealed no significant differences in growth.

Nitrogen sources. P. tinctorius can utilize ammonium ion, nitrate ion, urea, and peptone as nitrogen sources although not with equal facility (Table 3). Glutamate is very slowly utilized and this cannot be attributed to a change in pH brought about as a result of utilization.

Primary shunt metabolites. Neither spores nor mycelium grown in vitro contained detectable levels of reducing sugars, although both did contain at least four nonreducing polyhydroxy compounds (Fig. 5 and Table 4), which could serve as substrates for endogenous respiration or as endogenous nutrients for growth (7). The constituent that chromatographed as a disaccharide (Fig. 5) quite likely is the nonreducing trehalose so common in fungi (38,39,44). The constituent that chromatographed as a 6-carbon nonreducing compound may be mannitol, which also is very common in fungi (17,38). Experiments are in progress to isolate and identify these products. Both spores and mycelium contain lipids that are extractable in petroleum ether and diethyl ether; spores contain twice as much lipids as the mycelium (Table 4). Endogenous respiratory substrates other than lipids or carbohydrates can occur in basidiomycetes (18), and consequently, additional differences between spores and mycelium may exist. Similarly, the principal endogenous respiratory substrate may not be a polyol or an oligosaccharide (46).

DISCUSSION

Basidiospores of *P. tinctorius* collected and stored as described herein possessed a low, but detectable, endogenous and exogenous respiration. The existence of respiration was confirmed by exposing spores to ^{14}C-glucose and isolating ^{14}C-CO_2 from the vessel atmosphere. The maximum Q_{O_2} was 0.3 whereas typical values for fungi range from 5 to 50. In this study, the Q_{O_2} observed for spores of a representative fungus, *Penicillium frequentans*, was 7 and for mycelium of *P. tinctorius* was 5 based on glucose oxidation and 10 based on total oxygen uptake. Exogenous respiration was low; 0.0003 μmole (of 36 μmoles present) of glucose was respired per milligram (dry weight equivalent) of spores per hour with no increased rate of respiration following the addition of glucose. In contrast, 0.017 μmole of glucose was respired per milligram of mycelium (dry weight equivalent) per hour. Inhibition

TABLE 3. Growth of Georgia isolate (#246) of *Pisolithus tinctorius* mycelium on various nitrogen sources

Nitrogen source	Dry wt. (g/L)	Final pH	Days
NH_4NO_3	3.1 ± 0.7	2.9	30
$NaNO_3$	0.9 ± 0.1	6.4	30
Urea	0.6 ± 0.02	6.4	30
Peptone	1.5 ± 0.3	6.1	30
Glutamate	0.5 ± 0.1	6.0	103

TABLE 4. Some constituents of a Texas isolate of *Pisolithus tinctorius* mycelium and spores

	Percent dry weight	
Constituent[a]	Mycelium	Spores
Lipids	5.3	13.0
Polyols[b]	1.6	1.5
Polysaccharides (nonreducing)* and disaccharides	5.7	1.8
Residual solids	60.0	...[c]

[a] Lipids were measured gravimetrically, polyols were expressed as mannitol equivalents, and polysaccharides as trehalose equivalents (nonreducing oligosaccharide). See text for assay procedures.
[b] Other constituents of the hot water extract were not measured. No reducing sugars were present.
[c] Spores were ground with glass beads in a mortar and pestle so residual solids were not measurable.

of respiration of glucose and subsequent ^{14}C-CO_2 formation by a mixture of azide and iodoacetate, and absence of ^{14}C-CO_2 in vessels charged with heat-killed spores established that gas exchange was due to respiration and was not an artifact of the procedure. Azide and iodoacetate did not completely inhibit respiration, but reduced it to the rate exhibited by endogenously-respiring spores. The inhibitors may have completely inhibited glucose respiration, but not the respiration of an endogenous substrate. Alternatively, respiration may be only partially inhibited by these inhibitors. Some fungi have a cyanide-resistant respiratory path (8). Limited respiration may have been due to limited uptake of glucose, oxygen, water, or to limited efflux of natural inhibitors. The spores are hydrophobic and thick-walled (1) and influx or efflux may be difficult. Prolonged exposure to liquid did not favor increased respiration; suspending wetted and washed spores in buffer for 7 hr before adding ^{14}C-glucose did not result in increased respiration. Conceivably, respiration was limited by insufficient amounts of B vitamins, trace elements, or various constituents found in yeast extract. Increased respiration rates did not follow the addition of these nutrients to the spores, however. It is possible that the low rate of respiration observed to date is due to suppression by constitutional dormancy and that an appropriate factor can release this suppression.

