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Effects of Zinc, Iron, Cobalt, and Manganese on *Fusarium moniliforme* NRRL 13616 Growth and Fusarin C Biosynthesis in Submerged Cultures

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The influence of zinc, iron, cobalt, and manganese on submerged cultures of *Fusarium moniliforme* NRRL 13616 was assessed by measuring dry weight accumulation, fusarin C biosynthesis, and ammonia assimilation. Shake flask cultures were grown in a nitrogen-limited defined medium supplemented with various combinations of metal ions according to partial-factorial experimental designs. Zinc (26 to 3,200 ppb [26 to 3,200 ng/ml]) inhibited fusarin C biosynthesis, increased dry weight accumulation, and increased ammonia assimilation. Carbohydrate was found to be the principal component of the increased dry weight in zinc-supplemented cultures. Zinc-deficient cultures synthesized more lipid and lipidlike compounds, such as fusarin C, than did zinc-supplemented cultures. Microscopic examination showed that zinc-deficient hyphae contained numerous lipid globules which were not present in zinc-supplemented cultures. Addition of zinc (3,200 ppb) to 2- and 4-day-old cultures inhibited further fusarin C biosynthesis but did not stimulate additional dry weight accumulation. Iron (10.0 ppm) and cobalt (9.0 ppm) did not affect fusarin C biosynthesis or dry weight accumulation. Manganese (5.1 ppm) did not affect dry weight accumulation but did increase fusarin C biosynthesis in the absence of zinc. Maximum fusarin C levels, 32.3 μg/mg (dry weight), were produced when cultures were supplied manganese, whereas minimum fusarin C levels, 0.07 μg/mg (dry weight), were produced when zinc, iron, cobalt, and manganese were supplied. These results suggest a multifunctional role for zinc in affecting *F. moniliforme* metabolism.

Fusarium moniliforme NRRL 13616 (perfect *Gibberella fujikuroi*) is a fungal pathogen that infects a wide variety of plant hosts and is often isolated from corn (*Zea mays*) kernels and from animal feeds (18, 19). The presence of *F. moniliforme* in grains and feeds is correlated with leukoencephalamalacia in horses, esophageal cancer in humans, and experimentally induced hepatocarcinomas in ducks and mice (13, 18, 20, 22, 25). Moniliformin, fusariocin A, fumonisin B_1 and B_2, and fusarin C are examples of toxins that have been isolated from *F. moniliforme* culture extracts (2). Moniliformin and fusariocin A are acutely toxic, whereas fusarin C exhibits mutagenic activity comparable to that of aflatoxin B_1 (2, 9, 10, 30). Fusarin C is a polyketide produced by *F. moniliforme* strains isolated from corn kernels in areas of China and South Africa with high incidences of esophageal cancer (18, 20). Fumonisins are toxins with cancer-promoting activity that have recently been isolated from *F. moniliforme* (7). The interaction of these toxins in causing disease remains uncertain.

The biosynthesis of other fungal polyketides, such as alternarial and aflatoxin, is regulated by nutritional and environmental factors (1, 14, 21, 26). Using submerged cultures of *F. moniliforme* in a defined medium, we are investigating the effects of various nutritional environments on fusarin C biosynthesis and culture growth. Reported is the influence of zinc, iron, cobalt, and manganese on cultures of *F. moniliforme* as determined by measuring fusarin C levels, dry weight accumulation, ammonia assimilation, and cellular composition.

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MATERIALS AND METHODS

Culture inoculum. Spore inocula were produced on a V-8 agar plate from a single-spore isolate of *F. moniliforme* NRRL 13616. V-8 agar consisted of 200 ml of V-8 juice, 3.0 g of calcium carbonate, and 20 g of agar per liter. The V-8 agar plate containing spores was rinsed with deionized water, and the spores were dried on silica beads for long-term storage at 4°C.

For submerged-culture inocula, silica-dried spores were grown and sporulated on 25 ml of V-8 agar in 75-cm^2 tissue culture flasks (no. 25116; Corning Glass Works, Corning, N.Y.) at room temperature. Spores were harvested by rinsing the agar surface of V-8 agar flasks with sterile-filtered, deionized water. A concentration of 5×10^6 spores per ml was used in all submerged-culture experiments.

