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Conidiation Environment Influences Fitness of the Potential Bioherbicide, *Colletotrichum truncatum*

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Colletotrichum truncatum NRRL 13737 is a fungal pathogen of the noxious weed *Sesbania exaltata* and shows promise as a biological control agent. The commercial potential of a bioherbicide is dependent on numerous factors including a cost-effective method for producing propagules which can be stored and have the capacity to rapidly infect and kill the weed host. Our previous submerged culture studies showed that the carbon-to-nitrogen (CN) ratio of the sporulation medium significantly influences *C. truncatum* spore yield and spore fitness. Although spore yields were optimized in media with a CN ratio of 30:1, media with a CN ratio of 10:1 yielded spores which germinated more rapidly, formed appressoria more frequently, and incited more disease in *S. exaltata.* In addition to spore yield and fitness, amenability to storage and formulation is also a requirement for the development of a commercial biological control product. In this study, a comparison of harvesting and storage procedures suggests that lowered temperatures (4°C or lower) adversely affect the viability of both "10:1" and "30:1" spores although a more rapid loss of viability is observed in "30:1" spores. Under the conditions of these experiments, storing either "10:1" or "30:1" spores at 20°C leads to maximal viability (70% after 24 d). The relevance of these data in developing strategies to optimize production methods for biological control agents is discussed.

Introduction

Interest in using host-specific plant pathogens to control weedy plants has increased as a result of mounting difficulties and expenses in registering chemical herbicides and public demand for reducing chemical pesticide usage. Many factors influence the feasibility of commercializing a biological control agent. Initially, market potential, host-specificity, and weed-killing efficacy are important factors in selecting plant pathogens for development as biological control agents. Commercialization of biological control agents requires that 3 additional requirements be met: 1.) consistent weed control under field conditions; 2.) low-cost methods for producing the biological control agent; and 3.) stable formulations of the biological control agent with a minimum effective shelf-life of 6 months. In our laboratory, we are developing methods for producing conidial spores of *Colletotrichum truncatum* NRRL 13737. This *C. truncatum* strain is a promising fungal bioherbicide which is a pathogen of the noxious weed, *Sesbania exaltata* (Boyette 1991).

The use of submerged culture techniques for producing *C. truncatum* spores offers a

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convenient, controllable method for mass-producing this biological control agent. Our approach to optimizing production methods for *C. truncatum* spores considers spore yield relative to spore attributes such as rapid germination and appressoria formation, efficacy in inciting disease in *S. exaltata* and amenability to formulation. Our submerged culture studies using defined nutritional environments have identified carbon concentration and carbon-to-nitrogen (CN) ratio as factors which significantly influence spore yield (Jackson and Bothast 1990). Efficacy studies (Schisler *et al.* 1991) showed that the CN ratio of the medium in which spores are produced significantly influenced their ability to incite disease. Spores produced in media with a CN ratio of 10:1 germinated more rapidly, produced appressoria more frequently, and were more efficacious in inciting disease in *S. exaltata* compared to spores produced in media with a CN ratio of 30:1. Compositional analysis of these spores has revealed a significantly higher protein and lower lipid content in "10:1" spores compared to "30:1" spores (Jackson and Schisler 1991). The ability of "10:1" spores to germinate rapidly and form appressoria frequently should provide these spores a considerable advantage in infecting the weed host under field conditions.

In addition to spore yield and spore efficacy, amenability to formulation is also a critical requirement for developing a biological control agent. Methods for harvesting and storing *C. truncatum* spores must be developed which maximize viability. This is required for large-scale field testing and commercialization. To develop methods for storing and formulating *C. truncatum* spores derived from liquid cultures, we have examined environmental factors which influence spore viability and fitness during spore harvest and storage. In these initial studies, spores were maintained in an aqueous environment. Length of storage, storage medium, and temperature were the environmental conditions considered in regard to "10:1" and "30:1" spore viability and fitness. Viability and fitness were determined by measuring the ability of spores to germinate and form appressoria, respectively.

Materials and Methods

Organism

C. truncatum (Schw.) Andrus and Moore (NRRL 13737=ARS patent culture collection #18434 (Boyette 1991) was obtained from the U.S. Department of Agriculture, Agriculture Research Service, National Center for Agricultural Utilization Research Culture Collection. Stock cultures of *C. truncatum* were maintained and spore inocula produced for liquid-culture studies as previously described (Jackson and Bothast 1990).

Media and Culture Conditions

The defined basal salts medium supplemented with vitamins used in all liquid-culture experiments has been previously described (Jackson and Bothast 1990). Stock solutions of glucose (20% w/v; Difco) and vitamin-free Casamino acids (5% w/v; Difco) were added to the basal salts medium to provide a 4 g/l carbon concentration and CN ratios of 30:1 and 10:1. Carbon concentrations and CN ratios were calculated as the carbon and nitrogen present in glucose (40% carbon) and Casamino acids (53% carbon, 8% nitrogen).

