Research Note

Relationship between the Stages of Development of Fusarium moniliforme ATCC 38932 and Production of Fusarin C

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ABSTRACT

A study of the kinetics of fusarin C production by Fusarium moniliforme ATCC 38932, a known producer of fusarin C, was carried out. This strain was subcultured on an EG medium for an adequate sporulation, and a 4% inoculum was transferred to the 10% ICI N medium. The conditions for the production of fusarin C in this synthetic culture medium were optimized. The time-course study of fusarin C performed over 26 days with this strain showed three different developmental stages in which a maximum production of fusarin C was reached on the 8th day of incubation; thereafter this strain ceased growing exponentially and exhibited a sharp decrease of fusarin C from that moment on.

Key words: Development stages, fusarin C, Fusarium moniliforme, mycotoxin

Mycotoxins are thought to be produced during the logarithmic growing stage of a fungus (2). Fusarin C was first isolated from extracts of a North American strain of Fusarium moniliforme Sheldon by Wiebe and Bjeldanes (11). This mycotoxin is labile in UV light and heat, and it seems that the organisms produce fusarin C in response to various environmental stresses, such as restriction of nutrients (5). Ventilation, pH, and to a lesser extent temperature seem to play a critical role in fusarin C production in a liquid medium (3, 4). A critical concentration of O2 as well as CO2 is required to obtain the optimal production of fusarin C (3, 5). In bacterial systems, fusarin C has been shown to be a powerful mutagen with an activity comparable to that of aflatoxin B1 and sterigmatocystin (6, 7). The objective of this work was to study the kinetics of fusarin C production by F. moniliforme ATCC 38932 in a synthetic culture medium.

MATERIALS AND METHODS

Type culture

A Fusarium type culture, a known fusarin C producer, F. moniliforme ATCC 38932, was obtained by Dr. Kuhlman, South eastern Forest Experimental Station, USDA, and kindly supplied to us by the Department of Organic Chemistry of the University of Granada, Spain (1).

Fusarin C production

Fusarium moniliforme ATCC 38932 was subcultured on EG medium (1 g of glucose, 1 g of NH4NO3, 1 g of KH2PO4, 0.5 g of MgSO4·7H2O, 1 g of yeast extract, and 1,000 ml of distilled water; pH 5.5) for 5 to 6 days at 28°C for an adequate sporulation. A 0.05% Tween 80-sterile distilled water suspension of conidia (106 spores per ml) was prepared from this subculture, and a 4% inoculum was transferred to the medium for fusarin C production.

For the study of biological production of fusarin C, Fusarium ATCC 38932 was grown in a modified 10% ICI N medium (80 g of glucose, 0.48 g of NH4NO3, 5 g of KH2PO4, 1 g of MgSO4·7H2O, 2 ml of a solution of microelements, and 1,000 ml of distilled water; pH 5.5). The solution of microelements consisted of the following inorganic salts dissolved in 100 ml of distilled water: 0.1 g of FeSO4·7H2O, 0.15 g of CuSO4·5H2O, 0.161 g of ZnSO4·7H2O, 0.01 g of MnSO4·7H2O, and 0.0075 g of NaMoO4·2H2O. Triplicate flasks containing the defined medium were shaken continuously at 250 rpm from 3 to 26 days in the dark at 20°C.

Extraction and cleanup procedure

As fusarin C is not stable under UV light, precautions were taken to exclude light by operating under “gold” fluorescent lighting (Westinghouse 6YT 126D) during extraction, cleanup, and determination procedures. Cultures in liquid media were vacuum filtered, and the lyophilized mycelia ground and extracted several times with 3-pentanone. For the mycelium weight determination, 2.5 ml of diluted culture was passed through a washed and preweighed 13-mm-diameter 0.45-μm-pore-size Millipore membrane filter. The filter was washed with 2.5 ml of sterile distilled water, dried to constant weight, and weighed.

Fusarin C was extracted three times with 1.5 volumes of chloroform. The combined extracts were dried over anhydrous sodium sulfate, concentrated under reduced pressure at 45°C, and evaporated to dryness under a gentle stream of nitrogen.

For purification, we followed the method described by Barrero et al. (1); thus, the residue was resuspended in 0.1 ml of hexane/diethyl ether (1:1) and fractioned on a silica-gel column (1.0 by 10 cm, Kieselgel 40; 70/230 mesh ASTM) (E Merck, Darmstadt, Germany), which was sequentially washed with hexane/...
diethyl ether (1:1), diethyl ether/ethyl acetate (9:1), (3:1), and (1:1), fusarin C being eluted with the last wash. The eluant was concentrated under vacuum at 45°C, evaporated to dryness under a gentle stream of nitrogen, and stored at −12°C for less than 3 days to prevent fusarin C from starting degradation, as was confirmed by high-performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR).

