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Submerged culture conidial germination and conidiation of the bioherbicide *Colletotrichum truncatum* are influenced by the amino acid composition of the medium

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SUMMARY

Submerged culture experiments were conducted to determine the optimal nitrogen source for rapidly producing conidia of the bioherbicide, *Colletotrichum truncatum*. Germination of *C. truncatum* conidial inocula in submerged culture occurred most rapidly (>95% in 6 h) in media provided with a complete complement of amino acids. When $(NH_4)_2SO_4$, urea, or individual amino acids were provided as the sole nitrogen source, conidial germination was less than 20% after 6 h incubation. Conidia production was delayed in *C. truncatum* cultures grown in media with urea or individual amino acids as nitrogen sources compared to cultures supplied with Casamino acids or complete synthetic amino acid nitrogen sources. The use of methionine, lysine, tryptophan, isoleucine, leucine or cysteine as a sole nitrogen source severely inhibited *C. truncatum* conidia production. Media with synthetic amino acid mixtures less these inhibitory amino acids produced significantly higher conidia yields compared to media with amino acid mixtures containing these amino acids. When various amounts of each individual inhibitory amino acid were added to media which contained amino acid mixtures, cysteine and methionine were shown to be most effective in reducing conidiation. An optimal nitrogen source for *C. truncatum* conidiation in submerged culture should contain a complete mixture of amino acids with low levels of cysteine, methionine, leucine, isoleucine, lysine and tryptophan for rapid conidiation and optimal conidia yield.

INTRODUCTION

The 'bioherbicide' approach is a weed control strategy which uses an inundative application of fungal or bacterial propagules to selectively infect and kill a target weed [22]. While many microorganisms have been identified which show promise as bioherbicides, only three fungal bioherbicides are currently registered for use in North America [16,21]. A significant barrier to the commercial development of many of these plant pathogens as bioherbicides is the availability of low-cost methods for producing infective microbial propagules [22]. Submerged culture fermentations are considered to be the most economical method of production [4].

Colletotrichum truncatum NRRL 18434 is a specific fungal pathogen of the weed hemp sesbania (*Sesbania exaltata* [1]). Various research groups are currently evaluating the commercial potential of *C. truncatum* in regard to host-range specificity, field efficacy, and disease development [2, 24]. Our research has focused on developing submerged culture techniques for producing and formulating high concentrations of *C. truncatum* conidia. Previous studies have shown that the nutritional environment of the conidiation medium significantly impacts *C. truncatum* conidia yield and efficacy [8,9,17].

Since the commercial viability of microbial biological control agents depends on an economical production method, development of media or environmental conditions which reduce fermentation times and increase conidia yields should lower production costs. In this report, we describe the impact of various amino acids, amino acid mixtures, and inorganic nitrogen sources on the rate of *C. truncatum* conidial inocula germination, the onset of culture conidiation, and conidia yield.

MATERIALS AND METHODS

Organisms

C. truncatum (Schw.) Andrus and Moore (NRRL 13737, ARS Patent Culture Collection 18434, deposited by C.D. Boyette, USDA, Stoneville, MS) was used throughout this study. Stock cultures were obtained from single spore isolates and stored at −80 °C in 10% glycerol as previously described [8]. Conidial inocula were produced by growing glycerol stock cultures of *C. truncatum* on potato dextrose agar at

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room temperature. Sporulated potato dextrose agar plates were rinsed with deionized water and conidial suspensions were used to inoculate shake flask cultures. The initial conidial concentration for all liquid culture studies was 5×10^4 conidia ml^{-1}.

Culture conditions

Liquid culture experiments were carried out in triplicate in baffled 250 ml-Erlenmeyer flasks (no. 2543-0250; Bellco Glass, Inc., Vineland, NJ) at a 100 ml volume. The cultures were incubated at 28 °C and 300 rpm in a rotary shaker incubator. A pH of 5 was maintained by daily adjustments with sterile 2 N NaOH or 2 N HCl. Mycelial growth on the flask wall was minimized by frequent shaking.

