Biology of Conidia, Ascospores, and Microsclerotia of Calonectria crotalariae in Soil

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ABSTRACT

Among the three types of *Calonectria crotalariae* propagules, microsclerotia were the most infective to papaya and ohia seedlings while the infection potential of conidia and ascospores was about the same. Ability of these three propagule types to colonize dead papaya stems did not differ significantly. The population of conidia, ascospores, and microsclerotia decreased 87, 46, and 20%, respectively, after an 8 month incubation in soil. Only microsclerotia remained viable after 8 months when colonized papaya tissues containing conidia, ascospores, and microsclerotia of *C. crotalariae* were incubated in soil. Microsclerotia, but not conidia or ascospores, were recovered from soils collected from planting holes in which papaya seedlings were killed by *C. crotalariae* 3 years previously in an abandoned field.

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Additional key words: Carica papaya, Metrosideros collina subsp. *polymorpha.*

Calonectria crotalariae (Loos) Bell & Sobers was identified as the causal organism of peanut black rot in Georgia in 1965 (3). Subsequently this disease was reported in other states and has been a serious threat to peanut production in several areas (2, 5, 16, 17). Stem and root rot of blueberry incited by this fungus also has been reported in North Carolina recently (14). In Hawaii *C. crotalariae* causes collar rot of koa (*Acacia koa* Gray) and papaya (*Carica papaya* L.) (1, 12).

The fungus produces conidia, ascospores and microsclerotia on diseased tissues of various crops (1, 3, 7, 12, 18). However, very little is known about the behavior of these propagules in soil. We report here the infection, colonization and survival potential of conidia, ascospores and microsclerotia of *C. crotalariae* in soil.

MATERIALS AND METHODS.—A single conidial isolate (CK-2) of *C. crotalariae* used in this study was obtained from an infected koa seedling. Conidia, ascospores and microsclerotia of *C. crotalariae* were obtained as previously described (8, 9), and propagule concentrations were determined by the microsyringe method (11).

Substrate colonization.—Twenty ml of propagule suspensions at concentrations of 10^2, 10^3, 10^4, or 10^5/ml was mixed with 200 g of air-dried soil (a clay loam, pH 6.5) in a 500-ml beaker and the soil moisture was adjusted to about 30% (w/w). Twenty stem sections (10 × 4 mm) of 2-month-old papaya seedlings were sterilized by exposing them overnight to propylene oxide in a desiccator (10), and they were then mixed into the infested soil. A preliminary experiment, in which papaya stems were removed from soil and plated at various time intervals, indicated that the shortest time for the highest percentage colonization by conidia, ascospores, and microsclerotia of *C. crotalariae* was 48 hours at 24 C. Therefore, an incubation period of 48 hours was used. Stem sections were removed from soil after incubation, washed in running tap water for 30 minutes, and placed on acidified dextrose-peptone agar (9). Presence of *C. crotalariae* in a stem section was indicated by production of a brown colony with visible strands of microsclerotia. Three replicates were used and the experiment was repeated twice.

Infection potential.—Five papaya or ohia [*Metrosideros collina* Forst.) Gray subsp. *polymorpha* (Gaug.) Rock] seedlings 3 weeks and 5 months old, respectively, were established in field soil in plastic containers (9.5 × 9.5 cm). Thirty ml of the propagule suspension was evenly distributed over the soil surface in each container. For controls the same amount of distilled water was added. Soils in containers were saturated with water for 24 hours following treatment and then watered daily. Each treatment was replicated three times, and the experiment was repeated once.

Survival.—Thirty ml of conidial, ascospore, and microsclerotial suspensions at concentrations of 10^5/ml, 10^5/ml, and 5×10^4/ml, respectively, were separately mixed with 300 g of air-dried soil in 500-ml beakers. Soil moisture content was adjusted to 30% and maintained at about the same level by adding water periodically. The population of each propagule in the soil was determined by plating the diluted soil suspension on acidified dextrose-peptone agar. Three replicates with five plates per replicate were used.