**Analysis of fusarin C**

The analytical methods used for the detection and determination of fusarin C in the purified extracts were as follows.

**Spectrophotometry.** A Beckman DU-70 spectrophotometer (Beckman Instruments Division, Berkeley, CA) set at 340 to 370 nm was used for determination of UV spectra, and confirmation of the presence or absence of fusarin C in the purified extracts.

**Thin-layer chromatography (TLC).** TLC plates (silica gel IB2-F) (J.T. Baker Inc., Phillipsburg, NJ, USA) were spotted with different amounts of extract and developed with diethyl ether/ethyl acetate/methanol (5:5:2). Positive samples were identified by the presence of a bright yellow spot under 366 nm (Rf value 0.36).

**High performance liquid chromatography (HPLC).** After having tested various methods (1, 3, 4, 6, 10) in order to analyse fusarin C in the different purified extracts which contain this mycotoxin, we chose the method proposed by Tseng et al. (10), as we obtained a better resolution of the peaks of fusarin C and its isomer 8-Z. In this case, we used a Waters model 600E liquid chromatograph coupled to a Waters model 464 variable UV wavelength detector set at 365 nm, and a Waters C18 column (3.9 by 300 mm). The mobile phase used in the isocratic system was methanol/chloroform (1:19) at a 0.5 ml/min flow rate; injection, 20 μl. We optimized this method by changing the methanol/chloroform ratio in the mobile phase, using a 52:48 ratio instead of 5:95 as proposed by this method.

**Nuclear magnetic resonance (NMR).** NMR spectra were obtained at 300 MHz (Bruker Instr., MA, USA) in 100% CHCl₃ as an internal standard. Confirmation of fusarin C in the extracts was performed by comparison of its spectroscopic data with those in the literature (1).

**RESULTS AND DISCUSSION**

If we observe Figure 1, the low fusarin C production during the first 6 days is quite remarkable. In fact, no production of fusarin C on the second day of incubation was detected, whereas from the 3rd day onwards some production (3.56% on the 3rd day) was quantified. However, there was a sharp increase in the content of mycotoxin in the synthetic culture medium between the 6th and 8th day, having reached the maximum of fusarin C production on the 8th day (6.1 mg of fusarin C per g dry weight of strain ATCC 38932 mycelium). A gradual decrease in fusarin C production happened from the following day onwards, as 34.23% fusarin C was produced on the 11th day, 8.66% on the 15th, and 3.28% on the 26th day. Therefore, it can be stated the kinetics of fusarin C production is divided into three stages.

A. **First stage, day 0 to day 6.** This is a lag stage in which the fungus is growing and, therefore, barely produces any of the mycotoxin. The maximum growth of ATCC 38932 mycelia in 10% ICI N liquid medium was reached on the 6th day of incubation.

B. **Second stage, from day 6 to day 8.** After the fungus has fully grown, an exponential increase in the fusarin C production was observed. The calculations concerning the fusarin C content in the synthetic medium were carried out by means of area integration with HPLC (maximum production: 200 μg/liter of ICI medium).

This time-course study of fusarin C production performed with the Fusarium moniliforme ATCC 38932 that showed a maximum of production over days 6 to 8 is in agreement with the results obtained with a North American F. moniliforme strain, the B33A isolate (9). This strain showed a maximum of fusarin C production in a synthetic liquid culture during day 8, with a quick decrease from that time onwards. These results are significantly different from those described by Farber and Sanders (4), who found that in a synthetic liquid medium the largest amounts of fusarin C were produced at lag stage between days 2 and 6, with production stopping until a slight increase occurred between days 12 and 14. In this case, the pH dropped rapidly from around 6.2 to 3.0 within 48 h and then remained constant throughout the fermentation; whereas in our case, the initial pH value of the ICI medium was 6.0 to 6.1, reaching a pH of 4.3 after 26 days. Glucose decreased fairly rapidly between days 4 and 8, but was never completely utilized by the fungus (residual sugar was 1.7 mg/ml of glucose).

C. **Third stage, from 9th day onwards.** Levels of fusarin C were seen to decrease quickly and continuously, probably due to destabilizing biochemical reactions or enzymatic degradation associated with fungal growth (8, 12) (mycotoxin dry weight: 4.2 g/liter of ICI medium), given that this mycotoxin is very unstable over the course of time, with isomerizations and interconversions to other compounds derived from this mycotoxin being produced (9). Three isomers of fusarin C were identified and, on the basis of their 1H NMR chemical shifts and (H, H) coupling constants, were assigned the 8Z, 6Z, and 10Z stereochemistry, respectively (Figure 2). On the 21st day, more than half of the fusarin C present disappeared. After 26 days of incubation,
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REFERENCES