Media

A defined basal salts medium [8] was used in all liquid culture experiments. Stock solutions of glucose (20% w/v; Difco Laboratories, Detroit, MI) were added to the basal medium after autoclaving as required. Nitrogen sources and glucose were added to the basal medium to provide 8 g carbon L^{-1} and a carbon to nitrogen (CN) ratio of 15:1. The carbon concentration and CN ratio of the various media were calculated based on the following assumptions: glucose, 40% carbon; vitamin-free Casamino acids, 53% carbon and 8% nitrogen; synthetic amino acid mixtures (Table 1), 53% carbon and 16% nitrogen; urea and individual amino acids, per molecular formula. Nitrogen sources used included vitamin-free Casamino acids (Difco), ammonium sulfate, urea (EM Sciences, Gibbstown, NJ), proline, serine, alanine, tyrosine, arginine, glutamate, histidine, valine, aspartate, glycine, asparagine, threonine, glutamine, phenylalanine, methionine, lysine, tryptophan, isoleucine, leucine, and cysteine (Sigma Chemical, St Louis, MO). The synthetic amino acid mixtures are described in Table 1.

TABLE 1

Amino acid mixtures used as nitrogen sources

Amino acid	Amino acids (mg L^{-1})			
	Mix A[1]	Mix B[2]	Mix C[3]	Mix D[4]
Alanine	86	116	266	178
Arginine	122	164	266	178
Aspartate	190	254	266	178
Cysteine	10	—	—	178
Glutamate	668	894	266	178
Glycine	34	46	266	178
Histidine	74	100	266	178
Leucine	312	—	—	178
Isoleucine	160	—	—	178
Lysine	226	—	—	178
Methionine	92	—	—	178
Phenylalanine	182	244	266	178
Proline	322	432	266	178
Serine	186	250	266	178
Threonine	142	190	266	178
Tyrosine	146	196	266	178
Tryptophan	40	—	0	178
Valine	206	276	266	178
Total	3198	3162	3192	3204

[1]Mix A – like casein.
[2]Mix B – like casein minus inhibitory amino acids.
[3]Mix C – equal amounts of amino acids minus inhibitory amino acids.
[4]Mix D – equal amounts of all amino acids.

Analyses

Submerged culture *C. truncatum* conidial germination was assessed by removing a 3-ml sample at 3, 6, 12, and 24 h. The sample was immediately combined with 1 ml of concentrated HCl to inhibit further conidial germination. One hundred conidia from each sample were examined with an Olympus BH-2 microscope and those with a germ tube counted. Germination of conidia on water agar plates and measurement of conidia concentrations in liquid culture were determined by microscopic measurement, as previously described [8,17].

All experiments were repeated at least three times. Representative data were subjected to analysis of variance and the means of the various treatments were separated by Fisher's protected least significant difference (LSD).

RESULTS AND DISCUSSION

The nitrogen source had a profound influence on the rate of *C. truncatum* conidial germination. Under aerated submerged-culture conditions, conidial inocula germinated most rapidly when nitrogen was supplied as a complete complement of amino acids (Table 2). Essentially all the conidia in media containing Casamino acids or synthetic amino acid mixtures, like Casamino acids, germinated within 6 h. Conversely, less than 20% of the conidia in media containing urea, ammonium sulfate, or individual amino acids as a nitrogen source had germinated in 6 h.

In an analogous study, rapid conidial germination by *Glomerella cingulata* (anamorph, *Colletotrichum gloeosporioides*) in liquid culture required peptone as a nitrogen source when conidia concentrations exceeded 100 conidia ml^{-1} [13]. More than 90% of the *G. cingulata* conidia germinated in liquid culture without peptone addition when conidia concentrations were less than 100 conidia ml^{-1}. These authors suggested that trace compounds in the peptone, not amino acids, were enhancing *G. cingulata* germination by overcoming the activity of a germination self-inhibitor. Under the conditions of our study, low concentrations of rinsed *C. truncatum* conidia (30 conidia ml^{-1}) did not germinate in liquid culture without the addition of Casamino acids, indicating that factors other than a germination self-inhibitor were affecting germination. Our results showed that the amino acid component of Casamino acids was beneficial to germination since a mixture of purified amino acids supported a conidial germination rate like that of Casamino acid-supplemented cultures (Table 2).

