Effects of *Beauveria bassiana* and acephate on enzyme activities and microbial diversity in paddy soil

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ABSTRACT

To investigate the ecological safety of *Beauveria bassiana* in soil, we evaluated the effects of different concentrations of *B. bassiana* spores suspensions and acephate on paddy soil microbial flora and enzyme activities in a potted trial. Results showed that *B. bassiana* can increase the quantity of bacteria and fungi on day 10 and 30, while it showed inhibition on actinomycetes growth on day 10. However, acephate reduced the quantity of bacteria, fungi, and actinomycetes in soil. Investigation of enzyme activities revealed that invertase activity declined during prophase, while urease activity decreased later in *B. bassiana* treatment groups, and there were no significant changes in alkaline phosphatase or dehydrogenase activity. Acephate showed higher inhibition rates of enzymes. *B. bassiana* treatment at lower concentrations showed a higher yield. Overall, compared with the acephate, *B. bassiana* is an effective, environmentally friendly microbial pesticide in this system.

Keywords: DGGE; ecological safety; *Chilo suppressalis*; Shannon index; biological control

Chemical pesticides can cause potential adverse effect on plants and other non-target organisms when environmental exposure conditions are vulnerable. As an alternative to chemical pesticides, biological control agents (or microbial pesticide), such as *Beauveria bassiana*, is becoming increasingly popular to protect crop yields from losses due to disease and pest infestation. *B. bassiana* is a well-known entomopathogenic fungus that can parasitize more than 700 different kinds of insects (Meyling and Eilenberg 2007). Isolates of *B. bassiana* can antagonize a variety of soil and foliar plants pathogens (Vega et al. 2010) while they are in symbiosis with many plant species. However, knowledge about the impact of *B. bassiana* on soil microbial flora and enzyme activities after application is largely lacking.

Both microbial pesticide *B. bassiana* and chemical pesticide acephate are able to control *Chilo suppressalis* in rice paddies, but with different insecticidal mechanism. No studies have compared the differences in ecological function of these two pesticides in a rice paddy system to date. The objectives of this study were to (i) investigate and compare the influence of *B. bassiana* and acephate on paddy soil microorganisms, enzyme activities and paddy growth after inoculation with *C. suppressalis* larvae, and (ii) further understand the ecological safety of *B. bassiana* in soil and accumulate database for future development of safe use guidance for microbial pesticides in agriculture.

MATERIAL AND METHODS

Experimental design. A pot experiment was conducted in the garden at the Nanjing Normal University, Nanjing, China (32°16'N, 118°79'E).

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The soil sample was collected from the surface layer of a field (20 cm) located in the garden at the Nanjing Normal University. The collected samples were air-dried, and sieved (< 2 mm). Soil properties were characterized for: pH 5.97, organic matter 10.80 g/kg, alkalytic N 98.64 mg/kg, total N 1.21 g/kg, available P 24.67 mg/kg, total P 0.39 g/kg, available K 67.58 mg/kg, and total K 0.73 g/kg. The soil properties were analyzed according to the method of Bao et al. (2000).

Spore suspensions were prepared from *B. bassiana* on a potato dextrose agar medium as described by Malarvannan et al. (2010). Treatments were as follows: (1) CK – blank control group; (2) CS – *C. suppressalis* larvae (40 per pot); (3) B1 – *B. bassiana* at 7.5×10^4 spores/mL + CS; (4) B10 – 7.5×10^5 spores/mL + CS; (5) B100 – 7.5×10^6 spores/mL + CS; (6) B1000 – 7.5×10^7 spores/mL + CS; (7) AE – acephate + CS.

Fifteen kilograms of soil was transferred to each plastic pot (70 cm × 23 cm) and flooded with tap water. Similar size seedlings from 3-week-old healthy paddies were transplanted to each pot on May 20, 2012. Each treatment consisted of six replications and each pot contained four seedlings. *B. bassiana*, CS, and acephate treatment doses were applied on July 10, 2012. Each treatment group was covered with gauze (3.5 × 1 × 1.8 m) separately after treatments dosing.

Sampling and measurements. Soil samples were collected at 0, 10, 30, and 60 days after treatment application. Soils were collected from the surface layer (0–5 cm), placed into sterilized plastic bags and immediately proceeded for analysis as detailed below.

