Production of Host-Specific Toxins by *Helminthosporium victoriae* and *H. maydis* in Liquid Shake Culture

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ABSTRACT

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A method is described for growing a uniform suspension of *Helminthosporium victoriae* hyphae in liquid shake culture or in a small fermentor. Such cultures produced host-specific toxin within a few days and the titers were equivalent to those produced in surface cultures grown for 3–4 wk. *H. maydis* host-specific toxin also was produced in liquid-shake culture. In addition to saving time, an advantage of shake or fermentation cultures was the rapid depletion of residual nutrients in the medium.

The symptoms of several plant diseases are known to be caused by a host-specific toxin produced by the invading fungus. Examples include *Helminthosporium maydis* Nisikado and Miyake race T on corn (3) and *H. victoriae* Meehan and Murphy on oats (5). In general, purification and chemical structure determinations of these toxins have been slow. The toxin-producing organisms usually are grown as surface cultures on a liquid medium for 3–4 wk before the titer of the toxin is high enough to attempt purification. Apparently the general experience has been that host-specific toxins cannot be produced satisfactorily in liquid shake culture (4,8,12,14), although there are no compelling a priori reasons why this is so. A potential problem may be the lack of adequate interface between the fungal mycelium, growing in clumps, and the medium.

In this report, we show that host-specific toxins can be rapidly produced by growing uniform suspensions of *H. victoriae* or *H. maydis* in liquid shake cultures. Under such conditions, toxins (victorin and race T. toxin, respectively) can be produced in a few days which have titers comparable to those of surface cultures grown 2–4 weeks.

MATERIALS AND METHODS

Culture conditions. The two fungi were maintained on Difco potato dextrose agar plates and slants. Shake cultures were grown in Fries' medium consisting of 30 gm sucrose, 5 gm ammonium tartrate, 1 gm NH_4NO_3, 1 gm KH_2PO_4, 0.5 gm $MgSO_4 \cdot 7H_2O$, 0.1 gm NaCl, 0.1 gm $CaCl_2$, and 0.5 gm yeast extract per liter. The residue dry weight of this medium was 38.2 mg/ml.

Shake cultures were initiated by transferring several pieces of mycelium from an agar culture into 150 ml of medium in 500-ml unbaffled Erlenmeyer flasks and shaking the flasks at 200 rpm on a rotary shaker at 25 C for one to two days until mycelial growth was observed. The entire culture was then transferred to a sterile 400-ml Sorvall Omnimixer canister (Dupont Instruments, Newtown, CT 06470) and blended at top speed for 60 sec. The entire suspension was returned to the Erlenmeyer flask and shaken for an additional 2–3 days until a dense mycelial culture was obtained. The culture was then blended in the Omnimixer as before and 10- to 20-ml aliquots were seeded into 150 ml of fresh medium. This blend-and-seed procedure was repeated until the hyphae grew uniformly throughout the medium rather than forming large clumps (10).

To determine if a host-specific toxin could be produced on a larger scale, 8 L of medium in a Microferm (Model MF-114) laboratory Fermentor (New Brunswick Scientific Co., Edison, NJ 08817) was inoculated with 800 to 900 ml of a blended *H. victoriae* culture. The temperature of the fermentor was 25 C, the paddle speed was 800 rpm, and the airflow rate was 6 L/min at 1.76 kg/cm^2 (25 psi). In some instances ~2 ml of sterile antifoaming agent (SAG 4130, Union Carbide Chemical Co., Inc., Danvers, MA 01923) was added at the start of the fermentation. However, foaming usually was not a problem.

H. maydis was grown in still culture by inoculating 400 ml of medium in 1 L Corning low-form culture flasks with conidia as described previously (2). The same procedure was used to grow *H. victoriae* except that 1-L Erlenmeyer flasks containing 200 ml of medium were inoculated.

Processing of culture fluids. Aliquots periodically were removed from the shake flasks or the fermentor and the mycelium was separated from the culture fluid by filtration through four layers of cheesecloth and one layer of Miracloth (Chicopee Mills, Inc., New York, NY 10018). The filtrates were treated with an equal volume of acetone to precipitate extracellular macromolecules. Dry weights of the residual culture fluid were determined by placing measured volumes (2–4 ml) of fluid in small preweighed aluminum pans and heating at 90 C for 12–16 hr.

Bioassays. *Victorin.* The assay method was similar to that described by Luke and Wheeler (4). Dehulled oats were washed in distilled water for several hours, placed on two sheets of water-saturated filter paper in a petri plate, and covered with a single sheet of moistened paper previously blotted with a paper towel. After 20–30 hr at 23 C, germinated seeds with root tips 5–10 mm long were placed in 100-mm-diameter petri dishes containing 5 ml of filtrate diluted with water. Occasionally, 60-mm-diameter dishes (7) with 2 ml, not 5 ml (7), of solution were used.

Growth of the primary root was measured 24–36 hr later. Inhibition of growth was calculated after subtracting the initial root lengths.

