Microplate Assay for *Colletotrichum* Spore Production

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A simple microplate method was devised to assay spore production by *Colletotrichum gloeosporioides* by growing the fungus on 1 ml of solid media in the wells of tissue culture plates. Growth and sporulation on microplates were compared at days 4 and 8 with growth and sporulation in 100-ml liquid batch cultures that involved 11 common media. Spore production per unit volume of medium was the same for solid and liquid forms of the media. Qualitative assessment of mycelial growth measured on microplates agreed with that of growth measured in liquid cultures. The microplate assay indicated that V8 juice was the best medium and that an organic content of about 6 mg/ml was optimal for high sporulation and low mycelium production. The assay provides a convenient, rapid, and inexpensive means of screening media for the production of fungal conidia in large numbers, to be used, for example, in biological control programs.

The use of pathogenic microorganisms in biological control programs for pathogens, weeds, and insects is gaining increased attention (2, 6). Fungal propagules for use in biological control strategies must be produced in large quantities, cheaply, quickly, and easily (11). Medium composition is often chosen empirically (3) despite evidence indicating that composition has a major effect on sporulation (9, 10, 13, 19), and there is little comparative information concerning the effects of medium form (liquid or solid) (11). Whenever possible, mass production of spores is undertaken in large liquid culture fermentations (3). Once a liquid medium has been chosen, the optimal combination of nutritional and environmental components for production are identified in large-scale flask experiments (5, 10, 12, 13). This process wastes space, time, and raw materials. A small-scale, easy screen of the effects of culture variables on spore production would be beneficial.

Since the medium used in liquid culture is generally a broth version of the agar medium on which a given fungus is known to grow well (3), it is reasonable to assume that a solid medium assay can be developed. This assay would be done most conveniently on a small scale, but it would have to be demonstrated initially that the resulting small-scale production of spores on solid media accurately reflected proportional production in larger, liquid systems. A comparison of sporulation in solid and liquid media forms would indicate the best form for mass production (some biological control fungi sporulate much better on solid media [11, 15, 19]). If there was no difference in spore yields, the solid media assay would provide a simple screening method and would allow transfer of information between the media forms.

We report here the development and use of a microplate assay of the nutritional factors affecting spore production by *Colletotrichum gloeosporioides*, a fungus of considerable commercial interest for biological control programs (1, 3).

MATERIALS AND METHODS

Fungus. *C. gloeosporioides* was isolated in 1982 from diseased *Myriophyllum spicatum* plants growing in Madison,

Wisconsin, and was stored on silica gel crystals (14). Working cultures were maintained on potato glucose (PG) agar at 25°C in natural daylight. For spore production, 100-ml portions of liquid Richard's V8 medium (RV8) (J. T. Daniel, G. E. Templeton, and R. J. Smith, Jr., U.S. patent 3,849,104, November 1974) in 250-ml Erlenmeyer flasks were inoculated with spores taken from PG agar plates with a sterile loop. The flask cultures were harvested after 4 days of incubation on an orbital shaker operating at 100 rpm by filtering the medium through a double layer of sterile muslin into sterile centrifuge bottles. The spores were pelleted by centrifugation at 1.1×10^5 rpm for 6 min, washed by suspension and centrifugation, and diluted to the concentration required.

Media. The 11 media chosen for study were prepared on the basis of standard procedures (4, 7, 8, 17, 18; Table 1), with the exception of the medium that was developed for this study, which contained homogenized healthy *M. spicatum* leaves and stems (HM medium). The solid media were analogous to the liquid forms, with additions of 1.8% (wt/vol) agar (Difco Laboratories, Detroit, Mich.). We used typical fungal media, including asparagine-glucose (AG), Cooks no. 2 amended (CS2), Czapek (CZ) (Difco Laboratories), glucose-peptone (GP), PG, RV8, and V8 juice (V8) (Campbells Soup Co.) and media normally used to culture bacteria, including Casamino Acids-peptone-glucose (CPG), King's B medium (KSB), and nutrient broth (NB) (Difco). (See Table 1 for composition of the media.) They were selected because as a group they constituted a suitable range over which the ability of the fungus to grow and sporulate was anticipated to vary. RV8 is the most commonly used laboratory medium for *Colletotrichum* spp. spore production (J. T. Daniel, G. E. Templeton, and R. J. Smith, Jr., U.S. patent 3,849,104, November 1974).

