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Accumulation of type A trichothecenes in maize, wheat and rice by *Fusarium sporotrichioides* isolates under diverse culture conditions

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Abstract

Toxigenic isolates of *Fusarium sporotrichioides* were tested for the production of type A trichothecenes (T-2 toxin, HT-2 toxin, diacetoxyscirpenol and neosolaniol) when grown on three substrates (maize, rice and wheat) under various conditions of temperature and water activity in the laboratory for 3 weeks. Trichothecenes were determined by high-performance liquid chromatography with fluorescence detection, after derivatisation with coumarin-3-carbonyl chloride. This is the first time this analytical method has been applied to an extensive study of trichothecene accumulation. With minor exceptions, greater trichothecene production occurred when samples were incubated at 20 °C and moistened with 35% water (water activity 0.990) although incubation conditions affected the substrates studied in different ways. No correlation between the different pairs of trichothecenes was found except for neosolaniol and diacetoxyscirpenol ($r = 0.56$). Principal component analysis results show that the data points can be grouped in three rough clusters related to cereal type, which points out that the composition of these cereals can influence the production of type A trichothecenes. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Mycotoxins; Trichothecenes; *Fusarium sporotrichioides*; Cereal grains

1. Introduction

Fusarium species occur widely in nature as saprophytes and plant parasites; they are found in a great variety of plants and agricultural products (Bamburg and Strong, 1971; Anonymous, 1983). In addition to the losses caused by infection of plants by *Fusarium* before or during harvest, some species are capable of producing mycotoxins in affected products. The wide range and frequent presence of *Fusarium* toxins found naturally occurring on cereals reveal an increasing need for research on the toxigenic potential of *Fusarium* species (Bamburg and Strong, 1971; Anonymous, 1983; Beasley, 1989; Chu, 1991; Visconti et al., 1992).

Fusarium species reported as major producers of type A trichothecenes (e.g. T-2 toxin, HT-2 toxin,

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neosolaniol and diacetoxyscirpenol) are *Fusarium sporotrichioides*, *F. sambucinum*, *F. venenatum*, *F. acuminatum* and *F. compactum* (Chelkowski, 1989; Chu, 1991; Bosch and Mirocha, 1992; Miller and Trenholm, 1994). *F. sporotrichioides* is widespread on plants and in soil throughout the cold and cool temperate regions of the world (Visconti et al., 1985). At least two serious cases of mycotoxicoses have been associated with the ingestion of food and feed infected with this fungus. The first is Alimentary Toxic Aleukia which occurred in Russia during World War II (Yagen and Joffe, 1976) and the other is the bean-hull poisoning of horses in Japan (Ueno et al., 1972). In both cases, isolates of *F. sporotrichioides* isolated from grains and beans were found to produce T-2 toxin and its derivatives.

Most analytical methods for type A trichothecenes are based on ELISA (Park and Chu, 1993), gas chromatography (Lauren and Agnew, 1991; Bosch et al., 1992; Croteau et al., 1994; Furlong and Valente-Soares, 1995), and HPLC (Cohen and Boutin-Muma, 1992). This last method is complicated by the absence of a chromophore in the molecules. Coumarin-3-carboxylic acid chloride was shown to produce fluorescent derivatives with T-2 toxin and its hydroxyl derivatives and has been proposed as a derivatising agent for T-2 toxin (Karlsson et al., 1985; Cohen and Boutin-Muma, 1992). Recently, we have studied and optimised a method for determination of type A trichothecenes in fungal cultures of cereal grains that use coumarin-3-carbonyl chloride derivatisation and HPLC/fluorescence detection (Jiménez et al., 2000)

Mycotoxin production is influenced by external factors including water activity, extent of infection, temperature, substrate, microbial interactions and fungal isolates (Moss, 1984; Chelkowski, 1989; Mills, 1989). Nevertheless, little attention has been given to the range of environmental factors implicated in growth and accumulation of type A trichothecenes in grain substrates.

