J Ind Microbiol Biotechnol (2003) 30: 599–605
DOI 10.1007/s10295-003-0088-3

ORIGINAL PAPER

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Development of a submerged-liquid sporulation medium for the johnsongrass bioherbicide *Gloeocercospora sorghi*

Received: 23 March 2003 / Accepted: 15 August 2003 / Published online: 3 October 2003

Abstract Submerged culture experiments were conducted in three phases to determine the optimal medium for rapidly producing conidia of the fungal bioherbicide *Gloeocercospora sorghi*. In phase I, 18 crude carbon sources were evaluated to determine which would support sporulation. Under the conditions tested, butter bean and lima bean brines (1.5–4.6 mS/cm) provided best conidiation. In phase II, a fractional-factorial design was utilized to screen 76 different medium adjuncts in combination with butter bean brine for improved sporulation. D-Mannitol and carboxymethylcellulose (CMC) were the only acceptable factors that resulted in a significant improvement. In phase III, a central composite design with response surface methodology was used to optimize concentrations of these critical factors. The model predicted optimal sporulation in a medium composed of 2.69 mS/cm butter bean brine + 0.043 M D-mannitol + 0.37% w/v CMC with an expected titer of 1.51×10^7 conidia/ml. Actual mean titer attained with the model-derived medium was 1.91×10^7 conidia/ml. Optimal sporulation occurred at 25.5°C in this medium and conidia remained viable up to 2.71 days when stored at 12°C. No significant difference was observed in virulence of conidia produced on agar vs washed conidia produced in the model-derived (liquid) medium.

Keywords *Gloeocercospora sorghi* · *Sorghum* spp. · Bioherbicide · Response surface methodology · Factorial analysis

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Introduction

Johnsongrass [*Sorghum halepense* (L.) Pers.] is a large, perennial grass that propagates by seeds and rhizomes. A native of the Mediterranean region, johnsongrass has become well established in warm regions of all major agricultural areas of the world [15]. It has been reported as one of the world's ten worst weeds [10]. This weed reduces crop yields [1, 28], hosts insect and disease pests of grain sorghum [9, 15], and hybridizes with grain sorghum [15].

Gloeocercospora sorghi D. Bain & Edg. causes zonate leafspot in cereals and grasses. An isolate of the fungus collected from johnsongrass demonstrated it could be utilized as a bioherbicide to control this weed [17, 18].

Johnsongrass plants (1–5 leaf stage) were killed in greenhouse trials when sprayed with 5×10^6 conidia/ml and incubated in a dew-deposition chamber adjusted to 25°C for a minimum of 12 h. Post-inoculative air temperature had no effect on virulence of this bioherbicide. In one field trial 91% biomass loss occurred to infected johnsongrass [18]. Results demonstrated that the fungus is too virulent to be used as a bioherbicide in sorghum but is safe in dicotyledonous crops. Chiang et al. [5] also tested this fungus as a potential bioherbicide and recommended it not be used in corn and sorghum. Our tests demonstrated that 11 sweet and 16 dent corn varieties exhibited hypersensitive flecking but plants grew out of this symptom within 10–14 days [18].

Many pathogenic fungi are being investigated as potential bioherbicides [4, 11, 16, 21, 29]. Typically, when a fungal bioherbicide is applied as inundative inoculum with the same application technologies as chemical herbicides [25], it infects and kills the weed host within 1–2 weeks. After death of the host plant, the pathogen is reduced to background levels by natural constraints. The host is not killed by innate toxicity but by interaction of the host-pathogen metabolism. Consequently, no toxic residue remains in the crop or builds-up in the soil, water or food chains to result in adverse

effects on the environment as observed with chemical herbicides.

While many microorganisms that show promise as bioherbicides have been identified, only three fungal bioherbicides are currently registered for use in North America [19, 27]. A significant barrier to the commercial development of many of these plant pathogens as bioherbicides is the lack of low-cost methods for producing infective microbial propagules (e.g., spores). Submerged-culture fermentations are currently considered to be the most economical method of production [6].

