Ecology and Epidemiology

Factors Affecting the Biological Control of *Cercosporidium* Leaf Spot of Peanuts by *Dicyma pulvinata*

James K. Mitchell and Ruth A. Taber

Former graduate student and research scientist, respectively, Department of Plant Pathology and Microbiology, Texas Agricultural Experiment Station, Texas A & M University, College Station 77843. Present address of first author: Department of Plant Pathology, University of Arkansas, Fayetteville 72701.

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ABSTRACT

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The survival and/or growth of *Dicyma pulvinata*, a mycoparasite on *Cercosporidium personatum*, was determined in culture media and on host tissue invaded by *C. personatum*. Variables tested included temperature, pesticide concentration, pH, and relative humidity. Potential carriers for spray formulations of the mycoparasite were tested also. In broth culture, maximal growth occurred at 23–25 C. On detached peanut leaflets infected with *C. personatum* maximal colonization occurred at 23–28 C. No colonization was noted at constant temperatures above 30 C, and no sporulation occurred at 31.5 C. Of nine pesticides tested, *D. pulvinata* was most sensitive to benomyl, mancozeb, and triphenyltin hydroxide; however a benomyl-tolerant isolate was obtained that grew in culture media containing 10 μg/ml benomyl. *D. pulvinata* grew over a broad pH range with maximal growth in the range of 3.3–7.7. Viability of spores of *D. pulvinata* was assessed at relative humidities (RH) ranging near 0–100% RH at three different temperature regimes. At 10 C *D. pulvinata* remained viable for at least 72 days under all relative humidities tested.

Additional key words: Arachis, mycoparasite.

The fungus *Dicyma pulvinata* (Berk. & Curt.) v. Arx (= *Hansfordia pulvinata* (Berk. & Curt.) Hughes) is a mycoparasite (1). Hughes (3) recorded *H. pulvinata* colonizing *Cercospora personata* (Berk. & Curt.) Ellis and Everhart late leaf spot lesions on peanut (*Arachis hypogaea* L.). Rathaiah and Pavgi (12) described the fungus parasitizing several species of *Cercospora* in India. In the United States, *H. pulvinata* was first found parasitizing hyphae and spores of *Cercosporidium personatum* (Berk. & Curt.) Deighton (= *Phaeoisariopsis personata* (Berk. & Curt.) v. Arx.) on peanut by Taber (18,19). Cultures of this fungus obtained from late leaf spot of peanuts south of San Antonio, TX, were deposited in the culture collection of the Northern Regional Research Laboratory, Peoria, IL, as NRRL 12356, 12357, and 12358. Taber (19) suggested the use of *H. pulvinata* as a biological control agent for late leaf spot and it was successfully inoculated onto several lesions of *C. personatum* in a preliminary field trial in Bryan, TX (Taber and Mitchell, *unpublished*). Krishna (5) reported its presence that same year in India on the same host. Shokes and Taber (13) reported this parasitic association in Florida in 1983. Mitchell and Taber (8,9) and Mitchell et al (7) defined several environmental parameters under which *D. pulvinata* could be established in the field. *D. pulvinata* has also been observed on lesions incited by *Cladosporium fulvum* Cooke (= *Fulvia fulva* (Cooke) Ciferri) on *Lycopersicon esculentum* Mill. (11,20) and associated with leaves of *Shorea robusta* Gaertn. on the adaxial leaf surface (10).

