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Carbon Concentration and Carbon-to-Nitrogen Ratio Influence Submerged-Culture Conidiation by the Potential Bioherbicide *Colletotrichum truncatum* NRRL 13737

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We assessed the influence of various carbon concentrations and carbon-to-nitrogen (C:N) ratios on *Colletotrichum truncatum* NRRL 13737 conidium formation in submerged cultures grown in a basal salts medium containing various amounts of glucose and Casamino Acids. Under the nutritional conditions tested, the highest conidium concentrations were produced in media with carbon concentrations of 4.0 to 15.3 g/liter. High carbon concentrations (20.4 to 40.8 g/liter) inhibited sporulation and enhanced the formation of microsclerotiumlike hyphal masses. At all the carbon concentrations tested, a culture grown in a medium with a C:N ratio of 15:1 produced more conidia than cultures grown in media with C:N ratios of 40:1 or 5:1. While glucose exhaustion was often coincident with conidium formation, cultures containing residual glucose sporulated and those with high carbon concentrations (>25 g/liter) exhausted glucose without sporulation. Nitrogen source studies showed that the levels of *C. truncatum* NRRL 13737 conidiation were similar for all protein hydrolysates tested. Reduced conidiation occurred when amino acid and inorganic nitrogen sources were used. Of the nine carbon sources evaluated, acetate as the sole carbon source resulted in the lowest level of sporulation.

The use of fungal plant pathogens to control weeds which are not safely or economically managed by chemical herbicides is becoming a practicable option (7). Two commercial products are *Colletotrichum gloeosporioides* f. sp. *aeschynomene* spores (Collego; Ecogen, Inc.) for the control of northern jointvetch (*Aeschynomene virginica*) in Arkansas rice fields and *Phytophthora palmivora* (Devine; Abbott Laboratories) for the control of stranglervine (*Morrenia odorata*) in Florida citrus groves (2, 6).

Numerous *Colletotrichum* species show promise for use as mycoherbicides. Strains of *Colletotrichum malvarnum* and *Colletotrichum coccodes* which show promise as biocontrol agents against prickly sida (*Sida spinosa*) and velvetleaf (*Abutilon theophrasti*), respectively, have been identified (4, 9). Strains of *C. gloeosporioides* which specifically infect the noxious weeds *Clidemia hirta* and *Malva pussilla* have been discovered (5, 8).

Hemp sesbania (*Sesbania exaltata*) is a troublesome weed in the southern United States and causes considerable losses in production of cotton, soybeans, and rice. Boyette (1) recently discovered a strain of *Colletotrichum truncatum*, NRRL 13737, which specifically infects and kills *Sesbania exaltata* but which is not amenable to submerged-culture sporulation with traditional liquid culture media. The ability to economically produce fungal spores is a requirement for the commercial development of a bioherbicide.

Our research is directed toward the development of cost-effective methods for producing fungal spores. In order to optimize submerged-culture sporulation of *C. truncatum*, we evaluated the effects of various nutritional environments on conidium formation. In this paper we report the development of a semidefined medium for the growth and sporulation of *C. truncatum* NRRL 13737 in submerged culture and the influence of carbon concentration and carbon-to-nitrogen (C:N) ratio on sporulation.

MATERIALS AND METHODS

Organism. *C. truncatum* (Schw.) Andrus and Moore (NRRL 13737 [deposited by D. Boyette, USDA, Stoneville, Miss.]) was obtained from the U.S. Department of Agriculture/Agricultural Research Service Northern Regional Research Center Culture Collection. Stock cultures of *C. truncatum* NRRL 13737 were grown as single-spore isolates on potato dextrose agar at room temperature and stored as 1-mm potato dextrose agar plugs in 10% glycerol at −80°C. For liquid-culture studies, spore inocula were produced by growing glycerol stock cultures of *C. truncatum* on potato dextrose agar plates at room temperature. Spore inocula were obtained by rinsing the sporulated potato dextrose agar plates with deionized water.

Media. The defined basal salts medium was composed of 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; $ZnSO_4 \cdot 7H_2O$, 14 mg/liter; $MnSO_4 \cdot H_2O$, 16 mg/liter; $FeSO_4 \cdot 7H_2O$, 50 mg/liter; $CoCl_2 \cdot 6H_2O$, 37 mg/liter; thiamine, riboflavin, pantothenate, niacin, pyridoxamine, and thioctic acid, 500 μg of each per liter; and folic acid, biotin, and vitamin B_{12}, 50 μg of each per liter in deionized water. Stock solutions of glucose (20%, wt/vol; Difco Laboratories, Detroit, Mich.) and vitamin-free Casamino Acids (5%, wt/vol; Difco) were added to the basal salts medium as required. Glucose and other carbon source stock solutions were autoclaved separately. A pH of 5.5 was maintained by the addition of either 2 N NaOH or 2 N HCl.

