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Carbon requirements of some nematophagous, entomopathogenic and mycoparasitic Hyphomycetes as fungal biocontrol agents

ManHong Sun & XingZhong Liu
Key Laboratory of Systematic Mycology & Lichenology, Institute of Microbiology, Chinese Academy of Sciences, 100080, Beijing, P. R. China

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Abstract

Thirty-three carbon sources were evaluated for their effects on spore germination, hyphal growth and sporulation of 11 fungal biocontrol agents, i.e. the nematophagous fungi *Paecilomyces lilacinus*, *Pochonia chlamydosporia*, *Hirsutella rhossiliensis*, *H. minnesotensis* and Arkansas Fungus 18, the entomopathogenic fungi *Lecanicillium lecanii*, *Beauveria bassiana* and *Metarhizium anisopliae*, and the mycoparasitic fungus *Trichoderma viride*. Variations in carbon requirements were found among the fungal species or strains tested. All strains studied except for *T. viride* grew on most carbon sources, although *B. bassiana* had more fastidious requirements for spore germination. Monosaccharides and disaccharides were suitable for fungal growth. For most isolates, D-glucose, D-mannose, sucrose and trehalose were superior to pectin and soluble starch among the polysaccharides and lactic acid among the organic acids. Both ethanol and methanol could accelerate growth of most isolates but not biomass. D-mannose, D-fructose and D-xylose were excellent carbon sources for sporulation, while D-glucose, sucrose, cellobiose, trehalose, chitin, dextrin, gelatin and lactic acid were better for some isolates. Neither sorbic acid nor linoleic acid could be utilized as a single carbon source. These findings provided a better understanding of the nutritional requirements of different fungal biocontrol agents that can benefit the mass production process.

Key words: carbon sources, fungi, nutritional requirement

Introduction

Fungal biocontrol agents in the Hyphomycetes have showed great potential in pest management and received intensive studies. The entomopathogenic fungi *Beauveria bassiana* (Balsamo) Vuillemin, *Metarhizium anisopliae* (Metschnikoff) Sorokin, *Lecanicillium lecanii* (Zimmermann) Zare & Gams [= *Verticillium lecanii* (Zimm.) Viégas], the nematophagous fungi *Paecilomyces lilacinus* (Thom) Samson, *Pochonia chlamydosporia* (Goddard) Zare & Gams [= *Verticillium chlamydosporium* Goddard], *Hirsutella rhossiliensis* Minter & Brady, *H. minnesotensis* Chen, Liu & Chen, sterile mycelium Arkansas Fungus 18 (ARF18), and the mycoparasitic fungus *Trichoderma viride* Persoon ex Gray are well known potential biocontrol agents and have received increasing interest for their commercial applications [1–9]. Commercial products of *B. bassiana* and *M. anisopliae* have come into use in the USA, Former USSR, Brazil, Australia, France and other countries since mid-20th century. There have been two successful commercial products of *L. lecanii* conidia and blastospores in the control of whiteflies, thrips and aphids in Netherlands [10] and of soybean cyst nematodes in the USA [11]. Up to date, more than 60 products including 38 species or varieties of fungi have been or being developed worldwide [12]. However, the prerequisites for

fungal industrialization, such as mass-production, stability and long shelf-life, have hindered the commercialization of the fungal agents [13].

As fungal biocontrol agents, large amounts of biomass and spores, which act as effective components in management of plant diseases in field, are indispensable. It is still hard to obtain abundant biomass and spore output on a given artificial medium in large-scale fungal production. Since the 1960s, effects of nutrition factors on trap formation of nematode-trapping fungi *Arthrobotrys*, *Dactylaria* and *Monacrosporium* were studied [14–21]. Later many types of media were evaluated in the fermentation processes of some nematophagous, entomopathogenic and mycoparasitic Hyphomycetes [22–28]. *Paecilomyces lilacinus*, growing on a broad range of carbon and nitrogen media, was identified to have accelerated mycelial growth and sporulation with the addition of plant oil residue [29]. A variety of media were used to cultivate *Trichoderma*, and commercial production has been achieved in many countries [30, 31]. Despite the many attempts to screen commercially available, low-cost ingredients of industrialized biological pesticides, the research on nutritional requirements of fungal agents were overlooked, and a systematic investigation of fungal nutrition utilization is very much needed to improve mass production and accelerate commercialization. We studied the growth and propagation of eight genera of important and commonly used fungal biocontrol agents in various nutrition factors, including carbon sources, nitrogen sources, vitamins and minerals in order to achieve their further production and application. In this study, 11 isolates including seven nematophages, three entomopathogens, and one mycoparasite were selected to evaluate the effects of 33 carbon sources on their spore germination, hyphal growth, and sporulation.

Materials and methods

Fungal isolates and inocula

In total, 11 isolates of nine fungal species from different hosts and locations were used in the study (Table 1). These included seven nematophagous isolates (*Paecilomyces lilacinus* M-14, *P. lilacinus* Peruvian strain (IPC-P), *Pochonia chlamydosporia* HSY-12-14, *Hirsutella rhossiliensis* OWVT-1, *H. minnesotensis* HLJ-46, sterile mycelium ARF18 (ARF907), and *Metarhizium anisopliae* SQZ-1-21), three entomopathogenic isolates (*Lecanicillium lecanii* CA-1-G, *Beauveria bassiana* IBC1201, and *M. anisopliae* RS-4-1), and one mycoparasitic isolate (*Trichoderma viride* TV-1). The biocontrol potential of these isolates had been extensively evaluated in greenhouses and fields in China. All the isolates were maintained on slants of potato dextrose agar (PDA; Oxoid Ltd., Basingstoke, Hampshire, England) at 4 °C.