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Reports concerning biological control of foliar pathogens are sparse compared with reports concerning control of soilborne diseases. Few systems have been studied in detail. Spurr (15) demonstrated that when conidia of selected, nonpathogenic isolates of *Alternaria alternata* (Fries) Keissl. were applied to tobacco (*Nicotiana tabacum* L.) leaves before inoculation with conidia of pathogenic *A. alternata*, leaf spot development was decreased. In another example, a formulation of *Bacillus thuringiensis* Berliner, normally used to control insects, was effective in reducing Alternaria leaf spot of tobacco and early leaf spot of peanuts incited by *Cercospora arachidicola* Mori (16). Partial control of both *Septoria nodorum* Berk. and *Cochliobolus sativus* (Ito & Kuribay.) Drechsler & Dastur on wheat (*Triticum aestivum* L.) leaves was obtained by manipulation of saprophytic yeast microflora (2). When carnations (*Dianthus caryophyllus* L.) were inoculated with a suspension of a mixture of spores of *Uromyces dianthi* (Pers.) Niessl. and *Verticillium lecanii* (Zimm.) Viegas, 50% less rust resulted than when they were inoculated with the rust alone (14). *Ampellomyces quisqualis* Ces. was used to control *Sphaerotheca fuliginea* (Schlecht. & Fr.) Poll. on glasshouse *Cucumis sativus* L. (4). Sundheim (17) demonstrated that *A. quisqualis* was resistant to the fungicide triforine (used to control powdery mildew) and that it could be sprayed in combination with triforine to obtain integrated control.

The use of *D. pulvinata* as a biological control agent may represent a model system to study the effects of different environmental parameters that are pertinent to the development of a foliar biocontrol system. The purpose of this research was to study the effect of several different environmental factors involved in establishing *D. pulvinata* in lesions of *C. personatum*. These factors included: temperature, pH, spray formulation best suited for spore survival of *D. pulvinata*, effect of pesticides currently used in peanut pest management, and spore survival under varied temperature and relative humidity conditions.

MATERIALS AND METHODS

Cultivation of *Dicyma*. An isolate of *D. pulvinata* was obtained from parasitized hyphae and spores of *C. personatum* in a leaf spot of peanut (18) in Atascosa County, TX, during 1979. *D. pulvinata* was cultured on V-8 agar in plastic petri dishes for 7–14 days at 25 C. Spores were harvested by flooding plates with distilled water and scraping the surfaces with an inoculating loop.

Broth culture studies. A suspension (0.1 ml) of distilled H_2O-washed spores of *D. pulvinata* (1×10^6 spores per milliliter) was added to each of 40 125-ml Erlenmeyer flasks containing 37 ml of sterile yeast extract-mannitol broth (YMB). The YMB medium had the following composition: 10 g of mannitol, 0.5 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of NaCl, and 0.5 g of yeast extract per liter of distilled water. Stationary cultures were grown at different temperatures with each set of four flasks representing one temperature regime. Temperature regimes tested included 2, 5, 10.5, 16.5, 22.5, 25, 28, 30, 31.5, and 35 C. Growth was expressed as dry weight of mycelium collected on tared Whatman No. 1 filter paper after drying at 60 C in an oven for 24 hr.

Colonization of *C. personatum* in lesions. A total of 1,300 leaf spot lesions from naturally infected peanut leaflets (cultivar Tamnut-74) were collected from the field. Leaflets were sprayed with 150-ml suspension of spores of *D. pulvinata* (1×10^6 spores per milliliter) in methylcellulose (CMC) on the abaxial surface and air-dried. The leaflets were placed into glass petri dishes containing moistened filter paper to have 100 lesions total divided among three dishes. Petri dishes were incubated at different temperatures with each set of three dishes representing one temperature regime. Temperature regimes included 23, 25, 28, 30, 31.5, 35 C (constant) and 25/28, 25/30, 25/31.5, 25/35, 28/30, 28/31.5, and 30/31.5 C (12-hr intervals at each temperature). Percentage of lesions of *C. personatum* exhibiting visible signs of colonization and sporulation by *D. pulvinata* was monitored through time at each temperature regime.

Pesticide studies. Pesticides used in commercial peanut production were evaluated for inhibitory action toward growth of *D. pulvinata*. Pesticides tested included: benomyl, mancozeb, PCNB, carbofuran, chloropyrifos, carboxin, copper hydroxide, aldicarb, and triphenyltin hydroxide. Nonsterile stock solutions of pesticides were prepared and amounts calculated to contain 2.5–25,000 μg/ml of active ingredient (a.i.) were pipetted into 250-ml Erlenmeyer flasks containing 100 ml of sterilized V-8 agar. Four replicates of each pesticide concentration were prepared. Final concentrations (a.i.) of each pesticide in V-8 agar are listed in Table 1. Spores from 7-day-old V-8 agar slant cultures of *D. pulvinata* were suspended in sterile distilled H_2O to give a titer of 1×10^5 spores per milliliter. This suspension (0.1 ml) was spread onto pesticide-amended V-8 agar dishes and incubated at 25 C. Growth of *D. pulvinata* (number of spores germinating in pesticide amended medium) was expressed as: XXX = solid mycelial lawn, XX = 300–500 colonies per dish, X = fewer than 300 colonies per dish, and O = no germination and growth.

