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Bioassay for Deoxynivalenol Based on the Interaction of T-2 Toxin with Trichothecene Mycotoxins

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Fungi that contaminate agricultural products worldwide often produce numerous trichothecene mycotoxins (Scott 1989). However, the interaction of trichothecenes in mixtures has not been adequately researched (Fitzpatrick 1990). Additionally, it is not obvious if the limited data available are comparable as they come from diverse systems (Bergers et al. 1985, Hoerr et al. 1981, Schiefer et al. 1986, Thompson and Wannemacher 1986). The yeast *Kluyveromyces marxianus* is very sensitive to the trichothecene T-2 toxin (Baxter et al. 1987, Sukroongreung et al. 1984) and is used in trichothecene bioassays. Using *K. marxianus* we have investigated the interaction of two binary trichothecene combinations, roridin A - T-2 toxin (Koshinsky and Khachatourians 1991) and HT-2 toxin - T-2 toxin (Koshinsky and Khachatourians 1992). A quicker understanding of the interaction of trichothecenes with T-2 toxin was arrived at using a modification of the spectrophotometric assessment of antibiotic interaction with bacteria (King and Krogstad 1983). Based on this analysis deoxynivalenol is the only trichothecene tested which interacts antagonistically with T-2 toxin. This could be the basis for a simple qualitative and quantitative deoxynivalenol bioassay.

Sensitive, analytical methods for rapid quantitative deoxynivalenol detection exist. Using thin-layer chromatography 1 μg/mL deoxynivalenol can be detected in 1 hr (Shannon et al. 1985). More involved procedures allow detection of 0.5 μg/mL (Kostiainen et al. 1991) and 0.05 μg/mL deoxynivalenol (Trucksess et al. 1986). Terhune et. al. (1984) describe a method to detect 20 ppb deoxynivalenol where purified extracted deoxynivalenol is derivatized with N-heptafluorobutyrylimidazole and quantitated by gas chromatography using an electron-capture detector. After sample cleanup high-performance liquid chromatography with on-line postcolumn photolysis and oxidative amperometric detection can detect 10 ppb deoxynivalenol (Childress et al. 1990). While there is some interference, a direct enzyme-linked immunosorbent assay can detect 10 ppb of deoxynivalenol after acetylation to deoxynivalenol triacetate (Xu et al. 1988). However, ours is the first report of a simple biological method for qualitative and quantitative deoxynivalenol detection.

MATERIALS AND METHODS

The growth medium (YPD) consisted of, on a weight per volume basis, 1% yeast extract, 1% peptone, and 2% dextrose. T-2 toxin (>99% pure) was purchased from Myco-lab, Chesterfield, MO. The other trichothecenes, diacetoxyscirpenol, deoxynivalenol, HT-2 toxin, roridin A and verrucarin A (all 97 to 99% pure) were

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purchased from Sigma Chemical Co., St. Louis, MO. All trichothecenes were dissolved in 95% ethanol, and the solutions were stored at -20°C until used.

Serial dilutions of either diacetoxyscirpenol, deoxynivalenol, HT-2 toxin, roridin A or verrucarin A were prepared in 15 mm X 100 mm test tubes containing 2.5 mL of YPD with or without 0.1 μg/mL T-2 toxin. These test tubes were inoculated with 40 μL of a standardized *K. marxianus* GK1005 culture, prepared according to Schappert et al. (1986), mixed and incubated statically at 35°C. After 20 hr incubation the optical density at 610 nm was measured. Growth of the treated cultures is expressed as % inhibition when compared to growth of the untreated control cultures. The results are the average of duplicate experiments. This test may also be done in a tissue culture multi-well plate and the absorbance measured with a Titertek® multiscan. The small volumes in the wells of these plates make any evaporation significant. The evaporation increases the trichothecene concentration resulting in more inhibition in multi-well plates than in test tubes.

