Evaluation of Agar and Grain Media for Mass Production of Conidia of *Dactylaria higginsii*

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ABSTRACT

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Growth and sporulation of *Dactylaria higginsii* were quantified on complex agar media containing biological materials (group 1) and chemically defined agar media (group 2), as well as on grains, and the inoculum produced on these various substrates was tested for virulence on *Cyperus rotundus*. The fungus grew well between 25 and 30°C on potato dextrose agar (PDA), with 27°C being the optimum temperature. Generally, conidial production was highly variable and lower on complex agar media than on chemically defined media. Addition of purple nutsedge leaves to PDA did not increase colony growth or conidial production when compared with unamended PDA. Conidial production was lowest on brown rice compared with white rice or white rice with nutsedge leaves. Peak production on grain media occurred from day 12 in test 1 (2.4×10^6 spores/g of grain) and on day 16 in test 2 (2.5×10^6 spores/g of grain). Germination rate of conidia produced on white rice was 50% compared with the near 100% germination of conidia produced on PDA or on white rice amended with potato dextrose broth (PDB). Conidia produced on white rice or PDA, when tested fresh or after two washings, were less virulent on *C. rotundus* than conidia from white rice amended with PDB. After four washings, conidia from all three media produced the same level of disease severity. White rice supplemented with PDB and PDA in trays were suitable for mass production of conidia of *D. higginsii*.

Additional keywords: biocontrol, chemical stimulants, inhibitory substances

Cyperus rotundus L., purple nutsedge, severely decreases crop yields of more crops in more countries than any other weed (10). Presently, it is managed effectively by the soil fumigant methyl bromide, and less effectively by other chemicals, such as dazomet, metam sodium, halosulfuron, and sulfentrazone (26). It is expected that methyl bromide will be phased out in the year 2005, and the other chemicals are labeled only for a few crops. Therefore, biological control is being pursued as an additional option for the management of purple nutsedge (15). In this

regard, the foliar fungal pathogen *Dactylaria higginsii* (Luttrell) M. B. Ellis, a dematiaceous hyphomycete, is a promising biocontrol agent for this weed (15,18). It forms elliptical, pale-brown leaf spots surrounded by a narrow, dark-brown border. The spots coalesce to form larger irregular spots, causing the leaves to die back from the tips (14,19). The host specificity and weed-control efficacy of *D. higginsii* and the environmental factors conducive for disease development on purple nutsedge have been determined in laboratory and field studies (15–18).

Using *D. higginsii* as a bioherbicide requires a low-cost method of inoculum production. For commercial production, submerged fermentation is generally preferred over the use of living host plants (which is required for obligate parasites), solid-substrate fermentation, or biphasic fermentation (3,4,13,21,27). The registered bioherbicides Collego and DeVine, and *Colletotrichum gloeosporioides* f. sp. *malvae*, soon to be registered as Mallet WP (6), are produced by submerged fermentation. Hyphomycetes that do not sporulate readily in liquid culture (23) may sporulate on solid substrates including various cereal grain, straw, or inert materials supplemented with water or nutrients (28). Biphasic fermentation protocols may also produce good spore yields. In this method, the mycelial biomass is grown in liquid culture and the biomass is blended and

spread in a thin layer in a tray, then allowed to dry gradually to induce sporulation (31,32).

In addition to the fermentation method, the type of growth medium and the nutritional and physical environments of the mass-production system are of paramount importance because they greatly affect the number, type, stability, durability, and virulence of fungal propagules (10,11). For instance, conidia produced on solid substrate have been found to be more virulent and stable and have a longer half-life than conidia obtained from submerged culture (21,23). Luttrell (19) reported that *D. higginsii* sporulated abundantly on 2% malt agar but, after several transfers, cultures contained only sparse mycelial growth that totally lacked conidial production. Furthermore, *D. higginsii* does not sporulate in submerged liquid cultures.

Except for the early observations of Luttrell (19), the cultural requirements for mass production of *D. higginsii* have not been established, and the work presented here is the first detailed study in this regard. The objectives of this study were to develop an easy, rapid, and inexpensive method of mass production of *D. higginsii* inoculum on solid substrate and to evaluate the relative virulence of inoculum from the three most suitable media on purple nutsedge in the greenhouse. An abstract reporting some aspects of this study has been published (33).

MATERIAL AND METHODS

Fungal material. *D. higginsii* was isolated from *C. rotundus* in Gainesville, FL (15). A single-spore culture was grown on potato dextrose agar (PDA) and a conidial suspension was prepared from the PDA culture and used to inoculate sterile soil in glass test tubes. The inoculated soil tubes were stored at ambient temperature in the dark to serve as an inoculum source for all experiments. For inoculum production, a few milligrams of the infested soil were spread on PDA plates. After 1 week, newly developed mycelium was transferred to fresh PDA plates and grown for 2 weeks in the dark at $25 \pm 1^\circ\text{C}$. These plates served as the source for agar plugs or mycelial-spore suspensions for all experiments.