Submerged-culture sporulation must be optimized for the large-scale fermentation of a fungal bioherbicide. The classical method of optimization varies the level of one parameter at a time over a certain range and holds the remaining variables constant. This strategy is generally time-consuming and requires a large number of experiments. Statistical methodologies have been employed for optimization of the fermentative process because they offer the possibility of studying several variables simultaneously with a reduced number of experiments. Factorial designs can be applied to define both main effects and interactions of the factors that play fundamental roles in the fermentation.

Response surface methodology (RSM) can determine optimal settings for the critical factors [2, 26]. The Box-Wilson central composite design (CCD) using RSM in fermentation has been widely reported and allows optimization for polysaccharides [8, 20], enzymes [7, 13], surfactin [24], microbial growth [22], antibiotics [14, 23], ethanol [12], and citric acid [3]. The aim of this work was to apply a fractional factorial design followed by RSM to rapidly screen and optimize medium ingredients that would allow for submerged-liquid sporulation of *G. sorghi*.

Materials and methods

Organism

An isolate of *G. sorghi* was obtained from sporulating lesions on johnsongrass growing within Washington County (Ark.). A pure culture was obtained by aseptically removing sporodochia observed under a stereomicroscope with a sterile dissecting probe, placing them onto a potato-dextrose agar plate and streaking the inoculum with an inoculating loop for isolation. Stock cultures of the fungus were maintained in 5% w/v non-fat dry skim milk and 20% v/v glycerol at −80°C. Spore inocula were produced by aliquots of stock cultures placed on 23% v/v sweet pea brine agar (1.8% w/v) plates at room temperature (23–24°C) for 4 days under fluorescent lights (1:1, Gro-Lux: Cool White) adjusted to a 14-h photoperiod. To obtain inocula, pea agar plates were aseptically scraped with a sterile cotton swab and conidia were suspended in sterile deionized water.

Submerged culture

Liquid batch-culture experiments were performed in 250 ml Erlenmeyer flasks, each containing 50 ml medium. Cultures were incubated at 23–24°C on a rotary shaker adjusted to 240 rpm. The initial spore concentration for all submerged cultures was 2×10^4 conidia/ml. Flasks were shaken daily to remove mycelial growth on the flask wall. Flasks were harvested 6 days after inoculation and spores counted with a hemacytometer under the microscope. Spore titer was recorded as $\log_{10}$ conidia/ml.

Media and experimental design

The entire experiment was executed in three phases. The objective of phase I was to identify a crude carbon source that would provide sporulation of the fungus in submerged liquid culture. Potential carbon sources and concentrations tested are shown in Table 1. Canned vegetables were filtered through four layers of cheesecloth and the filtrate (brine) was used for the experiment. In phase II, medium adjuncts were evaluated for their capacity to increase spore titer. On the basis of carbon results of phase I, the phase II experiment screened inorganic salt, amino/organic acid, fatty acid/oil, emulsifiable oil/surfactant and several miscellaneous compounds (Table 2). Specific ingredients (factors) that improved the amount of sporulation were elucidated by application of the fractional factorial design available with the design of experiment (DOE) screening platform of JMP4 Statistical Discovery software (SAS Institute, Cary, N.C.). Each shaker batch screened five factors at a low and high numerical setting according to the fractional factorial design shown in Table 3. The low setting (−1) represented absence of a factor and high setting (1) the concentrations shown in Table 2. This allowed for estimation of all main effects and some two-factor interactions with a total of 16 trials plus 1 center point. All trials were performed in duplicate and the $\log_{10}$ conidia/ml was used as the response. In phase III, an orthogonal CCD was used to model critical factors identified in phase II. Contour and prediction profiler platforms of JMP4 were utilized to optimize concentrations of the critical factors. All trials were performed in duplicate and the $\log_{10}$ conidia/ml was used as the response.

