International Scholars Journals

African Journal of Parasitology Research ISSN 2343-6549 Vol. 7 (4), pp. 001-008, April, 2020. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Optimize culture conditions for biomass and sporulation of the nematophagous fungus *Pochonia chlamydosporia*, isolate HSY-12-14 by "two-step" cultivation

Li Gao

State Key Laboratory for Biology of Plant Disease and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, People's Republic of China. E-mail: xiaogaosx@hotmail.com.

Accepted 17 January, 2020

Biomass yields and sporulation of *Pochonia chlamydosporia* isolate HSY-12-14 was concerned on culture conditions including culture method, nutritional requirements together with environmental factors in this study. We optimized them for biomass yields of *P. chlamydosporia* HSY-12-14 with the "two-step" cultivation method as well as orthogonal matrix method: firstly cultured spore suspension on the basal medium (sucrose 19.00 g, soy peptone 4.06 g, K_2HPO_4 1.00 g, KCl 0.50 g, $MgSO_4$ 0.50 g, $FeSO_4$ 0.01 g and 17.00 g Bactor) for the first stage culture of 4 days under room condition, then transferred them to another defined medium (sucrose 19.05 g, soypeptone 0.01 g, $ZnSO_4$ $7H_2O$ 0.05 g/L, $MnSO_4$ H_2O 0.05 g/L, H_3BO_4 0.05 g/L and 17.00 g Bactor) for more 4 days cultivation, together with the environmental factors combination of water potential -3.9 MPa /pH 4 /24 h light cycle/23°C for biomass yields. We optimized the best culture conditions for sporulation of *P. chlamydosporia* under water potential -1.2 MPa /pH 4 /24 h light cycle/32°C wit h the same nutrition for biomass yields. These results is characterized in that it supplies the composition of media and environmental suitable for the mass production and provide important information industrialization of this great potential biocontrol fungus.

Key words: Nutrition, environment, sporulation, biological control, mass selection.

INTRODUCTION

Increasing social awareness on environmental and health concerns associated with the use of synthetic chemicals for nematode management, urged to search an alterna-tive with biosafety for better and economic management of nematodes. Biological management of plant parasitic with potentially useful antagonists in the groups of fungi is of current interest for sustainable production of crops. Opportunistic soil fungi are often studied for nematode management in a range of crops at various geographical locations on a variety of nematode taxonomic groups, since they are easy to handle and also efficient colonizer of nematodes and plant roots.

Pochonia chlamydosporia (syn. *Verticillium chlamydosporium* Goddard), an opportunistic soil fungus, has long been evaluated for nematode management in different countries with a view to identify potential field effective strains (Zare et al., 2001). The nematophagous fungus *P. chlamydosporia* var. *catenulate* is a widespread and naturally occurring facultative parasite of root-knot nematode eggs and has shown potential as a biocontrol agent against *Meloidogyne* spp and the fungus has since been extensively studied for its potential for development into a biological nematicide (De Leij et al., 1993).

So, the production of *P. chlamydosporia* as a biocontrol agent of root-knot nematodes has been investigated. The mycelial growth and sporulation of *P. chlamydosporia* are

Abbreviations: CGMCC, Center of general microorganisms culture collection**; ANOVA,** analysis of variances**; LSD,** least significant difference.

influenced by components of the medium and culture conditions (Kerry et al., 1986; Zaki and Maqbool, 1993). The effects of various nutrients on growth and sporulation of *P. chlamydosporia* have also been studied recently (Liu and Chen, 2003). The effects of carbon and nitrogen sources, carbon-to-nitrogen ratio (C:N ratio) and initial pH value on its growth and sporulation in liquid culture were examined on solid culture (Mo et al., 2007).

While, our lab have been system studied the solid growth conditions: including 30 carbon sources (Sun and Liu, 2006), 19 nitrogen sources and several mineral ele-ments, 3 carbon concentration and 15 carbon to nitrogen ratios with continuous cultivation method (Gao et al., 2007), together with novel "two-step" cultivation method on carbon concentration and carbon to nitrogen ratio (Gao and Liu, 2009), combinations of carbon and nitrogen sources under certain carbon concentration and C/N ratio with most sporulation, and also environmental factors (pH, water potential, light and temperature) (Gao et al., 2009), then we used orthologal method to optimize these factors for better sporulation. This paper will com-prehensively report these system works on sporulation of
P. chlamydosporia, isolate HSY-12-14, under the com-bination of novel "two-stage" cultivation method and orthologal method on solid.

