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# Production of fusaric acid by *Fusarium oxysporum*

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**Summary**

The aim of the study was to determine whether isolates of *Fusarium oxysporum* Schlecht. produce fusaric acid in culture and whether mycelial growth can be used as a measurement of fusaric acid production. Three isolates produced significant levels of fusaric acid in culture filtrate, the maximum concentration being reached after 18 days, whereafter it remained constant. Mycelial growth could not be used to measure or estimate the production of fusaric acid. Growth curves from specific isolates could be used to determine the concentration of fusaric acid in culture filtrate after specific lengths of time.

## Introduction

Plant pathogens produce various kinds of toxic compounds to culture media as well as in plant tissues (Drysdale, 1982). These compounds cause a series of morphological and biochemical changes in plant tissues and contribute to the pathogenicity or virulence of the organisms producing the toxin. *Fusarium* sp. are known to produce several toxins including fusaric acid (Drysdale, 1982; Hardborne, 1983). This toxin has been studied extensively (Julien, 1988; Kern, 1972; Löffler & Morris, 1992; Matsui & Watanabe, 1988; Mégnégneau & Branchard, 1988). The production of fusaric acid by a bulb rot producing *Fusarium* sp. was first reported by Löffler & Morris (1992). This paper is the first report of the production of fusaric acid by *Fusarium oxysporum* Schlecht, emend Snyd. & Hans, which causes rot of potato tubers.

## Materials and methods

*Fungal isolates.* We used three isolates of *Fusarium oxysporum* (F.o.1, F.o.4 and F.o.6) which had been isolated from potato tubers with dry rot symptoms. Their virulence had been verified by Theron & Holz (1989).

*Culture filtrate.* Erlenmeyer flasks containing 30 ml culture medium were inoculated with single agar plugs (1 $mm^3$) of one of the three isolates. The following culture medium was used:
0.74 g $MgSO_4 \cdot 7H_2O$; 0.045 g $MnSO_4 \cdot 4H_2O$; 0.018 g $ZnSO_4 \cdot 7H_2O$; 5.0 µg $CuSO_4 \cdot SH_2O$; 0.084 g $FeSO_4 \cdot 7H_2$); 0.11 g $Na_2EDTA$; 0.1 g myo-inositol; 2.0 mg glycine; 0.5 mg nicotinic acid; 0.5 mg pyridoxine.HCl; 0.1 mg thiamine.HCl; 10.0 g

glucose; 2.0 g L-asparagine; 1.0 g $KH_2PO_4$; 5.0 µg biotin; 1,000 ml distilled water. The flasks were incubated at 22±1 °C on an orbital shaker (50 r.p.m.) for the duration of the experiment.

*Mycelial growth.* Mycelial growth was determined every 24 h for 30 days. The mycelium and culture medium from five flasks were individually separated by a Buchner funnel, the mycelium weighed, and the mean fresh weight (g) recorded.

*Toxin preparation and extraction.* Crude filtrates and a control of culture medium only were acidified with 1 N HCl and extracted with an equal volume of ethyl acetate. The organic phase was removed and dried under vacuum, the residues dissolved with 5 ml methanol and stored at -20 °C. The fusaric acid peak of the samples was identified by co-elution with pure fusaric acid added to the sample. Extraction efficiency was determined by spiking half of the sample with 0.015 mg $ml^{-1}$ pure fusaric acid (5-butyl picolinic acid) (Sigma Chemical Company, U.S.A.) prior to acidification. Eight replicates were extracted.

*Analysis of fusaric acid.* Culture filtrates were collected every 24 h for 32 days and tested for the presence of fusaric acid using reversed phase High Performance Liquid Chromatography (HPLC) on a Bondclone 10 C18 column (10 µm, 300 x 3.9 mm). A 50 µl aliquot of culture filtrate, culture medium or pure fusaric acid was injected and eluted isocratically with eluting buffer. The buffer was prepared by mixing (v/v) 40% methanol and 60% of an aqueous solution of 0.62 mM $Na_2EDTA$ and 2% $H_3PO_4$ (Julien, 1988). The absorption was recorded with a UV detector at 254 nm, using a Beckman system Gold model injector, pump and UV detector Model 168. Standard curves were established with pure fusaric acid. The column was stored in 100% methanol and equilibrated with eluting buffer 1 h before use. Samples were injected as methanol solutions.

