Survival and Growth of the Mycoparasite Darluca filum

G. W. Rambo and G. A. Bean

Graduate student and Assistant Professor, respectively, Department of Botany, University of Maryland, College Park 20742.

Scientific Article A1593, Contribution No. 4312 of the Maryland Agricultural Experiment Station. Portion of an M.S. Thesis of the senior author.

Accepted for publication 20 April 1970.

ABSTRACT

After 5 months at 5 C, conidia of *Darluca filum* stored within pycnidia were 100% viable. Conidia released from pycndia after wetting did not germinate unless the spore masses were diluted in distilled water. Conidia that remained in the spore masses became greatly enlarged, vacuolated, and did not germinate when placed in distilled water. The addition of bean rust spores to water agar increased the width and amount of branching of *D. filum* germ tubes; penetration of rust spores was not observed. Adding the solvent extracts from rust uredospores or intact or ground bean rust uredospores to a complete medium increased mycelia and pycnidia production. Colony diam was greatest on a medium containing 10:0.1 g/liter of glucose-ammonium sulfate, and mycelial production increased with glucose level. Pycnidial production occurred only on a medium containing 0.1 g/liter of ammonium sulfate, and max pycnidial production resulted from the addition of 10 g/liter glucose. Phytopathology 60: 1436-1440.

Hyperparasitism or mycoparasitism, the parasitism of one fungus by another (3, 8), is frequently associated with diseased plant tissue above the ground, but evidence for its occurrence in the soil is circumstantial (3). The attempts to use mycoparasites to control soil-borne organisms have been unsuccessful (2). Studies on the physiology of mycoparasitism have dealt primarily with host range, mode of parasitism, and nutritional requirements of soil-inhabiting mycoparasites using dual culture (3). There is evidence that mycoparasites on aboveground parts can reduce disease severity. The fungi often mentioned are *Tuberculina* spp., *Gonotobotryum* spp., *Cladosporium* spp., and *Darluca* spp. (3, 7, 8). Of these fungi, *Darluca* spp. appear to be the most effective mycoparasites. For example, Bean (1) reported that after *Darluca filum* (Biv.) Cast. became established, it prevented further development of stem rust of bluegrass (*Puccinia graminis* f. sp. *poae*). Cunningham (6) found that many rust fungi were destroyed in Dominica, British West Indies, by mycoparasitic fungi, and that *Darluca* spp. was abundant in most uredial collections.

Only limited studies have been done on *Darluca* spp. because of difficulties in growing the organism in culture. Although Chester (5) concluded that *D. filum* was an obligate parasite, Calpouzas et al. (4) grew *D. filum* on potato-dextrose agar; Nicolas & Villanueva (12) used a semisynthetic medium to compare carbon and nitrogen utilization of *D. filum*, and recently Bean (1) compared growth of mycelial and pycnidial isolates on a chemically defined medium, medium 56. This study reports on factors that influence the survival and growth of *D. filum*.

MATERIALS AND METHODS, RESULTS.—Mycelial- and pycnidial-producing isolates of *D. filum* were used. The mycelial isolate (Isolate I) was obtained from the Centraal Bureau voor Schimmel Cultures, Baarn, The Netherlands (C.B.S. 234.51), and a mycelial-pycnidial isolate (Isolate IX) was obtained from stem rust pustules on infected bluegrass (*Poa pratensis* L.) leaves.

Cultures were maintained on medium 56 (10.0 g glucose, 1.5 g K_2HPO_4, 2.0 g KH_2PO_4, 1.0 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 1.0 ml trace minerals and 0.5 ml vitamins/liter) at 24 C. Subcultures were made every 4-5 weeks.

Survival of Darluca filum.—Bluegrass leaves, infected with stem rust which was also infected with *D. filum*, were placed in plastic bags and stored dry for varying periods at 5 C. The leaves were removed and placed on moist filter papers in petri dishes to stimulate conidial production. After 24 hr at 4 temp, the percentages of pycnidia producing spore masses were estimated.

Storage of *D. filum* on plant material for 20 weeks did not reduce spore mass production. The optimum temp for release of conidia, at each 2-week interval, was 30 C; over 90% of the pycnidia exuded a conidial mass. The production of spore masses at 20 and 25 C varied from 30 to 90%. Incubation of the pycnidia at 35 C greatly reduced the number of spore masses released.