MATERIALS AND METHODS

Fungal strain

The tested biocontrol fungi *P. chlamydosporia*, isolate HSY-12-14 was originally isolated from *Meloidogyne incognita* (Kofoid and White) Chitwood by M. H. Sun from Hainan province in China, and now was deposited in the Center of General Microorganisms Culture Collection (CGMCC) in the Institute of Microbiology, Chinese Academy of Sciences.

Nutrition requirements for the sporulation of *P. chlamydosporia*, isolate HSY-12-14 by this novel method

Sources of chemicals used

The chemicals used were sucrose, maltose, $NaNO_3$, urea, K_2HPO_4, $MgSO_4$, $FeSO_4$ (Beijing Chemical Reagents Company, Beijing China), soy peptone (Shanghai Chemical Reagents Company, Shanghai China) and KCl (Nanjing Chemical Reagents Company, Nanjing China).

Basal medium

The basal medium was composed of sucrose 19.00 g (equal to 8.00 g carbon), soy peptone 4.06 g (equal to 0.33 g nitrogen), K_2HPO_4 1.00 g, KCl 0.50 g, $MgSO_4$ 0.50 g, $FeSO_4$ 0.01 g and 17.00 g Bactor (Difco) agar per liter. We used this medium for the first stage vegetative fungi culture of 4 days.

Effects of carbon concentration and carbon to nitrogen ratio

Carbon concentrations were adjusted with sucrose (42% carbon) to 1, 2, 4, 8 and 16 g/l, and nitrogen concentrations were adjusted with soy peptone (8% nitrogen) to 0.2, 0.4, 0.8 and 1.6 g/l, which respectively replace the carbon and nitrogen source in basal medium. The combinations of different carbon and nitrogen concentrations resulted in C:N ratios from 0.625:1 to 80:1. This carbon concentrations and C:N ratios were used in the second stage culture for the sporulation of another 4 more days. After this experiment, we had the optimal carbon concentration of 8 g/l with C/N ratio of 10:1.

Effects of carbon and nitrogen source

The combinations of carbon sources including sucrose, maltose and nitrogen sources including of $NaNO_3$ and urea were tested. Based on the carbon concentration 8 g/l (pure carbon per liter calculated by percentage of carbon element in the molecule) and carbon to nitrogen ratio 10:1 (0.8 g/l nitrogen concentration, pure liter calculated by percentage of nitrogen element in the molecular), we had the combinations of different carbon and nitrogen sources for sporulation with this novel method. For each combination, we added them to the basal medium to replace the sucrose and soy peptone as sporulation medium for the second stage culture of more 4 days. The basal medium for sporulation of another 4 days was used as control.

Effects of mineral elements

After tested the components and concentration gradients of six mineral elements for sporulation of the isolate fungi with one-factor-at-a-time method, we had the optimal components for the sporulation of *P. chlamydosporia* HSY-12-14, including of $ZnSO_4$ $7H_2O$ 0.05 g/l, $MnSO_4$ H_2O 0.05 g/l, and H_3BO_4 0.05 g/l.

Effects of environmental factors on sporulation of *P. chlamydosporia*, isolate HSY-12-14 by this novel method

The novel method of "two-stage" cultivation in plates was applied to evaluate the effects of pH, water potential, light and temperature on the second stage culture of 4 more days on sporulation of the biocontrol fungus. Water potentials including -0.3, -0.8, -1.2, -2.1,-3.9 and -7.3 MPa; pHs including 3, 4, 5, 6, 7, 8 and 9; light including 24, 12 and 0 h; temperatures including 20, 23, 26, 29 and 32°C.

Orthogonal matrix method

The orthogonal $L_{16}(2^{15})$ was used to obtain the optimal culture conditions in solid for pH, water potential, light and temperature on the certain sporulation medium, successively, after the testing of carbon concentration, C/N ratio, the combination of carbon and nitrogen source, and environmental factors by one-factor-at-a-time with this novel method. We have been got the best nutrition combination by full experiment, now we try to get the optimal combination of nutrition together with environmental factors for sporulation of *P. chlamydosporia*, isolate HSY-12-14 by orthogonal matrix method under the selected two levels for sporulation of four environmental factors. All the treatments were replicated for three times.