## Results and discussion

*Extraction efficiency.* Fusaric acid eluted in a reproducible manner as a sharp peak with an elution time of 5.56 minutes and a temperature of 20±1 °C. Pure fusaric acid co-injected with the sample increased the height of the peak, and the product eluted with this peak had the characteristic UV spectrum of fusaric acid. The extraction efficiency (% fusaric acid recovered) was determined from day 0 to day 4 for all three isolates by spiking half of the sample with 0.015 mg/ml pure fusaric acid. Fusaric acid (85.46%) was recovered with a coefficient of variance of 8.1%.

The growth- and fusaric acid production curves for the three isolates are shown in Fig. 1A-C. The variation of 92.0%, 89.7% and 93.0% encountered for the fresh mass (Fig. 1A-C) can be described by quadratic curves, and the variation of 99.8%, 99.8% and 98.5% encountered for fusaric acid production (Fig. 1A-C) can be described by exponential curves. Isolate F.o.1 showed the least mycelium growth and isolate F.o.6 the most; isolate F.o.1 produced the most fusaric acid and F.o.6 the least. Both these

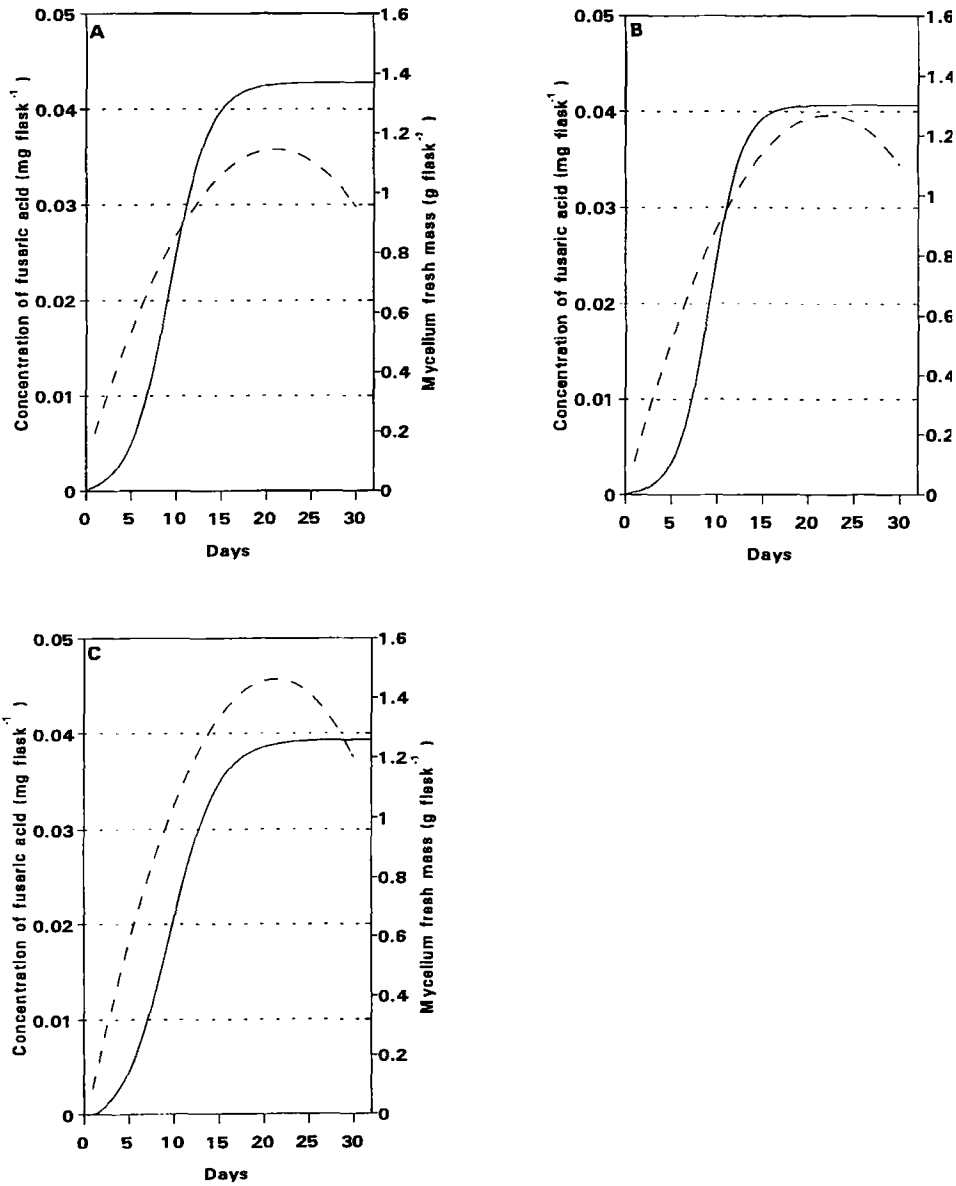


Fig. 1. Fusaric acid production (-) and mycelium growth (- - -) of isolates of *Fusarium oxysporum*. A = isolate F.o.1, B = F.o.4, C = F.o.6.

Table 1. Analysis of variance of the results obtained from the growth (g fresh weight) measured over 32 days of three isolates of *Fusarium oxysporum* with five replicates.

| Source of variation | Degrees of freedom | Sum of squares | Mean of squares | F-value | p-value |
|---|---|---|---|---|---|
| **Factors** | 23 | 45.2316 | | | |
| F. oxysporum isolates | 2 | 1.7550 | 0.7749 | 114.73 | <0.001 |
| Days | 21 | 43.4767 | 2.0703 | 270.70 | <0.001 |
| **Interaction** | 306 | 3.9915 | | | |
| F. oxysporum ´ days | 42 | 1.9724 | 0.0470 | 6.14 | <0.001 |
| Residual | 264 | 2.0191 | 0.0076 | | |
| **Total** | 329 | 49.2231 | | | |
| CV = 9.7% | | | | | |

differences were significant ($p<0.001$). The growth curves for all three isolates showed a logarithmic growth phase from days 2–18, after which a short stationary phase followed. Mycelium growth decreased after 20 days. At day 20 the fresh weight reached 1.46 g for isolate F.o.6, 1.26 g for isolate F.o.4 and 1.14 g for isolate F.o.1.