Plant material. A collection of purple nutsedge from Florida (FL01, Gainesville), clonally propagated in a greenhouse at $28 \pm 3^\circ\text{C}$, was used. For tests, two tubers were planted in each pot (9 cm in diameter by 8

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cm high) containing a commercial potting medium (Metro-Mix 300; Scott-Sierra Horticultural Products Co., Marysville, OH). Pots were placed on a greenhouse bench and watered regularly. Three-week-old plants were used in all experiments.

Agar and grain media. Agar media tested were divided into complex and chemically defined media (Table 1). The former group included those containing some undefined biological materials, including commercially available media, such as PDA, oatmeal agar (OMA), modified Sabouraud's agar (MSBA), or those readily prepared in the laboratory, such as dextrose-yeast extract agar (DA-YE), PDA and nutsedge leaf extract (PDA-NLE), malt agar and yeast extract (MA-YE), V8 juice agar (V8A), and cornmeal agar (CMA). To prepare PDA-NLE, 2.5 g of freshly cut purple nutsedge leaves boiled for 10 min in 100 ml of deionized water were used. Chemically defined media included Czapek-Dox agar (CZDOX), Sach's agar (SACH), and Sanderson and Serb medium (SSM). Tests with agar media were done in 9-cm-diameter plastic petri plates.

The grain media tested were white rice (WR; Long Grain Rice, Publix Super Markets, Inc., Lakeland, FL), WR supplemented with purple nutsedge leaves cut to approximately 1-cm in length (WR-NL), and brown rice (BR; Long Grain Brown Rice, Publix Super Markets). Grain (30 g) was placed in a 250-ml Erlenmeyer flask and autoclaved for 40 min at 121°C. The flasks were shaken vigorously soon after removal from the autoclave to prevent lumping of the grain. After cooling, 18 ml of deionized water was added to each flask of WR and BR (WR and BR media) and 20 ml of water and 1 g of cut nutsedge leaves were added to WR (WR-NL medium). The grain was allowed to sit over-

night and autoclaved again for 40 min before use.

Determination of carbon:nitrogen ratios of agar media. For chemically defined agar media, carbon-to-nitrogen (C:N) ratios were based on the amount of ingredients used and their chemical formulas (Table 1). For complex media, total organic carbon was defined by oxidation and total Kjeldahl nitrogen by colorimetric standard methods described in Environmental Monitoring and Support Laboratory (7). The analyses were conducted by Advanced Technologies & Testing Laboratories, Alachua, FL.

Effect of temperature on fungal growth on PDA. The optimum temperature during dew period, dew duration, illumination level, and photoperiod for *D. higginsii* disease development on purple nutsedge have been determined by Kadir et al. (16). These conditions were used in the following experiments. To determine the optimum temperature for in vitro growth, which had not been previously established (16), *D. higginsii* was grown on PDA in 9-cm-diameter petri plates. One 3-mm-diameter agar plug, obtained from a 14-day-old nonsporulating PDA culture, was placed centrally in each plate. Six replicate plates per temperature were incubated for 17 days in microbial incubators set at 10, 15, 20, 25, 30, and 35°C. Colony diameter was measured in millimeters, and the precise temperature inside the cabinet was recorded with a HOBO Pro Series relative humidity and temperature data logger (Onset Computer Corp., Pocasset, MA). The fungus grew best between 25 and 30°C; therefore, all further experiments were conducted within this temperature range.

Assessment of fungal growth and sporulation on agar and grain media. Agar plates were inoculated centrally with a 5-mm-diameter agar plug. Flasks with

grain were inoculated with three 0.75-cm-diameter agar plugs. There were four replicate plates or Erlenmeyer flasks per medium per test. Inoculated agar plates and flasks were arranged completely randomized in a growth chamber and incubated at $26 \pm 1^\circ\text{C}$ with a 12-h photoperiod and light intensity of $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ provided by fluorescent light bulbs (16).

On agar media, fungal growth was recorded by measuring the colony diameter (in millimeters) and reported as growth per milliliter of agar used (15 ml per plate). Colony diameter was measured 1, 2, 4, 5, 7, 11, and 14 days after inoculation.

Spore production on agar media was determined after 14 days of growth by flooding the plates with 2.5 ml of deionized water, gently rubbing the culture with a rubber spatula to detach the conidia, and filtering through a double layer of cheesecloth to remove large mycelial masses. Conidial concentration was determined with a haemocytometer and expressed as number of conidia per milliliter of agar.

Spore production on grain media was determined after 6, 8, 10, 12, 14, 16, and 21 days, by rinsing the culture with 40 ml of deionized water and filtering through a double layer of cheesecloth. The number of conidia per milliliter of the filtrate was determined and reported as number of spores per gram of grain.