Statistical analysis

Data 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

Effects of nutrition on sporulation of *P. chlamydosporia*, isolate HSY-12-14

The results indicated that the optimal carbon concentration for sporulation of *P. chlamydosporia* isolate HSY-12-14 by "two-step" cultivation method was 8 g/l and C/N ratio 10:1 (Table 1). There were significant effects of the combination of carbon and nitrogen sources on sporulation of the isolate of fungi (Table 2). In the following experiments, we used sucrose and soy peptone as carbon and nitrogen source together with other components in basal medium for optimization of nutrition conditions by the novel "two-step" cultivation method.

Effects of environmental factors on sporulation of *P. chlamydosporia*, isolate HSY-12-14

We selected two better levels for sporulation of each factor for the orthogonal research, respectively was water potential -3.9 and -1.2 MPa, pH 4 and 3, 24 and 12 h light, temperatures 23 and 32°C (Gao et al., 2009). The levels of environmental factors are shown in Table 3.

Optimization by orthogonal matrix method

To investigate the relationships between variables of environmental factors and certain medium components and optimize the culture conditions for sporulation, the orthogonal layout of $L_{16}(2^{15})$ was employed. Based on the design of four factors and two levels (Table 3), the experimental conditions for each experimental group were listed in Table 4 with the experimental results concluded in the last two columns. According to the orthogonal method, the effect of environmental factors, including pH, water potential, light, and temperature on growth and sporulation was evaluated and shown in the bottom five rows of Table 4. According to the magnitude order of R (maximum difference) in Table 5, the order of the effect of all factors on mycelia growth could be determined as 74.42 (water potential) > 44.25 (light) > 31.92 (temperature) > 16.75 (pH), the results indicated that the effect of 74.42 (water potential) was more important than that of the others three environmental factors; the order of effect of all factors on sporulation could be determined as 0.69 (temperature) > 0.13 (water potential) > 0.12 (light) > 0.08 (pH), the results indicated that the effect of 0.69 (temperature) was more important than that of the others three environmental factors.

To test the effects of four factors, ANOVA was used and shown in Table 6, the factor of water potential had significant effects on biomass yields and the factor of temperature had significant effects on sporulation. Table 7 shows the effect of combinations of four factors on biomass yields and sporulation for *P. chlamydosporia*, isolate HSY-12-14. It is demonstrated that the combinations of B_2/A_1, A_1/C_1, A_1/D_2, B_2/C_1, B_2/D_2, D_1/C_1 have the best effect on biomass yields, producing 219.50, 204.17, 181.58, 215.33, 183.25, 224.08 mg per colony biomass yields respectively. To obtain a high mycelial yields, the optimum factors should be water potential -3.9 MPa (A_1) /pH 3 (B_2) /24 h light (C_1) /23 ℃ (D_1). It is demonstrated that the combinations of B_1/A_2, A_2/C_1, A_2/D_1 or A_2/D_2, B_1/C_1, B_1/D_2, D_2/C_1 have the best effects on sporulation, producing 1.22, 1.24, 1.15, 1.20, 1.24, 1.24 $\times 10^5$ per colony spore yields respectively. To obtain a high spore yields, the optimum factors should be water potential -1.2 MPa (A_2) /pH 4 (B_1) /24 h light (C_1) / 32°C (D_2).

DISCUSSION

"Two-step" cultivation method

Biological control using entomopathogenic fungus will only become feasible if economic methods of mass production are available (Kleespies and Zimmermann, 1992). "Two-step" cultivation method on solid for spores can be produced in an easier step, and industrial scale-up could be enhanced and have a high spore yields compared with liquid fermentation, from liquid to solid or just solid-state production of aerial conidia.

After incubating for 4 days on basal medium firstly, fresh mycelium and its underlying cellophane were taken off from the agar plate to second stage medium for sporulation of another 4 days, then to determine its biomass before spore production was quantified. This method we have used in our previous study (Gao and Liu, 2009), we got the nutrition for sporulation of *P. chlamydosporia*, isolate HSY-12-14 on second stage medium, and also got the two better levels of 4 environ-mental factors on sporulation of *P. chlamydosporia*, isolate HSY-12-14, then we combined them together by orthogonal matrix method to got a better combinations including nutrition and environmental under "two-step" cultivation method based on biomass and spore yields, which indicating that fungal biomass could be well estimated by mycelia fresh weight (Sun and Liu, 2006), which lead to a high production than traditional method, and this method has been got patent in China of 2004.

