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Influence of water activity, temperature and incubation time on the simultaneous production of deoxynivalenol and zearalenone in corn (*Zea mays*) by *Fusarium graminearum*

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Abstract

The production of deoxynivalenol and zearalenone by *Fusarium graminearum* was studied under different culture conditions (water activity, temperature and incubation time). The maximum levels of both toxins were obtained at the 35th day of incubation, the zearalenone level being much higher than the deoxynivalenol. The culture conditions that gave higher yields of deoxynivalenol were at 22 and at 28 °C (6.0 and 5.5 mg/kg), after 35 days. At an incubation temperature of 28 °C 16 days, followed by 12 °C, for the same time, the production was low (1.1 mg/kg). The highest level of zearalenone was obtained at 28 °C for 16 days, followed by incubation at 12 °C (36.7mg/kg) at the 35th day. When the temperature was constant at 28 °C, the zearalenone production was lower (3.0 mg/kg) than when incubated at 22 °C (12.3 mg/kg), at the 35th day. *Fusarium graminearum* did not produce deoxynivalenol and zearalenone at 37 °C.

Keywords: Deoxynivalenol; Zearalenone; *Fusarium*; Temperature; Incubation time

1. Introduction

Fusarium species are widespread in nature and commonly contaminate many cereals grains and other plant tissues in the field. They have become of increasing notoriety for producing mycotoxins in foodstuffs. The most frequently isolated *Fusarium* species in temperate climates have been *F. graminearum* and *F. moniliforme,* followed by *F. culmorum, F. proliferatum* and *F. equiseti* (Sohn, Seo, & Lee, 1999). *F. graminearum* Schwabe are together with *F. culmorum,* the most important producers of the phytotoxic nonmacrocyclic trichothecenes, deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON) (Marasas, Nelson, & Toussoun, 1984) and zearalenone (ZEN) (Mirocha & Christensen, 1974).

Deoxynivalenol (DON, vomitoxin) is one of the toxic 12,13-epoxytrichothecenes, considered the most potent inhibitors of protein synthesis in eucaryotic cells. The epoxy group of trichothecenes can also react with sulfhydryl groups on enzymes, causing numerous biological disturbances. Swine are especially sensitive to these toxic effects. Rejection of contaminated feed (feed refusal), decreased weight gain and, if enough is consumed, vomiting will result. DON may also cause gastrointestinal disorders and haematological changes in most of the animal species (Ueno, 1987). Studies in chickens have shown a very slight effect on egg quality (Kubena & Harvey, 1988). DON has also been implicated in two outbreaks of gastrointestinal illness in humans in India and China (Truckess, Ready, Pender, Ligmond, Wood, & Page, 1996).

Zearalenone (ZEN), also known as F-2 toxin, 6-(10-hydroxy-6-oxo-trans-1-undecenyl)- β-resorcyclic acid lactone, is a very heat-stable compound, despite its large lactone ring (Ryu, Hanna, & Bullerman, 1999). ZEN is estrogenic and the action on the hypothalamus and pituitary glands appears to be the same as estrogen (Cheeke & Shull, 1985). This mycotoxin causes hyperestrogenism, especially in swine (Mirocha & Christensen, 1974). In young male swine, ZEN can cause feminization which includes testicular atrophy. Swine appear to be the most sensitive of the domestic animals.

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Enlargement, or swelling and reddening of the vulva in gilts and sows (vulvovaginitis), vaginal and rectal prolapses may also occur (Sydenham, Thiel, & Marasas, 1988)

Fungal growth and mycotoxin production depend on the complex interaction of several factors. Water activity, temperature, oxygen and substrate are primary environmental factors that influence mycotoxin production (Charmley, Rosenber, & Trenholom, 1994).

F. graminearum is most frequently isolated from maize in temperate climates and the zearalenone production often occurs during cold weather storage of high-moisture feeds carrying the mould (Hollinger & Ekperigin, 1999). This occurs frequently in some areas of Portugal; therefore we had good reason for carrying out the present study (Martins, 1994; Martins & Martins, 1995; Martins, Martins, & Gimeno, 1996).

