Acta Agraria Kaposváriensis (2006) Vol 10 No 2, 277-284
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Novel methods of Fusarium toxins' production for toxicological experiments

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ABSTRACT

Studies were performed to develop a novel, efficient and cost-effective method for fumonisin and T-2 toxin production, respectively, in sufficient quantities for animal toxicological experiments. On the basis of three earlier published fumonisin toxin production methods a novel method was developed. Three, absolutely necessary factors were taken into account and tested in a serial experiment. The Fusarium verticillioides strain MRC 826 was directly inoculated onto soaked, autoclaved, whole maize kernels (50 g/1.7 l jar). The inoculation was performed by standard spore suspension ($1 \cdot 10^6$ /ml), a 5/2 surface/volume culture was prepared and incubated at 25 °C for 5 weeks. To maintain the optimal a_w of approximately 1.00, the evaporated water was re-filled weekly. A final concentration of 4454±1060.9 mg kg^{-1} fumonisin B_1 was reached. T-2 toxin was produced experimentally on ground maize by Fusarium sporotrichioides strain NRRL 3299. With the method in this study similarly high T-2 toxin concentration can be reached as in case of fumonisin production. In the laboratory practice, applying the above settings, a high toxin production can be obtained.
(Keywords: fumonisin B_1, T-2 toxin, production methods)

INTRODUCTION

Fumonisins, a family of food-borne carcinogenic mycotoxins, were first isolated in 1988 (*Gelderblom et al*., 1988) from cultures of *Fusarium verticillioides* (Sacc.) Nirenberg (previously known as *Fusarium moniliforme* Sheldon). Fumonisin B_1 (FB_1) typically accounts for 70 to 80% of the total fumonisins produced, fumonisin B_2 usually makes up 15 to 25% and fumonisin B_3 usually from 3 to 8%, when cultured on corn or rice. (*Marín et al.,* 1999). FB_1 is responsible for several toxicological actions in animals. These include the neurotoxic syndrome, leukoencephalomalacia in horses, pulmonary edema in pigs, and hepatocarcinogenic and hepatotoxic effects in rats (cit. *Marasas et al*., 1993). FB_1 is carcinogenic and has been implicated in the pathogenesis of oesophageal cancer in humans (*Marasas et al*., 1993).

Although cereals are important substrates, moisture level and temperature are the critical abiotic factors regulating the growth of *F. verticillioides* and the production of fumonisins (*Cahagnier et al.,* 1995). The best temperature range is 20–28 °C for fumonisin production (*Alberts et al.,* 1990) but low kernel moisture content (less than 22%) should reduce or prevent toxin production during storage (*Le Bars et al*., 1994). *Marín et al.* (1995) surveyed the effects of different temperature and water activity (a_w; the water available for fungal growth) values on fungal growth and fumonisin production by *F. verticillioides* and *F. proliferatum* strains, observing that both increased when the

moisture and temperature increased. Since fumonisins have been known only for a short time, adequate information concerning their toxicology is lacking.

In the method of *Fazekas* (1998), the toxin was produced by *F. verticillioides* strain 14/A as inoculum, grown on maize. Since then the production ability of this strain was first decreased, and later discontinued, probably due to the successive inoculations. (*Fazekas*, 2004). Other methods (*Vismer et al*., 2004; *Alberts et al*., 1990) were previously tested in our laboratory, but little quantities of FB_1 were reached (*Fodor*, 2005). In this respect, the need to develop efficient methods for the production of fumonisin toxins in sufficient quantities is essential.

Table 1

Comparison of the reference data

Concerning reference		Methods		
		Fazekas, 1998	***Alberts et al.,1990***	***Vismer et al., 2004***
Strain		14/A	MRC 826	MRC 826
Growing the strain on any agar		On Czapek agar for a week and on new Czapek agar slants once again for the same duration	-	-
Inoculum		Unknown spore number	Unknown spore number	Standardized spore suspension ($1 \cdot 10^6$ spores per ml)
Medium		100 g of whole yellow corn kernels and 70 ml of water	400 g of whole yellow corn kernels and 400 ml of water	30 g ground yellow maize kernels and 30 ml water
Preparation of medium	Soaking	overnight	1 h	1 h
	Autoclaving	for 1 h at 121 °C and 120 kPa on each of two consecutive days	for 1 h at 121 °C and 120 kPa on each of two consecutive days	for 1 h at 121 °C and 120 kPa on each of two consecutive days
	Means of FB_1 production	2-liter glass fruit jars with a cover, in a closed room	2-liter glass fruit jars with a cover, in incubator	18 cm diameter petri-dishes
Conditions of production	Temperature	25 °C	25 °C	25 °C
	Light	darkness	darkness	darkness
	Duration	4 weeks	11 weeks	14 days
	Shaking	In the first week daily	No information	-