The numbers of soil culturable microorganisms were determined by counting CFU. The culture media used for bacteria, actinomycetes, and fungi were beef-extract peptone medium, Cause's No. 1 synthetic medium, and Rose Bengal medium, respectively. Denaturing gradient gel electrophoresis (DGGE): Bacterial 16S rRNA was amplified using universal primer (Tan and Ji 2010), and fungal 18S rRNA was amplified using universal primer as described by Duarte et al. (2010). The total soil DNA was extracted using an ultra clean soil DNA kit (Carlsbad, USA). Polymerase chain reaction (PCR) and DGGE were performed according to Chen et al. (2013). The PCR-DGGE primers and procedure are shown in Table 1.

Soil enzyme activity. Soil invertase and alkaline phosphatase activity was determined as described by Guan et al. (1986). Soil urease activity was measured as described in Klose and Tabatabai (1999). Soil dehydrogenase activity was analyzed as described by Casida et al. (1964).

Statistical analysis. Basic statistical analysis were performed by SPSS 13.0 using (ANOVA) followed by the Duncan's test ($P < 0.05$). The DGGE profiles were analyzed using the Gelcompar II software (Applied Math, Austin, USA).

RESULTS

Microbial quantity in soil. As shown in Table 2, the quantity of both bacteria and fungi increased throughout the study period. There was a significantly higher quantity of bacteria and fungi in the soils of the *B. bassiana* treatments. All *B. bassiana* treatment groups except B1000 showed increased bacteria population. The level of fungi in the B1000 group was by 42.5% and 101.6% higher than that of CK on days 10 and 30, respectively. *B. bassiana* treatments showed inhibition on actinomycetes growth on day 10.

The dynamics of actinomycetes growth in the CS group were consistent with the CK group. However, the levels of bacteria, fungi, and actinomycetes in AE group were by 52.1, 35.0, and 41.0% lower than CK on day 10, respectively. These indicate that acephate reduced microbial quantity.

Table 1. PCR-DGGE primers and conditions

Target	Primer	PCR conditions
Bacteria	518R(5'-ATTACCGCGGCTGCTGG-3') GC[a]-338F(5'-GACTCCTACGGGAGGCAGCAG-3')	94°C, 5 min; 30 cycles, 94°C, 45 s, 60°C, 30 s, 72°C, 45 s; 72°C, 10 min
Fungi	NS1(5'-GTAGTCATATGCTTGTCTC-3') GC[b]-Fung(5'-ATTCCCCGTTACCCGTTG-3')	94°C, 4 min; 35 cycles, 94°C, 30 s, 55°C, 30 s, 72°C, 1 min; 72°C, 10 min

[a](CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G); [b](CGC CCG CCG CGC CCC GCG CCG GCC CGC CGC CCC CGC CCC)

Table 2. Effect of different treatments on soil culturable microorganisms (n = 6)