Conidia of *D. filum* are released within a gelatinous matrix (10). The influence of the gelatinous matrix on survival of *D. filum* was investigated as follows. Spore masses were placed in test tubes of distilled water at 10 C to prevent germination. After 24, 48, 96, and 144 hr of storage at 10 C, the conidia were incubated at 25 C and the percentage germination determined after 12 hr. The percentage germination of conidia after 24 hr in distilled water was 100%. In other dilutions of conidia stored for 144 hr, only 25% of the conidia had germinated. Conidia remaining in the gelatinous matrix became enlarged, thin-walled, highly vacuolated, and did not germinate when placed in distilled water.

Factors influencing conidial germination.—Conidia of *D. filum* were applied to the surface of water agar, water agar containing 300 mg (fresh wt) of bean rust uredospores, or medium 56. The conidia were incubated for 24 hr at 24 C in total darkness, incandescent plus fluorescent light (400 ft-c), or a 12-hr light period

followed by 12 hr of darkness; and the percentages germination determined.

Light conditions or media used did not influence the rate or percentages of *D. filum* conidia that germinated, but the type of germ tube that occurred differed according to the medium. On water agar, germ tubes were thin, unbranched, and longer than germ tubes produced on water agar plus bean rust spores, or medium 56. On water agar plus rust spores medium, germ tubes of *D. filum* grew randomly in the medium, and although contact with a rust spore or its germ tube did occur, no penetration was detected.

Medium 56, inoculated with *D. filum* conidia, was incubated for 24 hr or 48 hr at 5, 25, 30, or 35 C to observe the influence of temp on speed and percentage of conidial germination. The percentage of germinated conidia was the same after 24 or 48 hr. The optimum temp for conidial germination were either 30 C (95%) or 25 C (93%). At 35 C, there was 88% germination and no conidia were germinated at 5 C after 48 hr. The speed of germination was the same at 25, 30, and 35 C.

Factors influencing growth rate.—Plastic petri dishes containing 20 ml of medium 56 were inoculated with a 6-mm disc of mycelium, and colony diam were measured. Mycelial wt were determined by cutting out colonies, placing them in a beaker containing distilled water, and heating in an Arnold Steam Sterilizer until the mycelial mats separated from the agar. The mats were collected on weighed filter paper, dried in an oven for 24 hr at 100 C, and placed in a desiccator until weighed. Cultures were also grown in 125-ml Erlenmeyer flasks containing 20 ml of liquid medium 56. Each flask was inoculated with a disc of mycelium and incubated for 30 days as stationary cultures. The mycelium was collected and weighed as above.

Mycelial and pycnidial production by *D. filum* on different glucose to ammonium sulfate ratios were studied to determine an explanation for (i) the limitation of *D. filum* to rust pustules; and (ii) the change in a sporulating isolate to mycelial isolate unless transferred every 4-5 weeks (1). Glucose was added to equal 5, 10, 15, 20, and 25 g glucose/liter; the amount of $(NH_4)_2SO_4$ was 0.1, 1.0, 2.0, and 5.0 g/liter for 20 different combinations. The agar was inoculated with Isolates I and IX, and after 30 days notes were taken on colony diam, mycelium, and pycnidial production (Table 1).

Growth rates of *D. filum*, as determined by colony diam, was greater at the 5- or 10-g level of glucose than at higher levels; increasing levels of $(NH_4)_2SO_4$ also tended to decrease colony diam. The optimum growth rate of both isolates occurred on a medium which contained a glucose—$(NH_4)_2SO_4$ ratio of 10:0.1. Mycelium production increased with increasing levels of glucose; the addition of 0.1 g/liter of $(NH_4)_2SO_4$ resulted in the least amount of mycelium produced. Pycnidial production by Isolate IX occurred only on a medium containing 0.1 g/liter of $(NH_4)_2SO_4$; the optimum amount of glucose for pycnidial production was 10 g/liter.

Bean (1) reported different growth rates for mycelial and conidial isolates using the same carbon source. Isolate IX, which produces both mycelium

TABLE 1. Mycelial dry wt and colony diam of *Darluca filum* grown on media containing various glucose-ammonium sulfate ratios