We have studied the influence of water activity, incubation temperature and cereal type (substrate) on the accumulation of type A trichothecenes by five isolates of *F. sporotrichioides*. Our aim was to know better the storage conditions that favour the production of these metabolites by the species in order to prevent their accumulation in maize, rice and wheat. This is the first time HPLC with fluorescence detection for type A trichothecenes has been applied to the study of cereal cultures of *Fusarium* spp.

2. Materials and methods

2.1. Chemicals

Organic solvents were of HPLC grade from Merck (Darmstadt, Germany). HPLC quality water was prepared with a Waters Milli-Q system (Waters Associated, Milford, MA, USA).

4-Dimethylaminopyridine (DMAP) and standards of T-2 toxin, HT-2 toxin, neosolaniol (NEO) and diacetoxyscirpenol (DAS) were purchased from Sigma (St. Louis, MO, USA) and coumarin-3-carboxylic acid and thionyl chloride from Aldrich Chemical (Milwaukee, USA).

2.2. Fungi

F. sporotrichioides isolates with references FSp9, FSp11, FSp12, FSp13 and FSp14 were maintained in Potato Dextrose Agar (PDA). These cultures are held at the Department of Microbiology, University of Valencia (Spain). *F. sporotrichioides* FSp9 and *F. sporotrichioides* FSp11 were isolated from *Zea mays*. *F. sporotrichioides* FSp12 and FSp13 were isolated from *Triticum aestivum* and *F. sporotrichioides* FSp14 was isolated from *Panicum miliaceum*. All these isolates have been shown to produce type A trichothecenes (unpublished data).

2.3. Culture preparation

Cereal samples were purchased commercially. Only those where the mycotoxins studied were below their limits of detection were used for the experiments. Two hundred grams of sample (maize, rice or wheat) was placed in 500-ml Erlenmeyer flasks with 70, 90 or 110 ml of deionized water to reach water activities (a_w) of 0.990, 0.995 and 0.999, respectively. Flasks were plugged with cotton, covered with aluminium foil and autoclaved for 20 min at 120 °C. The sterilised substrate was inoculated

with pieces of PDA single-spore cultures of the five *F. sporotrichioides* isolates, and maintained at 20, 26 or 33 °C. During the first 3 days the culture flasks were shaken periodically to disperse inoculum uniformly. After 3 weeks, the cultures were dried at 45 °C for 48 h and then, finally, ground to powder and analysed for type A trichothecene contents. All possible combinations of cereal, water activity, incubation temperature and *F. sporotrichioides* isolate were assayed.

2.4. Extraction

Ground samples were thoroughly mixed. Ten grams of the samples were blended with 50 ml of acetonitrile/water (84:16) using a high-speed blender (Ultraturrax T25, Ika, Staufen, Germany) for 5 min and filtered through Whatman no. 4 paper. The filtrate was defatted with hexane and then concentrated to dryness under vacuum at 40 °C. The residue was redissolved in 3.0 ml of chloroform/methanol (90:10) prior to cleanup procedure. All the experiences were performed in duplicate.

2.5. Cleanup

A 3-ml capacity Florisil Sep-Pak column (Waters Chromatography Division, Millipore, Milford, USA) was preconditioned with 5 ml of methanol followed by 5 ml of chloroform/methanol (90:10). Sample extract (1.5 ml) was passed through the column. The trichothecenes were eluted from the column with 20 ml of chloroform/methanol (90:10) and then concentrated to dryness under vacuum at 40 °C. The residue was transferred to a screw cap amber vial (2 ml) with dichloromethane (2×0.5 ml), and then the sample was evaporated to dryness under a slow stream of N_2.

2.6. Derivatisation conditions

DMAP solution (10 μl) in toluene (2 mg ml^{-1}) was added to screw cap amber vials (2 ml) containing evaporated samples, followed by addition of coumarin reagent (10 μl). The vial was closed and the mixture heated at 80 °C for 20 min. The mixture was cooled in ice water and redissolved in toluene (0.4 ml); 0.05 M dihydrogen phosphate buffer (0.4 ml; pH 5.5) was added. After vigorous mixing and phase separation, an aliquot (300 μl) of the upper organic phase was transferred to another vial and evaporated to dryness under a slow stream of N_2. HPLC mobile phase (75 μl) was added to the residue, the solution was filtered through 0.20-μm filter and 20 μl was injected into the HPLC system. The final concentration was adjusted by appropriate dilution when necessary in order to maintain mycotoxin levels within the linear range of calibration.