Defined medium. A defined medium (Table 1) for submerged-culture studies was developed by evaluating *F. moniliforme* growth requirements for vitamins, amino acids, and purines and pyrimidines as previously described (31). Cultures were grown at 100-ml culture volume in duplicate 250-ml baffled Erlenmeyer flasks (Bellco Glass, Inc., Vineland, N.J.) and incubated at 28°C and 220 rpm in a rotary shaker incubator (5). Amino acids or ammonium sulfate and vitamins were necessary for optimum *F. moniliforme* culture growth.

Submerged culture. Liquid-culture experiments were carried out with a defined medium (Table 1) in duplicate 500-ml baffled Erlenmeyer flasks (Bellco no. 2543-00500) at 250-ml volume. Glucose was autoclaved separately. Stock solutions of $ZnSO_4 \cdot 7H_2O$ (1.4 g/liter), $MnSO_4 \cdot H_2O$ (1.56 g/liter), $FeSO_4 \cdot 7H_2O$ (5.0 g/liter), and $CoCl_2 \cdot 6H_2O$ (3.66 g/liter) were used to supply trace metals. Stock solutions of ferrous

TABLE 1. Defined medium for submerged culture of *F. moniliforme* NRRL 13616

Glucose	30 g/liter
$(NH_4)_2SO_4$	1.4 g/liter
KH_2PO_4	2.0 g/liter
$MgSO_4 \cdot 7H_2O$	0.3 g/liter
$CaCl_2 \cdot 2H_2O$	0.4 g/liter
Thiamine, riboflavin, pantothenate, niacin, pyridoxamine, thiotic acid	500 μg of each/liter
Folic acid, biotin, vitamin B_{12}	50 μg of each/liter
Deionized H_2O	To bring to 1.0 liter

sulfate were made fresh daily. The cultures were incubated for 9 days at 28°C and 220 rpm in a rotary shaker incubator (5). A pH of 5.0 was maintained by daily adjustment with 2 N HCl or 2 N NaOH.

Partial-factorial experimental designs were used to analyze the effects of zinc, manganese, iron, and cobalt on *F. moniliforme* growth and fusarin C synthesis (Table 2) (27). Zinc, manganese, iron, and cobalt were supplied at concentrations of 49 μM (3.2 ppm [3.2 μg/ml]), 92 μM (5.1 ppm), 180 μM (10.0 ppm), and 154 μM (9.0 ppm), respectively. Experimental results were statistically analyzed by normal analysis of variance with SAS (SAS Institute, Cary, N.C.).

The defined medium supplemented with manganese (5.1 ppm) was used to assess the influence of zinc on *F. moniliforme* cultures. Cultures were supplemented with zinc at 3,200, 640, 128, 26, and 5 ppb (3,200, 640, 128, 26, and 5 ng/ml) to determine concentration effects on dry weight accumulation and fusarin C biosynthesis. Cultures used for cellular composition analysis and timed-addition studies were supplemented with zinc at 3,200 ppb.

Analytical methods. (i) Glucose. Glucose concentrations were determined in a high-performance liquid chromatographic system (Waters Associates, Inc., Milford, Mass.) equipped with an Aminex ion exclusion column (model HPX-87H; Bio-Rad Laboratories, Richmond, Calif.) and a refractive index detector (model 401; Waters). The column was operated at room temperature, and acidified water (0.0017 N H_2SO_4) was used as the mobile phase.

(ii) Dry weight. Dry weight was determined by filtering a 2-ml culture sample on a dried and weighed 2.5-cm filter (GF/A; Whatman, Inc., Clifton, N.J.). The cell mass was rinsed three times with deionized water, dried at 90°C for 4 h, and weighed.

(iii) Fusarin C. Pure fusarin C standard was obtained by semipreparative high-performance liquid chromatography of ethyl acetate extracts from *F. moniliforme* cultures grown on MYRO medium [1.0 g of $(NH4)_2HPO_4$, 3.0 g of KH_2PO_4, 2.0 g of $MgSO_4 \cdot 7H_2O$, 5 g of NaCl, and 30 g of glucose per liter] as previously described (5). Fusarin C was purified on a Dynamax silica column (21.4 by 250 mm; Rainin Corp.) by elution with chloroform-methanol (96:4) at a flow rate of 8 ml/min. Fusarin C eluted at 31.4 min and was detected by UV A_{365}. Fusarin C standard purity was assessed by thin-layer chromatography, nuclear magnetic resonance, and mass spectral analysis (5, 8).

