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The influence of nutrients on development, resting hyphae and aleuriospore induction of *Thielaviopsis basicola*

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Abstract: Two isolates of *Thielaviopsis basicola,* one from tobacco and one from cotton, were grown in crude host root extracts at varied dilutions. Aspects of hyphal morphology including hyphal diameter, hyphal length, and degree of branching were positively correlated with the level of available nutrients under these conditions. Atypical hyphal forms were produced by *T. basicola* under conditions of nutrient stress, and these forms were similar to two cultural mutants previously reported. Secondary chlamydospores also were observed under nutrient stress conditions. Hyphal segments that possessed cytological and morphological features analogous to endoconidia and aleuriospores of *T. basicola* were observed during later stages of culture development under nutrient conditions favorable for growth and reproduction. These structures were termed "resting hyphae" because *T. basicola* grew from them upon restoration of nutrients when other regions of hyphae were no longer viable. Natural and simulated depletion of nutrients in the culture environment following a period of vegetative growth induced aleuriospore production. Development of aleuriospores under these culture conditions demonstrated that *T. basicola* is able to produce a substantial number of reproductive structures from nutrient reserves held within the existing thallus.

Key Words: *Chalara elegans,* chlamydospore, hemibiotroph

INTRODUCTION

Thielaviopsis basicola (Berk. and Broome) Ferraris (synanamorph *Chalara elegans* Nag Raj and Kendrick) is a soilborne, plant-pathogenic fungus commonly found in cultivated and noncultivated soils (Yarwood, 1974; 1981). *T. basicola* has a wide host range (Otani, 1962) and significantly impacts production of such diverse plants as cotton, beans, carrot, pansy, peanut, and tobacco.

Development of *T. basicola* on a susceptible host involves differentiation of specialized infection structures (Hood and Shew, 1996a, b) and production of various hyphal forms and spore types characteristic of this fungus (Christou, 1962; Koch, 1936; Stover, 1950a). Host factors associated with the formation of infection structures have been investigated (Hood and Shew, 1997), but our understanding of factors that affect hyphal morphology and spore production is limited. Culture variants commonly develop in cultures of isolates of *T. basicola* obtained from different geographic regions and different hosts (Haung and Patrick, 1971; Johnson and Valleau, 1935; Rawlings, 1940; Stover, 1950a, b). Development of variant growth forms has been associated with factors of the culture environment (Punja, 1992; Stover, 1950a), but more often such development by *T. basicola* has been attributed to genetic changes that occur during growth in culture (Haung and Patrick, 1971; Rawlings, 1940; Stover, 1950a, b).

Aleuriospores (chlamydospores) are considered to be the primary overwintering propagules of *T. basicola* (Tsao and Bricker, 1966) and have been the subject of considerable investigation. Production of aleuriospores has served as a measure of pathogen fitness (Corbaz, 1971; Tosi and Zazzerini, 1991), and their structural details and nutritional requirements for production and germination have been described (Clough and Patrick, 1976; Mathre and Ravenscroft, 1966; Patrick et al., 1965; Stover, 1956; Tsao and Bricker, 1970; Tsao and Tsao, 1970). Factors that induce differentiation of aleuriospore from vegetative hyphae have not been reported.

The current study was conducted to elucidate how growth and differentiation of *T. basicola* are influenced by interactions with a natural nutrient source. Production of vegetative hyphae and reproductive structures in dilutions of crude host root extracts are reported and results discussed in reference to the ecology of the fungus.

MATERIALS AND METHODS

Fungal isolates, culture maintenance, and nutrient sources.—Two isolates of *T. basicola* were used

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throughout this study, including isolate SH-2 from tobacco (*Nicotiana tabacum* L.) and isolate COT-10 from cotton (*Gossypium hirsutum* L.). Each isolate was obtained from diseased roots of their respective host (Shew and Meyer, 1992). The isolates differed slightly in colony morphology, but each was pathogenic and typical of wild-type isolates of *T. basicola* from their respective hosts.

Cultures were maintained on 5% carrot agar (50 mL canned carrot extract [Hollywood Carrot Extract, Pet Inc., St. Louis] and 18 g agar/L deionized water) in the dark at 22–26 C. Endoconidia were harvested by washing 5 to 7-d-old colonies of *T. basicola* from plates with 2 mL of sterile deionized water. The endoconidia were trapped on a sterile 0.2 μm syringe filter (Nalgene, Rochester, New York), rinsed with 10 mL of sterile deionized water, and then resuspended in sterile deionized water. The concentration of endoconidia was determined with a hemacytometer and diluted with sterile deionized water as needed.