*Mycelial growth and fusaric acid production.* Most variation resulted from the main factor, which was days (Table 1). The variation explained by this factor was 96.1%, leaving 3.9% for the isolates. With reference to total variation, 88.3% of the variance was explained by days, with only 3.6% by isolates. The interaction of days and isolates explained 4.0% of the total variance. The variation between days and isolates and the interaction of days and isolates was statistically significant ($p<0.001$).

In Table 2 it is apparent that the most variation (99.6%) again occurred as a result of days against 0.37% for isolates. Of total variation, 99.2% of the variance was explained by days, while the interaction days x isolates explained 0.55%. The variation between days and isolates and the interaction of days and isolates was statistically significant ($p<0.001$). The interaction according to mycelial growth (4.0%) was more important than that according to fusaric acid production (0.04%), but in both cases it was low.

Table 2. Analysis of variance of the results obtained from fusaric acid production (mg $ml^{-1}$) measured over 32 days by three *Fusarium oxysporum* isolates with eight replicates.

| Source of variation | Degrees of freedom | Sum of squares | Mean squares | F-value | p-value |
|---|---|---|---|---|---|
| **Factors** | 20 | 0.1266 | | | |
| F. oxysporum isolates | 2 | 0.0005 | 0.0002 | 114.73 | <0.001 |
| Days | 18 | 0.126 | 0.0070 | 270.70 | <0.001 |
| **Interaction** | 425 | 0.0006 | | | |
| F. oxysporum- days | 36 | 0.0007 | 0.0000 | 6.14 | <0.001 |
| Residual | 389 | 0.0005 | 0.0000 | | |
| **Total** | 445 | | 0.127 | | |
| CV = 1.8% | | | | | |

The fusaric acid production curves showed a lag phase from days 0–5 and an exponential growth phase from days 6–18. After day 18 a stationary phase was reached and the fusaric acid concentration remained constant from then until day 32, when the experiment ended. The concentration of fusaric acid differed significantly between the different isolates. On day 20 the difference was 0.042 mg $ml^{-1}$ for isolate F.o.1, 0.040 mg $ml^{-1}$ for F.o.4 and 0.038 mg $ml^{-1}$ for isolate F.o.6. When determining the fusaric acid production it is important to determine the concentration after the stationary phase has been reached.

It is apparent that mycelial growth cannot be used to measure or estimate fusaric acid production, because the functions explaining them differ. It is possible that a growth curve from specific isolates might be used to determine fusaric acid production.

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