Effect of temperature on sporulation and spore survival

G. sorghi was cultured in the CCD-derived optimal medium at selected temperatures ranging from 15–36°C (five flasks/treatment). Batch cultures were prepared, grown, harvested, and conidia counted as described previously. The data were analyzed by utilizing the regression wizard platform of SigmaPlot 8.0 software (SPSS, Chicago, Ill.).

Table 1 Crude carbon sources evaluated for sporulation of *Gloeocercospora sorghi*

Type (concentration)	Specific ingredient
Canned vegetable Brine (5,10, and 15% v/v)	V8 juice[a], whole kernel corn[b], sweet peas[b], brown pinto beans[d], cut yams (in syrup)[d], butter beans[d], sliced beets[d], lima beans[b], sliced potatoes[d], sliced carrots[b], mustard greens[d], French style green beans[b], turnip greens[d], cut okra[d]
Syrups (1,2, and 4% w/v)	Corn (Karo dark and light)[f], sorghum molasses (full body)[c], and beet molasses[e]

[a]Campbell Soup Co. (Cambden, N.J.)
[b]Del Monte Foods (San Francisco, Calif.)
[c]Nabisco Inc. (East Hannover, N.J.)
[d]Marsh Supermarkets, LLC (Indianapolis, Ind.)
[e]Eli Lilly Company (Indianapolis, Ind.)
[f]CPC International (Englewood Cliffs, N.J.)

Table 2 Ingredients and concentrations evaluated with 2-level fractional factorial design. *MC* Methylcellulose, *CMC* carboxymethylcellulose

Type	Specific ingredient[a] (concentration)[b]
Inorganic salts	$MgSO_4 \cdot 7H_2O$ (0.4), K_2HPO_4 (0.5), $CaCO_3$ (0.5), NaCl (0.1), $CoCl_2 \cdot 6H_2O$ (0.0025), $MnCl_2 \cdot 4H_2O$ (0.05), $ZnSO_4 \cdot 7H_2O$ (0.05), $Fe_2(SO_4)_3 \cdot 7H_2O$ (0.01), $CaCl_2$ (0.1), KCl (0.05), $CaSO_4 \cdot 2H_2O$ (0.25), $Na_2MoO_4 \cdot 2H_2O$ (0.025), $CaH(PO_4)_2 \cdot H_2O$ (0.1), $MnSO_4 \cdot H_2O$ (0.1), $Na_2B_4O_7 \cdot 10H_2O$ (0.1), $NaHCO_3$ (0.25), KNO_3 (0.25), $BaCl_2 \cdot 2H_2O$ (0.0025), $Na_2SiO_3 \cdot 5H_2O$ (0.25), NH_4NO_3 (0.25), K_2SO_4 (0.25)
Amino/organic acid	NZ-amine A (0.25), peptone (0.25), Proflo (0.25)[c], Pharmamedia (0.5)[c], yeast extract (0.25), tryptone (0.2), casein (0.25), casamino acids (0.25), fish meal (0.5)[e], soybean meal (0.5)[e], canola meal (0.5)[e], beef extract (0.25), corn gluten (0.5)[e], soybean grits (0.5)[e], sulfanate (0.125), proprionate (0.05), citrate (0.3), ascorbate (0.2), alginate (0.1), L-glycine (0.25), L-alanine (0.25), L-phenylalanine (0.25), L-leucine (0.25), sodium acetate (0.5)
Fatty acid/oil	Stearic (0.1), palmitic (0.1), soybean (5% v/v), corn (2.5% v/v), canola (5% v/v), cottonseed (2.5% v/v)[c], sunflower (2.5% v/v), linseed (5% v/v)[e]
Emulsifiable oil/surfactant	Agrimul COS2 (0.1% v/v)[d], Agrimul RSO3 (0.1% v/v)[d], Agrimul RSO5 (0.05% v/v)[d], Tween 20 (0.03% v/v), Triton X100 (0.03% v/v)
Miscellaneous	Mannitol (0.25), ethephon (0.25% a.i. v/v)[f], urea (0.25), maltose (0.25), imidazole (0.05), inositol (0.25), xylose (0.25), galactose (0.25), sorbitol (0.5), sucrose (0.25), vanilla extract (0.3% v/v)[g], gum arabic (0.35), MC (0.1), lactose (0.25), soluble starch (0.25), corn steep liquor (0.5% v/v), CMC (0.1, medium viscosity), glycerophosphate (0.25)