Isolates of *T. basicola* were grown in root extract from their respective hosts. Seedlings of tobacco (Burley 21 × Kentucky 10 [courtesy of F. W. Rickard Seeds, Winchester, KY]) and cotton (Delta Pine 50 [courtesy of Novartis, Greensboro, NC]) were grown under greenhouse conditions. Roots of 1-mo-old seedlings of each host were rinsed free of soil particles in deionized water, cut to ca. 1 cm lengths, and blotted twice between several layers of filter paper to remove excess water. Two to 4 g of root pieces were packed into a 5-mL disposable syringe (needle removed) and pressed to exhaustion with the syringe plunger. The root pieces were pressed three times in such a manner, with removal, spreading, and repacking of root pieces between pressings. The resulting root extract was filtered twice through sterile 0.2 μm syringe filters (Nalgene) and diluted with sterile deionized water as needed. Extract dilutions ranged from 10^{-1} to 10^{-5} in 10-fold increments and included sterile deionized water as an extract-free control.

Hyphal morphology.—Endoconidia of *T. basicola* were germinated in extract dilutions on glass microscope slides, which were cleaned and autoclaved prior to use. Twenty μL of root extract of each dilution and sterile deionized water were applied to the slides (three separate sites per slide) and 2 μL of spore suspension (ca. 10 endoconidia/μL) were added to each site. Slides were incubated in 100% humidity in the dark at 22–26 C for 24 h. Specimens then were fixed by rapidly drying the slides on a slide warmer at 60 C and applying a drop of lactophenol and a cover slip to each application site. Slides were examined under phase-contrast microscopy for determination of hyphal diameter and under brightfield microscopy for hyphal length and degree of branching (number of apexes). Images of germinated endoconidia were captured through the microscope via a high-resolution, color-video camera (Sony CCD IRIS). Measurements were determined with the Image-Pro Plus 1.3 image analysis program (Media Cybernetics, Silver Spring, MD), and the data were exported to SigmaPlot (Jandel Corp., San Rafael, CA) for compilation. Five germinated spores were quantified per application site, treatments were replicated four times per run, and the experiment was conducted twice with the tobacco isolate of *T. basicola.* Data were analyzed by the GLM procedure of SAS (SAS Institute, Cary, NC USA) and combined across replications and runs of the test when possible. Significant differences in the analysis of variance were determined by F-tests with $P<0.01$, and by Waller-Duncan *K*-ratio test ($K=100$).

Hyphal length and degree of branching (number of apexes) of the tobacco isolate also were quantified at 60 and 120 h after inoculation in the manner described above. Quantification at these later time intervals was performed only at the 10^{-4} and 10^{-5} extract dilutions and in sterile deionized water.

Hyphal morphology also was determined during prolonged growth in dilutions of root extract. Two μL of a spore suspension (ca. 10 endoconidia/μL) were added to each of the wells of a 24-well plate (Becton Dickinson, New Jersey). Each well also contained 1 mL of diluted root extract (10^{-1} to 10^{-5} in 10-fold increments) or sterile deionized water. The plates were incubated in the dark at 22–26 C. Fungal development was observed daily for 14 d using an inverted microscope. After 14 d, extract dilutions were removed and replaced with a 10^{-1} dilution, and fungal development was observed for an additional 2 d. Some specimens were removed from the wells and examined by other microscopy methods, including tissue clearing (autoclaved in 1 M KOH for 15 min at 121 C) and differential interference contrast microscopy. Each treatment was replicated eight times per run in a completely randomized design. The experiment was run twice with both isolates of *T. basicola.* Quantitative data were not collected from this experiment, rather the morphological features of fungal structures were recorded.

