Effects of Separation of Spores from the Chain and of Culture Age on Germination of Chlamydospores of Thielaviopsis basicola

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ABSTRACT

Intact chains of chlamydospores of *Thielaviopsis basicola* were segregated from endoconidia and mycelial fragments by repeated centrifugation in 1% K_2HPO_4, and the individual chlamydospores of the chains separated from each other by chitinase. Washed, separated chlamydospores from 6- to 12-week-old cultures did not require exogenous nutrients for germination. They germinated either in deionized water (76-91% germination) or in the presence of nutrients (79-94% germination). Treatments which caused partial or complete separation of the chlamydospore chains increased the germinability of the chlamydospores in deionized water. Individual, young chlamydospores from 3- to 4-week-old cultures germinated less well than older spores in deionized water, but both spores germinated equally well in nutrients. The interacting effects of chain separation, age, and nutrients may reflect differences in the structure of the spore walls. Phytopathology 60:891-896.

Chlamydospores are the major resistant propagules of the root pathogen, *Thielaviopsis basicola* (Berk. & Br.) Ferr., in soil (12, 14, 20), although some endoconidia survive for relatively long periods (11, 12). Patrick et al. (16) demonstrated that chlamydospore chains (13, 15, 20). Separation of the cells in the upon exposure to soil. They suggested that either chain separation or the rupturing of the outer wall of the spore was a prerequisite for germination, although germination also occurs while the spores are still held in chains (13, 15, 20). Separation of the cells in the chlamydospore chain can be accomplished in vitro by chitinase (4). A problem associated with physiological and ecological studies of chlamydospores of *T. basicola* is the segregation or isolation of these propagules from a mixed suspension containing endoconidia and mycelial fragments, and several methods are available to accomplish this (1, 11, 13). This paper reports a new method for producing and harvesting chlamydospores aseptically and the effects of chain separation, chitinase treatment, and culture age on in vitro germination of chlamydospores of *T. basicola* in water and in nutrients. A preliminary report of portions of this work has been published (7).

MATERIALS AND METHODS.—*Isolates.*—In most experiments, isolate T35 from citrus was used. For comparative studies two additional isolates, T491 from cotton and T492 from tobacco, were included. Isolates T491 and T492 were supplied by T. A. Toussoun.

Production and harvesting of chlamydospores.—The culture medium (Difco potato-dextrose agar plus 0.2% yeast extract) in 9-cm petri dishes was inoculated with 1 ml of a suspension of endoconidia, and the dishes were rotated to ensure uniform coverage of the agar surface. A disc of cotton gauze was then placed in close contact with the agar surface by smoothing with a bent glass rod, and the dishes were incubated at 25 C in the dark. Chlamydospores began to form uniformly over the dish within 3-4 days, but maximum yields were obtained only after 4-6 weeks. Generally, 6- to 8-week-old gauze cultures were used to prepare a chlamydospore suspension. Each gauze piece, with adhering mycelium and spores, was removed from the agar surface and transferred to 200 ml of water in a capped bottle or other suitable container. The cultures were washed in three 200-ml changes of water by shaking for 5 min on a Burrell wrist action shaker. This treatment removed most of the endoconidia. The chlamydospore-bearing mycelium was removed from the wet gauze cultures by stretching them out on a large (140 mm diam) petri dish bottom and scraping with a rubber policeman. The scraped mycelium was suspended in water and blended for 1-2 min in a Waring Blendor to dislodge the chlamydospore chains from the mycelium.

Chlamydospores can be segregated from endoconidia and mycelial fragments by repeated low-speed centrifugation. The efficiency of this process is affected by the specific gravity of the suspending medium. Centrifugation in a 1% (w/v) solution of K_2HPO_4 (sp gr about 1.01) proved superior to other solutions tested. The blended suspension containing all three types of propagules (Fig. 1-A) was centrifuged six times in 1% K_2HPO_4 for 30 sec at 100 *g*. Virtually all the mycelial fragments and endoconidia were removed in the supernatant, leaving a pellet composed mainly of intact chlamydospore chains. The chlamydospore chains were then washed by centrifugation for 1 min at 1,400 *g* in three 10-ml changes of water and finally resuspended in 5 ml of water (Fig. 1-B). All operations were carried out under aseptic conditions with sterile deionized water or sterile solutions.