The results obtained from the above experiments were analyzed to determine the type of interaction trichothecenes have with T-2 toxin. The analysis used was a modification of a method described by King and Krogstad (1983). Figure 1 is a theoretical graph that visually presents this analysis by showing the lines which define and the areas which represent each type of interaction. A dose-response curve of the effects of progressively increasing concentrations of the trichothecene in question (Fig 1, x-axis) on growth of *K. marxianus* are plotted and a line (Fig 1, thick line) fitted to the exponential portion of the curve. To this graph is added a line (Fig 1, thin solid line) which represents the effect of 0.1 μg/mL T-2 toxin on growth of *K. marxianus*. From these two lines a third line (Fig 1, dashed line) is drawn. The first point which defines this line is the maximal concentration of the trichothecene in question which causes 0% inhibition and the % inhibition caused by 0.1 μg/mL T-2 toxin. The second point is the concentration and % inhibition where the dose-response curve of the trichothecene in question begins to plateau (at or earlier than 100% inhibition). This third line (Fig 1, dashed line) defines the effects predicted to occur if T-2 toxin and the trichothecene in question are combined and do not interact (zero interaction). On this graph the effects of a mixture of progressively increasing concentrations of the trichothecene in question with 0.1 μg/mL T-2 toxin on growth of *K. marxianus* are plotted. These effects are compared to the effects predicted to occur if T-2 toxin and the trichothecene in question are mixed and do not interact. If the effects observed when the two trichothecenes are combined are on or near the dashed line (Fig 1, white area) then they have zero interaction (observed effect equals predicted effect). If the effects observed when the two trichothecenes are combined are on the upper left of the dashed line (Fig 1, dark area) then they have a synergistic interaction (observed effect greater than predicted effect). If the effects observed when the two trichothecenes are combined are on the right or lower left of the dashed line (Fig 1, dotted area) then they have an antagonistic interaction (observed effect less than predicted effect). Figure 1 can be adapted to represent a mixture of any two toxins.

RESULTS AND DISCUSSION

Growth of *K. marxianus* is inhibited 19.5% ± 4% by 0.1 μg/mL T-2 toxin. This agrees with previous results which showed that 0.05 μg/mL T-2 toxin inhibited *K. marxianus* by 20% (Schappert et al. 1986). Deoxynivalenol - T-2 toxin is the only combination tested which has an antagonistic interaction (Fig 2). This type of antagonistic interaction is called autonomous, meaning that the effects of the

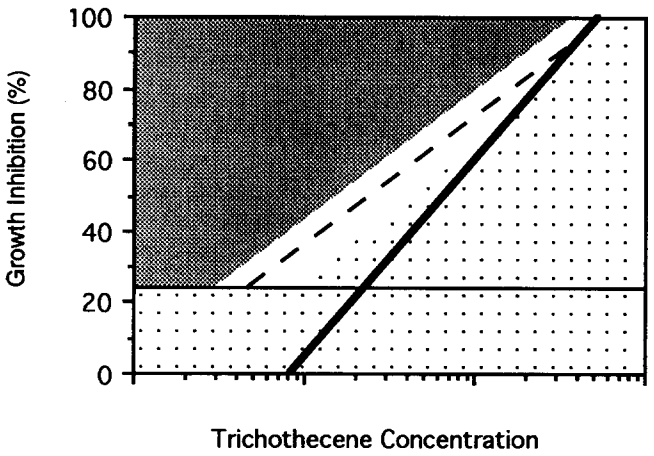

Figure 1. Domains of a theoretical graph showing the interaction that a mixture of two trichothecenes can have. Refer to the materials and methods for an explanation of the three lines and three areas.