TABLE 2. Partial-factorial experimental designs for *F. moniliforme* NRRL 13616 trace metal studies[a]

Trace metal (ppm)	Sample no.									
	1	2	3	4	5	6	7	8	9	10
10-Sample model										
Iron (10)	0	0	0	0	0	1	1	1	1	1
Manganese (5.1)	0	0	0	1	1	0	0	1	1	1
Cobalt (9.0)	0	0	1	0	1	0	1	0	1	1
Zinc (3.2)	0	1	0	0	1	0	1	1	0	1
8-Sample model										
Zinc (3.2)	0	0	0	0	1	1	1	1		
Iron (10)	0	0	1	1	0	0	1	1		
Cobalt (9.0)	0	1	0	1	0	1	0	1		
Manganese (5.1)	0	1	1	0	1	0	0	1		

[a] From reference 27. 0, Minus trace metal; 1, plus trace metal.

The A_{365} for 10 μg of purified fusarin C per ml in chloroform-methanol (95:5) was recorded at the time of purification to assess the spontaneous and UV-induced deterioration of measurable fusarin C standard over time (10, 24). Also recorded were the retention times for the decomposition products derived from purified fusarin C (10).

Culture samples were analyzed for fusarin C by extraction of 3-ml culture samples under gold fluorescent light (Westinghouse catalog no. 6YT126D) with 3 volumes of ethyl acetate as previously described (5, 9). High-performance liquid chromatography of culture extracts was performed with a 4.6-by-250-mm Zorbax silica column (Dupont Instruments). Samples of 20 μl, with phenothiazine (250 μg/ml) as an internal standard, were applied to the column with an autosampler (WISP 710B; Waters). Isocratic elution was performed with chloroform-methanol (95:5) at 1.5 ml/min. Fusarin C was detected by UV A_{365} (variable-wavelength detector; Kratos Instruments). Fusarin C levels were measured in samples by summing the peak areas which corresponded to retention times for purified fusarin C and its decomposition products and comparing this response with peak areas for known quantities of fusarin C standard.

(iv) Chemical composition. Whole-culture samples (2 ml) were centrifuged (3,000 × *g*) for 5 min. The supernatant was removed and analyzed for ammonium ion by the indophenol blue reaction (11). The pellet was suspended in 3 ml of deionized water and centrifuged three times. The rinsed cells were resuspended in deionized water. Rinsed-cell samples were incubated in 1 N NaOH at 90°C for 10 min to solublize cellular protein. Protein was measured by the method of Lowry et al. (16), with a bovine serum albumin standard. Total carbohydrate was estimated in rinsed-cell samples by the phenol reaction (11).

Total lipids were determined by a modification of the method of Borrow et al. (3). Whole-culture samples (10 ml) were centrifuged (800 × *g*) for 5 min. The pellet was suspended in 15 ml of 1 N HCl and incubated for 60 min at 90°C. The sample was filtered on a 2.5-cm filter (Whatman GF/A) and dried in vacuo at 80°C for 2 h. The dried cell mass was removed from the filter, coarsely ground, and extracted with 15 ml of ethyl ether for 48 h. The extract mixture was filtered on a 2.5-cm GF/A filter, and the filtrate was collected in a preweighed test tube. The ethyl ether was evaporated under nitrogen at 40°C, and the residual lipid was weighed.

Photomicroscopy. Microscopic examination was performed with an Olympus BH2 microscope with phase contrast. Photomicrographs were taken with an Olympus OM-4T camera with Plus-X black-and-white film (Eastman Kodak Co., Rochester, N.Y.). Lipid globules in hyphae were detected by their ability to take up Sudan Black B (15).

RESULTS

Results of partial-factorial-designed experiments (Table 2) showed that dry weight accumulation by *F. moniliforme* cultures was unaffected by manganese, iron, and cobalt at

