Inhibition of Trichothecene Biosynthesis in *Fusarium tricinctum* by Sodium Bicarbonate†

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The effect of 0.11 M sodium bicarbonate on trichothecene mycotoxin formation was determined by analyzing cultures of *Fusarium tricinctum* NRRL 13426 incubated in peptone- and saccharose-supplemented Czapek–Dox broth in the presence and absence of the base. Sodium bicarbonate-treated cultures exhibited a 7–51-fold decrease in the production of diacetoxyscirpenol (DAS) and T-2 toxin. The production of 15-acetoxyscirpenol, acetyl T-2 toxin, and neosolaniol was completely inhibited by the sodium bicarbonate. The analysis method (which could also detect 3-acetyl-DAS, deoxynivalenol, HT-2 toxin, scirpentriol, T-2 tetrol, T-2 tetraacetate, and T-2 triol) employed cold on-column capillary gas chromatography/isobutane chemical ionization ion trap mass spectrometry of the underivatized mycotoxins.

INTRODUCTION

Trichothecenes are fungal secondary metabolites biosynthesized predominantly by the genus *Fusarium*. These mycotoxins are isolated primarily in grain and produce toxicoses when contaminated grain is ingested by animals or humans. Trichothecenes have been implicated in the human toxicity described as alimentary toxic aleukia (Joffe, 1971) and akakabi sickness (Ueno, 1977). Poisoning of livestock is an ongoing problem with financially significant losses (Ueno, 1983; Scott, 1990). It has been determined that sodium bicarbonate inhibits the formation of aflatoxins by *Aspergillus parasiticus* (El-Nabarawy *et al.*, 1989). The biosynthesis of ochratoxin A has also been shown to be inhibited by sodium bicarbonate and potassium bicarbonate in corn (Montville and Shih, 1991). A subsequent investigation demonstrated that dilute solutions of sodium bicarbonate potentiate color and morphology changes in plate cultures of *Fusarium tricinctum* NRRL 13426 (DePasquale *et al.*, 1990). This study was initiated to determine if these changes also resulted in the impairment of trichothecene biosynthesis.

EXPERIMENTAL PROCEDURES

Media. Medium A (Greenhalgh *et al.*, 1988) consisted of 40 g of sucrose and 10 g of glycerol in 1 L of distilled water supplemented with 1 g of ammonium phosphate, 3 g of potassium phosphate, 0.2 g of magnesium sulfate, and 5 g of sodium chloride. Medium B consisted of a peptone (1%) and saccharose (10%) supplemented Czapek–Dox broth (Cullen *et al.*, 1982). Sodium bicarbonate-treated media were produced by aseptically adding 0.92 g of sodium bicarbonate to 100 mL of sterile media to obtain a 0.11 M concentration.

Inoculation and Incubation. Spore suspensions of *F. tricinctum* NRRL 13426 were used to inoculate all broth treatments in this study. Spore suspensions were prepared by inoculating 100 mL of sterilized potato dextrose agar (PDA) with *F. tricinctum* 13426 using a sterile loop. The PDA culture was incubated at 35 °C for 10 days. Twenty milliliters of sterile Tween 80 (0.5% solution) was then aseptically added to the fungal culture. One milliliter of the resulting spore suspension was removed from the PDA flask with a 5-mL serological pipet and released into 100 mL of sterile fungal broth. Broth treatments were incubated as shake or stationary cultures at times and temperatures specified under Results and Discussion.

Toxin Extraction. To extract trichothecenes from the fungal mass, 100 mL of HPLC grade acetone was added to the culture broth and the mixture was vigorously shaken and allowed to sit for 12 h before filtration. The filtrate was concentrated on a rotary evaporator to remove the acetone. The remaining aqueous portion was transferred to a 500-mL separatory funnel and partitioned with 3 × 50 mL of ethyl acetate. The ethyl acetate fractions were combined and dried through a glass funnel containing 50 g of sodium sulfate. The extract was concentrated on a rotary evaporator to approximately 5 mL and then evaporated to dryness under a gentle stream of nitrogen. The internal standard, fluorene (diphenylmethane), was added, and the extract was taken up to 1 mL with ethyl acetate. The extract was stored in a 4-mL sample vial with a Teflon-lined cap at −15 °C until analysis.