In a preliminary experiment, benomyl at concentrations ≥ 0.5 μg/ml was found to inhibit growth of *D. pulvinata*. Isolation of a benomyl-tolerant strain of *D. pulvinata* encompassed subjecting spores of *D. pulvinata* to chemical mutagenesis with ethyl methane-sulfonate (EMS) by a modification of the procedure of Lindegren et al (6). EMS-treated spores were transferred onto V-8 agar containing benomyl (0.5 μg/ml a.i.) and colonies formed at 25 C after 7 days were selected for further evaluation.

pH studies. The effect of pH on germination and growth of *D. pulvinata* was determined in potato-dextrose extract broth (Difco) adjusted to several hydrogen ion concentrations with sterile NaOH or HCl solutions after autoclaving. Spores from 7-day-old V-8 agar slant cultures of *D. pulvinata* were washed three times in sterile distilled H_2O, resuspended at a concentration of 1×10^6 spores per milliliter, and 0.1 ml was added to each flask. Growth at each pH value was determined in triplicate at pH values of 2.9, 3.2, 3.3, 4.0, 5.4, 6.5, 7.7, 8.7, and 9.2. Growth was expressed as dry weight of mycelium collected on tared Whatman No. 1 paper after drying at 60 C in an oven for 24 hr.

Spore survival in spray carriers. Spray carriers selected for use included: H_2O, Sunspray-7E, glycerol, Tween 80, CMC, citrus pectin, and ghatti gum. Final concentrations of each carrier are listed in Table 2. Fifty-milliliter stock solutions of each spray formulation to be tested were autoclaved in 125-ml Erlenmeyer flasks. On cooling, two 5-ml aliquots of each solution were pipetted into sterile test tubes. H_2O-washed spores of *D. pulvinata* (0.2 ml) was added to each tube. One set of tubes was incubated at 6 C and the other set at 25 C. The initial viable spore counts and viable counts through time were determined by serial dilution of spray carriers and culturing of 0.1 ml of each dilution on V-8 agar dishes. Four replicates of each dilution were prepared. Colonies of *D. pulvinata* after 7 days of incubation at 25 C were enumerated.

Spore survival under varied temperature and humidity conditions. Humidity chambers were prepared by placing varying concentrations of aqueous glycerol (w/w) into 460-ml Mason jars (100 ml per jar). Approximate relative humidity values were 10, 20, 40, 60, 80, and 100% (H_2O alone). For desiccated conditions, 100-ml volume of Drierite (Fisher Chemicals) per jar was added. Disks (5 mm) of dialysis tubing were cut with a hole puncher, autoclaved in a glass petri dish, and oven dried at 60 C for 2 hr. Disks were used as supports for spores of *D. pulvinata* in the humidity chambers. The disks were aseptically removed with sterile forceps and placed onto sporulating cultures of *D. pulvinata* grown in V-8 agar dishes. The disks with adhering spores were placed onto sterilized stainless steel screen wire cut to size to fit in the mouth of the jars suspended above their contents (50 disks per jar). The jars were then capped. One set each of different RH jars were incubated at 10, 25, and 37 C. Four disks from each jar were periodically removed and aseptically placed onto V-8 agar and observed for growth at 25 C after 7 days incubation.