The vast majority of trichothecenes are produced by the Fusarium species. Production of these metabolites depends, of course, on many factors, including substrate, temperature, humidity, etc. In general, the type A trichothecenes have been most frequently associated with the following *Fusarium* species: *F. tricinctum, F. sporotrichioides, F. poe, F. equiseti.*

Interest in these compounds stems from their occurrence as contaminants in major crops and their acute toxicity in humans and domesticated animals upon consumption (*Marasas et al*., 1984). Many of the cytotoxic characteristics of the trichothecenes are attributed to their ability to inhibit protein synthesis (*McLaughlin et al*., 1977) and to induce apoptosis in eukaryotic cells (*Okumwai et al*., 1999). Various methods for the production of trichothecene mycotoxins have been reported (*Burmeister*, 1971; *Bata et*

al., 1984) but none of them are satisfactory for mass production for the great number of animal experiments. Because of deficiencies the database, even in the quantification of NOEL (No Observed Effect Level), only a temporary TDI (Tolerable Daily Intakes) could be determined for trichothecenes (*Schlatter*, 2004). Thus, further longer-term animal studies are clearly justified with in which a NOEL would be identified.

The need to develop efficient and cost-effective methods for the production of fumonisin and trichothecene toxins in sufficient quantities for a variety of biological studies is essential, since amounts of these mycotoxins produced under natural conditions are far too low for this purpose. There are only few data available on the reproducible experimental conditions for the optimal production of these toxins in culture. The objective of the present study was to describe a novel, alternative method for fumonisin and T-2 production, respectively.

MATERIALS AND METHODS

The strain of *F. verticillioides* used in experiments was originally isolated from corn in Transkei, southern Africa and deposited in the culture collection of the South African Medical Research Council (MRC) as *F. verticillioides* MRC 826 (*Marasas et al*., 1984). Based on three different, earlier published methods (*Table 1*), a novel method was worked out. During this process three, absolutely necessary factors were taken into account and tested in a serial experiment, as follows: 1.Quantity of medium: 50 g; 2. Standardized spore suspension from the lyophilized conidia: $1 \cdot 10^6$/ml; 3. Water added weekly to flask: 10 ml.

The method developed based on the factors tested is the next: Maize was prepared in 1.7-liter wide-mouthed glass fruit jars (diameter: 11 cm) with a (cotton plug between 2 linen rags) cover by autoclaving 50 g yellow maize kernels in 50 ml water (>50% moisture content; ≈1.00 a_w) for 1 h at 121 °C on two consecutive days, after an overnight soaking. The prepared maize was inoculated with a standard spore suspension. The number of spores in the freeze-dried vial was counted (with making tenfold dilutions until the 10^{10} dilution), and adjusted to $1 \cdot 10^6$ spores per ml. Whole corn cultures (surface/volume ratio, approximately 5:2) were directly inoculated with 1 ml of the spore suspension. The flasks were closed, and shaken manually to homogenize the culture material. The inoculated cultures were placed in a pre-sterilized incubator (LP-122, Labor-MIM, Hungary) to avoid contamination of the cultures and were incubated at 25 °C for 5 weeks, in air-flow function. To prevent a stuffy condition and possible fermentation, furthermore to get a better fungus distribution on the medium, all jars were vigorously shaken daily for 7–8 days. According to our preliminary investigations, in order to keep the proper water activity (a_w 0.98–0.93; *Cahagnier et al*., 1995), 10 ml sterile distilled water was added to each flask weekly.

T-2 toxin was produced experimentally on corn grits by *Fusarium sporotrichioides* strain NRRL 3299 (Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL), as described below.