Aleuriospore induction.—Two μL of spore suspension (ca. 3 endoconidia/μL) were added to each well of a 96-well plate (Corning, New York); each well also contained 200 μL of 10^{-1} root extract. The plates were incubated in the dark at 22–26 C. All wells were scanned 24 h after inoculation to determine the number of germinated endoconidia, and only wells that contained five to seven germinated endoconidia

were selected for use. The root extract in the wells was removed 72 h after inoculation by aspiration and replaced with fresh root extract at dilutions ranging from 10^{-1} to 10^{-5} in 10-fold increments; one well per replication was not amended. The replacement of the 10^{-1} root extract with a range of further dilutions effectively decreased the concentration of nutrients available to *T. basicola*. The number of aleuriospores present per well was determined 24 h after induction (root extract replacement) using an inverted microscope. The cultures in nonamended wells were monitored daily to determine when aleuriospore production began in this treatment. Treatments were replicated four times per run and assigned in a completely randomized design. The experiment was run twice with both isolates. Data were analyzed by the GLM procedure of SAS (SAS Institute) and combined across replications and runs of the test when possible. Significant differences in the analysis of variance were determined by F-tests with $P<0.01$.

Time-course documentation of aleuriospore development following induction also was completed. Aleuriospore induction was performed as described above, except that sterile deionized water was used to replace the initial root extract dilution, and observations were conducted between 10 and 24 h after induction. Six replicate inductions were performed over time for this purpose, with multiple aleuriospore formations observed in each replication.

RESULTS

Hyphal morphology.—Hyphal diam, length, and degree of branching were positively influenced by the concentration of root extract ($P<0.01$ for each) (FIG. 1). Hyphal diam at or below the 10^{-4} dilution of extract did not differ significantly from hyphal diam in sterile deionized water alone. However, hyphal diam significantly increased from ca. 1.5 μm at the 10^{-4} dilution to 4 μm at the highest concentration of root extract (10^{-1}). Total hyphal length differed between runs of the test, but treatment effects were similar. Hyphal length at the lowest extract concentrations (10^{-3}, 10^{-4}, and 10^{-5}) after 24 h of incubation was not significantly different from hyphal length in sterile deionized water alone. Hyphal length significantly increased from 300 μm at the 10^{-3} dilution to 3700 μm at the highest level of available nutrients. The degree of branching by *T. basicola* also was affected by the concentration of extract; no significant change in hyphal branching was observed below the 10^{-3} dilution, but the number of apexes increased from 2 at the 10^{-3} dilution to 21 at the 10^{-1} dilution. Hyphae in extract dilutions of 10^{-4} and 10^{-5}, and in the absence of extract, remained unbranched through 60

