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A novel method to optimize culture conditions for biomass and sporulation of the nematophagous fungus *Metarhizium anisopliae* SQZ-1-21 and the entomopathogenic fungus *Metarhizium anisopliae* RS-4-1

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Metarhizium anisopliae **is a fungus that offers great potential for the biocontrol of a wide range of host species. In this study, we developed a novel two-stage cultivation method to optimize nutrition and environmental conditions for *M. anisopliae* SQZ-1-21 and *M. anisopliae* RS-4-1. Both species were cultured on basal medium (19.00 g sucrose, 4.06 g soy peptone, 1.00 g K_2HPO_4, 0.50 g KCl, 0.50 g $MgSO_4$, 0.01 g $FeSO_4$ and 17.00 g Bactor) during the first four days at room temperature, and then one of them was transferred to sporulation medium (38.09 g sucrose, 0.43 g urea, 0.05 gL^{-1} $ZnSO_4•7H_2O$, 0.05 gL^{-1} $CuSO_4•5H_2O$, 0.005 gL^{-1} H_3BO_4, 0.01 gL^{-1} $MnSO_4•H_2O$, and 17.00 g Bactor), and the other was transferred to a distinct sporulation medium (9.52 g sucrose, 10.00 g soy peptone, 0.05 g/L $ZnSO_4•7H_2O$, 0.05 gL^{-1} H_3BO_4 and 17.00 g Bactor) for an additional four days. Basal and sporulation medium of *M. anisopliae* SQZ-1-21 was cultured under the following environmental conditions separately: -1.2 MPa, pH 9, 12 h light, and 29°C and -1.2 MPa, pH 9, 0 h light and 29°C. Basal and sporulation medium of *M. anisopliae* RS-4-1 was cultured under the following conditions separately: -0.3 MPa, pH 8, 24 h light, and 29°C and -3.9 MPa, pH 5, 12 h light and 26°C. These results provide important information on the mass production of this potential biocontrol fungus.**

Key words: *Metarhizium anisopliae*, nutrition, environment, sporulation.

INTRODUCTION

The disadvantages of chemical insecticides include environmental concern and health risks, leading people to use fungi to perform the biocontrol of insects as an innovative alternative (Arthurs and Thomas, 2001). *Metarhizium anisopliae* (Metschnikoff) Sorokin is a fungus that is commonly found in the soil and can infect

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more than 200 species of insects. It is an entomogenous fungus that has become one of the main research targets. This species may have a promising future because of its broad host range (Gisbert, 1993), safe to humans, and the fact that insects are unlikely to develop resistance to it. Despite intensive efforts, biocontrol agents face problems primarily due to their poor efficacy (Amsellem et al., 2002) and inability to compete with the low cost of chemical pesticides.

Other factors have also limited the commercialization of this fungus. One of the main requirements for commercially available biocontrol agents is that they can be readily produced in large quantities at a low cost. Tryptophan, glutamic acid, and histidine are all effective at promoting the growth and sporulation of *M. anisopliae*. However, nitrogen sources containing sulfur are poorly utilized for sporulation by *M. anisopliae* (Campbell et al., 1978).

Lu et al. (2004) found that the proper temperature for growth of *M. anisopliae* WZW3 on solid culture was 25°C and that the optimal pH was 7. The presence of sucrose and yeast extract together with the microelement Mn produced the best mycelia and the largest sporulation. *M. anisopliae* M337 grew best on media containing maltose and lactose as carbon sources. This species also grew best when the media had a C/N ratio of 20/10 according to growth comparisons using 6 regular media, 4 carbon and nitrogen sources and 6 combinations of C and N (Song et al., 2008).

In the previous studies mentioned above, continuous culture on agar plates and/or in liquid media has generally been used to study the effects of nutrition and/or environmental factors on fungal growth and sporulation. This continuous culture approach was unable to define certain nutrients and environmental conditions that contributed to the growth and/or sporulation of the fungus. In this paper, we used a novel two-stage cultivation method to study the combined effects of culture conditions, including nutrition and environmental factors, on fungal growth and sporulation. Differences in the optimal conditions needed for growth versus sporulation were determined (Gao and Liu, 2010). The aim of this study was to determine the optimal combination of nutrition and environmental factors for sporulation using this novel method. The results of this study will improve our understanding of fungal physiology and various ecological characteristics, including mass production, colonization, survival, and competitive ability under field conditions.