Nutrition of mycelial cultures of *P. tinctorius* is unique in that very few carbon sources can be utilized for growth. Small concentrations of glucose added to favor induction of relevant enzymes did not permit eventual utilization of carbon sources that were not used when when they were present as sole carbon sources. The inability to use a variety of carbon sources may be a trait of mycorrhizal basidiomycetes (32). These strains resemble some *Claviceps* spp. in growing readily on nonionized succinic acid, but not on ionized succinate (36,37) and in not growing on either form of acetic acid. The significance, if any, of these patterns to the establishment and adaptation either fungus is not readily apparent. Otherwise, these two strains of *P. tinctorius* appear to possess conventional nutritional patterns. That is, they can utilize several nitrogen sources for growth and require an external source of thiamine.

Spores and mycelium of *P. tinctorius* both contain primary shunt metabolites (38,39) such as lipids, polyols, and a nonreducing disaccharide. In view of the known differences between spores and mycelium of a given fungus, differences might be expected here. These preliminary studies (Table 4 and Fig. 5) suggest some differences, but also a similarity in kinds of nonreducing polyhydroxy compounds. One or more of these constituents (Table 4) could be the substrate of endogenous respiration (47). Studies are in progress to determine if these compounds are metabolized. Polyols could be mainly hydrogen sinks formed from sugars in oxidation of NADH; thus, their presence would not necessarily predict their use in respiration. Alternatively polyols in mycorrhizal fungi may play a specific role in regulation of the carbohydrate enzymes of the tree roots (47) and may not be involved in fungal metabolism as such.

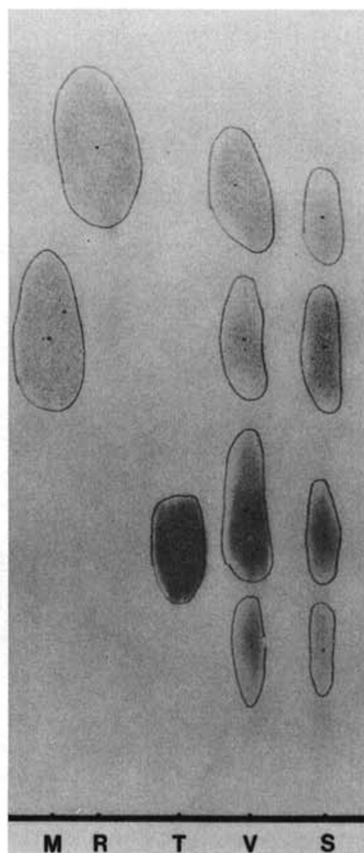

Fig. 5. Polyhydroxy compounds in mycelium and spores of *Pisolithus tinctorius*. Freeze-dried in vitro-grown mycelium (2.6 g) and defatted spores (0.5 g) ground with 200-μm glass beads were treated with hot water. The aqueous extracts were treated with Dowex 1 (Cl^-) and Dowex 50 (H^+) and the polyhydroxy compounds remaining in the water were adsorbed to charcoal then eluted with 20% aqueous ethanol. Aliquots were chromatographed along with reference standards on Whatman #1 paper by using the solvent system ethyl acetate:acetic acid:water (6:2:2, v/v). The paper was sprayed with alkaline silver nitrate (42). The letter M refers to mannitol, R to ribose, T to trehalose, V to vegetative phase mycelium extract, and S to spore extract. Spores and culture were from a Beaumont, TX, sporocarp.