[a]Compounds not referenced were purchased from Sigma-Aldrich (St. Louis, Mo.). CMC and MC viscosities of 2% w/v at 25°C were rated 400–800 and 400 centipoises, respectively
[b]All concentrations in % w/v except where indicated as % v/v. Each compound was evaluated using butter bean brine (2 mS/cm) as a basal medium
[c]Traders Protein (Memphis, Tenn.).
[d]Cognis Corporation (Cincinnati, Ohio).
[e]Eli Lilly Company (Indianapolis, Ind.)
[f]Fruit Eliminator by Bonide Products, Inc. (Yorkville, N.Y.)
[g]Marsh Supermarkets, LLC (Indianapolis, Ind.)

Following experimental assay, contents of the flasks cultured at 24°C were pooled. Aliquots (5 ml) were pipetted into 16×125 mm sterile screw cap test tubes and incubated at 3, 12 and 24°C (three tubes/temperature) to determine how long conidia remain viable. Samples were removed periodically from each tube after vortexing and aseptically pipetted onto sterile water agar (1.5% w/v) plates (10×100 mm) containing 10 ml agar. Five 50 µl drops were removed from each tube and evenly distributed 2 cm from the outer perimeter of a water agar plate. Plates were incubated at 24°C for 18 h and viability (% germination) of conidia was determined by microscopic examination.

Table 3 Sixteen-trial (plus one center point) fractional factorial design used to study five factors to improve sporulation

Trial	Coded setting for factor[a]				
	1	2	3	4	5
1	−1[b]	−1	−1	−1	1
2[c]	−1	−1	−1	1	−1
3	−1	−1	1	−1	−1
4	−1	−1	1	1	1
5[c]	−1	1	−1	−1	−1
6	−1	1	−1	1	1
7	−1	1	1	−1	1
8	−1	1	1	1	−1
9[c]	1	−1	−1	−1	−1
10	1	−1	−1	1	1
11	1	−1	1	−1	1
12	1	−1	1	1	−1
13	1	1	−1	−1	1
14	1	1	−1	1	−1
15	1	1	1	−1	−1
16	1	1	1	1	1
17	0	0	0	0	0

[a]Each of factors 1–5 represents a different specific ingredient listed in Table 2 and required a total of 16 shaker batches to screen
[b]Low (no factor) and high concentrations tested (shown in Table 2) represented as −1 and 1, respectively; 0 represents center point concentration
[c]These flasks contained only 1 factor

Disease characterization

Seeds of *S. halepense* were purchased from Azlin Seed Service (Leland, Miss.). Test plants were grown on greenhouse benches at 24–27°C in 7.5×9 cm plastic pots (five plants/pot) containing pasteurized field soil. The plants were inoculated at 17 days old (one to two-leaf stage). Seedlings (ten pots/treatment) were sprayed to runoff using an artist's air-brush atomizer (1.4 kg/cm^2). The spray contained conidia produced from either pea agar (spores suspended in deionized water) or the CCD-derived liquid culture. Liquid-culture-produced conidia were either used directly or centrifuged at 3,836 *g* for 5 min and the (washed) conidial pellet was resuspended in deionized water. Conidial suspensions were standardized to 5×10^6 spores/ml with a hemacytometer and the surfactant Tween 20 was added to all inocula (including controls) to a final concentration 0.05% v/v. Controls (ten pots) included no spray or plants sprayed with either deionized water or CCD-derived medium. Inoculated plants were air-dried, placed into a dew-deposition chamber (100% RH) at 24°C for 18 h, and then arranged in randomized complete block design on greenhouse benches. Following 14 days growth the leaves were removed, dried at 40°C for 3 days, and dry weights recorded. The data were analyzed by ANOVA using Minitab Statistical software (Minitab, State College, Pa.) to compare virulence of agar vs liquid-produced conidia on johnsongrass.