The fungi were transferred onto PDA Petri dishes (9-cm-diam.) and incubated at 25 °C for 1–2 weeks to produce conidia as inocula for carbon utilization test. A plug of fungal agar culture (*ca.* 5×5 mm^2) was placed into a 15 ml sterile plastic centrifuge tube (Miniplast, Israel). A homogeneous spore suspension of each fungal isolate was prepared by vibrating the agar piece with 5 ml sterile 0.05% Tween-80 solution. Spore

Table 1. Details of assay fungal strains

Species	Isolate number	Host	Locality	Isolated by
Beauveria bassiana	IBC1201	*Locusta migratoria*	Tianjin, China	C.S. Deng
Hirsutella minnesotensis	HLJ-46	*Heterodera glycines*	Jiansanjiang, Heilongjiang, China	R. Ma
H. rhossiliensis	OWVT-1	*Heterodera glycines*	Minnesota, USA	X.Z. Liu
Lecanicillium lecanii	CA-1-G	Aphid	Fujian, China	M. Xie
Metarhizium anisopliae	RS-4-1	*Alissonotum* sp.	Jiangsu, China	Z.A. Chen
M. anisopliae	SQZ-1-21	*Meloidogyne arenaria*	Qingzhou, Shandong, China	M. Xie
Paecilomyces lilacinus	M-14	*Heteroderaglycines*	Huanan, Heilongjiang, China	X.Z. Liu
P. lilacinus	Peruvian strain (IPC-P)	*Meloidogyne incognita*	International Potato Research Center, Peru	P. Jatala
Pochonia chlamydosporia	HSY-12-14	*Meloidogyne incognita*	Sanya, Hainan, China	M.H. Sun
Sterile hyphum ARF18	ARF907	*Heterodera glycines*	Arkansas, USA	D.G. Kim
Trichoderma viride	TV-1	*Alternaria alternata*	Yunnan, China	G. Wang

concentration was determined with a hemacytometer under a microscope (400×) and adjusted to 10^6 spores/ml. For sterile hyphum ARF907, the hyphal fragments were used as inoculum.

Preparation of media

The basal medium was composed of 20 g D-glucose (equivalent to 8.0 g carbon per litre), 2 g $NaNO_3$ (equivalent to 0.33 g nitrogen per litre), 1 g K_2HPO_4, 0.5 g $MgSO_4$, 0.5 g KCl, 0.01 g $FeSO_4$, and 17 g agar (Bacto agar; Difco, Becton Dickinson and Company, Sparks, USA) per liter of distilled water.

D-glucose in basal medium was substituted by various carbon sources conforming to a carbon concentration of 8.0 g/l. The carbon substitutes included 19 carbohydrates (D-mannose, D-galactose, D-fructose, L-sorbose, D-ribose, D-xylose, D-arabmose, maltose, sucrose, trehalose, cellobiose, soluble starch, dextrin, pectin, gelatin, agarose, chitin, methyl cellulose, and sorbitol), 11 organic acids (oxalic acid, lactic acid, malic acid, amber acid, citric acid, sorbic acid, tartaric acid, dodecanoic acid, tetradecanoic acid, stearic acid, and linoleic acid), and two simple hydroxyl compounds (ethanol and methanol). All the substitutes were tested separately for their effects on spore germination, hyphal growth and sporulation of each fungal isolate. The basal medium was used as a control.

Utilization of carbon sources in agar media

All media were adjusted to a pH of 6.5 by 1 N HCl or 1 N NaOH before autoclaving at 121 °C for 30 min. A volume of 15 ml of the medium was poured into each 9-cm-diameter plastic plate (Miniplast, Israel). After the agar cooled down, a piece of sterile cellophane (diam. 9 cm) was placed on the agar surface of each plate. Exactly 10 μl spore suspension (*ca.* 10^4 spores) of each isolate was inoculated with a pipette (Gilson, France) onto the centre of each agar plate supplemented with different carbon sources. Plates were sealed with parafilm and incubated at 25 °C. After 24 h, 30 spores of each plate were randomly examined for germination under a microscope at 100×. ARF907 was cultured on PDA for 4 weeks before testing for carbon utilization. Plugs (diam. 4 mm) of ARF907 from colony margins were inoculated onto the center of assay media.

Hyphal growth was determined by the mycelial growth rate and biomass production. The growth rate was estimated by measuring colony diameter (three measurements) at day 3 and 7 and colony extension (mm/day). Fungal biomass was estimated by measuring hyphal fresh weight, which had a good correlation to dry weight determined in a preliminary study. The fresh weights of fungal colony and cellophane disks were measured after 2 week incubation for all isolates except ARF907 whose colonies were measured at the end of 4 week incubation. The thallus and cellophane weighed then transferred to 15 ml 0.05% Tween-80 surfactant in a 50 ml plastic tube. Fungal spores from each colony were dislodged and suspended by vortexing with a vibrator for 30 s and counted using a hemacytometer under a microscope. The sporulation was determined by the number of spores per colony and per gram mycelium fresh weight (spores per gram). Three replicates for each medium and each isolate were applied.

Statistical analysis

The SAS system V8.0 (SAS Institute Inc., Cary, NC) was used for the analysis of variance (ANOVA). Least significance difference (LSD) at $P = 0.05$ was used for comparison of the means from each medium.

Results

The study focused on the spore germination, hyphal growth and sporulation properties of 11 fungal isolates grown on different carbon sources. Variations in carbon requirements were found among the fungal species or strains tested.

Effects of carbon sources on spore germination

The effects of carbon sources on fungal spore germination differed in the time elapsed before the appearance of germinal tubes (data not shown). Two isolates of *P. lilacinus*, M-14 and IPC-P, were obtained from *Heterodera glycines* Ichinohe and *Meloidogyne* sp., respectively. Spores of isolate M-14 germinated faster in the media supplemented

with gelatin and agarose whereas spores of isolate IPC-P germinated faster in trehalose and pectin media. Both isolates of *M. anisopliae*, SQZ-1-21 from egg of *Meloidogyne* and RS-4-1 from grub of *Alissonotum*, germinated faster in the media containing gelatin, dextrin, and simple alcohol. There was no difference in germination after 24 h among most isolates (Table 2). Sorbic acid and linoleic acid inhibited spore germination of all isolates tested; and dodecanoic acid inhibited spore germination of isolates IPC-P, HSY-12-14, and IBC1201. The spore germination of isolate TV-1 was inhibited by chitin and most of the organic acids. L-sorbose was a poor carbon source for spore germination of isolate SQZ-1-21 and RS-4-1. Compared with other 7 isolates, the spore germination rate of isolate IBC1201 was lower in most of the media (27 of 33 carbon sources).