Organism	Treatment	Time (days)			
		0	10	30	60
Bacteria (10^8 cfu/g)	CK	0.68 ± 0.04^{b}	0.71 ± 0.06^{bc}	0.93 ± 0.07^{b}	1.43 ± 0.07^{a}
	CS	0.69 ± 0.08^{b}	0.65 ± 0.10^{b}	0.96 ± 0.13^{b}	1.43 ± 0.08^{a}
	B1	0.70 ± 0.06^{b}	0.77 ± 0.07^{d}	1.14 ± 0.07^{c}	1.48 ± 0.08^{a}
	B10	0.69 ± 0.05^{b}	0.76 ± 0.09^{d}	1.11 ± 0.11^{c}	1.46 ± 0.11^{a}
	B100	0.70 ± 0.06^{b}	0.74 ± 0.08^{cd}	1.01 ± 0.08^{b}	1.46 ± 0.14^{a}
	B1000	0.68 ± 0.10^{b}	0.68 ± 0.05^{bc}	0.84 ± 0.09^{b}	1.39 ± 0.05^{a}
	AE	0.61 ± 0.06^{a}	0.34 ± 0.04^{a}	0.77 ± 0.09^{a}	1.43 ± 0.08^{a}
Fungi (10^4 cfu/g)	CK	0.30 ± 0.04^{a}	0.40 ± 0.02^{c}	0.62 ± 0.04^{b}	0.95 ± 0.08^{a}
	CS	0.30 ± 0.03^{a}	0.35 ± 0.02^{b}	0.69 ± 0.04^{b}	1.10 ± 0.05^{d}
	B1	0.29 ± 0.03^{a}	0.44 ± 0.06^{d}	0.87 ± 0.08^{c}	0.97 ± 0.07^{ab}
	B10	0.33 ± 0.05^{a}	0.49 ± 0.03^{e}	0.97 ± 0.07^{d}	1.02 ± 0.09^{bc}
	B100	0.38 ± 0.03^{b}	0.51 ± 0.02^{e}	1.06 ± 0.07^{e}	1.04 ± 0.09^{bc}
	B1000	0.45 ± 0.04^{c}	0.57 ± 0.05^{f}	1.25 ± 0.11^{f}	1.07 ± 0.08^{cd}
	AE	0.31 ± 0.03^{a}	0.26 ± 0.04^{a}	0.55 ± 0.08^{a}	0.93 ± 0.07^{a}
Actinomycete (10^7 cfu/g)	CK	0.36 ± 0.03^{a}	0.78 ± 0.07^{c}	0.66 ± 0.07^{bc}	0.74 ± 0.09^{a}
	CS	0.38 ± 0.05^{b}	0.77 ± 0.07^{c}	0.64 ± 0.08^{b}	0.76 ± 0.05^{a}
	B1	0.39 ± 0.05^{b}	0.57 ± 0.08^{b}	0.77 ± 0.06^{d}	0.75 ± 0.08^{a}
	B10	0.37 ± 0.04^{b}	0.57 ± 0.04^{b}	0.71 ± 0.03^{d}	0.74 ± 0.04^{a}
	B100	0.39 ± 0.03^{b}	0.58 ± 0.05^{b}	0.70 ± 0.05^{c}	0.75 ± 0.05^{a}
	B1000	0.39 ± 0.04^{b}	0.59 ± 0.03^{b}	0.63 ± 0.03^{b}	0.77 ± 0.03^{a}
	AE	0.33 ± 0.03^{a}	0.46 ± 0.05^{a}	0.52 ± 0.05^{a}	0.72 ± 0.02^{a}

Mean values (n = 6) ± SD. Different letters indicate significant differences among treatments (P < 0.05). CK – blank control group; CS – *Chilo suppressalis*; B1 – *Beauveria bassiana* at 7.5 × 10^4 spores/mL + CS; B10 – 7.5 × 10^5 spores/mL + CS; B100 – 7.5 × 10^6 spores/mL + CS; B1000 – 7.5 × 10^7 spores/mL + CS; AE – acephate + CS

Diversity of soil microbes by DGGE clustering. DGGE clustering analysis showed that both bacterial and fungal community structures changed over time (Figure 1, Table 3). Community structure similarity, Shannon index, and band number in the *B. bassiana*-treated group were higher than those in the negative control group on days 10 and 30. AE groups had a lower Shannon index and band number on days 10 and 30, which indicates that acephate could significantly influence the bacterial and fungal community structures in paddy soil.

Enzyme activities. The urease activity (Figure 2b) was inhibited by *B. bassiana* on day 60; however, the alkaline phosphatase (Figure 2c) and dehydrogenase (Figure 2d) activity were increased by 9.4% and 16.0% relative to CK on day 10. For acephate, the inhibition rates of the invertase, urease, alkaline phosphatase and dehydrogenase activities reached 45.4, 34.9, 47.3, and 51.7% on day 10, and inhibition of invertase and dehydrogenase lasted for 60 days.

Agronomic characters. Groups of B1, B10, and B100 showed increased rice yield (7.8, 7.2, and 4.3%, respectively), while B1000 showed lower yield than CK (Table 4). The yield of rice was improved significantly by applying reasonable concentrations of *B. bassiana* spore suspensions. However, due to the disease caused by *C. suppressalis*, the yield of the CS group was 31.6% lower than CK group.

