Improved Methods for Inducing Sporulation of *Pyrenochaeta lycopersici*

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ABSTRACT

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Factors affecting sporulation of *Pyrenochaeta lycopersici* obtained from tomato roots in 1980 and 1981 were studied. V-8 juice agar medium (V8A) was superior to 18 other media. Sporulation was enhanced by short incubation (3–4 days) of recent isolates on water agar before transfer to V8A, whereas isolation from diseased tomato roots directly onto V8A gave nonsporulating colonies. The optimum pH for sporulation was about 5.5. Light was required; fluorescent cool-white lamps were as effective as fluorescent BLB black light lamps, and constant exposure was superior to 12- or 8-hr photoperiods. The optimum temperature range for sporulation was 20–24 C. Isolates maintained for 2 yr on V8A by conidial transfer remained sporogenic and pathogenic. Conidia may be useful for testing resistance of seedlings.

Pyrenochaeta lycopersici Schn. & Ger., the cause of corky root (brown root rot) of tomato (*Lycopersicon esculentum* Mill.), occurs in greenhouse and field soils worldwide (1,4,6,19,20). In the past, *P. lycopersici* has been difficult to isolate, to induce to sporulate, and thus, to identify (7,16,24,25). No sporulation has been observed in nature, and for many years, isolates were referred to as the "grey sterile fungus" (16,20,24,25). It was not until 1964 that several isolates that readily formed pycnidia were found (7) and later used for the description of *P. lycopersici* (17). Later, Clerjeau (2) described a method for sporulation on hypocotyls of melon (*Cucumis melo* L.) or tomato seedlings. Near-ultraviolet radiation induces sporulation of many fungi, including *Phoma terrestris* (formerly *Pyrenochaeta terrestris* [18]) (13). Recently, C. M. Leach (*personal communication*) reported that sporulation of *P. lycopersici* was also induced by near-ultraviolet light. Nevertheless, sporulation and identification of *P. lycopersici* has remained a problem (1,22).

Other problems associated with cultures of *P. lycopersici* include loss of pathogenicity (3,10) and loss of ability to sporulate (2). Although a method for screening tomato seedlings for resistance using pure cultures has been described (3), reliable results are not always obtained (8).

In 1980, eight gray fungal isolates recently obtained from diseased tomato roots were transferred to 19 media. None sporulated on 18 media and only two sporulated on V-8 juice agar under fluorescent cool-white (FCW) lamps at room temperature. Both isolates were *P. lycopersici*. Starting with these cultures, the objective of this paper was to determine the factors affecting sporulation of *P. lycopersici* in order to have a reliable method for routine identification and for increase of conidial inoculum for use in pathogenicity trials.

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MATERIALS AND METHODS

Media. Five media used throughout this study were acidified potato-dextrose agar (APDA), water agar (WA), and V-8 juice agar (V8A = 200 ml V-8 juice, Campbell Soup Co., 800 ml distilled H_2O, 1 g $CaCO_3$, and 15 g agar modified from that of Miller [15]), double-strength V8A [(2×)V8A], and one-fourth-strength V8A [(¼×)V8A] containing 400 ml V-8 juice plus 2 g $CaCO_3$/ L or 50 ml V-8 juice plus 0.25 g $CaCO_3$/ L, respectively. When effects of pH on sporulation were tested, $CaCO_3$ was omitted.

Initially, several other media (21) were screened for possible use in sporulation of *P. lycopersici*: carrot agar, charcoal water agar, Czapek agar, Czapek plus caseine, peptone, or yeast extract, cornmeal agar, lima bean agar, malt agar, Matsushima agar plus cellophane (14), plant parts, potato agar, potato-dextrose agar, starch agar, tomato juice agar, Watson's low nutrient agar and straw (23), yeast-dextrose carbonate agar, and 523 agar (11).

Isolation, identification, and maintenance. Infected root pieces were immersed in 0.5% NaOCl for 2–3 min and chips of tissue from the lesion margins were plated on APDA. Isolates were transferred to WA and incubated 3–4 days in the dark at 24 C. Plugs cut from the margins of colonies on WA were inverted and placed on V8A. Plates were kept in the standard conditions used for this study: constant light at 21 ± 1 C for 15–20 days. Light was provided by four 40-W General Electric FCW lamps in a fixture 25 cm above the plates. The arrangement was nearly identical to that of Leach (13), except he suspended the lamps 56 cm above the cultures.

