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EXPERIMENTAL ARTICLES

Nutritional Requirements of Mycelial Growth and Sporulation of Several Biocontrol Fungi in Submerged and on Solid Culture[1]

Li Gao[a,b] and Xingzhong Liu[a2]

[a] *Key Laboratory of Systematic Mycology and Lichenology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, P. R. China*

[b] *State Key Laboratory for Biology of Plant Disease and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, P. R. China*

e-mail: liuxz@imac.cn

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Abstract—To develop fungal biopesticides, the nutritional requirements on available culture media that maximize mycelia and spore yields at low cost are essential for their mass-production. The objective of present study was to find the optimal combinations for the most mycelia yield in submerged and the highest spore yield on solid. Have a better understanding of their basic physiology, and have a better yield of mycelia and conidia with short time and low cost will help to provide valuable information for mass production and commercialization.

Key words: biocontrol, conidiation, *Metarhizium anisopliae*, mycelia, *Paecilomyces lilacinus*

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INTRODUCTION

Chemical insecticides can lead to environmental damage, pest resurgence and resistance, together with lethal effects on non-target organisms. Additionally, the cost of chemical insecticides is becoming increasingly inaccessible to farmers, particularly in developing countries. These negative effects of chemical pesticides have led to a worldwide resurgence of interest in the use of entomopathogenic fungi as microbial control agents [1–3]. Several entomopathogenic fungi, such as *Metarhizium anisopliae* was useful as a biological control agent because of its broad host range [4], *Paecilomyces lilacinus*, a soil Hypomycete, is known as an effective parasite of nematodes that cause diseases to plants [5–7] and shows potential as a biocontrol agent of plant parasitic nematodes [8].

Although biological control agents over an environmentally friendly alternative to chemical pesticides, their practical use is limited due to theirs mass production retards the commercialization and field application. The improvement of potential control agents often depends on an adequate mass-production method for producing the infective propagules on a suitably large scale [9]. The understanding of the basic aspects of growth of entomopathogenic hyphomyceteous fungi can greatly help programs seeking to use those organisms as biological control agents, and detailed knowledge of the nutritional requirements for their growth and sporulation is essential and important for mass production and commercialization. Various experiments have dealt with the influence of nutrition in basal media on fungal growth and sporulation in order to screen the optimal components and concentrations for mass production. Leite et al. [10] examined the effects of carbon and nitrogen sources on the growth of three genera of *Entomophthorales*: *Batkoa*, *Furia* and *Neozygites*, they concluded that these isolates are similar in growth patterns in media with various sources of carbon, but different in media with different sources of nitrogen. Liu and Chen [11, 12] reported the nutritional requirements of nematode endoparasitic fungus *Hirsutella rhossiliensis* together with nematode egg parasites *Pochonia chlamydosporia* and Arkansas fungus 18 (ARF18), and they found that some carbon and nitrogen sources were good for growth in liquid and solid, while some could not be utilized either in liquid or solid. Jackson and his colleagues also found that conidiation medium greatly influenced the sporulation of *Colletotrichum truncatum*, especially that carbon concentration and C/N ratio significantly affected the number of conidia produced and conidial attributes of *C. truncatum* in liquid culture [13–16]. The requirements of carbon concentrations, C/N ratios for fungal growth and sporulation have been extensively studied on *Helminthosporium solani* which caused silver scurf on potatoes storage, and they demonstrated that higher carbon concentrations or C/N ratios reduced its conidiation [17]. On the contrary, higher C/N ratios increased spore yield for the biolog-

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[2] Corresponding author; e-mail: liuxz@im.ac.cn

Table 1. Details of fungal isolates used

Species	Isolates	Host	Location	Isolated by
Paecilomyces lilacinus	IPC-P	*Meloidagyne incognita*	Lima, Peru	P. Jatala
P. lilacinus	M-14	*Heterodera glycines*	Huanan County, Heilongjiang, China	X.Z. Liu
Metarhizium anisopliae	SQZ-1-21	*Meloidogyne arenaria*	Qingzhou, Shandong, China	M.H. Sun
M. anisopliae	RS-4-1	Soil with *Galleria mellonella* baiting	Jiangsu, China	Z.A. Chen

