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Sporulation of Several Biocontrol Fungi as Affected by Carbon and Nitrogen Sources in a Two-Stage Cultivation System

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The development of fungal biopesticides requires the efficient production of large numbers spores or other propagules. The current study used published information concerning carbon concentrations and C:N ratios to evaluate the effects of carbon and nitrogen sources on sporulation of *Paecilomyces lilacinus* (IPC-P and M-14) and *Metarhizium anisopliae* (SQZ-1-21 and RS-4-1) in a two-stage cultivation system. For *P. lilacinus* IPC-P, the optimal sporulation medium contained urea as the nitrogen source, dextrin as the carbon source at 1 g/L, a C:N ratio of 5:1, with $ZnSO_4 \cdot 7H_2O$ at 10 mg/L and $CaCl_2$ at 3 g/L. The optimal sporulation medium for *P. lilacinus* M-14 contained soy peptone as the nitrogen source and maltose as the carbon source at 2 g/L, a C:N ratio of 10:1, with $ZnSO_4 \cdot 7H_2O$ at 250 mg/L, $CuSO_4 \cdot 5H_2O$ at 10 mg/L, H_3BO_4 at 5 mg/L, and $Na_2MoO_4 \cdot 2H_2O$ at 5 mg/L. The optimum sporulation medium for *M. anisopliae* SQZ-1-21 contained urea as the nitrogen source, sucrose as the carbon source at 16 g/ L, a C:N ratio of 80:1, with $ZnSO_4 \cdot 7H_2O$ at 50 mg/L, $CuSO_4 \cdot 5H_2O$ at 50 mg/L, H_3BO_4 at 5 mg/L, and $MnSO_4 \cdot H_2O$ at 10 mg/L. The optimum sporulation medium for *M. anisopliae* RS-4-1 contained soy peptone as the nitrogen source, sucrose as the carbon source at 4 g/L, a C:N ratio of 5:1, with $ZnSO_4 \cdot 7H_2O$ at 50 mg/L and H_3BO_4 at 50 mg/L. All sporulation media contained 17 g/L agar. While these results were empirically derived, they provide a first step toward low-cost mass production of these biocontrol agents.

***Keywords*:** *M. anisopliae*, *P. lilacinus*, nutrition, sporulation

The fungus *Metarhizium anisopliae* is a promising biocontrol agent of insect pests (Kang *et al*., 1998), and *Paecilomyces lilacinus* is a promising biocontrol agent of plant-parasitic nematodes (Jatala *et al*., 1979, 1980; Cabanillas and Barker, 1989; Zaki and Bhatti, 1990). Their practical use is limited, however, because of difficulties in the mass production required for their commercialization. The commercialization of potential control agents often depends on an efficient method for producing large numbers of infective propagules (Jenjins and Goettel, 1997). Mass production and comercialization of these organisms, therefore, will require an understanding of the nutritional requirements for their growth and sporulation.

Various researchers have examined how nutrition in basal media affects fungal growth and sporulation with the goal of determining the optimal components and concentrations for mass production of biocontrol agents. Leite *et al*. (2003) examined the effects of carbon and nitrogen sources on the growth of three genera of *Entomophthorales* (*Batkoa*, *Furia*, and *Neozygites*), and they reported that the growth of these isolates was similar with different sources of carbon but differed depending on the source of nitrogen. Liu and Chen (2002, 2003), who studied the nutritional requirements of nematode endoparasitic fungus *Hirsutella rhossiliensis* and nematode egg parasites *Pochonia chlamydosporia* and Arkansas fungus 18 (ARF18), found that some carbon and nitrogen sources supported good growth in liquid and solid media, while others could not be utilized. Jackson and his colleagues also found that the medium greatly influenced the sporulation of *Colletotrichum truncatum* and that the carbon concentration and C:N ratio significantly affected the number of conidia produced and conidial attributes of *C. truncatum* in liquid culture (Jackson and Bothast, 1990; Schisler *et al.*, 1991; Jackson and Schisler, 1992; Jackson and Slininger, 1993). The effects of carbon concentration and C:N ratio on growth and sporulation of the fungus *Helminthosporium solani*, which causes silver scurf on stored potatoes, has been extensively studied and they demonstrated high carbon concentrations or C:N ratios reduced *H. solani* conidiation (Elson *et al.*, 1998). In contrast, high C:N ratios increased spore yield for the biocontrol agent *Talaromyces flavus* (Engelkes *et al.*, 1997). Overall, these researches have demonstrated that the carbon and nitrogen sources, the carbon concentrations, and the C:N ratios of the culture medium may greatly affect fungal growth and sporulation.