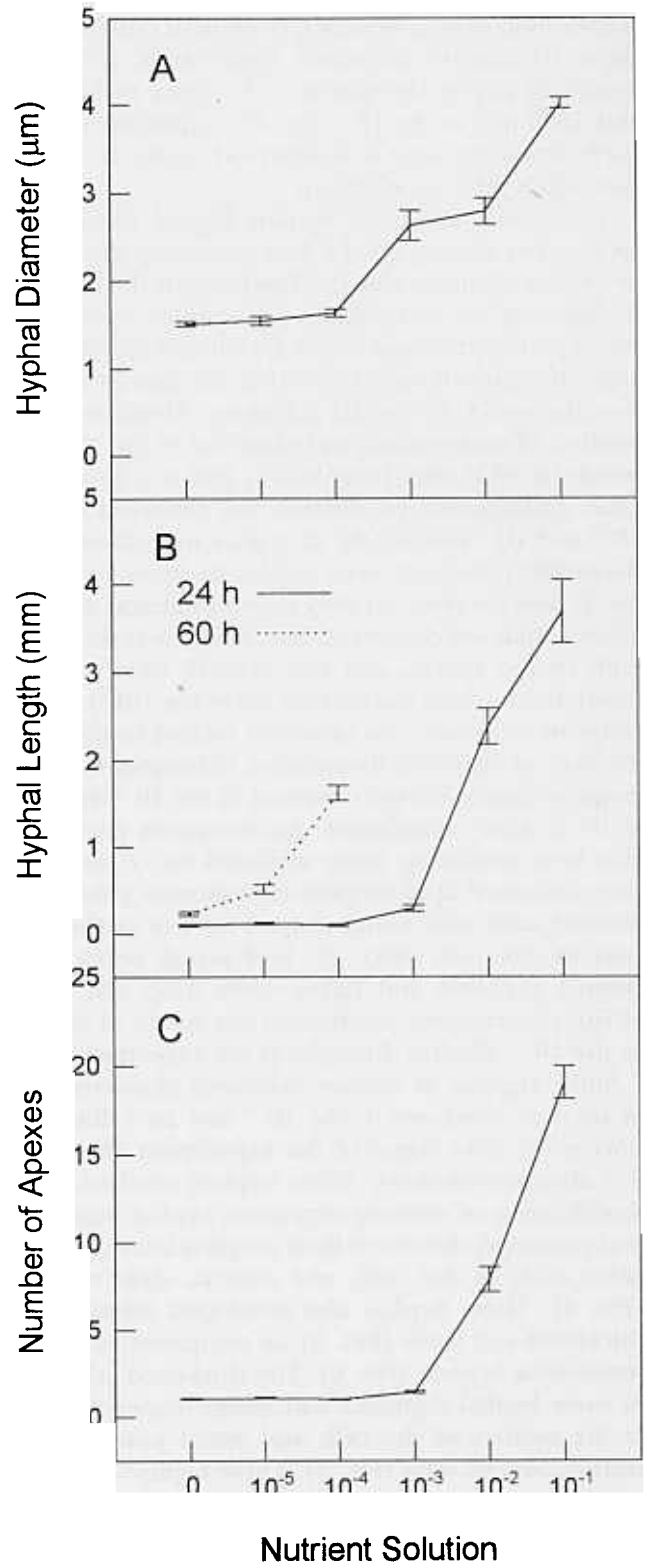


FIG. 1. Quantification of hyphal morphology of *Thielaviopsis basicola* after growth in a series of root-extract dilutions. Concentration "0" = sterile deionized water alone. Error bars = SE. A. Hyphal diam after 24 h; combined runs are shown. B. Hyphal length after 24 and 60 h; run 1 is shown. C. Degree of branching (number of apexes) after 24 h; combined runs are shown.

h after inoculation; however, the length of hyphae in these treatments increased significantly ($P<0.01$) from 230 μm in the absence of extract to 520 μm and 1630 μm at the 10^{-5} and 10^{-4} dilutions respectively. Branches were first observed in the 10^{-4} dilution 120 h after inoculation.

