Dimorphism in Verticillium albo-atrum as Affected by Initial Spore Concentration and Antisporulant Chemicals

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ABSTRACT

Verticillium albo-atrum exhibited increasing tendency to grow as spores in shaken liquid cultures when initial spore concentrations were increased from 10^4 to 10^8 spores/ml; at initial concentrations above 10^8 spores/ml, negligible amounts of mycelium were formed. In order of increasing activity, semicarbazide, phenylhydrazine, deoxyadenosine, gossypol, and 5-fluorodeoxyuridine (FUdR) were effective as antisporulants in shaken cultures initiated with less than 10^8 spores/ml. Except for gossypol, these compounds had little or no effect on total culture dry weight, but all caused greater accumulation of mycelium than in nonsupplemented cultures. Attempts to control Verticillium wilt of cotton with soil applications of 5-FUdR or deoxyadenosine were unsuccessful. Phytopathology 61: 1266-1269.

Additional key words: disease control.

In common with other vascular wilt fungi, wild-type isolates of *Verticillium albo-atrum* Reinke & Berth. readily produce spores in shaken liquid media and in infected plants. This tendency to sporulate is probably of importance in the pathogenic colonization of xylem in the plant (4, 6, 11). It was, therefore, hypothesized that chemicals which promote mycelial growth at the expense of spore production might control Verticillium wilt of cotton, although not necessarily inhibiting total growth of the fungus. This paper reports tests of that hypothesis.

MATERIALS AND METHODS.—A severe, defoliating strain of *V. albo-atrum* (V3H), was grown in 125-ml Erlenmeyer or 50-ml DeLong flasks containing 30 or 10 ml, respectively, glucose-ammonium nitrate synthetic medium (10). This medium will hereafter be referred to as the "standard medium". In some experiments, the fungus was cultured similarly on yeast extract-peptone broth (0.3% yeast extract [Difco], 1% peptone [Difco], and 2% glucose, pH 4.5), or a modified Czapek's medium (2% sucrose; 0.2% $NaNO_3$; 0.05 M potassium phosphate, adjusted to pH 6.1; 0.05% KCl; 0.05% $MgSO_4$; 1 ppm each Fe^{++}; Zn^{++}; Mn^{++}). When supplemental chemicals were added, media were sterilized by passage through 0.22-μ membranes (Nalge Co., Rochester, N.Y.). Cultures were initiated with spores obtained by increasing conidia from single spore cultures on potato-dextrose agar slants for one generation on the standard medium. The spores were washed and diluted to desired concentrations with sterile distilled water. Spore concentration was determined turbidimetrically by absorbance measurement at 400 nm or by direct hemocytometer counts. Cultures were grown in shaken culture (110 reciprocal strokes/min) at 25 C. The cultures were harvested by passing them through nylon cloth (1,200 mesh/cm^2) and washing with water. Spores which passed through the cloth were pelleted by centrifugation. The retained mycelium and the pelleted spores were washed with water, then washed into tared weighing pans, dried for 16-24 hr at 80 C, and weighed to the nearest milligram.

Cotton plants (cultivar SJ-1) were grown in 4-inch pots of sand in a growth chamber as previously described (5) until 4 weeks old, then inoculated with *V. albo-atrum* spores by the stem puncture (5) or root-drench (12) method. Solutions of deoxyadenosine and 5-fluorodeoxyuridine (5-FUdR, Hoffman-LaRoche) were applied to the pots in 50 ml water/pot.

RESULTS.—When V3H cultures were initiated with relatively low concentrations of spores, initial growth was primarily as mycelium, but later growth was predominantly as spores with relative depletion of mycelium (Fig. 1-A, B). Longer lag phases were observed in cultures containing low initial spore concentrations (Fig. 1-A), and more mycelium accumulated. Significant mycelial development was noted when cultures were initiated with up to ca. 10^8 spores/ml, but cultures containing initial spore concentrations above 1.6×10^8 cells/ml consisted entirely of spores (Fig. 2). Generation times became progressively longer as the initial spore concentration was increased to 3.25×10^8 cells/ml (3.6 hr at initial spore concentration of 6×10^7/ml or less, 13 hr at 3.25×10^8 cells/ml). Attempts to demonstrate fungus adaption or the accumulation in cultures of stable morphogenetic factors failed. Spores from cultures containing initial spore concentrations above 1.6×10^8 cells/ml grew at the same rates as spores from less dense cultures when added to fresh culture media; similarly, spores added to supernatant fluids from log phase cultures with cell populations above 1.6×10^8 cells/ml had the same growth rate and morphogenetic form as de novo cultures.