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

Species	Isolates	For mycelia growth in submerged culture		For sporulation on solid culture	
		Carbon (g/l)	C/N ratio	Carbon (g/l)	C/N ratio
P. lilacinus	IPC-P	6	20	12	20
P. lilacinus	M-14	12	20	8	10
M. anisopliae	SQZ-1-21	8	40	8	10
M. anisopliae	RS-4-1	8	40	8	160

ical control agent of *Talaromyces flavus* [18]. All of these researches have demonstrated that the carbon and nitrogen sources, together with carbon concentrations, C/N ratios of culture medium have great impact on fungal growth and sporulation.

However, all of these studies mentioned above demonstrated their conclusions with their basal media replaced by carbon or nitrogen sources, and or carbon concentrations together with C/N ratios with one-factor-at-a-time method, which had not systematically reported the effects of screened combinations of media components and contents on fungal growth and/or sporulation. In our previous studies, with one-factor-at-a-time method, total 33 carbohydrates, 20 nitrogen sources, 6 mineral elements have been evaluated for the mycelial growth and sporulation of these biocontrol fungi [19, unpublished data], together with the suitable carbon concentration and C/N ratio from 3 carbon concentrations and 15 C/N ratios for mycelial growth and sporulation of these biocontrol fungi [20]. Based on the results of suitable carbon concentration, C/N ratio and mineral elements, this effort will entail selecting the most favorable combinations of carbon and nitrogen sources for mycelia in submerged and conidia on solid for mass production of these agents as well as considering the cost of each isolate for the lowest concentrations that afford high yield, and together with help to develop these promising fungi as practical biopesticides.

MATERIALS AND METHODS

Fungi and inocula. Three isolates of nematophagous fungi *P. lilacinus* M-14 and IPC-P, and *M. anisopliae* SQZ-1-21, one isolate of entomopathogenic fungi, *M. anisopliae* RS-4-1 were used in this study (Table 1). All fungi were obtained from laboratory stocks grown as single-conidium isolates on potato dextrose agar (PDA; Oxoid Ltd., Basingstoke, Hampshire, England) slants at room temperature, and cultured on PDA plates at 25°C. Conidial inocula were prepared according to Gao et al. [20].

In Submerged Culture of These Agents

Media preparation. Based on the carbon concentration and C/N ratio for mycelial growth (Table 2) [20], combined with the screened minerals (Table 3, unpublished data) as well as carbon and nitrogen sources for mycelial growth of these fungi (Table 4, unpublished data), we try to grope for the combinations of carbon and nitrogen sources with those certain parameters mentioned above for mycelial growth of all tested fungi. The pH was adjusted to 7 by adding 1 N NaOH

Table 3. Selected mineral elements for mycelia 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-1-21	$ZnSO_4 \cdot 7H_2O$ 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	$ZnSO_4 \cdot 7H_2O$ 50 mg/l, H_3BO_4 50 mg/l

Table 4. Selected carbon and nitrogen sources for mycelia growth and sporulation of tested biocontrol agents

Species	Isolates	Selected carbon and nitrogen soerce for mycelia growth of tested biocontrol agents
		Carbon sources
P. lilacinus	IPC-P	D-(−)-Fructose, D-(+)-glucose, maltose, D-(+)-mannose, D-(+)-trehalose
P. lilacinus	M-14	D-(+)-Cellobiose, D-(−)-fructose, maltose, D-(+)-mannose, D-(−)-ribose, starch soluble, sucrose
M. anisopliae	SQZ-1-21	D-(−)-Fructose, D-(+)-glucose, D-(+)-mannose, sucrose
M. anisopliae	RS-4-1	D-(−)-Fructose, glutin, D-(+)-glucose, D-(+)-mannose, sucrose, D-(+)-xylose
		Nitrogen sources
P. lilacinus	IPC-P	Soy peptone, tryptone, urea, yeast extract, NH_4NO_3
P. lilacinus	M-14	Soy peptone, tryptone, urea, yeast extract, NH_4NO_3
M. anisopliae	SQZ-1-21	Soy peptone, tryptone, yeast extract
M. anisopliae	RS-4-1	Soy peptone, tryptone, yeast extract
Species	Isolate	Selected carbon and nitrogen sources for sporulation of tested biocontrol agents
		Carbon soures
P. lilacinus	IPC-P	D-(+)-Mannose, maltose
P. lilacinus	M-14	Maltose, sucrose, starch soluble
M. anisopliae	SQZ-1-21	D-(+)-Mannose, sucrose, D-(+)-glucose
M. anisopliae	RS-4-1	D-(+)-Mannose, glutin, sucrose
		Nitrogen sources
P. lilacinus	IPC-P	Tryptone, urea, yeast extract
P. lilacinus	M-14	Soy peptone, yeast extract
M. anisopliae	SQZ-1-21	Urea, yeast extract
M. anisopliae	RS-4-1	Soy peptone, yeast extract