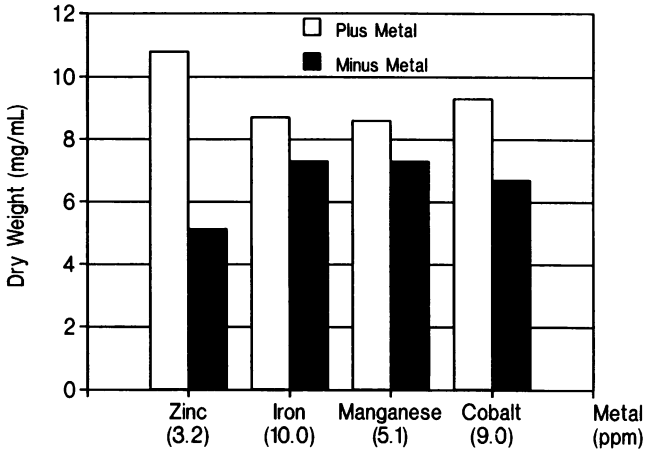

FIG. 1. Dry weight accumulated by 9-day submerged cultures of *F. moniliforme*. Dry weights are based on averages obtained from a 10-sample partial-factorial-designed experiment (Table 2). Standard deviations for dry weight mean values (in milligrams): ±1.5, plus zinc; ±0.8, minus zinc, ±3.2, plus iron; ±2.8, minus iron; ±2.4, plus manganese; ±3.5, minus manganese; ±3.2, plus cobalt; ±2.3, minus cobalt. Analyses of variance indicated that zinc significantly increased dry weight ($P = 0.0623$). At the concentrations used, iron, cobalt, and manganese had no significant effect on dry weight.

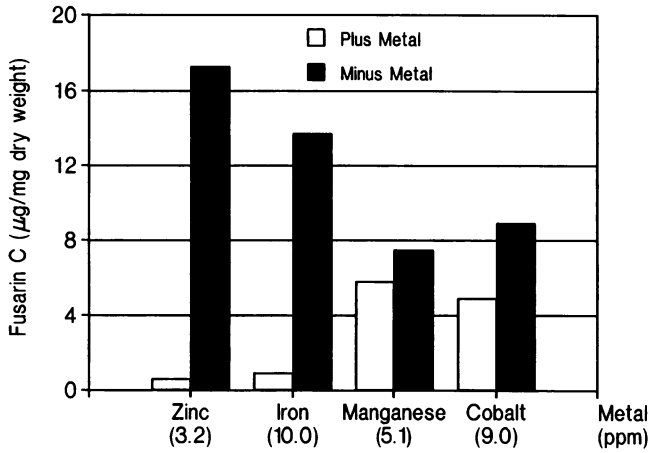

FIG. 3. Effect of trace metals on fusarin C biosynthesis in 9-day *F. moniliforme* submerged cultures. Fusarin C levels are averages obtained from a 10-sample partial-factorial-designed experiment (Table 2). Standard deviations for fusarin C mean values (micrograms per milligram [dry weight]): ±0.2, plus zinc; ±8.9, minus zinc; ±0.88, plus iron; ±11.8, minus iron; ±13.5, plus manganese; ±14.2, minus manganese; ±14.4, plus cobalt; ±13.3, minus cobalt. Analyses of variance indicated that zinc significantly decreased fusarin C biosynthesis ($P < 0.02$). Iron, cobalt, and manganese did not significantly influence fusarin C biosynthesis. The apparent effect of iron was not significant when statistically analyzed ($P > 0.1$). Additional experiments supported the finding that iron was ineffective in increasing fusarin C biosynthesis (Table 3).

the concentrations used (Fig. 1). When zinc was added at the start of culture growth, dry weights were significantly increased ($P = 0.06$). Zinc concentrations of 26 to 3,200 ppb significantly increased culture dry weights, whereas 5 ppb had little effect (Fig. 2). Addition of 3,200 ppb of zinc to 2- and 4-day-old cultures did not increase dry weight accumulation.

A 10-sample partial-factorial-designed experiment indicated that zinc and iron increased fusarin C biosynthesis by *F. moniliforme* (Fig. 3). Statistical analyses of these results showed that the influence of zinc on fusarin C biosynthesis was significant ($P < 0.02$) under all conditions tested, whereas the apparent effect of iron was not significant ($P > 0.1$). The mean fusarin C values (plus or minus standard deviation) were 13.4 ± 11.8 μg of fusarin C per mg (dry weight) for iron-deficient cultures and 0.89 ± 0.88 μg/mg (dry weight) for iron-supplemented cultures. Results of an eight-sample partial-factorial-designed experiment supported the finding that iron did not significantly affect fusarin C synthesis (Table 3). Zinc concentrations of 26 to 3,200 ppb inhibited fusarin C biosynthesis, whereas 5 ppb was slightly inhibitory (Fig. 2). The addition of zinc (3,200 ppb) to 2- and 4-day-old cultures inhibited further fusarin C biosynthesis (Fig. 4).