In another test, about 20 g of 3-month-old fresh papaya seedling stem sections were inoculated with conidia of *C. crotalariae*, and incubated at 24 C. After 2 weeks the stem sections, which contained numerous conidia, ascospores, and microsclerotia, were mixed with 500 g of soil in a 500-ml beaker. Moisture content of the soil was maintained at about 30% as described above. After 8 months, 10 g of infested soil was passed through a 38-μm sieve with 100 ml of distilled water. While allowing conidia and ascospores to pass through, the sieve retained

TABLE 1. Percentage of ohia seedlings killed and papaya seedlings killed or stunted by conidia, ascospores, and microsclerotia of *Calonectria crotalariae*

Inoculum level (no. of propagules per container)[b]	Seedlings killed or stunted (%)[a]					
	Ohia			Papaya		
	Conidia	Ascospores	Microsclerotia	Conidia	Ascospores	Microsclerotia
1×10^6	81	68	...	15	20	...
1×10^5	43	42	...	0	0	...
5×10^4	...	...	100	...	...	65
1×10^4	15	4	70	0	0	...
5×10^3	...	...	30	...	...	...
Control	0	0	0	0	0	0

[a]Average of 30 plants per treatment.
[b]Plastic containers (9.5 × 9.5 cm). Soil in containers was saturated with water for 24 hours following treatment (indicated inoculum in 30 ml of suspension) then watered daily.

microsclerotia on the screen. Filtrate and the material retained on the screen were plated separately on acidified dextrose-peptone agar to determine the presence of *C. crotalariae*.

The method of Lingappa and Lockwood (13) was used for studying propagule germination on the soil surface. To determine germination in soil, 2 ml of propagule suspension at the concentration of 10^7 conidia, 10^7 ascospores, or 10^5 microsclerotial/ml was mixed with 20 g soil. After incubation at 24 C for 24 hours for conidia, or 48 hours for ascospores and microsclerotia, 1 g of soil suspended in 5 ml of distilled water in a small petri dish (50 × 15 mm) was observed microscopically, and percentage germination of each propagule was determined.

RESULTS.—*Propagules produced in nature.*—When surface-sterilized stem sections (20 × 10 mm) of 3-month-old papaya seedlings were inoculated with conidia of *C. crotalariae*, conidia and perithecia were produced on stems in 1 and 2 weeks, respectively. After 3 weeks, microsclerotia were formed abundantly either on the surface or within the outer cortex of the stems. Microsclerotia were easily differentiated from perithecia of *C. crotalariae*; microsclerotia were dark brown, whereas perithecia were orange to red. Microsclerotia were composed of small round cells with thick walls, while perithecia were formed by large thin-walled cells of irregular shape. On papaya stems, a number of perithecia were produced on stromata which were morphologically similar to microsclerotia. Microsclerotia of *C. crotalariae* were also found on naturally-infected papaya tissues in soil samples collected from a papaya field. After incubation of these infected papaya tissues on acidified dextrose-peptone agar for 3 days at 24 C bundles of cylindrical conidia and stipes with globose vesicles at their tips, typical of *C. crotalariae*, emerged directly above these microsclerotia.

Infection potential.—Among the three propagule types of *C. crotalariae*, microsclerotia were the most infective, while the infection potential of conidia and ascospores was about the same (Table 1). For example, 70% of the ohia seedlings were killed by microsclerotia at the concentration of 1×10^4/container, whereas only 43 and 42% were killed by conidia and ascospores, respectively, at the concentration of 1×10^5/container. Similarly, 65% of the papaya seedlings were killed or stunted by microsclerotia at the concentration of 5×10^4/container, while only 15 and 20% were killed or stunted by conidia and ascospores, respectively, at the concentration of 1×10^6/container.

TABLE 2. Colonization of papaya stem sections[a] by conidia, ascospores, and microsclerotia of *Calonectria crotalariae*

Type of propagules	Colonization (%)[b]				
	Propagule density (no./g soil)				
	0	10	100	1,000	10,000
Conidia	0	10 A	43 A	75 A	95 A
Ascospores	0	3 A	15 A	60 A	90 A
Microsclerotia	0	10 A	53 A	93 A	100 A

[a]Stem sections (10 × 4 mm) were sterilized by propylene oxide.
[b]Data are average of three replicates of 20 papaya stem sections per replicate. Means followed by the same letter in the same column are not significantly different, $P = 0.05$.

