An Efficient Technique for Inducing Profuse Sporulation of *Alternaria* Species

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ABSTRACT

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A new technique was developed for inducing profuse sporulation of *Alternaria solani, A. alternata,* and *A. dauci* in pure culture without the concomitant production of aerial mycelium. Agar blocks from cultures on primary media were transferred to the surface of a second (sporulation) medium of water agar plus $CaCO_3$ and incubated in the dark at 18 C. Spores were harvested after 18–24 hr. Initial addition of sucrose to the sporulation medium allowed repeated harvest of conidia at 24-hr intervals. *Helminthosporium maydis* race T also sporulated well then subjected to this protocol, but a fourth *Alternaria* species (*A. zinniae*) that was tested did not.

Additional key words: potato early blight, *Solanum tuberosum,* sporulation medium.

Alternaria solani (Ell. & Mart.) Jones & Grout. causes early blight of potato and tomato and serious diseases of other important crops. Plant pathologists and breeders use conidia of the fungus as inoculum to screen plants for resistance to the fungus. However, since the pathogen produces relatively few conidia on agar media, considerable attention has been directed toward improving in vitro conidial production by induction with ultraviolet light (1,6), fluorescent light (1,5), and solar illumination (6,7,8). Sporulation has been enhanced by mycelial wounding (1,2,4,6,8), medium dehydration (1,4,6,8), or use of chemical additives (1,3). In our laboratory, however, none of these methods consistently produced sufficiently high numbers of spores for screening large numbers of plants. Moreover, many of those procedures required a long period for sporulation (7–20 days), produced copious amounts of aerial mycelium, or required extensive environmental manipulation.

In this paper, a simple method is described that induces prolific sporulation of *A. solani* within a short time period. Abundant conidia repeatedly were collected from the same culture dish at 24-hr intervals. Conidia obtained by this procedure resembled those from infected plant tissue and were highly pathogenic on potato.

MATERIALS AND METHODS

***Alternaria* spp. isolates.** Isolates of *Alternaria solani* (Ell. & Mart.) Jones & Grout. were obtained from G. D. Easton, Prosser, Washington, and from naturally infected potatoes through the courtesy of L. E. Claflin, Kansas State University, Manhattan. *Alternaria alternata* was provided by R. G. Grogan, University of California, Davis; *A. dauci* by J. Strandberg, University of Florida, Agricultural Research and Education Center, Sanford; and *A. zinniae* by L. Baxter, Clemson University, Clemson. The isolates were maintained on potato dextrose agar (PDA) plates sealed with Parafilm and stored at 5 C.

Primary culture media. Cultures were grown in 100-mm diameter petri plates containing 20 ml of medium. Six primary media were tested: 2% Difco cornmeal agar (CMA), lima bean agar (LBA), malt extract agar (MEA), rye agar (RA), 2% (w/v) water agar (WA), and potato dextrose agar (PDA). Plates were seeded either by spreading 0.8 ml of a suspension of conidia or mycelia in sterile distilled water over the agar surface, or with a centrally positioned 2-mm diameter plug taken from the edge of actively growing 4-day-old agar plate cultures. Cultures were maintained on primary media for varying times at 25 C in the dark. Comparable plates also were incubated under either constant or dirunal light until actively growing. Forty-eight to 72 hr after seeding, but before the appearance of aerial mycelium, the agar medium containing developing mycelium was cut with a sterile scalpel into approximately 4 mm^2 blocks, which were individually positioned on the surface of the sporulation medium (S-medium) (Fig. 1A). The number of blocks added to each S-medium plate was not critical, but usually at least one half of the growing colony in the primary medium plate was transferred to the surface of an S-medium plate. The S-medium was composed of 20 g sucrose, 30 g $CaCO_3$, and 20 g of agar per liter of distilled water (pH 7.4). Sterile distilled water (2 ml) was added to the plates to partially cover the mycelial blocks and the plates were incubated at 18 C in the dark. Older cultures (5 days or more) also could be used to inoculate S-medium plates after any fluffy aerial mycelium present was excised with a sterile razor blade and then washed away with sterile distilled water.

Spore collection. After a gentle rubbing of the agar surface with a rubber policeman, dislodged conidia were removed by repeatedly flooding plates with 10-ml aliquots of sterile distilled water. Resultant spore suspensions were filtered through two layers of sterile cheesecloth, combined, and the concentration of conidia was determined with a haemocytometer. Recovery of spores was still more efficient if the plates were flooded with 0.01% Triton X-100 (Sigma Chemical Co., St. Louis, MO 63178) which prevented the conidia from sticking to either the agar surface or the petri dish walls during collection. Spore suspensions were centrifuged at 400 *g* for 5 min and resultant pellets were diluted to the desired concentration(s) with sterile distilled water. Pathogenicity tests were done on potato (*Solanum tuberosum* 'Russet Burbank') plants regenerated from mesophyll cell protoplasts (9) as well as on the mother clone. Plants maintained under 100% relative humidity at 24 C were sprayed with conidial suspensions at known concentrations ranging 100–100,000 conidia per milliliter. The number of successful infections per leaf were recorded after 8 days.