Instrumental Conditions. A Varian Model 3400 gas chromatograph (Varian Associates, Walnut Creek, CA) interfaced to a Finnigan 800 ion trap detector (Finnigan MAT, San Jose, CA) and controlled by an IBM PC/AT was used to analyze trichothecenes. The analyses and quantifications were performed with Finnigan ion trap software (revision 3.15). A splitless on-column injector held at 50 °C was used, and a 2 m × 0.53 mm (i.d.) deactivated fused silica precolumn was fitted between the injector and capillary column. A 15 m × 0.25 mm (i.d.) J&W (Rancho Cordova, CA) DB-1 fused silica capillary column (1-μm film thickness) was held at 50 °C before being temperature-programmed from 50 to 280 °C at 20 °C/min and then held for 10 min. Carrier gas (He) velocity was 15 cm/s; injection volume was 1 μL. A 5-μL Hamilton syringe equipped with a 15-cm needle was used for the on-column injections. The mass spectrometer was operated in the chemical ionization mode using isobutane reagent gas at a source pressure that gave a 2:1 ratio for m/z 43 to m/z 57. The filament voltage and current were 70 eV and 80 μA, respectively. Electron multiplier gain was 10^5. Scan range was from 100 to 470 amu at 1 s/scan. Transfer line and manifold temperatures were 250 and 220 °C, respectively.

Calibration Curves. The initial stock solution of trichothecenes was made by adding T-2 toxin and DAS to methylene chloride at a concentration of 100 ng/μL for each toxin. From the stock solution, standards were prepared at levels of 50, 25, 10, 5, 2, and 1 ng/μL of standard solution. Fluorene was chosen as the internal standard because of its stability and was added to each standard at a concentration of 28 ng/μL. Each level of trichothecene standard was analyzed in triplicate, and calibration curves were constructed employing Finnigan ion trap software (revision 3.15). In addition, 40 ng/μL standards of 15-acetoxyscirpenol, 3-acetyl-DAS, acetyl T-2, deoxynivalenol, HT-2 toxin, neosolaniol, scirpentriol, T-2 tetrol, T-2 triol, and T-2 tetraacetate

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were prepared in ethyl acetate and analyzed using capillary gas chromatography/isobutane chemical ionization mass spectrometry.

RESULTS AND DISCUSSION

This investigation was initiated to determine if sodium bicarbonate could inhibit the formation of trichotecenes in a culture of *F. tricinctum* NRRL 13426. To help accomplish this work, a method for the analysis of trichothecenes that was suitable for our needs and appropriate for our available instrumentation was developed. Previous analytical methods for the quantitation of trichothecenes such as packed-column GC/MS (selected ion monitoring mode) of trimethylsilyl ethers (Rosen and Rosen, 1984) have been improved upon by separation of underivatized trichothecenes on capillary columns and detection by oxygen negative ion mass spectrometry (Miles and Gurprasad, 1985) or ammonia chemical ionization mass spectrometry (D'Agastino *et al.*, 1986). The first method requires an expensive mass spectrometer, while the second method relies on the carbon 13 isotope of the $M + NH_4^+$ ion for confirmation. Our method also does not require chemical derivitization and can be accomplished using a relatively inexpensive mass spectrometer, the Finnigan ITD 800 ion trap detector. Ion traps, because of their design, provide very sensitive full-scan mass spectra. This allows the analyst to quantitate by mass chromatography instead of selected ion monitoring, eliminating the need for difficult-to-obtain deuterated trichothecene internal standards. The thermal lability of the trichothecenes warranted that they be analyzed in a manner that minimized thermal exposure during injection and is the reason why cold on-column injection was employed. The trichothecenes were analyzed in the isobutane chemical ionization mode to maximize the quantitation ion masses as well as their intensities and to minimize chemical noise from the matrix (Mattern *et al.*, 1990). The ions obtained by this process, together with their relative intensities in parentheses, were 15-acetoxyscirpenol 307 (100), 229 (49), 247 (48), 265 (34), 201 (20); 3-acetyl-DAS 349 (100), 289 (25), 391 (14), 229 (12), 307 (11); acetyl T-2 toxin, 287 (100), 407 (31), 227, (15), 347 (12), 449 (11); deoxynivalenol 249 (100), 297 (60), 231 (52), 219 (35), 279 (20); DAS 247 (100), 307 (82), 349 (65), 229 (54), 289 (44); HT-2 toxin 263 (100), 215 (65), 245 (63), 305 (33), 407 (18); neosolaniol 305 (100), 215 (72), 245 (70), 365 (25), 257 (23); scirpentriol 217 (100), 247 (75), 265 (55), 229 (37), 235 (33); T-2 tetrol 215 (100), 281 (45), 263 (40), 251 (30), 253 (25); T-2 tetraacetate 287 (100), 407 (51), 347 (12), 227 (11), 257 (10); T-2 toxin 215 (100), 245 (95), 305 (93), 263 (52), 227 (45); T-2 triol 215 (100), 281 (34), 263 (32), 223 (21), 233 (12). The quantitation and confirmation ions that we used together with retention times and limits of detection in PSC broth are summarized in Table I.