Effects of nutrition on sporulation of *P. chlamydosporia*, isolate HSY-12-14

The nutrition combination with this method greatly accelerated the spore production without excess cost. Our results proved that different carbon concentrations and C/N ratios, together with combinations of carbon and nitrogen sources lead to different spore yields. According to the "two-step" cultivation method, we got about 30 times of spores on screened carbon concentration (8 g/l) and C/N ratio (10:1) than the control medium with of 8 g/l

Table 1. Effects of carbon concentration and carbon-to-notrogen ratio on the sporulation of *P. chlamydosporia* HSY-12-14 (10^5/ml) by "two-step" cultivation method.

Carbon concentration (g/l)	Carbon-to-nitrogen ratio	Spore yields	
1	5	4.00	de
1	2.5	2.20	fgh
1	1.25	1.90	ghi
1	0.625	1.70	ghi
2	10	1.30	hijk
2	5	4.00	de
2	2.5	4.90	de
2	1.25	3.50	ef
4	20	0.90	hij
4	10	1.40	ghij
4	5	1.20	ghij
4	2.5	4.00	de
8	40	0.50	ij
8	20	0.10	j
8	10	32.80	a
8	5	20.40	b
16	80	5.20	d
16	40	1.00	hij
16	20	2.50	fg
16	10	8.50	c
8(CK)	24	1.00	hij

*Values are means of three replicates. Values in the same column followed by a same letter are not significantly different (LSD; P ≤ 0.05).

Table 2. Effects of carbon and nitrogen source on the sporulation of *P. chlamydosporia* HSY-12-14 (10^5/ml).

Carbon source	Nitrogen source						CK	
	$NaNO_3$		Soy peptone		Urea			
Sucrose	47.0	b	359.0	a	47.7	b	13.00	d
Maltose	36.3	c	36.3	c	47.7	b		

* Values are means of three replicates. Values in the same column followed by a same letter are not significantly different (LSD; P ≤ 0.05)

Table 3. L_{16} (2^{15}) orthogonal design of optimization of culture environment of *P. chlamydosporia* HSY-12-14.

Factors	Water potential (MPa)	pH	Light (h)	Temperature (°C)
Level 1	-3.9	4	24	23
Level 2	-1.2	3	12	32

*Symbols A, B, C, and D represent factors of water potential, pH, light and temperature respectively.

carbon concentration with a C:N of 24:1; under the screened carbon concentration and C/N ratio, about 27.6 times more spore yields with screened carbon and nitrogen sources (359.0×10^5 spores/ml) than control plates by traditional culture method on CK media of 8 g/l carbon concentration with a C:N of 24:1 (13.0×10^5 spores/ml).

Effects of environmental factors on sporulation of *P. chlamydosporia*, isolate HSY-12-14

Environmental factors which have different effects on biomass and sporulation, in our research, water potential is important to biomass, while temperature was the key to sporulation for *P. chlamydosporia*, isolate HSY-12-14.

Table 4. Orthogonal experiment of L_{16} (2^{15}) of biomass yields and sporulation of *P. chlamydosporia* HSY-12-14.

Exp. group	A	B	A×B*	C	A×C	B×C		D	A×D	B×D		C×D				Biomass yields (mg per colony)	Sporulation (10^5 per colony)
1‡	1†	1	1	1	1	1	1	1	1	1	1	1	1	1	1	122.00 ± 39.95§	0.483 ± 0.357
2	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	140.00 ± 29.61	1.771 ± 0.152
3	1	1	1	2	2	2	2	1	1	1	1	2	2	2	2	133.67 ± 17.95	0.434 ± 0.154
4	1	1	1	2	2	2	2	2	2	2	2	1	1	1	1	146.67 ± 26.86	1.326 ± 0.188
5	1	2	2	1	1	2	2	1	1	2	2	1	1	2	2	128.00 ± 36.37	0.986 ± 0.097
6	1	2	2	1	1	2	2	2	2	1	1	2	2	1	1	178.67 ± 72.02	1.449 ± 0.145
7	1	2	2	2	2	1	1	1	1	2	2	2	2	1	1	92.00 ± 40.03	0.632 ± 0.608
8	1	2	2	2	2	1	1	2	2	1	1	1	1	2	2	114.67 ± 17.04	1.280 ± 0.087
9	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	209.33 ± 83.26	1.031 ± 0.296
10	2	1	2	1	2	1	2	2	1	2	1	2	1	2	1	114.00 ± 22.61	1.315 ± 0.577
11	2	1	2	2	1	2	1	1	2	1	2	2	1	2	1	230.33 ± 89.31	0.707± 0.296
12	2	1	2	2	1	2	1	2	1	2	1	1	2	1	2	324.33 ± 32.50	1.496 ± 0.056
13	2	2	1	1	2	2	1	1	2	2	1	1	2	2	1	143.67 ± 54.90	0.858 ± 0.151
14	2	2	1	1	2	2	1	2	1	1	2	2	1	1	2	140.67 ± 26.65	1.462 ± 0.165
15	2	2	1	2	1	1	2	1	2	2	1	2	1	1	2	166.67 ± 29.40	1.015 ± 0.239
16	2	2	1	2	1	1	2	2	1	1	2	1	2	2	1	322.00 ± 139.33	1.527 ± 0.172