Inoculum production for pathogenicity test. Based on preliminary results, WR was chosen as an effective and relatively inexpensive medium for scaling up inoculum production. Conidia were produced on WR in 2.8-l Fernbach flasks, with water or potato dextrose broth (PDB) added. PDA, which supported good colony growth as well as sporulation, was used for comparison. For grain media, 180 g of WR was autoclaved for 40 min at 121°C. Immediately after removal from the autoclave,

Table 1. Complex and chemically defined agar media tested, ingredients, carbon-to-nitrogen (C:N) ratio, and a source or reference

Medium	C:N ratio	Ingredients/liter of water	Source or reference
Complex			
Potato dextrose agar (PDA)	10:1	200 g of infusion of potatoes, 20 g of dextrose, 15 g of agar	Difco
Oatmeal agar (OMA)	4:1	60 g of oatmeal, 12.5 g of agar	Difco
Modified Sabouraud's agar (MSBA)	6:1	20 g of dextrose, 10 g of neopeptone, 20 g of agar	2
Dextrose-yeast extract agar (DA-YE)	8:1	7.5 g of yeast extract, 20 g of dextrose, 15 g of agar	5
PDA + nutsedge leaf extract (PDA-NLE)	21:1	200 g of infusion of potatoes, 20 g of dextrose, extract of 25 g of boiled purple nutsedge leaves	
Malt and yeast extracts agar (MA-YE)	8:1	3 g of malt extract, 0.5 g of KH_2PO_4 , 2 g of yeast extract, 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 20 g of agar	5
V8 Juice agar (V8A)	10:1	200 ml of V8 canned vegetable juice, 3 g of CaCO_3 , 14 g of agar	2
Cornmeal agar (CMA)	22:1	50 g of infusion of corn meal, 15 g of agar	Difco
Chemically defined			
Czapek-Dox agar (CZDOX)	3:1	2 g of NaNO_3 , 1 g of K_2HPO_4 , 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g of KCl, 0.01 g of FeSO_4 , 30 g of sucrose, 20 g of agar	5
Sach's agar (SACH)	1:1	4 g of CaNO_3 , 0.25 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, trace of FeCl_3 , 0.25 g of K_2HPO_4 , 4 g of CaCO_3 , 20 g of agar	5
Sanderson and Serb (SSM)	1:2	2 g of KH_2PO_4 , 0.6 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g of NaCl, 0.1 g of CaCl_2 , 2.4 g of NaNO_3 , 10 g of sucrose, 20 g of agar, 1 ml of trace element stock solution (TESS); TESS = 9 mg of H_3BO_3 , 1.95 mg of KI, 58.5 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 9 mg of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 7.6 mg of NaMoO_4 , 822 mg of $\text{ZnSO}_4 \cdot 6\text{H}_2\text{O}$, 139.8 mg of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 300 ml of water	5

lumps of grain were broken up by vigorously shaking the flasks. After cooling, 120 ml of deionized water or PDB was added and the grain was allowed to sit overnight at room temperature. The grain was autoclaved again for 40 min at 121°C, cooled to room temperature, and inoculated. To prepare the PDA medium, 500 ml of sterile PDA amended with antibiotics (50 mg of chloramphenicol and 74 mg of streptomycin sulfate per liter of medium) was poured into an autoclaved aluminum baking tray (29 by 42 cm). The medium was inoculated after it solidified.

Rice and PDA media were inoculated using the same 14-day-old culture grown on PDA. Rice was inoculated with 18 agar plugs (0.75-cm diameter) cut from the PDA culture. For the PDA trays, a suspension of mycelium and conidia in sterile water was prepared by carefully rinsing the PDA plates. Antibiotics were added to the suspension (at the previously mentioned concentrations), and 15 ml of the suspension were spread over the agar surface in the tray with a sterile pipette. The tray was then rocked slowly to cover the entire sur-

face with the suspension, packed in a sterile Reynolds oven bag (Reynolds Metals Company, Richmond, VA), placed, together with the Fernbach flasks, in a growth chamber at $26 \pm 1^\circ\text{C}$ in the dark for 2 weeks, and grown as still cultures.

Spore germination test. In preliminary trials, conidia produced on WR, despite their normal appearance under light microscope, germinated at lower rates than those produced on PDA, suggesting the possible presence of endogenous inhibitors of germination. To see whether washing conidia might improve their germinability, conidia produced on rice were filtered through a double layer of cheesecloth and a 20-ml sample of the suspension was adjusted with deionized water to 10^6 conidia/ml. This suspension was centrifuged at $12,000 \times g$ for 10 min and the supernatant was collected (wash 1). The remaining conidial suspension from rice was centrifuged similarly in 20-ml aliquots for two, three, or four times to obtain washes 2 through 4.