RESULTS

When blocks of primary medium impregnated with mycelium of *A. solani* were placed on the S-medium, the following pattern of events consistently was observed. After 18–24 hr in the dark at 18 C, the surface of each agar block was covered with conidia (Fig. 1B,C) without the concomitant presence of aerial mycelium. Mycelia proliferated from the agar blocks directly into the S-medium.

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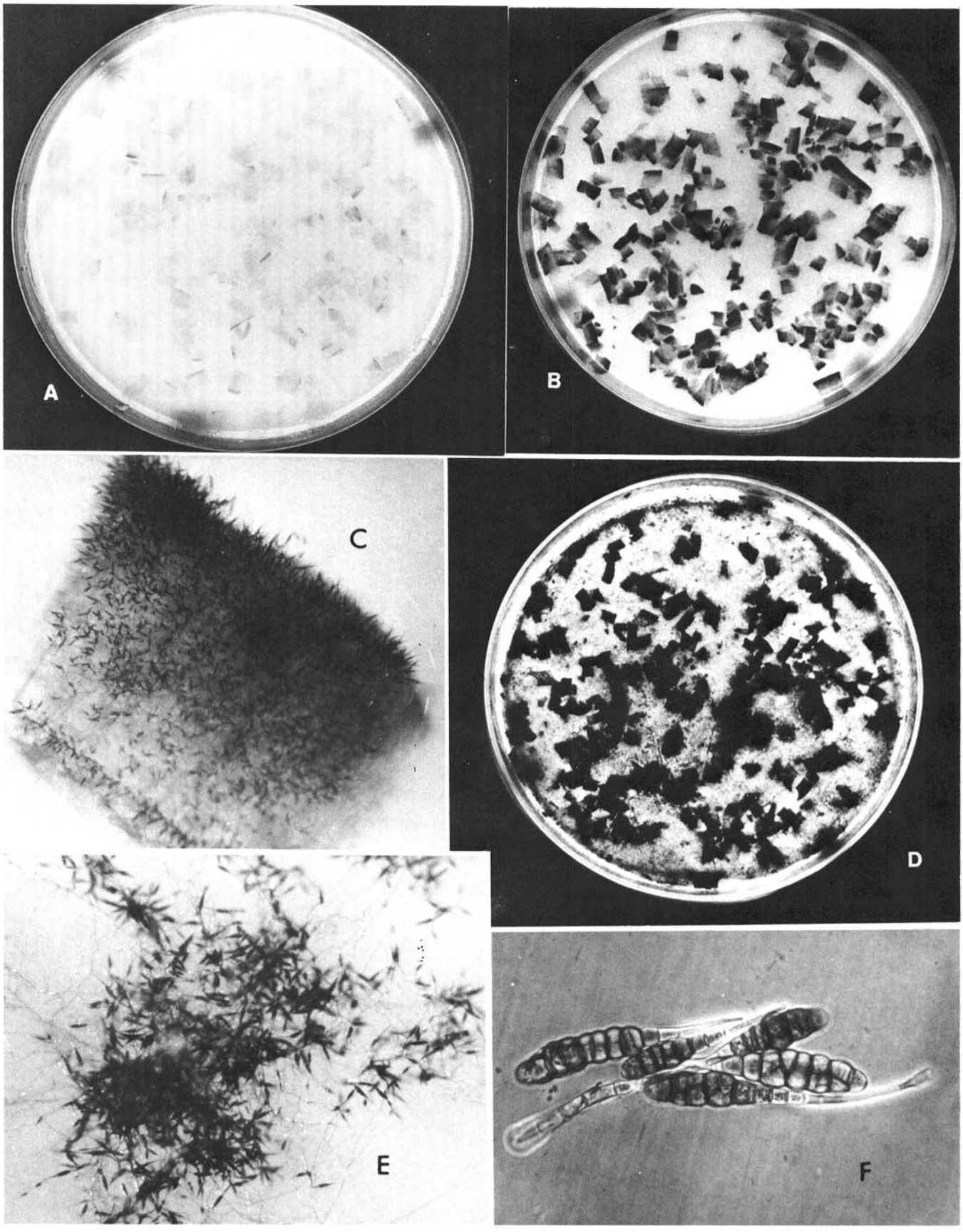

Fig. 1. Sporulation of *Alternaria solani.* **A,** Mycelial blocks from a 2-day-old culture on PDA spread over the S-medium. No spores were present. **B,** After 18 hr incubation at 18 C in the dark, the intensity of the dark color reflects abundant spores. **C,** An agar block showing sporulation. **D,** 72 hr after incubation at 18 C in the dark. Note the spores on the plate medium between the agar blocks. This plate was harvested twice at 24-hr intervals. **E,** A view of the mycelium embedded in the agar with conidia emerged above the agar surface. No aerial mycelium was evident throughout the sporulating process. **F,** Conidia of *A. solani.*

Within 48–72 hr, short conidiophores that produced abundant conidia without aerial mycelia, often covered the entire surface of the S-medium with spores (Fig. 1D,E). After conidia were collected by flooding with sterile distilled water, mycelia embedded in the S-medium repeatedly produced another crop of conidia when the plates were reincubated at 18 C in the dark. This permitted the repeated collection of spores from the same plate(s) at 24-hr intervals. Harvested spores were both mature and highly pathogenic when inoculated on potato leaves.