DISCUSSION

Soil microorganisms can reflect a complex environment and can be indicators for monitoring soil status. In our study, groups treated with *B. bassiana* showed increased bacterial and fungal quantities. Previous studies revealed a variety of carbon sources in the rhizosphere, and *B. bassiana* can interact with plant roots to survive and grow (St Leger 2008). We believe it is possible that *B. bassiana* can interact with soil microorganisms or alter the root exudates

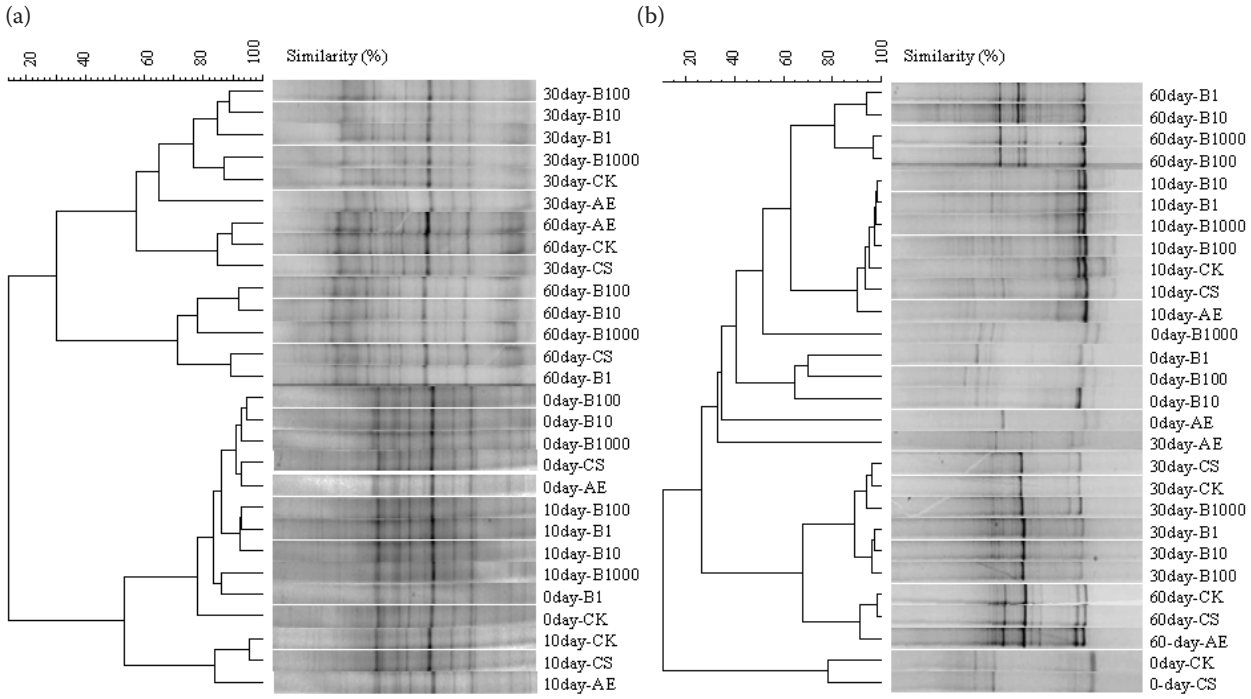

Figure 1. DGGE cluster analysis (unweighted pair group method with arithmetic mean) of 16S (a) and 18S rRNA (b) profiles of paddy soil microbial communities. CK – blank control group; CS – *Chilo suppressalis*; B1 – *Beauveria bassiana* at 7.5×10^4 spores/mL + CS; B10 – 7.5×10^5 spores/mL + CS; B100 – 7.5×10^6 spores/mL + CS; B1000 – 7.5×10^7 spores/mL + CS; AE – acephate + CS

of paddies by adjusting paddy growth and changing the soil microbial flora. Notably, the quantity of bacteria, actinomycetes, and fungi were reduced after application of acephate. A mechanistic explanation might be that acephate killed or inhibited the activity of certain groups of bacteria and fungi. However, the

Table 3. Diversity of soil bacteria and fungi by DGGE analysis

Organism	Treatment	Time (days)							
		0		10		30		60	
		N	S	N	S	N	S	N	S
Bacteria	CK	36	3.38	35	3.33	44	4.17	57	4.73
	CS	35	3.41	32	3.21	43	4.28	51	4.52
	B1	34	3.26	34	3.58	48	4.42	59	4.49
	B10	35	3.28	37	3.36	46	4.23	50	4.53
	B100	37	3.34	39	3.44	49	4.38	55	4.79
	B1000	36	3.46	36	3.48	42	3.96	54	4.45
	AE	35	3.37	28	2.89	35	3.15	58	4.51
Fungi	CK	27	2.82	32	2.55	33	3.29	38	3.67
	CS	28	2.9	31	2.49	34	3.38	41	3.76
	B1	29	2.87	38	2.72	35	3.52	48	3.94
	B10	24	2.79	36	2.68	41	3.73	42	4.08
	B100	25	3.29	37	2.94	38	4.07	46	3.79
	B1000	31	3.1	35	3.18	42	4.31	47	3.75
	AE	27	3.05	23	2.01	39	3.04	39	3.67

