Appl Microbiol Biotechnol (1994) 41:384–387

ORIGINAL PAPER

F. Altpeter · U. K. Posselt

Production of high quantities of 3-acetyldeoxynivalenol and deoxynivalenol

Received: 5 August 1993 / Received revision: 30 November 1993 / Accepted: 13 December 1993

Abstract The time course of 3-acetyldeoxynivalenol (AcDON) formation was analysed over a 11 day culture period of *Fusarium graminearum* (DSMO 4258) on rice. The maximum of 2840 mg/kg AcDON was detected after 9 days of culture. To overcome the complicated clean up of the solid substrate extract, 10% methanol in water was applied as extract solvent. After liquid-liquid partition with ethyl acetate, more than 75% of the toxin could be detected. After one simple clean up step (column chromatography over florisil) AcDON could be crystallised. After ion exchange chromatography deoxynivalenol (DON) could be crystallised and an overall yield of 1,44 g DON per kg of culture was obtained.

Introduction

The world-wide natural occurrence of the trichothecenes deoxynivalenol (DON) and 3-acetyldeoxynivalenol (AcDON) as toxic contaminants in cereal grains (Gruber-Schley 1987) are due to infections with several *Fusarium* species. To carry out feeding and toxicological studies, large quantities of these mycotoxins are needed. Additionally, DON is regarded as a factor of aggressiveness in the pathogenesis of *Fusarium* head blight of wheat (Snijders and Krechting 1992). DON tolerance in wheat and corn is based on trichothecene degradation, increased membrane stability and a modification in peptidyltransferase-activity. DON tolerance seems to be correlated with *Fusarium* resistance (Miller 1989; Snijders and Krechting 1992; Wang and Miller 1988).

In-vitro selection for DON tolerance could provide plants with increased *Fusarium* resistance and is practised by several authors (Maier and Oettler 1993; Menke-Milczarek and Zimny 1991; Posselt 1990; Shimada and Otani 1990). From the existing methods for producing high quantities of DON, the production in a fermentor (Miller and Blackwell 1986) is the most elegant but requires expensive equipment, whereas the method described by Ehrlich and Lillehoj (1984) requires numerous clean-up steps. A more convenient method, which requires only one labour intensive clean up step, was described by Witt et al. (1985).

The results reported here describe a simple, convenient and high reproducible method that needs only a minimum of technical equipment and organic solvents to enable a common tissue culture laboratory to produce gram quantities of crystalline AcDON or DON.

F. Altpeter
Research Centre of Biotechnology and Plant Breeding,
University of Hohenheim,
P.O. Box 700562,
D-70593 Stuttgart 70, Germany

U. K. Posselt (✉)
State Plant Breeding Institute,
University of Hohenheim,
P.O. Box 700562,
D-70593 Stuttgart 70, Germany

Materials and methods

Culture conditions

A 2% malt extract agar slant of *F. graminearum* DSMO 4258 (accession number of the collection of micro-organisms at Braunschweig, Germany) was macerated in 25 ml sterile distilled water in a 100 ml erlenmeyer flask. An aliquot (2.5 ml) of the resulting suspension was used to inoculate 300 ml erlenmeyer flasks, each containing 50 ml SNA medium (Nirenberg 1981) without agar but supplemented with 1% yeast extract (Merck, Darmstadt, Germany) and autoclaved at 121° C for 20 min. The cultures were kept on a rotary shaker (2.6 cm throw) at 250 rpm at 25° C for 20 h. Rice meal (20 g) (Schneekoppe, Naturreis aus kontrolliertem Anbau) was moistened with 7.5 ml solution of 38 g/l of saccharose and 2.5 g/l of casein hydrolysate in a baby-food jar and covered with magenta-B caps (Sigma). After 24 h at room temperature the substrate was autoclaved at 121° C for 20 min. The mycelium of the shaken culture was macerated and 2 ml inoculum added (final substrate humidity 40%) to each baby food jar. The batch culture was grown at 28° C in the dark (Vesonder et al. 1982). To identify the ideal culture time for maximal toxin formation two baby food

jars in the two independent experiments were analysed in duplicate for AcDON content 3, 5, 7, 9 and 11 days after culture initiation.

Reagents

The water used was distilled and put through a Milli-Q/UF-System (R = 18 MO). All chemicals and solvents used, were of analytical grade. AcDON and DON standards were obtained from Sigma. The AcDON standard for HPLC was used in a concentration of 10 µg AcDON/ml methanol and had been adjusted in ethanol by use of a photometer (Beckman model 24) set at 219 nm (E = 5900) (Yoshizawa and Morooka 1973).

