Glucose and zinc concentration influence fusarin C synthesis, ethanol synthesis and lipid composition in *Fusarium moniliforme* submerged cultures *

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Abstract: A fusarin C-producing *Fusarium moniliforme* strain was grown in submerged culture with defined media containing differing amounts of glucose (30 g/l or 90 g/l) and zinc (5 ppb or 3200 ppb). The influence of zinc on fusarin C synthesis and lipid composition was dependent on the initial glucose concentration. In cultures supplied with 30 g/l glucose, zinc inhibited fusarin C and lipid synthesis by diverting common substrates to ethanol synthesis. Zinc-supplemented cultures with 90 g/l glucose had ample carbon substrate to produce both ethanol and fusarin C. More total lipid with a higher unsaturated fatty acid content (more oleic acid and less stearic acid) was found in *F. moniliforme* biomass produced in zinc-deficient media.

Key words: *Fusarium moniliforme*; Polyketide; Mycotoxin; Fatty acid composition; Stearic acid; Oleic acid

Introduction

Strains of *Fusarium moniliforme* produce numerous disease-causing secondary metabolites [1–5] including the mycotoxin fusarin C [6]. Fusarin C, C_{23}H_{29}NO_{7}, is a polyketide secondary metabolite whose structure consists of a polyene with a substituted 2-pyrollidone moiety (Fig. 1) [7]. Biosynthetic studies indicate that the polyene portion of the fusarins is formed by the condensation of a C_{14}-polyketide, derived from an acetyl-CoA starter unit and six malonyl-CoA units, plus oxaloacetate [8]. The origin of the 2-pyrollidone moiety is uncertain.

It has been shown that a zinc-deficient medium enhanced fusarin C and lipid biosynthesis in *F. moniliforme* NRRL 13616 by shunting carbon into the above compounds at the expense of dry weight accumulation [9]. The association between lipid and fusarin C synthesis is not surprising since both compounds are synthesized from similar precursors. It has also been shown that, in a zinc-deficient medium, carbohydrate availability...
is the limiting factor for fusarin C synthesis [10]. That is, cultures grown in a zinc-deficient medium in which glucose was still available following 9 days growth (initial glucose concentration of 60 or 90 g/l) produced significantly more fusarin C than cultures grown in media with 30 g glucose/l (glucose was exhausted after 7 days growth).

In the present study, the interaction of zinc and glucose concentration on F. moniliforme metabolism was investigated. Since fusarin C is a lipid-like compound, the impact of zinc on the composition of fatty acids produced under differing nutritional conditions was evaluated. Ethanol biosynthesis was measured because it requires substrates common to lipid and fusarin C synthesis and because alcohol dehydrogenase, a key enzyme in the ethanol biosynthetic pathway, has a zinc cofactor requirement.

**Materials and Methods**

*Culture inoculum and media components*

Cultures of F. moniliforme NRRL 13616 were stored and spore inocula produced as previously described [9]. The initial spore concentration in all submerged cultures was $5 \times 10^6$ spores/ml.

The basal medium used in all submerged culture experiments has been previously described [10]. The basal medium was supplemented with glucose (30 g/l or 90 g/l) and ammonium sulfate (1.4 g/l with 30 g/l glucose or 4.2 g/l with 90 g/l glucose) as carbon and nitrogen sources. Glucose stock solutions were autoclaved separately.

A stock solution ($100 \times$) of zinc sulfate heptahydrate, 1.4 g/l, was used to supply zinc at a concentration of 49 $\mu$M (3200 ppb), as required. Media not supplemented with zinc contained 4 ppb zinc [10].

*Submerged culture*

Submerged culture experiments were carried out in triplicate in 500-ml baffled Erlenmeyer flasks (Belco Glass, Inc.) each containing a 250-ml volume. All experiments were performed at least twice. Cultures were grown for 9 days at 220 rev min$^{-1}$ and 28°C in a rotary shaker incubator.

A pH of 5 was maintained by daily adjustment with either 2 N hydrochloric acid or 2 N sodium hydroxide.

*Analytical methods*

Glucose, dry weight, and fusarin C were measured by previously described methods [9,11]. Ethanol was measured on a gas chromatograph (Hewlett Packard 5890A) equipped with a 6 ft Porapak Q$^\circ$ column and flame ionization detection (FID).