Numerous studies have shown that *de novo* protein

TABLE 2

Germination of *C. truncatum* conidial inocula in a defined liquid medium with differing nitrogen sources

Nitrogen source	% Germination (h)			
	3	6	12	24
Casamino acids	87	99	100	100
Mixture A*	76	99	100	100
Ammonium sulfate	0	0	0	12
Urea	0	0	0	4
Proline	0	1	12	57
Serine	0	1	10	61
Alanine	1	1	22	52
Tyrosine	1	1	18	72
Arginine	7	7	7	16
Glutamate	0	0	10	28
Histidine	1	4	10	34
Valine	0	0	0	20
Aspartate	8	12	23	26
Glycine	0	0	1	41
Asparagine	0	0	0	2
Threonine	0	1	0	9
Glutamine	0	0	14	76
Phenylalanine	5	8	15	65
Methionine	0	19	92	99
Lysine	0	0	0	1
Tryptophan	0	1	4	30
Isoleucine	0	0	3	23
Leucine	0	0	0	1
Cysteine	0	0	0	37

*Refer to Table 1 for amino acid composition.

synthesis is required for conidial germination and that amino acid pools must be available for this process [15,18]. Conidia which contain ample endogenous nutritional reserves for germination are considered 'nutrient-independent' spores [14]. When rinsed *C. truncatum* conidia were sprayed onto water agar plates, more than 80% of the conidia germinated in 6 h. Previous studies have also demonstrated that *C. truncatum* conidia with higher protein content germinated more rapidly on water agar plates [9,17]. These results suggested that *C. truncatum* conidia are 'nutrient-independent' since, on a nutrient-deficient solid substrate, they can utilize endogenous nutritional reserves for germination.

The question arises: 'Why is a complete complement of amino acids needed for rapid *C. truncatum* conidial germination in liquid culture and not needed when conidia are placed on solid substrates?'. One possibility is that during germination *C. truncatum* conidia may become leaky for various nutrients, including amino acids. In an aqueous environment, these endogenous nutrients would diffuse into the medium and become inaccessible to the conidium. On solid substrates, even though amino acids are leaked, high concentrations of amino acids would remain near the conidium due to the absence of an agitated aqueous environment. Furthermore, on solid substrates germinating conidia of *C. truncatum* have been shown to produce a water-soluble mucilagenous matrix which adheres the conidium to the host plant surface [24]. This matrix may also inhibit the diffusion of amino acids and other nutrients away from the conidium. In agitated liquid culture, this mucilagenous matrix is not detectable on germinating *C. truncatum* conidia.

Nutrient leaching of conidia in the soil and in aqueous environments has been shown to inhibit conidial germination by numerous fungi [7,11,14,19]. As conidia begin to germinate, nutrients begin to flow out of the conidium and are used by microorganisms in the soil or diffuse into an aqueous environment thereby becoming inaccessible to the conidium. The inhibitory influence of nutrient leaching on conidial germination by *Cochliobolus victoriae* and *Curvularia lunata* was overcome by bathing these conidia in dilute solutions of glucose and amino acids [3]. In a similar fashion, providing *C. truncatum* conidia a nutritional environment with relatively high concentrations of Casamino acids (>1.7 g L^{-1}) allowed for optimal liquid culture conidial germination (Table 3). Using relatively high concentrations of amino acids in the culture medium relieves the apparent inhibitory effects of nutrient leaching. Regardless of the mechanism of inhibition, *C. truncatum* conidia required the availability of a complete complement of amino acids for rapid germination under liquid culture conditions.

The nitrogen source provided by submerged cultures of *C. truncatum* not only influenced germination of conidial inocula but also affected conidiation. Cultures supplied with Casamino acids as the nitrogen source produced conidia more rapidly than cultures supplied with individual amino acids (Table 4). By day 7, many of the cultures provided by individual amino acids or urea as the nitrogen source had produced conidia concentrations similar to those obtained by Casamino acid-supplemented cultures. The slower germination of conidial inocula in cultures supplied with urea or individual amino acids was likely responsible, at least in part, for the lengthened fermentation times required for

TABLE 3

The effect of Casamino acid amendments on submerged culture *C. truncatum* conidial inocula germination in media containing ammonium sulfate as the nitrogen source