Carbon concentration and C:N ratio studies were conducted with the basal salts medium supplemented with various amounts of glucose and Casamino Acids as the carbon and nitrogen sources. Carbon concentrations and C:N ratios were calculated as the carbon and nitrogen present in the glucose (40% carbon) and Casamino Acids

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(53% carbon, 16% nitrogen). During C:N ratio studies, carbon concentrations were maintained at a constant level by varying the amount of glucose and Casamino Acids present.

For nitrogen source studies, our basal salts medium was supplemented with glucose and various nitrogen sources at a carbon concentration of 8 g/liter and a C:N ratio of 15:1. Nitrogen sources tested were vitamin-free Casamino Acids, malt extract, yeast extract (Difco), cottonseed hydrolysate, distillers grains and solubles, bovine serum albumin, corn steep liquor, corn gluten hydrolysate, asparagine, glutamate (Sigma Chemical Co., St. Louis, Mo.), digest of milk and meat protein (AY19BT), Enhancetone (PP90BT), papaic digest of soy (SE50BT), digest of casein (CE90BT), digest of rice flour (RE30BT), digest of animal tissue (AE80BT; Deltown Chemurgic, Greenwich, Conn.), sodium nitrate, urea, and ammonium sulfate (Fisher Scientific, Fairlawn, N.J.).

A basal salts medium was supplemented with Casamino Acids and various carbon sources at a carbon concentration of 8 g/liter and a C:N ratio of 15:1 in our carbon source studies. Carbon sources tested were glucose and sucrose (Difco); fructose, glycerol, and acetate (Fisher); galactose, cellulose, and corn starch (Sigma); and citrate (J. T. Baker Chemical Co., Phillipsburg, N.J.).

Modified Richard V-8 medium consisted of sucrose, 50 g/liter; KNO_3, 10 g/liter; KH_2PO_4, 5 g/liter; $MgSO_4 \cdot 7H_2O$, 2.5 g/liter; $FeCl_2$, 0.02 g/liter; and mixed vegetable juice, 150 ml/liter in deionized water, as previously described (2). The medium was adjusted to a pH of 6.0 with 5 N NaOH prior to autoclaving. Potato dextrose broth (Difco) was prepared according to the instructions of the manufacturer, with deionized water.

Submerged culture. Liquid-culture experiments were carried out in duplicate 250-ml Erlenmeyer flasks (no. 2543-00250; Bellco Glass, Inc., Vineland, N.J.) at a 100-ml volume. The cultures were incubated at 28°C and 300 rpm in a rotary shaker incubator. The initial spore concentration for all submerged cultures was 5×10^4 spores per ml. The pH of each medium was maintained by daily adjustments with either 2 N NaOH or 2 N HCl. Flasks were shaken frequently to inhibit mycelial growth on the flask wall.

Analyses. Glucose concentrations were determined by using a high-performance liquid chromatography system (Spectra-Physics, San Jose, Calif.) equipped with an Aminex ion exclusion column (model HXP-87H; Bio-Rad Laboratories, Richmond, Calif.) and a refractive index detector (model 410; Waters Associates, Inc., Milford, Mass.). The column was operated at room temperature, and acidified water (0.0017 N H_2SO_4) was used as the mobile phase.

Spore concentrations were determined microscopically on an Olympus BH-Z microscope using a Neubauer hemocytometer (Reichart-Jung, Buffalo, N.Y.). Homogeneous samples for counting spores were obtained by vortexing the sample for 30 s before the hemocytometer was loaded.

RESULTS

Nitrogen and carbon source studies. Nineteen nitrogen sources were evaluated for their influence on conidium formation in submerged cultures of *C. truncatum* NRRL 13737. All the nitrogen sources tested supported vigorous growth, with the exception of ammonium sulfate. Cultures supplemented with ammonium sulfate grew slowly and did not sporulate. The 14 cultures supplemented with complex nitrogen sources, those containing protein and protein hydrolysates, produced statistically similar conidium concentrations (6.5×10^6 to 3.9×10^7 spores per ml) following 6 days of growth (Table 1).