MATERIALS AND METHODS

Fungal strains

The nematophagous fungus *M. anisopliae* SQZ-1-21 was originally isolated from *Meloidogyne incognita* by M.H. Sun from Qingzhou, Shandong, China. The entomopathogenic fungus *M. anisopliae* RS-4-1 was originally isolated from soil with *Galleria mellonella* by Z.A. Chen from Jiangsu, China. Both of these strains were deposited in the Center of General Microorganisms Culture Collection in the Institute of Microbiology, Chinese Academy of Sciences.

Nutrition requirements for the sporulation of *M. anisopliae* SQZ-1-21 and RS-4-1

The source of chemicals used

The following chemicals were used in this study: yeast extract from Sigma Chemical Co.; glucose, sucrose, mannose, glutin, sucrose, urea, K_2HPO_4, $MgSO_4$, and $FeSO_4$ from Beijing Chemical Reagents Company (Beijing, China); soy peptone from Shanghai Chemical Reagents Company (Shanghai, China); and KCl from Nanjing Chemical Reagents Company (Nanjing, China).

The basal medium comprised 19.00 g sucrose (equal to 8 g carbon), 4.06 g soy peptone (equal to 0.33 g nitrogen), 1.00 g K_2HPO_4, 0.05 g KCl, 0.50 g $MgSO_4$, 0.01 g $FeSO_4$ and 17.00 g Bacto (Difco) agar per liter. We used this medium during the first culture stage, which lasted 4 days.

Effects of carbon concentration and the 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 to replace the carbon and nitrogen sources in the basal medium. Various combinations of carbon and nitrogen concentrations produced C:N ratios that ranged from 0.625:1 to 80:1. These various carbon concentrations and C:N ratios were applied during the second culture stage to induce sporulation over an additional 4 days. After this experiment, we used the optimal carbon concentration of 16 g/L with a carbon to nitrogen ratio of 80:1 for *M. anisopliae* SQZ-1-21 and a carbon concentration of 4 g/L with a carbon to nitrogen ratio of 5:1 for *M. anisopliae* RS-4-1 (Gao and Liu, 2009a).

Effects of the combination of carbon and nitrogen sources

For *M. anisopliae* SQZ-1-21, the carbon sources included mannose, sucrose, and glucose, and the nitrogen sources included soy peptone and urea. The various carbon and nitrogen source combinations used to induce sporulation in this strain all contained a carbon concentration of 16 gL^{-1} (pure carbon per liter calculated from the percentage of carbon atoms in the molecule) and a C/N ratio of 80:1 (with 1 gL^{-1} nitrogen calculated from the percentage of nitrogen atoms in the molecule).

The carbon sources applied to *M. anisopliae* RS-4-1 included mannose, sucrose, and glutin, and the nitrogen sources included soy peptone and yeast extract. The various carbon and nitrogen combinations used to induce sporulation in this strain all contained a carbon concentration of 4 gL^{-1} (pure carbon per liter calculated from the percentage of carbon atoms in each source molecule) and a C/N ratio of 5:1 (with 1 gL^{-1} of nitrogen calculated from the percentage of nitrogen atoms in the molecule).

For each combination, the carbon and nitrogen sources were added to the basal medium to replace the sucrose and soy peptone and form the sporulation medium for the second-stage culture period of 4 days. Cells were also grown on basal sporulation medium for 4 days as a control.

Effects of mineral elements

After testing the effects of various mineral elements and

Table 1. $L_{16}(2^{15})$ orthogonal desigh of optimization of culture environment of *Metarhizium anisopliae* SQZ-1-21 and RS-4-1.