The aim of this work was to evaluate, under laboratory conditions, the effect of a_w, temperature and incubation time on the DON and ZEN production in corn (*Zea mays)* inoculated with *F. graminearum,* isolated from corn.

2. Material and methods

2.1. Strain

F. graminearum obtained from corn was used for DON and ZEN production. Colonies of *F. graminearum* (wild) were applied to Czapek agar (Oxoid—CM 97, Portugal) plates, and incubated at 25 °C for 5 days. These isolates were identified, considering macroscopic and microscopic morphological aspects, compared to descriptions given by Gerlach and Nirenberg (1992).

F. graminearum have previously been shown to be DON and ZEN producers in corn.

2.2. "In vitro" DON and ZEN production

The culture conditions used in this experiment reflect earlier work reported by Martins, (1994); Martins and Martins, (1995) and Martins et al. (1996). The studies of mycotoxin production by *F. graminearum*, were carried out in duplicate on Erlenmeyer flasks containing 50 g of sterilised cracked corn, adding 20 ml of distilled water and adjusting a_w to 0.97. The corn contained no fungal infection, DON and or ZEN contamination.

Autoclaved substrate was inoculated with 2 ml of the spore suspension, according the following procedure: 5 ml of sterile distilled water were added to each slant of 5-day-old culture, gently scraping the agar surface to give a turbid suspension, corresponding to 1×10^8 spores/ml. Two millilitres of this suspension were added to the cracked corn. Inoculated flasks were shaken daily for the first 3 days. The incubation temperatures were 22, 28 and 28 °C for 14 days followed by incubation at 12 and 37 °C for 8 weeks (56 days). Cultures were examined for DON and ZEN at selected incubation times (14, 21, 28, 35, 42, 49 and 56 days). When the incubation temperature was 28 °C, followed by incubation at 12 °C, the cultures were examined at 21 days.

2.3. Mycotoxin determinations

All reagents and chemicals were purchased from Merck Portugal. Standards of DON (code: D-0156) and ZEN (code: Z-2125) were purchased from Sigma.

2.4. Deoxynivalenol determination

DON analysis was carried out following the method described by Cahill, Kruger, Brian, Ramsey, Prioli, and Ramsey (1999). A sample of 25 g was extracted in distilled water by blending for 3 min at high speed, filtered through both a fluted and glass microfibre filter paper and applied to an immunoaffinity column, (DON test HPLC—VICAM, Watertown, MA, USA). Subsequently, the column was washed with distilled water and the toxin was eluted from the column with methanol, evaporated to dryness in a rotary evaporator (Heidolph 4000) and re-dissolved in 300 μl of acetonitrile–water. Determination of DON was carried out by isocratic reverse-phase liquid chromatography (HPLC) using a LiChrospher 100 RP-18, 5 μm column, 25×4.6 mm EcoPack (Merck, Portugal). The mobile phase was acetonitrile–water, filtered through a 0.22 μm filter membrane, degassed and used at a flow rate of 0.6 ml/min. DON was detected using a Merck–Hitachi, L7420 UV detector set to 218 nm. Data were analyzed with a computing integrator Merck Hitachi (Compaq Deskpro).

Working standard solutions, limit of detection and percentage recovery were determined according to the method previously mentioned. The limit of detection was 0.100 mg/kg. Recovery was determined by spiking DON standards, at levels of 100, 250 and 500 μg/kg, to corn samples. Recovery averages were 98.0, 85.0 and 88.0%, respectively.