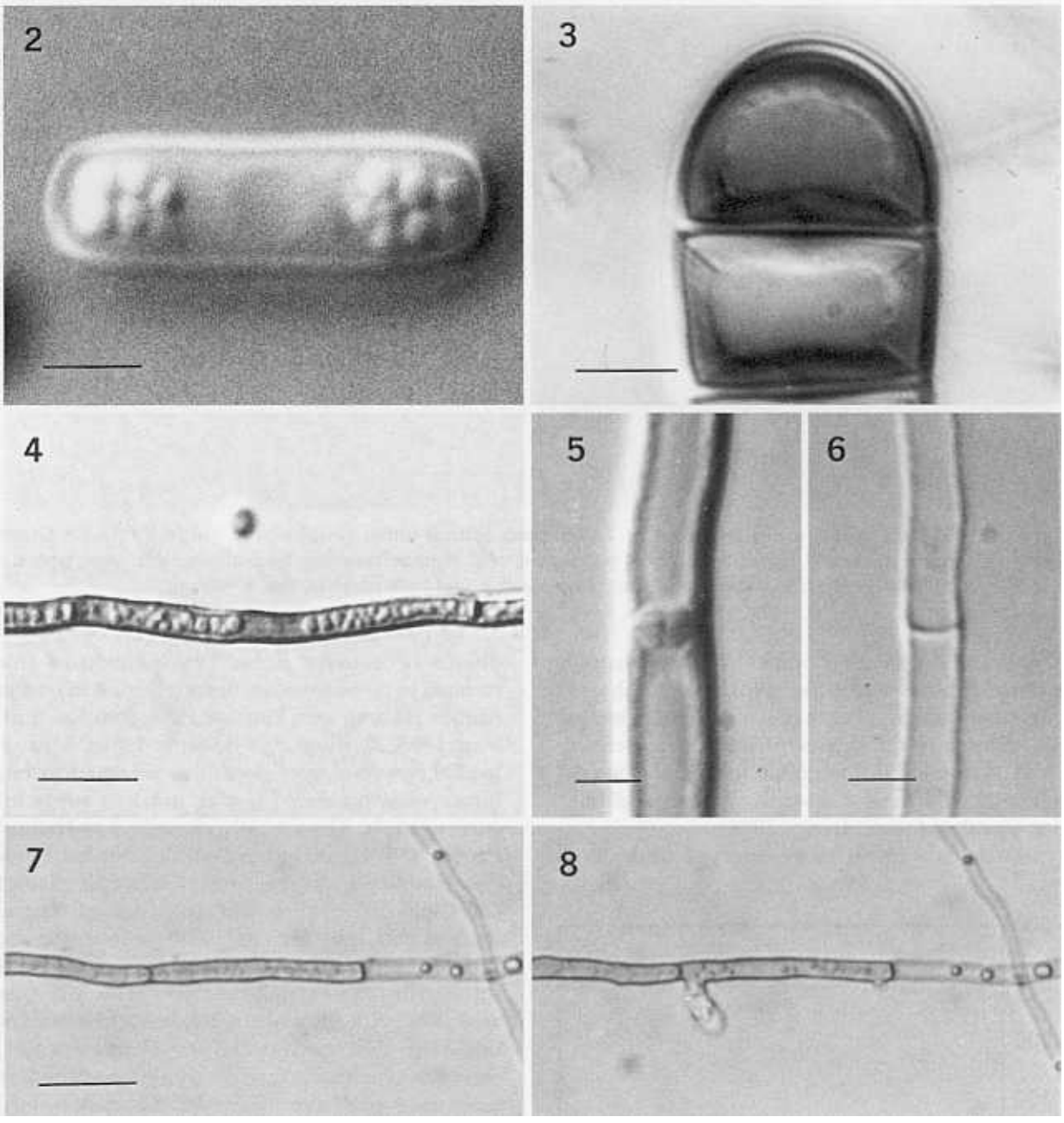
T. basicola developed various hyphal forms and spore types during the 14 d of growth in the range of extract dilutions tested. Observations did not differ between the two isolates, replications, or runs of the experiment except where specifically noted. Only vegetative growth was observed in the first 24 h after inoculation in all extract dilutions. Abundant production of endoconidia was observed in the 10^{-1} dilution by 48 h after inoculation, and to a lesser degree, endoconidia production was observed in the 10^{-2} and 10^{-3} dilution by 72 h after inoculation. Endoconidia produced were similar to those reported for *T. basicola* (FIG. 2); they were cylindrical and hyaline, contained clusters of lipid droplets at the polar ends of the spores, and had central, clear regions where the nucleus was located (Brierley, 1915). Aleuriospore formation was observed earliest in the 10^{-3} dilution, at 72 h after inoculation. Subsequently, aleuriospore formation was observed in the 10^{-2} dilution at 96 h after inoculation. Aleuriospores produced also were similar to those reported for *T. basicola*; they contained lipid droplets (not shown), thick, pigmented walls with wedge-shaped fissures at the corners of the cells (FIG. 3), and septal pores (not shown) (Christos and Baker, 1970; Tsao and Tsao, 1970). Aleuriospore production was sparse or absent in the 10^{-1} dilution throughout the experiment.

Some regions of hyphae exhibited characteristics of survival structures in the 10^{-3} and 10^{-2} dilutions during the later stages of the experiment (from ca. 9 d after inoculation). These hyphae resulted from modification of existing vegetative hyphal segments and possessed clusters of lipid droplets located at the polar ends of the cells, and central, clear regions (FIG. 4). These hyphae also developed substantially thickened cell walls (FIG. 5) as compared to undifferentiated hyphae (FIG. 6). The thickened cell walls of these hyphal segments had wedge-shaped fissures at the corners of the cells and septal pores in the contiguous cell walls (FIG. 5). These hyphae resumed growth upon restoration of nutrient availability 14 d after inoculation (FIG. 7, 8), while other regions of hyphae did not grow. Growth occurred at points along the length of the cell and gave rise to hyphae that were typical of *T. basicola*. During growth, the clusters of lipid droplets became disorganized and individual droplets migrated into the growing regions of hyphae.

Development of *T. basicola* often was atypical at low levels of nutrient availability. Some branches produced in the 10^{-4} dilution, 120 to 144 h after inoculation, were thicker than their parent hypha (FIG. 9). These branches matured over 48 to 72 h and gave rise to hyphal segments of uniformly thick diameters that often terminated in a single or pair of pigmented cells that resembled aleuriospore segments (FIG. 9). Upon restoration of nutrient availability 14 d after inoculation, hyphae that were typical of *T. basicola* grew from the terminal cell, as well as subtending cells. These structures were observed with both isolates of *T. basicola,* but they were more common and more often had terminal, pigmented cells with the tobacco isolate than with the cotton isolate. In the 10^{-4} dilution, hyphae were produced that exhibited irregularly swollen and bulbous cells (FIG. 10). This hyphal morphology was equally common across isolates and was observed to a lesser extent in the 10^{-3} dilution. Upon restoration of nutrient availability 14 d after inoculation, further growth was not observed. Secondary chlamydospores, described by Stover (1950a), were common in the 10^{-4} and 10^{-5} dilutions.