Experimental procedures. (i) **Liquid media.** Two 100-ml portions of each liquid medium were inoculated with freshly produced conidia to give a final concentration of 10^4 cells per ml. The flasks were incubated at 25°C on an orbital shaker operating at 100 rpm in natural daylight. One flask culture of each medium was harvested at day 4, and another was harvested at day 8. The experiment was conducted on 3 different days, giving three replicates of each medium and harvest time combination. At harvest, the contents of each

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TABLE 1. Composition of media

Medium	Component(s) (g/liter):				pH	Source or reference
	Carbon and nitrogen source(s)	Supplementary carbon source	Inorganic nitrogen source	Buffer		
AG	Glucose (20) Asparagine (15)			K ₂ HPO ₄ (0.5)	6.0	7
CPG	Glucose (10) Peptone (10) Casein (1)				6.4	4
CS2	Glucose (20)			K ₂ HPO ₄ (0.5)	6.6	17
CZ	Saccharose (30)		NaNO ₃ (2)	K ₂ HPO ₄ (1.0)	7.5	18
GP	Glucose (20) Peptone (5)			K ₂ HPO ₄ (0.25)	6.6	18
HM	Plant material (7)				5.3	Slade et al. ^a
KSB	Glycerol (10) Peptone (20)			K ₂ HPO ₄ (1.5)	7.3	8
NB	Beef extract (3) Peptone (5)				7.0	18
PG	Plant material (7)	Glucose (20)			5.8	18
RV8	Plant material (6)	Sucrose (50)			6.0	Patent, Daniel et al. ^b
V8 ^c	Plant material (8)		KNO ₃ (10)	KH ₂ PO ₄ (5.0)	4.3	18

^a Prepared for this study (500 g/l, wet weight).^b See Materials and Methods.^c No addition of CaCO₃.

flask were homogenized in a blender (ten-speed Pulse-matic Oster; Sunbeam Corp., Milwaukee, Wis.) for 10 s at blend speed and were poured through a double layer of preweighed muslin. The homogenate was rinsed with distilled water and then dried at 85°C overnight to constant weight. Mycelial dry weight was calculated by subtraction. The weights of plant solids in the HM, RV8, and V8 media, in which the plant solids appeared to have remained mainly undigested, were accounted for by subtracting the weight of plant solids present at the start of the experiment. Although some plant solids may have been digested, mycelial yield measurements would have been much less accurate if the weight of the solid material had not been taken into account. The actual amount of plant solids was very low compared with mycelium yield. The number of spores per milliliter of medium was calculated from the number of spores counted in the filtrate by using a hemacytometer with a 0.1-mm deep stage. The spore suspension was diluted as necessary, and the average number of spores present in two sets, each having five fields of view (either 0.0625, 0.05, or 0.04 mm²), was recorded for each replicate.

(ii) **Solid media.** Portions (1-ml each) of each molten medium were pipetted with wide-mouth pipettes into the wells of polystyrene, lidded, 24-well tissue culture plates (no. 25820; Corning Glass Works, Corning, N.Y.). The media were distributed randomly among the wells. One-ml plugs were easy to handle, and they provided data directly as counts of spores per milliliter. After solidification of the agar, each well was inoculated with a 25-μl droplet of a freshly prepared spore suspension containing 10⁴ spores per ml. A 25-μl droplet was used because it was sufficient to cover entirely the flat agar surface without forming a large meniscus and was absorbed within a few hours. The plates were sealed in plastic bags to avoid dehydration and were incubated at 25°C in natural daylight for up to 8 days. The experiment was repeated on 3 different days, independently of the liquid medium experiments, giving three replicates of each medium and harvest time combination. The relative mycelium formation was recorded at day 2, 4, or 8 (see Table 4). At harvest, the medium in each well was removed, and the agar was cut away from the base of each plug to leave

thin fungal mats, which were placed in centrifuge tubes containing 10 ml of sterile distilled water. The tubes were shaken for 18 h at 250 cycles per min on a lateral shaker. Lactophenol (25 μl) was added to each tube before shaking to inhibit further growth or contamination. Spore production per milliliter of initial medium was calculated from the number of spores counted in the tubes. There was no need to remove hyphal fragments before counting with the hemacytometer since the spores were clearly visible and easily counted. The minimum hemacytometer count possible was 1.25 × 10⁴ cells per ml, and this value was used when no spores were observed.

