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Screening for nematicidal activities of *Beauveria bassiana* and associated fungus using culture filtrate

Di Zhao[1], Bin Liu[2], Yuanyuan Wang[1], Xiaofeng Zhu[1], Yuxi Duan[1] and Lijie Chen[1]*

[1]Nematology Institute of Northern China, Shenyang Agricultural University, Shenyang, Liaoning, China.
[2]Liaoyuan Agricultural Academy of Science, Liaoyuan, Jilin, China.

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Using dilution and tissue block isolation methods, we obtained 105 isolates of *Beauveria bassiana* and seven isolates of its associated fungus. The filtrate of fermentation fluid of these isolates was tested for nematicidal activities against four targeted nematode species. Results indicate that nine isolates had high nematicidal activities against four targeted nematodes. Studies show that the culture filtrate of different isolates of *B. bassiana* and its associated fungus had different levels of activities against the same nematode, and the same culture filtrate had selective toxicity against different nematodes.

Key words: *Beauveria bassiana*, associated fungus, fermentation fluid, nematode, bioactivity.

INTRODUCTION

Plant parasitic nematodes (PPN) have been recognized as serious pathogens of many economic crops (Ruanpanun et al., 2010). Annual crop losses due to PPN damage exceed $10 billion in the United States alone (Koenning et al., 1999). Synthetic chemical pesticides have been widely used against parasitic nematodes. However, such pesticides can cause a series of problem including environment pollution and long-term residue issues. Therefore, fungal biological control is an exciting and rapidly developing research area and there is growing attention in the exploitation of fungi for the control of nematodes (Moosavi and Zare, 2012).

Beauveria bassiana is the most widely used entomopathogen. It has been reported to control many crop pests such as stem borers, beetles, aphids, mites, termites, white flies, mealy bugs, thrips etc. (Biswas et al., 2012). The major species *B. bassiana* and *Beauveria brongniatii* are often used in crop and forest pest prevention. Species of the genus *Beauveria* have been reported to produce the secondary metabolites bassianin, bassiacridin, beauvericin, bassianolide, beauverolides, tenellin and oosporein (Strasse et al., 2000; Quesada and Vey, 2004). Currently, there are few reports on the application of *Beauveria* in the control of nematode diseases. Junxianke, a fermentation product using a fungal isolate Snef907 (*B. bassiana*), is lethal to *Ditylenchus destructor*, *Heterodera glycines* and *Meloidogyne incognita* (Liu et al., 2007). Thus, it can be seen that *Beauveria* fungi can be potentially applied in prevention of plant parasitic pests and nematodes.

In studying the biological control of PPN, we have obtained a large number of fungal isolates of *B. bassiana* (Balsamo) Vuillemin that exhibit high nematicidal activity (Liu et al., 2008). After then, 105 isolates of *B. bassiana* have been isolated in our lab from soil and pests by selective mediums (potato sucrose agar added 0.8 g/L Kasumin-Bordeaux 47% WP and streptomycin) (Chen et al., 2008).

In the process of isolating *Beauveria*, one white colony (Snef2621) with different morphological characteristics was isolated simultaneously with *Beauveria* clones. Therefore, there were eight strains of *B. bassiana* and one strains of associated fungus (Snef2621) were isolated in our screening research against plant nematodes. We found the nine strains in total from *Antheraea pernyi* pupae or soil in China, of which 9

*Corresponding author. E-mail: chenlijie0210@163.com.

Table 1. Origin of test fungi and their corrected mortalities on *H. glycines* J2.

Species	Strains number	Origin	Corrected mortality (%) for 24 h
unknown	Snef2621	Liaoyuan, Jilin Province Chinese oak silkworm pupae	100
Beauveria bassiana	Snef2607	Xiahe, Gansu Province soil	91.38
Beauveria bassiana	Snef2615	Tieling , Liaoning Province soil	88.34
Beauveria bassiana	Snef2636	Harbin, Heilongjiang Province soil	74.23
Beauveria bassiana	Snef2637	Changtu, Liaoning Province soil	84.42
Beauveria bassiana	Snef2568	Shenyang, Liaoning Province soil	85.48
Beauveria bassiana	Snef2598	Liaoyuan, Jilin Province soil	100
Beauveria bassiana	Snef2626	Liaoyuan, Jilin Province soil	97.73
Beauveria bassiana	Snef2601	Liaoyuan, Jilin Province soil	100

strains exhibiting nematicidal activity.