All isolates identified as *P. lycopersici* possessed dark, setose pycnidia and hyaline, single-celled conidia (about 4.5 × 2 μm) borne on septate, ramose conidiophores (17,18). Sixteen isolates were maintained in short-term storage for use in experimentation, seven from 1980 (801–807) and nine from 1981 (811–819). Isolate 804, a strong sporulator forming numerous pycnidia in a wide range of conditions, and isolate 807 will be deposited with the American Type Culture Collection.

For short-term storage or increase of conidial inoculum, stock cultures were induced to sporulate on (2×)V8A as follows: conidial suspensions (3×10^3 spores per milliliter) were prepared by aseptically crushing three or four pycnidia in a sterile test tube and diluting with distilled water; 200 ml of molten, sterile (2×)V8A (about 38 C) were inoculated with 1 ml of conidial suspension in a flask equipped with a 20-ml dispenser (VWR Scientific Inc., San Francisco, CA 94119), and dispensed at 20 ml/ 100-mm-diameter plastic petri dish. Inoculated plates were incubated under standard conditions and used immediately as sources of conidia or stored as long as 4 mo at 5 C. For long-term storage, soil containing diced tomato roots was sterilized in test tubes, inoculated, and frozen after colonization by *P. lycopersici* (1).

Environmental conditions affecting sporulation. For comparison of treatments, replicate plates were either inoculated with plugs of mycelium cut from the periphery of a single colony on WA or with a conidial preparation as described earlier. In the latter case, the average number of colonies per plate within each trial did not vary significantly ($P = 0.05$). Intensity of sporulation was determined by counting setose pycnidia in a 10-cm^2 area defined by a template placed over the petri dish. The template was a circle of cardboard with three parallel, rectangular openings, each 1 cm wide. Results are reported as the average number of pycnidia per square centimeter.

The effect of the fungus on the pH of media was tested. Several plates were

inoculated with plugs of mycelium and incubated in the dark at 24 C. At intervals, inoculated and noninoculated plates were withdrawn from the incubator and the pH of the medium at the colony margin was measured with a flat-surface combination electrode (A. H. Thomas Co., Philadelphia, PA 19106). These plates were discarded. Several buffers [KH_2PO_4/K_2HPO_4, NaH_2PO_4/Na_2HPO_4, succinic acid/NaOH, malic acid/potassium phosphate, 2-(*N*-morpholino)-ethanesulfonic acid, and tris-(hydroxymethyl)aminomethane] were tested for their effect on sporulation and stabilization of the pH of the medium. The KH_2PO_4/K_2HPO_4 buffer was selected as the standard.

Effects of pH and nutrient concentration on sporulation were tested using 100-mm divided plastic petri dishes containing 25 ml of media in each half of the plate. One medium in each plate was inoculated with a plug of mycelium 5 mm from the center divider. After incubation in standard conditions for 25 days, the pycnidia in a 1-cm^2 area were counted on the second half of the plate directly opposite the mycelial plug.

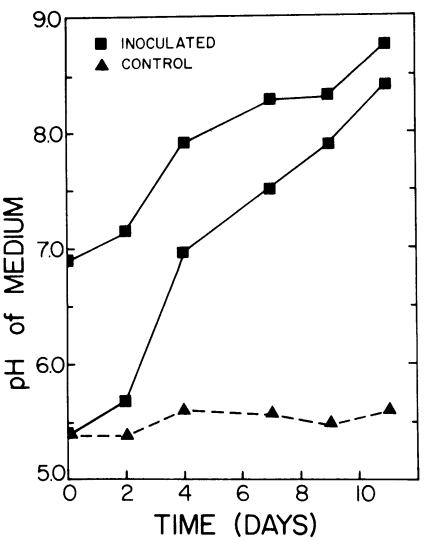

Fig. 1. Effect of *Pyrenochaeta lycopersici* isolate 803 at 24 C on pH of potato-dextrose agar initially adjusted with NaOH/HCl to pH 5.4 or 6.9.

