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NOTE

Li Gao · Xing-Zhong Liu · Man-Hong Sun
Shi-Dong Li · Jin-Li Wang

Use of a novel two-stage cultivation method to determine the effects of environmental factors on the growth and sporulation of several biocontrol fungi

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Abstract To supply essential information for improving mass production and biocontrol efficacy, two-stage cultivation on agar plates was used to evaluate the environmental conditions affecting mycelial growth and sporulation of seven biocontrol fungi. Maximum growth and sporulation occurred on acid media for *Paecilomyces* (*Pa.*) *lilacinus* IPC-P, *Pochonia* (*Po.*) *chlamydosporia* HSY-12-14, and *Lecanicillium lecanii* CA-1-G, and on alkaline media for *Metarhizium anisopliae* isolates. All fungi preferred a certain water potential and temperature for sporulation. Light greatly inhibited the growth of *P. lilacinus* IPC-P, *M. anisopliae* SQZ-1-21, and *L. lecanii* CA-1-G but enhanced the sporulation of *P. lilacinus* M-14, *P. chlamydosporia* HSY-12-14, and *L. lecanii* CA-1-G.

Key words Light · pH · Temperature · Water potential

Because of concerns about the negative effects of chemical pesticides on the environment and human health, people have increasingly shifted to the use of biological measures for control of insect pests, weeds, and diseases (Ortiz and Orduz 2001). Acting as biocontrol agents, many species of fungi have considerable potential for management of agricultural pests and have recently attracted considerable social and commercial interest. The nematophagous fungi *Paecilomyces lilacinus* and *Pochonia chlamydosporia* (syn. *Verticillium chlamydosporium*), the entomopathogenic fungi *Metarhizium anisopliae* and *Lecanicillium lecanii* (syn. *Verticillium lecanii*), and the mycoparasitic fungus *Trichoderma viride* have been commercialized or are being commercialized as biocontrol agents (Liu and Li 2004). Development and application of these fungi, however, has been hampered by incomplete information about how environmental conditions affect agent growth and sporulation during production and by a poor understanding of agent ecology after release.

Previous researchers have studied the influences of pH, water potential, temperature, light, and other environmental factors on fungal vegetative growth and/or sporulation. Hung and Trappe (1983) demonstrated that the optimal pH for growth varied greatly among fungal species. McQuiken et al. (1997) found that mycelial extension and pycnidial production of four isolates of the mycoparasite *Coniothyrium minitans* occurred over a pH range of 3–8, with optimum values between pH 4.5 and 5.6. Water is an important factor regulating fungal growth (Gervais and Molin 2003). The sporulation pattern of *M. anisopliae* was directly related to water content (Dorta and Arcas 1998). The optimum water potential for mycelial growth and sporulation of *Verticillium dahliae* was between –100 and –120 bars (Ioannou et al. 1977). Temperature is another important factor affecting fungal growth and sporulation (Magan and Lacey 1988). Hyphae of four isolates of *C. minitans* grew between 4° and 25°C, with maximum growth at 20°–25°C; the isolates produced pycnidia between 10° and 25°C, and the optimum was 20°C (McQuiken et al. 1997). Fluorescent light increased the radial mycelial growth of *Sphaeropsis pyriputrescens* (Kim et al. 2005) and the formation of pycnidia of some Sphaeropsidales species (Ashworth 1959; Knox-Davies 1965) such as *Ascochyta pisi* (Leach 1962) and *Botryodiplodia theobromae* (Ekundayo and Haskins 1969). Bennett et al. (1981) reported that *Aspergillus parasiticus* did not grow at 15°C if the culture broth was under light but grew slowly if the culture was in darkness. McQuiken et al. (1997) demonstrated that light increased the pycnidial production but not the vegetative growth of four isolates of *C. minitans*.

Information about how environmental factors affect the growth or sporulation of fungal biocontrol agents has been

L. Gao · X.-Z. Liu (✉)
Key Laboratory of Systematic Mycology and Lichenology, Institute of Microbiology, Chinese Academy of Sciences, Datun Road, Chaoyang District, Beijing 100010, P. R. China
Tel. +86-10-64807505; Fax +86-10-64807505
e-mail: liuxz@im.ac.cn

L. Gao · M.-H. Sun · S.-D. Li
State Key Laboratory for Biology of Plant Disease and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, P. R. China

J.-L. Wang
Beijing Municipal Forestry Protection Station, Beijing, P. R. China

useful for improving both mass production of their inocula and their biocontrol efficacy. However, many of these studies and especially those concerned with in vitro sporulation were conducted via traditional continuous culture on agar plates. In such cultures, the fungus usually grows vegetatively before sporulating, and in growing vegetatively, the fungus usually changes the nutritional content of the medium. Thus, the nutritional requirements for fungal spore production are unclear. To better define the nutritional requirements for sporulation, a two-stage cultivation method was applied for culturing fungi on solid media (Sun et al. 2009). In the first stage, the fungus was cultured on an agar plate for 4 days for vegetative growth. The fungus was then transferred to a new agar plate with defined nutrients or under different environment conditions for sporulation (secondary stage). The effect of environmental conditions such as pH, water potential, dark/light cycle, and temperature on mycelial growth and sporulation of seven biocontrol fungi by this two-stage culturing method is reported in this article.