Conidia produced in PDA trays were detached from the agar surface by carefully rubbing the culture with rubber-gloved hands. A 20-ml aliquot containing 10^6 conidia/ml of freshly harvested spore suspension was passed through a Nitex nylon filter (Tetko, Kansas City, MO) with a 30- μm -mesh opening. This conidial preparation was used as the fresh untreated conidia. Centrifugation was not possible in this case due to the lack of pelleting at $12,000 \times g$, perhaps due to the production of more buoyant conidia on this medium.

Suspensions of conidia from WR, WR-PDB, and PDA as well as the untreated and

washed conidia (washes 1 through 4) were sprayed on 1.5% water agar plates. The water agar plates, with four replicated plates per treatment, were placed completely randomized in a growth chamber at 25°C in the dark for 18 h. The percentage of germinated conidia was estimated 24 h after inoculation by counting 50 spores per plate. A conidium was considered as germinated when the germ tube was longer than conidial length.

Pathogenicity test. Trials with grain showed that the highest conidia production occurred on WR when compared with BR. However, the rate of germination of the WR-produced conidia was lower than that of PDA-produced conidia; therefore, pathogenicity of conidia produced on WR, WR and PDB, and PDA was tested on purple nutsedge in the greenhouse. In addition, conidia produced on these media and washed twice or four times (wash 2 and wash 4), as well as fresh untreated conidia (FS), were tested.

Purple nutsedge plants were sprayed to run-off with conidial suspensions at a concentration of 10^6 conidia/ml in 0.5% (wt/vol) Metamucil (a plant-derived hydrophilic mucilloid; Procter & Gamble, Cincinnati, OH) using an aerosol sprayer. Control plants were sprayed with water plus 0.5% (wt/vol) Metamucil (water). There were four replicate pots, each with two plants grown from tubers. The inoculated plants were placed in a dew chamber at $27 \pm 1^\circ\text{C}$ in the dark for 24 h, then transferred to the greenhouse, kept completely randomized, and maintained at 25 to 30°C dur-

Table 2. Slopes of *Dactylaria higginsii* mean colony diameters on complex and chemically defined agar media measured seven times over 14 days^v

Medium ^x	Slope (mean) ^w	
	Test 1 ^y	Test 2 ^z
Complex		
OMA	0.35 a	0.34 a
CMA	0.27 b	0.34 a
PDA	0.28 b	0.30 b
V8A	0.28 b	0.27 c
DA-YE	0.25 b	0.26 c
MA-YE	0.25 b	0.15 d
PDA-NLE	0.17 c	0.17 d
MSBA	0.06 ef	0.15 d
Chemically defined		
SACH	0.09 d	0.16 d
CZDOX	0.04 e	0.07 e
SSM	0.04 e	0.02 f

^v Growth was measured 1, 2, 4, 5, 7, 11, and 14 days after inoculation.

^w Mean values from four replicate petri dishes. Growth-curve data (slopes) were analyzed by random coefficient regression analysis. The slope values represent the rate of colony growth; high values signify profuse growth while low values indicate delayed or inhibited growth.

^x OMA, oatmeal agar; CMA, cornmeal agar; PDA, potato dextrose agar; V8A, V8 juice agar; DA-YE, yeast extract-dextrose agar; MA-YE, malt and yeast extracts agar; PDA-NLE, PDA and nutsedge leaf extract; MSBA, modified Sabouraud's agar; SACH, Sach's agar; CZDOX, Czapek-Dox agar; SSM, Sanderson and Serb medium.

^y Means in this column followed by different letters differ significantly at $P < 0.001$, according to the least significant difference (LSD) test ($\text{LSD} = 0.05$, $R^2 = 0.97$).

^z Means in this column followed by different letters differ significantly at $P < 0.001$, according to the LSD test ($\text{LSD} = 0.03$, $R^2 = 0.94$).

Fig. 1. Effect of temperature on growth of *Dactylaria higginsii* on potato dextrose agar (PDA) in petri plates. Cultures were grown from standard-sized inoculum discs for 17 days. Data were averaged from two trials, each with six replicate plates per temperature and plotted against the precise temperatures recorded inside incubators set at 10, 15, 20, 25, 30, or 35°C . Means denoted by different letters are significantly different according to Duncan's multiple range test ($\alpha = 0.01$).

ing the day and 20°C at night under ambient diurnal light conditions.