Photoperiods were compared in similar environments in a Scherer CEL growth chamber by dividing the chamber longitudinally with a partition. Identical fixtures were placed on either side of the partition and operated by separate time clocks. Four zones were defined under each light fixture by placing open boxes (20 × 27 × 8 cm deep) along their axis. Thermocouples were embedded in the agar of closed petri dishes that were placed at the center of each box and plates for experiments were arranged in a Latin square design in the four corners of each box. Petri dish lids were not removed (12,13). In each trial, there were three treatments that always included a 0- and a 24-hr photoperiod plus an intermediate photoperiod. Plates for the 0-hr treatments were stored within the chamber in closed and ventilate film boxes.

Fluorescent BLB black light lamps (BLB) (General Electric) and FCW lamps were compared in the same chamber. The spectral emissions of the FCW lamps (9) and of the BLB lamps (13) have been characterized; BLB lamps are standard sources of near-ultraviolet light (13). All replicate plates were placed in constant exposure at time zero. Sample plates were transferred at intervals to closed film boxes and kept in the dark until sporulation intensity was determined for all plates at the end of the trial.

The effect of temperature on sporulation was determined using the flat surface of a temperature gradient bar made for test tubes (11). The bar was used in a laboratory and a light fixture was suspended parallel to and 25 cm above the surface of the bar. Replicate 60-mm-diameter plastic petri dishes containing 10 ml of (2×)V8A (inoculated with conidia before pouring) were incubated in the dark at 24 C for 4 days and distributed at regular intervals along the bar. After the agar surfaces were thoroughly colonized, the petri dish lids were removed for short periods to measure the agar temperature at the cool and warm sides of each plate with a thermocouple probe. The probe was cleaned with 0.5% NaOCl and airborne contaminants were not a problem. Measurements were made two to six times per trial during periods when the room temperature was high and low as monitored by a recording thermograph. One to three trials were done per isolate and the temperature gradient was readjusted between trials so that a given temperature would occur at different places on the bar.

Pathogenicity of conidia. Spore suspensions were prepared by flooding stock cultures on (2×)V8A plates with sterile distilled water. After 30 min, the cultures were rubbed with a glass rod and the suspension was filtered through two layers of sterile cheesecloth. Inoculum densities were determined by dilution plating. Spore suspensions were applied to vermiculite with an atomizer while shaking the vermiculite in a polyethylene bag, or for larger batches, while tumbling the vermiculite in a cement mixer. Four replicate pots (11.5 cm diam.) per treatment containing 500 ml of vermiculite were sown with tomato seeds and arranged in randomized blocks in a plastic covered chamber. The chamber was cooled with an air conditioner to 19 ± 3 C and lighted by a bank of FCW lamps for 16 hr daily. One half of each pot was sown with a susceptible tomato cultivar (VF145-7879) and the other half with one of three tolerant tomato cultivars (Moboglan II, RS791705, or BL41-2). Pots were irrigated as needed with half-strength Hoagland's solution. After 5 wk, 20 seedlings of each cultivar per pot were rated for disease. A disease index of 0–4 based on discoloration and lesions on the taproots was used, where 0 = no discoloration, 1 = slight discoloration, 2 = discoloration and one or two small lesions (about 1 mm^2 diam.), 3 = general discoloration and several lesions, and 4 = general necrosis of the entire taproot.

RESULTS

Effects of isolation technique on sporulation. Colonies developed by plating diseased tissue directly onto V8A did not sporulate. If new cultures from isolation plates were transferred to WA for 3–4 days before transfer to V8A, about 90% of the isolates formed pycnidia on or near the inverted water agar plug.

