Production, Purification, and Bioassay of Tentoxin

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ABSTRACT

The chlorosis-inducing toxin produced by *Alternaria tenuis* was isolated from mycelia and culture filtrates of an isolate of *A. tenuis* which is pathogenic on beans (*Phaseolus vulgaris*). No toxin was produced in 28 days by the fungus grown in still culture at 16 or 36 C, or in shake culture for 28 days at 24 C. Maximum toxin production occurred at 28 C in still culture.

Routine purification procedures suitable for large numbers of toxin samples involved removal of polymers by ethanol precipitation, partitioning between diethyl ether and acidic and basic water solutions, and elution from a silicic acid column with ethyl acetate:acetone:*n*-hexane (2:1:1). Toxin prepared by this method was spectrophotometrically pure and free of interfering effects, such as stunting and germination inhibition of the bioassay plant, that were noted in crude toxin preparations.

The quantitative bioassay for toxin activity involved spectrophotometric estimation of chlorophyll extracted from cotyledons and hypocotyls of cucumber seedlings (*Cucumis sativus*) with hot ethanol, and is reliable over a concentration range of 1.5-20 μg/ml of toxin.

Chlorosis was visually detectable in seedlings germinated in continuous light at toxin concentrations as low as 0.2 μg/ml, and reached a maximum at a toxin concentration of 20 μg/ml. Seedlings that germinated 48 hr in the dark prior to illumination showed a fourfold increase in chlorosis at low toxin concentrations, as compared with seedlings germinated under continuous light. Phytopathology 60: 415-418.

A chlorosis-inducing toxin produced by *Alternaria tenuis* Nees. has been characterized by Templeton and co-workers as cycloleucyl-N-methylalanyl-glycyl-N-methyldehydrophenylalanyl (tentoxin) (4, 8, 9). They purified the toxin by ether extraction of culture filtrates and mycelial mats, followed by deionization on anion and cation exchange resins, filtration through alumina, and crystallization from benzene. The toxin has an ultraviolet absorption peak at 280 mμ with a molar extinction coefficient of 13,000 (8), and is heat stable.

Activity of the toxin was noted on most dicotyledonous plants tested, with the exception of tomato and members of the Cruciferae. No activity was noted on members of the Gramineae (2, 7). Effects of the toxin were noted at concentrations as low as 0.01 mg/ml (4, 7) in a visual bioassay on cucumber seedlings, and chlorosis was increased if seedlings were germinated in the dark, rather than in continuous light. No chlorosis was noted, however, if the toxin was added to cucumber seedlings 48 hr after the initiation of germination (7). Fulton & Bollenbacher (1) and Fulton et al. (2) noted that maximum production of the toxin occurred in 4-week-old still-grown cultures, and concluded that it is a product of fungal autolysis.

We first observed the effects of the chlorosis toxin in culture filtrates of an isolate of *A. tenuis* which had been shown to cause a leaf spot on beans. The present studies were undertaken to (i) develop a procedure for more rapid purification of the toxin; (ii) develop a reliable, quantitative bioassay for the toxin; (iii) study the effects of fungal growth conditions on toxin production; and (iv) evaluate quantitatively the reported increase in toxicity obtained by germination of seeds in the dark. A preliminary report of these studies has been published (6).

MATERIALS AND METHODS.—The fungus isolate used (H_5) was obtained from Robert Goth, Beltsville, Maryland. Unless otherwise noted, it was grown for 28 days in still culture at 24 C in Richard's solution as modified by Fulton et al. (2).