Effects of carbon sources on hyphal growth

The effects of different carbon sources on the hyphal growths of the fungal isolates tested are shown in Table 3. Most of the carbon sources were suitable for the growth of all the isolates except isolates HIJ-46 and TV-1, which were fastidious for carbon sources. In general, monosaccharides and disaccharides were more effective for fungal growth. D-glucose, D-mannose, sucrose and trehalose could promote the hyphal growth of most isolates; D-fructose, D-xylose, and maltose were also good carbon sources. Pectin and soluble starch were preferred carbon sources of polysaccharides; and other polysaccharides resulted in moderate level of growth. Among organic acids, lactic acid was a better carbon source, followed by stearic acid. The rest of acids were poor sources for fungal growth. Sorbic acid and linoleic acid could not support fungal growth when each of them was used as a single carbon source. Most of the fungal isolates achieved high daily growth rates in the media containing ethanol or methanol despite limited net biomass. The two growth measurements of fungal isolates were parallel for some substrates but not for others.

Paecilomyces lilacinus M-14 grew faster in the media containing methanol, methyl cellulose, stearic acid, agarose, and pectin, though the aerial hypha were sparse. Sorbitol supported much biomass of M-14, most of the monosaccharides and all disaccharides were fair carbon sources, while organic acids and alcohols were unsuitable. Maltose, mannose, D-xylose, and pectin promoted the growth of *P. lilacinus* isolate IPC-P, and mannose and maltose were the best for hypha biomass production. D-glucose, D-fructose, trehalose, and sorbitol were also good carbon sources for biomass yield. Compared with isolate M-14, isolate IPC-P stopped growing when dodecanoic acid was supplied as the single carbon source, and was nearly incapable of utilizing most of the organic acids and simple hydroxyl compounds. Rapid colony extension of *P. chlamydosporia* isolate HSY-12-14 was observed in the media supplemented with soluble starch, trehalose, agarose, methyl cellulose, and two hydroxyl compounds. However, maximum hyphal fresh weight was harvested from the media containing D-glucose, sucrose, maltose, and trehalose. Both isolates of *Hirsutella*, HLJ-46 and OWVT-1, grew slowly and produced little biomass in all of the media. D-glucose and D-mannose were the best monosaccharides for *Hirsutella* cultivation. Trehalose and gelatin could greatly increase daily growth of both isolates, while mannose and citric acid enhanced biomass accumulation of HLJ-46 and OWVT-1, respectively. The growth of ARF907 was promoted by D-xylose, pectin, or agarose. Some disaccharides, such as sucrose and trehalose, were also good carbon sources for ARF907; whereas most of the organic acids and methyl cellulose were not suitable for ARF907 hyphal growth.

Trichoderma viride TV-1 produced the most biomass in media containing D-glucose, D-fructose, D-xylose, cellobiose, and pectin. On the contrary, its biomass production was inhibited by chitin or such organic acids as oxalic acid, lactic acid, amber acid, sorbic acid, tartaric acid, and stearic acid.

Two isolates of *M. anisopliae*, SQZ-1-21 and RS-4-1, differed in growth rate. SQZ-1-21 grew faster than RS-4-1 in most media. D-mannose and gelatin were good carbon sources for the colony extension of RS-4-1, while gelatin, pectin, and D-mannose were good for SQZ-1-21. For biomass production, D-fructose and D-mannose for isolate RS-4-1, while sucrose, D-glucose, D-mannose, and D-fructose for SQZ-1-21 were required. Polysaccharides, various acids and alcohols were difficult to be utilized by *M. anisopliae* for biomass yield. In general, disaccharides were excellent carbon sources for *B. bassiana* IBC1201, among them

Table 2. Effcts of various carbon sources on spore germination rate of biocontrol fungi (%)