RESULTS

Temperature studies. In broth culture, a typical growth curve was observed with maximal growth of *D. pulvinata* occurring at 23–25 C. At 28 C, growth rate declined to 22% of maximal and at

30 C 5% of maximal growth developed. On detached peanut leaflets (Fig. 1), maximal colonization of lesions of *C. personatum* occurred at 23–28 C and followed a logistic growth pattern. At 30 C, the asymptotic level of lesion colonization had only decreased to 60% of maximal (compared with 5% of maximal observed in broth culture at this temperature). The time lag in colonization increased and the asymptotic level declined markedly at maximal temperatures greater than 28 C. No colonization was observed at constant temperatures ⩾31.5 C (although growth did occur in cycling temperatures in which the minimum temperature was <31.5 C).

Maximal sporulation of *D. pulvinata* in leaf spot lesions occurred at 23–28 C (Fig. 2). No sporulation occurred in colonized lesions incubated at maximal temperature of 31.5 C, regardless of minimal temperatures. As with growth, the time lag in sporulation increased and the asymptotic level declined markedly at maximal temperatures >28 C.

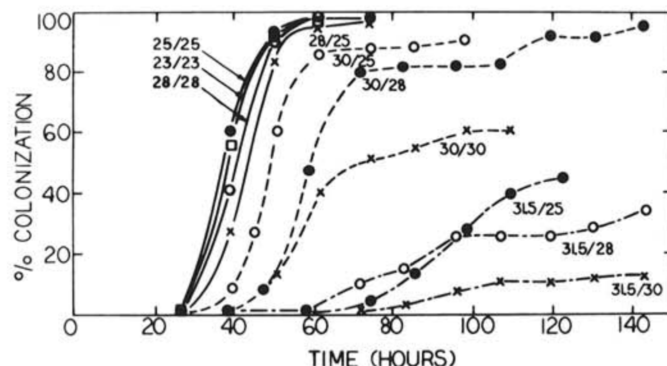

Fig. 1. Effect of temperature on colonization of *Cercosporidium personatum* lesions of infected peanut leaflets by the mycoparasite *Dicyma pulvinata*. Visible signs of colonization by *D. pulvinata* were recorded at 12-hr temperature intervals of 23/23, 25/25, 28/25, 28/28, 30/25, 30/28, 30/30, 31.5/25, 31.5/28, and 31.5/30 C.

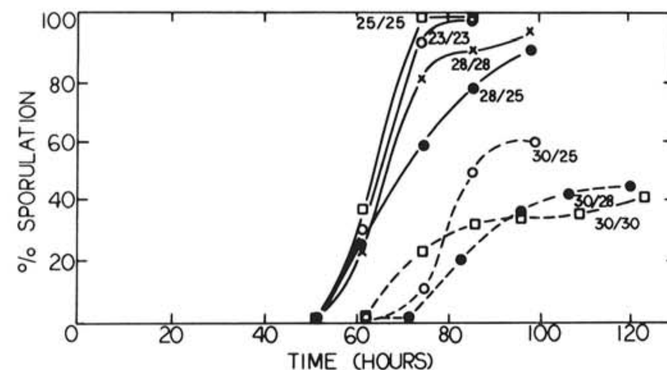

Fig. 2. Effect of temperature on sporulation of *Dicyma pulvinata* on lesions of *Cercosporidium personatum* on detached peanut leaflets. Visible signs of sporulation of *D. pulvinata* were recorded at 12-hr temperature intervals of 23/25, 25/25, 28/25, 28/28, 30/25, 30/28, and 30/30 C.

Effect of selected pesticides on growth of *Dicyma*. Benomyl, mancozeb, and triphenyltin hydroxide (Table 1), when tested at concentrations similar to those used during commercial field applications, completely inhibited germination and growth of *D. pulvinata*. Slight inhibition of growth of *D. pulvinata* was observed with carbofuran, quintozene, and carboxin.

A chemically induced mutant of *D. pulvinata* (BR30) was selected that was tolerant of benomyl at concentrations up to 10 μg/ml a.i. In growth chamber studies, BR30 was shown to colonize lesions of *C. personatum* as efficiently as the wild-type isolate in the absence of benomyl; however, if benomyl (10 μg/ml a.i.) was applied to plants infected with *C. personatum* only the BR30 isolate of *D. pulvinata* was observed to colonize lesions. Similar rates of lesion colonization by isolate BR30 occurred with or without application of benomyl to plants.