Isolate	Factors	Water potential (MPa)	pH	Light (h)	Temperature (℃)
M. anisopliae SQZ-1-21	Level 1	-1.2	9	0	29
	Level 2	-0.3	8	12	26
M. anisopliae RS-4-1	Level 1	-3.9	8	24	32
	Level 2	-0.3	5	12	29

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

concentration gradients on the sporulation of these two isolates, we identified the optimal components to induce sporulation in both strains. For *M. anisopliae* SQZ-1-21, the following media components produced the best sporulation: 0.05 gL^{-1} $ZnSO_4 \bullet 7H_2O$, 0.05 gL^{-1} $CuSO_4 \bullet 5H_2O$, 0.005 gL^{-1} H_3BO_4, and 0.01 gL^{-1} $MnSO_4 \bullet H_2O$. For *M. anisopliae* RS-4-1, the optimal media contained 0.05 gL^{-1} $ZnSO_4 \bullet 7H_2O$ and 0.05 gL^{-1} H_3BO_4.

Effects of environmental factors on the sporulation of *M. anisopliae* SQZ-1-21 and RS-4-1

A novel two-stage cultivation method on plates was applied to evaluate the effects of pH, water potential, dark/light cycle and temperature during the 4 days of the second-stage culture period on the sporulation of the biocontrol fungi. The following water potentials: -0.3, -0.8, -1.2, -2.1, -3.9 and -7.3 MPa were tested, and pH values of 3, 4, 5, 6, 7, 8 and 9 were evaluated. Sporulation was evaluated with the following dark/light cycles: 24 h/0 h, 12 h/12 h, and 0 h/24 h. Ambient temperatures of 20, 23, 26, 29, and 32°C were evaluated. We selected two levels of each factor to carry out the orthogonal research. The selected levels of the environmental factors are shown in Tables 2 (Gao et al., 2009b).

Orthogonal matrix method

The orthogonal $L_{16}(2^{15})$ was used to identify the optimal solid media culture conditions for the following variables: pH, water potential, dark/light cycle, temperature, C/N ratio, and carbon and nitrogen source combination. Additional environmental factors were evaluated individually using this novel method. After identifying the best nutrition balance, we determined the optimal combinations of nutrition and environmental factors to induce sporulation in *M. anisopliae* SQZ-1-21 and RS-4-1 using the orthogonal matrix method with two levels for each of four environmental factors.

Statistical analysis

The data were analyzed using one-way ANOVA. Significant differences were evaluated using Duncan's multiple range test at $P = 0.05$ with the Statistical Analysis System software (Version 8.2, SAS Institute, Cary, NC).

RESULTS

Nutritional requirements to induce sporulation in *M. anisopliae* SQZ-1-21 and RS-4-1

To investigate the relationships between environmental factors and certain medium components and to 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 1), the experimental conditions for each experimental group are listed in Table 2, with the experimental results summarized in the last two columns (biomass and sporulation) separately for SQZ-1-21 and RS-4-1. According to the orthogonal method, the effects of various environmental factors, including pH, water potential, dark/light cycle, and temperature, on growth and sporulation were evaluated and are shown Table 3. According to the magnitude order of R (maximum difference) for *M. anisopliae* SQZ-1-21 in Table 3, the order of effects of all of the factors on biomass yields was determined to be 15.63 (pH) > 15.38 (water potential) > 8.13 (light) > 7.04 (temperature); these results indicate that the effect of 15.63 (pH) was more important than those of the other three environmental factors. The order of the effects of all of the factors on sporulation was found to be 0.86 (water potential) > 0.33 (light) > 0.23 (pH) > 0.14 (temperature); these results indicate that the effect of 0.86 (water potential) was more important than those of the other three environmental factors.

For *M. anisopliae* RS-4-1, the order of the effects of all of the factors on the biomass yield was found to be 46.25 (water potential) > 20.00 (pH) > 3.17 (temperature) > 1.17 (light); these results indicate that the effect of 46.25 (water potential) was more important than those of the other three environmental factors. For this strain, the order of the effects of all of the factors on sporulation was found to be 0.53 (pH) > 0.26 (temperature) > 0.23 (light) > 0.04 (water potential); these results indicate that the effect of 0.53 (pH) was more important than those of the other three environmental factors. To test the effects of each of the four factors, ANOVA was applied. As shown in Table 4, water potential significantly affected the sporulation of *M. anisopliae* SQZ-1-21, and pH significantly affected the sporulation of *M. anisopliae* RS-4-1 (Table 4). Table 5 shows the effects of combinations of the four factors on biomass yields and sporulation for each fungus separately. For *M. anisopliae* SQZ-1-21, we found that the following combinations: B_1/A_1, A_1/C_2, A_1/D_1, B_1/C_2, D_1/B_1, D_1/C_2 had the best effects on biomass yields, producing the following yields: 188.00, 189.42, 190.42, 196.88, 214.92, 185.42 mg per colony,

Table 2. Orthogonal experiment of $L_{16}(2^{15})$ of biomass yields and sporulation of *Metarhizium anisopliae* SQZ-1-21 and RS-4-1.