Aleuriospore induction.—A decrease in the level of available nutrients following 3 d of vegetative growth induced *T. basicola* to produce a significantly greater amount of aleuriospores than nondecreasing nutrient controls ($P<0.01$); the magnitude of the decrease in nutrients was positively correlated with the abundance of aleuriospores produced (FIG. 11). Aleuriospore formation was not induced by replacement of the nutrient environment with root extract of equal concentration; however, by 24 h after replacement with the 10^{-5} dilutions, the mean number of aleuriospores per well was 250 with the tobacco isolate and 50 with the cotton isolate. Aleuriospore production was absent or sparse in the nonamended (n.a.) control treatments 24 h after induction; only 0 and 3 aleuriospores per well were observed for the tobacco and cotton isolates. Substantial aleuriospore production in nonamended wells did not begin until 48 h after induction.

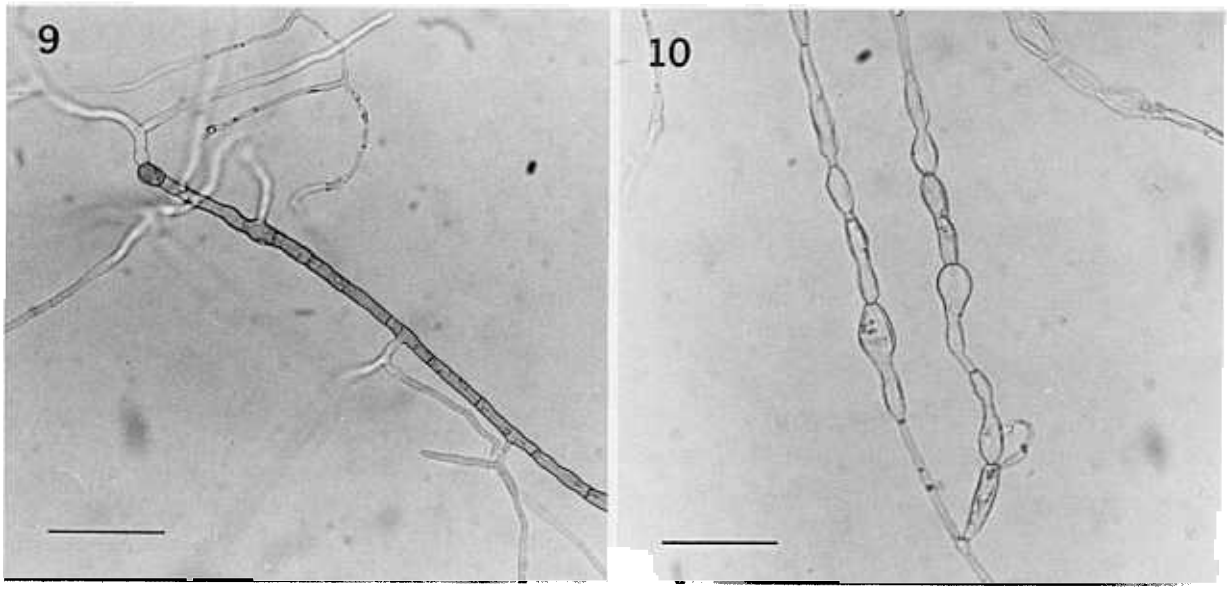
Differentiation of aleuriospores began 12 to 18 h after induction and occurred terminally and on short branches. Initial morphological differentiation consisted of a slight swelling of the vegetative apex (time 0 for initiation). A septation was formed basal to the swelling 20 min after initiation. The distal cell continued to swell, and 140 min after initiation an additional septation was formed within the cell. The distal-most segment of the developing aleuriospore continued to enlarge and successive septations were formed, resulting in a chain of 3 to 5 segments within 300 min of initiation. Pigmentation of the aleuriospore segments was complete within 12 h of initiation.



