# Relationship between flavours and deoxynivalenol concentrations in wheat samples infected by *Fusarium culmorum*

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## ABSTRACT

Volatile metabolites that might be associated to production of deoxynivalenol (DON, vomitoxin) by *Fusarium culmorum* were investigated. Isolates of seven strains were grown for 3, 7, 15, and 21 days on sterile wheat grains and checked for DON excretion by GC-electron capture detection (ECD). Among these seven fungi, six emerged as DON producers after 21 days (from 0.2 to 112 ppm). Volatile organic compounds (VOCs) excreted in the media were analyzed by GC-O and GC-MS. Their amount proved to be considerably influenced by both incubation time and strain type. At 21 days, the β-farnesene concentration could reach up to 5 ppm in the medium inoculated with the DON non-producer strain whereas it did not exceed 0.7 ppm with the other strains, leading to an apparent inverse relationship between *β*-farnesene and DON concentrations. Additional analyses indicated, however, that kinetic parameters are also strain-dependent, with the consequence that VOCs must be used very carefully as mycotoxin-producer indicators.

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## INTRODUCTION

DON or deoxynivalenol is a sesquiterpenic mycotoxin belonging to the group-B trichotecenes. It is mainly produced by *Fusarium spp* and more particularly by *F. graminearum* and *F. culmorum* (Ueno, 1983). Many papers have been devoted to acquiring a better understanding of trichotecenes biosynthesis (Beremand and Mc Cormick, 1992 ; Sweeney and Dobson, 1999 ; Tamm and Breitenstein, 1980). Interestingly, recent studies have shown that it might be easier to monitor fungal contamination or to assess the mycotoxin occurrence by analysing VOCs or volatile organic compounds (Keshri *et al.*, 1998 ; Magan and Lee, 2000 ; Olsson *et al.*, 2002). For example, Olsson *et al.* (2002) have shown that high concentrations of aldehydes (nonanal, 2-hexanal) and alcohols (1-penten-3-ol, 1-octanol) are typically found in cereal grains containing less than 5 µg/Kg OTA (Ochratoxin A). On the contrary, ketones such as 2-hexanone and 3-octanone are abundantly detected when the OTA concentration exceeds 5 µg/Kg. Regarding DON, levels of some alcohols (1-octanol, 1-nonanol, 1-heptanol, *etc.*) correlate negatively with this mycotoxin whereas pentane, 3-pentanone, 3-octen-2-ol, *i*-octylacetate, *etc.* show a positive correlation (Olsson *et al.*, 2002). Sesquiterpenes, commonly used to classify different strains or to identify *Penicillium* species, are also closely related to the production of sesquiterpenic mycotoxins. As previously mentioned, aristolochene and *P. roqueforti* toxin T1 evolve quite similarly (Jelen, 2002) whilst trichodiene can be used to follow the generation of trichotecenes excreted by *F. sambucinum* and *F. sporotrichioides* (Demyttenaere *et al.*, 2004 ; Jelen *et al.*, 1997). Other sesquiterpenes are more specifically associated with the development of aflatoxinogenous strains of *Aspergillus flavus* (Zeringue *et al.*, 1993).

The aim of the present work was to assess the toxinogenic features of seven *Fusarium culmorum* strains after 3, 7, 15, and 21 days of incubation on wheat at 28°C. Compiling the results obtained by GC-MS, GC-O, and GC-FID, we tried to correlate the occurrence of several VOCs with the DON content in order to find reliable and easily measurable indicators. The relationship existing between β-farnesene and DON was particularly studied. Our results concerning the production kinetics of this odorant lead us to question the validity of using this alternative approach to evaluate food toxicity.