Effect of pH on growth of *D. pulvinata*. *D. pulvinata* grew over a broad pH range of 3.2–8.7. Maximal growth rates were observed between pH values of 3.3–7.7. A sharp decrease in growth occurred at pH values below 3.3, whereas a more gradual decline occurred at pH values >7.7.

Survival of spores of *D. pulvinata* in spray carriers. Spores of *D. pulvinata* survived for 29 days (Table 2) in carriers consisting of H_2O, 0.1–0.2% CMC, 0.2–0.4% citrus pectin, or 0.25% ghatti gum incubated at 25 C; or 60 days (data not shown) at 6 C, with little loss in viability. Viability of spores of *D. pulvinata* was higher after 29 days in the above carriers when incubated at 25 C compared with 6 C. Whether this pattern occurs after 60 days of incubation is not known because the 25 C experiment was terminated after 29 days. At either temperature, poorest survival was noted with spores suspended in Tween 80, glycerol (all concentrations), and 0.4–0.8% CMC formulations.

Spore survival under varied temperature and humidity conditions. Viability of spores of *D. pulvinata* was maintained for a longer period of time at 10 C (irrespective of RH), compared with 25 and 37 C (Fig. 3). Spores of *D. pulvinata* maintained at 10 C survived for at least 72 days regardless of the RH, except under desiccation (Fig. 3). Desiccated spores retained viability for 55 days. At 25 C, spores remained viable for 21 days under desiccation, 60 and 80% RH; 43 days at 20 and 40% RH; and 72 days at 10% RH. At this temperature, spore survival was more dependent on RH than at 10 C. Spores survived best if maintained between 10–40% RH. The prolonged viability of spores of *D. pulvinata* maintained at both 10 and 25 C under 100% RH appeared to be due to the viability of vegetative mycelium. Microscopic examination of spores maintained at 100% RH demonstrated that spores of *D. pulvinata* had germinated within 6 days. Spores failed to germinate at all other relative humidities and temperatures. At 37 C, spores maintained under desiccation, 80 and 100% RH remained viable for only 1 day; spores maintained at 10, 20, 40 and 60% RH remained viable for 2 days.

TABLE 1. Growth of *Dicyma pulvinata* in the presence of pesticides

	Growth index[a] (μg/ml a.i.)											
Pesticide	0.025	0.050	0.100	0.250	0.500	1.000	2.500	5.000	10.00	50.00	100.0	500.0
Benomyl	XXX	XXX	XX	0	0	0	0	0	0	0	0	0
Mancozeb	XXX	XXX	XXX	XXX	XXX	XX	X	0	0	0	0	0
Quintozene	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XX	X	0	0	0
Carbofuran	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XX	X	X	X	X
Chloropyrifos	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XX	XX	XX	X	X
Carboxin	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XX	XX	0	0	0
Copper hydroxide	XX	XX	XX	XX	XX	XX	XX	XX	XX	XX	XX	XX
Aldicarb			XX	XX	XX	XX	XX	XX	XX	XX		
Triphenyltin hydroxide				XX	XX	XX	XX	0	0	0	0	0

[a] XXX = Solid mycelial lawn, XX = 300–500 colonies/plate, X = <300 colonies/plate, 0 = no growth. Blank spaces indicate either untested or interpretation problem.

