Evaluation of a Chemical Method for Assay of Helminthosporium maydis Race T Toxin

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Olen C. Yoder and Vernon E. Gracen, Jr.
Departments of Plant Pathology and Plant Breeding, Cornell University, Ithaca, New York 14853

ABSTRACT

A chemical test reported by Karr et al. (Plant Physiol. 55:727) to assay for host-specific toxin produced by Helminthosporium maydis race T was evaluated. Preparations from culture filtrates of both race T isolates, containing host-specific toxin, and similar preparations from race O isolates, containing no detectable host-specific toxin, gave positive reactions in the chemical assay. Also, preparations containing active or inactive toxin gave equal responses in this test. The procedure does not provide a reliable method for assaying H. maydis race T toxin.

Detection and quantification of host-specific toxins depend on the use of bioassays. Such tests accurately detect host-specific toxin activity in either crude or purified preparations since the criterion for toxin activity is observable effects on tissues, cells, or organelles of susceptible plants with similar effects on resistant plants. Chemical assays have not been reported until recently when a chemical method for quantifying Helminthosporium maydis Nisikado and Miyake race T (HMT) toxin in partially purified preparations was described (4). The test is based upon use of the Liebermann-Burchard reagent (sulfuric acid-acetic anhydride) which is known to react with steroids and other classes of compounds (2, 5). The rationale for the use of a non-specific chemical test was that after an appropriate purification procedure (acetone precipitation, butanol extraction, and diethyl ether extraction), the "host-specific toxins were the only compounds in the preparations which reacted with the reagents used in the assay. Using such a procedure, it was possible to measure total toxin concentrations without subjecting the toxin preparations to extensive purification" (4). Comparable preparations without host-specific toxin activity were not included in the study. We used the purification and chemical assay procedures as described (3, 4) to determine whether or not this non-specific chemical test could be used reliably to assay for host-specific toxin and found that the test did not distinguish between preparations containing host-specific toxin activity and those that did not.

MATERIALS AND METHODS

Bioassays. Biological activity of HMT toxin was detected by its specificity for corn (Zea mays L.) inbred W64A containing Texas male sterile (T) cytoplasm as compared with the same inbred containing nonsterile (N) cytoplasm. Preparations were tested with the seedling root growth bioassay described by Wheeler et al. (6) or with the dark CO2 fixation bioassay described by Bhuller et al. (1). Concentrations required for 50% inhibition (ED50) were estimated from dosage response curves.

Colorimetric Assay. The Liebermann-Burchard color reagent was prepared under carefully controlled conditions to reduce variability between batches. Acetic anhydride (50 ml) was cooled to OC on a cold plate. Concentrated sulfuric acid (10 ml) was added dropwise with constant stirring at a rate which did not allow the temperature of the reagent to exceed 5 C; this procedure took 30 to 35 min. The color reagent was cooled to O C (about 10 min) and added (2 ml) to each sample or lanosterol standard with constant stirring. Samples and standards were heated (56 C) in a water bath for 15 min, cooled, and A of each was measured with a Bausch and Lomb 505 or Bausch and Lomb 200 spectrophotometer. For best quantification, at least three concentrations of each sample were assayed and dosage response curves were plotted. Absorbance was converted to µg lanosterol equivalents/ml of original culture filtrate using the lanosterol included in each experiment as a standard. Reactions of color reagent with samples or with lanosterol varied from batch to batch. The color reagent was added to samples only if, in a preliminary test, it formed a product with lanosterol (200 µg/ml) which gave an A540 value of 0.5 to 0.6; additional lanosterol standards were run simultaneously with samples.

Helminthosporium maydis Isolates. Field isolates (MT [race T] and MO [race O]) were obtained from G. A. Strobel of Montana State University. Ascospore isolates were obtained by backcrossing race O to a recurrent race T ascospore-derived parent for six (cross 115) or nine (cross 118) generations to obtain isolates of race T and race O which were genetically similar but not identical. In these crosses, a single gene segregated for the race T characteristic (7), resulting in equal numbers of race T and race O isolates in each progeny. The procedures for mating and analyzing progenies have been described (7). Isolations were made from asci that contained eight ascospores and the four sets of twins in each ascus were identified using mating type, race, and/or albinism as markers. One member of each set of twins (two race T isolates and two race O isolates from each ascus) was selected and analyzed for ability to produce HMT toxin, as determined by bioassay, and to produce materials which reacted in the colorimetric assay (4).