2.7. HPLC analysis

Trichothecene derivatives were analysed using a reversed phase HPLC with fluorescence detector system. The HPLC system consisted of a Waters 600 pump connected to a Waters 474 Scanning Fluorescence detector and a Digital Celebris 590 PC (Millenium Software was used). Chromatographic separations were performed on a stainless steel LiChrospher 100, C-18 reversed-phase column (250×4 mm, 5-μm particle size) provided with a guard column (4×4 mm, 5 μm particle size) filled with the same phase. The mobile phase was acetonitrile: water containing 0.75% acetic acid (65:35) at 1.0 ml min^{-1} and was degassed by passing through an on line vacuum degassing device (Waters). The column was kept at room temperature. For fluorescence detection, the excitation and emission wavelengths were set at 292 and 425 nm, respectively, with a slit width of 18 nm.

2.8. Statistical analysis

The concentration levels found in samples were statistically treated with the SPSS for Microsoft Windows (release 6.1) package or the Statgraphics Plus for Windows 2.1 programme. The existence of significant differences was inferred by analysis of variance (ANOVA). In addition, multivariate analysis was used to perform principal component analysis (PCA).

The experiment was planned as a factorial design. The first factor was the substrate where the fungi were grown (maize, rice and wheat grains). The second factor was the water activity level (0.990, 0.995 and

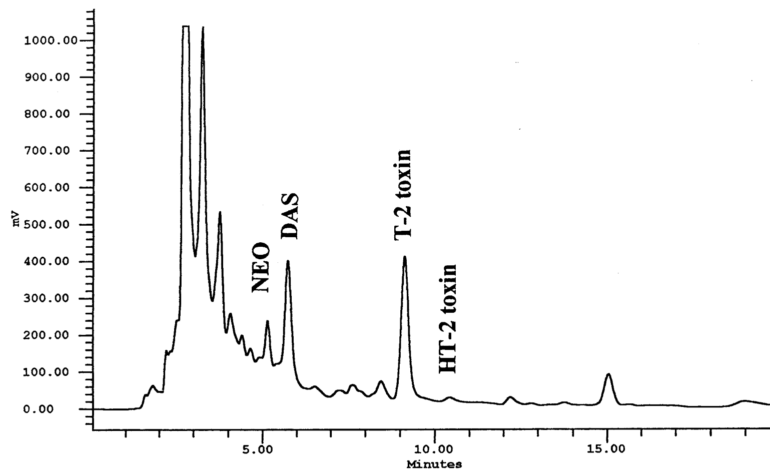

Fig. 1. Liquid chromatogram showing the type A trichothecenes produced in a rice sample culture inoculated with *F. sporotrichioides* isolate FSp 9. Chromatographic conditions: see text.

0.999) and the third one was the incubation temperature (20, 26 and 33 °C).

3. Results

Under the analysis conditions used, the four mycotoxins (T-2 toxin, HT-2 toxin, DAS, NEO) give rise to four well resolved peaks (Fig. 1) and there were no interfering peaks from samples. Good sensitivity and linearity, and low detection limits were reported earlier (Jiménez et al., 2000).

Table 1 lists the mean levels of the four type A trichothecenes found in the cultures (expressed as milligrams of toxin per kilogram of cereal grain) under the different incubation conditions. Each

Table 1
Mean levels of type A trichothecenes (mg/Kg^{-1}) in cereal cultures of *F. sporotrichioides* under different incubation conditions