Toxin was purified from mycelia by the following procedure. Mycelia were homogenized with five volumes (w/v) of water in a Sorvall Omni-Mixer, the homogenate centrifuged at 6,000 *g* for 10 min, and the pellet discarded. The supernatant was evaporated to one-tenth its original volume under reduced pressure, then combined with seven volumes of absolute ethanol. The ethanolic solution was clarified by filtration, and the ethanol removed by evaporation under reduced pressure. The resulting aqueous solution was adjusted to pH 1.0 with HCl and shaken with an equal volume of diethyl ether; the aqueous phase was discarded and the ether phase was shaken with an equal volume of 5% $NaHCO_3$. The aqueous phase was discarded, the ether solution evaporated to dryness under reduced pressure, and the residue redissolved in anhydrous diethyl ether (4 ml/liter of original homogenate). The ether solution was loaded onto the top of a 1.5 × 14-cm column of silicic acid, and the toxin eluted with ethyl acetate:acetone:*n*-hexane (2:1:1). The toxin was purified from culture filtrates by the same procedure; however, the initial homogenization and centrifugation steps were omitted. For experiments involving routine bioassays of large numbers of samples, the silicic acid chromatography was omitted, and dried residues following ether extraction were dissolved in water.

Estimation of toxin concentrations in silicic acid-purified samples was based on the assumption that the toxin has a molecular wt of 414.5 and a molar extinction coefficient of 13,000 at 280 mμ (8) in aqueous solution. Absorption spectra were measured using a Cary Model 15 spectrophotometer.

Cucumber seeds (*Cucumis sativus* L. 'SMR-18')

TABLE 1. Dry wt of material and degree of purification in active fractions at various steps in the purification of tentoxin from the mycelia and culture filtrate of *Alternaria tenuis* produced in 1 liter of medium

Step in purification	Dry wt (g)	Purification factor
Crude filtrate and homogenate	67.10	1.00
Crude, after centrifugation	61.20	1.10
After ethanol precipitation	38.40	1.75
Ether, after acid extraction	0.125	536.00
Ether, after $NaHCO_3$ extraction	0.054	1,240.00
After silicic acid chromatography	0.012	5,590.00

were germinated at 24 C for 4 days under 200 ft-c of continuous illumination from cool-white fluorescent lamps, unless otherwise noted. Nine-cm diam petri dishes containing 3 discs of Whatman No. 1 filter paper, 10 ml of toxin solution, and 15 seeds were used. These conditions, however, were not essential to the bioassay. Chlorosis of seedlings was measured by the following procedure. Cotyledons and hypocotyls were weighed and immersed in absolute ethanol and maintained at 70 C for 3 hr with occasional shaking. Absorption spectra of the ethanolic solutions were measured and chlorophyll content expressed as $OD_{663\ m\mu}/g$ (fresh wt), with values corrected for absorbance at 720 mμ.

RESULTS.—*Purification of toxin.*—The degree of purification of the toxin in a combined sample of mycelium and culture filtrate from 1 liter of original growth medium is presented in Table 1. These results are based on the assumption that 100% of the activity was retained in each of the active fractions. However, traces of activity were detected in each of the aqueous fractions following extraction with diethyl ether. It was impossible to reliably bioassay toxin activity at each step, due to interference by other fungal metabolites which either stunted seedlings or prevented seed germination. No such side effects were noted on samples following the two ether extraction steps. Spectrophotometric and visual estimates of chlorosis-inducing activity of fractions eluted from a silicic acid column are presented in Fig. 1. Each 20-ml fraction collected represents one retention volume of the column. Traces of toxin which remained on the column after elution with 20 retention volumes of ethyl acetate:acetone:*n*-hexane (2:1:1) were eluted in the first retention volume of 50% aqueous ethanol. The ultraviolet absorption spectra of purified toxin fractions are shown in Fig. 2-A. The absorption spectrum of fractions 7-9 is nearly identical to that reported by Grable (3) for crystallized toxin, whereas that of a composite of the other active fractions shows a shift of the major peak to a shorter wavelength and the appearance of a new peak at 245 mμ, indicating impurities. Purified toxin is ninhydrin-negative, but becomes ninhydrin-positive upon hydrolysis with HCl. The hydrolysate contains leucine, glycine, and two unknown amino acids, thus providing further evidence that it is identical with the toxin described by Templeton et al. (8).