Isolate	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)
M. anisopliae SQZ-1-21	1‡	1†	1	1	1	1	1	1	1	1	1	1	1	1	1	1	218.00 ± 62.19§	2.53 ± 0.25
	2	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	140.00 ± 11.36	2.53 ± 0.21
	3	1	1	1	2	2	2	2	1	1	1	1	2	2	2	2	217.00 ± 40.60	2.51 ± 0.16
	4	1	1	1	2	2	2	2	2	2	2	2	1	1	1	1	177.00 ± 23.64	2.51 ± 0.18
	5	1	2	2	1	1	2	2	1	1	2	2	1	1	2	2	172.00 ± 23.07	2.46 ± 0.09
	6	1	2	2	1	1	2	2	2	2	1	1	2	2	1	1	138.67 ± 47.01	2.29 ± 0.12
	7	1	2	2	2	2	1	1	1	1	2	2	2	2	1	1	115.33 ± 63.95	2.22 ± 0.46
	8	1	2	2	2	2	1	1	2	2	1	1	1	1	2	2	158.00 ± 48.77	2.37 ± 0.10
	9	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	207.67 ± 26.76	1.31 ± 0.13
	10	2	1	2	1	2	1	2	2	1	2	1	2	1	2	1	156.00 ± 46.36	1.54 ± 0.10
	11	2	1	2	2	1	2	1	1	2	1	2	2	1	2	1	173.67 ± 27.02	1.91 ± 0.28
	12	2	1	2	2	1	2	1	2	1	2	1	1	2	1	2	170.67 ± 21.20	2.08 ± 0.32
	13	2	2	1	1	2	2	1	1	2	2	1	1	2	2	1	140.00 ± 46.36	0.57 ± 0.26
	14	2	2	1	1	2	2	1	2	1	1	2	2	1	1	2	253.33 ± 55.19	1.43 ± 0.65
	15	2	2	1	2	1	1	2	1	2	2	1	2	1	1	2	186.33 ± 54.50	1.91 ± 0.26
	16	2	2	1	2	1	1	2	2	1	1	2	1	2	2	1	171.33 ± 25.01	1.81 ± 0.13
M. anisopliae RS-4-1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	103.00 ± 28.58§	1.82 ± 0.16
	2	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	82.00 ± 31.24	2.15 ± 0.09
	3	1	1	1	2	2	2	2	1	1	1	1	2	2	2	2	80.67 ± 14.58	1.10 ± 0.30
	4	1	1	1	2	2	2	2	2	2	2	2	1	1	1	1	99.67 ± 49.65	2.18 ± 0.17
	5	1	2	2	1	1	2	2	1	1	2	2	1	1	2	2	100.00 ± 21.52	1.24 ± 0.13
	6	1	2	2	1	1	2	2	2	2	1	1	2	2	1	1	130.00 ± 41.62	1.87 ± 0.05
	7	1	2	2	2	2	1	1	1	1	2	2	2	2	1	1	155.67 ± 29.67	1.44 ± 0.51
	8	1	2	2	2	2	1	1	2	2	1	1	1	1	2	2	64.67 ± 5.77	1.77 ± 0.06
	9	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	138.33 ± 14.15	2.25 ± 0.07
	10	2	1	2	1	2	1	2	2	1	2	1	2	1	2	1	153.00 ± 15.13	2.24± 0.13
	11	2	1	2	2	1	2	1	1	2	1	2	2	1	2	1	136.33 ± 15.50	1.98 ± 0.10
	12	2	1	2	2	1	2	1	2	1	2	1	1	2	1	2	127.67 ± 36.30	2.13 ± 0.10
	13	2	2	1	1	2	2	1	1	2	2	1	1	2	2	1	127.33 ± 15.01	1.69 ± 0.05
	14	2	2	1	1	2	2	1	2	1	1	2	2	1	1	2	162.33± 73.65	1.39 ± 0.17
	15	2	2	1	2	1	1	2	1	2	2	1	2	1	1	2	172.00 ± 16.09	1.19 ± 0.45
	16	2	2	1	2	1	1	2	2	1	1	2	1	2	2	1	168.67 ± 19.09	1.04 ± 0.15

*A×B, A×C, B×C, A×D, B×D, C×D represent the interactions 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.