Many of these studies and especially those concerned with *in vitro* sporulation were conducted via traditional continuous culture on agar plates. In such culture, the fungus usually grows vegetatively before sporulating, and in growing vegetatively, the fungus usually changes the nutritional content of the medium. In these cases, the nutritional requirements for fungal spore production are unclear. In addition, all of the studies mentioned in the previous paragraph based their conclusions on experiments in which

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Table 1. Selected mineral elements for mycelial growth and sporulation of tested biocontrol agents

Species	Isolates	Mineral elements
P. lilacinus	IPC-P	$ZnSO_4 \cdot 7H_2O$ 10 mg/L, $CaCl_2$ 3 g/L
P. lilacinus	M-14	$ZnSO_4 \cdot 7H_2O$ 250 mg/L, $CuSO_4 \cdot 5H_2O$ 10 mg/L, H_3BO_4 5 mg/L, $Na_2MoO_4 \cdot 2H_2O$ 5 mg/L
M. anisopliae	SQZ-1-12	$ZnSO_4 \cdot 7H_2O$ 50 mg/L, $CuSO_4 \cdot 5H_2O$ 50 mg/L, H_3BO_4 5 mg/L, $MnSO_4 \cdot H_2O$ 10 mg/L
M. anisopliae	RS-4-1	$ZnSO4 \cdot 7H_2O$ 50 mg/L, H_3BO_4 50 mg/L

only one factor in the basal medium (carbon or nitrogen sources, and or carbon concentrations together with C:N ratios) was changed at a time. These studies did not screen the effects of combinations of media components and contents on fungal growth and/or sporulation.

In our previous study, we used a two-stage cultivation method to measure the effects of carbon concentration and C:N ratio on sporulation of several biocontrol fungi, including *P. lilacinus* and *M. anisopliae* (Gao and Liu, 2009). In the current study, we used the two-stage cultivation method to determine how different combinations of carbon and nitrogen sources affect sporulation of the biocontrol agents *P. lilacinus* and *M. anisopliae*.

Materials and Methods

Fungi and inocula

The study used three isolates of the nematophagous fungus *P. lilacinus* (M-14, IPC-P) *M. anisopliae* (SQZ-1-21), and one isolate of the entomopathogenic fungus *M. anisopliae* (RS-4-1). The isolates were deposited in the Center of General Microorganisms Culture Collection (CGMCC) at the Institute of Microbiology, Chinese Academy of Sciences. *P. lilacinus* M-14 (CGMCC 3.10031) was isolated from *Heterodera glycines* in Huanan County, Heilongjiang, China, by X.Z. Liu, and IPC-P (CGMCC 3.10032) was isolated from *Meloidogyne incognita* in Lima, Peru, by P. Jatala. *M. anisopliae* SQZ-1-21 (CGMCC 3.10033) was isolated from *Meloidogyne arenaria* in Qingzhou, Shandong, China, by M.H. Sun, and RS-4-1 (CGMCC 3.10035) was isolated from *Galleria mellonella* that had been added to soil in Jiangsu, China, by Z.A. Chen). All fungi were single-conidium isolates and were maintained on potato dextrose agar (PDA; Oxoid Ltd., England) slants at room temperature. For experiments, the fungi were cultured on PDA plates at 25°C. Conidial inocula were prepared according to Gao *et al*. (2007).

Media preparation

One liter of the basal medium for mycelial growth was made according to Gao and Liu (2009). The entire defined media was autoclaved at 121°C for 30 min. The pH was adjusted to 7.0 by adding 1 N NaOH or 1 N HCl. For the sporulation medium of each isolate, mineral elements were added as indicated in Table 1, carbon concentrations and C:N ratios (Table 2) were adjusted with different carbon and nitrogen sources (Table 3). Selection of carbon concentrations and C:N ratios were based on Gao and Liu (2009).