FIGS. 2–8. Spores and resting hyphae produced by *Thielaviopsis basicola* under nutrient concentrations favorable for growth and reproduction. 2. Conidia. Bar = 3 μm. 3. Cleared specimen of aleuriospore showing thickness of spore wall with wedge-shaped fissures at corners of cells. Bar = 4 μm. 4. Resting hypha showing clusters of lipid droplets at either end of cell with central, clear region. Bar = 20 μm. 5. Cleared specimen of resting hypha showing thickness of cell wall with wedge-shaped fissures at corners of cells and septal pore. Bar = 6 μm. 6. Similarly cleared specimen of hypha segment not differentiated into resting hypha. Bar = 6 μm. 7 and 8. Growth of resting hypha upon exposure to nutrient source (root extract). Bar = 30 μm.

DISCUSSION

The nutritional status of the rhizosphere or host environment should be considered a primary factor in determining hyphal morphology of *T. basicola* during parasitic establishment. Previous studies of *T. basicola* have associated patterns of hyphal morphology with conditions that influence nutrient availability. Hood and Shew (1997) reported differences between the diam of hyphae growing from carrot agar plugs and germ tubes of endoconidia, Linderman and Toussoun (1967) reported variation in germ tube diam and branching characteristics in soil amended with carrot juice and unamended controls, and Baard and Laubscher (1985) and Linderman and Toussoun



FIGS. 9, 10. Atypical hyphal forms produced by *Thielaviopsis basicola* under conditions of nutrient stress. 9. Growth of hyphae from mature thickened branch with distal pigmented cell. Hyphae resulting from this growth were typical of *T. basicola*. Bar = 50 μm. 10. Hyphae consisting of irregularly swollen and bulbous cells. Bar = 40 μm.

(1968) reported increased branching of hyphae on the surface of host roots subsequent to establishment of infection courts. In the current study, hyphal length, diam, and the degree of branching were influenced by the level of available nutrients. This developmental pattern of *T. basicola* agrees with similar studies on other fungi (Trinci, 1984).

Atypical hyphal forms were observed under conditions of nutrient stress. Development of cultural variants by *T. basicola* has been reported in numerous studies (Haung and Patrick, 1971; Johnson and Valleau, 1935; Rawlings, 1940; Stover, 1950a, b), and two hyphal morphologies previously reported as mutant forms were produced under nutrient stress in the current study. The variants, described by Haung and Patrick (1971), included (i) thick hyphal branches that contained a single or pair of distal, pigmented cells, and (ii) hyphae that consisted of irregularly swollen and bulbous cells. The genetic changes of variants observed by Haung and Patrick (1971) likely resulted in stress conditions similar to low nutrient availability. In addition, secondary chlamydospores, originally described by Stover (1950a), were produced under nutrient stress conditions. These structures were originally reported in association with bacterial antagonism (Stover, 1950a) and may reflect conidial development of *T. basicola* under unfavorable conditions.

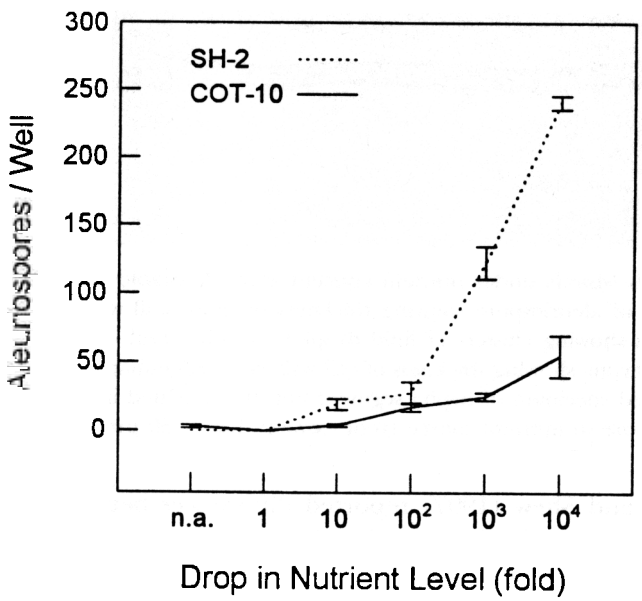


FIG. 11. Aleuriospore induction of isolates of *Thielaviopsis basicola* by a decrease in the concentration of available nutrients; n.a. indicates nonamended treatment. Error bars = SE. Combined runs are shown.