Glucose/ ammonium[a] sulfate	Isolate I		Isolate IX		
	Mycelium[b]	Colony diam[c]	Mycelium	Colony diam	Pycnidia[d]
	mg	*mm*	*mg*	*mm*	
5/0.1	132 a	45	144 a	47	>60
5/1.0	145 a	44	195 b	43	0
5/2.0	118 a	44	246 c	47	0
5/5.0	150 a	42	158 a	42	0
10/0.1	150 a	58	186 b	57	>90
10/1.0	206 b	49	348 d	49	0
10/2.0	225 b	46	331 d	31	0
10/5.0	286 c	45	328 d	39	0
15/0.1	195 b	46	268 c	43	>30
15/1.0	276 c	44	361 d	38	0
15/2.0	278 c	39	330 d	35	0
15/5.0	221 b	42	398 e	35	0
20/0.1	192 b	40	273 c	35	>30
20/1.0	302 c	38	618 f	30	0
20/2.0	293 c	40	580 f	29	0
20/5.0	270 c	36	438 f	30	0
25/0.1	185 b	39	290 c	31	>30
25/1.0	363 d	37	718 h	31	0
25/2.0	365 d	38	698 g	32	0
25/5.0	330 d	35	518 f	28	0

[a] g of glucose/g ammonium sulfate per liter.

[b] Means in the same column not followed by the same letter differ significantly at the 1% level of probability; data represent avg of 3 replicates.

[c] Avg. colony diam after 30 days.

[d] 0 = No pycnidia; >30 = greater than 30%/colony; >60 = greater than 60%/colony; >90 = greater than 90%/colony.

TABLE 2. Mycelial dry wt and pycnidial production by *Darluca filum*, grown on medium containing different carbon sources

Carbon source	Mycelium[a]	Pycnidia[b]
	mg	
Maltose	680 a	>30
Fructose	637 a	0
Sucrose	515 a b c	0
Cellulose	482 a b c d	>30
Galactose	283 b c d	0
Glucose	269 b c d	>90
Xylose	218 c d	0
Lactose	108 d	0

[a] Treatment means followed by the same letter are significantly different at the 1% level; data represent the avg of 3 replicates.
[b] 0 = No pycnidia.
>30 = Greater than 30%/colony.
>90 = Greater than 90%/colony.

and pycnidia, was grown on 8 different carbon sources to determine the influence of carbon on the ratio of mycelium-pycnidia. The amount added was equal to 10 g carbon/liter, and mycelial-pycnidial production on solid and liquid was determined after 30 days. Table 2 summarizes the results using solid medium.

The largest number of pycnidia occurred on a medium containing glucose; pycnidia were also produced on maltose and cellulose medium, but none on the other carbon sources. Mycelium production was highest when maltose was the carbon source, and lowest with lactose in the medium. In liquid medium, the results were the same.

The influence of nitrogen sources on growth of *D. filum* was also studied. Nicolas & Villanueva (12) compared nitrogen utilization of *D. filum*, using a semisynthetic medium. Sodium nitrate ($NaNO_3$) or ammonium sulfate $(NH_4)_2SO_4$ were added to medium 56 at 0.1 g or 1.0 g/liter. The media were inoculated with Isolate IX; the cultures were incubated for 30 days at 20, 25, or 30 C, and colony diam measurements and number of pycnidia were determined (Table 3).

Although the optimum temp for spore release was 30 C, growth and pycnidial production of *D. filum* was greater at 20 or 25 C than at 30 C. The addition of 1.0 g/liter rather than 0.1 g/liter of either nitrogen source resulted in greater mycelium production. The use of $(NH_4)_2SO_4$ instead of $NaNO_3$ also increased mycelium production. Whereas mycelium production increased with the amount of nitrogen added, the colony diam and number of pycnidia were less at the higher nitrogen level. Pycnidial production was highest at 20 C on a medium containing 0.1 g/liter $(NH_4)_2SO_4$.

The influence of rust uredospores or uredospore extracts on growth of *D. filum* was determined. Growth of *D. filum* is confined to rust pustules (10), indicating that uredospores may be the primary source of nutrients. To observe the influence of uredospore extracts on growth and pycnidial production, 200 mg (fresh wt) of bean uredospores (*Uromyces phaseoli* [Pers.] Wint. var. *typica* Arth.) were extracted for 3 hr with either hexane, acetone, 95% ethanol, or a combination of hexane-95% ethanol at 24 C. The suspensions were centrifuged to remove the spores, and the supernatants were evaporated to dryness. Residues were dissolved in 15 ml of 95% ethanol and added to modified medium 56 (0.1 g $[NH_4]_2SO_4$/liter) before or after autoclaving, at the rate of 1 ml/liter.

Two hundred-mg quantities of rust uredospores were homogenized in a 15-ml Ten Broech glass-tissue homogenizer containing 10 ml of distilled H_2O. Samples were examined periodically with a microscope, and grinding was continued until at least 75% of the spore walls were ruptured. The suspension was sterilized by filtering through a 0.2 μ Nalgene filter unit (Nalgene Labware, N.Y.), and the filtrate and spore residue were added to media before autoclaving. Intact rust spores were also added to media at the rate of 200 mg/liter prior to autoclaving. After 30 days' incubation at 20 C, mycelium, pycnidial production, and colony diam were determined.