DISCUSSION

D. pulvinata exhibited a broader growth range in terms of maximal growth rates (23–28 C) on peanut leaflets infected with *C. personatum* (Fig. 1) compared with growth rates in broth culture (23–25 C). At 30 C, only 5% of maximal growth was observed in broth culture, whereas the asymptotic level of colonization on leaflets infected by *C. personatum* decreased to only 50% of maximum. These results demonstrate the necessity of using a test system that more closely resembles natural conditions (bioassay) in studies involving environmental parameters of mycoparasites. The time lag in colonization of *C. personatum* by *D. pulvinata* increased and the asymptotic level declined markedly at temperatures greater than 28 C. No growth was apparent at 35/25 C or constant temperatures $\geqslant$ 31.5 C. In the detached leaflet study, growth response of *D. pulvinata* subjected to different temperature regimes could be divided into three sections (Fig. 1) based on the time lag in colonization and asymptotic level achieved: maximal temperatures of (I) 23–28 C, (II) 30 C, and (III) 31.5 C. Temperature regimes $<$ 23 C were not tested in these experiments because higher temperatures normally occur during peanut production; however, colonization rates at temperatures $<$ 23 C would most likely fit into sections I, II, or III. No sporulation was observed to occur at temperature regimes in section III (Fig. 2). The time lag in sporulation increased and the asymptotic level declined (as demonstrated with colonization) markedly as temperature regimes increased through section I to section II (Fig. 2). This information may be of value in the development of future modeling schemes of *D. pulvinata* mycoparasitism, as affected by temperature. The results herein indicate that only the maximum and minimum daily temperatures attained need be measured and used in calculations (disregarding for the present the length of time for which this temperature is maintained). The section (I, II, or III) under which this maximal temperature resides may then be used to estimate the rate of colonization of *C. personatum* by *D. pulvinata*.

Pesticide studies demonstrated that *D. pulvinata* could be incorporated with the insecticides chloropyrifos and carbofuran onto peanut plants without any adverse effects (Table 1). Furthermore, treatment of the soil with the nematicide aldicarb should have no deleterious effect on saprophytic growth of *D. pulvinata*. Growth of *D. pulvinata* was inhibited by the fungicides benomyl, mancozeb, and triphenyltin hydroxide. An isolate of *D. pulvinata* (BR30) was selected in the lab which was tolerant to benomyl at 10 μg/ml concentration. This isolate could be incorporated into an existing pest management system that uses benomyl.

When *D. pulvinata* is used on a commercial basis it will be important to determine the type of spray formulation in which spores of *D. pulvinata* survive best. Best survival of spores of *D. pulvinata* was in the carriers H_2O, 0.1–0.2% CMC, 0.2–0.4% citrus pectin, and 0.25% ghatti gum. At the temperatures tested, poorest survival was observed with spores suspended in Tween 80, glycerol (all concentrations), and 0.4–0.8% CMC formulations; hence, these carriers would not be appropriate in commercial production of inoculum formulations of *D. pulvinata*.

A study was conducted under controlled temperature and relative humidity conditions in the laboratory to relate how well spores of *D. pulvinata* might survive in the field. Viability of spores of *D. pulvinata* was maintained for a longer period of time at 10 C (irrespective of RH), compared with 25 and 37 C (Fig. 3). Viability

TABLE 2. Number of colonies of *Dicyma pulvinata* produced after 29 days incubation in different spray formulations

Formulation	Concentration	Colony forming units ($\times 10^4$)[a] 6 C	25 C
H_2O		89.0	158.0
Tween 80		82.0	12.0
Sunspray-7E	10^{-2}	81.0	5.0
	10^{-3}	86.0	8.0
	10^{-4}	104.0	87.0
Glycerol (%)	5	17.0	1.0
	10	16.0	3.0
	20	10.0	2.0
	40	25.0	0.1
Methyl cellulose (%)	0.1	77.0	135.0
	0.2	66.0	115.0
	0.4	66.0	6.0
	0.8	25.0	11.0
Citrus pectin (%)	0.2	134.0	121.0
	0.4	95.0	125.0
	0.8	109.0	15.0
	1.6	22.0	6.0
Ghatti gum (%)	0.25	106.0	129.0
	0.5	134.0	18.0
	1.0	92.0	2.0
	2.0	85.0	1.0

[a] Original population at time = 0 was 142×10^4 cfu/ml.