MATERIALS AND METHODS

Culture and isolation

The fungus was isolated using a soil dilution plate method directly from soil. Each soil samples (5 g) was mixed with 45 mL sterile distilled water and shaken for 5 min by vortex oscillator. The soil suspension was diluted 1000×. An aliquot (0.5 mL) of the diluted soil suspension was spread over a 9 cm selective plate and cultured in selective medium (potato sucrose agar medium added 0.8 g/L Kasumin-Bordeaux 47% WP and 0.625 g/L Streptomycin) at 25°C for two weeks. Colonies of *B. bassiana* were selected and purified by single spore purification method (Chen et al., 2008). The nine strains of bio-control fungi, named Snef2621, Snef2607, Snef2615, Snef2636, Snef2637, Snef2568, Snef2598, Snef2626 and Snef2601, identified as *B. bassiana*, were cultured in PSA media (potato 200 g, sucrose 20 g, agarose 20 g, and sterile distilled water 1000 mL) at 25°C for two weeks. Although, Snef2621 have not been identified, it was obtained by single spore purification method (Chen et al., 2008).

Insects infected by *B. bassiana* were collected and those with spores or mycelium were used directly for spore and hyphae isolation. Tissue blocks were used for isolation from those without spores and hyphae. When tissue block was used, the bug was rinsed with 75% alcohol for 3 to 5 s, disinfected with 0.5% NaClO for 3 to 5 min, washed with sterile distilled water three times, then cut into 0.5 cm pieces, which were then laid out on 9 cm PSA plates and incubated at 25°C for five to seven days until colonies emerged (Wang et al., 2007). After then, it was obtained by single spore purification method (Chen et al., 2008).

Fungal culture filtrates

The fungus were cultured in 250-ml flasks containing 50 ml of Modified Czapek medium ($(NH_4)_2SO_4$, 3.67g; K_2HPO_4, 0.64 g; $MgSO_4 \cdot 7H_2O$, 1.04 g; KCl, 0.96 g; $FeSO_4$, 0.02 g; sucrose, 28.3 g; sterile distilled water, 1000 mL). Each flask received a 1 cm square from a two-week-old fungal colony growing on potato sucrose agar (PSA) plates. The flasks were shaken at 150 rev/min speed for seven days in darkness at 25°C. Then the culture broth was passed through two layers of filter paper (Whatman No.1) and then through a filter of 0.45 µm pore size to remove bacteria and fungal spores. The filtrates were used at concentrations of the original preparation (Liu et al., 2008).

Effect on J2

As target nematodes, *Meloidogyne incognita* second-stage juveniles (J2), *Heterodera glycines* J2, *Aphelenchoides besseyi* and *Caenarhabditis* sp. were chosen and maintained in the Nematology Laboratory, Shenyang Agricultural University, Shenyang, China. To determine the effect of culture filtrates on nematodes, 800 µl filtrates of different isolates and 200 µl sterilized distilled water were transferred to wells of 24-well tissue culture plates to which about 40 J2 of *M. incognita* were added. Czapek-Dox broth served as control. Three replicates were used per treatment. After 24 and 48 h at 25°C, the dead J2 bodies were counted under an inverted microscope and the corrected mortality was calculated. The J2 were considered dead when they did not move on probing with a fine needle (Cayrol et al., 1989). The same methods were used to determine the effects of culture filtrates on *H. glycines*, *A. besseyi* and *Caenarhabditis* sp.

Statistical analysis

One-way analysis of variance (ANOVA) was used for statistical analysis in SPSS. Duncan's New multiple range test was employed to test for significance of differences between treatments at ($p < 0.01$).

RESULTS

Initial screening of *B. bassiana* and associated fungus

From soil samples and infected insects collected from Northeast China and Gansu Province China, we obtained 105 isolates of *B. bassiana* and seven isolates of the associated fungus. Toxicities of these isolates were initially tested using culture filtrates against *H. glycines* J2 as the target. Through this test, we obtained nine isolates that could cause a mortality rate of greater than 60% (Table 1). Further analysis was performed using four targeted nematodes.

Effect on targeted nematodes

The culture filtrates of nine isolates showed various

Table 2. Nematicidal activity of *B. bassiana* and associated fungal isolates.