C Sources	*P. lilacinus* M-14	*P. lilacinus* IPC-P	*P. chlamydosporia* HSY-12-14	*T. viride* TV-1	*M. anisopliae* RS-4-1	*M. anisopliae* SQZ-1-21	*B. bassiana* IBC1201	*L. lecanii* CA-1-G	LSD[a]
D-Glucose	96.7[b]	97.8	96.7	98.9	100.0	97.8	81.1	98.9	4.9
D-Mannose	98.9	98.9	94.4	100.0	100.0	98.9	90.0	100.0	3.7
D-Galactose	98.9	98.9	95.5	100.0	100.0	98.9	81.1	98.9	3.5
D-Fructose	95.6	95.6	85.6	98.9	97.8	93.3	55.6	94.4	8.0
L-Sorbose	91.1	94.4	82.2	97.8	71.1	82.2	3.3	83.3	7.6
D-Ribose	96.7	97.8	86.7	97.8	96.7	97.8	47.8	96.7	7.2
D-Xylose	94.4	96.7	92.2	98.9	96.7	96.7	40.0	96.7	7.1
D-Arabinose	94.4	98.9	96.7	100.0	97.8	94.4	97.8	98.9	3.5
Maltose	97.8	98.3	97.8	100.0	95.6	100.0	53.3	94.4	11.6
Sucrose	100.0	98.9	98.9	100.0	100.0	98.9	95.6	98.9	2.6
Trehalose	100.0	98.9	95.6	100.0	100.0	98.9	97.8	100.0	2.4
Cellobiose	96.7	98.9	97.8	100.0	100.0	97.8	92.2	100.0	4.3
Soluble starch	97.8	97.8	96.7	100.0	100.0	96.7	96.7	100.0	2.6
Dextrin	98.9	100.0	95.6	100.0	100.0	100.0	96.7	100.0	2.6
Pectin	98.9	100.0	96.7	100.0	100.0	100.0	76.7	100.0	4.3
Gelatin	98.9	100.0	97.8	100.0	100.0	100.0	98.9	100.0	2.0
Agarose	100.0	100.0	98.9	100.0	100.0	100.0	95.6	100.0	2.6
Chitin	98.9	98.4	93.3	0.0	100.0	94.4	ND[c]	100.0	3.6
Mythyl cellulose	98.9	100.0	98.9	98.9	ND	ND	ND	100.0	2.7
Sorbitol	98.9	100.0	97.8	100.0	100.0	100.0	98.9	100.0	2.0
Oxalic acid	95.6	91.1	98.9	1.1	98.9	91.1	37.8	97.8	8.6
Lactic acid	97.8	98.9	95.6	0.0	98.9	93.3	75.6	100.0	12.4
Malic acid	97.8	97.8	93.3	37.8	100.0	98.9	38.9	98.9	7.9
Amber acid	94.4	96.7	95.6	73.3	100.0	95.6	23.3	94.4	8.8
Citric acid	97.8	95.6	92.2	93.3	100.0	97.8	80.0	97.8	6.3
Sorbic acid	0.0	0.0	2.2	2.2	0.0	1.1	0.0	0.0	2.0
Tartaric acid	98.9	94.4	95.6	1.1	100.0	96.7	71.1	100.0	4.2
Dodecanoic acid	ND	0.0	0.0	78.9	8.9	7.8	0.0	46.7	9.6
Tetradecanoic acid	97.8	96.7	94.4	100.0	100.0	94.4	71.1	100.0	7.2
Stearic acid	94.4	98.9	98.9	3.3	100.0	98.9	60.0	98.9	7.0
Linoleic acid	0.0	0.0	1.1	0.0	1.1	0.0	0.0	0.0	1.7
Ethanol alcohol	100.0	100.0	95.6	100.0	100.0	100.0	92.2	100.0	1.7
Methyl alcohol	96.7	100.0	96.7	97.8	100.0	95.6	87.8	100.0	7.1
LSD	3.6	3.6	5.4	4.8	2.3	3.8	11.8	4.2	

[a] LSD is the least significant difference at $p = 0.05$.
[b] Each value is the mean of three replicates.
[c] Not determined.

Table 3. Effects of various carbon sources on hyphal growth in terms of colony growth rate (GR) and colony fresh weight (FW) of biocontrol fungi