## EXPERIMENTAL PROCEDURES

Chemicals

Mycosep 227 was purchased from Coring system (Bruxelles, Belgium). 1-Chloroheptane 97% came from Acros Chimica (Geel, Belgium) and both 2-acetylthiazole 99% and 4-phenylpyridine 97% were from Aldrich (Bornem, Belgium). *β*-Farnesene 90% was provided by Bedoukian (Danbury, CT, USA), dichloromethane >99.8%, by Lab-Scan (Dublin, Ireland) and acetonitrile >99.8% by Romil (Gent, Belgium). Heptabutyric-fluoroanhydride (HBFA) 98%, deoxynivalenol (DON) and 4-dimethylaminopyridine (DMAP) 99% were supplied by Sigma (St Louis, MO, USA). Aldrine 99.8% was from Riedel de Haen (Seelze, Germany). Merck (Darmstadt, Germany) supplied toluene and $NaHCO_3$.
Dichloromethane was distilled twice and water was filtrated on a Milli-Q purification system (Millipore, Bedford, MA, USA) before use.

Culture conditions and spore inoculation on wheat

The seven strains of *F. culmorum* (*MUCL43791*, *MUCL43792*, *MUCL43793*, *MUCL43794*, *MUCL43796*, *MUCL43797* and *MUCL43798*) came from the MUCL collection (Université catholique de Louvain, Belgium). They were grown on malt agar. After 10 days at 28°C in the dark, the Petri dishes were washed with sterile water and spores were counted. Two 250-mL flasks containing autoclaved, humidified wheat were inoculated with each *Fusarium* strain in order to reach $10^3$ spores/g. Later (28°C – 3, 7, 15 or 21 days), the wheat grains were dried for 48 h at 50 °C. Then, they were ground and stored at –18°C. A reference sample was obtained in the same way but without spore inoculation.

DON concentration in wheat after *Fusarium* inoculations

Ten grams of ground wheat was extracted by an 80:20 acetonitrile-water solution for 3 minutes in an UltraTurrax (T25) at 9500 rpm. The mixture was then centrifuged at 2000 rpm for 3 minutes (Sorvall RC2B, rotor GSA). Afterwards, 10 mL of the upper phase was purified on a Mycosep 227. Of this, 7 mL was collected and evaporated under nitrogen at 40°C. Then DMAP (1 mL) and HBFA (50 µL) were added to the residue under stirring. The whole was heated at 60°C during 20 minutes. After cooling, 1 mL of $NaHCO_3$ (3 %) was poured into the system. The aqueous phase was then eliminated and 1 mL of ultra-pure water was added. The organic phase was finally recovered and 0.5 µL of this phase was analyzed twice by GC-ECD.

Gas chromatography hyphenated to electron capture detector (GC-ECD)

The GC system (Chrompack CP9001) was equipped with a split/splitless injector (split mode/260°C) and an ECD ($^{63}$Ni/300°C) connected to an integrator (Shimadzu CR8A). DON analysis was performed with a 50 m × 0.32 mm i.d., wall-coated open tubular (WCOT) apolar CP-SIL 5 CB capillary column (film thickness, 1.2 µm). The oven temperature, initially set and maintained for 5min at 170°C, was then programmed to rise from 170-200°C at 2°C/min followed by 200-260°C at 50°C/min. The final temperature was maintained for 20 min. Nitrogen was used as carrier gas at a flow rate of 1.2 mL/min.

Volatile extraction method

Many techniques are suitable for isolating this kind of metabolite (Demyttenaere *et al.*, 2003). In this case, an improved Likens-Nickerson method was applied. As previously shown by Bouseta and Collin (1995), this extraction procedure yields, when cautiously performed (especially no sugar and no oxygen in the medium), very representative extracts which perfectly mimic the initial sensorial properties of the matrix (Bouseta and Collin, 1995).

Five grams of ground wheat was extracted with 3 × 10 mL of $CH_2Cl_2$ in an UltraTurrax for 3 minutes. The liquid phase was recovered and then evaporated at 45°C in a Kuderna to 1 mL. To remove the non-volatile compounds, Likens-Nickerson extraction was further applied during 45 minutes (Bouseta and Collin 1995). After addition of 20 µL of a 2078-ppm chloroheptane solution, the extract was concentrated to 0.5 mL, of which 2 µL were injected on GC and analyzed by olfactometry, flame ionization or mass spectrometry. This experiment was repeated at least twice for each strain.