or 1 N HCl. These defined medium were put in 50-ml tubes for 10 ml and was autoclaved for 30 min at 121°C.

Culture conditions. Liquid culture experiments were carried out in triplicate in 50-ml sterile plastic tubes at 10 ml volume. The cultures were incubated at 25°C and 180 rpm in a rotary shaker incubator. Mycelial growth on the tube wall was minimized by frequent shaking.

Assays of the net dry mycelial weight. Each treatment was carried out in triplicate by a 50-ml plastic tube containing 10 ml of culture medium. Each tube was inoculated with 1 ml spore suspension made before. The tubes were loosely capped to allow gas exchange, and incubated at a 180 rpm shaker at 25°C.

Filter papers used for this research was put in 80°C for 48 h to get the constant weight. Mycelia of each treatment after 1 week culture were collected by one filter paper treated as above which was put on a sterile tube, washed the mycelia of cultured tube three times by sterile water on the filter paper, then held these tubes which covered by filter paper at 80°C for 24 h to the dry constant weight, then the net dry biomass (primarily mycelium) weight for each culture medium was determined by minis the constant weight of filter paper got before [10].

On Solid Cultivation

Media preparation. Based on the certain carbon concentration and C/N ratio for sporulation (Table 2) [20], combined with screened mineral elements (Table 3, unpublished data) as well as carbon and nitrogen sources for sporulation of these tested fungi (Table 4, unpublished data), we try to grope for the best combination of carbon and nitrogen sources with those certain parameters mentioned above for sporulation of all these fungi. Seventeen grams of Bacto (Difco) agar was added to these defined basal salts medium and was autoclaved at 121°C for 30 min. The pH was adjusted to 7 by adding 1 N NaOH or 1 N HCl solutions. Fifteen milliliter of each experimental medium was poured to sterile plastic plates. Apiece of sterile cellophane (3.5 cm diameter) was overlaid on each plate 2 days before inoculation avoiding the inoculation disperse out the cellophane.

Assays of sporulation. Five microlitre of homogenous conidial suspension (about 5×10^4 conidia) prepared above was transferred onto membrane center of each treatment plate and cultured at 25°C for 2 weeks. The colonies with the membrane were transferred to a 50-ml centrifuge tube containing 10 ml sterile 0.05% Tween-80 surfactant. Spores were dislodged from colony and the number of spores per colony was deter-

mined using a haemacytometer as above. Each treatment was carried out in triplicate.

Statistical analysis. All data were subjected to one-way analysis of variances (ANOVA). Means at various treatments were separated using Fisher's protected least significant difference (LSD) at $P = 0.05$ by Statistical Analysis System (Version 8.2, SAS Institute, Cary, NC).

RESULTS

In submerged culture. The nematophagous fungus *P. lilacinus* IPC-P had the highest dry mycelia yield (91.0×10^{-3} g) on the medium with D-(+)-glucose and NH_4NO_3 as carbon and nitrogen source respectively, D-(+)-mannose as carbon source with nitrogen sources of soy peptone (85.0×10^{-3} g) or yeast extract (83.0×10^{-3} g) and tryptone as nitrogen source with carbon sources of D-(–)-fructose (83.0×10^{-3} g) or D-(+)-glucose (87.0×10^{-3} g) also had satisfactory production. However, the combination of maltose with urea as carbon and nitrogen source greatly inhibited its mycelial growth and had much less production than other treatments (31.0×10^{-3} g). So, the optimal medium for mycelial growth of *P. lilacinus* IPC-P was NH_4NO_3 as nitrogen source and from D-(+)-glucose at 6 g/l, C/N ratio 20 : 1 with $ZnSO_4 \cdot 7H_2O$ 10 mg/l and $CaCl_2$ 3 g/l (Table 5).