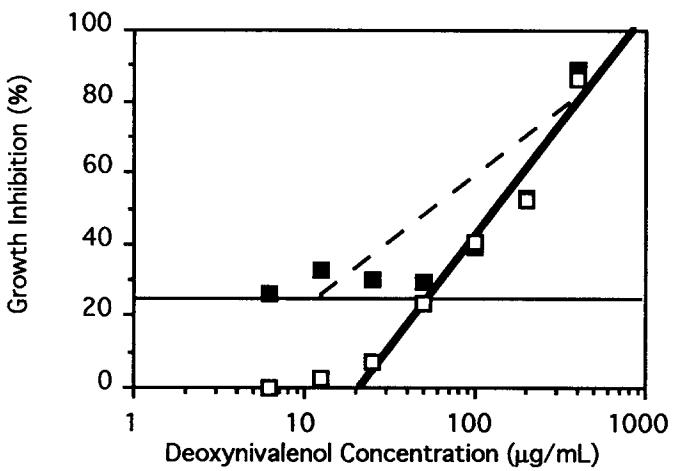

Figure 2. Growth inhibition of *K. marxianus* caused by 0.1 µg/mL T-2 toxin (thin solid line) or deoxynivalenol (□, thick line), and their combination (■). The dashed line indicates the effects predicted if a combination of T-2 toxin - deoxynivalenol have zero interaction.

combination are the same as the effects of the most active trichothecene alone. Diacetoxyscirpenol - T-2 toxin interact synergistically (Fig 3A). When with T-2 toxin either HT-2 toxin or roridin A or verrucarin A have zero interaction (Fig 3B-D). Similar patterns of interaction occur if the T-2 toxin concentration is 0.2 or 0.3 µg/mL which alone cause 30.6 or 36.6% inhibition of growth of *K. marxianus*, respectively (data not shown). All five cases tend to agree with the report that trichothecenes interact synergistically at a high % inhibition (Koshinsky and Khachatourians 1991). The method described here could be used to consolidate and expand the knowledge of how trichothecenes interact.