Colonization potential.—Percentage of dead papaya stems colonized by conidia, ascospores, and microsclerotia of *C. crotalariae* was directly correlated with propagule density and the colonization potential of these three types of propagules was about the same (Table 2). When dead papaya stems were buried in soil artificially infested with conidia or ascospores of *C. crotalariae*, microsclerotia were produced in the epidermis in 1 month. These microsclerotia germinated readily on acidified dextrose-peptone agar.

Survival potential.—When propagules grown on nutrient agar were used, the population of viable conidia and ascospores declined gradually over an 8-month period, while that of microsclerotia remained more or less constant (Fig. 1). Within this period, the population of conidia, ascospores, and microsclerotia decreased 87, 46, and 20%, respectively. At the beginning of the experiment, colonies developed from conidia were about 7 mm in diameter after 1-week incubation at 24 C, whereas the average colony size was only about 4 mm when conidia were incubated in the soil for more than 2 months. However, the size of colonies originating from ascospores and microsclerotia remained unchanged throughout the experiment. Ascospores of *C. crotalariae* that survived 8 months in the soil were changed from the

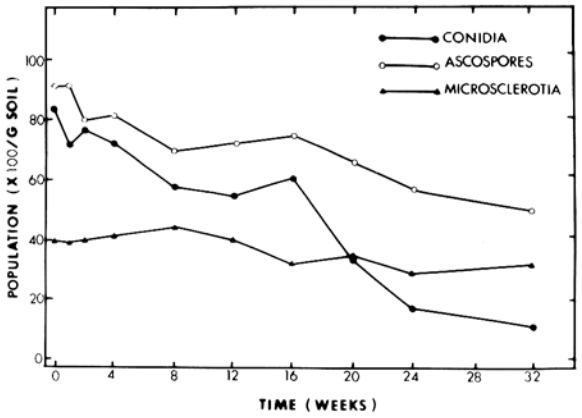

Fig. 1. Survival of conidia, ascospores, and microsclerotia of *Calonectria crotalariae* in soil in an 8-month period.

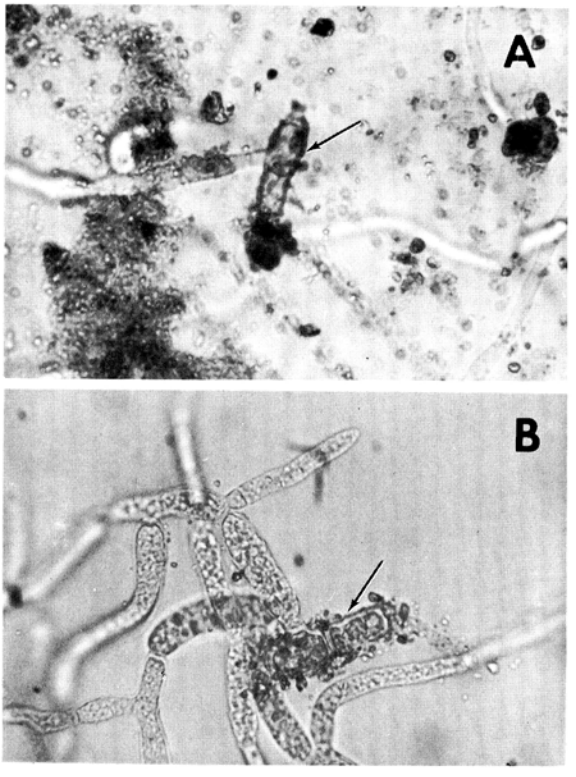

Fig. 2. Appearance of an ascospore and a conidium germinating on acidified dextrose-peptone agar. **A)** An ascospore (arrow) recovered from soil after 8 months of incubation. **B)** A conidium (arrow) recovered from soil after 1 month of incubation.

original four cells to one or two cells, and from hyaline to slightly brownish in color (Fig. 1-A). It was not possible to observe the origin of the *C. crotalariae* colonies due to poor survival of the conidial inoculum after 8 months of incubation in soil. However, when soil infested with 10^6 *C. crotalariae* conidia per g soil was plated after 1-month incubation, two or three cells of each conidium were lysed and there was a color change from hyaline to slightly brownish (Fig. 1-B).