Results

Phase I

We evaluated 18 carbon sources for their influence on conidium formation in submerged-liquid cultures of *G. sorghi* (Table 1). Sporulation was observed in all concentrations tested of pea, butter bean and lima bean

Table 4 Submerged-culture conidiation of *G. sorghi* with three crude carbon (brine) sources[a]

Carbon	Concentration % v/v	Conductivity (mS/cm)	Mean no. of $\log_{10}$ conidia/ml[b]
Pea	2.5	0.232	5.09 b
	5.0	0.413	5.39 c
	10.0	0.774	5.66 cd
	15.0	1.130	5.53 c
Butter bean	2.5	0.945	4.61 a
	5.0	1.670	5.84 de
	10.0	3.130	6.12 ef
	15.0	4.590	6.37 f
Lima bean	2.5	0.914	4.70 a
	5.0	1.561	5.86 de
	10.0	2.864	6.11 ef
	15.0	4.166	6.04 e

[a]Six-day-old cultures placed stationary for 1 min prior to removing samples for counts
[b]Values followed by different letters are significantly different ($P < 0.05$) using Fisher's protected least significant difference test

Table 5 Parameter estimates and *P* values for phase II fractional factorial sporulation study

Term[a]	Estimate[b]	*P* value
Mannitol	0.325	< 0.0001
MC	0.345	< 0.0001
CMC	0.253	< 0.0001
Peptone	0.382	0.0149
Ethephon	0.333	0.0440
Mannitol×sunflower oil	−0.030	0.0499
MC×gum arabic	0.198	0.0388
CMC×galactose	0.059	0.0078
Peptone×leucine	−0.253	< 0.0001
Peptone×imidazole	0.284	0.0200
Peptone×soybean meal	0.228	0.0108
Ethephon×yeast extract	−0.416	0.0207

[a]All main effect and interaction terms where $P > 0.05$ are not shown
[b]Positive (e.g., improved sporulation) significant main effect estimates are shown with corresponding interaction terms. All other significant negative estimates are not shown

brines. To determine which of these three carbon sources to use in phase II, we grew the fungus in selected brine concentrations and counted spores on 6-day-old cultures (Table 4). Butter bean (3.13–4.59 mS/cm) and lima bean (2.86–4.17 mS/cm) resulted in significantly greater conidiation compared to pea. There was no significant difference in conidiation between butter bean vs lima bean which was of no surprise since both of these represent the plant species *Phaseolus lunatas* L. Canned butter bean was less expensive than lima bean; therefore, we chose butter bean brine for phase II experiments.

Phase II

Butter bean brine (2 mS/cm) was evaluated with a total of 76 different compounds (factors) to determine if any would improve sporulation in submerged-liquid culture (Table 2). Factor level (concentration) selection was a difficult part of this experimental process and was based upon prior experience in growing plant pathogenic fungi. Five factors were evaluated with each shaker batch until all compounds were tested (which required a total of 16 shaker batches). The r^2 value ranged from 0.91–0.99 for all batch runs. The high value for r^2, together with the many significant effects, provides strong evidence that the model accurately quantified the process. Five individual [D-mannitol, methylcellulose (MC), carboxymethylcellulose (CMC), peptone and ethephon] and four interaction (MC×gum arabic, CMC×galactose, peptone×imidazole, and peptone×soybean meal) effects resulted in a significant increase in sporulation. *P* values are shown in Table 5. Although peptone and ethephon significantly improved sporulation, the resulting conidia exhibited atypical morphology. Conidia produced in peptone appeared as thin-walled ghosts. Conidia produced in ethephon were very short and not the typical filiform shape. No significant difference in conidiation was observed between MC and CMC factors. CMC dissolved more easily and therefore it and mannitol were chosen for phase III.

Only 3 of 76 compounds screened resulted in an acceptable significant improvement in submerged-culture sporulation of the fungus. This represented a total of 52 significantly negative and 114 non significant main effect and interactions of tested factors (not shown in Table 5).