Semicarbazide (10^{-2} M), phenylhydrazine (10^{-3} M), deoxyadenosine (10^{-3} M), gossypol (10^{-4} M), and 5-fluorodeoxyuridine (5-FUdR, Hoffman-LaRoche) (10^{-5} M) were the most effective antisporulants against *V. albo-atrum* in liquid media (Table 1, Fig. 3) when an initial spore concentration of 10^6/ml was

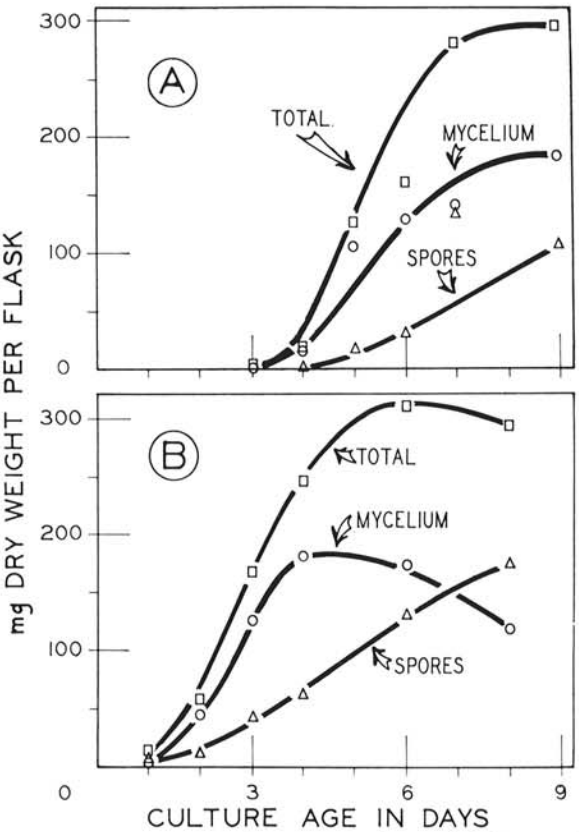

Fig. 1. Growth of the spore and mycelial forms of *Verticillium albo-atrum* in shaken cultures (30 ml) at 24 C. Cultures were grown on the standard glucose-ammonium nitrate medium. **A)** 1.2×10^2 spores/ml initial cell density; **B)** 8×10^5 spores/ml.

used. However, no condition was found that produced total conversion of the culture to mycelial growth. Zinc sulfate had a slight effect, and the use of tryptophan as a sole nitrogen source resulted in considerable mycelial development (Table 1). A number of compounds reported in the literature to have antisporulant properties against various fungi were not effective against *V. albo-atrum;* they included aminopterin (Lederle Labs) up to 10^{-4} M; 6-azauracil up to 5×10^{-3} M; deoxyribose up to 5×10^{-3} M; deoxyuridine up to 10^{-3} M; dimethylglyoxime up to 3.4×10^{-3} M; benzidine hydrochloride up to 3.8×10^{-4} M; 5-fluorouracil up to 10^{-5} M; α-ketoglutaric acid up to 10^{-3} M; hexachloroisopropanol up to 3.6×10^{-4} M; potassium cyanide up to 10^{-5} M; sodium azide up to 5×10^{-5} M; sodium bisulfite up to 5×10^{-2} M; and sodium cyanate up to 4.5×10^{-3} M. Results similar to those in Table 1 were obtained when the glucose concentration in the standard medium was reduced from 2.5 to 0.5%, or when the phosphate concentration was reduced from 0.2 to 0.05 M. Fluorodeoxyuridine also produced similar morphogenetic responses in cultures grown on Czapek's medium (27% mycelium at 10^{-6} M; 55% mycelium at 10^{-5} M). The morphogenetic effects of 5-FUdR were partially reversed by 5-bromodeoxyuridine and by high concentrations of thymidine (Table 1). Confirming Bell (1), gossypol was inhibitory to growth at the concentration that produced morphogenetic effects (ED_{50} varying between 50 and 150 μg/ml), but the other compounds with antisporulant properties were largely without effect on total dry weight accumulation (Table 1).