Gas chromatography hyphenated to a flame ionization detector or a sniffing port (GC-FID or GC-O)

Volatiles analysis was performed with a Thermo Finingan Trace 2000 gas chromatograph equipped with a splitless injector maintained at 225°C and opened after 0.5 min. VOCs were separated on a 50 m × 0.32 mm i.d., wall-coated open tubular (WCOT) apolar CP-Sil 5 CB capillary column (film thickness, 1.2 µm). The oven temperature was programmed to start and be maintained for 2 min at 36°C, and then to rise from 36 to 85°C at 20°C/min, then to 145°C at 1°C/min, and finally to 250°C at 3°C/min. The final temperature was maintained for 30 minutes. The eluent was sent to a FID detector maintained at 275°C and allowing peak integrations (Spectra Physic, Chromjet DP-700) or to a sniffing outlet for identification confirmation.

Gas chromatography hyphenated to a mass spectrometer (GC-MS)

Chromatographic conditions were the same as those mentioned for FID detection. Mass spectra (m/z = 40 to 380) were recorded at 70 eV on a ThermoFinnigan Trace MS mass spectrometer connected to a ThermoFinnigan Trace GC 2000 gas chromatograph. Spectral recording was done automatically throughout elution with the Xcalibur software.

## RESULTS AND DISCUSSION

Among the seven *F. culmorum* strains grown on 40 % humidified and sterile wheat grains, six were able to produce DON over a 21-day incubation period at 28°C. The exception was *MUCL43793* (table 1). To validate our results, we confirmed the absence of any DON co-eluting compound in the reference sample.

| *F. culmorum strain* | *Origin of the strain* | *DON [ppm] after 3, 7, 15, and 21 incubation days at 28°C* | | | |
|---|---|---|---|---|---|
| | | *3d* | *7d* | *15d* | *21d* |
| *MUCL43793* | hyacinth roots / Holland | ND | - | - | ND |
| *MUCL43792* | asparagus / Belgium | ND | - | - | 0.2 |
| *MUCL43798* | wheat / Belgium | ND | - | - | 0.3 |
| *MUCL43794* | wheat / Belgium | ND | - | - | 1.1 |
| *MUCL43791* | oat / Canada | ND | - | - | 5.4 |
| *MUCL43796* | wheat / Belgium | ND | - | - | 54.2 |
| *MUCL43797* | wheat / Belgium | ND | 50.3 | 84.4 | 112.3 |

ND = undetected ; - = not determined

**Table 1:** Production of deoxynivalenol on wheat grains by different *F. culmorum* strains of the MUCL collection

An improved Likens-Nickerson method was used to extract VOCs from wheat samples infected separately with each *F. culmorum* strain and incubated for 3 to 21 days at 28°C (Bouseta and Collin, 1995). The highly representative extracts obtained were analyzed by GC-FID, GC-O, and GC-MS. Most compounds were undetectable after 3 days of incubation at 28°C but easily quantified after 7, 15 or 21 days. The identified VOCs belonged to various chemical classes and had typical odours such as greenery (hexanal/(*Z*)-3-hexenal, nonanal), flower (β-phenylacetaldehyde, β-phenylethanol), oil ((*E, E*)-2, 4-decadienal, (*E, E*)-2, 4-nonadienal), or unpleasant notes (trimethylbenzene). 3-Methylbutanol, 2-methylbutanol, and 1-octen-3-ol, being *Fusarium* metabolites (Jelen *et al.*, 1997), were found as expected whatever the strain. MS identifications were confirmed in most cases by co-injection of commercial standards.