Separation of chlamydospore chains into individual spores.—A modification of the method developed by Christias & Baker (4) was used to separate spore chains into individual spores or cells. The 5-ml suspension of chlamydospore chains was added to 5 ml of 0.2 M potassium phosphate buffer (pH 7.0) containing 2 mg of a chitinase preparation (Calbiochem). The mixture was then incubated at 25 C for 24 hr, during which time the spores did not germinate. The suspension was then blended for 1 min in a Waring Blendor and washed by centrifugation in three 10-ml changes of water. The final suspension consisted primarily of individual chlamydospores (Fig. 1-C).

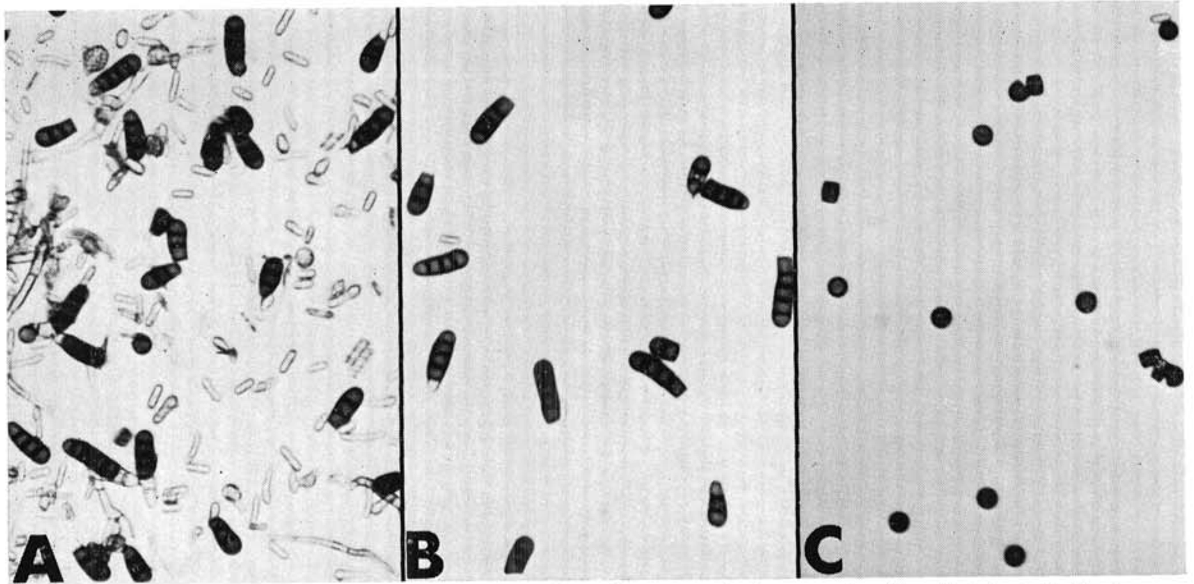

Fig. 1. Chlamydospores of *Thielaviopsis basicola* at the various stages in the procedure for obtaining individual spores. **A)** A starting suspension containing endoconidia, mycelial fragments, and intact chlamydospore chains. **B)** Chlamydospore chains after six centrifugations of the mixed suspension in 1% K_2HPO_4, showing the drastic reduction in the concentrations of endoconidia and mycelial fragments. **C)** Individual chlamydospores separated from the chains after chitinase and blending treatments.

Germination in vitro.—All tests were conducted in sterilized, acid-washed glass microbeakers (19 mm inside diam, 5 mm deep at edges and 4 mm deep at center). The volume of the combined test solutions and spore suspension was standardized at 0.4 ml (1 mm in depth at center), and the number of spores at 2,000/microbeaker. Unless otherwise stated, all tests were run for 24 hr at 25 C and were terminated by adding, to each beaker, two drops of a phenolic rose bengal solution (rose bengal, 1 g; $CaCO_3$, 50 mg; 5% phenol, 100 ml) which stained the germ tubes red in addition to killing the spores. To prevent evaporation loss, the microbeakers were placed in 15 × 140 mm circular moist chambers during incubation.