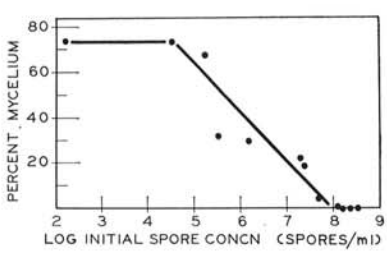

Fig. 2. Per cent mycelium after 5 days in shaken 10-ml cultures of *Verticillium albo-atrum* initiated with various spore concentrations.

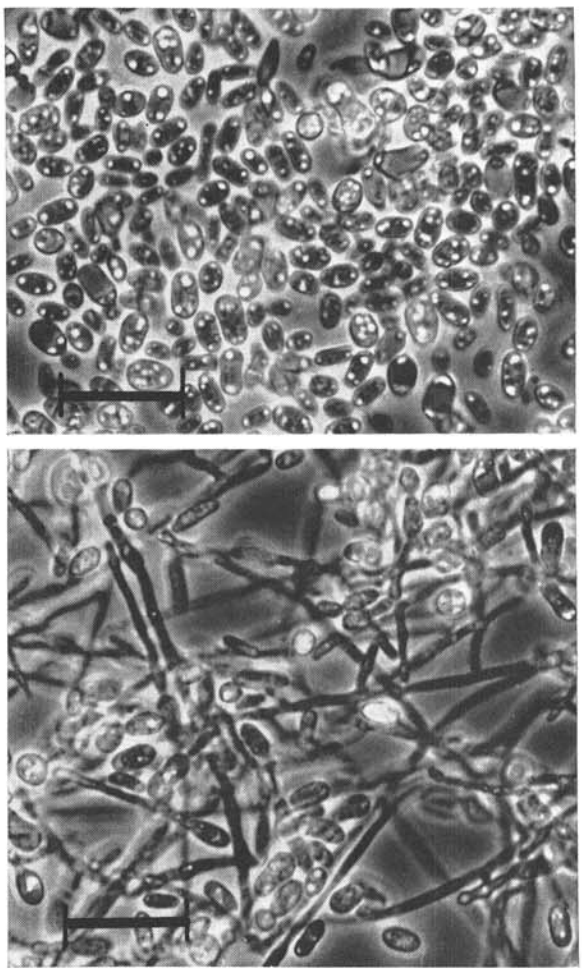

Fig. 3. Growth of *Verticillium albo-atrum* on the standard medium only (upper) and on the standard medium supplemented with 10^{-5} M 5-fluorodeoxyuridine (lower). Cultures (10-ml) were initiated with 10^7 spores/ml and grown for 3 days in shaken culture. Photographed with phase contrast optics. Lines denote 10 μ.

TABLE 1. Percentage mycelium in shaken cultures of *Verticillium albo-atrum* grown on the standard medium supplemented with various chemicals[a]

Medium supplement	% Mycelium[b]	Growth as % of control[c]
Nonsupplemented control	5	100
5-Fluorodeoxyuridine (5-FUdR) (10^{-7} M)	10	94
5-Fluorodeoxyuridine (5-FUdR) (10^{-6} M)	41	82
5-Fluorodeoxyuridine (5-FUdR) (10^{-5} M)	67	91
Thymidine (10^{-3} M)	7	97
5-FUdR (10^{-5} M) + thymidine (10^{-3} M)	18	91
5-Bromodeoxyuridine (5-BUdR) (10^{-5} M)	5	108
5-FUdR (10^{-5} M) + 5-BUdR (10^{-5} M)	48	48
Zinc sulfate (10 ppm)	10	105
Tryptophan (sole N source at 0.847 g N/liter)	29	101
Semicarbazide (10^{-2} M)	36	88
Phenylhydrazine (10^{-3} M)	65	88
Gossypol (2.6×10^{-4} M)	62	18
Deoxyadenosine (2×10^{-4} M)	28	90
Deoxyadenosine (10^{-3} M)	38	85
Deoxyadenosine (5×10^{-3} M)	41	101

[a] Cultures were initiated with ca. 10^6 spores/ml and grown for 3-4 days in shaken culture on 10 ml of medium. Data are representative of at least two experiments with each chemical.