Interestingly, four compounds (β-farnesene [$RI_{CPSil5CB}$: 1458 – odour: green, terpenic – 5 major ions m/z : 41, 69, 93, 43, 55], 2-acetylthiazole [$RI_{CPSil5CB}$: 978 – odour: nutty – 5 major ions m/z : 43, 127, 99, 58, 112], 4-phenylpyridine [$RI_{CPSil5CB}$: 1434 – odour: unpleasant – 5 major ions m/z : 155, 154, 127, 102, 51], and (Z)-α-bisabolene trans-1,2-epoxide [$RI_{CPSil5CB}$: 1470 – odour: pleasant, terpenic – 5 major ions m/z : 43, 57, 41, 55, 121]) revealed to be much more concentrated in the extract of the DON non-producer (*MUCL43793*) culture (Figure 1).

Besides, it was important to look at the evolution of VOCs levels as a function of time. It turned out that most strains produced larger amounts of β-farnesene after 7 or 15 days than after 21 days (Figure 2). The four best DON-producing fungi (from 1 to 112 ppm of DON) showed the maximum β-farnesene concentration after 15 days. Both strains generating 0.2 to 0.3 ppm of DON were characterized by higher β-farnesene production at 7 days, like the DON non-producer strain (*MUCL43793*). This observation cannot be strictly generalized to other sesquiterpenes such as β-bisabolene as shown in Figure 3. It proves that it is quite dangerous to assess strain toxicity solely by measuring VOCs concentrations, especially in real foods where each strain can be at different kinetic point. Culture conditions and incubation time can significantly affect the residual amounts of secondary metabolites like VOCs, leading to poor differentiation of strains.

Because DON is also a secondary metabolite issued from terpenes (Demyttenaere *et al.*, 2004 ; Rynkiewicz *et al.*, 2002), its concentration was determined for the high-producer strain (*MUCL43797*) at different growth stages. As depicted in Figure 4, the DON concentration gradually increased with time (linear between 7 and 21 days of incubation, $R^2$=0.9994). This indicates that no catabolism occurred in the medium, as is known for other mycotoxines such as OTA (El-Nezami *et al.*, 2002). It is noteworthy that trichodiene, known as a potential DON-marker in *F. sambucinum* and *F. sporotrichioides* cultures (Demyttenaere *et al.*, 2004), was not detected in our extracts (inoculated with *F. culmorum*).

In conclusion, determination of VOCs appears insufficiently reliable as a means of avoiding the strict quantification of toxins like DON. Some compounds like β-farnesene might, however, be used as potential indicators of DON contamination if kinetic data are available.