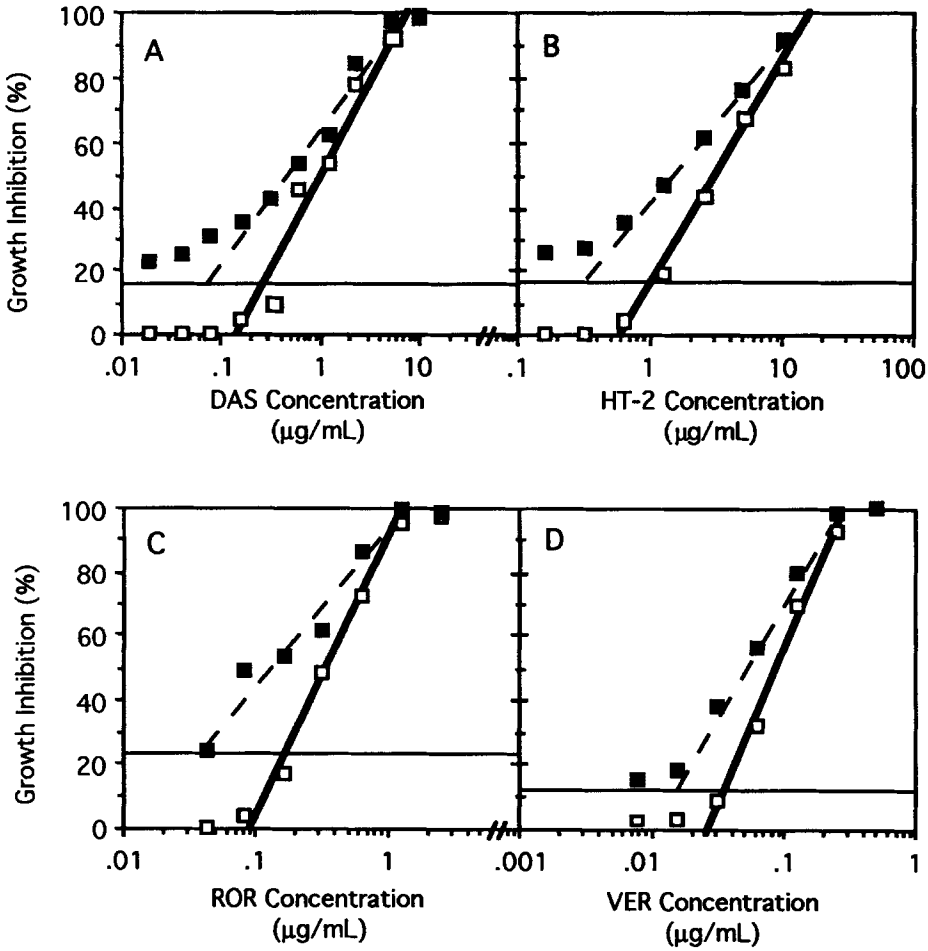

Figure 3. Growth inhibition of *K. marxianus* caused by 0.1 µg/mL T-2 toxin (thin solid line) or (A) diacetoxyscirpenol or (B) HT-2 toxin or (C) roridin A or (D) verrucarin A alone (□, thick line) and in combination with 0.1 µg/mL T-2 toxin (■). The dashed line indicates the effects predicted if the combination has zero interaction. Abbreviations are DAS, diacetoxyscirpenol; HT-2, HT-2 toxin; ROR, roridin A; VER, verrucarin A.

Based on the above data and arguments a bioassay for deoxynivalenol is conceivable. Hypothetically the bioassay would involve four test tubes containing YPD and: a) nothing; b) 0.1 µg/mL T-2 toxin; c) unknown trichothecene; and d) 0.1 µg/mL T-2 toxin + unknown trichothecene (same concentration as in tube c). All tubes would be inoculated with standardized *K. marxianus*, statically incubated for 20 hr at 35°C, and the optical density at 610 nm measured. The % growth inhibition in tubes b, c, and d as compared to tube a would be determined and the results interpreted from Table 1. When the unknown trichothecene is deoxynivalenol the % inhibition in tube c is logarithmically proportional to the deoxynivalenol concentration from 50 to 400 µg/mL, and defined by y = -82.606 +

62.253x; r = 0.967. In repeated experiments the standard error of the % inhibition in Figure 2 never exceeded 8% of the mean.

Table 1. Interpretation of results from the hypothetical deoxynivalenol bioassay.

Growth inhibition (%)		
Tube c	Tube d	Interpretation; next step
< tube b	≤ tube b	Concentration of unknown is below detection limit; concentrate unknown and retest
100	100	Concentration of unknown is above detection limit; dilute unknown and retest
25 - 100[a]	25 - 100[a]	Unknown is deoxynivalenol; determine concentration
Y[b]	>Y[b]	Unknown is not deoxynivalenol; use another method to identify unknown
Z[c]	<Z[c]	Unknown is not deoxynivalenol; use another method to identify unknown

[a] inhibition is between 25 and 100% and the same in both tubes.

[b] inhibition is between 25 and 100% and larger in tube d.

[c] inhibition is between 25 and 100% and smaller in tube d.

Of the naturally occurring trichothecenes, deoxynivalenol is the most common. Although deoxynivalenol is not as toxic as other trichothecenes, safety concerns arise because of its frequent occurrence (Scott 1989). Bioassays can be used as inexpensive, technically easy methods to screen for compounds. Currently there is no bioassay for the detection of deoxynivalenol (Khachatourians et al. 1989). A method for routine screening of commodities intended for consumption by animals or humans and for diagnosis of suspected deoxynivalenol toxicosis is needed (Casale et al. 1988). A survey of the natural occurrence of deoxynivalenol in U. S. and Canadian grains, feeds, and grain foods showed that in 78% of the contaminations deoxynivalenol occurred alone (Scott 1989). As there are usually no other trichothecenes present, there could be no interference with detection of the unique interaction of deoxynivalenol - T-2 toxin. It follows that the interaction of deoxynivalenol - T-2 toxin could be exploited to develop a rapid and simple bioassay for determining if an unknown trichothecene is deoxynivalenol.

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