Table 2. Selected carbon concentrations and C:N ratios for sporulation of tested biocontrol agents

Species	Isolates	For sporulaiton on solid culture	
		Carbon (g/L)	C:N ratio
P. lilacinus	IPC-P	1	5
P. lilacinus	M-14	2	10
M. anisopliae	SQZ-1-12	16	80
M. anisopliae	RS-4-1	4	5

Effects of combinations of carbon and nitrogen sources on sporulation in a two-stage cultivation system

Sterile basal medium (15 ml) was poured into 9.0-cm diameter plastic plates (Miniplast, Israel). After agar solidified and 2 days before inoculation, a sterile cellophane membrane disc (3.5 cm diameter) was placed on the surface of the agar in each plate. A conidial suspension (5 µl containing about 5×10^4 conidia/ml) was transferred onto the center of the sterile cellophane disk, and the plate was then sealed with double Parafilm (Pechiney Plastic Packaging, USA). After 4 days at 25°C, the cellophane disks and associated colonies were transferred to fresh medium (sporulation medium) and cultured for another 4 days, i.e., the colonies were subjected to two-stage cultivation. Continuous cultivation for 8 days on the basal medium with the cellophane disk was used as the control (CK). To quantify sporulation, both the colony and disk were transferred into a 50-ml centrifuge tube containing 10 ml of 0.05% Tween 80, and the tube was shaken. Spores were counted with a hemacytometer. Each combination of isolate and sporulation medium for two-stage cultivation was replicated three times. The control was also replicated three times.

Statistical analysis

Data for fungal sporulation were subjected to one-way analysis of variances (ANOVA), and means were separated using Fisher's protected least significant difference (LSD) at $P=0.05$ with SAS software (Version 8.2, SAS Institute, USA).

Results

For *P. lilacinus* IPC-P, spore yield was highest on the sporulation medium containing dextrin as the carbon source and urea as the nitrogen source (Table 4), while spore yield was lowest on the sporulation medium containing dextrin and tryptone. Among the nitrogen sources, urea generally

Table 3. Selected carbon and nitrogen sources for sporulation of tested biocontrol agents

Species	Isolates	Carbon sources
P. lilacinus	IPC-P	Mannose, maltose, dextrin
P. lilacinus	M-14	Maltose, sucrose, starch soluble
M. anisopliae	SQZ-1-12	Mannose, glucose, sucrose
M. anisopliae	RS-4-1	Mannose, glutin, sucrose
		Nitrogen sources
P. lilacinus	IPC-P	Tryptone, urea, yeast extract
P. lilacinus	M-14	Soy peptone, yeast extract
M. anisopliae	SQZ-1-12	Urea, soy peptone
M. anisopliae	RS-4-1	Soy peptone, yeast extract

Table 4. Combinations of carbon and nitrogen sources for sporulation on solid of tested biocontrol agents

Species	Isolates	Carbon sources	Nitrogen sources								CK		LSD
			Tryptone		Yeast extract		Soy peptone		Urea				
P. lilacinus	IPC-P	Mannose	173[a]	h	672	c			1051	b			
		Maltose	587	d	87	i			416	f			
		Dextrin	64	i	455	e			1375	a	270	g	28
P. lilacinus	M-14	Starch soluble			73	d	79	d					
		Maltose			40	e	319	a					
		Sucrose			143	b	126	c			45	e	12
M. anisopliae	SQZ-1-21	Mannose					38	bcd	71	ab			
		Sucrose					13	cd	91	a			
		Glucose					6	d	53	abc	6	d	46
M. anisopliae	RS-4-1	Mannose			42	b	12	de					
		Glutin			12	de	15	d					
		Sucrose			24	c	54	a			5	d	8

[a] Values are means of three replicates. Four each isolate, values followed by the same letter are not significantly different (LSD; $P \leq 0.05$)

supported the highest spore yield. Overall, spore yield for *P. lilacinus* IPC-P was highest when the isolate was grown on the basal medium for 4 days, and then grown on sporulation medium containing urea as the nitrogen source, a carbon concentration (from dextrin) of 1 g/L, a C:N ratio of 5:1, the mineral elements listed in Table 1, and 17 g of Bacto (Difco) agar per liter (Tables 1-4).

For *P. lilacinus* M-14, spore yield was highest on the sporulation medium containing maltose as the carbon source and soy peptone as the nitrogen source, while spore yield was lowest on the sporulation medium containing maltose and yeast extract (Table 4). Overall, spore yield for *P. lilacinus* M-14 was highest when the isolate was grown on the basal medium for 4 days, and then grown on sporulation medium containing soy peptone as the nitrogen source, a carbon concentration (from maltose) of 2 g/L, a C:N ratio of 10:1, the mineral elements listed in Table 1, and 17 g of agar per L (Tables 1-4).