Four nematophagous fungi (*Paecilomyces lilacinus* IPC-P and M-14, *Metarhizium anisopliae* SQZ-1-21, and *Pochonia chlamydosporia* HSY-12-14), two entomopathogenic fungi (*M. anisopliae* RS-4-1 and *Lecanicillium lecanii* CA-1-G), and one mycoparasitic fungus (*Trichoderma viride* TV-1) were used in the present study. The fungi were deposited in the Center of General Microorganisms Culture Collection (CGMCC), Institute of Microbiology, Chinese Academy of Sciences (Table 1).

Single-spore cultures of the fungi were transferred from slants of potato dextrose agar (PDA; Oxoid, Basingstoke, UK) to PDA in 9.0-cm-diameter Petri plates (Miniplast Ein-shemer, Israel). After 8 days at 25°C, conidia suspensions were prepared by transferring a 5.0-mm-diameter plug from the colony edge into a 50-ml centrifuge tube containing 10 ml sterile 0.05% Tween 80 and shaking for 5 min. Conidial concentration was determined with a hemocytometer and was adjusted to 10^6 spores/ml for use in the following experiments.

Disks (3.5 cm diameter) of sterile cellophane were placed on the surface of PDA plates (9.0 cm diameter, one disk per plate, pH 7) 2 days before addition of conidial suspensions. Disks were 3.5 cm in diameter for all fungi except *T. viride* TV-1; larger disks (9.0 cm diameter) were used for *T. viride* TV-1 because it grows very quickly. A conidial suspension (5 µl) was transferred onto the center of the cellophane disk, and plates were kept at 25°C (20°C for plates used in the light experiment) for 4 days in the dark. Then, in a purgative table under room condition, the cellophane disk with the fungal colony was quickly transferred to a new PDA plate (9.0 cm diameter), which had been adjusted to a specific pH or water potential or which was exposed to specific dark/light cycles or temperature (see section on treatments). The new plates with the colonies and disks were double sealed with Parafilm (Pechiney Plastic Packaging, Chicago, IL, USA) for another 4 days. Each colony with the cellophane disk was removed from the plate, weighed, and then placed in a 50-ml centrifuge tube containing 10 ml sterile 0.05% (v/v) Tween 80. Spores were dislodged from the colony, and the number of spores cm^{-2} per colony was determined with a haemocytometer. The net fresh weight of the colony was calculated by subtracting the weight of the cellophane disk. Each treatment was replicated three times (one plate per replication).

The effects of pH, water potential, dark/light cycle, and temperature were studied independently. Except as noted, treatments used PDA made following the instructions (PDA; Oxoid), and PDA plates were kept at 25°C under room conditions. For pH treatments, PDA was adjusted to a pH range of 3–9 with various combinations of Na_2HPO_4 and citric or tris-(hydoxymethyl) aminomethane, which were added to the agar after sterilization but before solidification (Gomori 1955). For water potential treatments, the water potential of PDA was adjusted with potassium chloride to –0.3, –0.8, –1.2, –2.1, –3.9, or –7.3 MPa (Robinson and Stokes 1959). Dark/light cycle treatments were applied in an environmental-controlled cabinet equipped with 18 W/m^2 white fluorescent tubes (Philips, China) located about 60 cm above the plates. For the dark/light cycle of 24 h/0 h, the plates were covered with black paper and kept in a sealed, opaque plastic box. For the dark/light cycles of 0 h/24 h or 12 h/12 h, plates were not covered with black paper and were kept in a sealed, clear plastic box. The dark/light cycle treatments were carried out by a microcomputer timer switch (KG-316T; Zhengchuang, Shanghai, China). Temperature treatments were 20°, 23°, 26°, 29°, and 32°C.

Data were subjected to one-way analyses of variance (ANOVA), and means were compared with Fisher's protected least significant difference (LSD) at $P = 0.05$; SAS software (Version 8.2; SAS Institute, Cary, NC, USA) was used. A logarithmic transformation was applied to the sporulation data before statistical analysis.