Sensitivity was influenced by the polarity of the toxin. Trichothecenes with more than two hydroxyl groups (scirpentriol, T-2 triol, and T-2 tetrol) were the most insensitive, with limits of detection of 0.5 ppm. Toxins containing several acetyl groups (acetyl T-2, T-2 tetraacetate, and 3-acetyl-DAS) were the most sensitively detected trichothecenes, suggesting that the analytical method presented here is suitable for all but the most polar (and, fortunately, least toxic) trichothecenes.

Several extraction schemes were tested to determine optimal conditions for the recovery of trichothecenes from fungal broth. Acetone extraction followed by ethyl acetate partitioning was determined to optimally extract trichothecenes and resulted in average recoveries ($n = 3$) of 99.0 ± 11.4% and 107.1 ± 6.5% for DAS and T-2 toxin, respectively, from fungal media.

Table I. Retention Times, Limits of Detection, and Quantitation and Confirmation Ions Used for Analyses of Trichothecenes

trichothecene	retention time (min)	LOD[a] (ppb)	Q(C)[b] ions (*m/z*)
15-acetoxyscirpenol	14.2	20	307 (229)
3-acetyl-DAS	14.3	0.1	349 (289)
acetyl T-2 toxin	18.4	0.5	287 (407)
deoxynivalenol	13.8	100	249 (297)
DAS	14.3	15	307 (247)
HT-2 toxin	19.5	20	263 (215)
neosolaniol	15.8	25	305 (245)
scirpentriol	14.0	500	217 (247)
T-2 tetrol	15.8	500	281 (263)
T-2 tetraacetate	15.9	5	287 (407)
T-2 toxin	19.5	20	305 (245)
T-2 triol	18.9	500	281 (215)
fluorene[c]	9.2		167

[a] LOD, limit of detection in PSC broth (ng of toxin/mL of broth). [b] Q, quantitation ion; C, confirmation ion. [c] Internal standard.

Two media cited in the literature as promoting trichothecene production were compared. Medium A produced 10 ppm of T-2 toxin when inoculated with *F. tricinctum* NRRL 13426 and incubated as a stationary culture for 14 days. DAS was not detected. No T-2 toxin was detected in the 0.11 M $NaHCO_3$ treated fungal broth.

Incubation of a stationary fungal culture medium B at 19 °C for 14 days resulted in extensive production of mycotoxins. T-2 toxin, DAS, neosolaniol, 15-acetoxyscirpenol, and acetyl T-2 toxin were measured at 913 (average of 1210 and 616), 36 (average of 39 and 32), 19 (average of 25 and 13), 11 (average of 16 and 5), and 10 (average of 10 and 9) μg/g cell dry weight, respectively. Another culture, containing 0.11 M sodium bicarbonate [previously shown to cause color and morphology changes in *F. tricinctum* NRRL 13426 without reduction in fungal mass (DePasquale *et al.*, 1990)], contained 18 (average of 24 and 13) μg/g T-2 toxin (a 51-fold reduction) and 3 (average of 5 and 1) μg/g DAS (a 12-fold reduction). The other three mycotoxins could not be detected after incubation in the presence of bicarbonate.

Incubation in medium B (shake culture) at 28 °C for 15 days resulted in the formation of 103 (average of 107 and 99) μg/g T-2 toxin and 2 (average of 2 and 2) μg/g DAS. None of the other mycotoxins in Table I could be detected. The 15-day shake culture that was incubated in the presence of 0.11 M sodium bicarbonate contained T-2 toxin at a level of 15 (average of 18 and 12) μg/g (7-fold reduction) and DAS at a level below the detection limit of 0.15 μg/g.

The final average pH values of stationary and shake control PSC fungal cultures were 6.9, while the final pH values for sodium bicarbonate-treated stationary and shake PSC cultures averaged 8.7. Addition of sodium bicarbonate to trichothecene-spiked PSC broth did not reduce trichothecene concentrations, ruling out the remote possibility that the trichothecenes were hydrolyzed during incubation.

In conclusion, we have shown that sodium bicarbonate is an effective inhibitor of trichothecene biosynthesis in cultures of *F. tricinctum* NRRL 13426. Determination of inhibition in various crops is a logical extension of this investigation that should be undertaken.

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