Maximum germinability of chlamydospores was determined in a 0.5% carrot juice preparation made from a commercial canned carrot juice (Diamond A brand) similar to the method of Linderman & Toussoun (12). Most particulate matter was removed from the juice by centrifuging for 5 min at about 1,400 *g*. The cleared juice was then diluted 1 in 5 with water and autoclaved for 15 min at 121 C. Final concentrations were prepared from this 20% stock. All germination data were recorded as an average per cent germination based on counts of 100 propagules in each of three replicate microbeakers. For tests with intact chlamydospore chains, a single unbroken chain was regarded as the propagule unit; for separated chlamydospores, the individual chlamydospore cell was regarded as the propagule unit. A spore was considered as germinated when the length of the germ tube surpassed the width of the germ tube.

RESULTS.—*Segregation of chlamydospores from other propagules.*—The centrifugation procedure with 1% K_2HPO_4 provided adequate yields of chlamydospores relatively free from endoconidia and mycelial fragments, although there was a loss of all three types of propagules. In a typical experiment, the procedure reduced mycelial fragments by more than 1,000-fold, endoconidia by 425-fold, and chlamydospore chains by only 10-fold. The efficiency of this segregation procedure is illustrated in Fig. 1-A, B. When two gauze cultures were used as starting material, as in most experiments, the yield of chlamydospore chains was usually between 1-2 million, with an average ratio of chlamydospore chains to endoconidia of 15:1. The final suspension contained even fewer mycelial fragments than endoconidia.

Effect of spore chain separation on germination.—Individual chlamydospores separated by chitinase treatment from intact chains produced on 37- to 54-day-old cultures generally germinated equally well (usually above 80%) in deionized water and in 0.5% carrot juice (Table 1). The germ tubes formed in carrot juice, however, were longer and thicker, and maximum germination was reached after about 12 hr compared with 22-24 hr in deionized water. Exceptions sometimes occurred; for example, there was a significant increase in germination in 0.5% carrot juice compared with that in deionized water for chlamydospores of isolate T491 (Table 1). This may reflect an intrinsic difference between isolates in the capacity of individual chlamydospores to germinate in deionized water.

Comparisons were made of the effects of no blending (nontreated), two blendings, and the complete chitinase treatment (including the two blendings) on subsequent germination of chlamydospores in deionized water and in 0.5% carrot juice (Table 2). The nontreated chlamydospore chains and the spore chains receiving two blendings were incubated in 0.1 M phosphate buffer

TABLE 1. Germination of washed, chitinase-treated chlamydospores of *Thielaviopsis basicola* in deionized water and in 0.5% carrot juice

Exp.	Isolate	Culture age (days)	% Germination[a] Water	Carrot juice
1	T35	54	82	84
2	T35	50	91	94
3	T35	54	83	80
4	T35	44	84	85
5	T35	43	76	90*
6	T35	37	84	86
	T491	37	57	79**
	T492	37	82	89

[a] There was no significant difference (at both the 0.01 and 0.05 levels) between germination in deionized water and in 0.5% carrot juice, except in Exp. 5 and for isolate T491 in Exp. 6, which were significant at 0.05 (*) and 0.01 (**) levels, respectively.

without chitinase. Isolate T35 was used and cultures of different ages were included in each of three experiments. In deionized water, nontreated chlamydospore chains and chains subjected only to two blendings germinated poorly in contrast to the chitinase-treated chlamydospores (Table 2). Also, nontreated chlamydospore chains germinated less well than those receiving two blendings. There was an obvious correlation between the degree of chain separation and germination in deionized water. This was so despite the criterion used for assessing germination of intact spore chains in which one germ tube/three or four cells in the chain was counted as one propagule germinated.

Effect of washing on germination.—Experiments were conducted to determine the effect of increased washing on germination of chitinase-treated chlamydospores in deionized water. The per cent germination of the spores washed 3, 4, 5, and 6 times ranged from 62-71 in deionized water and 74-78 in carrot juice. There was no significant difference (at both 0.01 and 0.05 levels) among the four washing treatments in the per cent germination of spores incubated either in water or in carrot juice. This indicates that the high germination in deionized water obtained with spores that were normally washed by centrifuging three times was not due to any residual exogenous nutrients in the washed spore suspension.