Strains number	Corrected mortality (%)							
	Meloidogyne incognita		*Aphelenchoides besseyi*		*Heterodera glycines*		*Caenarhabditis* sp.	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48h
Snef2621	94.20^{Aab}	95.96^{Aa}	20.47^{Bd}	64.85^{Aa}	82.22^{ABab}	90.65^{ABa}	99.05^{Aa}	100.00^{Aa}
Snef2607	66.83^{Ab}	96.30^{Aa}	13.37^{ABbcd}	45.47^{ABab}	56.19^{ABCDbc}	85.30^{ABa}	100.00^{Aa}	100.00^{Aa}
Snef2615	64.39^{Ab}	93.46^{Aa}	3.75^{Bd}	22.47^{BCcd}	16.35^{DEFde}	74.50^{ABCab}	98.64^{Aa}	100.00^{Aa}
Snef2636	4.88^{Bc}	73.41^{ABCab}	29.73^{Aa}	37.54^{ABCbc}	35.80^{CDEFcd}	52.10^{BCbc}	100.00^{Aa}	100.00^{Aa}
Snef2637	25.39^{Bc}	61.63^{ABCabc}	18.90^{ABabc}	19.96^{BCcd}	61.01^{ABCab}	78.47^{ABCab}	98.33^{Aa}	100.00^{Aa}
Snef2568	73.42^{Aab}	90.42^{ABa}	4.95^{Bcd}	23.39^{BCcd}	44.96^{BCDEc}	51.68^{BCbc}	97.07^{Aa}	100.00^{Aa}
Snef2598	99.39^{Aa}	99.79^{Aa}	6.39^{Bbcd}	15.02^{Cd}	92.01^{Aa}	98.32^{Aa}	100.00^{Aa}	100.00^{Aa}
Snef2626	5.62^{Bc}	33.07^{BCbcd}	1.32^{Bd}	64.74^{Aa}	65.81^{ABCabc}	97.48^{Aa}	99.05^{Aa}	100.00^{Aa}
Snef2601	22.10^{Bc}	15.39^{Cd}	2.41^{Bd}	64.25^{Aa}	55.10^{ABCDbc}	100.00^{Aa}	79.12^{Aab}	100.00^{Aa}

Values in the table are corrected mortality. Alphabet following a value indicates the result of Duncan's new multiple range tests. Capital letter denotes $P < 0.01$ and lowercase letter denotes $P < 0.05$.

activities against *M. incognita* J2. The culture filtrates of nine isolates exhibited nematicidal effects of various degrees on J2 of M. *incognita* (Table 2). Among them, five isolates were exhibited highly nematicidal effects on *M. incognita* J2. The activity of Snef2598 was the highest, with corrected mortality 99.39 and 99.79% for 24 and 48 h, respectively. The activity of Snef2621 took second place, with corrected mortality 94.20 and 95.96% for 24 and 48 h treatments, respectively. Next, isolates Snef2607, Snef2615 and Snef2568 also caused greater than 90% mortality upon treatment for 48 h, being significantly greater than the controls. Isolate Snef2601 showed the lowest toxicity, caused only 15.39% corrected mortality ($p < 0.01$).

The nine isolates showed low activities against *A. besseyi*. The percentage mortality of three isolates (Snef2621, Snef2626 and Snef2601) was 64.85, 64.74 and 64.25%, respectively in *A. besseyi* for 48 h. The corrected mortality showed no different from each other, but the corrected mortality showed significance compared with the water control ($p < 0.01$) (Table 2).

Four isolates showed high nematicidal activities against *H. glycines* J2. The percentage mortality of isolate Snef2598 was 92.01 and 98.32% for 24 and 48 h, respectively. Next to Snef2598, the percentage mortality of isolate Snef2601 was 55.10 and 100.00% for 24 and 48 h, respectively. The percentage mortality of isolate Snef2621 was greater than 80% for 24 h and 48 h. The culture filtrate exhibited significant differences compared to the water control ($p < 0.01$) (Table 2).

Caenorhabditis sp. is a saprophytic species that generally does not cause damages to crops and trees. However, it has specific significance to be used as a nematode target. The nine isolates showed high activities against *Caenorhabditis* sp, with the percentage mortality greater than 79% ($p < 0.01$, compared with controls). There were little differences among these isolates (Table 2).