The influence of manganese on fusarin C biosynthesis was dependent on the presence of zinc. When zinc was absent, manganese increased fusarin C biosynthesis (Fig. 5). When zinc was present, manganese did not affect fusarin C biosynthesis. At the concentrations tested, cobalt did not significantly affect fusarin C biosynthesis (Fig. 3).

Of all metal ion combinations used in the partial-factorial experimental design (Table 2), maximum fusarin C biosyn-

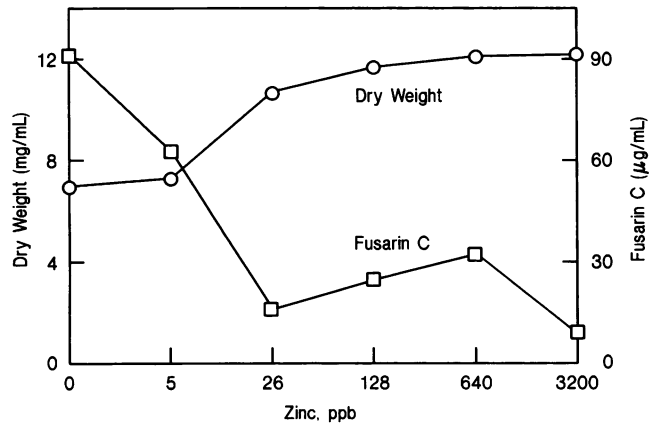

FIG. 2. Influence of zinc concentration on fusarin C biosynthesis and dry weight accumulation in *F. moniliforme*. Zinc levels of 26 ppb significantly increased dry weight and decreased fusarin C biosynthesis, whereas the effect of 5 ppb was not significant.

TABLE 3. Effects of zinc, iron, cobalt, and manganese on fusarin C biosynthesis by *F. moniliforme* NRRL 13616 in an eight-sample partial-factorial experimental design

Trace metal	Fusarin C biosynthesis (mean μg/mg [dry wt] ± SD)	Analysis of variance (*P*)
Zinc		
Added	3.4 ± 3.4	0.034
Not added	24.9 ± 9.3	
Iron		
Added	12.4 ± 14.7	0.458
Not added	16.4 ± 9.8	
Cobalt		
Added	18.3 ± 14.2	0.998
Not added	10.5 ± 9.5	
Manganese		
Added	10.5 ± 9.8	0.990
Not added	18.3 ± 13.9	

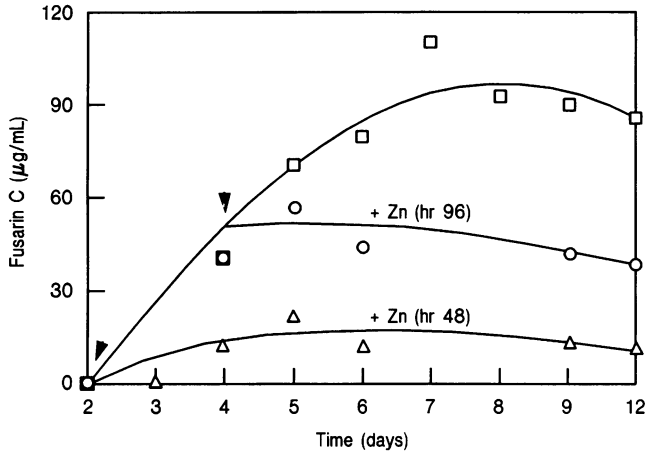

FIG. 4. Effect of addition of 3.2 ppm of zinc to *F. moniliforme* submerged cultures after 48 and 96 h of growth. Fusarin C biosynthesis was inhibited by the addition of zinc.

thesis, 32.3 μg/mg (dry weight) or 92.2 μg/ml, occurred when manganese was the only metal ion present in the defined medium (Fig. 6A). Minimum fusarin C biosynthesis, 0.07 μg/mg (dry weight) or 0.6 μg/ml, occurred when all four metal ions were present (Fig. 6B). Dry weight accumulation by 9-day-old cultures was 2.9 mg/ml in the presence of manganese and 8.2 mg/ml when all four metals were present.