The pH of the medium significantly affected fungal growth and sporulation of the seven fungal isolates (Table 2). Growth of *Pa. lilacinus* IPC-P was enhanced by pH 3 but sporulation was enhanced by pH 6; similar phe-

Table 1. Fungi used in this study

Species	Isolate	Host	Location	Isolated by
Lecanicillium lecanii	CA-1-G	*Myzus persicae*	Fujian, China	M. Xie
Metarhizium anisopliae	SQZ-1-21	*Meloidogyne arenaria*	Qingzhou, Shandong, China	M.H. Sun
Metarhizium anisopliae	RS-4-1	Soil with *Galleria mellonella* baiting	Jiangsu, China	Z.A. Chen
Paecilomyces lilacinus	IPC-P	*Meloidogyne incognita*	Lima, Peru	P. Jatala
Paecilomyces lilacinus	M-14	*Heterodera glycines*	Huanan County, Heilongjiang, China	X.Z. Liu
Pochonia chlamydosporia	HSY-12-14	*Meloidogyne incognita*	Sanya, Hainan, China	M.H. Sun
Trichoderma viride	TV-1	*Alternaria alternata*	Yunnan, China	G. Wang

Table 2. Effects of pH on mycelial growth and sporulation of the tested biocontrol fungi

	pH	Isolate						
		IPC-P	M-14	SQZ-1-21	HSY-12-14	RS-4-1	CA-1-G	TV-1
Mycelial growth (mg cm^{-2})	3	52.00a	45.78b	71.15b	53.23a	46.43bc	47.68a	21.23b
	4	27.03c	30.01c	53.62c	29.43c	48.61abc	40.43abc	52.13a
	5	30.85bc	36.69bc	47.54cd	39.41abc	40.71c	27.62d	26.70b
	6	34.89bc	34.05bc	35.44d	33.01bc	47.05bc	32.17cd	37.80ab
	7	32.03bc	37.29bc	37.19d	47.80ab	58.69ab	35.13bcd	30.33b
	8	38.48b	38.26bc	61.36bc	41.17abc	61.05a	45.95ab	20.32b
	9	36.13bc	62.40a	179.14a	35.91bc	48.85abc	38.35abcd	27.56b
	LSD	9.50	12.60	15.00	17.00	13.10	11.70	21.10
Sporulation (10^5 conidia cm^{-2})	3	22.84ab	10.68b	31.93b	18.73ab	6.39c	54.27ab	28.52c
	4	16.57ab	4.79bc	19.52bc	21.15a	18.99bc	84.75a	52.84b
	5	14.30b	18.82a	18.63bc	10.88ab	21.57ab	35.98b	14.88cd
	6	32.94a	6.08bc	13.06c	10.62ab	16.30bc	24.55b	11.77cd
	7	23.87ab	6.19bc	32.21b	17.91ab	21.61ab	13.62b	14.92cd
	8	17.00ab	1.73c	51.29a	18.28ab	34.86a	24.27b	81.65a
	9	19.92ab	4.77bc	53.41a	8.63b	17.96bc	41.45ab	13.77cd
	LSD	18.21	7.74	14.22	12.04	14.99	44.77	15.76

LSD, least squares difference
Values are means of three replicates; values in the same column followed by the same letter are not significantly different (LSD; $P \leq 0.05$)

Table 3. Effects of water potential on mycelial growth and sporulation of the tested biocontrol fungi

	Water potential (MPa)	Isolate						
		IPC-P	M-14	SQZ-1-21	HSY-12-14	RS-4-1	CA-1-G	TV-1
Mycelial growth (mg cm^{-2})	–0.3	30.00b	47.90a	43.00a	38.54bc	52.69ab	40.88a	28.00a
	–0.8	42.08b	36.30b	48.47a	29.23c	37.03b	37.54a	31.00a
	–1.2	28.29b	32.00b	40.35a	32.01c	35.32b	42.83a	33.00a
	–2.1	29.20b	36.60b	38.69a	36.66c	48.18ab	45.41a	29.00a
	–3.9	53.75b	37.00b	44.00a	55.25a	51.99ab	59.91a	36.00a
	–7.3	168.05a	37.90b	44.00a	49.76ab	63.87a	61.46a	33.00a
	LSD	67.80	23.70	21.50	13.10	25.00	33.00	19.00
Sporulation (10^5 conidia cm^{-2})	–0.3	16.42ab	19.03a	5.30a	14.64b	9.04a	43.18b	101.83a
	–0.8	15.76ab	3.40a	4.87ab	15.67b	2.24b	43.35b	28.45ab
	–1.2	8.68b	4.60a	5.52a	19.38b	7.85a	24.85b	3.30b
	–2.1	12.78ab	2.21a	5.24a	15.30b	8.15a	62.37b	8.00b
	–3.9	23.31a	3.76a	3.86ab	111.10a	12.95a	143.93a	34.75ab
	–7.3	4.28b	2.14a	0.15b	1.49b	0.33b	68.69b	2.44b
	LSD	12.66	17.93	4.87	33.89	5.57	56.61	84.26