*A×B, A×C, B×C, A×D, B×D, C×D represent the interac tions between the factors water potential and pH, water potential and light, pH and light, water potential and temperature, pH and temperature, light and temperature, successively.

‡ Every row of the experimental group number represents one experimental replicate, and every experimental group was replicated thrice.

§ Values are mean ± SD of triple determinations.

So, we could culture them under different environmental conditions to get certain yields we want to have, for example, biomass or spores. It is demonstrated that the combinations of different environmental factors have different effects on biomass and spore yields.

Optimization by orthogonal matrix method

Many reports concern the orthogonal matrix method just on nutritional components, or just environmental factors, while our research combined them together. The first level we choose was the best for sporulation of *P. chlamydosporia*, isolate HSY-12-14 by one-factor-at-one-time method, while after the orthogonal matrix method, we found that certain nutrition not all combined the first level of environment, mostly was the mixture of these two levels, which also means that the orthogonal method was necessary for opti-mize the sporulation culture conditions including nutritional and environment factors. In this study, we used the former two levels of environmental factors with certain nutrition to optimize the culture conditions with orthogonal matrix method after doing the full experiment of nutrition and environmental conditions.

Combinations of the three fields

For a fungal pesticide, hyphae and conidia are the main biocontrol entities and generally a large mass of inoculum 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 (Coleman and Hodges, 1990; Engelkes et al., 1997; Leite et al., 2003). This provides opportunities to find the most effective and commercially available

Table 5. Analysis of environmental factors on biomass production and sporulation of *P. chlamydosporia* HSY-12-14 with this novel method.

		A	B	A×B	C	A×C	B×C		D	A×D	B×D		C×D			
B*	K_1	1055.7	1420.3	1315.4	1176.3	1612.0	1280.7	1307.7	1225.7	1376.7	1451.3	1297.7	1510.7	1163.0	1380.3	1349.3
	K_2	1651.0	1286.4	1391.3	1530.3	1094.7	1426.0	1399.0	1481.0	1330.0	1255.3	1409.7	1196.0	1543.7	1326.3	1357.3
	k_1	131.96	177.54	164.42	147.04	201.50	160.08	163.46	153.21	172.08	181.42	162.21	188.83	145.38	172.54	168.67
	k_2	206.38	160.79	173.92	191.29	136.84	178.25	174.88	185.13	166.25	156.92	176.22	149.50	192.96	165.79	169.67
	R	74.42	16.75	9.50	44.25	64.67	18.17	11.42	31.92	5.83	24.50	13.92	39.33	47.58	6.75	1.00
	O	2	1	2	2	1	2	2	2	1	1	2	1	2	1	2
S†	K_1'	8.36	8.56	8.87	9.35	9.43	9.06	8.69	6.14	8.34	8.38	8.32	8.96	8.58	8.90	8.30
	K_2'	9.41	9.21	8.96	8.42	8.34	8.72	9.08	11.62	9.44	9.40	9.44	8.78	9.20	8.80	9.47
	k_1'	1.05	1.07	1.11	1.17	1.18	1.13	1.09	0.77	1.04	1.05	1.04	1.12	1.07	1.11	1.04
	k_2'	1.18	1.15	1.12	1.05	1.04	1.09	1.14	1.45	1.18	1.18	1.18	1.10	1.15	1.10	1.18
	R'	0.13	0.08	0.01	0.12	0.14	0.04	0.05	0.69	0.14	0.13	0.14	0.03	0.08	0.01	0.15
	O'	2	2	2	1	1	1	2	2	2	2	2	1	2	1	2