The influence of zinc on glucose uptake, ammonium assimilation, and culture composition was analyzed by growing *F. moniliforme* cultures in a defined medium supplemented with manganese. Glucose uptake was not influenced by zinc (Table 4). In zinc-supplemented cultures, ammonium was not detected in culture supernatants after 1 day of culture growth, whereas ammonium (52 μg/ml) was detected in zinc-deficient cultures after 2 days of culture growth (Table 4).

Compositional analyses of 9-day-old cultures showed that zinc-supplemented cultures contained more carbohydrate than did zinc-deficient cultures (52.8 and 29.8%, respectively; Table 5). Increased carbohydrate in zinc-supplemented cultures accounted for ~80% of the greater dry weight accumulation seen in these cultures. Conversely, the lipid content in zinc-deficient cultures was significantly higher than in zinc-supplemented cultures (53.5 and 23.7%,

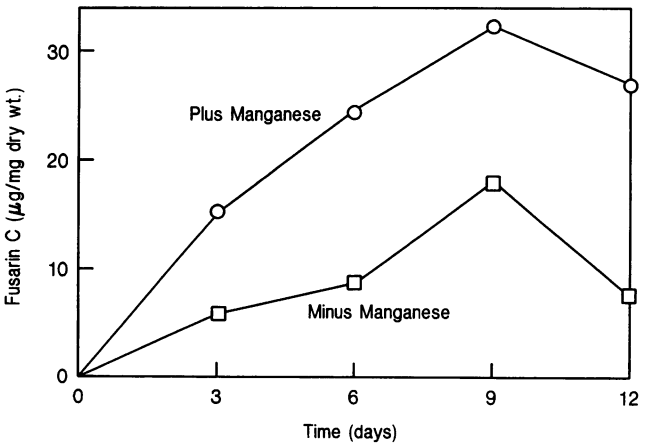

FIG. 5. Effect of manganese on fusarin C biosynthesis in the absence of zinc. *F. moniliforme* cultures supplied with manganese produced 32.3 μg of fusarin C per mg (dry weight), whereas those without manganese produced 18.1 of μg fusarin C per mg (dry weight).

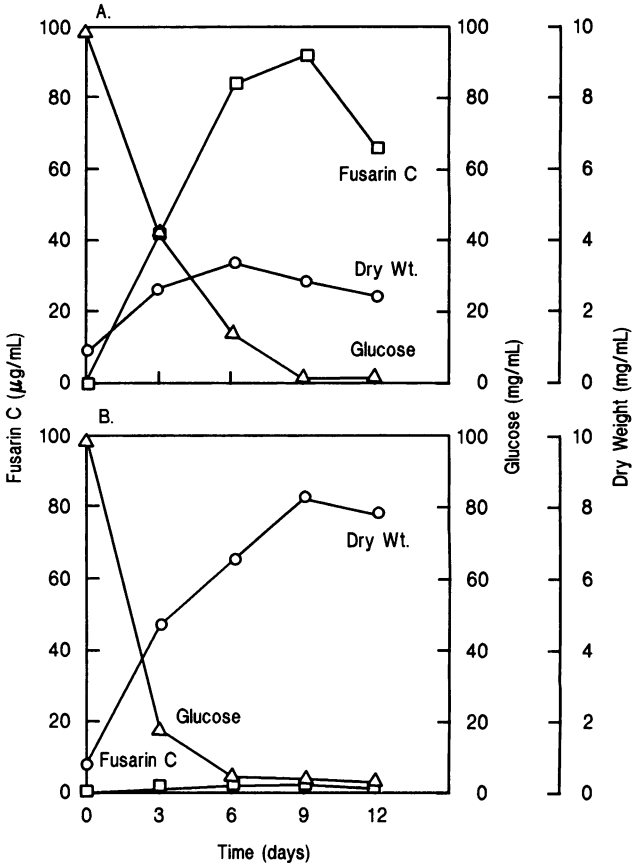

FIG. 6. Influence of trace metals on fusarin C biosynthesis, dry weight accumulation, and glucose consumption by *F. moniliforme*. Of the metal ion combinations used in the partial-factorial-designed experiment, maximum fusarin C biosynthesis occurred when manganese was present and cobalt, iron, and zinc were absent (A); minimum fusarin C biosynthesis occurred when all four metals were present (B). Glucose was utilized at a similar rate in both cultures. Dry weight accumulation was significantly higher in the presence of all four metals.