Hyphal segments that exhibited features analogous to spore types of *T. basicola* were produced under treatments conducive to growth and reproduction of the fungus. These hyphal segments contained clusters of lipid droplets located in either end of the cells and central, clear regions. This arrangement of cellular components is similar to that of endoconidia (Brierley, 1915). Also, these hyphal segments possessed substantially thickened cell walls with wedge-shaped fissures at the corners of the cells and septal pores that are analogous to morphological features of aleuriospores (Christos and Baker, 1970; Tsao and

Tsao, 1970). In addition, regrowth from these differentiated hyphal segments upon restoration of nutrients occurred when the remaining regions of hyphae were no longer viable. The thick-walled hyphal segments reported in the current study are perhaps best termed "resting hyphae". However, they are described by the definition of chlamydospores: viable, asexually produced spores, resulting from structural modification of existing hyphal segments that possess an inner, secondary wall (Griffith, 1974; Hawksworth, 1995). Confusion may arise concerning the description of resting structures and spores of *T. basicola* because the pigmented, overwintering propagules often are referred to as chlamydospores (Shew and Meyer, 1992) in addition to aleuriospores (i.e., Barnett and Hunter, 1987; Baard and Laubsher, 1985; Mauk and Hine, 1988; Punja, 1992; Tsao and Bricker, 1970). For this reason, the pigmented, overwintering propagules of *T. basicola* have been referred to as aleuriospores in this study: terminal, often thick-walled spores similar to true conidia (Griffith, 1974). This terminology may be more appropriate because aleuriospores, unlike chlamydospores, are not formed by modification of existing hyphal segments. Tsao and Bricker (1970) discussed terminology for aleuriospores based upon time-course documentation of differentiation. Aleuriospore differentiation in the current study was analogous to the description provided by Tsao and Bricker (1970), but with the additional detail that the initial swelling of the hyphal apex occurred before the structure was basally delimited by a septation.

The ecological role of resting hyphae of *T. basicola* has not been determined, and their occurrence in soil deserves investigation. Their production in vitro was during the later stages of culture development, generally after resources within the thallus available for aleuriospore production had been depleted. It is possible that the resting hyphae represent a consolidation of resources within the remaining living hyphae.

The effect of nutrient depletion on aleuriospore production provided evidence that these spores are formed in response to a decrease in the level of available nutrients in the environment, and this hypothesis was further supported by the aleuriospore induction experiment. Earliest production of aleuriospores occurred in the 10^{-3} dilution and later in the 10^{-2} dilution. The lack of aleuriospore production in the 10^{-1} dilution possibly is due to the accumulation of staling products during rapid fungal growth at this high concentration of nutrients. Also, *T. basicola* had the ability to produce a substantial number of aleuriospores solely from nutrient resources held within the existing thallus, which was observed when the nutrient solution was replaced with the lowest concentration of root extract or sterile deionized water. This observation may have important implications to understanding the ecology of *T. basicola,* particularly concerning the mechanisms of nutrient acquisition.

The nutritional classification of *T. basicola* is that of a hemibiotroph (Hood and Shew, 1996b, 1997; Nan et al., 1992). After an initial biotrophic phase of parasitism, further development and reproduction by hemibiotrophs occurs in necrotic tissue (Luttrell, 1974). This later development in necrotic tissue has been considered evidence of necrotrophic nutrient acquisition from the host (Luttrell, 1974). During pathogenesis by *T. basicola,* aleuriospore production begins several days after infection, which corresponds with the death of host cells in the infection court. However, the current study demonstrated that aleuriospore production may occur in the absence of additional nutrient acquisition and provides evidence that nectrotrophic behavior is not required by *T. basicola* to produce substantial numbers of aleuriospores.

The aleuriospore induction method developed in this study may be a valuable tool for experimental manipulation of *T. basicola.* Aleuriospores constitute the primary inoculum of *T. basicola* (Tsao and Bricker, 1966) and therefore represent an important stage in development of the organism. For example, disease severity is dependent on the initial population of *T. basicola* (Holtz and Weinhold, 1994; Meyer et al., 1989; Shew and Shoemaker, 1993). In addition, these propagules constitute an individual's genetic contribution to the next generation, and how an individual responds to factors responsible for aleuriospore induction will significantly influence its fitness. An improved understanding of the environmental and biotic factors that influence aleuriospore induction also may provide for novel approaches to the development of methods or compounds for control of *T. basicola* and black root rot in the field. Furthermore, by timing treatment exposures concurrent with induction, effects on aleuriospore production can be investigated without the confounding influence of treatments on prior vegetative growth.

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