(iii) **V8 juice concentration study.** On the basis of the first experiments, the V8 medium was designated as the best for sporulation and was chosen for further study. Eight concentrations of V8 juice in water were prepared: 5, 10, 15, 20 (normal), 30, 40, 50, and 60%, representing 2.05, 4.09, 6.14, 8.18, 12.27, 16.36, 20.24, and 24.54 mg of organic material per ml, respectively, calculated after ashing at 550°C for 1 h. The experimental procedure with liquid and solid forms of the medium was the same as that described above.

(iv) **Analysis.** Analysis of the logarithmically transformed spore production data (the data were transformed to provide better variance homogeneity) was performed by using a split-plot model (16). The form of the medium, liquid or solid, was the whole-plot experimental factor, and there were two crossed-subplot experimental factors, medium type and harvest time, nested within the whole plots. The design structure is indicated by the analysis of variance (ANOVA) in Table 2. In addition to the ANOVA, the difference between day 4 and day 8 harvest time counts for each combination of medium and medium form and the difference between spore production on solid and liquid media forms for each combination of medium and harvest time were assessed by least significant difference (LSD) tests (16). The appropriate error term for the harvest time comparisons was the subplot error; for the comparisons of solid and liquid media, the appropriate error term was a combination of the subplot and whole-plot error. We also compared the mycelium production in liquid media at each time. The analyses of data for the V8 medium were performed in

TABLE 2. ANOVA of logarithmically transformed data for spore production in 11 different solid or liquid media after days 4 and 8

Source or error	Sum of squares	Degrees of freedom	Mean square	F ratio
Whole plot				
Liquid or solid (L)	0.528	1	0.528	4.400 ^a
Whole-plot error	0.480	4	0.120	
Subplot				
Medium (M)	129.079	10	12.908	53.346 ^b
Day (D)	6.283	1	6.283	25.967 ^b
M × L	18.407	10	1.841	7.607 ^b
M × D	9.368	10	0.937	3.872 ^b
L × D	0.400	1	0.400	1.653 ^a
M × L × D	1.986	10	0.199	0.821 ^a
Subplot error	20.325	84	0.242	

^a Not significant at $P = 0.05$.^b Significant at $P = 0.01$.

a manner similar to the analyses of data for the different media. An ANOVA of spore production in solid and liquid forms of the V8 media is shown below (see Table 6).

RESULTS

Different media. (i) Spore production. Spore yields showed similar trends for liquid and for solid media at days 4 (Fig. 1) and 8. An ANOVA (Table 2) and an LSD analysis showed that a difference between systems of 0.79 in the logarithmically transformed data was required to be detectable at a significance level of 0.05. (This corresponds to a 6.2-fold difference in actual spore numbers.) This finding enabled us to rank the media on the basis of spore production in liquid form and on solid form into the following three categories: best media (10^6 to 10^7 spores per ml), RV8, V8, and PG; intermediate media (10^5 to 10^6 cells per ml), CZ, HM, AG, and NB; and poor media (10^4 to 10^5 cells per ml), KSB, CPG, GP, and CS2. The solid system showed the same relative ranking of the media as did the liquid system; in addition, spore yields for the two systems were, in general, not significantly different when taken as a whole (indicated by the low F ratio for the liquid/solid comparison in Table 2). The significant (liquid/solid) × medium interaction was due primarily to differences between spore production for the

TABLE 3. ANOVA of data on mycelium production in 11 different liquid media after days 4 and 8

Source or error	Sum of squares	Degrees of freedom	Mean square	F ratio
Medium (M)	128.148	10	12.815	24.535 ^a
Day (D)	25.519	1	25.519	48.859 ^a
M × D	33.575	10	3.358	6.428 ^a
Blocks ^b	13.990	24	0.583	1.116 ^c
Error	10.446	20	0.522	

^a Significant at $P = 0.01$.^b Includes the Blocks main effect and all Block × treatment interactions, none of which were significant individually.^c Not significant at $P = 0.05$.

liquid and the solid forms of the poor media. In individual comparisons of LSD test data for spore production on solid forms and in liquid forms of the best media (conducted separately for the day 4 and day 8 data), the only significant difference was a higher spore production in liquid RV8 medium at day 4, a difference which disappeared by day 8. Whenever differences were significant among the other media tested, spore production in liquid forms was greatest for the intermediate media, whereas production on solid forms was greatest for the poor media.