*Lipid analyses*

Total lipids were extracted from F. moniliforme cells hydrolyzed in 1 N HCl, as previously described [9]. Lipid extracts of F. moniliforme cells were centrifuged (1000 rpm x 5 min) at 12°C to remove solids. Aliquots of clarified lipid samples were dissolved in hexane and injected onto a Dynamax 60-A silica HPLC column (250 mm x 10 mm i.d., Rainin Instruments, Woburn, MA). Samples were gradient-eluted using a SP8800 pump (Spectra Physics, San Jose, CA) as follows: hexane/acetone (97:3 v/v) to hexane/acetone (93:7 v/v) in 40 min, hold 5 min, then to 100% acetone at 46 min, hold 34 min. The mobile phase flow rate was 1 ml/min and was post-column split, 30% to the detector and 70% for collection. Column eluant fractions were collected corresponding to peaks detected using an evaporate light scattering detector (ELSDII, Varex Corporation, Rockville, MD) operated at 105°C with 60 cm$^3$/min nitrogen flow through the nebulizer. Solvent was removed from samples containing lipid components using a rotatory evaporator. Lipid components were dissolved in ethyl ether and transferred to tared vials. The ethyl ether was evaporated under a stream of nitrogen and lipid component weights were determined.

Identification of the major lipid fractions as triglycerides was made by thin-layer chromatography (TLC) using lipid standards. Collected fractions were spotted on silica gel 60 TLC plates (5 cm x 20 cm x 0.25 mm, Brinkman Instruments, Westbury, NY) and developed in benzene/ petroleum ether/ethyl ether (8:2:1, v/v/v). The percentage of triglycerides in the collected lipid
fractions was calculated using the weights of the various fractions.

Fatty acid profiles for the triglyceride fractions were determined using fatty acid methyl esters. Methyl esters of the fatty acids were prepared by heating (65°C) samples in 5% HCl-MeOH (HCl gas bubbled into dry methanol) for 2 h. Methyl esters were separated and measured by gas chromatography (Hewlett Packard 5890 with FID) using a cyano silicone capillary column (SP-2330, 30 m × 0.25 mm, 0.2 μm coating thickness, Supelco, Bellefonte, PA) operated isothermally at 190°C. Known methyl ester standards of palmitic, stearic, oleic and linoleic acids were used to identify major peaks.

Results and Discussion

These studies suggest that zinc inhibits fusarin C synthesis in cultures provided with limited glucose (30 g/l), in part, by funneling available carbon into ethanol synthesis. In zinc-supplemented media, *F. moniliforme* cultures utilized approximately one-third of the available glucose for ethanol production. In 9-day-old cultures, an ethanol concentration of 5.3 mg/ml was reached with only 3 μg fusarin C/mg dry weight (Table 1). Without zinc supplement, the fusarin C concentration was 28 μg/mg dry weight with no ethanol production.

Regardless of the zinc content of the medium, fusarin C synthesis in cultures grown in media with 90 g/l glucose was similar (Table 1). The excess carbohydrate available in media with 90 g glucose/l provided adequate substrate for fusarin C and ethanol synthesis (Table 1). Analogous studies with *Rhizopus nigricans* showed that high levels of glucose negated the inhibitory effect of zinc on fumaric acid biosynthesis [12].

Previous studies [9] have shown that zinc supplementation not only inhibited fusarin C synthesis in *F. moniliforme*, but also inhibited lipid synthesis while enhancing biomass accumulation. The present study showed that *F. moniliforme* biomass produced in zinc-deficient media contained significantly more lipid than did biomass from zinc-supplemented media (Table 2). Analyses of the lipid fractions in the current study indicated that approximately 85% of the total lipid was triglyceride, regardless of the zinc or glucose concentration of the medium (Table 2). The presence of numerous highly refractive lipid droplets reported earlier in *F. moniliforme* hyphae produced in zinc-deficient media [9] supports the present compositional data which show that triglycerides comprise over 55% of the dry weight in these cultures (Table 2).

The principal fatty acids present in the triglyceride fraction were palmitic (16:0), stearic (18:0), oleic (18:1) and linoleic (18:2). These four fatty acids were approximately 96% of the total fatty acids measured. The fatty acids obtained from cultures grown in zinc-deficient media with 30 g or 90 g glucose/l were significantly more unsaturated compared to fatty acids obtained from cultures grown in a zinc-supplemented medium with 30 g glucose/l (Table 2). This increase in unsat-

Table 1
Effect of zinc on ethanol and fusarin C synthesis by *Fusarium moniliforme* submerged cultures