CK – blank control group; CS – *Chilo suppressalis*; B1 – *Beauveria bassiana* at 7.5×10^4 spores/mL + CS; B10 – 7.5×10^5 spores/mL + CS; B100 – 7.5×10^6 spores/mL + CS; B1000 – 7.5×10^7 spores/mL + CS; AE – acephate + CS; N – band number; S – Shannon index

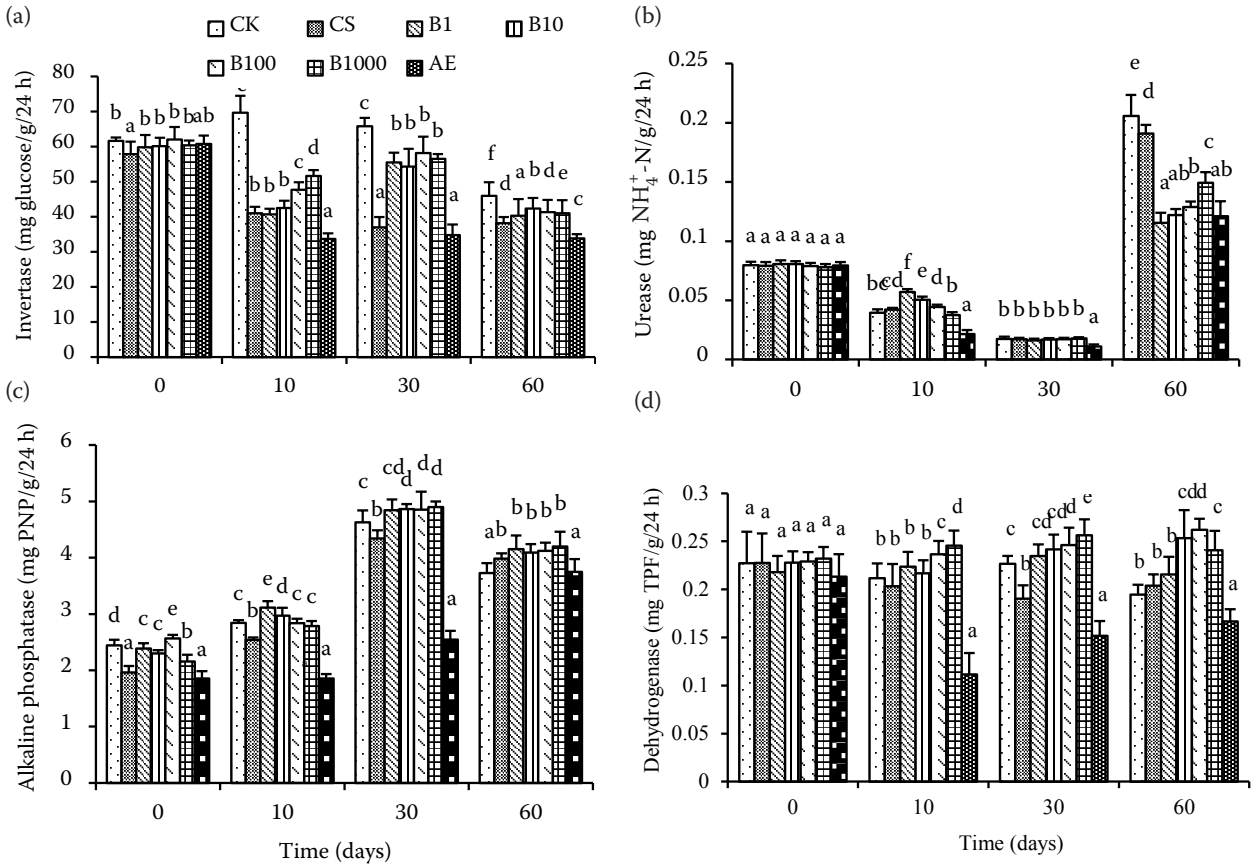

Figure 2. Effect of different treatments on paddy soil enzyme activity. (a) Invertase activity; (b) urease activity; (c) alkaline phosphatase activity, PNP – paranitrophenol, and (d) dehydrogenase activity, TPF – triphenylformazan. Error bars represent the standard error of means. Mean values (n = 6) ± SD. Different letters indicate significant differences among treatments (P < 0.05). CK – blank control group; CS – *Chilo suppressalis*; B1 – *Beauveria bassiana* at 7.5 × 10^4 spores/mL + CS; B10 – 7.5 × 10^5 spores/mL + CS; B100 – 7.5 × 10^6 spores/mL + CS; B1000 – 7.5 × 10^7 spores/mL + CS; AE – acephate + CS