Table 3. Analysis of environmental factors on biomass production and sporulation of *Metarhizium anisopliae* SQZ-1-21 and RS-4-1 with this novel method.

Isolate			A	B	A×B	C	A×C	B×C		D	A×D	B×D		C×D			
M. anisopliae SQZ-1-21	B*	K_1	1336.00	1460.01	1502.99	1425.67	1370.67	1352.66	1369.00	1430.00	1473.66	1537.67	1384.67	1414.67	1494.32	1467.00	1290.00
		K_2	1459.00	1334.99	1292.01	1369.33	1424.33	1442.34	1426.00	1365.00	1321.34	1257.33	1410.33	1380.32	1300.67	1328.00	1505.00
		k_1	167.00	182.50	187.87	178.21	171.33	169.08	171.13	178.75	184.21	192.21	173.08	176.83	186.79	183.38	161.25
		k_2	182.38	166.87	161.50	171.17	178.04	180.29	178.25	170.63	165.17	157.17	176.29	172.54	162.58	166.00	188.13
		R	15.38	15.63	26.37	7.04	6.71	11.21	7.13	8.13	19.04	35.04	3.21	4.29	24.21	17.38	26.88
		O	1	2	1	2	2	1	1	2	2	2	2	1	1	2	2
	S†	K_1'	19.42	16.92	15.80	14.64	17.52	16.24	15.64	15.42	16.56	16.16	15.80	15.64	16.66	16.28	16.60
		K_2'	12.56	15.06	16.18	17.32	14.46	15.76	16.34	16.56	15.44	15.84	16.18	16.34	15.32	15.70	16.60
		k_1'	2.43	2.12	1.98	1.83	2.19	2.03	1.96	1.93	2.07	2.02	1.98	1.96	2.08	2.04	1.92
		k_2'	1.57	1.88	2.02	2.17	1.81	1.97	2.04	2.07	1.93	1.98	2.02	2.04	1.92	1.96	2.08
		R'	0.86	0.23	0.05	0.33	0.38	0.06	0.09	0.14	0.15	0.04	0.05	0.09	0.17	0.07	0.15
		O'	1	2	2	2	1 or 2	1	1	2	1	2	2	1	1	2	1
M. anisopliae RS-4-1	B*	K_1	815.7	920.7	995.7	995.6	1019.7	1037.3	959.0	1013.3	1051.0	984.0	958.3	929.3	991.0	1088.7	1073.7
		K_2	1185.7	1080.7	1005.7	1005.4	981.7	964.0	1042.3	988.0	950.3	1017.3	1043.0	1072.0	1010.3	912.7	927.7
		k_1	101.96	115.08	124.46	124.45	127.46	129.67	119.88	126.67	131.38	123.00	119.79	116.17	123.88	136.08	134.21
		k_2	148.21	135.08	125.71	125.67	122.71	120.50	130.29	123.50	118.79	127.17	130.38	134.00	126.29	114.08	115.96
		R	46.25	20.00	1.25	1.17	4.75	9.17	10.42	3.17	12.59	4.17	10.58	17.83	2.42	22.00	18.25
		O	2	2	1	2	2	1	1	2	2	2	2	1	1	2	2
	S†	K_1'	13.60	15.85	12.56	14.65	13.42	13.90	14.37	12.71	12.40	13.22	13.81	14.12	13.81	14.27	14.26
		K_2'	13.91	11.63	14.92	12.83	14.06	13.58	13.11	14.77	15.11	14.26	13.67	13.36	13.67	13.21	13.22
		k_1'	1.70	1.98	1.57	1.83	1.68	1.74	1.80	1.59	1.55	1.65	1.73	1.77	1.73	1.78	1.78
		k_2'	1.74	1.45	1.87	1.60	1.76	1.70	1.64	1.85	1.89	1.78	1.71	1.67	1.71	1.65	1.65
		R'	0.04	0.53	0.30	0.23	0.08	0.04	0.16	0.26	0.34	0.13	0.02	0.10	0.02	0.13	0.13
		O'	2	1	2	1	2	1	1	2	2	2	1	1	1	1	1