<table>
<thead>
<tr>
<th>Initial glucose concentration (g/l)</th>
<th>Zinc (3200 ppb)</th>
<th>Ethanol (mg/ml)</th>
<th>Fusarin C (μg/mg dry weight)</th>
<th>Residual glucose (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>Plus</td>
<td>5.3±0.1 b</td>
<td>3.0±2.9</td>
<td>0.9±0.7</td>
</tr>
<tr>
<td>90</td>
<td>Minus</td>
<td>0.0±0.0</td>
<td>28 ± 9.5</td>
<td>1.1 ± 0.6</td>
</tr>
<tr>
<td>30</td>
<td>Plus</td>
<td>17.0±2.4</td>
<td>26 ± 3.2</td>
<td>30.0±2.3</td>
</tr>
<tr>
<td>90</td>
<td>Minus</td>
<td>0.0±0.0</td>
<td>29 ± 4.8</td>
<td>49.3±4.7</td>
</tr>
</tbody>
</table>

a Analyses performed on 9-day-old culture samples. Values are the average of three replicates.
b ± Standard deviation.
Table 2

The influence of zinc and glucose concentration on the lipid composition of 9-day-old *F. moniliforme* cultures

<table>
<thead>
<tr>
<th>Nutritional environment</th>
<th>3% Glucose</th>
<th>9% Glucose</th>
<th>LSD (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(-) Zn (^a)</td>
<td>(+) Zn (^a)</td>
<td>(-) Zn (^a)</td>
</tr>
<tr>
<td>Lipid (% of dry weight)</td>
<td>43</td>
<td>26</td>
<td>40</td>
</tr>
<tr>
<td>Lipid (% triglyceride)</td>
<td>89</td>
<td>73</td>
<td>91</td>
</tr>
</tbody>
</table>

Fatty acids \(^c\)

| % Palmitic (16:0) | 19.8 | 22.3 | 19.8 | 21.6 | NSD |
| % Stearic (18:0) | 6.2 | 11.0 | 6.7 | 10.8 | 3.8 (\(P < 0.05\)) |
| % Oleic (18:1)   | 47.8 | 35.8 | 46.0 | 42.1 | 8.3 (\(P < 0.10\)) |
| % Linoleic (18:2) | 22.7 | 26.0 | 24.4 | 21.3 | NSD |
| % Unsaturated    | 70.5 | 61.8 | 70.4 | 63.4 | 7.5 (\(P < 0.01\)) |

\(^a\) Zinc supplied at 3200 ppb.

\(^b\) Mean values within rows were subjected to analysis of variance using Fisher's least significant difference. NSD, not significantly different.

\(^c\) Fatty acids obtained from the triglyceride fraction.

Lipid synthesis and fatty acid composition studies in other *Fusarium* species have provided values similar to those obtained for the present *F. moniliforme* strain grown in zinc-supplemented media. Fiore [13] grew *F. lini* Bolley and *F. lycopersici* on a zinc-supplemented defined medium with 83.5 g/l glucose. His total lipid and fatty acid values were similar to the values we obtained for *F. moniliforme* cultures grown in zinc-supplemented media with 90 g/l glucose. The fatty acid profiles for *F. solani* f. *phaseoli* cultures grown on potato dextrose broth (20 g/l glucose) were similar to those we found in the present *F. moniliforme* strain grown in zinc-supplemented media with 30 g/l glucose [14].

Reports on the fatty acid composition of cultures grown in zinc-deficient media are lacking. This study showed that *F. moniliforme* cultures grown in zinc-deficient media produced more total lipid, principally triglycerides, which contained more unsaturated fatty acids compared to biomass produced in zinc-supplemented media. It has been suggested that fatty acid unsaturation occurs in response to environmental factors [15]. It is possible that the high lipid content of *F. moniliforme* cultures grown in zinc-deficient media necessitates increased lipid fluidity leading to the synthesis of more unsaturated fatty acids. Due to the highly unsaturated nature of fusarin C (Fig. 1), we suggest that conditions which enhance the synthesis of unsaturated fatty acids may also increase fusarin C synthesis.

Fusarin C synthesis is nutritionally regulated by zinc and carbohydrate availability. Zinc inhibits fusarin C synthesis under glucose-limited conditions by shunting available carbon into biomass accumulation and ethanol synthesis. Under zinc-deficient conditions or nutritional conditions that provides excess carbohydrate, fatty acid unsaturation is enhanced and ample substrate is
provided for fusaric C synthesis. The requirement of high carbon concentrations for enhanced fusaric C synthesis by *F. moniliforme* is ideally satisfied by the nutritional composition of corn kernels, a favoured habitat of this organism.

Acknowledgements

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References