Disease severity was used to estimate the virulence of inocula. Disease severity

Table 3. Spore production by *Dactylaria higginsii* on complex and chemically defined media 14 days after inoculation

Media ^x	Spores/ml of agar ^w	
	Test 1 ^y	Test 2 ^z
Complex		
MA-YE	123,438 a	10,000 bcde
PDA	85,650 a	175,000 a
V8A	60,850 a	66,250 bcd
OMA	18,750 ab	131,667 ab
DA-YE	11,667 abc	27,500 bcde
MSBA	2,833 abc	5,833 cde
PDA-NLE	7,813 abc	3,125 e
CMA	9,375 abc	45,625 bcd
Chemically defined		
CZDOX	14,063 ab	4,833 de
SSM	4,688 abc	77,333 bc
SACH	850 abc	2,125 e

^w Each value represents the combined means of four replicate petri dishes. Nontransformed data are shown.

^x MA-YE, malt and yeast extracts agar; PDA, potato dextrose agar; V8A, V8 juice agar; OMA, oatmeal agar; DA-YE, yeast extract-dextrose agar; MSBA, modified Sabouraud's agar; PDA-NLE, PDA and nutsedge leaf extract; CMA, cornmeal agar; CZDOX, Czapek-Dox agar; SSM, Sanderson and Serb medium; SACH, Sach's agar.

^y Means in this column followed by different letters differ significantly at $P < 0.05$, based on the least significant difference (LSD).

^z Means in this column followed by different letters differ significantly at $P < 0.001$, based on the LSD.

(total leaf area diseased) per plant was assessed 14 days after inoculation with the modified Horsfall-Barratt scale, where 1 = 0, 2 = 1 to 5%, 3 = 6 to 10%, 4 = 11 to 15%, 5 = 16 to 30%, 6 = 31 to 55%, 7 = 56 to 65%, 8 = 66 to 75%, 9 = 76 to 85%, 10 = 86 to 95%, and 11 = 96 to 100% necrosis and death.

Statistical analysis. Experiments were conducted twice with four or six replicates per experiment as described. Data from experiments (referred to as tests 1 and 2) were pooled and analyzed together because there was no significant difference for experiment, or for the experiment-treatment interaction. Variances were transformed, if required, using the $\log_{10}$ of data points. Means were compared using the least significant difference (LSD) test or the Duncan's multiple range test as mentioned. Growth-curve data (Table 2) were analyzed by random coefficient regression analysis (9). All statistical analyses were performed with the Statistical Analysis System (SAS Institute, Cary, NC).

RESULTS

Effect of temperature on fungal growth on PDA. *D. higginsii* grew well between 25 and 30°C on PDA, with the maximum growth occurring at 27°C (Fig. 1). Growth was moderate at 22°C, but poor at 10 to 20°C and 32 to 35°C. The following studies were therefore carried out within the conducive temperature range of 24 to 30°C.

Assessment of fungal growth and sporulation on agar and grain media. The effect of media on colony growth (diameter) of *D. higginsii* was highly sig-

nificant (analysis of variance [ANOVA] $P < 0.001$, $R^2 = 0.97$ and 0.94 for tests 1 and 2, respectively). The highest growth rate was seen on OMA in test 1 and on OMA and CMA in test 2. Generally, colony growth was limited on chemically defined media (Table 2). Fungal growth on all media tested was linear over time (data not shown).

Generally, the number of conidia produced on the complex agar media was low and the production was even more sparse and erratic on the chemically defined media (Table 3). There were no significant differences among the media tested in test 1. In test 2, conidium production on PDA was higher than on MA-YE, V8A, DA-YE, MSBA, PDA-NLE, CMA, and the chemically defined media, but not different from OMA (LSD test, $P < 0.001$). In both tests, variability in conidium production was high among the agar media (Table 3). Also, the number of conidia produced was not correlated with the rate of colony growth (Tables 2 and 3) or the C:N ratios of the agar media (Table 1).

Grain medium and fungal harvest day (both $P < 0.0001$, $R^2 = 0.79$ and 0.78 for tests 1 and 2, respectively) influenced conidium production. Conidium production in both tests was significantly less on BR than on WR and WR-NL (LSD test, $P < 0.05$; Fig. 2A). In test 1, the highest production on all three media combined reached nearly 2.5×10^6 conidia/g of grain on day 12 (LSD test, $P < 0.05$; Fig. 2A) and this level did not significantly change during the remaining days of the trial (LSD test, $P < 0.05$; Fig. 2B). In test 2, however, highest conidial production occurred on

Fig. 2. Effect of **A**, grain media on *Dactylaria higginsii* spore production 21 days after inoculation and **B**, on spore production over time (days after inoculation) on all three media combined. Differences among the grain media or among the harvest days were significant at $P < 0.05$, according to a least significant difference test. Each bar in each test represents the mean of four replicated Erlenmeyer flasks. WR = white rice, WR-NL = white rice with purple nutsedge leaves, and BR = brown rice. Nontransformed data are shown.

day 16 after inoculation (LSD test, $P < 0.05$; Fig. 2B).