Extraction for AcDON analysis

Each 20 g rice was extracted three times with 50 ml of 10% methanol in water for 30 min on a rotary shaker at 100 rpm. The extract was filtered through a Schleicher and Schuell 520 a 1/2 filter and saturated with NaCl. The extract was liquid-liquid partitioned three times with ethyl acetate with a volume ratio of 1:2 (ethyl acetate:extract). For the second and third partitions ethyl acetate from rotary evaporation was recycled. The ethyl acetate layer was collected, taken to dryness and dissolved in methanol for HPLC quantification. To determine the recovery of this extraction procedure, 4 ml AcDON solution containing 0.5 mg AcDON/ml methanol was added to 20 g rice meal, prepared like for inoculation and extracted after evaporation of the solvent. This experiment was repeated three times.

Scale up

A total of 1.4 kg culture from 70 baby food jars was macerated and transferred to a 10 l bucket 9 days after culture initiation, 3.15 l water was added, followed by 0.35 l methanol 20 min later, then all shaken for 30 min at 100 rpm. The supernatant was put through a sieve (1 mm mesh) and the residue was extracted twice more with 3.50 l of 10% methanol in water. The collected liquid was kept in an aspiration bottle overnight at 1° C to sediment the rice particles. The supernatant liquid was filtered through a Schleicher and Schuell 520 a 1/2 filter and extracted with ethyl acetate as for AcDON analysis.

The ethyl acetate layer was collected and taken to dryness. The crude extract from 1.4 g culture was taken up in 25 ml methylene chloride and chromatographed on a column (5.0 cm × 30.0 cm) packed with 100 g florisil (100–200 mesh) in methylene chloride (Miller personal communication). The AcDON was eluted with 1200 ml of 0.3% methanol in methylene chloride (600 ml/h). The product was recrystallised three times from hot ethanol under addition of hexane and cooling to give AcDON as fine colourless needles up to 5 cm in length.

Hydrolysis of AcDON

The transfer of AcDON to DON was carried out on an ion exchange column as described by Greenhalgh et al. (1986).

Analysis

The AcDON content was analysed by HPLC. Separation was performed on a prepacked Beckmann Ultrasphere octadecyl silane (ODS) 4.6 mm × 250 mm column, particle size 5 µm. The compounds were eluted with 50% methanol in water at a flow rate of 1.0 ml/min. Detection was performed with a Merck-Hitachi L 4000 UV detector set at 218 nm used in connection with an LDC-Milton Roy Constametric 3 HPLC pump. Chromatograms were recorded and evaluated by an LDC-Milton Roy CI-10 data system. The retention time for AcDON was 5.2 min.

The purity of DON crystals was assessed by gas chromatography-electron capture detection (GC-ECD). The analysis of trimethylsilyl (TMS) ethers of DON, prepared as described by Scott et al. (1986), were made by ECD on a C-R4AX Chromatopac, Shimadzu Chromatograph fitted with a 25 m × 0.25 mm Permabond SE-54-DF-0.25 column (Macherey-Nagel). The carrier gas was helium (80 kPa). The make-up gas was nitrogen (45 ml/min). The injection was splitless. The injector temperature was maintained at 250° C. The temperature of the column was held at 60° C for 1 min, then heated to 120° C at 10° C / min and held for 2 min, then heated to 210° C at 10° C / min, then heated to 280° C at 4° C / min and held for 20 min. The detector temperature was 330° C. The retention time for DON was 29.6 min.

The identity of crystalline DON was confirmed by comparison of its mass spectrum with that of the DON standard. The underivatised DON dissolved in ethyl acetate dissolved DON (1 mg/ml) was, after splitless injection, performed with a Model 3700 gas chromatograph in connection with a Varian Model 44S MS. Separation was achieved on a 30 m × 0.25 mm DB 1 column (J. & W. Scientifics). The carrier gas was helium (1.2 bar). The column temperature was programmed from 140° C to 180° C at 4° C / min, the injector temperature was maintained at 250° C. Electron-impact (EI) mass spectrometry (MS) was carried out at 70 eV with a Varian 44S.

Safety Note

Contact with DON and crude extract was always avoided. Contaminated glassware was soaked in 10 % bleach solution overnight.