LITERATURE CITED

1. Alexopoulos, C. J., and Mims, C. W. 1979. Introductory Mycology. John Wiley & Sons, New York. 632 pp.
2. Anonymous. 1960. Chromatography. E. Merck AG, Darmstadt, Germany. 185 pp.
3. Bowen, G. D., and Theodorou, C. 1979. Interactions between bacteria and ectomycorrhizal fungi. Soil Biol. Biochem. 11:119-126.
4. Bulmer, G. S. 1964. Spore germination of forty-two species of puffballs. Mycologia 56:630-632.
5. Corte, A. 1969. Research on the influence of the mycorrhizal infection on the growth, vigor, and state of health of three *Pinus* species. Arch. Bot. Biogeogr. Ital. 45:1-11.
6. Davis, W. C., Wright, E., and Hartley, C. 1942. Diseases of forest tree nursery stock. U.S. Civ. Cons. Corps, Forest Publ. 9.
7. Dawes, E. A., and Ribbons, D. W. 1962. The endogenous metabolism of microorganisms. Annu. Rev. Microbiol. 16:241-265.
8. Edwards, D. L., and Unger, B. W. 1978. Cyanide and hydroxamate-resistant respiration in *Neurospora crassa*. J. Bacteriol. 133:1130-1134.
9. Garrett, S. D. 1960. Biology of root-infecting fungi. Cambridge Univ. Press, New York. 393 pp.
10. Gottlieb, D. 1978. The germination of fungus spores. Meadowfield Press, London. 165 pp.
11. Grand, L. G. 1976. Distribution, plant associates, and variation in basidiocarps of *Pisolithus tinctorius* in the United States. Mycologia 68:672-678.
12. Hacskaylo, E. 1967. Mycorrhizae: Indispensible invasions by fungi. Agric. Sci. Rev. 13-20.
13. Hile, N., and Hennen, J. F. 1969. In vitro culture of *Pisolithus tinctorius* mycelium. Mycologia 61:195-198.
14. Hung, L-L., and Chien, C. Y. 1979. Two new mycorrhizal syntheses: *Pisolithus tinctorius* and *Suillus bovinus* with Taiwan red pine. Mycologia 71:202-206.
15. Krywolap, G. N., Grand, L. F., and Casida, L. E., Jr. 1964. The natural occurrence of an antibiotic in the mycorrhizal fungus *Cenococcum graniforme*. Can. J. Microbiol. 10:323-328.
16. Levisohn, I. 1954. Aberrant root infections of pine and spruce seedlings. New Phytol. 53:284-290.
17. Lewis, D. H., and Smith, D. C. 1967. Sugar alcohols (polyols) in fungi and green plants. I. Distribution, physiology and metabolism. New Phytol. 66:143-184.
18. Li, S-M. 1980. Endogenous respiration of *Polyporus sulphureus*. Mycologia 72:64-72.
19. Marks, G. C., and Kozlowski, T. T., eds. 1973. Ectomycorrhizae—Their Ecology and Physiology. Academic Press, New York. 444 pp.
20. Marx, D. H. 1966. The role of ectotrophic mycorrhizal fungi in the resistance of pine roots to infection by *Phytophthora cinnamoni* Rands. Ph.D. dissertation. North Carolina State University, Raleigh.