Race T toxin. Inhibition of dark CO_2 fixation (2) was measured in thin (1 mm) leaf sections obtained from the center 8-mm region of the fourth true leaves of corn (the main vein was included in the section). The thin sections from W64A-T (susceptible) and W64A-N (resistant) corn were prepared by the procedure of Rathnam (9). Ten leaf sections were preincubated for 30 min in 0.5 ml of buffer, pH 7.6 (9), containing toxin. The tubes were sealed with serum

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stoppers and 10 μl of 60 mM $NaH^{14}CO_3$ were added (3×10^5 dpm). After 2 hr of incubation, trichloroacetic acid was added to give a final concentration of 17% (w/v) and the vials were subjected to a stream of air for 20–30 min. Seven milliliters of Handifluor (Mallinckrodt Chemical, St. Louis, MO 63160) scintillation fluid was added and the radioactivity was determined in a Packard Scintillation Spectrometer at 70–80% counting efficiency.

RESULTS

The repetition of the blend-and-transfer steps over a period of several days led to rapidly growing homogeneous cultures of *H. victoriae* and *H. maydis*. The micrograph in Fig. 1A shows an aliquot of a *H. victoriae* culture shortly after seeding with the blended mycelium; such cultures consist of short hyphal fragments, all of which are capable of growing into filaments. Fig. 1B shows an aliquot of the same culture after 24–30 hr growth. Growth of *H. victoriae* comparable to that shown in Fig. 1B was also obtained after 1–2 days in the fermentor.

The data reported in Table 1 establish that *H. victoriae* produced a host-specific toxin in both the fermentor and in shake culture. The toxin probably was victorin. Culture aliquots were removed daily from the fermentor and assayed for the production of *H. victoriae* toxin (Table 2). Dry weights of the culture fluid were determined on the same samples and these values also are listed in Table 2. Note that toxin production was observed as early as 3 days after the start of the fermentation. Continued fermentation resulted in a slightly higher titer of toxin, but more importantly, it resulted in a further decrease in the residual dry weight of the cultural fluid. Results similar to those reported in Table 2 were obtained from shake flasks incubated for the same time periods. In contrast, *H. victoriae* must be grown in still culture for 3–4 wk to obtain comparable titers of toxin.

It is obvious that a variety of conditions can be tested in a reasonably short time in order to increase toxin production and/or lower the residual dry weight of the culture fluid. These conditions include temperature, pH, aeration, and alterations in the composition of the medium. The results in Table 3 show the effect of decreasing the sucrose and/or yeast extract concentration in Fries' medium on *H. victoriae* toxin production and residual dry weight. Reducing the sucrose concentration to 15 gm/L and keeping the yeast extract at 0.5 gm/L resulted in slightly higher victorin production. However, reducing the yeast extract concentration resulted in a slightly lower amount of toxin. Toxin production appears to be coupled to fungal growth since cultures with reduced yeast extract also had reduced growth.

H. maydis was grown in liquid-shake culture to determine if other host-specific toxins could be produced by this method. The data in Table 4 indicate that its host-specific toxin was produced after 6 days of growth. The titer of the toxin produced was comparable to cultures grown for 16 days in still culture.

DISCUSSION

The data reported in this paper indicate that at least two host-specific toxins (ie, those from *H. victoriae* and *H. maydis*) are produced in liquid-shake cultures. The method used requires repeated blending of the cultures so that many small seed pieces can serve as starting points for growth. Consequently one obtains cultures that grow rapidly and are homogeneous.

The major advantages of the current procedure are that reasonable titers of host-specific toxin are produced within a few days. At the same time, a rapid depletion of the residual nutrients in the medium are obtained. Since the majority of the cells are young and actively growing, the residual medium probably contains fewer products resulting from the death and lysis of older cells. Consequently, the toxin in the original cultural filtrates may be contaminated with fewer extraneous natural products. Finally, since the cultures contain a relatively homogeneous population of cells, all of which may be producing toxin, it should facilitate

TABLE 1. Specificity of *Helminthosporium victoriae* toxin produced after 7 days of growth in a fermentor or in liquid shake culture

Culture method and oat type	Oat hypocotyl inhibition (%) with culture fluid dilution of:			
	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Fermentor				
Park (susceptible)	97	93	55	32
Otee (resistant)	20	2	0	0
Bates (resistant)	18	0	0	0
Shake culture				
Park	94	92	68	47
Otee	23	0	0	0
Bates	31	8	0	0

TABLE 2. Production of *Helminthosporium victoriae* toxin in a fermentor. Samples were taken at daily intervals and assayed for toxin activity with the Park cultivar and the residual dry weights were determined

Day	Residual dry weight of a culture fluid[a] (mg/ml)	Oat hypocotyl inhibition (%) with culture fluid dilution of:			
		10^{-2}	10^{-3}	10^{-4}	10^{-5}
3	15.2	100	91	65	30
4	6.8	100	92	65	55
5	6.2	100	91	67	45
6	4.5	100	95	76	54
7	4.5	100	97	81	58
25 (still culture)	12.7	92	55	34	22

[a]Initial residue dry weight was 38.2 mg/ml.