Processing of Culture Filtrates. Each fungal isolate was grown in 300-ml Erlenmeyer flasks (50 ml medium/flask) containing modified Fries medium (7) or the defined medium (D) of Karr et al. (3). Media were seeded with pieces (3 mm x 3 mm) of potato juice agar bearing mycelium and spores, and incubated in darkness at 25 C for 14 days in the case of Fries medium or for 21 days in the case of medium D. Mycelium was removed from medium by filtration through cheesecloth and filter paper. The filtrate was adjusted to pH 4, tested for HMT toxin activity by bioassay, and processed by concentration, acetone precipitation, butanol extraction, and diethyl ether extraction following exactly the procedure described by Karr et al. (3, 4) except that all flash evaporation was done at temperatures below 40 C. After the diethyl ether extraction step in the procedure of Karr et al. (3,
4), residual ether was removed by flash evaporation and volumes of extracts were adjusted to 0.01 times the volumes of the original culture filtrates. Aliquots were removed from each preparation, dried in vacuo over P₂O₅, and dissolved in 1 ml of 2-propanol. This purification procedure resulted in loss of approximately 90% of the host-specific toxin activity in each preparation from race T, as determined by seedling root growth and dark CO₂ fixation bioassays.

Host-specific toxin activity in extracts was destroyed by adjusting to pH 10 (after diluting 1:30) and incubating at 60°C for 7 days. Samples were cooled and adjusted to pH 5, aliquots (1 ml) were dried, and residues were dissolved in 2-propanol (1 ml) for the colorimetric assay or in water (1 ml or 10 ml) for the dark CO₂ fixation bioassay. For controls, extracts either were not processed or were processed as described above except that pH adjustment and heat treatment were omitted and for the dark CO₂ fixation bioassay the dried residues were dissolved in 166 ml water.

All chemicals used were reagent grade, all organic solvents were freshly redistilled before use, and distilled H₂O was passed through resins that removed anions, cations, and organic materials before glass distillation. Lanosterol was recrystallized from 2-propanol prior to preparation of standards.

RESULTS

Fungal growth was more extensive and more uniform on Fries medium than on medium D. For example, with isolates 115-3-3 (race T), 115-3-6 (race T), 115-3-7 (race O), and 115-3-8 (race O) the corresponding mycelial dry wt (mg/mycelial pad/flask) for Fries medium were 189, 169, 186, and 186; for medium D they were 62, 118, 74, and 76. After removal of mycelium from the growth medium, each filtrate was assayed for HMT toxin activity by the seedling root growth test. Filtrates from race T isolates grown on Fries medium caused 50% inhibition of root growth of T cytoplasm corn at dilutions of 1:100 to 1:500, whereas those from medium D caused 50% inhibition at dilutions of 1:35 to 1:75; there was no effect on root growth of N cytoplasm corn except that some of the isolates caused slight inhibition at 1:10 dilution. Filtrates from race O isolates grown on either Fries medium or medium D caused no inhibition of root growth of T or N cytoplasm corn at dilutions of 1:35 or greater; some of the filtrates caused moderate inhibition of root growth of both T and N corn at a 1:10 dilution.

Filtrates from Fries medium and medium D were processed as described by Karr et al. (3, 4) and were tested by both the colorimetric assay and the dark CO₂ fixation assay. Extracts of filtrates from both media contained about equal amounts of dry matter. For example, dry wt (mg/ml) of extracts from filtrates of isolates MT and MO were 22.5 and 20.1 for Fries medium and 21.4 and 23.7 for medium D. The specific activity (based on dry wt) of HMT toxin was greater in the preparation from Fries medium (ED₅₀ = 4.5 μg dry wt/ml) than in the preparation from medium D (ED₅₀ = 306.3 μg dry wt/ml). Such differences were observed in all experiments in which the two media were compared.