Casamino acids		% Germination (6 h)
% Of total nitrogen	g L^{-1}	
100	6.6	95
50	3.3	81
25	1.7	62
12	0.8	25
6	0.4	29
3	0.2	21
2	0.1	26
1	0.1	13
0	0.0	0
LSD ($P < 0.05$)		18

TABLE 4

Submerged culture growth and conidiation of *C. truncatum* cultures grown in a defined medium with differing nitrogen sources

Nitrogen source	10^6 conidia ml^{-1} (Day)				Dry weight (mg ml^{-1})
	4	5	6	7	
Casamino acids	15.8	23.6	25.5	19.0	7.5
Urea	8.0	15.4	14.0	19.3	5.9
Proline	0.6	12.0	17.5	19.0	10.8
Serine	7.0	15.2	16.3	17.8	7.9
Alanine	3.0	15.5	14.9	17.3	8.8
Tyrosine	0.0	12.5	12.5	12.0	7.9
Arginine	0.0	4.9	12.1	15.8	10.8
Glutamate	0.9	14.7	11.1	14.9	8.7
Histidine	0.0	1.2	10.3	21.0	8.0
Valine	0.0	0.2	9.5	29.4	8.2
Aspartic acid	9.5	10.0	8.4	14.7	7.0
Glycine	1.1	6.5	7.4	13.7	7.7
Asparagine	0.0	1.0	6.5	12.3	8.9
Threonine	1.5	6.1	5.7	9.5	8.0
Glutamine	1.1	2.8	4.8	7.9	8.8
Phenylalanine	0.0	0.1	4.2	9.3	5.1
Methionine	0.0	0.0	3.8	1.8	6.2
Lysine	0.0	0.0	0.1	0.7	1.2
Tryptophan	0.0	0.0	0.0	0.0	5.1
Isoleucine	0.0	0.0	0.0	0.0	5.6
Leucine	0.0	0.0	0.0	0.0	5.5
Cysteine	0.0	0.0	0.0	0.0	9.6
LSD ($P < 0.01$)	4.6	13.3	13.8	16.4	3.1

maximum conidiation in some cultures. Conversely, cultures supplied with methionine, lysine, tryptophan, isoleucine, leucine, or cysteine as sole nitrogen sources conidiated very poorly by day 7 (Table 4). Only in lysine-supplemented cultures was poor growth associated with poor conidiation. Why cysteine, methionine, tryptophan, isoleucine, and leucine inhibit *C. truncatum* conidiation is unclear. Studies comparing the amino acid composition of the medium and fungal sporulation are limited and those which have been conducted showed that the influence of amino acids on sporulation is dependent on the organism being evaluated [6,12,20].

To evaluate the impact of these inhibitory amino acids on *C. truncatum* conidiation, media were supplemented with synthetic amino acid mixtures (Table 1) in which methionine, lysine, tryptophan, isoleucine, leucine and cysteine were omitted or included. After 6 h incubation, conidial germination rates were significantly higher in cultures provided with a complete amino acid mixture (Table 5). By 12 h, more than 80% of the conidia in all cultures had germinated, regardless of the composition of the synthetic amino acid mixture. Cultures provided with amino acid mixtures without inhibitory amino acids produced significantly higher conidia concentrations after 6 days compared to Casamino acid-supplemented cultures (Table 5). Also, media containing

TABLE 5

Influence of synthetic amino acid mixtures on *C. truncatum* submerged culture conidial inocula germination, growth, and conidiation

Nitrogen source	% Germination (h)			10^6 conidia ml^{-1} (Day)			Dry weight (mg ml^{-1})
	3	6	12	4	5	6	
Casamino acids	93	100	100	8.4	18.2	22.6	8.3
Mix A*	62	89	99	5.5	9.5	17.0	9.4
Mix B*	16	42	97	23.2	38.4	38.3	7.8
Mix C*	14	38	81	8.9	21.8	35.2	8.6
Mix D*	0	95	100	0.2	0.3	0.3	9.1
LSD ($P < 0.05$)	8.5	12.6	7.8	4.7	7.0	12.4	NSD