[b] $\frac{\text{Dry wt of mycelium only}}{\text{Total dry weight of spores + mycelium}} \times 100.$

[c] Based on total dry wt of washed cultures.

In cultures containing initial spore concentrations above 1.6×10^8 spores/ml, neither gossypol nor 5-FUdR had any detectable effect on growth rate (102 and 97% of the nonsupplemented control, respectively) or morphogenetic form (1% mycelium for FUdR at 10^{-5} M; less than 1% mycelium for gossypol at 2.6×10^{-4} M; less than 1% mycelium in the control).

Applications of deoxyadenosine or 5-FUdR to the roots of cotton plants did not produce significant reductions in Verticillium wilt symptoms. Use of 5-FUdR at the highest tested level of 10 mg/pot resulted in slightly delayed (2-4 days) symptom expression in root-inoculated plants, but this dosage also produced severe leaf epinasty and stunting of both control and inoculated plants. Deoxyadenosine gave neither phytotoxic effects nor reduction in wilt symptoms when applied at rates up to 100 mg/pot.

DISCUSSION.—*Verticillium albo-atrum* tended to grow initially as mycelium in shaken cultures (Fig. 1), with later growth primarily as spores. The fungus exhibited a progressively greater tendency to grow as spores rather than mycelium when initial spore concentrations were increased, and growth was entirely as spores above initial concentrations of 1.6×10^8 spores/ml (Fig. 1, 2). The basis of this phenomenon is unknown, but does not appear to result from the production of nonvolatile, extracellular morphogenetic factors because replacement cultures grew in similar fashion to cultures on original medium.

Semicarbazide (9), phenylhydrazine (9), deoxyadenosine (8), and 5-FUdR (8) are recognized antisporulants in phytopathogenic fungi, but this property had not been reported for gossypol. Extensive work by Bell (1, 2, 3) indicates that gossypol and certain gossypol-related compounds may constitute a defense mechanism in cotton plants to *V. albo-atrum*. It has been assumed that these compounds act by direct inhibition of fungus growth. The antisporulant property of gossypol raises the possibility that any resistance conferred by such compounds could be due in part to reduced sporulation and therefore decreased colonization of the plant by the fungus. Bell (2) has proposed that decreased colonization rates by the parasite greatly increase the effectiveness of active plant defense factors.

Although the antisporulant properties of 5-FUdR and gossypol were readily observed in cultures initiated with 10^7 spores/ml or less, neither compound had any such effect on cultures initiated with 1.6×10^8 spores/ml or higher concentrations. Further, gossypol was not inhibitory to growth at these spore concentrations. The fact that mycelium was present in nonsupplemented cultures originating from low inoculum and was not present in those with high inoculum may indicate that the tested antisporulants exaggerate a pre-existing tendency to form mycelium, but do not convert totally spore-containing cultures to mycelial growth. These considerations suggest that spore-mycelium dimorphism in *V. albo-atrum* may be controlled by multiple factors.

Similar to *Ophiostoma multiannulata* (8), *V. albo-atrum* grew as mycelium in the presence of deoxyadenosine and 5-FUdR, and the effects of 5-FUdR were at least partially reversed by thymidine and 5-bromodeoxyuridine (Table 1); furthermore, neither fungus was affected by deoxyuridine and 5-fluorouracil. The effect of 5-FUdR in *V. albo-atrum* may therefore be mediated by an antagonistic effect on DNA biosynthesis as proposed for *Ophiostoma* (7, 8).

The failures of deoxyadenosine and 5-FUdR to provide significant reduction in disease symptoms may have been due to varied factors such as lack of uptake, chemical transformation in the plant, insufficient concentration in xylem vessels, or insensitivity of the fungus when in the xylem. Whatever the reason, the negative data obtained in this work offer no practical support for the rationale that antisporulants could be used to minimize fungus colonization of the plant and thereby provide control of Verticillium wilt of cotton.

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