For *M. anisopliae* SQZ-1-21, spore yield was highest on the sporulation medium containing sucrose as the carbon source and urea as the nitrogen source, while spore yield was lowest on the sporulation medium containing glucose and soy peptone (Table 4). Urea generally supported higher spore yield than soy peptone. Overall, spore yield for *M. anisopliae* SQZ-1-21 was highest when the isolate was grown on the basal medium for 4 days, and then transferred to sporulation medium containing urea as nitrogen source, a carbon concentration (from sucrose) of 16 g/L, a C:N ratio of 80:1, the mineral elements listed in Table 1, and 17 g of agar per L (Tables 1-4).

For *M. anisopliae* RS-4-1, spore yield was highest on the sporulation medium containing sucrose as the carbon source and soy peptone as the nitrogen source, while spore yield was lowest on the sporulation medium containing glutin and yeast extract or mannose and soy peptone (Table 4). Overall, spore yield for *M. anisopliae* RS-4-1 was highest when the isolate was grown on the basal medium for 4 days and then transferred to sporulation medium containing soy peptone as the nitrogen source, a carbon concentration (from sucrose) of 4 g/L, a C:N ratio of 5:1, the mineral elements listed in Table 1, and 17 g of agar per L (Tables 1-4).

Discussion

Our previous study used the two-stage cultivation method to determine the optimal carbon concentrations and C:N ratios for sporulation of six fungal isolates, including two isolates of *P. lilacinus* (IPC-P and M-14) and two isolates of *M. anisopliae* (SQZ-1-21 and RS-4-1) (Gao and Liu, 2009). The current study, which used the previously identified optimal carbon concentrations and C:N ratios, evaluated how different combinations of carbon and nitrogen sources affected sporulation of *P. lilacinus* IPC-P and M-14 and *M. anisopliae* SQZ-1-21 and RS-4-1 by the two-stage cultivation method. Researchers have previously reported that the influence of amino acids on fungal sporulation is species-dependent (Evans and Black, 1981; Latgé and Sanglier, 1985) or strain-dependent (Elson *et al*., 1998). The results of the current study were consistent with these reports in that the optimal combinations of carbon and nitrogen sources were different for the different isolates of *P. lilacinus* and *M. anisopliae*. In general, however, maltose and yeast extract did not support abundant sporulation of either of the two *P. lilacinus* isolates, and sucrose supported abundant sporulation of both *M. anisopliae* isolates.

Spore yields were greatly affected by the combination of carbon and nitrogen sources and by the two-stage cultivation method. Thus, using the best combination of carbon and nitrogen sources and the two-stage cultivation method increased spore yield relative to the control (continuous cultivation in the basal medium) by 5.1 times for *P. lilacinus* IPC-P, by 7.1 times for *P. lilacinus* M-14, by 15.2 times for *M. anisopliae* SQZ-1-21, and by 10.8 times for *M. anisopliae* RS-4-1. These results demonstrate that commercial production of these and other fungi will depend on a detailed understanding of their nutritional requirements.

The production of fungal biopesticides will probably be based on the growth of mycelia and/or conidia on relatively

simple media (Gray and Markham, 1997; Shah *et al*., 1998). Although one goal could be the maximizing of sporulation (which was the focus of the current study), the medium that produces the most spores may not produce the highest quality of spores. Nutritional factors can affect the efficacy (propagule quality) of biocontrol agents (Zhang *et al*., 2005). For example, the carbohydrate component of the medium affects the quantities of specific polyols and trehalose in conidia of entomopathogenic fungi, and trehalose can enhance desiccation tolerance (Harman *et al*., 1991; Gornova *et al*., 1992). In view of how media can affect both the quantity and quality of mass-produced biocontrol agents, it is prudent to evaluate a wide range of media in order to optimize nutritional conditions for the commercial production of these fungal biocontrol agents (Jackson *et al*., 1997).

To achieve these goals, further work needs to be accomplished to test the biocontrol efficacy of the harvested production from media at different combinations of nutrition factors, which will aid future research by providing a means of efficient production and biocontrol.

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