Spore germination. Conidial germination was affected by the production medium (Fig. 3A) (ANOVA; $P < 0.0001$, $R^2 = 0.96$) but not by the washing steps ($P = 0.117$; data not shown). Conidia produced on WR germinated at a significantly lower rate (50%) than those from PDA (100%) and WR-PDB (95%) ($P < 0.05$, LSD = 3.139; data of tests 1 and 2 combined; Fig. 3A).

Pathogenicity test. Disease severity was affected by the washing step, the medium (ANOVA, $P < 0.04$ and 0.0001, respectively, $R^2 = 0.61$), and the interaction between the two factors ($P < 0.0015$). When tested fresh (FS) or after washing twice (wash 2), the WR-PDB-produced conidia caused higher levels of disease severity compared with conidia produced on PDA or WR (Fig. 3B). Conidia washed four times produced the same level of disease severity irrespective of the production medium. Aerial parts of the purple nutsedge plants were killed at the disease severity range of 30 to 55% achieved in this study.

DISCUSSION

Kadir et al. (16) reported some critical environmental conditions that are conducive for *D. higginsii* to cause high levels of disease on purple nutsedge. In their study, the highest levels of disease developed at 25 to 30°C. Our results of the temperature effects on in vitro growth further confirmed that temperatures in excess of 30°C are not congenial for the growth of this fungus.

Carbon concentration and C:N ratio are known to affect spore yield and quality, including germinability, pathogenicity, and virulence (12,24). There are significant compositional differences in conidia produced in different nutritional environments, such as variation in protein and lipid content (12). Any attempt to optimize spore production must take into account not only the number of spores but also spore efficacy. It is possible that a medium that supports heavy sporulation may not be the best to assure spore quality. It has been reported that *Colletotrichum truncatum* (Schwein.) Andrus & W. D. Moore produced significantly more conidia on a medium with C:N ratio of 15:1 than on media with C:N ratios of 40:1 and 5:1 (12). Data on production of conidia of *Beauveria bassiana* (Bals.) Vuill. GK2016 under submerged growth in TKI broth revealed that the C:N ratio of 5:1 promoted high sporulation, whereas increasing nitrate concentration decreased spore yield (30). Neopeptone as a nitrogen source, which stimulates the otherwise sparse and erratic sporulation of *C. lindemuthianum* (Sacc. & Magnus) Lams.-Scrib. (20), had no effect on *D. higginsii*. Although addition of plant extracts to agar media can promote fungal growth and sporulation (25,29), the addi-

tion of purple nutsedge leaf extract to PDA had no stimulatory effect on growth and sporulation of *D. higginsii*. OMA and CMA, which supported good radial growth of *D. higginsii*, were not the most suitable for conidial production.

Sporulation of *D. higginsii* on agar media and grain was highly variable, and there was no correlation with the C:N ratio of the medium. The inconsistency in conidium yield in this study may have resulted, at least in part, from somatic variability of the isolate studied or variation in growing conditions, including minor differences in essential nutrients (e.g., vitamins), moisture, and light. We observed differences between single-conidia isolates of *D. higginsii* in spore production, pathogenicity, and pigmentation in culture dur-

ing this study. Moreover, a low-glucose supplement to WR (dextrose at 10 g/liter) increased conidiation, whereas a high-glucose supplement (dextrose at 100 g/liter) inhibited sporulation (G. S. Wyss and R. Charudattan, unpublished results). Spore production of *Phomopsis convolvulus* Ormeno differed on various solid media, including barley grain, oat bran, lentil seed, flax seed, and wheat bran (22). Spore production of *P. convolvulus* also was stimulated by low concentrations of carbohydrate (Ormeno-Nunez 1987 in 22). Further studies on conidium production of *D. higginsii* should include a comparison of several single-spore isolates as affected by pH, light, moisture, and other features.

Conidial production on WR was 10 to 100 times greater than on complex and

Fig. 3. A, Effect of grain media on germination of *Dactylaria higginsii* conidia on 1.5% water agar. Conidia were produced on potato dextrose agar (PDA) or white rice supplemented with potato dextrose broth (WR-PDB) or water (WR). Pooled data from two tests ($n = 8$; 50 conidia counted per plate) are presented. B, Virulence (disease severity on purple nutsedge) of conidia produced on PDA, WR-PDB, or WR and after washing treatments (FS = freshly harvested, untreated; wash 2 = washed twice; wash 4 = washed four times before inoculations; water = control treatment with 0.5% Metamucil). Disease severity, based on Horsfall-Barratt rating scale, was measured 14 days after inoculation ($n = 16$; data from two tests pooled). Differences between growth media (lower case letters) were significant according to a least significant difference (LSD) test at $P < 0.05$. A, LSD = 3.139; B, LSD_{FS} = 0.713, LSD_{wash2} = 0.550, LSD_{wash4} = 0.780, and LSD_{water} = 0. In B, the differences between FS and wash 2 or wash 4 (all media combined) were significant at $P < 0.05$ (*) or not significant (ns). Vertical bars indicate standard deviation.

chemically defined media, respectively, and resulted in more than 2.5×10^6 spores/g of grain (test 2, Fig. 2A). Addition of host leaves to grain preparations increased sporulation in test 1 compared with WR and BR. In comparison, *P. convolvulus* produced 4.8×10^8 conidia/g on barley grain (21), *Alternaria macrospora* A. Zimmern. produced 2 to 4×10^8 conidia/g on mycelial homogenate (30), and *Beauveria brongniartii* (Sacc.) Petch produced 2×10^9 conidia/g on barley (1).