Another nematophagous fungus *P. lilacinus* M-14 had the highest production of dry mycelia on the medium of starch soluble and urea as carbon and nitrogen source (143.0×10^{-3} g), tryptone as nitrogen source had moderate production with all carbon sources, soy peptone promoted mycelial growth with all carbon sources except with D-(+)-mannose and NH_4NO_3 accelerated mycelial growth with all carbon sources except with D-(+)-mannose; D-(+)-mannose had poor production with all nitrogen sources except with yeast extract, the combination of D-(+)-cellobiose, D-(+)-fructose, starch soluble and sucrose as carbon source with all nitrogen sources promoted its mycelial growth, and D-(–)-ribose as carbon source with all nitrogen sources supported moderate mycelial growth. The least production was on the medium of D-(+)-mannose with soy peptone (2.0×10^{-3} g) as carbon and nitrogen source respectively. For mycelial growth of *P. lilacinus* M-14, the tested optimal medium was urea as nitrogen source and from starch soluble at 12 g/l, C/N ratio 20:1 with $ZnSO_4 \cdot 7H_2O$ 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 (Table 5).

The nematophagous fungus *M. anisopliae* SQZ-1-21 had the most dry mycelia production on the media of D-(–)-fructose with tryptone (129.0×10^{-3} g), D-(–)-fructose as carbon source with all tested nitrogen source accelerated mycelial growth, others carbon sources with tested nitrogen sources supported moderate mycelial growth. Soy peptone and yeast extract as nitrogen source with tested carbon sources had similar abilities for mycelial growth, while tryptone had great difference effects between the different combinations with all tested carbon sources. For mycelial growth of *M. anisopliae* SQZ-1-21, the tested optimal medium was tryptone as nitrogen source and from D-(–)-fructose at 8 g/l, C/N ratio 40 : 1 with $ZnSO_4 \cdot 7H_2O$ 50 mg/l, $CuSO_4 \cdot 5H_2O$ 50 mg/l, H_3BO_4 5 mg/l and $MnSO_4 \cdot H_2O$ 10 mg/l (Table 5).

The entomophagous fungus *M. anisopliae* RS-4-1 had the most dry mycelial production on the medium of glutin with soy peptone (68.0×10^{-3} g) as carbon and nitrogen source respectively, D-(+)-mannose as carbon source with all tested nitrogen sources greatly inhibited mycelial growth, D-(+)-glucose as carbon source with all tested nitrogen sources supported moderate mycelial growth, other carbon sources with all tested nitrogen sources accelerated mycelial growth. Soy peptone as nitrogen source with all tested carbon sources supported better mycelial growth than other two kinds of nitrogen sources. The least production was on the medium of D-(+)-mannose with tryptone (11.0×10^{-3} g) as carbon and nitrogen source, others treatments had little differences on mycelia production. For mycelial growth of *M. anisopliae* RS-4-1, the tested optimal medium was soy peptone as nitrogen source and from glutin at 8 g/l, C/N ratio 40:1 with $ZnSO_4 \cdot 7H_2O$ 50 mg/l and H_3BO_4 50 mg/l (Table 5).

On solid culture. For the nematophagous fungus *P. lilacinus* IPC-P, the highest spore yield was (283.4×10^5 conidia/ml) on the medium with D-(+)-mannose and tryptone as carbon and nitrogen source respectively, to all nitrogen sources, tryptone accelerated sporulation, yeast extract supported moderate sporulation and urea with all tested carbon sources differed a lot on its sporulation. Combined the other information we had gotten, the suitable nutritional requirements of *P. lilacinus* IPC-P sporulation was composed of tryptone as nitrogen source and carbon concentration from mannose at 12 g/l, C/N ratio 20 : 1 and $ZnSO_4 \cdot 7H_2O$ 10 mg/l, $CaCl_2$ 3 g/l in addition to 17 g Bactor (Difco) agar per liter (Table 5).