Cereal	Mycotoxin	Water activity								
		0.990			0.995			0.999		
		Temperature (°C)			Temperature (°C)			Temperature (°C)		
		20	26	33	20	26	33	20	26	33
Maize	T-2 toxin	0.221	0.167	0.043	0.355	0.203	0.048	0.454	0.300	0.051
	HT-2 toxin	0.177	0.268	0.261	0.057	0.076	0.094	0.128	0.146	0.205
	DAS	0.047	0.133	0.040	0.181	0.057	0.025	0.226	0.119	0.062
	NEO	0.043	0.036	0.029	0.036	0.037	0.035	0.040	0.030	0.035
Wheat	T-2 toxin	0.941	0.243	0.125	0.836	0.228	0.181	0.258	0.047	0.042
	HT-2 toxin	0.076	0.037	0.035	0.049	0.047	0.042	0.039	0.034	0.033
	DAS	0.042	0.056	0.031	0.048	0.070	0.063	0.086	0.163	0.113
	NEO	0.037	0.064	0.023	0.076	0.057	0.051	0.067	0.042	0.046
Rice	T-2 toxin	0.788	1.019	1.083	0.315	0.652	0.161	0.273	0.454	0.092
	HT-2 toxin	0.062	0.044	0.044	0.100	0.077	0.082	0.061	0.045	0.025
	DAS	0.068	0.054	0.084	0.174	0.192	0.112	0.134	0.075	0.066
	NEO	0.024	0.028	0.028	0.114	0.073	0.048	0.029	0.024	0.028

value accounts for the arithmetic average of the five isolates assayed under the same conditions. Accuracy of values near the limits of detection of the analytical method (0.015–0.045 mg kg^{-1}) is low.

The data (T-2 toxin, HT-2 toxin, DAS and NEO levels) were statistically treated by ANOVA in order to infer the influence of the different factors and their interactions on the accumulation of the four mycotoxins.

3.1. T-2 toxin

Considered to be the most toxic of the studied mycotoxins on the basis of its LD_{50} in rats (Cole and Cox, 1981), T-2 toxin concentrations were in the range not detected to 3.31 mg kg^{-1}. Incubation conditions affected in a different way the levels of T-2 toxin depending on the substrate studied. Therefore, in maize, T-2 toxin accumulation increased when a_w level increased and incubation temperature decreased. The same effect, regarding temperature, was observed in wheat but mycotoxin production diminished at higher a_w levels. In rice, biosynthesis of T-2 toxin was favoured at 26 °C and low a_w values.

3.2. HT-2 toxin

HT-2 toxin levels were low (not detected to 0.45 mg kg^{-1}). Two significant first order interactions were seen, between cereal type and moisture grade, and between cereal type and temperature. The biosynthesis of this toxin was relatively common in maize (15 results of greater than 0.2 mg kg^{-1}) and lower, but quite similar, in both rice and wheat. Accumulation of HT-2 toxin increased in maize at higher incubation temperatures and lower a_w values. In