WR promoted conidium production, but the germination rate of conidia grown on WR supplemented with water was lowered by almost 50% compared with conidia grown on PDA or WR amended with PDB. Germination with PDA was almost 100%. The improvement in spore germinability and increased virulence associated with the addition of PDB also was observed in inoculation tests on purple nutsedge plants in the greenhouse (G. S. Wyss and R. Charudattan, unpublished results).

Endogenous reserves are initially the sole source of nutrients available to conidia sprayed on plates during the processes of germination. Our data suggested unidentified factors that promote germination when grown on WR-PDB and PDA in contrast to WR. It might be that accumulation of lipid reserves, carbon, or other sources of energy for early fungal development is limited on WR. Another explanation for reduced germination of conidia produced on WR could be the accumulation of inhibitory substances or induction of dormancy when grown in the rice medium. Our results supported this theory only partly because, although washing did not affect conidial germination, conidia washed four times produced the same level of disease regardless of their production medium. Furthermore, the twice-washed WR-produced conidia caused a higher level of disease severity than freshly harvested WR conidia, suggesting an enhancement in virulence after washing (Fig. 3B). However, chemicals known to stimulate spore germination (8), namely 1-nonanol, 2-heptanone, 5-methyl-2-hexanone, α -ionone, β -ionone, and 1-octanal, did not promote conidial germination of *D. higginsii* (G. S. Wyss and R. Charudattan, unpublished results).

WR with the addition of appropriate supplements and PDA in large trays are suitable media for noncommercial large-scale production of *D. higginsii*. The WR medium is less expensive than PDA, but far less expensive materials than WR may be required for commercial mass production. Further studies should measure the effect of various carbon and nitrogen sources on accumulation of exogenous reserves in the conidium and how those

reserves may affect viability and virulence of conidia.