Effects of pH and nutrient concentration on sporulation. Determination of the optimal pH for sporulation was complicated by the effect of the fungus on the sporulation medium. *P. lycopersici* rapidly raised the pH of unbuffered media (Fig. 1). Three other isolates behaved similarly. High pH values also were measured in plates at the end of several experiments. Furthermore, attempts to buffer pH failed because buffering capacities were overcome at 0.2 M and sporulation was inhibited at 2.0 M. If the nutrient concentration of buffered medium was reduced, isolate 804 sporulated at pH values below 7 but

Table 1. Effect of pH and nutrient concentration on the sporulation of four isolates of *Pyrenochaeta lycopersici* in divided petri dishes[a]

Medium inoculated[b]	Second medium[b] (no. of isolates sporulating [range of sporulation])[c]			
	A	B[d]	C	D
A	0/4	1/4 (8)	0/4	3/3[e] (105–169)
B	0/4	1/4 (31)	0/4	4/4 (12–45)
C	0/4	1/4 (6)	0/4	3/4 (6–84)
D	0/4	1/4 (17)	0/4	4/4 (22–55)

[a] Sixteen treatments with single replicates of four isolates: 803, 804, 807, and 816.
[b] All media were buffered with 0.1 M potassium phosphate buffer. A = double-strength V-8 juice agar (V8A), pH 7.5; B = double-strength V8A, pH 5.5; C = one-fourth strength V8A, pH 7.5; and D = one-fourth strength V8A, pH 5.5.
[c] Figures in parentheses show the number of pycnidia per square centimeter for sporulating isolates.
[d] Isolate 804 sporulated on medium B.
[e] Data for 807 unavailable for this treatment.

growth was poor and other isolates failed to sporulate. These problems were largely overcome by using divided petri dishes, which allowed the fungus to grow from the inoculated medium that was conducive to vegetative growth onto a buffered medium conducive to sporulation.

In the initial experiment, four isolates were tested in all possible reciprocal patterns between (2×)V8A at pH 7.5 or 5.5 and (¼×)V8A at the same pH values. All four isolates sporulated when the second medium was (¼×)V8A at pH 5.5 but not at pH 7.5 (Table 1). Only one isolate (804) formed pycnidia on (2×) V8A at pH 5.5. The optimum pH for sporulation of 10 isolates was determined. Nine of 10 isolates sporulated on (¼×) V8A at pH 5.1–5.5 but none did at pH 7.3 (Fig. 2). Pycnidia of all isolates were most abundant at pH 5.5, as exemplified by isolate 815. The pH of the second medium was raised 0.3–0.5 pH units at the end of the trial.

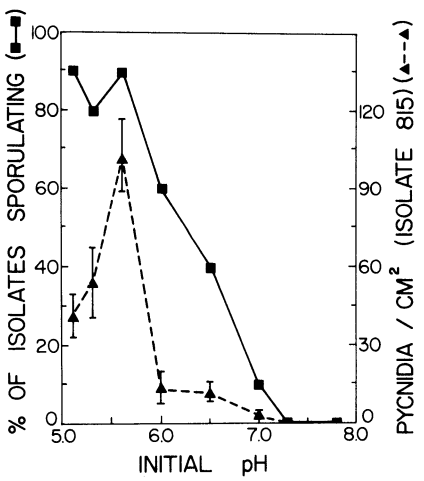

Fig. 2. Effect of pH on sporulation of 10 isolates of *Pyrenochaeta lycopersici*. Neutral (2×)V8A on one side of the divided petri dishes was inoculated and sporulation was determined on the second side containing (¼×)V8A buffered at pH 5.1–7.8 with 0.1 M potassium phosphate buffer. Data for a representative isolate, 815, are averages and standard deviations (vertical bars) from four replicate plates per treatment.

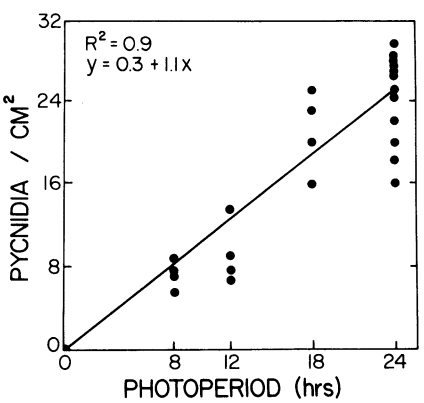

Fig. 3. Effect of photoperiod on the sporulation of *Pyrenochaeta lycopersici* isolate 805 at 21 ± 1 C. The data point at 0 represents 12 replicates.

Effect of light on sporulation. Light was required for sporulation; 18- and 24-hr photoperiods were superior to 8- and 12-hr photoperiods (Fig. 3). With an 8-hr photoperiod, pycnidia formed in concentric circles corresponding to periods of light.