2.5. Zearalenone determination

The analysis of ZEN was carried out according to the procedure of Kruger, Kohn, Ramsey, and Prioli (1999). Corn samples (20 g) were extracted in acetonitrile–water by blending for 3 min at high speed, filtered through a fluted paper, applied to an immunoaffinity column (ZearalaTest HPLC—VICAM, Watertown, MA, USA) eluted with methanol and water and injected into a liquid chromatographic (HPLC) system with a fluorescence detection set at 274 nm excitation and 440

Table 1
Production of deoxynivalenol and zearalenone by *Fusarium graminearum* on cracked-corn

Incubation time (days)	Incubation temperature (°C) and levels (mg/kg) of DON[a] and ZEN[a]							
	22		28		28 + 12		37	
	DON	ZEN	DON	ZEN	DON	ZEN	DON	ZEN
14	0	2.3	0	1.2	ND[b]	–[c]	ND	ND
21	2.6	4.5	2.2	2.8	0.6	13.1	ND	ND
28	0.7	12.0	2.3	2.8	0.9	35.7	ND	ND
35	6.0	12.3	5.0	3.0	1.1	36.7	ND	ND
42	4.5	11.8	4.4	0.7	0.9	30.3	ND	ND
49	4.0	6.8	4.0	0.5	0.5	28.3	ND	ND
56	3.7	4.2	3.8	0.2	0.3	27.8	ND	ND

[a] Average of two determinations.
[b] ND, not detected.
[c] – Not analysed.

emission using a LiChrospher 100 RP-18, 5 μm column 25×4.6 mm, EcoPack (Merck, Portugal). The mobile phase was acetonitrile–water–methanol, filtered through a 0.22 μm filter membrane, degassed and used at a flow rate of 1.0 ml/min. Data were analyzed with a computing integrator, Merck Hitachi (Compaq Deskpro).

Working standard solutions, limit of detection and percentage recovery were determined according to the method previously mentioned. The limit of detection was 0.005 mg/kg. Recovery was determined by spiking ZEN standards, at levels of 0.005, 0.010 and 0.10 μg/kg in corn samples. Recovery averages were 93.0, 95.0 and 96.0%, respectively.

3. Results and discussion

The effects of incubation temperatures, 22 and 28 °C, during 15 days, followed by 12 and 37 °C, over 56 days, on the production of DON and ZEN by *F. graminearum,* were compared. At 37 °C these mycotoxins were not detectable.

DON production for the other tested temperatures (22, 28 and 28 °C followed by 12 °C) was detected at the 21st day: 2.6, 2.2 and 0.6 mg/kg, respectively, increasing until the 35th day (6.0, 5.5 and 1.1 mg/kg), followed by a slight decrease (Table 1). Maximum production of DON by *F. graminearum* occurred at 22 °C and 35 days of incubation (6.0 mg/kg). Only small amounts of DON were produced when incubated at 28 °C during 15 days, followed by 12 °C.

ZEN production was detected earlier than DON, at the 14th day at 22 °C and at 28 °C (2.3 and 1.2 mg/kg), and at the 21st day (when incubated at 13.1 mg/kg). The maximum yield of ZEN was always observed when incubated at the temperature combination reaching a peak at the 35th day of incubation (36.7 mg/kg), and showed a slight decrease until the 56th day of incubation (27.8 mg/kg; Table 1).

Greenhalgh, Neish, and Miller (1983), studied the ability of three isolates of *F. graminearum* to produce DON and ZEN on rice and corn. On corn they all produced DON and ZEN and, on rice, only two strains produced these mycotoxins. They also studied the effects of the incubation temperature and time on production of DON and ZEN by *F. graminearum,* and verified that higher temperatures (28 °C) favoured DON formation after 24 days. The maximum level of ZEN was also produced at the same temperature. The DON results agree with ours. For ZEN, we obtained better results at 28 °C during 15 days, followed by 12 °C. An other study, carried out by Jiménez, Máñez, and Hernández (1996), verified that two isolates of *F. graminearum,* ZEN producers, produced higher amounts (5150 and 7100 mg/kg) when they were incubated at 28 °C for 2 weeks, followed by incubation at 12 °C. Our data show that the production of DON and ZEN are strongly dependent on the incubation temperature and time, which is in agreement with the results reported by Jiménez et al. (1996) and Di Menna, Lauren, and Smith (1991). According to our results we can suggest that the optimum temperature incubation for DON production is 22–28 °C and, for ZEN, 28 °C for 15 days, followed by 12 °C, reaching the peak at the 35th day of incubation.

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