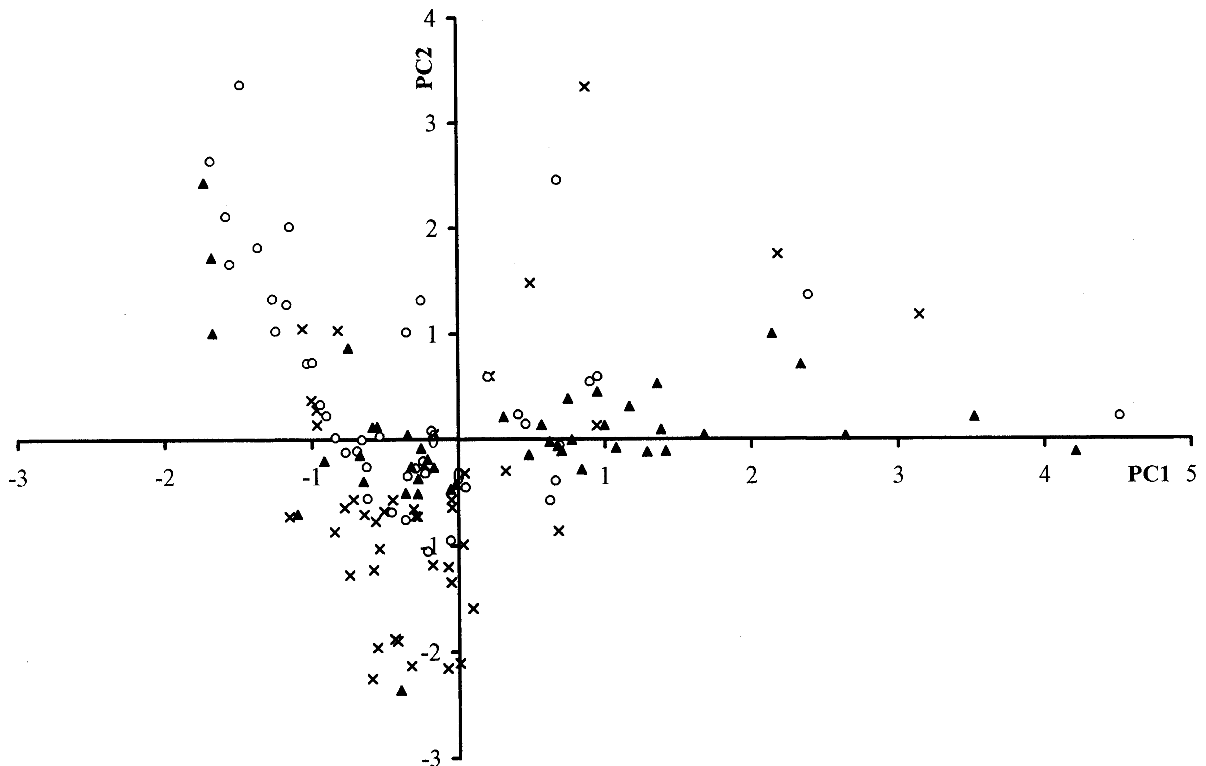

Fig. 2. Scatter diagram of the experimental data points obtained by principal component analysis: (×) maize; (○) rice; (▲) wheat.

wheat and rice, HT-2 toxin production increased as the incubation temperature decreased. In wheat, HT-2 toxin levels increased at lower a_w values whereas in rice the production was highest at a_w 0.995.

3.3. Diacetoxyscirpenol

Levels of DAS ranged between not detected and nearly 0.9 mg kg^{-1}. However, only three of 135 values were higher than 0.5 mg kg^{-1}. The results for wheat showed the lowest values (always less than 0.3 mg kg^{-1}). In both maize and rice, the biosynthesis of DAS increased as incubation temperature decreased, whereas in wheat, the temperature for maximum accumulation was 26 °C. Regarding a_w, the production of this toxin was higher in both maize and wheat when a_w value increased, but in rice the optimum a_w value was 0.995.

3.4. Neosolaniol

Overall, the accumulation of NEO was quite low (not detected to 0.365 mg kg^{-1}). It was relatively high in wheat (although the two highest values were found in rice) and low in maize, but significant differences were not apparent. In all three substrates, the biosynthesis of NEO decreased as incubation temperature increased. Water activity did not affect NEO accumulation in both maize and wheat but in rice the optimum a_w value for production was 0.995.

The production of the four mycotoxins was not correlated ($r < 0.2$), except between DAS and NEO where some correlation is apparent ($r = 0.56$).

The overall level of type A trichothecenes was highest in rice and lowest in maize. Principal components analysis (PCA) of the data provides only three rough groupings as a function of the cereal substrate used (Fig. 2). Most data from maize show negative values for both principal components PC1 and PC2. Data from rice are grouped mainly in a cluster with PC1 < 0 and PC2 > 0, whereas those from wheat spread along PC1. When PCA is applied to search for grouping the data by the four trichothecenes studied, only DAS and NEO are relatively close, especially by influence of PC1, which agrees with the highest correlation coefficient indicated above (data not shown). This grouping may be due to a common parental metabolite in the biosynthesis pathway of both toxins.