TABLE 1. Submerged-culture conidiation of *C. truncatum* NRRL 13737 with various nitrogen sources[a]

Nitrogen source	Mean no. of conidia/ml[b]	Mean residual glucose (mg/ml)
Casamino Acids	3.9×10^7 a	0.7
Digest of milk & meat protein	3.3×10^7 ab	2.3
Cottonseed hydrolysate	3.3×10^7 ab	0.7
Enhancetone	2.7×10^7 ab	0.8
Papaic digest of soy	2.6×10^7 ab	0.6
Distillers' grains and solubles	1.8×10^7 abc	0.8
Digest of casein	2.2×10^7 abc	0.8
Malt extract	2.5×10^7 abc	17.2
Bovine serum albumin	1.6×10^7 abc	ND[c]
Yeast extract	1.2×10^7 abc	0.9
Digest of rice flour	1.0×10^7 abc	3.3
Corn steep liquor	9.0×10^6 abc	ND
Digest of animal tissue	1.4×10^7 abc	0.8
Corn gluten hydrolysate	6.5×10^6 abc	1.1
Sodium nitrate	6.0×10^6 bc	ND
Asparagine	3.5×10^6 c	ND
Glutamate	2.7×10^6 c	ND
Urea	1.0×10^6 d	ND
Ammonium sulfate	—[d]	

[a] Six-day-old cultures with a carbon concentration of 8 g/liter and a C:N ratio of 15:1.
[b] Values followed by different letters are significantly different ($P < 0.05$) using Fisher's protected least significant difference on the $\log_{10}$ of the values shown.
[c] ND, Not done.
[d] —, Poor growth (no value obtained).

Although cultures supplemented with sodium nitrate, asparagine, and glutamate produced significantly fewer spores than cultures supplemented with Casamino Acids, spore levels were not significantly lower than those of many of the complex nitrogen-supplemented cultures (Table 1). Cultures supplemented with urea and ammonium sulfate produced significantly fewer conidia than cultures supplemented with all other nitrogen sources tested.

Seven of the nine carbon sources tested gave similar levels of sporulation in submerged cultures of *C. truncatum* NRRL 13737 (Table 2). Spore production in acetate-supplemented cultures was significantly reduced compared with spore production in cultures with the other carbon sources tested.

TABLE 2. Submerged-culture conidiation of *C. truncatum* NRRL 13737 in a basal salts medium supplemented with Casamino Acids and various carbon sources[a]

Carbon source	Mean no. of conidia/ml[b]
Corn starch	2.3×10^7 a
Fructose	1.9×10^7 a
Sucrose	1.5×10^7 a
Glycerol	1.3×10^7 a
Glucose	1.0×10^7 a
Cellulose	2.5×10^6 ab
Citrate	1.5×10^6 ab
Galactose	4.5×10^4 b
Acetate	5.0×10^3 c

[a] Six-day-old culture with a carbon concentration of 8 g/liter and a C:N ratio of 15:1.
[b] Values followed by different letters are significantly different ($P < 0.05$) using Fisher's protected least significant difference on the $\log_{10}$ of the values shown.

TABLE 3. Influence of carbon concentration on *C. truncatum* NRRL 13737 conidia production[a]

Carbon (g/liter)	Initial glucose (g/liter)	Day 5		Day 6		Day 7	
		Glucose (g/liter)	Conidia (10^6/ml)[b]	Glucose (g/liter)	Conidia (10^6/ml)[b]	Glucose (g/liter)	Conidia (10^6/ml)[b]
5.1	10	0.2	3.1 b	0.2	8.4 a	0.0	9.8 a
10.2	20	0.2	8.8 a	0.3	11.9 a	0.0	9.3 a
15.3	30	0.7	2.9 b	1.0	6.4 a	0.0	12.1 a
20.4	40	1.4	0.1 c	1.6	1.1 b	0.0	3.3 b
25.5	50	2.0	0 d	1.7	0 c	0.0	0 c
30.6	60	2.6	0 d	2.7	0 c	0.0	0 c
35.7	70	2.9	0 d	3.2	0 c	0.0	0 c
40.8	80	7.8	0 d	3.9	0 c	0.0	0 c

[a] C:N ratio, 15:1.
[b] Values followed by different letters are significantly different ($P < 0.05$) using Fisher's protected least significant difference on the $\log_{10}$ of the values shown.

In general, cultures supplemented with acetate, galactose, citrate, and cellulose produced low levels of conidia compared with those supplemented with glucose, sucrose, fructose, glycerol, and corn starch.