Another nematophagous isolate *P. lilacinus* M-14 had better spore production (1530.0×10^5 conidia/ml) on the medium containing maltose and yeast extract as carbon and nitrogen source respectively. Maltose as carbon source with all tested nitrogen sources accelerated sporulation, starch soluble with all tested nitrogen sources supported moderate growth, sucrose with all tested nitrogen sources had limited spore yield. Yeast extract as nitrogen source with all carbon sources had better spore yield than soy peptone. The least production of 133.0×10^5 conidia/ml was on the medium of sucrose and soy peptone as carbon and nitrogen sources respectively. So, the best medium for the highest sporulation of *P. lilacinus* M-14 was composed of yeast extract as nitrogen source and carbon concentration from maltose at 8 g/l, C/N ratio 10:1 with the supplement of $ZnSO_4 \cdot 7H_2O$ 250 mg/l, $CuSO_4 \cdot 5H_2O$

Table 5. Combinations of carbon and nitrogen sources for mycelia growth in submerged (10^{-3} g/test tube) and sporulation on solid of tested biocontrol agents (10^5 conidia/ml)

Species	Isolates	Combinations of carbon and nitrogen sources in surbmerged culture of tested biocontrol agents						
		Carbon sources	Nitrogen sources					LSD
			Soy peptone	Tryptone	Urea	Yeast extract	NH_4NO_3	
P. lilacinus	IPC-P	D-(−)-Fructose	41.0 bcd	83.0 ab	75.0 abcd	72.0 abcd	32.0 cd	48.3
		D-(+)-Glucose	40.0 bcd	87.0 ab	69.0 abcd	72.0 abcd	91.0 a	
		Maltose	72.0 abcd	68.0 abcd	31.0 d	74.5 abcd	68.0 abcd	
		D-(+)-Mannose	85.0 ab	61.5 abcd	79.0 abcd	83.0 ab	56.0 abcd	
		D-(+)-Trehalose	66.0 abcd	49.0 abcd	62.0 abcd	76.5 abcd	64.0 abcd	
P. lilacinus	M-14	D-(+)-Cellobiose	137.0 ab	81.0 abcdefg	87.0 abcdefg	128.0 abc	117.0 abcd	89.2
		D-(−)-Fructose	120.0 abc	88.0 abcdefg	81.0 abcdefg	89.0 abcdefg	100.0 abcdef	
		Maltose	100.0 abcde	124.0 abc	135.0 ab	23.0 efg	115.0 abcd	
		D-(+)-Mannose	2.0 g	68.0 abcdefg	21.0 efg	102.0 abcdef	13.0 fg	
		D-(−)-Ribose	69.0 abcdefg	91.0 abcdefg	40.0 abcdefg	32.0 defg	45.0 cdefg	
		Starch soluble	84.0 abcdefg	48.0 bcdefg	143.0 a	83.0 abcdefg	119.0 abcd	
		Sucrose	101.0 abcdef	83.0 abcdefg	57.0 abcdefg	113.0 abcde	114.0 abcd	
M. anisopliae	SQZ-1-21	D-(−)-Fructose	45.0 def	129.0 a		87.0 b		31.1
		D-(+)-Glucose	64.0 bcde	56.0 dcdef		54.0 cdef		
		D-(+)-Mannose	36.0 ef	40.0 ef		74.0 bcd		
		Sucrose	85.0 bc	26 f		35.0 ef		
M. anisopliae	RS-4-1	D-(−)-Fructose	46.0 abcd	34.0 abcd		39.0 abcd		42.8
		Glutin	68.0 a	39.0 abcd		24.0 bcd		
		D-(+)-Glucose	42.0 abcd	23.0 bcd		38.0 abcd		
		D-(+)-Mannose	21.0 bcd	11.0 d		19.0 cd		
		Sucrose	50.0 abcd	63.0 ab		32.0 abcd		
		D-(+)-Xylose	30.0 abcd	43.0 adcd		61.0 abc		