Phase III

An orthogonal CCD in the RSM platform of JMP4 was utilized to maximize concentrations of butter bean brine, mannitol and CMC required for sporulation of *G. sorghi*. Concentration values of butter bean brine (1 mS/cm, 3.5 mS/cm), mannitol (0.027 M, 0.082 M), and CMC (0.3, 0.9% w/v) were entered and the program adjusted to 16-trials and 3-center points (axial value = 1.287). Sporulation results of butter bean vs mannitol and butter bean vs CMC interactions are shown in Fig. 1. Optimal sporulation is predicted to occur in a medium composed of 2.20–3.03 mS/cm butter bean brine +0.024–0.058 M mannitol +0.22–0.59% w/v CMC. The prediction profiler tool of JMP4 was used to maximize desirability of the concentrations for each of these medium factors that would result in the highest spore titer and narrowed ingredient amounts for optimal sporulation to 2.69 mS/cm butter bean brine +0.043 M mannitol +0.37% w/v CMC. An overall desirability of 0.91 (range 0–1) was attained with a predicted maximum spore concentration of 1.51×10^7 conidia/ml.

RSM model verification

The predicted optimal medium (2.69 mS/cm butter bean brine +0.043 M mannitol +0.37% w/v CMC) was compared to 2 mS/cm butter bean brine (control) med-

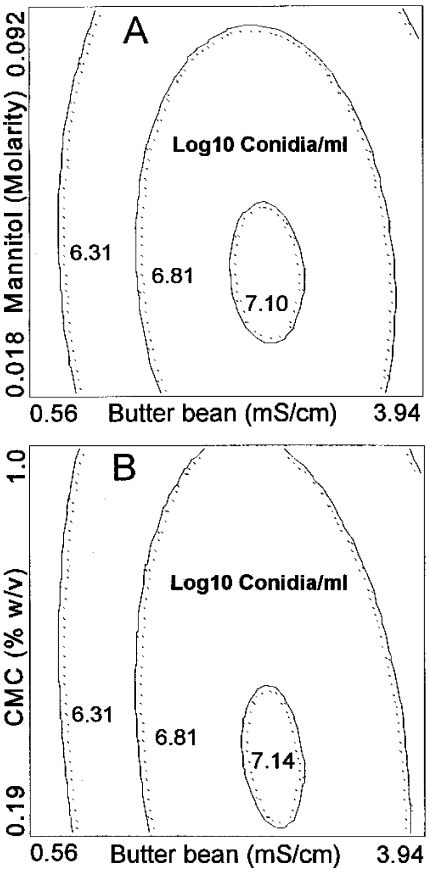

Fig. 1 Contour plot of spore yield: the effect of **A** mannitol and **B** carboxymethylcellulose (CMC) within butter bean brine and their mutual interaction on conidiation of *Gloeocercospora sorghi*

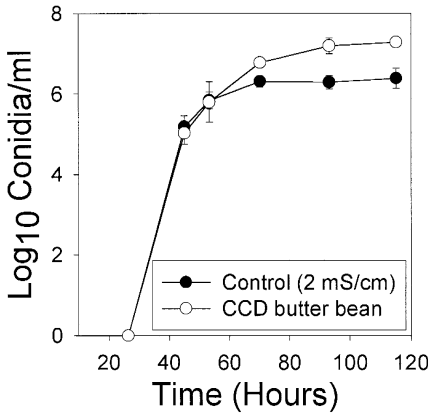

Fig. 2 Sporulation of *G. sorghi* in 2 mS/cm butter bean brine (control) vs the central composite design (CCD)-derived optimal medium (2.69 mS/cm butter bean brine +0.043 M D-mannitol +0.37% w/v CMC)

