Influence of culture medium and incubation time on the simultaneous synthesis of deoxynivalenol and zearalenone by Fusarium graminearum

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SUMMARY

Fusarium graminearum is a fungal species able to contaminate many substrates and to produce several mycotoxins including deoxynivalenol (DON) and zearalenone (ZEA). The acceptable level of these mycotoxins is regulated for foods and European Union published recommendation for feeds. The aim of this study was to evaluate the effect of substrate, incubation time and conditioning of culture medium on simultaneous production of DON and ZEA in laboratory conditions. Rice appeared to be a more favourable substrate for both DON and ZEA production than maize and wheat. Indeed, production is more rapid and toxins are produced in larger quantities after culture on this medium. The conditioning of the culture medium (Petri dishes vs Erlenmeyer flasks) did not influence both DON and ZEA production. No correlation was found between toxin production by Fusarium graminearum strain cultivated on rice and fungal biomass measured by ergosterol quantification. This result suggested that, on that culture medium, fungal metabolism is mainly oriented towards mycotoxin production to the detriment of mycelium development.

Keywords: Deoxynivalenol, zearalenone, Fusarium graminearum, culture medium, toxinogenesis.

Introduction

Fusarium graminearum is a fungal species that is able to develop in a wide range of environmental conditions (pH, temperature, nutrients contents,...) and therefore contaminate many different grains and feeds [11; 17; 33]. F. graminearum is known to produce mycotoxins among which deoxynivalenol and zearalenone are the most important and toxic compounds [12; 30]. Since they are produced by the same fungal species, these two mycotoxins can be found simultaneously on cereal grains, feeds and foods [23; 24; 33]. Tolerable levels of DON and ZEA have been established in human food, whereas recommended levels are provided by the EC for the animal feeds and cereals grains used for this purpose [36, 37].

The main ecologic particularity of Fusarium graminearum is that this fungal species is hydrophilic and requires high water activity (> 0.95 to grow) [15; 19; 29]. As a consequence, F. graminearum usually develop on living plants, in the field, where it can lead to plant diseases [9; 30; 39]. It may also grow in peri-harvest period, before the drying process of cereal grains. Many studies aimed to characterize the impact of abiotic parameters on both Fusarium graminearum development and toxinogenesis. For instance, it has been demonstrated that a water activity > 0.97 and a temperature of about 20-25°C were the optimal conditions for DON or ZEA production [19; 21; 24; 25; 29]. However, only few studies were devoted to study the simultaneous DON and ZEA production and specially the influence of the substrate on this synthesis [13; 24].

The aim of this study is to characterize, in laboratory conditions, the influence of substrate, conditioning and incubation time on both DON and ZEA production by a toxigenic strain of Fusarium graminearum grown at water activity and
temperature known to be favourable for toxinogenesis. The determination of optimal conditions for toxinogenesis may allow producing the large quantities of mycotoxins that are required for determination of their toxicity in farm animals, especially in the course of multiple contaminations of feeds.

Materials and Methods

CHEMICALS

All solvents and reagents were analytical grade and purchased from Prolabo (Paris, France); Deoxynivalenol and zearalenone pure standards were purchase from Sigma (Saint Louis, MO).

FUNGAL STRAIN

Fusarium graminearum strains INRA 159 was isolated from wheat harvested in France. Identification was done by morphological examination of strains cultured on PDA according to NELSON et al. [27] and confirmed by PCR-based DNA analysis using the Fg16NF (ACAGATGACAGATTCAGGCACA) and Fg16NR (TTCTTTGACATCTGTTCACCCA) primers by the method of NICHOLSON et al. [28].

Strain was maintained on PDA medium and sub cultured each 2 weeks. For mycotoxin production assays, 3 pieces (9 mm² each) of a one-week culture on PDA medium were used for inoculation of culture medium.

DEOXYNIVALENOL AND ZEARALENONE PRODUCTION

Mycotoxin production assays were carried either on 250 ml Erlenmeyer flasks containing 25 g of cereals added with 25 ml water or on Petri dishes (15 cm diameter) containing 50 g of cereal added with 50 ml distilled water. Water content of culture medium was assessed according to AFNOR norm NF ISO 711 [2]. Results showed that water content of all media were ranging from 51.5 to 54% and therefore corresponded to aw > 0.95 [26].

Recipients were filled with rice, wheat or roughly grounded maize. These substrates were tested for DON and ZEA content before use. All were found to be below LOD. They were autoclaved at 121°C for 20 minutes and cooled down before inoculation.