DISCUSSION

Fungal natural products are very promising potential sources of new chemicals to manage plant-parasitic nematodes (Anke and Sterner, 1997). Culture filtrates of many fungi possess activity against nematodes, and the nematicidal action of these culture filtrates may involve the production of toxic metabolites by the fungi (Caroppo et al., 1990; Liu et al., 2008; Lin et al., 2009).

As a classic fungal biocontrol agent, *Beauveria* possesses great potential for the control of sucking insect pests (Feng et al., 2004, Hatting et al., 2004; Jean et al., 2008). Although there have been numerous reports of toxicity of *B. bassiana* to insects, similar investigations with plant-parasitic nematodes have been very limited. Mayer (1995) reported that beauvericin produced by *B. bassiana* had weak nematicidal activity against *M. incognita*. Chen et al. (1996) found that *B. bassiana* showed little parasitism of nematode eggs but reduced hatch of *Heterodera glycines*.

In the current study, we have obtained 105 isolates of *B. bassiana* and seven isolates of its associated fungus. These isolates were tested for toxicity against *A. besseyi, Caenorhabditis* sp, *M. incognita* J2, and *H. glycines* J2. Results indicate that nine isolates had high nematicidal activities. Isolates Snef2598 and Snef2621 had high activities against *M. incognita* and *H. glycines*. Isolate Snef2601 also had high nematicidal activity against *H. glycines*. We are further analyzing these isolates. Results show that these isolates have different bioactivities against four targeted nematodes. There are two reasons for these differences. First, different nematodes have differential resistance due to the differences in habitat and feeding activities. Second, these isolates may have more than a single bioactive metabolite that are responsible for nematicidal activities, and each metabolite may act on a different site. Studies have shown that *Beauveria* can produce beauvericin, bassiana

beauvericin and oosporin. Beauvericin has a weak activity against *M. incognita* (Hamill et al., 1969; Suzuki et al., 1977; Anke et al., 1995). In this study, isolates of *B. bassiana* and its associated fungus showed strong activities against *Caenorhabditis* sp. Most isolates could cause 90% mortality to *Caenorhabditis* sp. The dead worms exhibited disintegrated body wall and internals, suggesting that these isolates produced certain common metabolites that are toxic to *Caenorhabditis* sp. Therefore, *Caenorhabditis* sp. as a target is not perfect for screening for bioactive components against phytoparasitic nematodes.

The results show that isolates Snef2598 and Snef2621 are potential candidate for biological control of plant parasitic nematodes, as are *Paecilomyces lilacinus* and *Verticillium chlamydosporium*. *P. lilacinus* (Thorn) Samson is an effective parasite of the eggs of *M. incognita* and other nematodes like *Globodera pallida* and *Heterodera* sp. *V. chlamydosporium* had been studied extensively as a potential biological control agent of *Heterodera* sp. and *Meloidogyne* sp. (Kerry and Jaffee, 1997). *V. chlamydosporium* infects nematode eggs and sedentary females of cyst nematodes by hyphae produced on actively growing mycelium (De Leij et al., 1991). It colonizes the rhizosphere, which facilitates the infection of egg masses protruding from female root-knot nematodes on infected roots (De Leij and Kerry, 1993). Survival and spread of the fungus occur through chlamydospores, microconidia, and mycelium. The culture medium for *V. chlamydosporium* had high parasitic rate on *Meloidogyne incognita* eggs (Liu et al., 2006). Compared with the mycoparasitism of *V. chlamydosporium* and *P. lilacinus*, Snef2621 and Snef2598 had high toxicity to phytoparasitic nematodes. Therefore, the nematicidal activity of Snef2598 and Snef2621 is worth of further study.

In summary, this is the first study of the nematicidal activity of culture filtrate of *B. bassiana* and associated fungus on four targeted nematodes. We believe *B. bassiana* and its associated fungus (Snef2621) are the new biological control factor that may be a potentially good source of a microbial nematicide that can be harnessed for successful nematode control. And the identification of Snef2621 by morphological characteristics and molecular phylogenetic analysis has being done, but the nematicidal compound of associated fungus is too difficult to isolate and purify, so we continue researching for a good method to isolate and purify bioactive compounds from culture filtrates in the following experiment.

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