*Biomass yields (mg per colony); † Sporulation (10^5 conidia per colony); K_1 and K_2 are the total 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 total 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.

respectively. To obtain high mycelia yields, the optimal factors included a water potential of -1.2 MPa (A_1), pH 8 (B_2), 12 h light (C_2), and 26°C (D_2), which were not consistent with the intuitive analysis results of a water potential of -0.3 MPa (A_1), pH 3 (B_2), 12 h light (C_2), and 29°C (D_2) shown in Table 3. The combinations B_1/A_1, A_1/C_1, A_1/D_1, B_1/C_1, B_2/D_1, and D_1/C_1 had the best effect on sporulation, producing 2.52, 2.26, 2.17, 2.20, 2.08, and 2.22 mg per colony spore yields, respectively. To obtain high spore yields, the optimal factors included a water potential of -1.2 MPa (A_1), pH 9 (B_1), 0 h (C_1) light, and 29°C (D_1), which were not consistent with the intuitive analysis results of a water potential of -1.2 MPa (A_1), pH 8 (B_2), 12 h light (C_2), and 26 °C (D_2) listed in Table 3. For *M. anisopliae* RS-4-1, the effects of various combinations of the four factors on biomass yields and sporulation demonstrated that the combinations of B_1/A_2, A_1/C_2, A_2/D_1, B_1/C_1, D_1/B_2, and D_1/C_2 had the best effect on biomass yields, producing 157.58, 142.67, 146.67, 131.42, 134.09, and 137.92 mg per colony biomass yields, respectively. To obtain high mycelia yields, the optimal factors included a water potential of -0.3 MPa (A_2), pH 8 (B_1), 24 h light (C_1), and 29 °C (D_2), which were not consistent with the intuitive analysis results of a water potential of -0.3 MPa (A_2), pH 5 (B_2), 12 h light (C_2), and 29°C (D_2) listed in Table 3. The combinations of B_2/A_1, A_1/C_1, A_1/D_2, B_2/C_2, B_2/D_2, and D_2/C_2 had the best effect on sporulation, producing 2.15, 2.02, 2.14, 1.93, 1.97 and 1.97 mg per colony spore yields,

Table 4. The variance analysis of L16(215) orthogonal test on optimation of environmental factors for biomass yields and sporulation of *Metarhizium anisopliae* SQZ-1-21 and RS-4-1.

Isolate		Variance source	Sum of square deviation (SS)	Degree of freedom (v)	Mean square (MS)	*F*-ratio	Significance level†
M. anisopliae SQZ-1-21	Biomass yields (mg per colony)	A	945.56	1	8555.78	0.71	
		B	976.88	1	1764.21	0.73	
		C	198.39	1	1.00	0.15	
		D	264.06	1	1024.16	0.20	
		A×B	2782.14	1	2782.14	0.42	
		A×C	179.94	1	179.94	0.03	
		A×D	1447.29	1	1447.29	0.22	
		B×C	499.86	1	499.86	0.07	
		B×D	4912.05	1	4912.05	0.73	
		C×D	70.96	1	70.96	0.01	
		Error	6684.85	5			
	Sporulation (10^5 conidia per colony)	A	2.94	1	0.22	55.28	***
		B	0.22	1	0.01	4.06	
		C	0.44	1	0.00	8.31	
		D	0.08	1	0.01	1.52	
		A×B	0.00	1	0.00	0.01	
		A×C	0.61	1	0.61	2.26	
		A×D	0.17	1	0.17	0.62	
		B×C	0.10	1	0.10	0.38	
		B×D	0.10	1	0.10	0.35	
		C×D	0.02	1	0.02	0.09	
		Error	0.27	5			
M. anisopliae RS-4-1	Biomass yields (mg per colony)	A	8555.33	1	8555.33	10.25	**
		B	1600.00	1	1600.00	1.92	
		C	5.48	1	5.48	0.01	
		D	40.07	1	40.07	0.05	
		A×B	7.25	1	7.25	0.01	
		A×C	91.25	1	91.25	0.02	
		A×D	632.53	1	632.53	0.15	
		B×C	335.13	1	335.13	0.08	
		B×D	70.49	1	70.49	0.02	
		C×D	1272.92	1	1272.92	0.30	
		Error	4173.68	5			
	Sporulation (10^5 conidia per colony)	A	0.01	1	0.01	0.15	
		B	1.11	1	1.11	23.29	***
		C	0.207	1	0.207	4.33	*
		D	0.27	1	0.27	5.54	*
		A×B	0.348	1	0.35	6.96	**
		A×C	0.053	1	0.05	1.06	