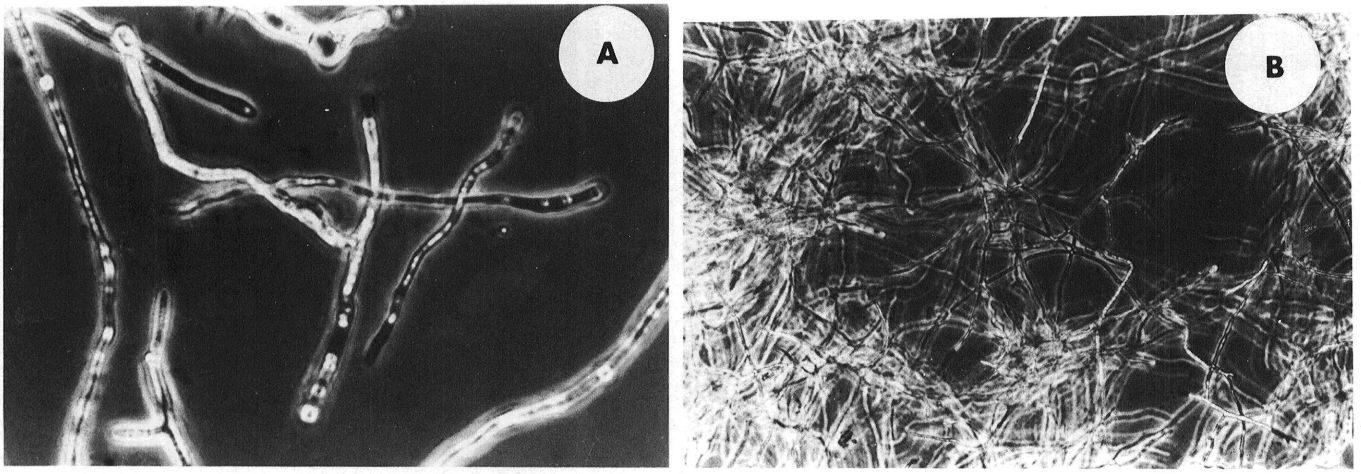

Fig. 1. Phase-contrast microscopy of *Helminthosporium victoriae:* **A,** immediately after blending (× 500) and **B,** after 24 hr of growth (× 125).

TABLE 3. Effect of sucrose and yeast extract concentrations in the growth medium on victorin production and residual dry weight of the culture fluid from *Helminthosporium victoriae* grown for 5 days

Sucrose (gm/L)	Yeast extract (mg/L)	Residual dry weight of culture fluid (mg/ml)	Oat hypocotyl inhibition (%) with culture fluid dilution of:		
			10^{-2}	10^{-3}	10^{-4}
15	500	5.5	100	89	70
30	500	6.7	96	79	64
15	100	8.6	91	58	33
30	100	13.5	94	66	35
15	0	11.8	83	66	31
30	0	19.3	84	50	30

studies on the biosynthesis of the toxins. That is, suspected intermediates which are isotopically labeled may be rapidly incorporated into a toxin. Although the description of the blending technique might lead to the conclusion that there is more labor involved than with previous methods, we have not experienced such a disadvantage. For large volumes (eg, 8–12 L) the reverse is true.

If one defines the specific activity of the toxin as the amount of toxin to produce 50% inhibition in the bioassay, then the specific activity of the *H. victoriae* crude culture filtrates from the fermentor is about 45 ng/ml (Table 2). At the present time we have no reason to think that the titers of victorin obtained by us are much different from other values reported in the literature (6,7,11,13,14). However, direct comparisons are difficult because of differences in the isolates of *H. victoriae* used, differences in assay conditions, and that some values reflect results with partially purified toxin. Likewise, the titers for *H. maydis* toxin reported in Table 4 cannot be compared with an earlier report of toxin production in still culture (1). The high-yielding, light-sensitive (1) *H. maydis* isolate used in that study was lost after several transfers to agar slants in 1976. However, as shown in Table 4, comparable titers of race T toxin were obtained from shake and still cultures; the advantage of the shake culture method is that toxin is produced sooner.

TABLE 4. Toxin production of *Helminthosporium maydis* race T in liquid shake or still culture as measured by inhibition of dark CO_2 fixation in corn leaf section

Corn type	Control	$^{14}CO_2$ fixed per leaf slice (nmoles) culture fluid dilutions of:				
		1/250	1/500	1/1,000	1/2,500	1/5,000
Shake culture (6 days)[a]						
N corn	3.9	4.0	3.7	4.0	NT[b]	NT
Inhibition (%)	–	0	5	0	–	–
T corn	3.9	1.6	2.3	2.5	3.3	3.6
Inhibition (%)	–	59	41	36	15	8
Still Culture (16 days)[c]						
T corn[d]	3.0	1.2	1.9	2.2	2.4	NT
Inhibition (%)	–	60	37	27	20	–

[a] Residual cultural fluid was 4.1 mg/ml.
[b] NT was not tested.
[c] Residual cultural fluid was 13.1 mg/ml.
[d] Assayed on a different day.

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