Carbon concentration and C:N ratio studies. When media with a C:N ratio of 15:1 were used, conidiation was measured in cultures with carbon concentrations between 5.1 and 40.8 g/liter (Table 3). Conidiation in 5-day-old cultures was highest, with a carbon concentration of 10.2 g/liter, while a range of carbon concentrations (5.1 to 15.3 g/liter) gave the highest levels of conidiation in 6- and 7-day-old cultures. Cultures with carbon concentrations of 25.5 g/liter or more did not sporulate. Glucose was absent from the supernatant of all cultures following 7 days of growth, regardless of the initial glucose concentration.

Under the conditions of this study, increased carbon concentration was correlated to increased hyphal melanization (Fig. 1). Microscopic examination of these cultures showed that hyphal melalization occurred in microsclerotiumlike, compact hyphal masses. An increase in the number of these highly melanized hyphal masses was correlated to reduced conidium formation.

The effect of C:N ratio on conidium formation was assessed by growing *C. truncatum* NRRL 13737 cultures at constant carbon concentrations (2, 4, and 8 g/liter) with C:N ratios of 40:1, 20:1, 15:1, 10:1, 7.5:1, and 5:1. A comparison of conidium production for 6-day-old cultures grown in media with the same carbon concentration and different C:N ratios showed that a medium with a C:N ratio of 15:1 produced significantly more conidia than media with C:N ratios of 40:1 and 5:1, regardless of the carbon concentration (Table 4). Conidium production in media with C:N ratios from 20:1 to 10:1 was not significantly different for cultures grown with 2 g of carbon per liter. In cultures with 4 or 8 g of carbon per liter, media with C:N ratios between 20:1 and 7.5:1 produced similar numbers of conidia (Table 4).

TABLE 4. Influence of C:N ratio on conidiation by *C. truncatum* NRRL 13737 at various carbon concentrations

C:N ratio	Spores/ml (10^5)[a] at carbon concn (g/liter)		
	2	4	8
40:1	5.5 a	13 a	65 a
20:1	34 c	104 c	138 ab
15:1	57 c	150 c	209 b
10:1	31 c	143 c	132 ab
7.5:1	11 b	99 c	88 ab
5:1	13 b	41 b	62 a

[a] Values followed by different letters are significantly different ($P < 0.05$) using Fisher's protected least significant difference on the $\log_{10}$ of the values shown.

A comparison of conidium production for cultures grown with various carbon concentrations and the same C:N ratio showed that media with 4 and 8 g of carbon per liter produce similar concentrations of conidia which are significantly higher than those of cultures grown with 2 g of carbon per liter (Fig. 2). Only at a C:N ratio of 40:1 did cultures grown with 2 and 4 g of carbon per liter produce similar levels of conidia which were lower than those produced by cultures with 8 g of carbon per liter.

DISCUSSION

The evaluation of nutritional factors on sporulation by *C. truncatum* NRRL 13737 in submerged culture required the

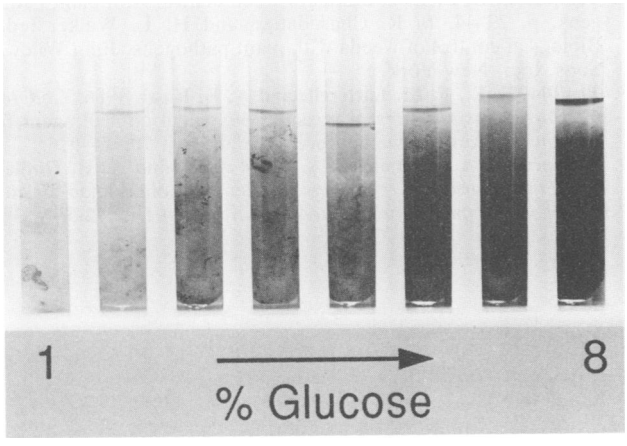

FIG. 1. Six-day-old *C. truncatum* NRRL 13737 submerged cultures grown at a C:N ratio of 15:1 with various glucose concentrations. Increasing glucose concentrations inhibit conidiation (Table 3) and increase the production of melanized, microsclerotiumlike hyphal masses.