Liquid-culture experiments were carried out in triplicate 500-ml baffled, Erlenmeyer flasks at a 200-ml volume. The cultures were incubated at 28°C and 300 rpm in rotary shaker incubator. The initial spore concentration for all submerged cultures was 5×10^4 spores/ml. A pH of 5 was maintained by daily adjustments with either 2N HCl or 2N NaOH. Flasks were shaken frequently to inhibit mycelial growth on the flask wall.

Spore Harvest and Storage

Aseptic procedures and sterile materials were used throughout the spore harvesting and storage process. Spores were harvested from 5-d-old *C. truncatum* liquid cultures. Triplicate culture flasks were pooled and filtered through single layers of cheesecloth. Spore suspensions were then filtered through 2 layers of cheesecloth and examined microscopically to

ensure that the suspensions were essentially free of mycelia.

Aliquots (20 mL) of filtered *C. truncatum* spores in spent culture medium were removed for storage experiments. The remaining spore suspension was centrifuged (10 min @ 6500 RCF) and the spore pellet rinsed with deionized water. This rinsing procedure was repeated and the final spore pellet was suspended in deionized water for storage.

Twenty mL spore suspensions (= 2 x 10^6 spores/mL) were stored in sterile, plastic 50 mL screw-cap conical tubes (Sarstedt #620547). Screw-caps were kept tightly closed throughout storage. Spore suspensions in spent culture media and deionized water were stored at various temperatures (28, 20, 4 and -20°C).

Germination and Appressoria Analyses

Spore germination and appressoria formation was assessed by spraying spore suspensions on 2% water agar plates overlayed with cellophane membranes, as previously described (Schisler *et al.* 1991). Briefly, 1 mL samples were periodically removed from the stored spore suspensions, diluted 1:1 with sterile deionized water and sprayed onto 2 membrane agar plates. Following a 6-h incubation at 25°C, spore germination and appressoria formation in 100 spores/plate were assessed microscopically.

Results and Discussion

The production of biological control agents which are amenable to storage and formulation is a requirement for commercialization. Since our previous work has shown that the nutritional environment present in the conidiation medium influences spore yield and efficacy, we have compared spores derived from media with CN ratios of 10:1 and 30:1 in regard to their ability to remain viable under various harvesting and storage conditions. Wet storage was used throughout this study to alleviate the deleterious affects of drying. Previous studies have chronicled the sensitivity of various Colletotricha to drying (Blakeman and Hornby 1966, Farley 1976) and preliminary attempts to dry submerged-culture *C. truncatum* spores in our laboratory were unsuccessful (R. Silman, personal communication).

The temperature at which *C. truncatum* spores are held during the harvesting and storage processes influences viability. In our initial tests, spore suspensions were held in ice baths during harvesting (~3 h) and stored for 2 d at 4°C in either spent culture media or deionized water. During the harvesting procedure, "30:1" spores which were rinsed with deionized water were less viable than "30:1" unrinsed spores (Table 1). Subsequent experiments in which conidia were held at room temperature rather than on ice baths during the harvesting process showed that viability was not lost in rinsed "30:1" spores (Table 2). The holding of spore suspension near 0°C (ice bath) for 3 h was sufficient to kill a significant percentage of "30:1" spores which were rinsed with deionized water.

Storing *C. truncatum* spore suspensions at lowered temperatures (4 for -20°C) reduced the viability of spores compared to those stored at 20°C, regardless of storage medium (Table 2). Storing "30:1" spores at lowered temperatures led to a much more rapid loss in viability compared to "10:1" conidia (Tables 1 and 2). After 24 h storage at 4 or -20°C, <10% of the "30:1" spores were viable. Storing "10:1" and "30:1" spores at 20°C in spent media or deionized water led to a maintenance of viability for 2 d (Table 2).

Long-term storage of "30:1" and "10:1" spores at 20 or 28°C in deionized water produced spore suspensions which retain ~70% viability following 24 d storage. By 90 d, all spore suspension were essentially nonviable. Once again, storage at lowered temperatures led to a rapid loss of viability (Table 3). The detrimental influence of lowered temperatures on spore viability, particularly the rapid loss in viability by "30:1" spores, was unexpected. Differences in the protein and lipid content of "30:1" (15% protein, 33% lipid) and "10:1" (32% protein, 17% lipid) spores have been observed (Jackson and Schisler 1991), but how these differences could influence susceptibility to cold temperatures is uncertain. Studies with the entomopathogenic fungus, *Beauveria bassiana*, have shown that blastospores produced in nitrogen-limited media contained more lipid and glycogen reserves and remained viable longer

when stored at 25°C compared to blastospores produced in carbon-limited media (Lane *et al.* 1991). The basic inability of *C. truncatum* spores to survive cold temperatures, regardless of the conidiation medium in which they are produced, suggests that a general susceptibility to cold temperatures exists.

Table 1. Viability of *Colletotrichum truncatum* spores stored in spent culture media or deionized water at 4°C. During the spore harvest procedure (~ 3 h), spore suspension were held in ice baths. % Germ = % spore germination; % Appr. = % appressoria formation.