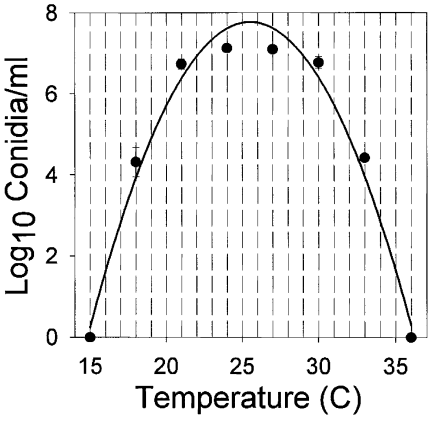

Fig. 3 Effect of temperature on conidiation in shake culture with CCD-derived optimal medium

ium. Final conductivity (following addition of mannitol and CMC) of the predicted optimal medium before autoclaving was 2.82 mS/cm (pH =6.42). After autoclaving final conductivity and pH values were 3.27 and 6.33, respectively. Sporulation results are shown in Fig. 2. Sporulation first appeared in both media after 45 h growth. The model-derived medium resulted in a significantly greater spore titer compared to the control medium. A mean titer of 1.91×10^7 conidia/ml was attained after 116 h (4.8 days) growth in the model-derived medium, 1.26-fold higher than the predicted value of 1.51×10^7 conidia/ml. The control medium resulted in a maximum titer of 2.4×10^6 conidia/ml. The CCD model accurately predicted and resulted in identifying a medium that improved submerged-culture sporulation of this fungus.

Effect of temperature on sporulation and spore survival

Measurements of *G. sorghi* sporulation at different temperatures were recorded at 15, 18, 21, 24, 27, 30, 33 and 36°C. The best-fit model (quadratic), including standard deviations, is presented in Fig. 3. Validation of this model resulted in an r^2 value of 98% and the equation $y = -0.068x^2 + 3.47x - 36.55$, where $y = \log_{10}$ conidia/ml and x = temperature. Optimal sporulation of *G. sorghi* in the CCD-derived liquid medium occurs at 25.5°C.

Survival of conidia produced in liquid culture stored at selected temperatures is shown in Fig. 4. No viable spores were found after 11.7 days at any temperature (not shown in Fig. 4). Best survival occurred at 12°C (100, 75% germ), followed by 24°C (52, 16% germ) and 3°C (11, 0% germ), after 2.71 and 5.7 days storage, respectively.

Disease characterization

Virulence of plate vs liquid culture produced conidia against johnsongrass is presented in Table 6. In addition to the butter bean-based CCD-derived liquid medium, we tested a brine prepared from dried lima bean (Hurst's Brand, Indianapolis, Ind.). Dried beans (454 g) were hydrated by adding 2 l tap water and soaking the beans

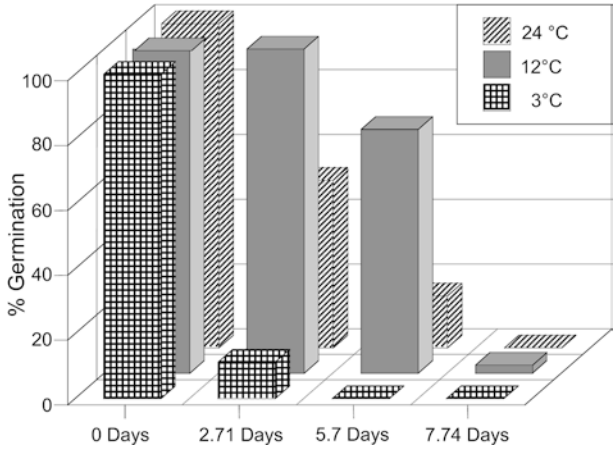

Fig. 4 Survival of shake-culture-produced conidia at selected temperatures (3, 12, and 24°C)

Table 6 Comparison of in vitro germination and virulence of plate (pea agar) vs central composite design (CCD)-derived liquid media conidia on johnsongrass[a]

Treatment[b]	% Germination[c]	Mean dry weight (g)[d]
Plate-produced	100 a	0.000 a
CCD-produced	100 a	0.155 b
CCD-produced (washed conidia)	100 a	0.000 a
CCD (dry lima bean)-produced (washed conidia)	100 a	0.000 a
Controls (no conidia):		
CCD medium		0.300 c
Deionized water		0.246 c
CCD produced (supernate only)		0.310 c
Uninoculated plants (no spray)		0.318 c