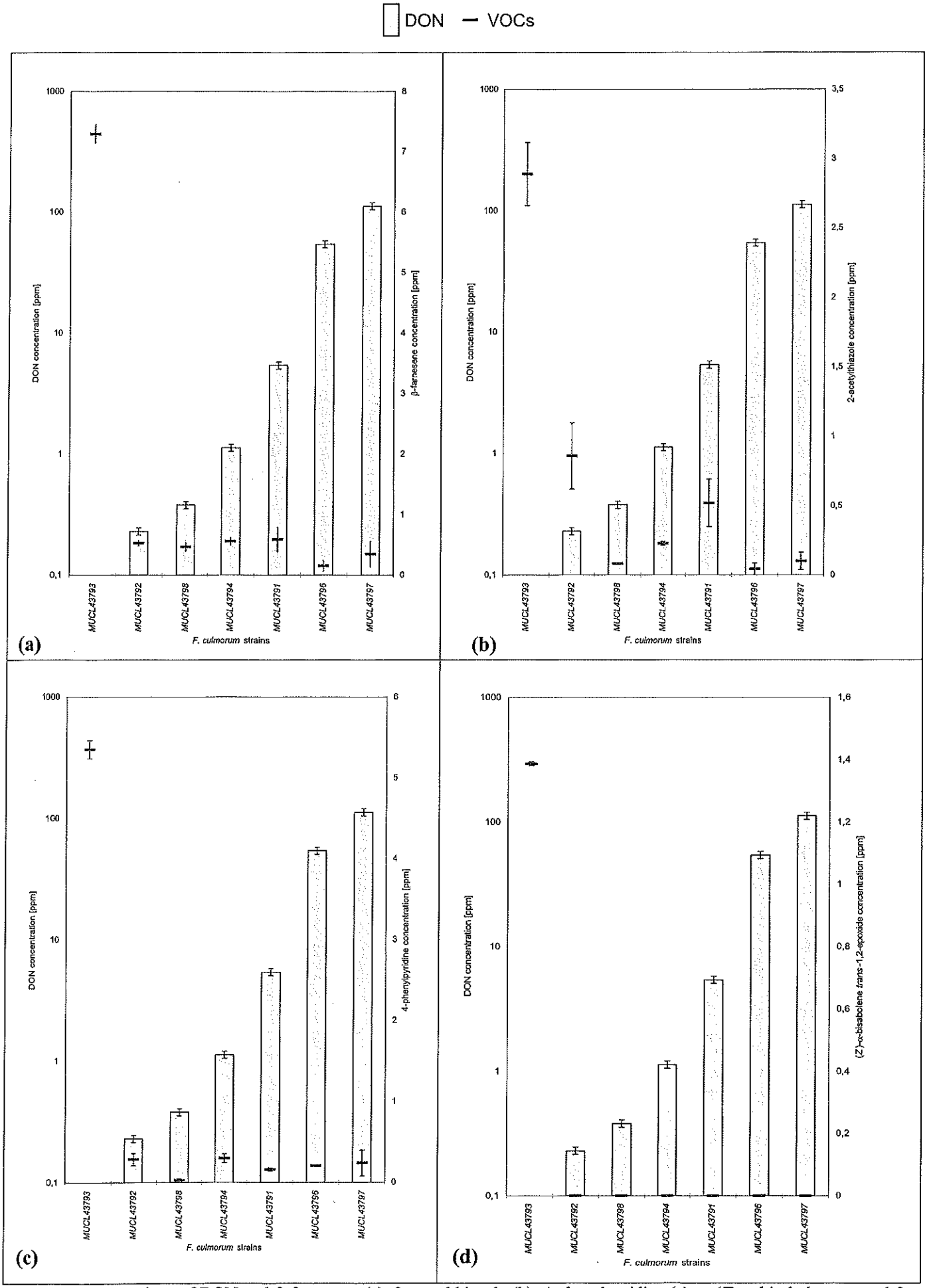


**Figure 1:** Comparison of DON and β-farnesene (a), 2-acetylthiazole (b), 4-phenylpyridine (c) or (Z)-α-bisabolene *trans*-1,2-epoxide (d) concentrations after 21 days of incubation at 28°C for different *F. culmorum* strains