In fact, under our experimental conditions it appeared that the usual three washings of the chitinase-treated spores removed inhibitory materials as well as nutrients. In a separate experiment, spores washed once and twice did not germinate in deionized water nearly so well (3% and 18%, respectively) as those spores washed three times (82%). In addition, the supernatant saved after the first washing (water added to the pellet after the buffer had been decanted) was highly inhibitory to germination. The nutrient medium (0.5% carrot juice) counteracted the inhibition, since germination was uniformly high (81-84%) for spores washed one, two, or three times.

Additional experiments showed that the potassium phosphate buffer used in the incubation medium was completely inhibitory to germination of washed, chitinase-treated chlamydospores, when tested at both 0.1 M and 0.01 M. This inhibition was fungistatic in nature, as the chlamydospores germinated well after washing. When tested individually, both KH_2PO_4 and K_2HPO_4 were inhibitory at 0.01 M (16% and 47% germination, respectively), and to a lesser degree at 0.001 M (63% and 68% germination, respectively), compared with germination in deionized water (82%). Results on inhibition of spore germination by these and other mineral salts will be reported elsewhere.

The chitinase preparations normally contained some particulate material that would sediment with the spores during centrifugation, and may have provided nutrients to the treated chlamydospores. To test this, intact chlamydospore chains were incubated in a chitinase-phosphate buffer mixture that had been centrifuged for 10 min at 1400 *g* to remove particulate material. There was no difference in germination, in deionized water or

TABLE 2. Influence of the degree of spore chain separation and of culture age on germination of chlamydospores of *Thielaviopsis basicola* in deionized water and in 0.5% carrot juice

Exp.	Culture age (days)	% Germination[a] Water: Non-treated	Two-blending treatment	Chitinase treated	Carrot juice: Non-treated	Two-blending treatment	Chitinase treated
1	45	14	27	79	[b]	61	87
	18	1	4	21	[b]	42	85
2	70	19	31	82	79	85	86
	40	23	20	82	87	79	83
	21	1	9	55	79	78	87
3	82	5	10	79	69	63	77
	40	19	16	54	78	80	80
	19	6	16	45	93	91	91

[a] In all cases there was a significant difference (at both the 0.01 and 0.05 levels) between germination, in deionized water, of chitinase treated spores and spores of the other two treatments. Additionally, there was a significant difference (at 0.05 level) between germination of young spores (18- to 21-day-old) and older spores in deionized water in all 3 experiments.

[b] Accurate readings were not obtained because of the presence of excessive numbers of germinated endoconidia in the nontreated spore suspension. Germination was, however, greater than 40% in these two treatments.

0.5% carrot juice, between chlamydospores treated with the two preparations.

Effect of culture age on germination.—The ability of chlamydospores, even the individual ones, to germinate in deionized water was affected by culture age. Germination in deionized water at 24 hr was much less in the case of individual chlamydospores from young spore chains (e.g., 21%) than from older spore chains (e.g., 79%) (Table 2). In carrot juice, germination of young chlamydospores at 24 hr was similar to, or even higher than, germination of older spores. These results indicate that young chlamydospores have a requirement for exogenous nutrients for germination, whereas older spores do not.

Age also affected the rate of germination in nutrient solution. Germination of individual chlamydospores from 3-, 6-, and 12-week-old cultures was compared in 0.5% carrot juice. After 8 hr, the per cent germination was 92, 85, and 63, respectively; after 24 hr, the per cent germination was 94, 89, and 92, respectively. Chlamydospores from the 3- and 6-week-old cultures reached near maximum germination after 8 hr, whereas chlamydospores from the 12-week-old culture reached maximum germination only after 20-24 hr.

DISCUSSION.—Spores of most fungi require exogenous nutrients for germination (5, 9), although there are notable exceptions such as uredospores of many rusts and ascospores of several *Neurospora* spp. Mathre & Ravenscroft (13) reported that intact chlamydospore chains of *T. basicola* required small amounts of simple sugars for germination. Patrick et al. (16) also reported that single chlamydospore cells, obtained by "aging" chlamydospore chains on Millipore filters placed on natural soil for 2-4 months, did not germinate well in distilled water. It appears from our data, however, that individual chlamydospore cells require exogenous nutrients for germination when they are young and/or when they are linked together in the chlamydospore chain, but not when they are older (over 6 weeks old) and separated from the chain. Age and chain separation, therefore, are important factors influencing the requirement of *T. basicola* chlamydospores for nutrients during germination.