Maize was prepared in 4.2-liter wide-mouthed glass fruit jars with a cover by autoclaving 800 g yellow corn grits (size: 2–3 mm) for 2 h at 121 °C, after an overnight soaking and filtering process. The inoculum was produced by growing the fungus on Czapek agar for 8 days at 25 °C. Spore suspensions were prepared by adding 2·5 ml of sterile, distilled water to the sporulated cultures. After dislodging of the conidia by gently scraping the agar surface with a sterile inoculation loop, the suspension was transferred into sterile, autoclaved maize. The culture was incubated in darkness at 24 °C

for a week, then at 8 °C for 2 weeks. After opening, the fungus-infected maize was dried at room temperature for some days, then ground. The resulting meal was stored at 4 °C until it was used in chemical analysis.

All treatments and culture methods were performed in triplicate (i.e. three cultures were homogenized together), and each experiment was repeated three (FB_1) and six (T-2) times, respectively. Determination of the actual fumonisin toxin concentration of the samples was carried out by a HPLC fluorescence detection method (LOD 0.05 mg kg^{-1}) based upon pre-column derivatisation with *ortho*-phthalyldialdehyde (*Fazekas*, 1998), while T-2 mycotocin was determined with GC-MS (LOD: 0.01 mg kg^{-1}).

RESULTS AND DISCUSSION

Fumonisin production

The experimental series are illustrated in *Table 2*. According to the three, previously published methods (see *Table 1*), the optimum constant incubation temperature for FB_1 production was identical, i.e. 25 °C, therefore this parameter was not altered. The use of ground kernels for the production was omitted, since in our pre-experiments with maize patties, the culture became stuffy, namely the necessary air amount for the mycelium growth was not met in the interior of the culture. Taking our experiences into considerations, the medium quantity (Factor 1) in the methods of *Alberts et al*. (1990) and *Fazekas* (1998) seemed to be far too high, accordingly, this parameter was reduced to 50 g, so the resulting surface/volume ratio strongly exceeded those in the published methods. It is also necessary to inoculate the corn cultures with a standardized spore suspension (Factor 2), otherwise the production of fumonisins will be negatively influenced. If the freeze-dried vials' contents are prepared from carnation leave agar (CLA) cultures, the same, i.e. standardized inoculum can be used each time. In order to reach almost equal production per culture, cultures were inoculated with standard spore suspension, since without this criteria, the standard deviation of the toxin concentration per jar was very large. Fumonisin biosynthesis by *F. verticillioides* is very much dependent on a_w. A 10% decrease in a_w i.e., from 1 to 0.90, reduced the quantity of FB_1 produced 300-fold (*Nelson et al.,* 1991). Based on this fact, in order to avoid water vapour elimination from the jars, sterile water was continuously dosed (Factor 3). *LeBars et al.* (1994) found that the total FB_1 concentration per batch after 3 weeks was lower than that after 2 weeks; this reduction was attributed to the decrease of oxygen during incubation. Namely, the fungus may also break down its own toxin after producing it. Therefore, in order to avoid an oxygen deprived status in the jars and the consequent decrease of fumonisin content, in our experiment the incubation period was 5 weeks. Applying all the above mentioned factors (Factor 1, 2 and 3), high production was reached. The toxin production started on the first week (*Figure 1*) and continued to increase after this phase, contrasting with the results reported previously (*Alberts et al.,* 1990), i.e. that the production commenced at 2 week. The mean and the relative standard deviation of the results obtained were 4454.6 mg kg^{-1} FB_1 and ±1060.9, respectively.

T-2 production

The first stage in the present study was to find a satisfactory strain for T-2 production. As shown in *Table 3*, the strain is able to produce T-2 toxin on corn. In comparison with the conditions of FB_1 production, the *F. sporotrichioides* strain is relatively non-sensitive to medium quantity (800 g). The corn grits were inoculated with a spore suspension of the fungus prepared on Czapek agar. The count of spores was unknown.