* Biomass yields (mg per colony).
† Sporulation (10^5 conidia per colony).
K_1 and K_2 are the totel content of biomass yields from the level 1 and level 2 separately; k_1 and k_2 are the mean value of levels 1 and 2 separately.
K_1' and K_2' are the totel spore yields from the level 1 and level 2 separately; k_1' and k_2' are the mean value of levels 1 and 2 separately.
R is the maximum of k_1, k_2 minus the minimum of k_1, k_2 and R' is the maximum of k_1, k_2 minus the minimum of k_1, k_2 respectively. O is the optimal level of biomass yields and O' is the optimal value of spore yields.

Table 6. The variance analysis of $L_{16}(2^{15})$ orthogonal test on optimation of environmental factors for biomass yields and speculation of *P. chlamydosporia* HSY-12-14.

	Variance source	Sum of square deviation (SS)	Degree of freedom (v)	Mean square (MS)	*F*-ratio	Significance level†
Biomass yields (mg per colony)	A	22150.37	1	8555.33	10.06	*
	B	1121.92	1	1600.00	0.53	
	C	7832.25	1	5.48	3.72	
	D	4074.91	1	40.07	1.93	
	A×B	360.77	1	360.77	0.03	
	A×C	16726.25	1	16726.25	1.59	
	A×D	136.10	1	136.10	0.01	
	B×C	1320.16	1	1320.16	0.13	
	B×D	2403.71	1	2403.71	0.23	
	C×D	6185.95	1	6185.95	0.59	
	Error	10538.57	5			

Table 6. Contd.

Sporulation (10^5 conidia per colony)	A	0.07	1	0.01	1.74	
	B	0.03	1	1.11	0.66	
	C	0.06	1	0.21	1.39	
	D	1.88	1	0.27	47.40	***
	A×B	0.13	1	0.13	0.67	
	A×C	0.07	1	0.07	0.33	
	A×D	0.09	1	0.09	0.43	
	B×C	0.02	1	0.02	0.08	
	B×D	0.07	1	0.07	0.37	
	C×D	-0.06	1	-0.06	-0.30	
	Error	0.20	5			

†$F_{0.1}$ (1,5) = 4.06, $F_{0.05}$ (1,5) = 6.610, $F_{0.01}$ (1,5) = 16.3, * *F*-ratio >$F_{0.1}$, **$F_{0.1}$ < *F*-ratio < $F_{0.05}$, *** *F*-ratio > $F_{0.01}$.

Table 7. Effects of combinations of environmental factors on biomass yields and sporulation of *P. chlamydosporia* HSY-12-14.

B, C or D	A				B				C			
	A_1		A_2		B_1		B_2		C_1		C_2	
	B†	S‡	B	S	B	S	B	S	B	S	B	S
B_1	135.59	1.00	193.25	1.22								
B_2	219.50	1.14	128.34	1.09								
C_1	204.17	1.11	198.84	1.24	187.67	1.20	215.33	1.16				
C_2	150.92	1.03	122.75	1.06	141.17	1.02	132.50	1.06				
D_1	173.50	0.93	170.67	1.15	179.59	0.98	164.58	1.11	224.08	1.12	120.09	0.96
D_2	181.58	1.21	150.92	1.15	149.25	1.24	183.25	1.12	178.92	1.24	153.59	1.12

A_1, A_2, B_1, B_2, C_1, C_2, D_1, D_2 represent the 1 and 2 levels of water potential, pH, light and temperature.
† Represent the biomass yields (mg per colony).
‡ Represent spore yields (10^5 conidia per colony).

nutritional and environmental factors, through screening the combination of carbon and nitrogen source, carbon concentration, C/N ratio, together with environmental factors including water poten-tial, pH, light and temperature to facilitate the mass production of a potential high-virulence biocontrol isolate. Fungal growth was determined on dry weight basis for mycelia cultured in liquid by other researchers (Satchuthananthavale and Cooke, 1967a,b,c; Saxena et al., 1989; Li and Holdom, 1995), however, the measure-ments 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, biomass was determined by fresh mycelia weight and conidiation was determined by spore numbers per colony.

In this paper, we also found that fungal biomass was not necessarily correlated with fungal sporu-lation under the orthogonal matrix method, which have been proved in our previous study, such as separately the environmental factors and nutrition factors on mycelia growth and sporulation. This phenomenon proved that our method is the right way to separate the mycelia growth and sporu-lation, and the result could help us have better spore yields under lower cost.