Table 4. Contd.

	A×D	0.57	1	0.57	11.39	**
	B×C	0.01	1	0.01	0.12	
	B×D	0.067	1	0.07	1.33	
	C×D	0.04	1	0.04	0.72	
	Error	0.050	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 5. Effects of combinations of environmental factors on biomass yields and sporulation of *Metarhizium anisopliae* SQZ-1-21 and RS-4-1.

Isolate	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
M. anisopliae SQZ-1-21	B_1	188.00	2.52	187.75	1.43								
	B_2	177.00	1.71	146.00	2.34								
	C_1	175.59	2.26	167.08	2.12	178.92	2.20	163.75	2.19				
	C_2	189.42	1.97	166.67	1.65	196.88	1.78	159.25	1.86				
	D_1	190.42	2.17	178.00	1.98	214.92	2.07	153.50	2.08	183.00	2.22	185.42	1.93
	D_2	174.59	2.07	155.75	1.79	160.08	1.88	169.50	1.97	159.67	2.16	170.67	1.69
M. anisopliae RS-4-1	B_1	91.34	1.81	157.58	1.33								
	B_2	138.83	2.15	112.59	1.58								
	C_1	112.25	2.02	142.67	1.34	131.42	1.55	123.50	1.81				
	C_2	117.92	1.94	127.50	1.57	117.50	1.59	127.92	1.93				
	D_1	116.09	1.82	146.67	1.28	128.67	1.34	134.09	1.76	124.84	1.56	137.92	1.54
	D_2	114.08	2.14	123.50	1.63	120.25	1.80	117.33	1.97	130.08	1.80	107.50	1.97

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).

respectively. To obtain high spore yields, the optimal factors included a water potential of -3.9 MPa (A_1), pH 5 (B_2), 12 h (C_2) light, and 29°C (D_2), which were not consistent with the intuitive analysis results of a water potential of -0.3 MPa (A_2), pH 8 (B_1), 24 h light (C_1), and 29°C (D_2) listed in Table 3.

DISCUSSION

"Two-stage cultivation" method

A novel "two-stage cultivation" method was used to improve the biomass and sporulation of the two biocontrol agents. A basal medium during the first stage of fungal growth (four days) and a second medium for the second stage of sporulation (another four days) were developed with the aid of supporting membranes of cellophane, a non-biodegradable wrapping paper. After incubating the fungi for four days on basal medium, fresh mycelium and its underlying cellophane were

removed from the agar plate and transferred to a second medium to undergo sporulation for an additional 4 days. The biomass was evaluated before spore production was quantified. We used this method in a previous study. In the present study, we optimized the nutritional components to induce the sporulation of both fungi on the second medium while also optimizing four environmental factors. We then combined the results of these studies using the orthogonal matrix method to identify better combinations of the nutritional and environmental variables for the “two-stage cultivation” method according to biomass and spore yields. We found that fungal biomass could be estimated from the mycelial fresh weight and that our new method improved production over that of traditional methods; this method was patented in China in 2004. The method is based on the induction of mycelial growth for a short time at a low cost to produce a large amount of mycelia, followed by incubation on the sporulation medium to produce spores that are able to provide the basis for commercial production.