*Refer to Table 1 for amino acid composition.
Mix A – like casein.
Mix B – like casein minus inhibitory amino acids.
Mix C – equal amounts of amino acids less the inhibitory amino acids.
Mix D – equal amounts of all amino acids.
NSD = not significantly different.

the synthetic amino acid mixture like casein less the inhibitory amino acids (mixture B, Table 1) supported more rapid conidiation compared to Casamino acid-supplemented cultures. The omission of these inhibitory amino acids in the conidiation medium appears to have a beneficial impact on the onset of conidiation and conidial yields. When a mixture containing equal amounts of all the amino acids (mixture D, Table 1) was used as the nitrogen source, conidiation was severely inhibited (Table 5). It is notable that mixture D contained higher concentrations of cysteine, methionine, and tryptophan compared to Casamino acids.

When various amounts of individual inhibitory amino acids were added to media with a synthetic amino acid mixture less these amino acids, cysteine and methionine were shown to be most effective in inhibiting conidia production by *C. truncatum* cultures (Table 6). Studies with other fungi have suggested that ethylene synthesis in cultures supplied with the sulfur-containing amino acids, methionine and cysteine, was responsible for reduced sporulation [5,10, 23]. Methionine and cysteine effectively reduced conidiation when as little as 500 mg L^{-1} was added. Conversely, isoleucine and lysine did not reduce conidiation when added at 2.5 g L^{-1} (Table 6). When various amounts of cysteine and methionine were added to cultures with a complex nitrogen source, Casamino acids, as little as 250 mg methionine L^{-1} or 500 mg cysteine L^{-1} were shown to significantly reduce conidial yields (Table 6). These results suggest that conidiation media containing low concentrations of cysteine and methionine are essential for optimal conidia production regardless of whether a complex or defined nitrogen source is used.

When *C. truncatum* cultures were grown in a medium with a synthetic amino acid mixture containing 178 mg of each amino acid including the six inhibitory amino acids

TABLE 6

Influence of various concentrations of inhibitory amino acids on *C. truncatum* conidiation, 6-day-old cultures

Nitrogen source	10^6 conidia ml^{-1}			
	250[1]	500	1000	2500
Amino acid mix C[2]	34.2	38.3	38.3	29.7
+ Cysteine	30.4	5.8	3.3	5.7
+ Methionine	23.0	4.4	8.1	1.8
+ Tryptophan	12.0	24.5	20.6	5.7
+ Leucine	37.6	29.7	20.4	12.8
+ Isoleucine	ND	ND	ND	25.7
+ Lysine	ND	ND	ND	27.1
LSD ($P < 0.05$)	14.1	16.4	9.4	6.7
Casamino acids	16.2	16.2	16.2	ND
+ Cysteine	18.0	5.4	0.0	ND
+ Methionine	5.3	4.5	2.0	ND

[1]Amount of individual amino acid (mg L^{-1}) added to Mix C or Casamino acids. A total (N) of 0.53 g L^{-1} was maintained.
[2]Refer to Table 1 for amino acid composition.
ND = not done.

(Table 1, mixture D), conidia production was severely inhibited ((Table 5). The addition of 178 mg of any single inhibitory amino acid was ineffective in reducing conidia production. These results suggested significant interactions between the various inhibitory amino acids to reduce conidiation. This suggestion was confirmed by a full-factorial evaluation of the influence of three of the most inhibitory amino acids (methionine, tryptophan, and cysteine) which showed considerable interaction between these amino acids in reducing conidiation (data not shown). It is also likely that interactions with the other inhibitory amino acids were occurring since small amounts of all the inhibitory amino acids had such a dramatic impact on conidiation (Table 5). The extent of interactions between these inhibitory amino acids on *C. truncatum* conidiation remains unclear and the elucidation of these interactions was beyond the scope of this study.

In conclusion, nitrogen nutrition is an important consideration in the development of liquid media to produce conidia of the biocontrol fungus, *C. truncatum*. Selecting complex or defined nitrogen sources which provide a complete complement of amino acids and are low in cysteine, methionine, lysine, tryptophan, leucine, and isoleucine is needed for rapid *C. truncatum* conidiation and high conidial yield in liquid culture.

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