There was no difference in the time for pycnidial formation or in the number of pycnidia formed when FCW and BLB lamps were compared (Fig. 4). Isolates 806 and 807 gave similar results.

Effects of temperature on sporulation. The optimum temperature for sporulation of isolate 813 was 21 C (Fig. 5). Eleven other isolates were tested, each in one to three trials per isolate, and had optima of 20–24 C. For all 12 isolates, sporulation declined sharply as temperatures approached 25–26 C and failed at 27 C or higher.

Pathogenicity of conidial inoculum. In four trials with conidia, five isolates of *P. lycopersici* were tested and found pathogenic (Table 2). With inoculum densities higher than 10^2 conidia per gram of vermiculite, the susceptible seedlings were severely diseased but tolerant seedlings were not.

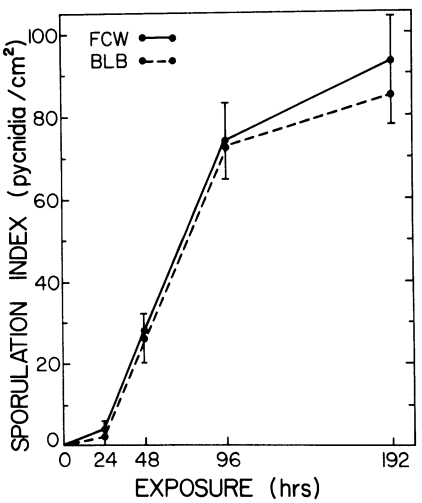

Fig. 4. Comparison of fluorescent cool-white (FCW) and BLB black light (BLB) lamps for sporulation of *Pyrenochaeta lycopersici*. Each value is an average and standard deviation (vertical bars) from four replicate plates of isolate 804.

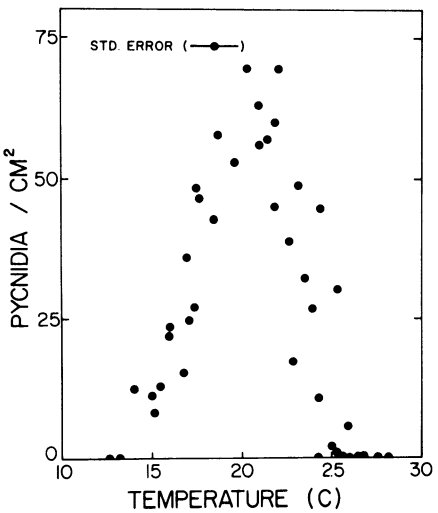

Fig. 5. Effect of temperature on sporulation of *Pyrenochaeta lycopersici* isolate 813 in three trials. Each point is from a single 60-mm-diameter petri dish. The standard error (horizontal line) is the average temperature variation caused by daily fluctuation of ambient temperature and the gradient across the plate.

Table 2. Pathogenicity of five isolates of *Pyrenochaeta lycopersici*

Trial	Isolate	Colony-forming units/g vermiculite	Disease index[w]	
			Susceptible[x]	Tolerant[y]
A	Nil	0.0	0.0	···
	804	0.2 ± 0.3	0.4 ± 0.5[z]	···
		2.1 ± 0.2	1.2 ± 0.9	···
		20.0 ± 4.0	2.9 ± 0.5	···
		2×10^2	3.5 ± 0.3	···
		2×10^3	3.7 ± 0.1	···
		2×10^4	3.9 ± 0.1	···
B	Nil	0.0	0.0 a	0.0 a
	803	1×10^5	4.0 ± 0.0 c	1.5 ± 0.2 b
C	Nil	0.0	0.0 a	0.0 a
	803	2×10^6	3.8 ± 0.1 d	1.3 ± 0.7 b
	806	5×10^6	3.9 ± 0.1 d	1.9 ± 0.4 c
D	Nil	0.0	0.0 a	0.0 a
	807	3×10^5	3.9 ± 0.1 c	2.2 ± 0.7 b
	817	2×10^6	3.9 ± 0.1 c	2.1 ± 0.3 b

[w] Expressed as the average disease severity ± standard deviation from 20 seedlings per replicate in four replicates rated on the disease severity scale: 0 = no discoloration, 1 = slight discoloration, 2 = discoloration and one or two small lesions (about 1 mm^2 diameter), 3 = general discoloration and several lesions, and 4 = general necrosis of the entire taproot. In trials B–D, each replicate contained susceptible seedlings sown in one-half of each pot and a tolerant line sown in the other half. Numbers followed by the same letters in each experiment did not differ significantly by Duncan's multiple range test ($P = 0.05$).