Combined environmental and nutritional factors identified using an orthogonal matrix will contribute to better yields and efficacy in the field

The performance of *M. anisopliae* products is affected by various environmental factors, such as soil moisture, air and soil temperatures, relative humidity of the air, and solar UV radiation (Chen et al., 2014). The successful development of entomopathogenic fungi as biocontrol agents depends upon the selection of highly efficient isolates, and the fungi must be adapted to the environmental conditions of the area where they are to be employed (McCoy, 1990).

M. anisopliae plays an essential role in the regulation and control of soil pests and is one of the most widely used biological insecticides. However, its efficiency in controlling soil pests is not constant, and one reason for the fluctuations in the activity of *M. anisopliae* is the complexity of edaphic factors. Temperature, moisture, pH and light were considered in this research, and the results will provide a reference for further studies on the influence of soil factors on *M. anisopliae* and on the control of soil pests using *M. anisopliae*. When isolates of *M. anisopliae* are applied to the soil, temperature and humidity play an important role in determining the success of their colonies. Research into the optimal combinations of both nutrients and environmental factors is not only essential for the mass production of these fungi but will also contribute to the success of the fungal colonies in the fields and improve their biocontrol efficiency when applied to soil.

Metarhizium spp. can parasitize more than 200 types of insects, mites and nematodes; it is one of the most broadly applied insecticide species in the world because of its broad host range and easy culture conditions (Meyling andEilenberg, 2007; Zimmermann, 2007). Most of these fungi colonize the root of the host (Bruck, 2005), and species of this genus are among the most common soil entomopathogens (Nishi et al., 2011; Scheepmaker and Butt, 2010). An optimal good soil environment could increase the production and activity of spores, thereby contributing to better biocontrol efficiency (Garrido-Jurado et al., 2011). *Metarhizium* spp. plays an important role in controlling the number of pests in the soil (Bruck, 2005; Scheepmaker and Butt, 2010; Guzmán-Franco et al., 2012). However, when applied to fields, the fungi do not typically display satisfactory biocontrol efficiency. The most likely explanation for this discrepancy is that it is difficult to evaluate the effects of soil conditions, such as soil temperature, pH, and water potential, on fungal growth. Soil is a complex environment, and *Metarhizium* spp. face many environmental factors that affect their success (Quesada-Moraga et al., 2007). Few previous reports have evaluated the effects of soil texture, temperature, water potential, and pH on the number, activity and infection rate of this fungus (Scheepmaker and Butt, 2010). *Metarhizium* spp. grow well in soil at temperatures of 20-30°C (Ekesi et al., 2003), with optimal growth at 30°C (Hallsworth and Magan, 1999). A previous study found that the optimal temperature for the germination of spores was 33-34°C (Faria et al., 2009), and sporulation was greatly reduced at temperatures below 15**°**C (Sookar et al., 2008). Ekesi et al. (2003) found that at the optimal temperature, a water potential between -0.1 and -0.01 Mpa led to the highest infection rate, and water potentials between -0.0055 and -0.0035 Mpa led to the lowest infection rate. This result was consistent with that of other reports indicating that the growth of *Metarhizium* spp. is reduced when exposed to a high water potential for a long time (Garcia et al., 2011). For example, during the rainy season, the water potential of the soil is increased, which serves to reduce the number at that location (Scheepmaker and Butt 2010). According to the report of Ekesi et al. (2003), little effect was observed on the activity of *Metarhizium* spp. below 15°C in dry soil, whereas in wet soil, the number was reduced even at the optimal temperature (30**°**C). Hallsworth and Magan (1996) reported that the optimal pH for *Metarhizium* spp. was 5-8, and Quesada-Moraga et al. (2007) reported that the highest number of *Metarhizium* spp. in the soil were observed at a pH < 7 and at pH values between 8-8.5 compared to other types of soil with different pH values. Although pH can affect the activity of *Metarhizium* spp., certain isolates may also regulate the soil pH. For example, the production of oxalic acid can be used to regulate the soil pH (St Leger et al., 1999). In conclusion, the use of an orthogonal matrix to evaluate environmental factors and nutrients provided important information on the production and application of these fungi that will serve to improve efficiency in the fields.

Conflict of interests

The authors did not declare any conflict of interest.

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