Values are means of three replicates; values in the same column followed by a same letter are not significantly different (LSD; $P \leq 0.05$)

nomena of higher pH for sporulation and lower pH for mycelial growth were observed for *Po. chlamydosporia* HSY-12-14, *L. lecanii* CA-1-G (both from pH 3 to pH 4), and *T. viride* TV-1 (from pH 4 to pH 8), whereas the opposite trend was found for *Pa. lilacinus* M-14 (from pH 9 to pH 5). For *M. anisopliae*, growth and sporulation were both favored by alkaline media: SQZ-1-21 had good mycelia growth at pH 9 and had good sporulation at pH 8 or pH 9; RS-4-1 had both good mycelia growth and sporulation at pH 8.

Water potential affected the growth and sporulation of most fungi (Table 3). The optimal water potential for growth was –0.3 MPa for *Pa. lilacinus* M-14, –3.9 MPa for *Po. chlamydosporia* HSY-12-14, and –7.3 MPa for *Pa. lilacinus* IPC-P and *M. anisopliae* RS-4-1. The optimal water potential for sporulation was the same for the optimum for growth of *Po. chlamydosporia* HSY-12-14 (both at –3.9 MPa), and higher than the optimum for growth of *Pa. lilacinus* IPC-P. Spore production was the highest at –0.3 MPa for *T. viride* TV-1, at –0.3, –1.2, and –2.1 MPa for *M. anisopliae* SQZ-1-21, at –0.3, –1.2, –2.1, and –3.9 for *M. anisopliae* RS-4-1, at –3.9 MPa for *Pa. lilacinus* IPC-P, *Po. chlamydosporia* HSY-12-14, and *L. lecanii* CA-1-G (see Table 3).

Darkness enhanced the mycelial growth of *Pa. lilacinus* IPC-P, *M. anisopliae* SQZ-1-21, and *L. lecanii* CA-1-G whereas the mycelial growth of the other fungi was insensitive to dark/light cycles (Table 4). Light enhanced the sporulation of *Pa. lilacinus* M-14, *Po. chlamydosporia* HSY-12-14, and *L. lecanii* CA-1-G. In contrast, *T. viride* TV-1 produced the most spores with a 12 h dark/12 h light cycle and 24 h dark/0 h light cycle, and *Pa. lilacinus* IPC-P and *M. anisopliae* SQZ-1-21 sporulated well with a 24 h dark/0 h light cycle. *M. anisopliae* RS-4-1 sporulated well with a wider range of dark/light cycles (see Table 4).

All seven fungi grew and sporulated over a wide range of temperatures (Table 5). The optimum temperature for

Table 4. Effects of dark/light cycle on mycelial growth and sporulation of the tested biocontrol fungi

	Dark/light cycle	Isolate						
		IPC-P	M-14	SQZ-1-21	HSY-12-14	RS-4-1	CA-1-G	TV-1
Mycelial growth (mg cm^{-2})	0 h/24 h	38.00b	44.72a	29.06b	45.87a	32.98a	33.26b	17.17a
	12 h/12 h	29.75b	55.05a	33.43ab	57.73a	36.84a	33.55b	25.16a
	24 h/0 h	49.96a	56.58a	39.38a	44.88a	39.12a	42.46a	21.23a
	LSD	10.60	28.50	6.60	24.90	7.10	7.10	10.20
Sporulation (10^5 conidia cm^{-2})	0 h/24 h	58.69b	130.64a	87.49b	61.39a	102.32a	70.54a	3.05b
	12 h/12 h	46.79b	121.57ab	108.20ab	35.84ab	90.50a	48.36ab	28.93a
	24 h/0 h	91.70a	55.91b	122.88a	23.85b	65.38a	30.68b	20.82a
	LSD	32.48	74.47	22.89	28.02	39.32	20.07	11.68

Values are means of three replicates; values in the same column followed by the same letter are not significantly different (LSD; $P \leq 0.05$)

Table 5. Effects of temperature on mycelial growth and sporulation of the tested biocontrol fungi