179 pp.
21. Marx, D. H. 1969. The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infection. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathology 59:153-163.
22. Marx, D. H. 1972. Ectomycorrhizae as biological deterrents to pathogenic root infections. Annu. Rev. Phytopathol. 10:429-454.
23. Marx, D. H. 1973. Mycorrhizae and feeder root diseases. Pages 351–382 in: G. C. Marks and T. T. Kozlowski, eds. Ectomycorrhizae—Their Ecology and Physiology. Academic Press, New York. 444 pp.
24. Marx, D. H. 1973. Field observations relating mycorrhizae to decreases in diseases of feeder roots. Pages 353–354 in: G. C. Marks and T. T. Kozlowski, eds. Ectomycorrhizae—Their Ecology and Physiology. Academic Press, New York. 444 pp.
25. Marx, D. H. 1976. Synthesis of ectomycorrhizae on loblolly pine seedlings with basidiospores of *Pisolithus tinctorius*. For. Sci. 22:13-20.
26. Marx, D. H. 1977. The host range and world distribution of the ectomycorrhizal fungus, *Pisolithus tinctorius*. Can. J. Microbiol. 23:217-223.
27. Marx, D. H., and Bryan, W. C. 1975. Growth and ectomycorrhizal development of loblolly pine seedlings in fumigated soil infested with the fungal symbiont, *Pisolithus tinctorius*. For. Sci. 21:245-254.
28. Marx, D. F., Bryan, W. C., and Cordell, C. E. 1967. Growth and ectomycorrhizal development of pine seedlings in nursery soil infested with the fungal symbiont, *Pisolithus tinctorius*. For. Sci. 22:91-100.
29. Marx, D. H., and Davey, C. B. 1969. The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. III. Resistance of aseptically formed mycorrhizae to infections by *Phytophthora cinnamomi*. Phytopathology 59:549-558.
30. Marx, D. H., Morris, W. G., and Mexal, J. G. 1978. Growth and ectomycorrhizal development of loblolly pine seedlings in fumigated and non-fumigated nursery soil infested with different fungal symbionts. For. Sci. 24:193-203.
31. Neish, A. C. 1952. Analytical methods for bacterial fermentations. Nat. Res. Counc. Can. Rep. 46-8-3, 2nd revision. 69 pp.
32. Palmer, J. G. 1971. Techniques and procedures for culturing ectomycorrhizal fungi. Pages 132–144 in: E. Hacskaylo, ed. Mycorrhizae. Proc. First N. Am. Conf. on Mycorrhizae, April 1969. U.S. Dep. Agric. Misc. Publ. 1189. Washington, DC.
33. Powell, W. M., Hendrix, F. F., and Marx, D. H. 1968. Chemical control of feeder root necrosis of pecans caused by *Pythium* species and nematodes. Plant Dis. Rep. 52:577-578.
34. Richard, C., Fortin, J. A., and Fortin, A. 1972. Protective effect of an ectomycorrhizal fungus against the root pathogen *Mycelium radicis atrovirens*. Can. J. For. Res. 1:246-251.
35. Ruehle, J. L., and Marx, D. H. 1979. Fiber, food, fuel and fungal symbionts. Science 206:419-422.
36. Taber, W. A. 1973. Uptake and metabolism of succinic acid by washed mycelium of *Claviceps purpurea*. Mycologia 65:558-579.
37. Taber, W. A. 1976. Uptake and efflux of succinic acid by uninduced mycelium of *Claviceps purpurea*. Can. J. Microbiol. 22:245-253.
38. Taber, W. A., and Taber, R. A. 1978. Ascomycetes. Pages 97–205 in: A. I. Laskin and H. A. Lechevalier, eds. CRC Handbook of Microbiology. CRC Press, West Palm Beach, FL. 874 pp.
39. Taber, W. A., and Vining, L. C. 1963. Physiology of alkaloid production by *Claviceps purpurea* (Fr.) Tul. Correlation with changes in mycelial polyol, carbohydrate, lipid, and phosphorus-containing compounds. Can. J. Microbiol. 9:1-14.
40. Tan, K. H., Sinanowth, P., and Todd, R. L. 1978. Formation of humic acid-like compounds by the ectomycorrhizal fungus, *Pisolithus tinctorius*. Soil Sci. Soc. Am. J. 42:906-908.
41. Trappe, J. M. 1977. Selection of fungi for ectomycorrhizal inoculation in nurseries. Annu. Rev. Phytopathol. 15:203-222.
42. Trevelyan, W. E., Proctor, D. P., and Harrison, J. S. 1950. Detection of sugars on paper chromatograms. Nature 166:444.
43. Umbreit, W. W., Burris, R. H., and Stauffer, J. F. 1972. Manometric methods. Burgess Publ. Co., Minneapolis, MN. 387 pp.
44. Vining, L. C., and Taber, W. A. 1964. Analysis of the endogenous sugars and polyols of *Claviceps purpurea* (Fr.) by chromatography on ion exchange resins. Can. J. Microbiol. 10:647-657.
45. Vozzo, J. A. 1971. Field inoculations with mycorrhizal fungi. Pages 187–196 in: E. Hacskaylo, ed. Mycorrhizae. Proc. First N. Am. Conf. on Mycorrhizae, April 1969. U.S. Dep. Agric. Misc. Publ. 1189. Washington, DC.
46. Weber, D. J., and Hess, W. M., eds. 1976. The Fungal Spore. John Wiley & Sons, New York. 985 pp.
47. Wedding, R. T., and Harley, J. L. 1976. Fungal polyol metabolites in the control of carbohydrate metabolism of mycorrhizal roots of beech. New Phytol. 77:675-689.
48. Wingfield, E. B. 1968. Mycotrophy in loblolly pine. I. The role of *Pisolithus tinctorius* and *Rhizoctonia solani* in survival of seedlings. II. Mycorrhizae formation after fungicide treatment. Ph.D. dissertation. Virginia Polytechnic Inst., Blacksburg, VA.
49. Zak, B. 1964. Role of mycorrhizae in root disease. Annu. Rev. Phytopathol. 2:377-392.