4. Discussion

Isolates were identified as *F. sporotrichioides* according to the taxonomy of Booth (1971, 1984), accepted by Nelson et al. (1981, 1983). Isolates producing T-2 toxin and its metabolites have sometimes been named as *F. tricinctum* (sensu Snyder and Hansen, 1945), which includes the species *F. tricinctum*, *F. poae* and *F. sporotrichioides* in the system of Booth (1971). A few cases of *Fusarium* isolates able to produce more than three trichothecenes related to T-2 toxin have been reported (Kotsonis et al., 1975; Swanson, 1980; Cole et al., 1981; Ishii and Ueno, 1981; Schmidt et al., 1982). *F. solani* M-11 (Ishii and Ueno, 1981), *F. poae* NRRL 3287 (Kotsonis et al., 1975) and *F. tricinctum* NRRL 3299 (Thrane, 1991) have been classified as *F. sporotrichioides* (sensu Booth) in a taxonomical revision (Ichinoe and Kurata, 1983). Similar problems have been found in other *Fusarium* species (Leslie, 1996).

Analytical data for total type A trichothecenes in our samples were consistent with those from earlier studies in which *F. sporotrichioides* was more commonly found in wheat, barley and maize (Marasas et al., 1986; Miller and Trenholm, 1994). Among several samples specifically analysed for T-2 toxin (Park, 1995), authors found that this toxin accounted on average for about 30%, but ranged from 9.5% to 76.7%, of the total type A trichothecenes; similar data were found in our study. The data shown by different authors for these mycotoxins are quite scattered. Some of them found levels similar to those found by us (Jelinek et al., 1988; Gleissenthal et al., 1989; Stratton et al., 1993; Park et al., 1996) whereas others found higher levels (Visconti et al., 1985; Bosch and Mirocha, 1992; El-Maghraby, 1996).

The experiment did not permit a clear deduction of the influence of the tested factors on the production of these mycotoxins in maize, wheat and rice. However, the influence of temperature appears to be important for the accumulation of T-2 toxin: 20 °C favoured accumulation. The water activity of the

grain seems to have also some influence on the production of T-2 toxin. It has been reported that *F. sporotrichioides* has a low optimal temperature for T-2 toxin production (Cullen et al., 1982; Ueno, 1983; Mirocha, 1984; Park and Chu, 1993) and produces mycotoxins during overwintering under snow cover in the field and/or during storage (Joffe, 1986; Marasas et al., 1986; Miller and Trenholm, 1994). Poor storage of grains contaminated with this mould at mild ambient temperatures (around 20 °C) usually during summer and the beginning of autumn in many cereal-producing areas might cause an increase in the T-2 toxin content. The problem is not as important in countries with hot summers (average temperature around or higher than 30 °C). Low moisture levels are associated with storage of grains in these countries, which decreases the possibility of T-2 toxin accumulation.

Taking this fact in consideration together the results from PCA, the composition of the substrate may play a role in the biosynthesis of the studied type A trichothecenes. As far as we know, this is the first time that results support the great influence of the cereal substrate on the production of these mycotoxins.

The biosynthesis (and accumulation) of the each of the four mycotoxins by *F. sporotrichioides* in cereals is complicated to be foreseen as it is very dependent on the storage conditions, most precisely on the particular combination of cereal, and water activity or incubation temperature, because of the strong interaction between the factors. Thus, it is difficult to design a storage programme for *F. sporotrichioides*-contaminated maize, wheat or rice grains in the laboratory so that initial levels of all four type A trichothecenes will not increase. Therefore, the conditions that hinder bioproduction of T-2 toxin (high temperature/water activity) should be followed owing to its greater presence in the three substrates and its high toxicity. However, these conditions may cause spoilage by another fungal species.

In the present work, we have shown the first data on the influence of temperature, water activity and type of cereal substrate on the production of four type A trichothecenes by *F. sporotrichioides*. The incubation conditions clearly influence the accumulation of each mycotoxin. There was not a set of factors affecting in the same way the production of different toxins. The nature of each factor's influence could not be deduced due to the complicated interactions among all implicated factors. Although not shown, some degree of intraspecific variability concerning the production of each of these toxins was found. Variation in the accumulation of a particular trichothecene may be favoured by culturing at low (or high) temperatures but changes in water activity can reverse the expected results.

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