Carbon sources	M-14		IPC-P		HSY-12-14		HIJ-46		OWVT-1		ARF907TV-1		RS-4-1		SQZ-1-21		IBC1201		CA-1-G		LSD[a]	
	GR (mm/d)	FW (g)	GR (mm/d)	FW (g)	GR (mm/d)	FW (g)	GR (mm/d)	FW (g)	GR (mm/d)	FW (g)	GR (mm/d)	FW (g)	GR (mm/d)	FW (g)	GR (mm/d)	FW (g)	GR (mm/d)	FW (g)	GR (mm/d)	FW (g)	GR (mm/d)	FW (g)
D-Glucose	5.15[b]	1.44	4.60	1.49	3.46	0.89	0.95	0.04	1.55	0.04	1.15	0.21	2.89	0.19	4.27	0.46	4.05	0.23	2.81	0.40	0.43	0.24
D-Mannose	4.70	1.11	4.95	1.79	3.15	0.57	0.88	0.04	1.48	0.04	1.54	0.13	3.37	0.44	4.36	0.44	4.32	0.24	2.74	0.29	0.48	0.39
D-Galactose	4.92	1.17	4.09	0.88	3.12	0.64	ND[c]	ND	0.76	0.04	0.93	0.14	2.98	0.09	3.19	0.05	4.34	0.08	2.51	0.22	0.44	0.16
D-Fructose	4.41	1.15	4.34	1.49	2.99	0.55	0.14	0.01	0.79	0.04	ND	0.19	3.10	0.51	3.71	0.43	4.21	0.15	2.60	0.16	0.52	0.31
L-Sorbose	ND	0.70	2.96	0.85	1.59	0.19	0.00	0.00	0.77	0.03	0.10	0.07	2.12	0.03	1.64	0.07	0.85	0.05	1.24	0.10	0.18	0.13
D-Ribose	4.09	1.20	4.31	1.26	3.18	0.61	0.13	0.00	0.99	0.02	2.08	0.14	2.87	0.05	4.05	0.09	2.48	0.09	2.46	0.21	0.57	0.32
D-Xylose	5.37	1.33	4.93	1.37	2.90	0.60	0.59	0.01	1.05	0.03	2.82	0.17	2.53	0.17	3.59	0.29	1.58	0.07	2.11	0.19	0.42	0.28
D-Arabinose	3.86	0.93	4.25	0.46	3.37	0.27	0.18	0.01	0.96	0.03	1.20	ND	2.83	0.09	3.93	0.05	4.36	0.26	3.16	0.31	0.47	0.22
Maltose	4.80	1.25	5.33	1.59	3.57	0.74	1.07	0.01	2.04	0.04	1.03	0.05	2.97	0.05	4.32	0.22	2.42	0.05	3.40	0.07	0.34	0.10
Sucrose	5.17	1.13	4.33	1.21	3.59	0.91	1.17	0.03	1.66	0.04	2.33	0.08	3.19	0.21	4.24	0.52	4.52	0.54	2.99	0.36	0.29	0.19
Trehalose	5.02	1.35	4.25	1.48	3.90	0.79	1.24	0.02	2.08	0.04	2.37	0.08	2.80	0.13	4.32	0.24	4.73	0.44	3.17	0.38	0.28	0.29
Cellobiose	4.74	1.30	4.23	1.24	3.44	0.55	1.04	0.02	1.36	0.04	1.21	0.16	3.18	0.07	4.27	0.05	4.21	0.20	2.65	0.25	0.34	0.14
Soluble starch	4.97	0.91	4.69	0.46	4.35	0.21	1.10	0.02	2.12	0.04	1.69	0.07	2.93	0.08	3.69	0.08	4.35	0.04	3.60	0.06	0.29	0.16
Dextrin	4.98	0.88	4.67	0.63	3.84	0.22	1.15	0.02	2.11	0.03	1.44	0.05	2.83	0.12	3.50	0.12	4.26	0.07	3.37	0.08	0.42	0.08
Pectin	5.44	0.15	4.92	0.04	3.79	0.10	0.54	0.03	1.96	0.03	2.78	0.17	3.18	0.03	4.34	0.04	3.38	0.04	3.27	0.07	0.18	0.03
Gelatin	3.43	0.45	4.08	0.28	2.62	0.24	ND	ND	2.71	0.04	2.31	0.05	3.23	0.09	4.65	0.16	3.98	0.16	3.03	0.16	0.35	0.16
Agarose	5.50	0.04	4.53	0.02	4.03	0.03	0.94	0.01	1.79	0.03	2.82	0.02	3.08	0.01	3.82	0.02	3.50	0.04	3.26	0.03	0.23	0.01
Chitin	3.82	0.36	3.83	0.09	2.11	0.08	0.79	0.00	1.74	0.03	0.73	0.00	2.62	0.02	3.13	0.01	2.23	0.05	3.35	0.05	0.26	0.03
Mythyl cellulose	5.89	0.21	4.26	0.05	3.94	0.05	1.03	0.02	2.00	0.02	0.38	0.11	1.57	0.03	2.03	0.01	3.59	0.02	3.13	0.05	0.30	0.03
Sorbitol	3.87	1.51	4.23	1.45	2.56	0.31	1.14	0.01	1.63	0.04	1.81	0.05	2.35	0.07	3.85	0.25	4.10	0.24	2.48	0.19	0.25	0.30
Oxalic acid	2.47	0.04	1.87	0.01	2.00	0.15	0.00	0.00	0.75	0.02	0.00	0.00	1.78	0.03	3.21	0.04	2.52	0.02	2.19	0.03	0.30	0.01
Lactic acid	5.15	0.51	4.54	0.08	2.73	0.07	0.80	0.02	1.44	0.04	1.07	0.00	2.93	0.04	4.21	0.02	4.23	0.04	3.49	0.05	0.38	0.02
Malic acid	ND	0.11	2.41	0.37	2.91	0.03	0.00	0.00	1.49	0.03	0.51	0.01	2.95	0.03	3.76	0.01	3.55	0.03	2.50	0.02	0.32	0.04
Amber acid	4.38	0.05	2.16	0.12	2.62	0.05	0.89	0.01	1.55	0.03	0.47	0.00	1.78	0.02	3.56	0.02	3.31	0.03	2.25	0.04	0.38	0.03
Citric acid	2.90	0.07	2.52	0.04	3.08	0.07	0.00	0.00	1.41	0.04	0.06	0.00	2.92	0.03	4.03	0.02	3.20	0.02	2.74	0.02	0.25	0.03
Sorbic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tartaric acid	ND	0.25	2.81	0.23	3.18	0.05	1.00	0.02	0.81	0.04	0.53	0.00	2.74	0.03	3.86	0.02	3.30	0.01	2.90	0.03	0.24	0.05
Dodecanoic acid	2.06	0.19	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.07	1.71	0.05	1.95	0.03	2.15	0.04	1.70	0.08	0.36	0.02
Tetradecanoic acid	3.97	0.17	3.32	0.02	2.11	0.06	0.00	0.00	ND	ND	ND	0.11	2.71	0.03	3.20	0.01	3.04	0.01	3.12	0.03	0.24	0.03
Stearic acid	5.99	0.11	4.24	0.02	3.66	0.03	1.00	0.02	1.94	0.03	1.51	0.00	3.15	0.02	3.27	0.01	3.48	0.02	3.11	0.03	0.71	0.04
Linoleic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ethanol	4.76	0.36	4.27	0.26	3.98	0.08	1.27	0.02	1.93	0.03	1.29	0.07	2.88	0.01	4.04	0.02	3.68	0.02	3.24	0.06	0.18	0.09
Methanol	6.43	0.08	4.22	0.03	4.01	0.04	1.19	0.01	2.02	0.03	1.15	0.04	3.01	0.01	3.98	0.01	3.84	0.01	3.43	0.02	0.29	0.02
LSD	0.35	0.24	0.45	0.34	0.43	0.17	0.15	0.01	0.09	0.01	0.32	0.06	0.26	0.04	0.37	0.05	0.22	0.04	0.20	0.03		

[a] LSD is the least significant difference at $p = 0.05$.
[b] Each value is the mean of three replicates.
[c] Not determined.

trehalose and sucrose were the best carbon sources for both mycelium growth and biomass production. Most polysaccharides, organic acids, and the two hydroxyl compounds were poor sources for isolate IBC1201 growth, and some monosaccharides, such as D-sorbose and D-xylose, inhibited the growth. For *L. lecanii* CA-1-G, soluble starch, lactic acid, and methanol supported the greatest amount of colony extension. Polysaccharides were also fair for colony growth, but were not good for biomass production. In addition, abundant mycelia would be obtained when it was incubated with glucose, trehalose, or sucrose. Arabinose, mannose and cellobiose were also good carbon sources for mycelial growth.

Effects of carbon sources on sporulation

Carbon compounds had significant effects on the spore count on each agar plate (Table 4). D-mannose, D-fructose and D-xylose were excellent carbon sources for most of the isolates, while D-glucose, sucrose, trehalose, cellobiose, dextrin, gelatin, and chitin were fair sources for some isolates. Most organic acids did not support fungal sporulation. Both hydroxyl compounds were not good carbon sources, either. Various carbon sources also had significant effects on sporulation per gram mycelium. Both hydroxyl compounds, agarose, chitin, and stearic acid were good for some fungi. Lactic acid, gelatin, dextrin, maltose, and cellobiose were also fair for some isolates.