Species	Isolates	Combinations of carbon and nitrogen sources in surbmerged culture of tested biocontrol agents						
		Carbon sources	Nitrogen sources					LSD
			Soy peptone	Tryptone	Urea	Yeast extract	NH_4NO_3	
P. lilacinus	IPC-P	D-(+)-Mannose		283.4 a	41 c	177.5 abc		145.3
		Maltose		120.9 bc	240.9 ab	123.4 bc		
P. lilacinus	M-14	Maltose	647.2 bc			1530.0 a		
		Sucrose	133.0 c			780.5 b		259.2
		Starch soluble	423.4 bc			266.7 bc		
M. anisopliae	SQZ-1-21	D-(+)-Mannose			74.2 bc	27.7 cd		
		Sucrose			318.5 a	11.4 d		51.7
		D-(+)-Glucose			120.4 bc	14.5 d		
M. anisopliae	RS-4-1	D-(+)-Mannose	302.7 b			1986.7 a		
		Glutin	119.7 b			639.2 b		863.6
		Sucrose	137.4 b			1525.9 a		

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

10 mg/l, H_3BO_4 5 mg/l, $Na_2MoO_4 \cdot 2H_2O$ 5 mg/l and 17 g/l Bactor (Difco) agar (Table 5).

The nematophagous isolate of *M. anisopliae* SQZ-1-21 had higher conidia production on the medium containing sucrose as carbon and urea as nitrogen source (318.5×10^5 conidia/ml). Urea as nitrogen source with all tested carbon sources greatly accelerated sporulation compared with yeast extract. The least production was on the medium of sucrose combined with yeast extract (11.4×10^5 conidia/ml). The tested optimal medium for sporulation of *M. anisopliae* SQZ-1-21 was that urea as nitrogen source and carbon concentration from sucrose at 8 g/l and C/N ratio 10 : 1 with supplement of $ZnSO_4 \cdot 7H_2O$ 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 and 17 g/l Bactor (Difco) agar (Table 5).

For the entomopathogenic fungus of *M. anisopliae* RS-4-1, which had the highest spore yield of 1986.7×10^5 and 1525.9×10^5 conidia/ml on the media containing mannose as carbon and yeast extract as nitrogen source or sucrose as carbon and yeast extract as nitrogen source respectively. D-(+)-mannose as carbon source with all nitrogen sources greatly promoted sporulation, soy peptone as nitrogen source with all carbon sources supported moderate sporulation, the ability of yeast extract as nitrogen source accelerated sporulation differed a lot compared with different carbon sources. The least spore production of 119.7×10^5 conidia/ml was on the medium of glutin as carbon and soy peptone as nitrogen source. The optimal medium for sporulation of *M. anisopliae* RS-4-1 was composed of yeast extract as nitrogen and carbon concentration from D-(+)-mannose or sucrose at 8 g/l and C/N ratio of 160 : 1 with supplement of $ZnSO_4 \cdot 7H_2O$ 50 mg/l, H_3BO_4 50 mg/l and 17 g/l Bactor (Difco) agar (Table 5).

DISCUSSION

We evaluated the suitability of various combinations of carbon and nitrogen sources for the mycelial growth and sporulation of four potential biocontrol fungi based on screened several carbon and nitrogen sources, several minerals and the suitable carbon concentrations in addition with C/N ratios for mycelial growth and sporulation. It has been reported that the influence of amino acids on fungal sporulation is species-dependent [21, 22] or strain-dependent [17]. In this study, our result was consisted with the latter phenomena, which is strain-specific on the combinations of components and contents for mycelial growth and sporulation. Concretely, in submerged for mycelial growth, the tested nitrogen sources for *P. lilacinus* IPC-P and *P. lilacinus* M-14 were the same, while other components and contents differed and these different combinations lead to different mycelia production. There existed similar phenomena to *M. anisopliae* RS-4-1 and *M. anisopliae* SQZ-1-21, the same tested nitrogen sources in different combinations lead to different mycelia yields too. On solid for sporulation, the same carbon source of maltose with different combinations for *P. lilacinus* IPC-P and *P. lilacinus* M-14 lead to different spore yields, the same carbon source of D-(+)-mannose and sucrose with different combinations for *M. anisopliae* RS-4-1 and *M. anisopliae* SQZ-1-21 had great difference in sporulation. The results we got before had indicated that the carbon source, nitrogen source, carbon concentration, C/N ratio and mineral elements were greatly different from each strain [19, 20, unpublished data], and from present experiment which indicated that each strain had their certain nutrition requirements (including components and contents) for mycelial growth and sporulation. In addition, it is also demonstrated that there are great necessary to have a better understanding of their basic physiology characters for better yields of mycelia and conidia with short time and low cost, this knowledge would be great necessary on the way of mass-production and commercialization for these biocontrol agents.