	Temperature (°C)	Isolate						
		IPC-P	M-14	SQZ-1-21	HSY-12-14	RS-4-1	CA-1-G	TV-1
Mycelial growth (mg cm^{-2})	20	39.13a	47.97a	35.91a	39.03a	33.87a	29.00b	86.24a
	23	43.98a	36.15a	24.26a	34.18a	35.00a	37.57a	125.41a
	26	32.62a	36.64a	31.82a	34.11a	31.13a	39.43a	77.75a
	29	33.00a	36.22a	31.58a	36.22a	36.00a	20.70c	87.01a
	32	35.11a	34.66a	27.56a	34.66a	35.00a	20.63c	87.73a
	LSD	21.59	18.28	14.25	14.47	22.39	23.22	71.07
Sporulation (10^5 conidia cm^{-2})	20	26.00b	62.39ab	20.88b	31.20bc	19.06b	42.64b	46.45b
	23	52.34ab	38.13b	30.50b	76.26a	45.41ab	46.45b	79.03a
	26	33.97b	98.79a	42.38b	24.70c	59.79ab	33.76b	77.02a
	29	71.75a	104.34a	71.93a	20.87c	50.35ab	86.05a	71.06ab
	32	32.24b	69.67ab	37.73b	41.80b	94.02a	30.85b	46.80b
	LSD	27.96	58.77	28.93	10.90	51.34	27.49	28.74

Values are means of three replicates; values in the same column followed by the same letter are not significantly different (LSD, $P \leq 0.05$)

growth was 23° or 26°C for *L. lecanii* CA-1-G. The growth of the other isolates was insensitive to temperature; they grew well from 20° to 32°C. For *L. lecanii* CA-1-G, the optimal temperature was higher for sporulation than for growth. The highest sporulation occurred at 23°C for *Po. chlamydosporia* HSY-12-14, at 23° or 26°C for *T. viride* TV-1; 26° or 29°C for *Pa. lilacinus* M-14; 29°C for *Pa. lilacinus* IPC-P, *M. anisopliae* SQZ-1-21, and *L. lecanii* CA-1-G; and 32°C for *M. anisopliae* RS-4-1 (Table 5).

Previous studies have usually used continuous culture on agar plates or in liquid media to measure the effects of environmental factors on fungal growth and sporulation. In this study, we used a novel method of two-stage cultivation to study the effects of different pH, water potential, dark/light cycle, and temperature conditions on fungal growth and sporulation in the second stage. One of the obvious advantages of this method compared with the traditional one was that under cellophane we successfully defined environmental conditions for sporulation on the second stage, and differences were detected in the optimal conditions for growth versus sporulation. Much more information was obtained by this two-stage cultivation method in comparison with the traditional one. This two-stage cultivation method is not only useful to determine the nutritional requirements and environmental conditions for fungal sporulation but also is significant for fungal biotechnology and industry.

The response of fungi to nutrition and other environmental factors can differ among species within a genus (Evans and Black 1981; Hung and Trappe 1983; Abellana et al. 2001) and even among isolates within a species (Boyle and Hellenbrand 1991; Elson et al. 1998). This distinction was also true in the current study in which isolates of *Pa. lilacinus* and *M. anisopliae* were no more similar to each other than they were to the other fungi. In addition, much more detailed information was obtained by the two-stage cultivation method than the traditional method, indicating another superiority of this novel method. We also have found that some isolates seems to show a bimodal response, such as sporulation of TV-1 and SQZ-1-21 in the pH gradient and sporulation of RS-4-1 in the water potential gradient. A possible reason is that the conditions below or above the optimal ones could reduce fungal growth and increase sporulation.

Studies of colony formation, growth, and sporulation of two isolates of *M. anisopliae* conducted by Li and Holdom (1995) indicated that soil environment greatly affected the survival of both isolates. Survival of *Beauveria bassiana* conidia was primarily dependent on temperature and soil water content when the conidia were applied to sterile and nonsterile soil under various temperature, soil water content, and pH regimes (Lingg and Donaldson 1981). Results in our study will contribute to a better understanding of the ecological characters of these biocontrol agents.

The two-stage cultivation method was first described and used to determine optimal nutrition for growth and sporulation of several biocontrol fungi (Gao et al., unpublished data). That information together with the results in this article will be useful for improving the mass production of these biocontrol agents, for helping us to understand colonization, survival, competitive ability, and biocontrol efficacy under field conditions, and are greatly helpful in developing better strategies for successful application of these fungi in natural conditions. Many more studies to optimize the combinations of environmental factors and nutritional requirements for their mass production, spore shelf-life, and pathogenicity are very necessary in the future (Papavizas 1987; Sayre and Walter 1991).

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