Although fungal spores may become less germinable and eventually nonviable with increasing age, it is also known that immature spores of many fungi germinate poorly. Some known examples are conidia of *Botrytis cinerea*, aeciospores of *Gymnosporangium* spp., and oospores of many Oomycetes (5, 19). The so-called "resting spores" of some other fungi also require an aging period to overcome constitutive dormancy. Although no detailed data were provided in their paper, Patrick et al. (16) stated that spore maturation or age could affect germination of chlamydospores of *T. basicola*. Our data indicate that age is a factor affecting chlamydospore germination in water, but not in the presence of adequate exogenous nutrients.

When an individual chlamydospore cell of *T. basicola* germinates, the germ tube emerges at the junction of the lateral and end walls at either end of the cell, indicating that there is an area of weakness at the rim of the end wall. This phenomenon of germination can be clearly seen whenever separated chlamydospores are used, and was described in detail by Patrick et al. (16). The existence of weak areas in the spore wall has been recently substantiated by Tsao & Tsao (21) in their electron microscopy study. They reported that a wedge-shaped area and a narrow "weak junction" exist between the lateral and end walls of the chlamydospore cell, and that an electron-transparent lamella layer separates the spore walls of two adjacent spores. In addition, their study (21) revealed that, in the formation of the chlamydospore chain, an extension of the hyphal wall forms a distinct outer wall that envelopes the entire chlamydospore chain (the chain envelope). Christias & Baker (4) suggested that the individual cells in the chlamydospore chain are cemented together with a material that might contain chitin, since chitinase treatment separated the individual chlamydospores but did not appear to affect any other structures of the fungus. Chitin, however, is probably present also in the spore wall, in the chain envelope, and in the mycelium, but is perhaps protected from large scale chitinase digestion if it is overlaid or interlaced with other cell wall constituents such as glucans as known in other fungi (2, 18, 22) or melanins in dark-pigmented fungi (3, 6, 10, 17). Melanins in pigmented spore walls are reportedly in the electron-dense layer of the wall (6). Kuo & Alexander (10) demonstrated that a synthetic melanin markedly inhibited the hydrolyzing activity of several enzymes including chitinase, and that melanin could protect certain substances from enzyme attack. Chitinase activity on the dark wall of *T. basicola* chlamydospores, therefore, could well be restricted to the electron-transparent layer of the spore wall and of the chain envelope. Perhaps enzymatic activity also occurs, to a lesser extent, at the weak junction (20) connecting the lateral and end walls of each spore.

Thus, a possible explanation of the interacting effects of age, chain separation, and nutrients could lie in a consideration of the weak junction and the wedge-shaped area formed in the wall (20). Apparently, during the germination process sufficient internal pressure is developed within the spore to cause one of the end walls to split in circumscissile fashion from the lateral walls, thereby permitting the emergence of the germ tube. The mechanical weakness of this junction in the wall might very well be negated when the individual spores in a chain are enveloped by an outer wall. Also, the requirement for exogenous nutrients for germination by young chlamydospores may reflect a greater strength at the wall junctions than that which occurs in older chlamydospores. Alternatively, or additionally, the differing capabilities of young and old chlamydospores to germinate in deionized water may reflect differences in membrane permeability or in the rate of transport of materials to and from the cell surface across the cell wall. Permeability changes are known to occur during the early stages of the germination process (19), and it is conceivable that young and old chlamydospores may differ in the changes which occur. Another possibility is that young and old chlamydospores might contain a self-inhibitor in different degrees. All the above speculations lack experimental evidence, and elucidation of

the actual mechanisms involved in germination of young and old chlamydospores of *T. basicola* awaits further study.

As pointed out by Christias & Baker (4), numerous soil microorganisms have the ability to produce chitinase, and the separation of chlamydospore chains in soil is likely due in part to enzymatic action. In fact, chlamydospores of *T. basicola* found in soil are mainly individual spores (15, 20). The knowledge that older, individual chlamydospores do not require exogenous nutrients for germination under in vitro conditions has proved useful in interpreting data concerning soil fungistasis and spore germination in soil (7, 8).

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