Cultures were incubated in darkness at 21°C for 5 weeks and were investigated for mycotoxin production and ergosterol content at days 7, 14, 28, 35, 42. Each experiment was done in triplicate.

MYCOTOXIN ANALYSIS

Deoxynivalenol and zearalenone were extracted from homogenised culture material by mechanical agitation overnight with respectively 75 and 150 acetonitrile/water (90:10 v/v) for Erlmeneyer flasks and Petri dishes. Extract were then filtered through Whatmann n°1 filter and tested for mycotoxin content.

Deoxynivalenol was quantified according to TRUCKSESS et al [35]. In brief, 5 µl of filtered extracts were potted on thin layer chromatography (TLC) plates (Merck n°5553, VWR, Fontenay sous bois, France). Separation was carried out in ethylic ether-methanol-water (96:3:1 v/v/v). After drying at room temperature, plate was sprayed with ammonium chloride (20% in ethanol 50%) and heated for 10 minutes at 110°C. Quantification was performed with a Shimadzu fluorodensitometer (CS930, Shimadzu corp., Kyoto, Japan) at 330 nm. Linear response was obtained over a range of 15 to 250 ng DON. Limit of quantification of the method is 3 µg DON/g of culture medium.

Zearalenone was analyzed according to HADIANI et al. [14]. For that, 3 µl of filtered extracts were spotted on TLC plates. Separation was done in toluene-acetate-formic acid (6:3:1 v/v/v). Quantification of the toxin was performed by fluorodensitometry at 313 nm. Linear response was obtained over a range of 18 to 300 ng ZEA. Limit of quantification of the method is 6 µg ZEA/g culture medium.

ERGOSTEROL QUANTIFICATION

Ergosterol content was assessed by fluorodensitometry as already described [5]. Extraction of ergosterol was performed using the method of Schwardorf and Müller [31] with slight modifications according to AFNOR norm NFV 18-112 [3]. 5 µl of the concentrated extract were spotted on TLC plates. They were developed in toluene-acetonitrile (85:15, vol/vol). After drying, plates were heated at 130°C for 30 min and ergosterol was quantified by fluorodensitometric detection at λ 365 nm (Shimadzu CS 930 fluorodensitometer).

STATISTICAL ANALYSIS

Results were expressed as mean +/- SD. ANOVA was used to assess statistical differences in mycotoxin production depending on the culture medium and incubation time. Significant differences are indicated on tables and figures.

Results and Discussion

For all experiments, water content was adjusted to get a water activity higher than 0.95 and temperature was maintained during all the experiments at 21°C. These values are known to be favourable for both DON [17; 25] and ZEA [18] production.

INFLUENCE OF CULTURE CONDITIONING

Since fungi are strictly aerobic micro organisms, the difference in oxygenation of the culture may influence the toxin...
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To assess the influence of different conditioning for culture medium on DON and ZEA production, two containers widely used for fungal culture were used: Petri dishes of 15 cm diameter and 250 ml Erlenmeyer flasks. These two kinds of container display a surface of exchange with oxygen that is different: 176 cm$^2$ for Petri dishes versus 50 cm$^2$ for Erlenmeyer flasks. However, in the later, volume of gaseous atmosphere above the colony was more important (600 vs 350 cm$^3$ for Petri dishes). The effect of container on DON and ZEA production is reported in table 1. No significant difference (ANOVA, $P < 0.05$) was obtained.

**INFLUENCE OF SUBSTRATE**

Fungal culture can be performed in laboratory conditions on classical agar medium, liquid medium or on solid medium containing cereal grains. For *Fusarium* species, it has been shown that solid culture medium was more favourable for toxinogenesis [18, 38]. That is why we tested the influence of three different cereal substrates on mycotoxin production by our *Fusarium graminearum* strain. The production of DON and ZEA according to the culture medium is presented on figure 1. Rice appeared to be the best substrate for both DON and ZEA production. Indeed, DON production on rice appeared to be 1.6 and 2.1 times higher than production on maize and wheat respectively. For ZEA, differences between culture media were even more important. Indeed, ZEA level observed after culture of INRA 159 strain on rice was found to be 10 fold higher than after culture on maize whereas this mycotoxin was not detected after culture on wheat.