actual mechanism by which this occurred is unclear from this study. Tejada et al. (2011) presented the differences in the response of microbial populations to chemical pesticides in soils which can occur due to different soil properties, community structure and vegetation present.

Soil enzymes are potentially important quality parameters for monitoring and evaluation of soil

Table 4. Agronomic characters after different treatments (n = 6)

Treatment	Height (cm)	Tiller (number/pot)	1000 seed weight	Shoot dry weight	Rice yield
			(g/pot)		
CK	116.42 ± 3.35^a	23.83 ± 1.47^a	26.22 ± 0.81^{bc}	45.32 ± 1.84^b	85.60 ± 1.35^c
CS	114.38 ± 2.58^a	22.33 ± 1.63^a	20.10 ± 1.12^a	31.93 ± 1.35^a	58.57 ± 1.00^a
B1	117.75 ± 2.18^a	23.16 ± 1.47^a	27.48 ± 1.43^{cd}	50.56 ± 1.60^{cd}	92.21 ± 2.24^e
B10	116.29 ± 4.35^a	24.16 ± 1.67^a	28.18 ± 1.75^d	52.34 ± 1.01^d	91.77 ± 0.88^e
B100	117.35 ± 3.41^a	23.50 ± 2.59^a	27.01 ± 0.85^{cd}	49.19 ± 3.43^c	89.30 ± 2.47^d
B1000	115.75 ± 2.91^a	23.17 ± 1.83^a	24.94 ± 0.58^b	51.33 ± 2.61^{cd}	76.20 ± 1.27^b
AE	117.53 ± 3.56^a	24.16 ± 1.60^a	26.58 ± 1.64^c	45.84 ± 1.88^b	82.47 ± 1.12^c

Mean values (n = 6) ± SD. Different letters indicate significant differences among treatments (P < 0.05). CK – blank control group; CS – *Chilo suppressalis*; B1 – *Beauveria bassiana* at 7.5 × 10^4 spores/mL + CS; B10 – 7.5 × 10^5 spores/mL + CS; B100 – 7.5 × 10^6 spores/mL + CS; B1000 – 7.5 × 10^7 spores/mL + CS; AE – acephate + CS

health. High concentrations of acephate were harmful to dehydrogenase and urease activity in two groundnut (*Arachis hypogaea* L.) soils (Mohiddin et al. 2011). In our study, *B. bassiana* did not induce a significant change in enzyme activities. However, acephate contributed to the decrease of several enzyme activities obviously. We assumed that acephate can interact with the enzyme-substrate complex. Changes by acephate in the plant growth status and microbial community structure can bring the enzyme activities changes. In order to evaluating the mechanisms of enzyme activities changes, more crop species, soil types and enzyme species should be included for the future studies.

B. bassiana spores can provide protection to cotton and tomato seedlings against damping off and improve the height and percentage survival of seedlings (Ownley et al. 2004, Griffin et al. 2005). In this study, the yields of B1, B10, and B100 were higher than CK, but those of CS and AE were lower. *B. bassiana* can induce host plant resistance and inhibit an array of soil-borne and plant pathogens and kill *C. suppressalis* then changed the paddy growth states. Therefore, it is possible that *B. bassiana* improved the yield of B1, B10, and B100 group by adjusting paddy growth, while disease caused by *C. suppressalis* led to the decreased yield in the CS group.

This paper has shown that, when compared with the chemical pesticide acephate, *B. bassiana* had less influence on the soil micro-ecological system, indicating that it can be potentially used as an environmental friendly microbial pesticide. Understanding the role of *B. bassiana* in the ecosystem could facilitate more effective exploitation of entomopathogenic fungi for development of better pest control strategies. The role of *B. bassiana* in other ecosystems and its effects on other crop soils need to be investigated.

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