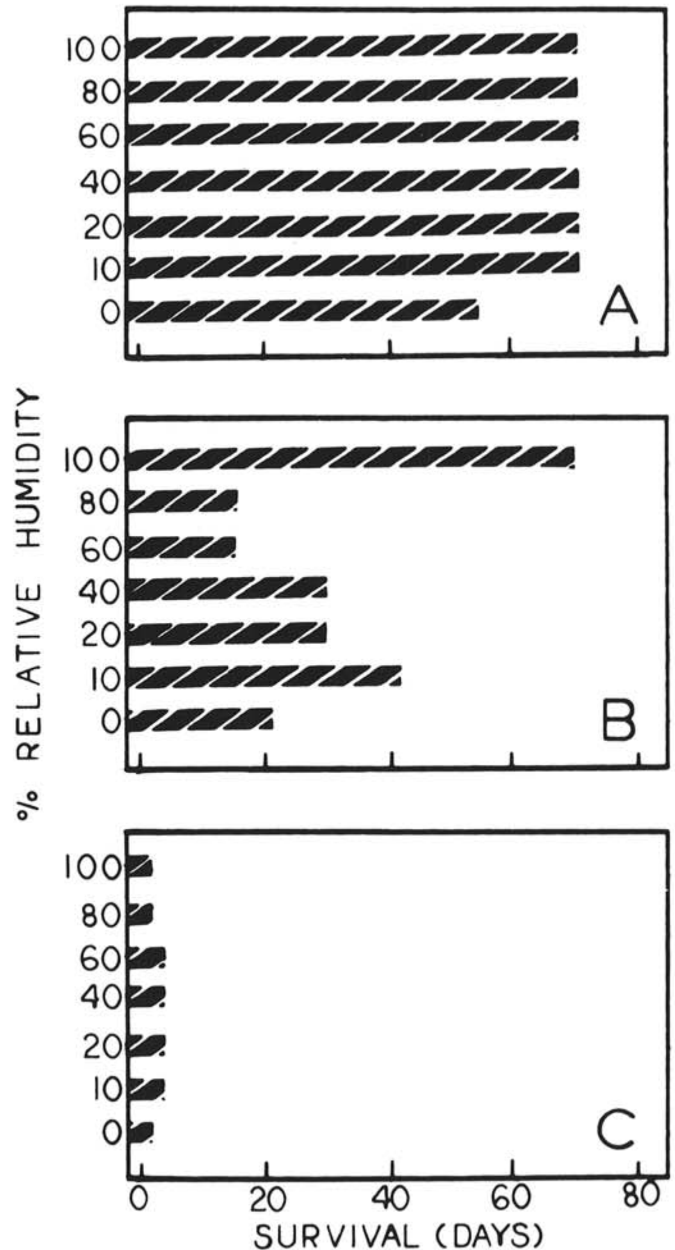

Fig. 3. Survival of spores of *Dicyma pulvinata* under varied temperature and relative humidity conditions. Spores of *D. pulvinata* supported on 5-mm disks of dialysis tubing were placed at different relative humidities (0–100%) and incubated at either **A**, 10; **B**, 25; or **C**, 37 C. Disks were periodically removed from jars and aseptically placed onto V-8 agar to observe for fungal growth.

of spores incubated at 25 C was more dependent on RH than spores incubated at 10 C. At all three temperature regimes tested, spores of *D. pulvinata* retained viability longer at relative humidities between 10–40%. Under field conditions in which germination of *D. pulvinata* would not occur (RH less than 88%), spores of *D. pulvinata* would survive for at least 14–43 days if the maximal daily temperature were maintained near 25 C. If the RH became greater than 88% within this time frame, then germination of *D. pulvinata* would occur. On the other hand, if temperatures suddenly rose to 37 C, then spores of *D. pulvinata* would only survive for 2 days maximum. In the latter situation, another application of spores of *D. pulvinata* would be recommended. More data must be collected on temperature vs. RH interactions to develop a more accurate appraisal of spraying schedules for this biocontrol agent.

Several factors affecting the biological control of *C. personatum* by *D. pulvinata* have been discussed. Information collected dictates the importance of using a bioassay, whenever possible, when studying environmental parameters affecting the use of this mycoparasite, compared with in vitro studies. Furthermore, it is possible to select for a mycoparasite better equipped for integration into existing pest management systems. Finally, the spray formulation should be composed of compounds which, in certain concentrations, maintain spore shelf life and survival in the field, as well as enhance mycoparasitic activity.

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