ACKNOWLEDGEMENTS

This project is supported by National Natural Scientific Foundation of China (No.30800709). The authors also thank Prof. B. Jaffee (*University of California at Davis*) for serving as a pre-submission reviewer and for his valuable comments and suggestions.

REFERENCES

Coleman LW, Hodges CF (1990). Growth and conidiation of *Bipolaris sorokiniana* in response to methionine and ethylene. Mycol. Res., 94: 1013–1016.

De Leij, Kerry BR, Dennehy JA (1993). *Verticillium chlamydosporium* as a biological control agent for *Meloidogyne incognita* and *M. hapla* in pot and micro-plot tests. Nematologica, 39: 115–126.

Engelkes, CA, Nuclo, RL, Fravel, DR (1997). Effect of carbon, nitrogen, and carbon-to-nitrogen ratio on growth, sporulation and biocontrol efficacy of *Taloromyces flavus*. Phytopathology., 87: 500-505.

Gao L, Liu XZ (2009). A novel two-step cultivation method to optimize carbon concentration and carbon-to-nitrogen ratio for sporulation of biocontrol fungi. Folia. Microbiol., 54(2):142-146.

Gao L, Sun MH, Liu XZ, Che YS (2007). Effects of carbon concentration

and carbon to nitrogen ratio on the growth and sporulation of several biological control fungi. Mycol. Res., 111: 87-92.
Gao L, Liu XZ, Sun MH, Li SD, Wang JL (2009). Effects of environmental factors on the sporulation of several biocontrol fungi. Mycoscience, 50: 317-321.
Kerry BR, Irving F, Hornsey JC (1986). Variation between strains of the nematophagous fungus, *Verticillium chlamydosporium* Goddard. Factors affecting growth *in vitro*. Nematologica, 32: 461–473.
Kleespies RG, Zimmermann G (1992). Production of blastospores by three strains of *Metarhizium anisopliae* (Metch.) Sorokin in submerged culture. Biocontrol Sci. Techn., 2: 127–135.
Leite, LG, Alves, SB, Batista Filho, A, Roberts, DW (2003). Effect of salts, vitamins, sugars and nitrogen sources on the growth of three genera of *Entomophthorales*: *Batkoa, Furia*, and *Neozygites*. Mycol. Res., 107: 872-878.
Li DP, Holdom DG (1995). Effects of nutrients on colony formation, growth, and sporulation of *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes). J. Invertebr. Pathol., 65: 253–260.
Liu XZ, Chen SY (2003). Nutritional requirements of P. chlamydosporia and ARF18, fungal parasites of nematode eggs. J. Invertebr. Pathol., 83: 10–15.
Mo MH, Xu CK, Zhang KQ (2007). Effects of carbon and nitrogen sources, carbon-to-nitrogen ratio, and initial pH on the growth of nematophagous fungus *Pochonia chlamydosporia* in liquid culture. Mycopathologia, 159: 381–387.
Satchuthananthavale V, Cooke RC (1967a). Carbohydrate nutrition of some nematode-trapping fungi. Nature, 214: 321–322.
Satchuthananthavale V, Cooke RC (1967b). Nitrogen nutrition of some nematode-trapping fungi. Trans Br. Mycol. Soc., 50: 423–428.
Satchuthananthavale V, Cooke RC (1967c). Vitamin requirements of some nematode-trapping fungi. Trans Br. Mycol. Soc., 50: 221–228.
Saxena G, Dayal R, Mukerji KG (1989). Nutritional studies on nematode-trapping fungi. Folia. Microbiol., 34: 42–48.
Sun MH, Liu XZ (2006). Carbon requirements of some nematophagous, entomopathogenic and mycoparasitic Hyphomycetes as fungal biocontrol agents. Mycopathologia, 161: 295–305.
Zaki MJ, Maqbool, MA (1993). Effect of temperature and culture media on the growth of Verticillium chlamydosporium, an egg parasite of root-knot nematode and cyst nematodes. Pak. J. Phytopathol., 86: 102–105.
Zare R, Gams W, Evans HC (2001). A revision of *Verticillium* section *Prostata*. V. The genus *Pochonia*, with notes on *Rotiferophthora*. Nova Hedwigia, 73: 51–86.