[x] VF145-7879.

[y] Moboglan II, RS791705, and BL41-2 were sown in the opposite half of the pot from the susceptible cultivar in trials B, C, and D respectively.

[z] The log of inoculum concentration starting at 0.2 cfu/g vermiculite and disease index were highly correlated ($r^2 = 0.88$) with $Y = 1.16 + 1.35X - 0.16x^2$.

Degeneration of *P. lycopersici* in culture. When *P. lycopersici* was incubated at room temperature on agar media, pale sectors rapidly developed. In these areas, hyphae were matted and distorted. Mycelial transfer to fresh medium did not reverse this trend. After 3–4 mo on V8A or PDA, isolates failed to sporulate when hyphae were transferred to V8A or melon seedlings (*Cucumis melo* L. 'Honeydew'). Similarly, isolates lost their ability to sporulate when incubated on WA for only 5 wk. Attempts were made to revive isolates by inoculation to and reisolation from tomato seedlings but sterile isolates remained sterile. Some nonsporulating isolates had also lost their pathogenicity.

Maintenance of sporulating cultures of *P. lycopersici.* Five isolates stored in soil tubes in 1977–1978 (1), two stored in 1980, and two in 1981 were tested with these standard methods in April 1982. Four of five isolates from 1977–1978, including one that failed to sporulate on melon seedlings in 1977, sporulated. The remaining 1977 isolate had been cultured for more than 3 mo on PDA before storage and remained sterile. The 1980–1981 isolates all sporulated well. Five isolates that had been in continuous culture as long as 2 yr, maintained by conidial transfer, and cycled as many as 10 times through short-term storage remained viable, sporogenic, and pathogenic (Table 2).

DISCUSSION

The utility of this technique was demonstrated with 35 isolates collected in 1980 and 20 collected in 1981. All sporulated and were identified as *P. lycopersici.* These were all from tomato, except one (805) from pepper (*Capsicum annuum* L.), and came from 29 fields in eight counties including Fresno, Monterey, Sacramento, and Yolo, where the pathogen had not been identified previously. The ability to form pycnidia varied markedly. Four of 55 isolates sporulated readily, forming numerous pycnidia over a range of conditions, but the others formed fewer pycnidia within a narrower range of conditions.

Growth of *P. lycopersici* was fastest at pH 4.7 and declined markedly above pH 7.0 (20). Sporulation in these tests also was favored by a narrower range of low pH values. The fungus rapidly raised the pH of agar medium to a level not conducive to sporulation.

Although FCW lamps emit more total energy than the BLB lamps (13), both types of lamps produce adequate amounts of the critical wavelengths, presumably in the near-ultraviolet spectrum, to induce sporulation. Given their high price (about $18 each), BLB lamps are not cost-effective.

Sporulation was depressed by high temperatures that occur in closed petri dishes under FCW lights at room temperature. During July to September, when most corky root samples arrive in our laboratory, average daily maximum temperatures were 25 C or higher in our air-conditioned laboratory in 1981. Using a thermocouple probe, temperatures as high as 29 C were measured in closed petri dishes under lights. Thus, sporulation of cultures was markedly improved by exposing cultures to FCW light in a growth chamber (21 ± 1 C).

P. lycopersici competes poorly with other fungi as a saprophyte and Davet (5) has suggested that it is "ecologically an obligate parasite." It rapidly degenerates in culture if maintained by mycelial transfers (3,8,10). In their study of "pycnidium producing strains" of *P. lycopersici*, Gerlach and Schneider (7) reported no correlation between ability to form pycnidia and pathogenicity. With conidial transfer, however, we have maintained 16 sporogenic cultures for more than 2 yr and each of the five tested were pathogenic. Conidial inoculum should improve the reliability of seedling screening tests (8) because it is easily quantified and incorporated even at high inoculum densities.

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