TABLE 4. Effects of zinc (3,200 ppb) on ammonium ion and glucose uptake by *F. moniliforme* cultures grown in manganese-supplemented defined medium

Culture age (days)	Uptake[a]			
	Glucose (mg/ml)		Ammonium ion (μg/ml)	
	− Zn	+ Zn	− Zn	+ Zn
0	30.0	30.0	1,400	1,400
1	13.4	11.3	183	—[b]
2	9.8	7.0	52	—
4	7.0	6.6	—	
6	5.3	4.6	—	—
9	1.1	0.9	—	—

[a] Culture supernatant levels.
[b] —, Levels too low to detect; assay sensitivity, 1.0 μg/ml.

TABLE 5. Effects of zinc (3,200 ppb) on dry weight and chemical composition of 6-day-old *F. moniliforme* submerged cultures grown in manganese-supplemented defined medium

Culture condition	Determination (mg/ml [%], mean ± SD)			
	Dry wt	Protein	Carbohydrate	Lipid
+Zinc	11.7 ± 0.3 (100)	1.3 ± 0.2 (11.5 ± 1.8)	6.2 ± 0.3 (52.8 ± 2.9)	2.8 ± 0.1 (23.7 ± 1.0)
−Zinc	6.3 ± 0.0 (100)	0.8 ± 0.0 (12.0 ± 0.5)	1.9 ± 0.1 (29.8 ± 1.9)	3.4 ± 0.3 (53.5 ± 4.7)

respectively). Zinc did not affect protein levels (~12% dry weight) in 9-day cultures.

Microscopic examination of zinc-deficient hyphae showed the presence of numerous lipid globules which were not seen in zinc-supplemented cultures (Fig. 7). Globules were determined to contain lipid by their refractile nature and ability to take up Sudan Black B stain.

DISCUSSION

Zinc has been shown to be an important cofactor in numerous fungal enzymes, with activities ranging from intermediate metabolism to RNA and DNA synthesis (6, 28). It has also been found to be a key metal in the biosynthesis of fungal toxins and antibiotics (4, 12, 23, 28, 29). Our results show that zinc has important regulatory effects on primary and secondary metabolism in *F. moniliforme*. Zinc increased ammonium uptake, carbohydrate synthesis, and dry weight accumulation while inhibiting fusarin C biosynthesis, which suggests a multifunctional role for zinc in *F. moniliforme* metabolism.

The transport of ammonia into *Fusarium graminearum* and other fungi occurs by passive diffusion (17), which indicates that the role of zinc in increasing ammonia uptake in *F. moniliforme* cultures is related to ammonium assimilation into amino acids. Although protein levels in zinc-supplemented and zinc-deficient cultures were similar (≈12% dry weight), the increased dry weight of zinc-supplemented cultures indicates a more efficient utilization of nitrogen for protein synthesis. Based on the addition of 1.4 g of ammonium sulfate per liter, zinc-supplemented cultures converted 72% of the available nitrogen into protein, compared with a 40% conversion rate for zinc-deficient cultures (Table 5). Increased protein synthesis would require increased amino acid synthesis. Whether zinc increases am-