Both isolates of *P. lilacinus* sporulated similarly on most of the substrates. Lactic acid and D-mannose were the best carbon sources for isolates M-14 and IPC-P sporulation, respectively. Chitin, D-xylose and dextrin were fair for both isolates. Methanol and chitin promoted sporulation per gram mycelium by M-14 and IPC-P, respectively. Sucrose, soluble starch, dextrin, pectin, agarose and lactic acid were easy to be utilized by *P. chlamydosporia* isolate HSY-12-14 for sporulation, as most of them were polysaccharides. However, the highest number of spores per gram mycelium was obtained in the media of agarose, stearic acid, and methanol. For *Hirsutella minnesotensis* isolate HLJ-46, stearic acid resulted in the highest yields of total spores and spores per gram. D-mannose was fine, and sucrose, dextrin, and methyl cellulose were also fair sources. Compared to HLJ-46, *H. rhossiliensis* isolate OWVT-1 had different preference to carbon sources for sporulation. Maltose, trehalose, and sucrose were excellent sources, while the most spores per gram was produced on methyl cellulose plates, followed by maltose, pectin, sucrose, and trehalose.

Similar to the carbon requirements for hyphal growth, cellobiose, D-fructose, and D-glucose were outstanding carbon sources for sporulation of *T. viride* isolate TV-1. The most spores per gram were yielded in the media of gelatin. Agarose and dextrin were also fair sources.

Between the two isolates of *M. anisopliae*, sporulation by RS-4-1 was more affected by carbon sources than was SQZ-1-21. Only three carbon sources, D-mannose, gelatin, and D-fructose, were good for isolate RS-4-1 sporulation. Mannose and fructose, which were optimal carbon sources for its hyphal growth, also greatly favored sporulation. However, sporulation was inhibited when glucose, sucrose, or xylose was used as the single carbon source, though these sugars were suitable for vegetative growth by isolate RS-4-1. Agarose was a suitable carbon source for RS-4-1 with the highest spores per gram yield. Several monosaccharides, such as D-mannose, D-xylose, and D-glucose, were best, and most of the disaccharides were also good for isolate SQZ-1-21 sporulation, while methanol was a good source to obtain a large number of spores per gram biomass. In media containing most of the polysaccharides, various acids, and hydroxyl compounds, few spores of both isolates were detected. Cellobiose and D-fructose were beneficial for the sporulation of *B. bassiana* isolate IBC1201, and sorbitol, sucrose, D-glucose, trehalose, and gelatin were also good sources. The greatest production of spores per gram was detected in methanol, tetradecanoic acid, and dextrin media, although the total spores were not high. A great quantity of spores were obtained when *L. lecanii* isolate CA-1-G was cultured in trehalose, cellobiose, D-arabinose, and gelatin media. D-mannose, sucrose and D-glucose also supported good sporulation. Lactic acid was the best carbon source for isolate CA-1-G in terms of sporulation per gram followed by gelatin and ethanol.

In general, spore germination, hyphal growth and sporulation differed significantly among the fungal strains grown on a given carbon source medium or among different carbon sources for a given fungal isolate. In addition, significant

Table 4. Effects of various carbon sources on sporulation in terms of the number of spores per colony (S/colony) and the number of spores per gram mycelium fresh weight (S/g) of biocontrol fungi[a]