Except for water agar, the compositions of the primary culture media were of relatively minor influence on the efficiency of *A. solani* sporulation on S-medium (Table 1). Nevertheless, inocula obtained from primary PDA and MA cultures did produce more spores than other cultures, and except for water agar, rye agar produced the lowest spore yields. Repeated subculturing of the isolates whether started from single spores or en masse, did not affect the capactiy of mycelia to sporulate.

In similar tests, both *Alternaria alternata* and *A. dauci* produced abundant conidia on S-media, but *A. zinniae* did not.

Several tests were conducted to investigate the significance of $CaCO_3$ and other factors of the sporulation system (Table 2). Addition of 10, 20, 30, 40, and 50 g/L of $CaCO_3$ in the S-medium gave comparable results. However, when $CaCO_3$ was eliminated from the S-medium, or when either Na_2CO_3 or $CaCl_2$ was substituted for $CaCO_3$, no sporulation occurred. Elimination of sucrose from the S-medium did not affect initial sporulation, but continued fungal growth and sporulation did not occur over prolonged periods. The addition of a nitrogen source, such as casamino acids or yeast extract, although supportive of mycelial growth was detrimental to sporulation (Table 2). Experiments also were done in which S-medium plates with agar blocks were incubated at 18 and 25 C in the dark, 18 C in the presence of UV light, and at 18 C in fluorescent light. No sporulation occurred at 25 C in the dark or at 18 C in UV light, and very little at 18 C in fluorescent light (Table 2).

TABLE 1. Influence of primary medium on sporulation of *Alternaria solani* on S-medium[x,y]

Medium	Colony diameter	Spores per plate[z]
Potato dextrose agar	3.86 cm	2.8×10^6 a
Maltose extract agar	3.40 cm	2.6×10^6 a
Cornmeal agar	3.00 cm	2.4×10^6 b
Lima bean agar	3.53 cm	2.3×10^6 b
Rye agar	4.30 cm	1.2×10^6 c
2% water agar	2.60 cm	1.8×10^5 d

[x] Plates of primary medium were centrally inoculated with 2-mm diameter plugs taken from the edge of actively growing 4-day-old cultures, and then incubated at 25 C. After 72 hr, colony diameters were measured and agar blocks then transferred to the S-medium. Five replicates were used per treatment. Spore yields were determined with a haemocytometer after 24 hr of incubation on the S-medium.

[y] S-medium consisted of: 2% water agar containing 20 g/L sucrose and 30 g/L $CaCO_3$, pH 7.4.

[z] Data followed by the same letter do not differ significantly, $P = 0.05$, according to Duncan's multiple range test.

TABLE 2. Summary of various treatments on sporulation of *Alternaria solani* in S-medium[a]

Treatments	Growth Habit	
	Sporulation	Vegetative
Casein hydrolysate[b] (1.0%)	−	+
Yeast extract[b] (2.0%)	−	+
Potato broth[b] (10.0%)	−	+
Fluorescent light (5,380 lux) at 18 C	±	+
UV-light at 18 C	−	+
Darkness at 25 C	−	+
Darkness at 18 C	+	−
Na_2CO_3[c]	−	+
$CaCl_2$[c]	−	+

[a] S-medium consisted of: 2% water agar containing 20 g/L sucrose and 30 g/L $CaCO_3$, pH 7.4.

[b] Chemicals were added to the S-medium.

[c] Added equimolar to the S-medium to replace $CaCO_3$.

[d] The symbol, ±, denotes the presence of only a few spores.

DISCUSSION

A simple water agar medium containing $CaCO_3$ promoted extensive sporulation of *A. solani* when the fungus first was established on any of several primary media. The addition of sucrose to the S-medium, while unnecessary for the initial development of conidia on the surface of the inoculum blocks, nevertheless permitted the continued growth and sporulation of the fungus over prolonged periods. Although the presence of $CaCO_3$ seemed critical for sporulation (none occurred in its absence), its absolute concentration was not. Moreover, several physical and environmental parameters were involved, including cool temperature (18 C), moisture (2 ml of water added to the surface of the S-medium), wounding of the mycelium, and absence of light. These factors are in agreement with some of those suggested by Waggoner and Horsfall (10) as essential for *A. solani* sporulation.

Our procedure allows production of abundant quantities of *A. solani* conidia under aseptic conditions during a relatively short period (72 hr). The technique also may find application for certain other fungi which normally sporulate poorly in vitro. For example, *Helminthosporium maydis* Nisikado and Miyake race T. (provided by C. A. Martinson, Iowa State University) also sporulated well when subjected to this protocol.

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