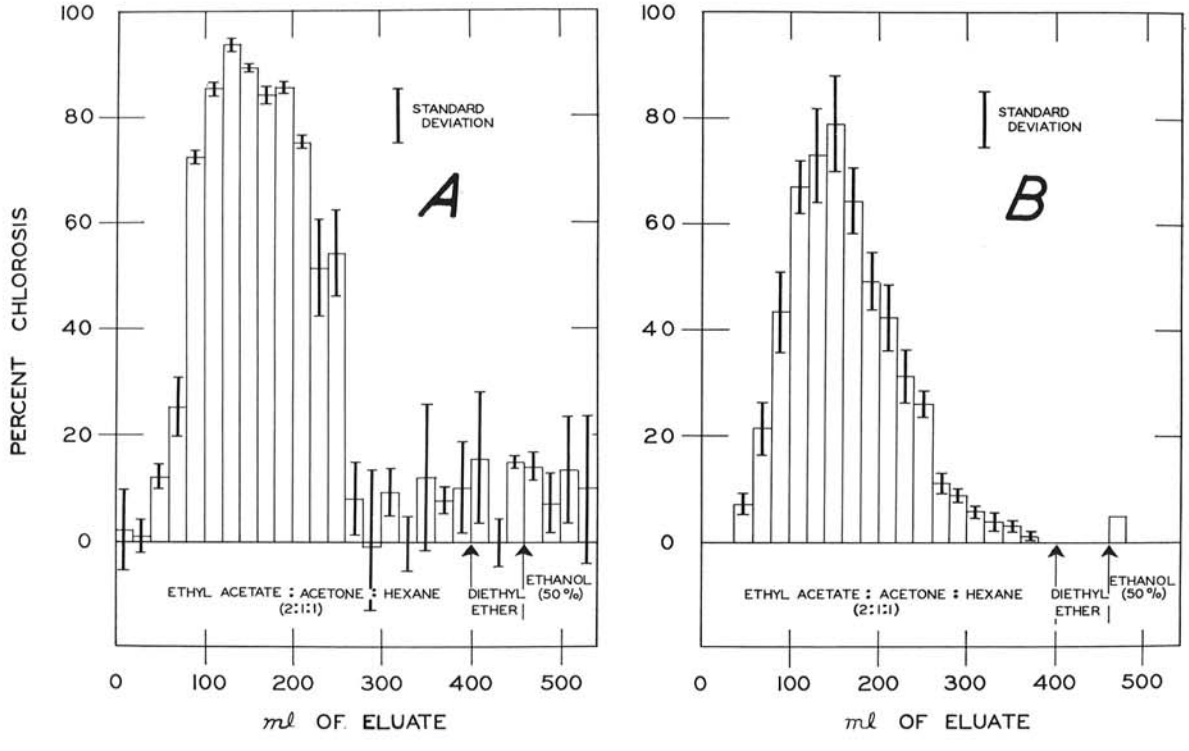

Fig. 1. A) Spectrophotometric and **B)** visual estimates of chlorosis induced in cucumber seedlings by tentoxin in various fractions eluted from a silicic acid column. Each fraction represents one retention volume of the column. Vertical lines indicate ± one standard deviation of the mean.