Sporulation Medium (CN Ratio)	Storage Environment	Storage Time (h)					
		0 h		24 h		48 h	
		% Germ.	% Appr.	% Germ.	% Appr.	% Germ.	% Appr.
10:1	Spent Media	95	75	50	17	30	13
	Deionized H_2O	87	54	40	10	32	9
30:1	Spent Media	94	36	1	0	0	0
	Deionized H_2O	65	30	1	1	2	0
S.E.		9.3	9.3	11.4	4.0	5.0	4.0

Table 2. Viability of *Colletotrichum truncatum* spores stored at various temperatures in spent culture media and deionized water. During the spore harvest procedure, spores were held at room temperature. % Germ = % spore germination; % Appr. = % appressoria formation.

Sporulation Medium (CN Ratio)	Storage Liquid	Storage Temperature (°C)	Storage Time (h)					
			0		24		48	
			% Germ.	% Appr.	% Germ.	% Appr.	% Germ.	% Appr.
10:1	Spent Media	20	100	52	98	39	98	48
		4	100	52	91	23	72	9
		-20	100	52	74	25	37	12
10:1	Deionized H_2O	20	99	53	99	41	90	35
		4	99	53	84	31	81	19
		-20	99	53	66	14	49	6
30:1	Spent Media	20	97	19	97	23	98	7
		4	97	19	5	0	1	0
		-20	97	19	0	0	0	0
30:1	Deionized H_2O	20	98	11	84	11	75	7
		4	98	11	7	0	3	0
		-20	98	11	2	0	1	0

Table 3. Long-term storage of *Colletotrichum truncatum* spores at various temperatures in deionized H_2O. Spore suspensions were held at room temperature during the harvesting procedure. % Germ = % spore germination; % Appr. = % appressoria formation.

Sporulation Medium (CN Ratio)	Storage Time (d)	Temperature (°C)							
		28		20		4		-20	
		% Germ	% Appr.	% Germ	% Appr.	% Germ	% Appr.	% Germ	% Appr.
10:1	0	98	55	98	55	98	55	98	55
	8	75	24	89	30	57	9	0	0
	24	39	4	71	7	7	0	-	-
	48	6	1	13	1	0	0	-	-
	84	-	-	1	0	-	-	-	-
30:1	0	95	16	95	16	96	16	95	16
	8	85	1	88	5	4	0	0	0
	24	68	6	72	8	0	0	-	-
	48	28	1	41	1	-	-	-	-
	84	-	-	10	0	-	-	-	-
S.E.		7.4	11.4	5.6	4.8	2.9	4.8	2.5	4.7

Previous studies with *C. coccodes* showed that spores do not survive overwintering (Blakeman and Hornby 1966). Sclerotia were shown to be the overwintering structures. Colletotricha produce acervular conidial spores which serve as infective propagules during the growing season (Dillard 1988). Since they typically germinate during the warmer growing season, the ability to withstand cold temperatures is not a requirement. It is also possible that the inability of liquid-culture produced spores to surround themselves with a mucilagenous matrix, a water-soluble glycoprotein which prevents spore death during desiccation, reduces their ability to withstand cold temperatures (Nicholson and Moraes 1980). Preliminary experiments with *C. truncatum* spores produced in acervuli on potato dextrose agar plates suggest that spores surrounded by a mucilagenous matrix are less susceptible to cold storage (data not shown). Further work is needed to determine how endogenous reserves or exogenous matrix material influence the temperature sensitivity of liquid-culture produced *C. truncatum* spores.

During this study, we also assessed the fitness of spores based on their ability to germinate and form appressoria on water agar overlayed with cellulose membranes. While the ability to form appressoria may not necessarily translate to increased spore efficacy in inciting disease in *S. exaltata* (Schisler *et al.* 1992), it does provide an indication of fitness since appressoria formation is required for hyphal penetration through the plant cuticle. Long-term storage experiments show that the ability to form appressoria decreases with length of storage for "30:1" and "10:1" spores (Table 3). Studies to determine if reduced appressoria formation in stored spores is correlated with reduced efficacy in inciting disease in *S. exaltata* are needed.

These studies demonstrate the importance of controlling temperature during the spore harvest and storage process. Lowered temperatures reduce the ability of *C. truncatum* spores to survive storage. The heightened sensitivity of "30:1" spores to lowered temperatures and rinsing with deionized water compared to "10:1" spores suggests that the nutritional history of *C. truncatum* conidia can alter not only spore efficacy and yield but also their amenability to storage.

Further work is needed to develop methods which will allow *C. truncatum* spores to be stored for up to 6 months without losing efficacy. These studies have shown that *C. truncatum* spores derived from liquid cultures can be harvested and stored for short periods of time (2 d) without loss in viability or fitness provided they are not subjected to cold temperatures. We are currently evaluating various drying protocols and amendments to liquid storage media to improve the shelf-life of *C. truncatum* spore formulations.

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