The development as bioinsecticides is based on mycelia and/or conidia produced in relatively simple media [23, 24]. In this study, the components and contents of carbon source, nitrogen source and mineral elements for tested were all screened by one-factor-at-a-time from 33 carbohydrates, 20 nitrogen sources, 6 minerals, 3 carbon concentrations and 15 C/N ratios, together with considering the cost for solid culture to optimize medium for choosing the tested optimal combination based on their mycelial growth and sporulation. Taken together, results from this research suggested the potential of improving the product quantity of these strains by managing the nutritional environment of production media without compromising biocontrol fungi.

While, the attempts to optimize mycelial growth and sporulation of these fungi in the mass production will be not necessarily result in the production on good quality propagules. Nutritional factors were also associated with agent efficiency during production of biomass of biocontrol agents [25]. For example, firstly about carbon concentration and C/N ratio, Jackson and Schisler [15] found that conidia of *C. truncatum* produced in a medium with a C/N ratio of 10 : 1 were longer and thinner than those produced in 30 : 1 or 80 : 1 media, germinated more rapidly, and formed appressoria more frequently in comparison with conidia produced in media with C/N ratios of 30:1 or 80:1. Secondly, carbon and nitrogen sources that slightly increased the number of ascospores of *T. flavus* reduced the efficacy of biological control of verticillium wilt compared with ascospores produced on PDA [18]. In addition, fungal biocontrol agents can be ineffective if conidia are unable to germinate because water availability is suboptimal [26] or if they have inadequate carbohydrate reserves [27, 28]. One way to overcome these problems is to modify the carbohydrate content of the fungal inocula [27, 29]. Carbohy-

drate component significantly affect the quantities of specific polyols and trehalose in conidia of entomopathogens, trehalose is known to enhanced desiccation tolerance, for example, conidia of *T. harzianum* and *Aspergillus japonicus* with an increase trehalose content retained viability during storage better than those contained less trehalose [28]. Conidia of *B. bassiana* and *P. farjnosus* were found to accumulate trehalose significantly more than those of *M. anisopliae*, the potential to improve the storage life of agriculture inocula may vary between species. The ability to manipulate polyol and trehalose content of fungal propagules may be critical in the development of inocula of enhanced quality which have important desiccation tolerance and storage life, and greater efficacy in the field. There is little research about mentioned above on the efficacy of mycelia, maybe which is difficult to describe their contribute compared to spores, but further research about this is necessary for good products from different combinations of carbon and nitrogen sources together with carbon concentrations and C/N ratios in defined medium in the future. In view of these differences, it is prudent to evaluate a wide range of media in order to optimize nutritional conditions for mass production and biocontrol efficacy of these fungal biocontrol agents [30].

Variations in biocontrol efficacy may result from specific changes in cell physiology and is likely due to many interacting factors [25]. Culture environment is also an important parameter for the quality and quantity of conidiation. Efficient biocontrol of these fungi requires maintenance of viability and virulence of the conidia after field application, numerous environmental factors may reduce growth and establishment of biocontrol fungi in ecosystems [31], studying the environmental factors on the propagate is essential to improve the survival ability of these agents and get a better biocontrol efficacy. Whether nutritional and environmental conditions that maximize mycelial growth and sporulation optimize the potential of these productions to germinate and virulence should receive more attention in the future.

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

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