Consistent with the trends shown in Fig. 1, medium type was the most important parameter affecting spore yields (shown also by the large sum of squares values in Table 2). There was no apparent relationship between spore production and either organic content, buffer, or pH of the media, although a formal statistical analysis was not undertaken (Table 1 and Fig. 1). Except for the two least sporulent of the poor media (GP and CS2 media), there was no significant increase in spore production from days 4 to 8 (the large increases for GP and CS2 media explain the significant effects of harvest time and the harvest time by media interaction factors in Table 2). Spore yields were at maximum levels by day 8 on GP and CS2 media since no increase occurred with an additional 4 days of incubation (data not shown).

(ii) Mycelium production. Mycelium production in liquid media was influenced by both the time of harvest and the medium type, the effects of which varied from combination to combination (Table 3). A difference of 1.23 mg of mycelium per ml in an LSD test was needed for significance at $P = 0.05$ (16). Comparisons of individual media treatments by using the LSD test showed a significant increase in mycelium production in liquid media from days 4 to 8 for RV8, PG, AG, KSB, GP, and CS2 media, along with a significant decrease for HM medium, and no change for V8, CZ, NB, and CPG media. Of the latter four, the V8 medium had by far the best spore yield, in terms of both spores per milliliter of medium and spores per milligram of organic material (due to the low organic content of V8 medium; Table 1). V8 was better than RV8 and PG media (which also produced a large number of spores) because of its lower mycelium production, and thus was designated the best medium and selected for further study. The visual mycelial growth trend on solid media (Table 4) was similar to that seen in liquid media (Fig. 1). However, the surface areas of most of the media plugs were covered with mycelium by day 4, making it difficult to distinguish any increase from days 4 to 8. Only poor media, such as CS2, showed any increase (Table 4). A measure of the relative growth rate and relative mycelium formation was obtained by recording trends up to day 4, before the plugs were covered. Mycelial formation

FIG. 1. Spore production and mycelium formation in different media after 4 days.

TABLE 4. Relative mycelium formation on solid media

Medium ^a	Relative mycelium production ^b on day:		
	2	4	8
RV8	4	5	5
PG	4	5	5
V8	3	4	4
CZ	1	3	3
HM	2	3	3
NB	1	3	3
CS2	2	3	4
GP	3	4	4

^a Media not listed include AG (results were similar to those for GP) and CPG and KSB (results were similar to those for CS2).

^b Scale: 1, very sparse growth; 2, clearly visible but sparse cover; 3, moderate growth over whole area; 4, dense cover; and 5, very dense cover.

trends on the solid media reflected trends in liquid forms, thereby allowing forecasting of relative mycelium production in the liquid systems.

Optimization of V8 juice concentration. Day 4 spore production trends in liquid and solid media were similar, showing increased spore production with increased organic content, reaching maximum levels of between 4.1 and 8.2 mg of organic material per ml, with considerably less sporulation in media with higher organic content (Fig. 2A and 3).

The ANOVA for spore production in the V8 media with different juice concentrations is shown in Table 6. At least a 2.6-fold difference in spore production (LSD value of 0.41 for log-transformed data) between any two values was necessary to be detectable at a confidence level of $P = 0.05$. Although there is some disagreement as to the appropriateness of using LSD analysis and of doing multiple comparisons when there is a quantitative variable (such as V8 juice concentration), in this study LSD analysis provided the best means to make the comparisons of interest in this study. Only the media with a higher-than-optimum organic content (in the range of 8.2 to 16.4 mg/ml) showed any significant difference in sporulation between the two media forms at day 4, but the spore production trend at day 8 was very different for the solid and liquid forms. Calculations of probability values by LSD analysis (data not shown) indicated that there was no significant difference between V8 media forms at low juice concentrations, but at higher juice concentrations spore yields were significantly different, since no increase in spore yield occurred from days 4 to 8 in liquid V8 media. On solid V8 media after 8 days, the spore production was directly proportional to the organic content of the media (Fig. 3). A comparison of spore production counts at days 4 and 8 for each medium showed that there was no significant increase for media with ≤ 6.1 mg of organic material per ml, with very significant ($P \leq 0.001$) increases occurring for media with higher organic content.