Results

Toxin Formation

The concentration of AcDON reached a maximum of 2840 mg/kg from an average of two duplicate analyses, two replicates and two independent experiments on day 9 (Fig. 1). According to statistical analysis neither the two experiments nor the two replicates of these experiments differed significantly from each other in toxin concentration after 9 days of culture. From 1.4 kg culture grown in 70 baby food jars after 9 days of culture 3520 mg AcDON was detected.

Toxin purification

With 10% methanol in water 76% of the added 100 µg AcDON/g was extracted. In the large scale experiment 93% of the AcDON in the ethyl-acetate fraction was obtained after chromatography over florisil. 81% of the column chromatographed AcDON could be crystallised. Overall, from 1.4 kg culture grown in 70 baby food jars, 2650 mg AcDON could be crystallised. After ion exchange chromatography 2020 mg DON crystals could be obtained. Purity of the DON crystals assessed by GC indicated a purity of 95%. GC-MS confirmed this result.

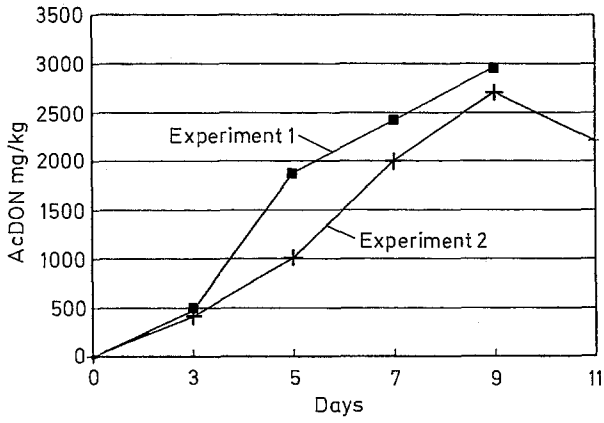

Fig. 1 3-Acetyldeoxynivalenol (AcDON) formation of *Fusarium graminearum* DSMO 4258 on rice

Table 1 Analysis of variance of the 3-acetyldeoxynivalenol content after 9 days of culture in independent experiments (*DF* degrees of freedom, *MS* mean squares, *F* F-test)

Source of variance	DF	MS	F
Replicates	1	327.77	2.24[a]
Experiments	1	65.28	0.45[a]
Error	1	146.31	

[a] Not significant at the 0.05 level

Discussion

A suitable method for AcDON and DON production is required to fulfil the following criteria. The toxin formation should reproducibly provide a highly concentrated source of toxin. A simple and convenient clean up should be possible. The method should be inexpensive. Ideally both toxins should be produced by one method.

The maximum after 9 days of 2840 mg AcDON/kg was detected on average from two duplicate analyses, two replicates and two independent experiments, which did not differ significantly from each other, indicating high reproducibility. This was confirmed by detecting 2514 mg AcDON/ kg culture in the large scale experiment. Schuster et al. (1987) detected a maximum of 170 mg AcDON/l from *F. graminearum* DSMO 4257 in shaken rice meal broth. Miller and Blackwell (1986) reported 710 mg AcDON/l after a fermentation of *F. culmorum* HLX 1503. Engelhardt et al. (1986) detected 917 mg AcDON/kg from *F. graminearum* DSMO 4258 (described as *F. tricinctum* 434), the same isolate as ours, after 10 days of culture on rice, indicating that with the described inoculation and culture method we could increase the toxin concentration more than threefold. Unfortunately, none of the cited authors gave information concerning reproducibility.

Schuster et al. (1987) regarded the existing methods for preparation of gram quantities of DON from solid substrate (Ehrlich and Lillehoj 1984; Witt et al. 1985) to be less suitable than the shaken liquid culture due to the labour intensive clean up. These methods and especially the shaken liquid culture do not provide a highly concentrated source of toxin, which is a basic requirement for convenient and time-saving production. Ehrlich and Lillehoj (1984) reported an overall yield of 450 mg of 71% pure DON/kg culture, and Witt et al. (1985) detected 600–700 mg DON/kg culture, but both authors did not give an overall yield of the pure DON. With the method described here, after a simple clean-up, an overall yield of 1890 mg crystallised AcDON/kg culture was obtained, which could be changed into 1440 mg of 95% pure DON/kg culture. AcDON production with a fermentor (Miller and Blackwell 1986) is an elegant method, but a fermentor is too expensive for most laboratories.