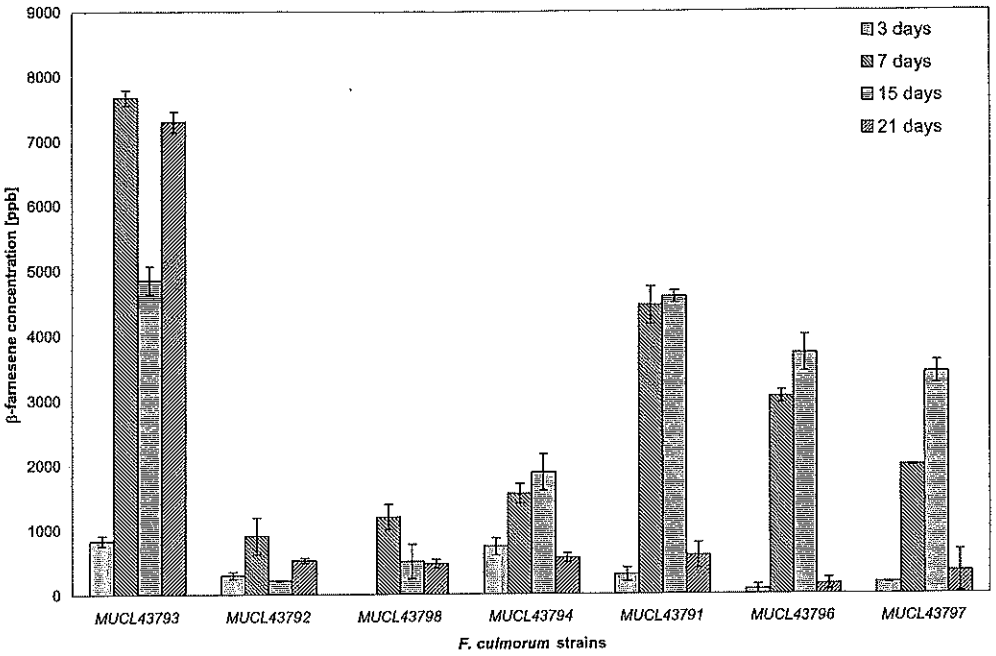


**Figure 2:** Evolution of β-farnesene in accordance with incubation time at 28°C for different *F. culmorum* strains

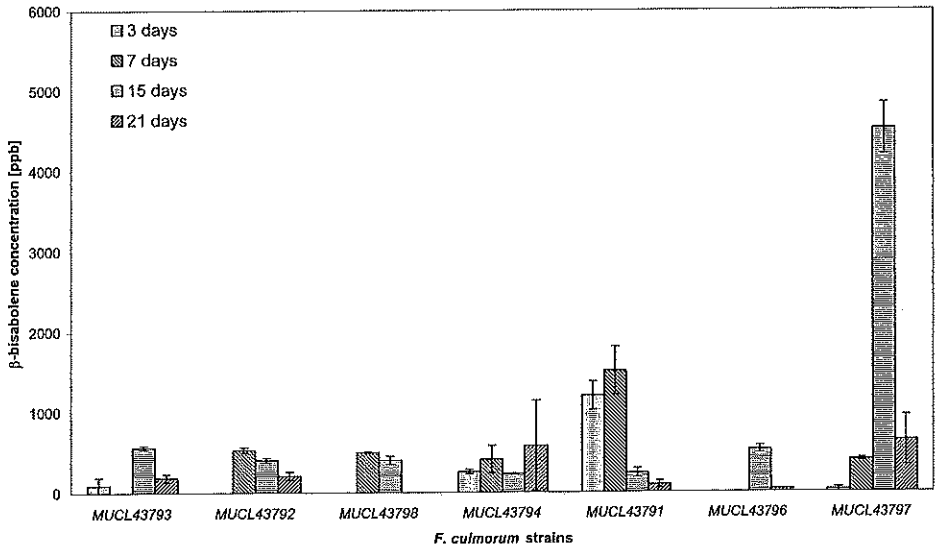


**Figure 3:** Evolution of β-bisabolene in accordance with incubation time at 28°C for different *F. culmorum* strains

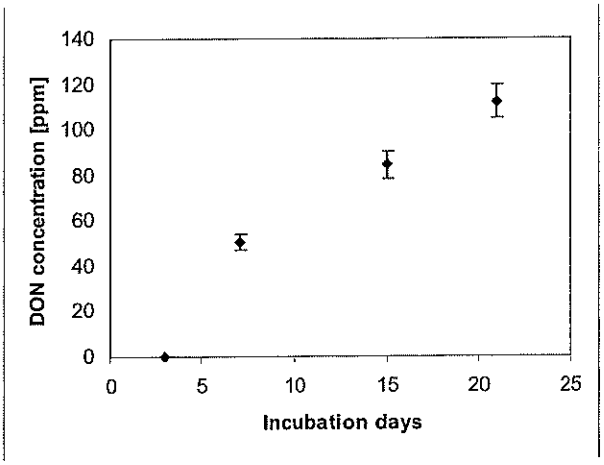


**Figure 4:** DON concentration in the *MUCL43797* (*F. culmorum*) culture medium at four different incubation times

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