Extracts from each of the race T isolates contained host-specific toxin activity, although the amount of activity varied among isolates (Table I). Some of the variation in cross 118 was attributed to segregation of a gene for albinism (a₁₂). In this cross (between race T a₁₂ and wildtype race O), all race T a₁₂ progeny produced more toxin in Fries medium than did the race T wild type progeny; isolate 118-3-6 a₁₂ is an example. We do not yet have an explanation for this correlation. None of the race O extracts contained detectable host-specific activity (Table I). Preparations from race O isolates, at a dilution of 1:10, either had no effect on T or N cytoplasm corn or caused equal inhibition (up to 20%) of both T and N cytoplasm corn. Extracts from both race T and race O isolates contained materials which reacted with color reagent (Table I). Reaction products from race T had absorption spectra similar to those from race O. Spectra of reaction products produced by isolates from cross 115 are shown in Figure 1. The A₅₀ of products of reaction between color reagent and lanosterol or extract was linear with concentration (Fig. 2). The slight differences in absorbance (Fig. 1) could not be attributed to the presence or absence of toxin because (a) in some cases extracts containing no detectable toxin caused as much or more absorbance than those that did (Table I, Fig. 2); (b) increasing the concentration of toxin-negative extract from race O caused an increase in absorbance (Fig. 2) but did not result in detectable toxin activity as determined by bioassay (Table I); and (c) in some cases, extracts from race T...
isolates grown on Fries medium contained relatively high toxin activity but low amounts of color-reactive material whereas extracts from the same isolates grown on medium D contained low toxin activity and high amounts of color-reactive material (Table I, Fig. 2).

Noninoculated culture media subjected to the extraction procedure used for culture filtrates also reacted with color reagent but water processed by the same procedure did not. Fries medium and medium D contained 18 and 5 μg lanosterol equivalents/ml of medium, respectively. The spectra of the reaction products were similar to those shown in Figure 1. All data presented here were corrected for the influence of culture medium alone.

Host-specific toxin activity was destroyed when extracts containing it were adjusted to pH 10 and held at 60°C for 7 days. Extracts were assayed by the dark CO₂ fixation and the colorimetric tests. Although treatment with high pH and heat abolished host-specific activity, it had essentially no effect on the response of extract to the colorimetric test (Table II). Results with race O extract, included as a control, were similar to those with race T extract, except that no host-specific toxin activity was detectable.

**DISCUSSION**

Since the Liebermann-Burchard reaction detects a wide array of chemical compounds, it would not be expected to detect a functional group or chemical configuration that confers host-specificity to HMT toxin. Host-specific toxicity could be lost without a concomitant change in response to the chemical test. Since HMT toxin is known to be chemically unstable, accurate tests for it cannot be based upon nonspecific chemical reactions until the chemical structure and nature of the instability are known. Nevertheless, we evaluated the Liebermann-Burchard test experimentally using preparations partially purified as described by Karr et al. (3, 4). The test was unreliable because: (a) preparations containing inactive toxin gave the same result as those containing active toxin; and (b) preparations from race T (which produces toxin) gave the same result as preparations from race O (which does not produce toxin). In the previous work (4), these comparisons were not made.

Although our biological evaluation is in variance with the conclusion of Karr et al. (4), our spectral data agree with theirs; the spectra of products from the reaction of color reagent with samples or with lanosterol are similar, and the absorbance maximum at 330 nm for lanosterol is comparable. These similarities rule out the possibility of significant differences between our procedures and theirs. We conclude from our assessment that bioassays provide, at this time, the only accurate means of detecting HMT toxin activity. Of the bioassays commonly employed for HMT toxin, we find that those based on seedling root growth, dark CO₂ fixation, mitochondrial respiration, and protoplast death are most useful to detect and/or to quantify toxin activity (8, 9).

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**LITERATURE CITED**

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