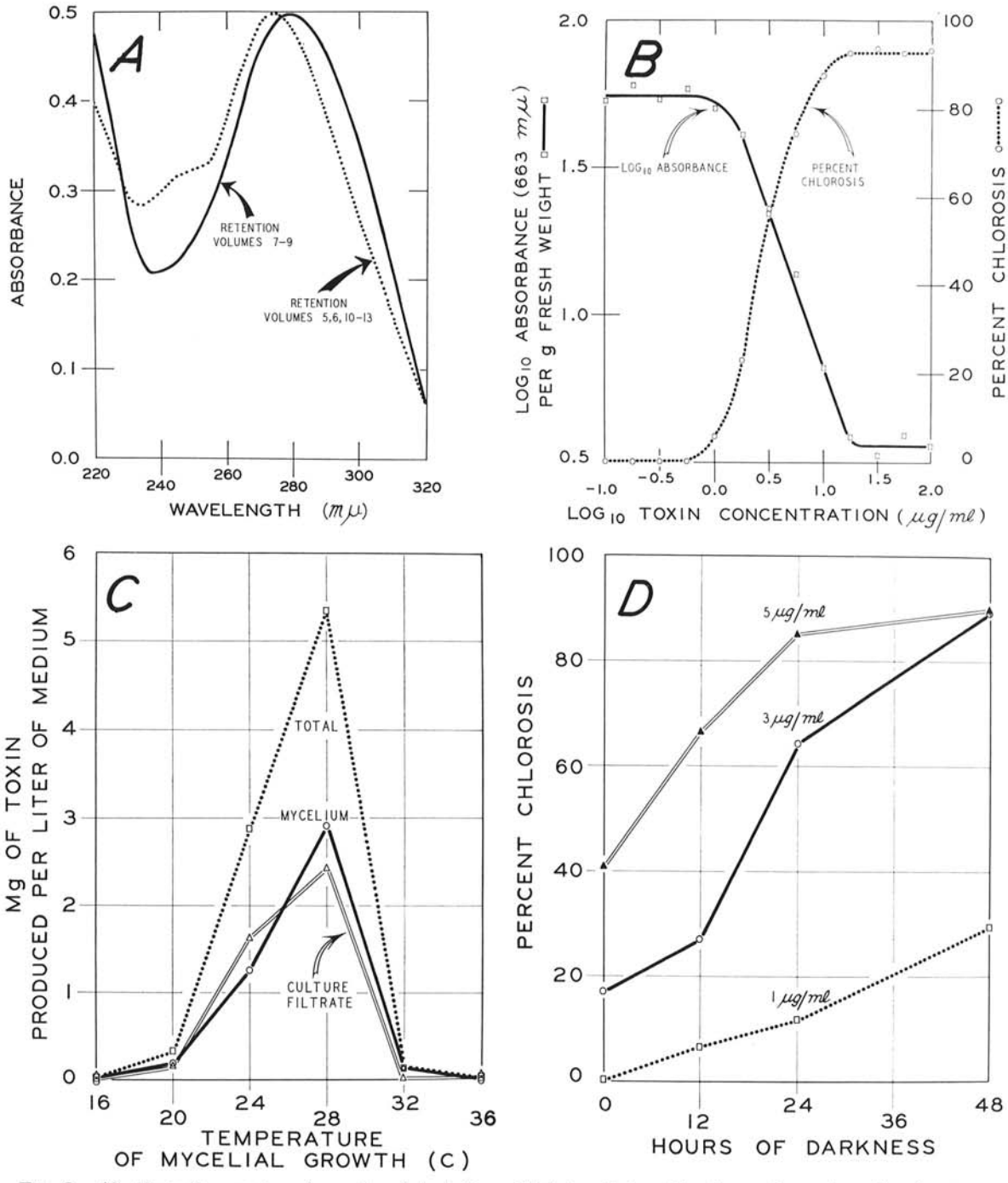

Fig. 2. **A)** Absorption spectra of samples of tentoxin purified by silicic acid column chromatography. Spectra are those of pooled samples from retention volumes 7-9 (solid line) and retention volumes 5, 6, and 10-13 (dashed line), as shown in Fig. 1. **B)** Effect of concentration of tentoxin on chlorophyll concentration and per cent chlorosis in cucumber seedlings. **C)** Effect of growth temperature on the production of tentoxin by *Alternaria tenuis*. The fungus was incubated for 28 days in still culture. **D)** Effect of various dark germination periods and toxin concentrations on chlorosis in cucumber seedlings. Chlorophyll content was determined 96 hr after initiation of the experiment.

Because of this apparent impurity in fractions 5 and 6 and 10-13, only the material from retention volumes 7-9 was used for experiments involving precise quantification of the toxin in developing the quantitative bioassay.