Mycelium production in the liquid V8 media with different juice concentrations is shown in Fig. 2B. A difference of 0.62 mg/ml between values was needed to be significant at $P = 0.05$. An ANOVA (data not shown) indicated that the concentration of medium had a large effect on mycelium formation and that it did not change with time. At both days 4 and 8 mycelium formation was directly proportional to V8 juice concentrations in the medium (Fig. 2B). By day 8, the flasks of liquid V8 media at the higher V8 juice concentrations contained so much mycelium that they had essentially solidified. Extensive mycelium production preceded sporulation for the media with high concentrations of V8 juice (Fig. 2A and B). Oxygen availability in the static

FIG. 2. (A) Spore production in liquid V8 medium with varying organic content. (B) Mycelium formation in liquid V8 medium with varying organic content.

mycelial clumps would probably have been greatly reduced, thus potentially inhibiting sporulation (3). This did not occur on solid media, thereby allowing for continued sporulation. At days 4 and 8, the relative mycelium production trends for the liquid media were the same as those for the solid media (Table 5), at least for the media with the five lowest concentrations of V8 juice. This occurred because, on the solid media with high juice concentrations, the small surface areas were quickly covered with mycelium, which did not allow further noticeable growth; however, further growth was possible in the 100-ml liquid system. The recording of mycelium formation for the solid media up to day 4 showed that the production trend did not change with time, a finding which was in agreement with that for the liquid system.

The microplate assay technique allowed for prediction of the best V8 juice concentration range for spore production in liquid V8 media at day 4 and at day 8 for media in the lower concentration range, but it did not account for the peculiar

FIG. 3. Spore production on solid V8 medium with varying organic content.

characteristics of highly concentrated liquid V8 media at day 8.

DISCUSSION

The microplate assay provided a simple approach for screening 11 different media and eight concentrations of one selected medium for rate and extent of spore production and for relative mycelium formation. Conclusions from microplate assay data with respect to the ranking of media and V8 concentrations were similar to those obtained with data from the more laborious conventional liquid method. Not only was the same relative ranking of media obtained by using the microplate method, but, in most cases (particularly for media in the best media category), spore yields were quantitatively comparable to those of the liquid system.

Harvesting the media plugs after day 4 rather than after day 8, permitted the most useful prediction of production in the liquid analog, particularly for media in the best media category. The few media with relatively poor correlations between spore production in liquid and solid forms allowed a large increase in mycelium production from days 4 to 8 in addition to having poor spore yields. Other poor correlations between results in solid and liquid media forms occurred under the completely uneconomical conditions of very high medium concentration.

The variability in spore yield data (that was responsible for the lack of sensitivity for comparative purposes) was due in part to the experimental design (we used one replicate on 3 separate days rather than three same-day replicates) to account for day-to-day variability in growth yields. The inherent biological variability of spore formation (particularly of spore numbers, which reflect spore size as well as spore biomass yield) was probably the primary source of the high variation in test results. The conventional method of separating spores from mycelium by using muslin (18) probably also contributed to the variation in the results; however, this was not quantitatively studied and, in any event, was not a factor influencing solid media data. Test counting of larger numbers of subsamples for selected systems resulted in a very minor decrease in spore yield variation. Low precision is characteristic of the measurement of fungal spore yields, including spore production by fungi used for biological control (10).