Our results are in agreement with those of GREENHALGH *et al.* and MEGALLA *et al.* that observed that rice was a better medium for DON production compared to maize [13, 22]. They also found that their isolates produced more ZEA after culture on maize that was not the case in our study. However, the levels of both DON and ZEA production reported in this study were much lower than those reported here (maximum production of about 150 ppm). Therefore, differences in strain metabolism and toxigenic potential may be responsible for these results.

The composition of rice may be responsible for higher and more rapid toxin production. Indeed, rice contents about 6% of proteins and 78% of carbohydrates whereas these values are approximately of 8 and 76% for maize and about 12 and 66% for wheat. It has been demonstrated that a high carbohydrate/protein ratio is favourable for mycotoxin production [12].

The observed differences in mycotoxin production according to the culture medium used could also be related to phenolic compounds content of each substrate. Indeed, it has been showed that phenolics concentration in grains may modulate mycotoxin synthesis [6, 16]. Ferulic acid, the most abundant phenolic compounds of cereal grains, has been showed to inhibit trichothecene synthesis by repressing *Tri* gene expression [7, 8]. Rice, wheat and corn grains have different phenolic compounds and especially ferulic acid contents and this could play a role in mycotoxin accumulation [1].

These results demonstrates that the choice of the culture media used for the screening of toxigenic potential of *Fusarium graminearum* strains may lead to underestimation of mycotoxin production.

**TABLE I: Mycotoxin production after 5 weeks of culture on different media conditioned in Petri dishes or Erlenmeyer flasks. Results are expressed as mean of three experiments ± SD. a : µg/g. different letters indicate significant differences ($P < 0.01$).**

<table>
<thead>
<tr>
<th></th>
<th>DON$^a$</th>
<th>ZEA$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Petri dishes</td>
<td>Erlenmeyer flasks</td>
</tr>
<tr>
<td>Rice</td>
<td>1022 ± 45$^a$</td>
<td>1118 ± 47$^a$</td>
</tr>
<tr>
<td>Maize</td>
<td>644 ± 21$^b$</td>
<td>531 ± 19$^b$</td>
</tr>
<tr>
<td>Wheat</td>
<td>463 ± 66$^b$</td>
<td>436 ± 43$^b$</td>
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</tbody>
</table>

**FIGURE 1: DON (A) and ZEA (B) production by F6G10 strain on rice (●), maize (▲) and wheat (■) as a function of incubation time. Results are expressed as mean +/- SD of three experiments. * indicates significant differences ($P < 0.01$).**
INFLUENCE OF INCUBATION TIME

Production kinetics of DON was different according to the substrate used for culture. On rice, DON production was very rapid with a peak obtained after 2 weeks of culture before a stabilisation of the toxin content. On maize and wheat, accumulation of DON was more progressive with a plateau obtained after 3 or 4 weeks of culture (Figure 1A).

For ZEA, there was a regular accumulation of the toxin from 1 to 3 or 4 weeks on rice and maize respectively (figure 1B). These results are in agreement with a previous study reporting a more rapid accumulation of DON compared to ZEA [24].

RELATION BETWEEN TOXIN PRODUCTION AND FUNGAL GROWTH

In order to evaluate if differences of mycotoxin production could be related to differences in fungal development, fungal biomass was evaluated using ergosterol measurement. Results are presented on figure 2. It appears that although rice was the best substrate for both DON and ZEA production, ergosterol content of rice cultures was found to be significantly lower than the one observed on maize and wheat whatever the incubation time. Moreover, a correlation between ergosterol and mycotoxin production during culture can be observed on maize and wheat whereas this relation does not exist on rice, whatever the toxin considered. Although no explanation can be provided to date to explain these differences, it has to be noted that ergosterol, ZEA and DON synthesis require the same precursors such as acetyl-Co-A and mevalonate. Therefore, the high rate of DON and ZEA production on rice may reduce the availability of these precursors for ergosterol synthesis [10; 20].

As a conclusion, it appeared from this study that DON and ZEA synthesis could be strongly influenced by the medium used for fungal development. The determination of the influence of abiotic parameters on simultaneous DON and ZEA production may also allow producing in laboratory conditions the high amounts of toxins required for experimental reproduction of mycotoxicosis and the study of the effect of a multi-contaminated feed on animal’s health. The substrate used for evaluating toxigenic potential of fungi may also be considered for characterisation of fungal strains isolated from grains or feeds.

References


FIGURE 2: relationship between DON (A) and ZEA (B) production and fungal biomass measured by ergosterol content. ◆: rice, ▲: maize, ■: wheat.
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