Bioassay.—Chlorosis was visually detectable in cucumber seedlings at toxin concentrations as low as 0.2 μg/ml, and increased to a maximum of about 90% at a concentration of 20 μg/ml (Fig. 2-B). Although individual seedlings within a treatment were completely chlorotic, groups of more than 10 seedlings seldom exceeded 90% chlorosis. No increase in chlorosis was

noted in seedlings germinated in more than 20 μg/ml of toxin. Spectrophotometric analyses of chlorosis are most reproducible at high toxin concentrations and are not useful at low concentrations (Fig. 1). Conversely, visual estimates based on the per cent of the cotyledon surface which appears to be chlorotic are most reliable at very low toxin concentrations. Spectrophotometric estimates of chlorosis were reliable only with toxin concentrations greater than 1.5 μg/ml; in all subsequent bioassays for toxin quantification, therefore, volumes of active fractions were adjusted to give between 20 and 80% chlorosis.

Effects of fungal growth conditions on toxin production.—Flasks of modified Richard's solution (2) were inoculated with *A. tenuis* and incubated for 28 days in still culture at temperatures ranging from 16 to 36 C. Mycelia and culture filtrates were collected and assayed for toxin activity as described previously. Similar amounts of toxin were isolated from both mycelia and culture filtrates, although the mycelia represented only a small portion of the total material (about 35 g/liter at 24-36 C). These results suggest that the toxin is produced within living mycelia and is released into the medium (Fig. 2-C).

An attempt was made to evaluate the effect of mycelial age on toxin production. The fungus was grown in shake culture at 24 C in modified Richard's solution. Mycelia and culture filtrates were collected at periods ranging from 2-28 days of growth and extracted as described previously. Although maximum growth of the fungus was achieved after 9 days, no toxin was detected in any of the samples, even after 28 days of growth, indicating that no toxin is produced by fungus grown in shake culture.

Effects of dark germination on expression of toxicity.—Cucumber seedlings were placed in water or in solutions containing 1, 3, or 5 μg/ml of tentoxin and kept in darkness at 24 C for periods ranging from 0-48 hr. Following the dark period, seedlings were exposed to 200 ft-c of light at 24 C until 96 hr had elapsed from the time they had been placed in the various solutions. At that time they were assayed for chlorophyll content. No differences were noted in physical appearance or in chlorophyll content of control seedlings grown under continuous light and those given initial dark periods (Fig. 2-D). Seedlings given 48 hr of darkness in the presence of 3 or 5 μg/ml of toxin were 90% chlorotic, the level normally achieved with 18-20 μg/ml of toxin under continuous illumination, and those germinated in 1 μg/ml of toxin with a 48-hr dark period showed a response which corresponds to that expected from about 4 μg/ml under continuous illumination. These results indicate that toxicity is increased about 4 times by an initial dark period of 48 hr.

DISCUSSION.—The procedure developed for purification of tentoxin provides a rapid method for the preparation of large numbers of toxin samples that are free of interfering side effects from other fungal metabolites inhibiting germination or stunt seedlings. Ultraviolet spectra indicate that pure toxin is obtained when the entire purification procedure, through silicic acid chromatography, is followed.

Bioassays involving extraction of chlorophyll from cucumber seedlings and subsequent spectrophotometric determination of per cent chlorosis should include hypocotyls as well as cotyledons, as the hypocotyl is the area most affected by low concentrations of toxin. The bioassay is not valid when applied to individual seedlings; samples of 10 or more seedlings should be used.

Toxin is produced at a maximum rate at 28 C in still culture, and production at various temperatures corresponds roughly to the growth rate of the fungus as reported by Saad (5). Possibly the toxin is produced only after the fungus attains maximum growth and, given sufficient time, equal quantities of toxin could be produced by the fungus at any temperature suitable for its growth. These results are in agreement with the proposal of Fulton & Bollenbacher (1) and Fulton et al. (2) that the toxin is a product of fungal autolysis. Seemingly in conflict with this proposal is the observation that the toxin is approximately 20 times as concentrated in the mycelium as in the culture filtrate. It is possible that the toxin is produced and retained within living cells, and is released into the medium upon autolysis. The fact that toxin is produced in still culture only after a dense mycelial mat has formed on top of the growth medium, and that no toxin is produced by fungus grown in shake culture, suggests that reduced O_2 tension may contribute to toxin production.

Application of the bioassay procedure to dark-germinated seedlings indicates that toxicity of tentoxin is increased about 4 times by maintaining seedlings in darkness through the first 48 hr of germination.

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