The advantages of examining a larger number of replicates at each stage of spore production counting and the variability caused by different factors (e.g., the use of muslin for liquid media systems and differences in shaking time for solid systems) will be analyzed in a later paper. The issue of

TABLE 6. ANOVA of log-transformed data for spore production after day 4 or 8 for different concentrations of V8 juice in solid or liquid V8 media

Source or error	Sum of squares	Degrees of freedom	Mean square	F ratio ^a
Whole plot				
Liquid or solid (L)	2.475	1	2.475	36.803
Whole-plot error	0.269	4	0.067	
Subplot				
Concn (C)	12.378	7	1.768	27.256
Day (D)	3.704	1	3.704	57.093
C × L	4.492	7	0.642	9.891
C × D	4.415	7	0.631	9.721
L × D	2.717	1	2.717	41.879
C × L × D	6.040	7	0.863	13.300
Subplot error	3.893	60	0.065	

^a All significant at $P = 0.01$.

whether replicates should be taken at one time or separately will also be addressed.

Spore production on V8 medium in the microplates was apparently less variable than in the flasks of liquid V8 medium. Independent analyses of data for solid and liquid V8 media showed that a difference of at least 3.3-fold in actual spore numbers was needed to indicate significance at $P = 0.05$ for liquid media, but only a 2-fold difference was needed for solid media. Although a minor difference in variability existed between the solid and liquid media systems, our aforementioned analysis (Table 6) is still valid since slight inhomogeneities of variance have negligible effect on the levels of significance. This means that for media such as V8 the microplate assay is particularly useful, since differences in the effects of culture variables on solid media can be distinguished from data derived with fewer replicates than would be needed for liquid media, and smaller differences can be resolved with the same number of replicates.

The microplate assay allows a large range of factors to be easily analyzed on a broad level, and poor media can be easily screened out. However, unless more replicates are done for each treatment combination (which is readily accomplished with the microplate assay), the assay does not allow accurate predictions of spore yields to be made which consider the effects of small changes in culture variables on spore production.

The characteristics of a good medium include both high spore productivity and associated low vegetative biomass. Relatively low mycelial growth with high spore production, as shown by the V8 medium, is preferable to production of unwanted mycelium accompanying good sporulation (e.g., RV8 and PG media). Commercially, excessive mycelium formation not only means that potentially less substrate is available for spore production but it also creates separation and waste disposal problems. It should be recognized that, because of the low precision of spore yield, a significant increase in mycelium from days 4 to 8 in a medium for which there was no significant difference in spore yield (Tables 4 and 5) does not imply that the nutrients available for fungal growth from days 4 to 8 were used exclusively for mycelial and not spore production or that a factor other than nutrient deprivation (such as the accumulation of a sporulation inhibitor) had limited sporulation.

For our systems, a 25- μ l inoculum of suspension (10^4 spores per ml) on the surface of a 1-ml plug of medium (2.5×10^2 spores per ml of medium) gave results that were

TABLE 5. Relative mycelium formation on solid V8 media at different concentrations of V8 juice

V8 concn		Relative mycelium production ^a on day:		
% (vol/vol)	Amt (mg/ml) of organic material	2	4	8
5	2.1	1	1	1
10	4.1	2	2	2
15	6.1	3	3	3
20 ^b	8.2	3	4	4
30	12.3	3	5	5
40	16.4	3	4	5
50	20.2	4	5	5
60	24.5	4	5	5

^a Scale: 1, very sparse growth; 2, clearly visible but sparse cover; 3, moderate growth over whole area; 4, dense cover; and 5, very dense cover.

^b Standard concentration of V8 juice.

comparable to those of a 1-ml inoculum (10^6 spores per ml) added to 100 ml of liquid medium (10^4 spores per ml of medium). An examination of a range of spore inoculum densities on the microplate indicated that spore production capacity was not affected by inocula over the range 2.5×10^1 to 2.5×10^4 spores per ml of medium (S. J. Slade, unpublished data). The microplate assay is currently being used to study the effect of inocula with very high spore densities on spore production.

For each fungus to be screened, a limited comparison of solid and liquid medium forms should initially be made, since even races of one fungal species may have quite different sporulation optima (3). The microplate technique is suitable for evaluation of the effect on spore yield of factors such as temperature, oxygen availability, and incident radiation, as well as media composition.

Even if appreciable differences exist between spore yields on solid media and those in liquid media, the production trend in liquid media may be reflected by that of the solid media. In such cases, the microplate method could be used successfully to predict the best media in a range of media to be tested, or it could be useful for screening studies of factors affecting sporulation, in liquid media forms.