In summary, the procedure described herein, reproducibly provides a highly concentrated source of toxin. The clean up in a single convenient step required a minimum amount of technical equipment and chemicals. Compared to other reported methods not only crystallised DON (Ehrlich and Lillehoj 1984, Witt et al. 1985) but also crystallised AcDON was produced.

Acknowledgements The authors thank J.D. Miller, PRC Ottawa, for introduction to toxin production. The following members of our university kindly supplied equipment (H.M. Müller, HPLC), GC analysis (J. Reimann), or GC-MS analysis (I. Klaiber). This work was supported by a grant of the Federal Ministry of Research and Technology, Bonn.

References

Ehrlich KC, Lillehoj EB (1984) Simple method for isolation of 4-deoxynivalenol from rice inoculated with *Fusarium graminearum*. Appl Environ Microbiol 48:1053–1054

Engelhardt G, Schuster M, Lepschy J, Wallnöfer PR (1986) Production of mycotoxins by *Fusarium* species isolated in Germany. 1. Time course of deoxynivalenol, 3-acetyldeoxynivalenol and zearalenone formation on solid substrates. Z Lebensm Unters Forsch 182:123–126

Greenhalgh R, Levandier D, Adams W, Miller JD, Blackwell BA, McAllees AC, Taylor A (1986) Production and characterisation of deoxynivalenol and other secondary metabolites of *Fusarium culmorum* (CMI 14764,HLX 1503). J Agric Food Chem 34:98–102

Gruber-Schley S (1987) Fusarien und ihre Toxine im Getreide unter besonderer Berücksichtigung von Mais. Ph.D. thesis. University Hohenheim, Stuttgart

Menke-Milczarek I, Zimny J (1991) Phytotoxity of deoxynivalenol to wheat calli. Mycotoxin Research 7, part 2. pp 146–149

Maier FJ, Oettler G (1993) Selection for *Fusarium* toxin deoxynivalenol in callus cultures of triticale. Hod Rosl Aklim Nasien (special edition) 37:43–49

Miller JD (1989) Effects of *Fusarium graminearum* metabolites on wheat cells. In: Graniti A et al (eds) Phytotoxins and plant pathogenesis (NATO ASI Series, vol H27). Springer, Berlin Heidelberg New York pp 449–452

Miller JD, Blackwell BA (1986) Biosynthesis of 3-acetyldeoxynivalenol and other metabolites by *Fusarium culmorum* HLX 1503 in a stirred jar fermenter. Can J Bot 64:1–5

Nirenberg HI (1981) A simplified method for identifying *Fusarium* spp. occurring on wheat. Can J Bot 59:1599–1609

Posselt UK (1990) In vitro selection in forage crops. In: Proceedings of the EUCARPIA Fodder Crops Section Meeting, 18–22 November, Pudoc Wageningen, The Netherlands, 117–121

Schuster M, Lepschy J, Biber A, Engelhardt G, Wallnöfer PR (1987) Production of mycotoxins isolated in Germany. 2. Time course of deoxynivalenol and 3-acetyldeoxynivalenol formation by *Fusarium graminearum* in different liquid media. Z Lebensm Unters Forsch 185:477–480

Scott PM, Kanhere SR, Tarter EJ (1986) Determination of nivalenol and deoxynivalenol in cereals by electron-capture gas chromatography. J Assoc Off Anal Chem 69:889–893

Shimada T, Otani M (1990) Effects of *Fusarium* mycotoxins on the growth of shoots and roots at germination in some Japanese wheat cultivars. Cereal Res Commun 18:229-232

Snijders CHA, Krechting CF (1992) Inhibition of translocation and fungal colonisation in *Fusarium* head blight resistant wheat. Euphytica 50:11–18

Vesonder RF, Ellis JJ, Kwolek WF, Demarini DJ (1982) Production of vomitoxin on corn by *Fusarium graminearum* NRRL 5883 and *Fusarium roseum* NRRL 6101. Appl Environ Microbiol 43:967–970

Wang YZ, Miller JD (1988). Effects of *Fusarium graminearum* metabolites on wheat tissue in relation to *Fusarium* head blight resistance. J Phytopathol 122:118–125

Witt MF, Hart LP, Pestka JJ (1985) Purification of deoxynivalenol (vomitoxin) by water-saturated silica gel chromatography. J Agric Food Chem 33:745–748

Yoshizawa T, Morooka N (1973) Deoxynivalenol and its monoacetate: new mycotoxins from *Fusarium roseum* and moldy barley. Agric Biol Chem 37:2933–2934