The growth rate of *D. filum*, as determined by colony diam, was not affected by the addition of ex-

TABLE 3. Colony diam, mycelial and pycnidial production by *Darluca filum* grown on media containing different nitrogen sources at three temp

	Nitrogen g/liter											
	$NaNO_3$						$(NH_4)_2SO_4$					
	0.1 g			1.0 g			0.1 g			1.0 g		
Temp	Colony diam[a]	Mycelium[b]	Pycnidia[c]	Colony diam	Mycelium	Pycnidia	Colony diam	Mycelium	Pycnidia	Colony diam	Mycelium	Pycnidia
C	*mm*	*mg*		*mm*	*mg*		*mm*	*mg*		*mm*	*mg*	
20	71	225 a	>30	52	725 a	0	69	351 a	>90	57	925 a	>30
25	57	427 a	>30	35	454 b	0	68	246 a	>30	35	683 b	0
30	33	89 b	0	24	108 c	0	33	89 b	0	25	240 c	0

[a] Avg colony diam after 30 days.
[b] Means in a column not followed by the same letter differ significantly at the 1% level of probability; data represent the avg of 3 replicates.
[c] 0 = No pycnidia.
>30 = Greater than 30%/colony.
>90 = Greater than 90%/colony.

TABLE 4. Colony diam, mycelial and pycnidial production by *Darluca filum* grown on media containing extracts from *Uromyces phaseolus* uredospores

Solvent	Colony diam[a]	Mycelium[b]	Pycnidia[c]
	mm	*mg*	
Acetone			
autoclaved	73	210 a	14 a
nonautoclaved	64	210 a	33 b
Ethanol			
autoclaved	72	164 b	17 a
nonautoclaved	72	236 a	27 b
Hexane-ethanol			
autoclaved	73	210 a	22 a
nonautoclaved	70	222 a	34 b
Hexane			
autoclaved	68	163 ab	31 b
nonautoclaved	69	204 a	23 a
Water			
autoclaved	67	99 c	20 a
nonautoclaved	69	134 c	27 b
Control	71	124 c	32 b

[a] Avg colony diam after 30 days.
[b] Means in the same column not followed by the same letter differ significantly at the 1% level of probability; data represents the avg of 3 replicates.
[c] Avg no. pycnidia/colony.

tracts from rust uredospores (Table 4). But mycelial production was greatly accelerated by the addition of all extracts used, whereas pycnidial production was reduced when acetone and alcohol extracts were added to the medium before autoclaving.

The addition of either intact or ruptured uredospores increased mycelial growth and pycnidial production (Table 5). Mycelial production was increased approximately three times, whether intact or ruptured spores were added, indicating that the increased growth response observed was probably the result of nutrients present in the rust spore wall.

DISCUSSION.—The survival of *D. filum* is dependent on the production of pycnidia. While the conidia remain in the pycnidia they do not germinate and can probably survive for long durations. When the conidia are released in the gelatinous matrix, there must be sufficient moisture to reduce the concn of conidia or the matrix before they can germinate. Lingappa & Lingappa (11) demonstrated this with *Glomerella cingulata*. This would insure conidial germination only after sufficient moisture is available for infection to occur.

TABLE 5. Colony diam, mycelial and pycnidial production by *Darluca filum* grown on media containing *Uromyces phaseolus* uredospores

Isolate	Medium	Mycelium[a]	Colony diam[b]	Pycnidia[c]
		mg	*mm*	
Conidial	Whole spores	395 a	76	50
Conidial	Broken spores	400 a	79	45
Conidial	Control (medium 56)	134 b	68	18
Mycelial	Whole spores	340 a	77	0
Mycelial	Broken spores	390 a	79	0
Mycelial	Control (medium 56)	160 b	70	0

[a] Means in the same column not followed by the same letter differ significantly at the 1% level of probability; data represents the avg of 3 replicates.
[b] Avg colony diam after 30 days.
[c] Avg no. pycnidia/colony.

Although temp did not influence the release of conidia from pycnidia, the growth rate of *D. filum* was affected by temp. The optimum temp for mycelial production by *D. filum* was lower than that reported for *P. graminis* f. sp. *triticii* (13), which may explain the occurrence of *D. filum* primarily in late fall, but rarely during the warmer months. The ability of *D. filum* to grow at a lower temp than its host may prevent the rust fungus from becoming established by infecting either rust uredospores or germ tubes before plant tissue is invaded.