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LITERATURE CITED

- Aregger, E. 1992. Conidia production of the fungus *Beauveria brongniartii* on barley and quality evaluation during storage at 2°C. J. Invertebr. Pathol. 59:2-10.
- Atlas, R. M. 1993. Handbook of Microbiological Media. CRC Press, Boca Raton, FL.
- Auld, B. A. 1991. Mass production, formulation and application of fungi as biocontrol agents. Pages 219-229 in: Biological Control of Locusts and Grasshoppers. C. J. Lomer and C. Prior, eds. CAB International, UK.
- Churchill, B. W. 1982. Mass production of microorganisms for biological control. Pages 139-156 in: Biological Control of Weeds with Plant Pathogens. R. Charudattan and H. L. Walker, eds. John Wiley & Sons, New York.
- Dhingra, O. D., and Sinclair, J. B. 1995. Basic Plant Pathology Methods. CRC Press, Boca Raton, FL.
- Encore Technologies LLC. 2001. Research and Development: Mallet WP. Encore Technologies LLC, Minneapolis, MN.
- Environmental Monitoring and Support Laboratory. 1983. Methods for Chemical Analysis of Water and Wastes. Environmental Monitoring and Support Laboratory. Office of Research and Development. U.S. Environmental Protection Agency, Cincinnati, OH.
- French, R. C. 1985. The bioregulatory action of flavor compounds on fungal spores and other propagules. Annu. Rev. Phytopathol. 23:173-199.
- Gumpertz, M., and Pantula, S. G. 1989. A simple approach to inference in random coefficient models. Am. Stat. 43:203-210.
- Holm, L. G., Plucknett, D. L., Pancho, J. V., and Herberger, J. P. 1977. The World's Worst Weeds: Distribution and Biology. University Press of Hawaii, Honolulu.
- Ibrahim, Y. B., Lim, T. K., Tang, M. K., and Teng, H. M. 1993. Influence of temperature, pH and selected growth media on germination, growth and sporulation of *Aschersonia placenta* and *Hypocrella raciborskii*. Biocontrol Sci. Technol. 3:55-61.
- Jackson, M. A., and Bothast, R. J. 1990. Carbon concentration and carbon-to-nitrogen ratio influence submerged-culture conidiation by the potential bioherbicide *Colletotrichum truncatum* NRRL 13737. Appl. Environ. Microbiol. 56:3435-3438.
- Jackson, M. A., Schisler, D. A., Slininger, P. J., Boyette, C. D., Silman, R. W., and Bothast, R. J. 1996. Fermentation strategies for improving the fitness of a bioherbicide. Weed Technol. 10:645-650.
- Kadir, J. B., and Charudattan, R. 1999. Occurrence of *Dactylaria higginsii* on purple nutsedge in Florida. Plant Dis. 83:588.
- Kadir, J. B., and Charudattan, R. 2000. *Dactylaria higginsii*, a fungal bioherbicide agent for purple nutsedge (*Cyperus rotundus*). Biol. Control 17:113-124.
- Kadir, J. B., Charudattan, R., and Berger, R. D. 2000. Effects of some epidemiological factors on levels of disease caused by *Dactylaria higginsii* on *Cyperus rotundus*. Weed Sci. 48:61-68.
- Kadir, J. B., Charudattan, R., Stall, W. M., and Bewick, T. A. 2000. Effect of *Dactylaria higginsii* on interference of *Cyperus rotundus* with *L. esculentum*. Weed Sci. 47:682-686.
- Kadir, J. B., Charudattan, R., Stall, W. M., and Brecke, B. J. 2000. Field efficacy of *Dactylaria higginsii* as a bioherbicide for the control of purple nutsedge (*Cyperus rotundus*). Weed Technol. 14:1-6.
- Luttrell, E. S. 1954. An undescribed species of *Piricularia* on sedges. Mycologia 46:810-814.
- Mathur, R. S., Barnett, H. L., and Lilly, V. G. 1949. Sporulation of *Colletotrichum lindemuthianum* in culture. Phytopathology 40:104-114.
- Morin, L. 1992. Mass-production of fungi for bioherbicides. Plant Prot. Q. 7:143-148.
- Morin, L., Watson, A. K., and Reeleder, R. D. 1990. Production of conidia by *Phomopsis convolvulus*. Can. J. Microbiol. 36:86-91.
- Roberts, D. W., and Sweeney, A. W. 1982. Production of fungi imperfecti with vector control potential. Pages 409-413 in: Invertebrate Pathology and Microbial Control. 3rd Int. Colloq. Invertebr. Pathol. Brighton, England.
- Schisler, D. A., Jackson, M. A., and Bothast, R. J. 1991. Influence of nutrition during conidiation of *Colletotrichum truncatum* on conidial germination and efficacy in inciting disease in *Sesbania exaltata*. Phytopathology 81:587-590.
- Shekhawat, P. S., and Prasada, R. 1971. Antifungal properties of some plant extracts. I. Inhibition of spore germination. Indian Phytopathol. 24:800-802.
- Stall, W. M. 1994. Effect of integrating alternative fumigant rates and methods with herbicides on nutsedge control. Pages 12-1 and 12-2 in: Proc. Annu. Int. Res. Conf. Methyl Bromide Alternatives and Emissions Reduction, Vol. 12. Kissimmee, FL.
- Stowell, L. J. 1991. Submerged fermentation of biological herbicides. Pages 225-261 in: Microbial Control of Weeds. D. O. TeBeest, ed. Chapman and Hall, New York.
- Stowell, L. J., Nette, K., Heath, B., and Shutter, R. 1989. Fermentation alternatives for commercial production of a mycoherbicide. Pages 219-227 in: Novel Microbial Products for Medicine and Agriculture. A. L. Damain, G. A. Somkuti, J. C. Hunter-Cervera, and H. W. Rossmore, eds. Society for Industrial Microbiology, Elsevier, Amsterdam.
- Tewari, S. N., and Dath, A. P. 1984. Effect of leaf extract media of some plants on the growth of three fungal pathogens of rice. Indian Phytopathol. 37:458-461.
- Thomas, K. C., Khachatourians, G. G., and Ingledew, W. M. 1987. Production and properties of *Beauveria bassiana* conidia cultivated in submerged culture. Can. J. Microbiol. 33:12-20.
- Walker, H. L. 1980. *Alternaria macrospora* as a potential biocontrol agent for spurred anoda: Production of spores for field studies. Pages 1-5 in: U. S. Dep. Agric. Adv. Agric. Technol. South, Ser. 12 (ISSN 0193-3728).
- Walker, H. L., and Riley, J. A. 1982. Evaluation of *Alternaria cassiae* for the biocontrol of sicklepod (*Cassia obtusifolia*). Weed Sci. 30:651-654.
- Wyss, G. S., Henkel, T., Charudattan, R., and DeValerio, J. T. 1999. Mass-production of conidia of *Dactylaria higginsii* (Luttrell) M. B. Ellis, a potential biocontrol agent for nutsedges (*Cyperus* spp.) on natural and synthetic media and grains. WSSA Abstr. 39:74.