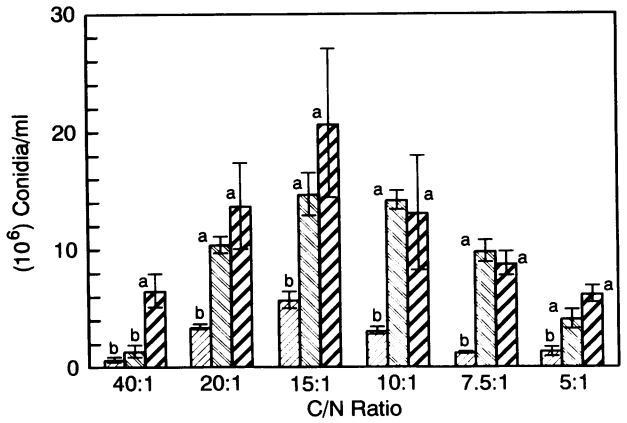

FIG. 2. The influence of carbon concentration on conidiation by 6-day-old *C. truncatum* NRRL 13737 submerged cultures grown at various C:N ratios. Within each C:N ratio grouping, mean spore concentrations with different letters are significantly different ($P < 0.05$) using Fisher's protected least significant difference on the $\log_{10}$ of the values shown. Symbols (grams of carbon per liter): ▨, 2; ▧, 4; ◪, 8.

development of a defined medium which could support good growth and sporulation. Using a vitamin-enriched basal salts medium which had been shown to support good growth by other fungi (3), we evaluated the suitability of various carbon and nitrogen sources (Tables 1 and 2). Results from these experiments showed that nitrogen sources containing protein or protein hydrolysates supported similarly high levels of conidium production, while inorganic nitrogen sources produced lower levels of conidiation. Vitamin-free Casamino Acids were used in all other experiments because of their vitamin-free nature and their relative consistency in composition compared with other proteinaceous nitrogen sources. Glucose was used as the carbon source because of its ability to support good conidium formation and because of its ease of measurement.

Under the conditions of these experiments, carbon concentration and C:N ratio were shown to influence submerged-culture conidium production by *C. truncatum* NRRL 13737. Optimum sporulation occurred when cultures were supplied with Casamino Acids and glucose with a total carbon concentration of 4.0 to 15.3 g/liter at a C:N ratio between 20:1 and 7.5:1. A C:N ratio of 15:1 consistently produced the highest levels of conidiation.

The inhibition of conidium formation by high carbon concentrations (Table 3) was unexpected. The possibility that residual glucose was inhibiting sporulation was dismissed when glucose was found to be absent from the culture supernatants following 7 days of growth. Nitrogen source studies also indicated that glucose depletion was unnecessary for sporulation (Table 1).

The production of numerous microsclerotiumlike, highly melanized, compact hyphal masses rather than conidia in *C. truncatum* NRRL 13737 cultures grown at high carbon concentrations suggests that carbon concentration may play an important developmental role. As a comparison, we tested conidium production by *C. gloeosporioides* f. sp. *aeschynomene* at various carbon concentrations. Cultures of *C. gloeosporioides* f. sp. *aeschynomene* produced more conidia as carbon concentrations were increased beyond those levels which inhibited *C. truncatum* sporulation, without the production of microsclerotiumlike hyphal masses (data not shown). These results suggest that regulation of differentiation by carbon concentration is not universal in *Colletotrichum* species.

Richard V-8 medium is typically used to grow and produce spores of various *Colletotrichum* species in submerged culture. Boyette found that *C. truncatum* NRRL 13737 sporulated poorly in Richard V-8 medium and produced numerous microsclerotia (1). Under our culture conditions, *C. truncatum* NRRL 13737 produced significantly fewer spores (3×10^6 spores per ml) in Richard V-8 medium than in our defined medium (2×10^7 spores per ml). Analysis of Richard V-8 medium shows that it supplies approximately 23 g of carbon per liter, a concentration which inhibited conidium formation and enhanced microsclerotium development by *C. truncatum* NRRL 13737 in our defined-medium studies. Conversely, potato dextrose broth (Difco) provides approximately 10 g of carbon per liter and supports submerged-culture sporulation by *C. truncatum* at levels comparable to those obtained with our defined media. The influence of carbon concentration on the submerged-culture sporulation of *C. truncatum* NRRL 13737 in these complex media followed the pattern we expected from our defined-medium studies.

An understanding of how the nutritional environment influences sporulation is required in order to predict what types of low-cost complex media may be suitable for spore production. Our results with a semidefined medium show that carbon concentration and C:N ratio are important nutritional factors regulating the submerged-culture sporulation of *C. truncatum* NRRL 13737. Recent studies indicate that these nutritional factors influence *C. truncatum* conidial viability and conidial efficacy in inciting disease in *Sesbania exaltata* (D. A. Schisler, M. A. Jackson, and R. J. Bothast, submitted for publication). The commercial use of fungal spores as bioherbicides will require cost-effective production techniques which maximize both conidial numbers and conidial efficacy.

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