Darluca filum grew equally well on either ammonium or nitrate nitrogen sources, but it did not produce pycnidia on the nitrate nitrogen medium. The occurrence of nitrate nitrogen in living tissue and ammonium nitrogen in infected tissue could explain the limitation of *D. filum* to rust pustules and occasionally the infected tissue surrounding the rust pustule.

A low glucose to ammonium sulfate ratio induced pycnidial production of *D. filum*. This could result from a lack of nutrients; a rapid mycelial growth rate normally results in a reduction in spore production. As the amount of glucose was increased, mycelial production increased and pycnidial production decreased. The minimal amount of nutrients available to *D. filum*, which cannot infect living tissue, probably stimulates pycnidial production and explains the absence of *D. filum* mycelial growth under field conditions.

The source of carbon may be the rust fungus as we have suggested for other nutrients needed for growth of *D. filum*, or else the rust-infected plant tissue may provide nutrients as well. Fedorinchik (9) reported that 98% of rust pustules, *P. rubigo-vera triticii*, were damaged when *D. filum* conidia were applied 1-10 days after rust inoculation. Since *D. filum* has not been observed to parasitize plant tissue, it must survive on the leaf surface until infection of the plant by the rust fungus takes place. Following infection of plant tissue by the rust fungus, breakdown products such as glucose or cellulose would become available. It is unlikely that the rust fungus is the sole source of nutrients for *D. filum*. Uredospores or rust germ tubes may provide the initial source of nutrients, but after pustule formation has occurred, nutrients from the infected plant tissue would then become available for growth of *D. filum*.

Darluca filum has characteristics of the ideal mycoparasite. It is widespread in distribution, easily disseminated, can survive for long periods, and does not infect healthy tissue. Although it may not be practical or possible to control the rust fungus with *D. filum* under field conditions, studies on the physiology of this fungus should aid in our understanding of the development of parasitism by fungi.

LITERATURE CITED

1. BEAN, G. A. 1968. Growth of the hyperparasite *Darluca filum* on chemically defined media. Phytopathology 58:252-253.
2. BOOSALIS, M. G. 1956. Effect of soil temperature and green-manure amendment of unsterilized soil on parasitism of *Rhizoctonia solani* by *Penicillium vermiculatum* and *Trichoderma* sp. Phytopathology 46:473-478.
3. BOOSALIS, M. G. 1964. Hyperparasitism. Annu. Rev. Phytopathol. 2:363-376.
4. CALPOUZOS, L., T. THEIS, & C. M. RIVERA BATILLE. 1957. Culture of the rust parasite, *Darluca filum*. Phytopathology 47:108-109.
5. CHESTER, K. S. 1946. The cereal rusts. Chronica Bot. Waltham, Mass. 269 p.
6. CUNNINGHAM, J. L. 1967. Natural enemies of the rust fungi in the tropics. Phytopathology 57:645 (Abstr.).
7. DARPOUX, H. 1960. Biological interference with epidemics, p. 521-565. *In* J. G. Horsfall & A. E. Dimond [ed.] Plant Pathology, An Advanced Treatise, Vol. III. Academic Press, N.Y.
8. DEVAY, J. E. 1956. Mutual relationships in fungi. Annu. Rev. Microbiol. 10:115-140.
9. FEDORINCHIK, N. S. 1952. Virulence and effectiveness of a culture of the rust parasite *Darluca filum* (Biv.) Cast. [In Russian] Microbiology 21:711-717. Rev. Appl. Mycol. 34:141 (Abstr.).
10. KEENER, P. D. 1933. Some characteristics of *Darluca filum* in culture. Pennsylvania Acad. Sci. 7:1-8.
11. LINGAPPA, B. T., & Y. LINGAPPA. 1965. Effects of nutrients on self-inhibition of germination of conidia of *Glomerella cingulata*. J. Gen. Physiol. 44:1029-1045.
12. NICOLAS, G., & J. R. VILLANUEVA. 1965. Physiological studies on the rust hyperparasite *Darluca filum*. I. Carbon and nitrogen nutrition. Mycologia 57: 782-788.
13. WILLIAMS, P. G., K. J. SCOTT, & J. L. KUHL. 1966. Vegetative growth of *Puccinia graminis* f. sp. *tritícii* in vitro. Phytopathology 56:1418-1419.