When soil was infested with colonized papaya stems that contained conidia, ascospores, and microsclerotia of *C. crotalariae*, microsclerotia alone remained viable after 8 months. Only the material retained on the 38-μm sieve contained viable propagules of *C. crotalariae*. No *C. crotalariae* propagules were detected in the filtrate. Presence of microsclerotia in the material retained on the sieve was confirmed by microscopic examination.

To determine the survival structure of *C. crotalariae* in nature, soils were collected from planting holes in which papaya seedlings were killed by this fungus 3 years earlier in an abandoned field. The material retained on the 38-μm sieve, but not the filtrate, contained viable propagules of *C. crotalariae*, and microsclerotia were observed.

Chinn and Tinline (4) reported that survival of spores of different *Cochliobolus sativus* isolates was correlated with their ability to germinate in soil. Germination of different propagules of *C. crotalariae* in soils was, therefore, tested. Percentage germination of conidia, ascospores, and microsclerotia was 89, 78, and 46%, respectively, on the soil surface, and was 60, 45, and 26%, respectively, in soil. Conidia and ascospores that germinated on the soil surface or in soil produced one to two germ tubes each; while each germinated microsclerotia had one to five germ tubes. All three types of propagules germinated completely on water agar. Each conidium and ascospore produced two to four germ tubes, while each microsclerotium produced 10 to 30 germ tubes on agar.

DISCUSSION.—Rowe et al. (17) reported that *C. crotalariae* survived 3 years in soil in the absence of host plants. Our results suggest that the survival structure of this fungus in soil is microsclerotia, and that both conidia and ascospores are capable of only short-term survival. Schreiber and Green (19) also showed that only microsclerotia of *Verticillium albo-atrum* were able to survive for a long period of time in soil, while conidia of this fungus were not detectable after 21 weeks in soil. Microsclerotia apparently are also important survival structures for *Cylindrocladium floridanum* (15) and *Cylindrocladium scoparium* (20, 21) because viable microsclerotia of both species have been isolated from naturally infested fields. Both conidia and ascospores of *C. crotalariae* can colonize dead papaya tissues and result in production of microsclerotia. Therefore, in the presence of organic matter both types of propagules may also contribute indirectly to the long-term survival of this fungus in soil.

Microslcerotia of *C. crotalariae* are multicellular, pigmented, and thick-walled, while conidia and ascospores are four-celled, hyaline, and thin-walled. This may be the reason why microsclerotia, but not conidia or ascospores can survive in soil for a long period of time. When conidia and ascospores of *C. crotalariae* grown on papaya stems were added to soil, both disappeared after 8 months. However, probably because excess amounts of spores were used, both types of spores grown on nutrient agar were still detectable in soil after the same period of time. Ascospores were better than conidia in short-term survival, and the percentage germination of the former was lower than the latter. These results are in accord with those of Chinn and Tinline (4) who also showed that *C. sativus* isolates that germinated in soil disappeared from soil faster than those that either failed to germinate or

germinated poorly in soil.

Conidia and ascospores of *C. crotalariae* were changed morphologically due to lysis of some cells in the spores during incubation in the soil. The size of colonies originating from conidia but not ascospores also decreased after incubation for more than 2 months in the soil. The basis for this change is still unknown.

Microsclerotia are multicellular and produce more germ tubes at germination than conidia and ascospores. This may explain why microsclerotia of *C. crotalariae* were much more effective in killing papaya and ohia seedlings than conidia and ascospores. Green (6) also showed that the minimum number of conidia of *V. albo-atrum* for 100% infection of tomato seedlings was much higher than that of microsclerotia. However, microsclerotia of *C. crotalariae* were not more effective in colonizing dead organic matter than conidia or ascospores. This suggested that the ability of a propagule for host infection may be different from that for substrate colonization.

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