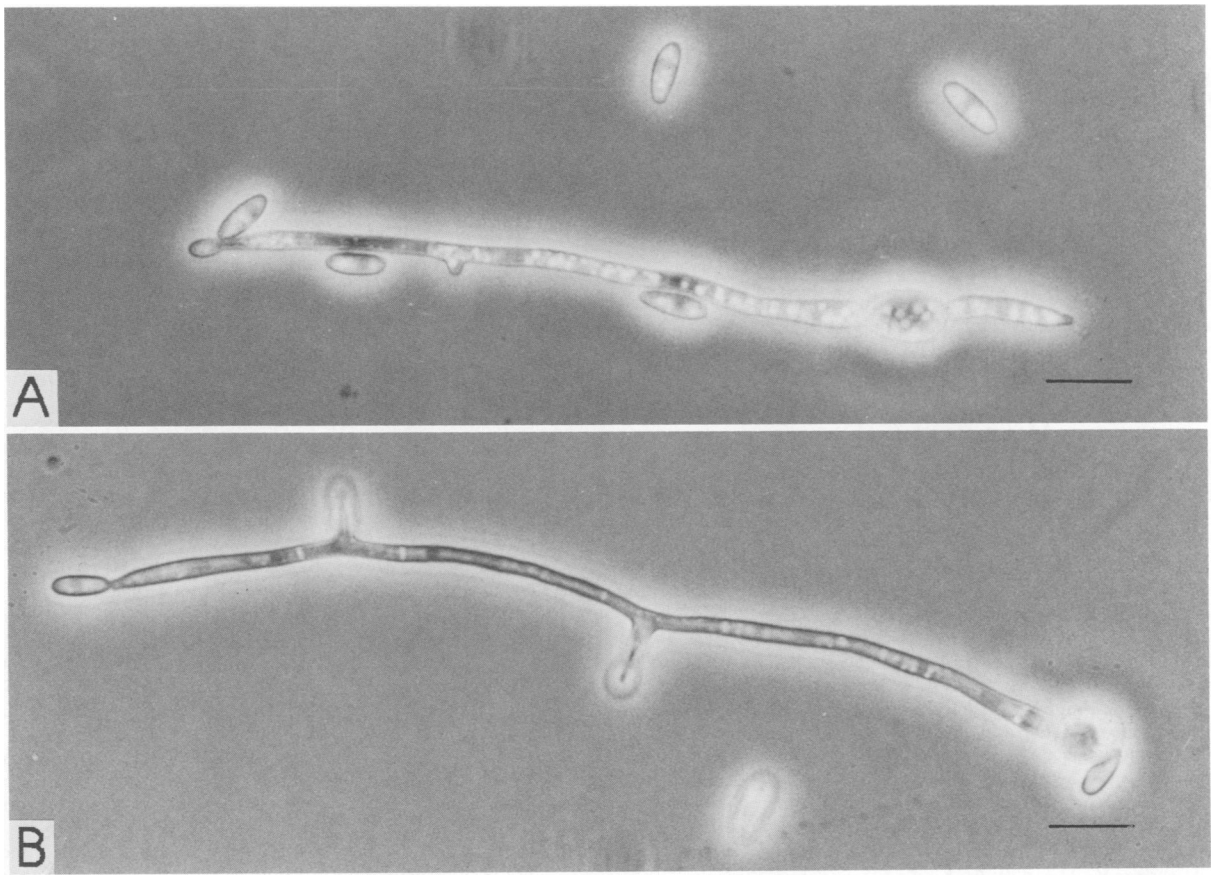

FIG. 7. Photomicrographs of 4-day-old *F. moniliforme* hyphae grown in submerged culture on a manganese-supplemented defined medium containing 3% glucose. Hyphae grown in the absence of zinc (A) contain numerous refractile lipid globules which were not seen in zinc-supplemented cultures (B). Bar = 10 μm.

monia assimilation by stimulating amino acid synthesis, protein synthesis, or the synthesis of other nitrogen-containing compounds (cell wall polymers or nucleic acids) has not been determined.

Inhibition by zinc of fusarin C biosynthesis and stimulation of dry weight accumulation appear to occur by mutually exclusive processes. Zinc concentrations that stimulate dry weight accumulation inhibit fusarin C synthesis. A similar zinc effect was reported for cynodontin biosynthesis in *Helminthosporium cynodontin* growth (29). Since zinc does not influence the consumption of glucose under the nitrogen-limited conditions of this study, these results suggest that zinc-supplemented cultures convert excess glucose into a carbohydrate component of dry weight, whereas zinc-deficient cultures convert glucose into lipids and lipidlike compounds such as fusarin C.

The mechanism by which zinc exerts its influence on fusarin C biosynthesis and carbohydrate synthesis is unclear. If zinc-deficient cultures are supplemented with zinc after 2 and 4 days of culture growth, fusarin C biosynthesis is inhibited without increased dry weight accumulation (Fig. 4). The inability of zinc to stimulate additional dry weight accumulation, presumably in the form of carbohydrate, may be due to the lack of available nitrogen for synthesizing required enzymes. Conversely, the ability of zinc to inhibit additional fusarin C biosynthesis suggests that the processes of carbohydrate synthesis and fusarin C biosynthesis may be regulated independently.

Our results show that zinc regulates ammonium assimilation, carbohydrate synthesis, and fusarin C synthesis in nitrogen-limited submerged cultures of *F. moniliforme*. The utilization of excess glucose for lipid and fusarin C biosynthesis occurs at the expense of carbohydrate synthesis by *F. moniliforme* cultures when zinc concentrations are limited to 5 ppb. The mechanism by which zinc regulates these metabolic processes is unknown but is currently being investigated in our laboratory.

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