[a]CCD medium contained butter bean brine carbon source except where indicated as dried lima bean
[b]All treatments (except uninoculated plants) contained 0.05% v/v Tween 20 surfactant
[c]Incubation was 18 h at 24°C on 1.5% w/v water agar plates
[d]Weight represents five johnsongrass plants. Values followed by different letters are significantly different ($P < 0.05$) using Fisher's protected least significant difference test

for 4 h at room temperature. This mixture was autoclaved (121°C) for 5 min, cooled and filtered through four layers of cheesecloth. Brine solution was added to 90 ml deionized water and total volume adjusted to 100 ml with water until 2.69 mS/cm conductivity. The amount of brine was then extrapolated to prepare larger volumes. Calculated brine, CMC and mannitol were mixed in a vessel containing deionized water and water added to total volume desired.

Disease symptoms appeared within 1 day after removing inoculated plants from the dew-deposition chamber. No significant difference was observed with in vitro germination of conidia produced on either pea agar vs CCD-derived liquid medium (Table 6). All treatments containing conidia significantly lowered plant biomass compared to control treatments. Both plate- and liquid-produced (washed) conidia killed johnsongrass seedlings (mean dry weight = 0.0 g). A significantly higher johnsongrass biomass (0.155 g) resulted when plants were inoculated with unwashed conidia from the CCD-derived (butter bean) liquid cultures (Table 6). There was no significant difference among control treatments including uninoculated plants (0.318 g) and plants inoculated with either Tween 20 (0.246 g), autoclaved liquid medium (0.300 g), and the supernatant separated from sporulated liquid cultures (0.310 g).

Discussion

The production of spores requires that nutritional conditions should be satisfactory to vegetative growth, supportive of sporophore development, and appropriate for induction of sporulation. It has been found that an appropriate change in the medium can induce some terrestrial fungi to sporulate in submerged culture. Results of this experiment demonstrate that autoclaved butter bean or lima bean brine simulates the nutritional conditions required by *G. sorghi* (a foliar plant pathogen of johnsongrass) to sporulate in submerged culture.

A rapid screening process using a fractional factorial design identified two compounds, mannitol and CMC, that improved sporulation of the fungus in the butter bean brine basal medium. The ample number of significantly negative and non-significant factors observed in this experiment might be a result of testing a critical total ion level above which no sporulation may occur. One must consider that when utilizing a fractional factorial design it is possible that the wrong concentration of a single variable could cause an apparent reversal of other variables in that batch. Although each microbe should be considered unique in its requirements for growth and reproduction, it is important to choose proven concentration ranges of test factors for a particular organism (e.g., fungus, bacterium, etc.), if not genus/species, when using this tool. In anticipation of this potential issue, different combinations of factors were evaluated when the experiments were repeated. Similar results were obtained.

RSM provided a very simplified approach to optimization of critical factors and accurately predicted maximum spore titer. Results suggest optimal sporulation of *G. sorghi* will occur at 25.5°C in a medium composed of 2.69 mS/cm butter bean (or lima bean) brine + 0.043 M mannitol + 0.37% w/v CMC (or MC). Canned brine or a less expensive brine prepared from dried beans may be used. Inoculum may be stored up to 2.7 days at 12°C without any loss in viability (% germination). Furthermore, there was no significant difference in the virulence of (washed) conidia produced within the model-derived liquid medium vs solid agar medium. The primary objective of this experiment—to determine if a statistical tool could be used to rapidly identify medium factors that would improve sporulation of the fungus—was accomplished. Future work will investigate scale-up procedures and the interactions of seed medium, aeration, and shear-force on submerged-

culture sporulation using a 2-l stirred-tank minifermenter. Results of spore survival in liquid medium suggests the development of a carrier formulation that will provide an adequate shelf-life. This work is in progress.

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