Table 2

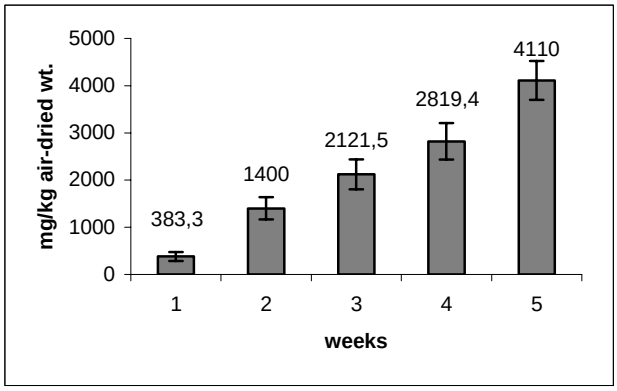
FB_1 produced in the experimental series for the development of the novel method

Factors applied*	Factor 1	Factor 1 and 2	Factor 1,2 and 3
FB1 Content of triplicate jars (mg kg^{-1} air-dried wt.)	621.4	1600	4110
	942.1	2185.7	3609
	611.8	1059.2	5645
Mean	725.1	1614.9	4454.6
±S.D.	±187.9	±563.3	±1060.9

*Factor 1.Quantity of medium: 50 g; Factor 2. Standardized spore suspension from the lyophilized conidia: $1 \cdot 10^6$/ml; Factor 3. Water added weekly to flask: 10 ml.

Figure 1

Time course of FB_1 production by the strain MRC 826, taking all factors into consideration (means of triplicate jars; n=3)



T-2 toxin production by Fusarium spp. has been evaluated previously on liquid (*Ueno et al*., 1975) and solid media, including autoclaved cereal grains (*Burmeister*, 1971). However, these methods have been criticized for lengthy, low-temperature incubations required (*Cullen et al*., 1982), poor yields and inordinate amount of incubator space required (*Richardson et al.,* 1984). Growing the fungi on a liquid medium required a shorther incubation period, but yields of T-2 toxin were low and variable, and the method required greater space in the incubator. Rice cultures of known toxigenic isolates grown yielded oily extracts containing compounds which interfered with qualitative and quantitative analysis for the toxin.

As compared with prior methods, our procedure resulted high and consistent amounts of T-2, moreover relatively rapidly. Therefore, the results suggested that this method has advantages over the others already reported.

According to our preliminary work, the a_w seems to be influential in toxin production because a great increase in the moisture content resulted much higher toxin concentration (5.87 g kg^{-1} T-2) in the cultures.

Thus, further studies are needed to investigate those potential factors which may cause a significant increase in the toxin production.

Table 3

T-2 toxin production by *Fusarium sporotrichioides* strain NRRL 3299

T-2 content of triplicate jars (mg kg^{-1} air-dried wt.)	591.5
	548.2
	706.0
	731.3
	531.9
	1180.4
Mean	714.8
S.D.	242.2

CONCLUSIONS

Infection of maize with *Fusarium* species and its contamination by different mycotoxins are generally influenced by many factors including environmental conditions (temperature, humidity) and pre-and postharvest handling. These factors do not influence infection independently but most often there are complex interactions. The higher concentration of nutrients and the loss of consistency due to the temperature treatment may enable the moulds to colonize the corn easily. Furthermore, the moisture conditions during the growing season as well as during storage are often pointed out to affect maize infection by *Fusarium spp*. and mycotoxins synthesis. In this context, water activity plays a key role. A possible reason for this lag is that the morphology and physiological functions of the fungi are dependent on a_w, and changes in a_w affect the ability of the fungi to produce mycotoxins because mycelial growth (at a_w 0.90) is less mycotoxigenic than sporodoquia (at a_w 0.98) production (*Nelson et al*., 1991). Moreover, from the viewpoint of toxin production, the number of infective spores and the type and availability of nutrients for fungus is also necessary. It is unfortunately a fact that not all batches of culture produce equally well, but with the following of standardized method, the production should not differ too much. In the laboratory practice, taking the above mentioned facts into consideration, a high toxin production can be reached both in fumonisin and T-2 production.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. W.C.A. Gelderblom (Medical Research Council, PROMEC Unit, Tygerberg, South Africa) for providing the strain MRC 826 of *Fusarium verticillioides*. Dr. Béla Fazekas, Dr. Gábor Sályi and Dr. András Szabó provided much help. This research was supported by the Hungarian Academy of Sciences (project no. B04074) and the Ministry of Education (NKFP 4/024/2004).

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