Carbon sources	M-14		IPC-P		HSY-12-14		HIJ-46		OWVT-1		TV-1		RS-4-1		SQZ-1-21		IBC1201		CA-1-G		LSD[b]	
	S/colony	S/g	S/colony	S/g	S/colony	S/g	S/colony	S/g	S/colony	S/g	S/colony	S/g	S/colony	S/g	S/colony	S/g	S/colony	S/g	S/colony	S/g	S/colony	S/g
D-Glucose	13.4[c]	9.6	29.3	20.0	12.4	13.9	12.5	345.2	4.5	103.0	85.7	402.6	1.1	5.7	109.5	241.4	192.0	881.3	150.7	379.1	46.3	248.1
D-Mannose	2.7	2.5	150.5	106.4	11.1	19.0	20.0	571.1	8.5	224.3	50.7	424.5	92.7	209.8	132.7	310.5	96.7	396.7	165.3	571.8	45.1	244.0
D-Galactose	20.1	17.0	62.6	77.8	9.3	14.3	ND[d]	ND	4.0	105.3	38.3	295.9	7.9	87.9	20.9	450.2	83.5	1063.1	84.8	413.8	19.7	210.3
D-Fructose	6.2	5.3	86.7	63.7	8.9	18.9	0.0	0.0	0.0	0.0	105.0	634.9	61.0	110.2	79.7	200.4	234.7	1617.6	62.3	393.6	38.3	351.9
L-Sorbose	8.2	11.6	34.7	42.0	5.7	29.9	0.0	0.0	0.0	0.0	29.3	451.6	4.4	184.6	1.5	21.5	3.0	57.2	13.0	131.0	8.0	130.5
D-Ribose	19.5	17.7	20.1	15.3	4.9	7.8	0.0	0.0	0.0	0.0	30.6	225.6	22.3	473.7	64.2	681.4	68.0	733.8	95.2	459.0	17.9	142.1
D-Xylose	139.0	105.8	128.7	101.2	6.6	11.8	1.8	258.3	4.7	146.9	35.7	394.7	1.5	8.7	113.0	395.0	50.8	794.2	64.3	339.5	41.6	229.9
D-Arabinose	7.1	5.7	10.7	24.3	10.3	50.9	0.0	0.0	0.0	0.0	23.6	ND	3.4	55.8	7.0	144.0	71.0	264.0	304.7	1037.5	56.4	335.3
Maltose	26.3	21.3	50.5	31.4	7.7	10.5	2.4	326.8	15.5	399.3	38.5	841.5	6.1	113.0	96.9	492.4	35.7	957.8	34.6	495.4	26.1	476.7
Sucrose	15.8	13.7	41.8	35.0	19.5	20.7	15.0	603.0	13.5	397.2	42.6	639.9	1.3	6.0	96.0	188.6	194.0	360.5	163.3	465.2	39.3	339.9
Trehalose	19.7	14.5	31.0	24.5	14.6	18.7	4.7	292.2	14.5	393.3	33.0	442.0	1.9	14.5	102.1	427.6	179.3	407.4	385.0	1057.4	37.9	187.2
Cellobiose	63.2	48.8	46.4	37.6	7.2	13.4	2.3	123.8	3.0	77.9	115.7	764.4	13.9	187.0	43.0	906.5	254.0	1275.0	322.7	1301.2	48.7	226.3
Soluble starch	108.3	121.1	58.3	160.8	21.3	103.4	7.0	530.3	4.0	117.1	58.0	851.4	8.2	101.4	31.4	408.8	29.4	787.8	17.8	312.0	26.8	215.6
Dextrin	137.3	189.4	116.5	186.7	19.1	85.4	15.6	734.6	2.5	88.8	44.1	974.0	7.5	64.9	66.9	546.8	113.7	2054.5	62.8	793.4	37.3	953.0
Pectin	14.9	105.7	15.8	395.0	21.2	208.2	1.8	62.7	11.0	399.2	65.3	388.4	2.6	76.2	6.6	154.1	15.1	410.7	14.8	216.8	6.7	135.7
Gelatin	5.9	16.1	12.5	50.1	6.6	28.7	ND	ND	6.0	145.8	67.0	1546.0	88.3	1005.5	53.4	377.0	149.3	959.8	250.5	1858.4	36.0	396.2
Agarose	15.3	352.3	15.4	695.0	19.7	703.4	4.0	666.7	3.5	136.9	17.9	1003.7	3.6	1702.5	6.7	485.0	10.0	279.2	13.3	492.4	3.8	1406.6
Chitin	147.3	367.7	114.0	1290.4	12.8	150.9	4.0	2714.3	5.0	187.5	0.0	0.0	2.2	119.3	2.6	310.0	19.9	395.8	17.3	342.2	28.8	1607.6
Mythyl cellulose	22.2	106.7	10.7	220.4	11.8	245.9	16.0	695.0	9.5	505.6	8.9	89.9	3.7	137.8	3.4	546.4	10.3	539.4	12.6	285.3	3.9	248.5
Sorbitol	26.6	18.2	72.0	51.8	3.5	12.8	12.7	0.0	4.0	108.9	23.0	492.0	2.3	32.0	23.0	93.3	204.7	844.8	106.7	553.0	20.1	2731.6
Oxalic acid	8.8	238.8	5.8	854.6	0.2	1.3	0.0	0.0	0.0	0.0	0.0	0.0	3.1	123.9	1.4	34.5	12.8	511.6	8.5	267.7	4.5	338.0
Lactic acid	180.0	330.2	21.8	298.5	19.2	265.9	2.9	135.6	7.5	211.5	0.0	0.0	5.9	169.7	11.4	813.9	56.5	1515.2	104.3	2267.8	14.2	436.7
Malic acid	61.0	610.4	17.8	48.7	6.8	271.6	0.0	0.0	3.0	97.2	0.0	0.0	3.6	139.6	5.2	509.9	11.5	424.7	18.0	767.3	4.6	264.4
Amber acid	27.0	519.5	6.4	53.8	8.8	185.9	0.4	45.8	1.0	27.8	0.0	0.0	4.1	222.4	5.7	348.7	16.3	609.1	24.3	606.3	5.3	173.3
Citric acid	21.6	294.4	5.6	167.1	4.3	82.8	0.0	0.0	1.5	35.0	0.0	0.0	3.8	142.3	4.2	236.9	16.3	856.5	14.9	653.1	4.6	219.4
Sorbic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Tartaric acid	98.7	394.8	6.4	28.6	10.4	232.9	1.5	63.4	0.0	0.0	0.0	0.0	2.4	75.9	3.2	129.8	8.8	1290.7	11.4	415.7	22.8	875.0
Dodecanoic acid	21.5	114.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.9	36.9	0.2	10.3	26.7	793.0	13.6	167.0	9.4	306.6
Tetradecanoic acid	38.2	214.0	2.3	154.7	6.4	107.7	0.0	0.0	ND	ND	4.2	41.6	2.9	93.2	2.8	435.9	29.3	2088.0	8.3	257.6	7.8	330.7
Stearic acid	40.2	394.5	10.6	479.7	23.8	612.9	59.0	3790.6	3.5	133.3	0.0	0.0	5.0	302.9	3.4	599.7	10.7	545.4	12.5	441.2	9.7	461.6
Linoleic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	18.3	228.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.4	13.2
Ethanol	20.1	60.9	27.2	109.9	4.3	54.1	5.2	227.1	2.0	70.7	13.3	180.5	5.6	839.1	10.5	839.3	27.9	1777.7	99.5	1842.1	16.0	781.4
Methanol	57.3	757.1	14.5	647.8	22.3	611.6	13.1	1310.0	1.0	38.5	13.3	341.1	4.8	365.0	7.2	1381.1	13.7	2513.1	14.1	692.9	14.7	956.4
LSD	29.5	157.6	22.3	201.9	3.7	122.2	7.5	2467.7	3.3	121.7	18.6	438.0	9.1	661.1	20.7	462.6	36.3	781.6	39.0	327.1		

[a] Conidial numbers of M-14 is $\times 10^6$/colony; conidial numbers of IPC-P, HSY-12-14, TV-1, RS-4-1, SQZ-1-21, IBC1201 and CA-1-G are $\times 10^7$/colony, respectively; and conidial numbers of HIJ-46H and OWVT-1 are $\times 10^6$/colony, respectively.

[b] LSD is the least significant difference at $p = 0.05$.

[c] Each value is the mean of three replicates.

[d] Not determined.