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LITERATURE CITED

1. Boyette, C. D., G. E. Templeton, and R. J. Smith. 1979. Control of winged water primrose (*Jussiaea decurrens*) and northern jointvetch (*Aeschynomene virginica*) with fungal pathogens. *Weed Sci.* 27:497-501.
2. Charudattan, R., and H. L. Walker (ed.). 1982. Biological control of weeds with plant pathogens. John Wiley & Sons, Inc., New York.
3. Churchill, B. W. 1982. Mass production of microorganisms for biological control, p. 139-156. In R. Charudattan and H. L. Walker (ed.), Biological control of weeds with plant pathogens. John Wiley & Sons, Inc., New York.
4. Cuppels, D., and A. Kelman. 1974. Evaluation of selective media for isolation of soft-rot bacteria from soil and plant tissue. *Phytopathology* 64:468-475.
5. DeTilly, G., D. G. Mou, and C. L. Cooney. 1983. Optimisation and economics of antibiotics production, p. 190-209. In J. E. Smith, D. R. Berry, and B. Kristiansen (ed.), The filamentous fungi, vol. 4. Fungal technology. Edward Arnold Ltd., London.
6. Freeman, T. E. 1981. Use of conidial fungi in biological control, p. 143-165. In G. T. Cole and B. Kendrick (ed.), The biology of conidial fungi, vol. 2. Academic Press, Inc., New York.
7. Harper, D. B., T. R. Swinburne, S. K. Moore, A. E. Brown, and A. H. Graham. 1980. A role for iron in germination of conidia of *Colletotrichum musae*. *J. Gen. Microbiol.* 121:169-174.
8. King, E. D., M. K. Ward, and D. E. Raney. 1954. Two simple media for the demonstration of pyocyanin and fluoroscin. *J. Lab. Clin. Med.* 44:301-307.
9. Latge, J.-P., G. Remaudiere, R. S. Soper, C. D. Madore, and M. Diaquin. 1978. Growth and sporulation of *Entomophthora virulenta* on semidefined media in liquid culture. *J. Invertebr. Pathol.* 31:225-233.
10. Latge, J.-P., R. S. Soper, and C. D. Madore. 1977. Media suitable for industrial production of *Entomophthora virulenta* zygospores. *Biotechnol. Bioeng.* 19:1269-1284.
11. Lisansky, S. G., and R. A. Hall. 1983. Fungal control of insects, p. 327-345. In J. E. Smith, D. R. Berry, and B. Kristiansen (ed.), The filamentous fungi, vol. 4. Fungal technology. Edward Arnold Ltd., London.
12. Mather, R. S., H. L. Barnett, and V. G. Lilly. 1950. Sporulation of *Colletotrichum lindemuthianum* in culture. *Phytopathology* 40:104-114.
13. McCoy, M., T. L. Couch, and R. Weatherwax. 1978. A simplified medium for the production of *Hirsutella thompsonii*. *J. Invertebr. Pathol.* 31:137-139.
14. Perkins, D. D. 1962. Preservation of *Neurospora* stock cultures with anhydrous silica gel. *Can. J. Microbiol.* 8:591-594.
15. Sansing, G. A., and A. Ciegler. 1973. Mass production of conidia from several *Aspergillus* and *Penicillium* species. *Appl. Microbiol.* 26:830-831.
16. Snedecor, G. W., and W. G. Cochran. 1980. Statistical methods, 7th ed. Iowa State University Press, Ames.
17. Swinburne, T. R. 1976. Stimulants of germination and appressoria formation in *Colletotrichum musae* (Berk. & Curt.) Arx. in banana leachate. *Phytopathol. Z.* 87:74-90.
18. Tuite, J. (ed.). 1969. Plant pathological methods—fungi and bacteria. Burgess Publishing Co., Minneapolis.
19. Walker, H. L. 1980. *Alternaria macrospora* as a potential biocontrol agent for spurred anoda (*Anoda cristata*). Production of spores for field studies. Advances in agricultural technology 12, Science and Education Administration, U.S. Department of Agriculture, Washington, D.C.