Table 5. Analysis of variance of spore germination, hyphal growth and sporulation of fungal strains on agar media with various carbon sources

Test	Spore germination[a]		Hyphal growth[b]				Sporulation[c]			
			Colony growth rate		Colony fresh weight		Sporulation per colony		Sporulation per gram fresh weight	
	df	Pr > F	df	Pr > F	df	Pr > F	df	Pr > F	df	Pr > F
Carbon source (C)	32	< 0.001	32	< 0.001	32	< 0.001	32	< 0.001	32	< 0.001
Strain (S)	7	< 0.001	9	< 0.001	9	< 0.001	9	< 0.001	9	< 0.001
C×S	219	< 0.001	280	< 0.001	284	< 0.001	285	< 0.001	284	< 0.001

[a] The percentage of germinated spores (%).
[b] Two measurements were used to evaluate hyphal growth, colony growth rate (mm/day) and colony fresh weight (g).
[c] Two modes were used to evaluate sporulation, sporulation per colony and sporulation per gram mycelium fresh weight.

interactive effects of carbon sources and strains were found (Table 5).

Discussion

Variations of carbon requirements in some fungi in biocontrol system

Previous studies have demonstrated that carbon nutrition has important isolate-dependent effects on fungal cultivation. In this study, D-mannose, sucrose, and soluble starch, being monosaccharide, disaccharide and polysaccharide, respectively, are all excellent carbon sources for most of the fungi tested. The entomopathogenic species, *B. bassiana*, *M. anisopliae*, and *L. lecanii*, preferred monosaccharides and disaccharides, such as sucrose, trehalose, cellobiose, mannose and fructose, for their growth and sporulation. Nematophagous fungi had diverse carbon requirements, including carbohydrates and their derivatives, organic acids, fatty acids, and hydroxyls, as well.

Fungal nutritional requirements might be strain dependent. The carbon sources required for hyphal growth by *P. lilacinus* isolate M-14 from *H. glycines* in China were different from that by isolate IPC-P from *Meloidogyne* sp. in Peru. Isolate M-14 had the ability to utilize many more carbon sources than did isolate IPC-P. Besides carbohydrates, tartaric acid and methyl alcohol were also good carbon sources for isolate M-14 sporulation. *M. anisopliae* isolates, SQZ-1-21 and RS-4-1, parasites of plant nematodes and insects, respectively, were similar in the utilization of various kinds of carbon sources. Most oligosaccharides, such as D-mannose and D-fructose, were good carbon sources for both growth and sporulation. Whereas, polysaccharides, organic acids and hydroxyls were poor carbon sources for both growth and sporulation.

Differences exist among species and isolates from different locations and hosts. This may lead to varying nutritional requirements for fungal growth and sporulation. Since late 1990s, the utilization of various nutrient substances by some biocontrol fungi has been studied in detail [32–34]. Comparing sterile mycelium ARF907 with isolate ARF908, which has been tested previously [34], contrasting conclusion is drawn: the prior grew fast in xylose and ribose media, while the latter could hardly utilize them. Furthermore, in this study, low-cost commercially available polysaccharides were also tested, and among them, pectin and gelatin were suitable carbon sources. It had already been noted in our test that the isolates of ARF907 and ARF908 were quite different in morphological characteristics and growth rate in PDA plates, indicating diversity existed in isolates of sterile mycelium ARF18. The isolate of *P. chlamydosporia* in the previous study was isolated from *Meloidogyne* spp. in USA [34], and its sporulation was compatible with oligosaccharides. While in this study, for the isolate HSY-12-14 of *P. chlamydosporia* from China, polysaccharides were much better; such disaccharides as trehalose and sucrose were also good carbon sources. The effects of carbon nutrients on growth and sporulation of *M. anisopliae* were similar among the isolates from plant nematode and insect in China

and the ones from *Inopus rubriceps* in Australia [32]. In this study, more carbon sources were screened, and a less costly and industrial product of gelatin was preferred.

Methods for determination of fungal growth

Two parameters, daily growth rate and mycelium fresh weight of fungal growth were adopted in this study. Sometimes they were consistent, but difference often occurred for some substrates and fungal isolates. For example, isolate HSY-12-14 and CA-1-G grew faster in media supplemented with polysaccharides, but produced less biomass. Growth rate is a direct and simple measurement that can be used to estimate fungal growth. However, hyphal fresh weight is a better indicator of the requirement and utilization of carbon.

Fungal growth was determined on dry weight basis for mycelia cultured in liquid by other researchers [17–19, 21, 32]; however, the measurements of hyphal growth and conidiation had to be conducted in two separate experiments (on agar and in liquid cultures). In order to simplify testing procedures, agar media were used for both fungal growth and sporulation measurements with the aid of supporting membranes of cellophane, a non-biodegradable wrapping paper in the present study. After incubating for a few days, fresh mycelium and its underlying cellophane were taken off from the agar plate to determine its biomass before spore production was quantified. The relationships between dry weight and fresh weight of mycelia were analyzed in a preliminary study (data not shown), and the results of both methods were very comparable on sealed plates and under equal moisture condition, thus indicating that fungal biomass could be well estimated by mycelial fresh weight.

Determination of fungal sporulation

Fungal conidiation was determined by two measurements, spore numbers per colony and spore numbers per gram mycelium fresh weight in this study. In mass production of biocontrol fungi, great numbers of conidia are required, and the total number of spores produced is widely used to evaluate sporulation. Generally, fungal sporulation is closely correlated with its biomass; however, our study showed that sporulation was not necessarily correlated with unit biomass. With the development of intensive industrial production, especially for fungal solid fermentation and sporulation, the parameter of sporulation in unit biomass, which reveals the ability of sporulation, is also likely to be adopted.

For a fungal pesticide, hyphae and conidia are the main biocontrol entities and generally a large mass of inocula of a biocontrol fungus is necessary for efficient application in the fields. However, their limited production outputs restrict the development of fungal agents to a great extent. It has been shown that alternative nutritional components can significantly influence growth and sporulation of many fungi [35–37]. This provides opportunities to find the most effective and commercially available ingredients, through systematically screening carbon requirements, to facilitate the mass production of a potential high-virulence biocontrol isolate.

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Address for Correspondence: Xingzhong Liu, Key Laboratory of Systematic Mycology & Lichenology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100080,
P. R. China
Phone: +86-10-62658587; Fax: +86-10-62656920;
E-mail: liuxz@sun.im.ac.cn.