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Biocontrol Potential of Some Entomopathogenic Fungal Strains Against Bean Aphid *Megoura japonica* (Matsumura)

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Abstract: This research reported the in vitro pathogenicity of *Verticillium lecanii* strains, L2 and L5, and *Beauveria bassiana* strains, B76 and B252, against *Megoura japonica* using leaf-dip method. The virulence potential of these four entomopathogenic fungal strains of *V. lecanii* and of *B. bassiana* were compared between fungi conidia (concentrations 1×10^6, 1×10^7, and 1×10^8 conidia mL^{-1}) and culture filtrate. Moreover, binary combination of four different fungal strains (L2 + B76, B76 + L5, L2 + B252, and L2 + B76 + B252 + L5) were evaluated against *M. japonica* under control condition. Aphid mortality was recorded after two, four, six, and eight days of post-treatment. In the conidial concentration bioassay, strain B76 showed maximal mortality (85.3%) against bean aphid, and strain L5 showed the lowest effect (60.0%) at the highest concentration (1×10^8 conidia mL^{-1}) at eight days post-treatment. Whereas, binary combinations of B76 and L2 strains showed the highest effect against *M. japonica* (90.5%) than other combinations. Moreover, in comparison with the effect of filtrate and conidia bioassay, 91.4% and 84.1% were achieved in strain B76, and the lowest effect (63.8% and 55.1%) was recorded in strain L5.

Keywords: *Verticillium lecanii*; *Beauveria bassiana*; *Megoura japonica*; binary combination; filtrates; conidial

1. Introduction

The bean aphid *Megoura japonica* (Matsumura) is one of the most dangerous agricultural insect pests on legume plants, such as common bean, soybean, and mung bean [1]. Bean aphid is distributed widely all over the world [2]. They suck cell sap from plants and also transmit various viruses in different crops [3]. The management of *M. japonica* is through synthetic pesticides. However, the unselective application of pesticides has resulted in many visible problems, such as resistance to pesticides, killing natural enemies, environmental pollution, and human health issues [4]. To overcome problems related to widespread use of chemical pesticides, alternative methods such as biocontrol substances have extensively been researched in the world. Many microbial insecticides based on pathogenic organisms, such as virus, bacteria, fungus, and nematode, have played a significant role in the field of crop protection and are being used to control an extensive range of insects [5–9].

Entomopathogenic fungal strains, including *Verticillium lecanii*, *Beauveria bassiana*, *Isaria fumosorosea*, and *Metarhizium anisopliae*, were used as the specific biological pesticides, which are environmentally friendly and can be used against many sucking insect pests [5,10–12]. Spores germinated after attaching to the epidermis of the host insects, and the hyphae penetrate the body of the insects, which causes the death of the host within a few days [13,14]. In addition, these entomopathogenic fungi have no or

little harmfulness on mammals. Their residuals are target specific and less vulnerable to resistance evolution [15,16]. These virulent fungi were focused on by researchers in the past decades for their potential as biological control agents around the world, and these could exist at the epizootic or enzootic levels in their host population.

V. lecanii and *B. bassiana* are among the most well researched virulent entomopathogenic fungi belonging to order of Hypocreals. They have a wide range of insect pest colonization [17,18]. These two kinds of fungal strains are easily collected from the phylloplane of vegetation, as well as from infected insects and soil [19,20]. As a bio-insecticide, *V. lecanii* has been used to control black bean aphid *Aphis fabae* (Hemiptera: Aphididae) under control conditions [21]. The entomopathogenic fungi have been developed as one of the major new bioactive agents for plant pathogen and insect pest control [22–24]. Fungal conidia are produced asexually and become the basis of infection in insect pests of crops. Infection through conidia starts when they are attached to the host cuticle, then germinate following the activation of the enzymatic reaction and invaded the body of the insects by germ tube, appressoria, and penetration pegs [25]. Refined culture filtrates of entomopathogenic fungi, *V. lecanii* and *B. bassiana*, decreased the reproductive rate of aphids [26,27] and prevented feeding of the larva of *Spodoptera littoralis* and *Bemisia tabaci* [28,29]. Increased fungus concentration decreased the number of adult parasitoids and also negatively affected its developmental stages [30]. Filtrate culture contains many enzymes like chitinases, lipases, and protease, and these enzymes help in the infection process by degrading the cuticle of insects. The concentration of an enzyme can be enhanced by the use of different additives in the culture media, like colloidal chitin [27].

This paper focused on the evaluation of the efficacy of our collected fungal strains (*V. lecanii* and *B. bassiana*) with different application materials (conidia, filtrate) on bean aphids. Furthermore, we also aimed to determine the combined effect of different entomopathogenic fungal strains against bean aphids. This result could be helpful for establishing an effective integrated pest management method which could reduce bean aphid population below economic thresholds, while minimizing the use of synthetic chemical pesticides.

2. Results

2.1. Inhibition Effect of Different Concentrations of the Fungal Spore on Bean Aphid

The conidial bioassay results showed the significant effect of the different fungal trains ($p < 0.01$; Table 1). Moreover, the factorial analysis of variance revealed a significant effect of different concentrations (F = 296.72, $p < 0.01$), different time intervals (F = ******, $p < 0.01$), and their interaction ($p < 0.01$, Table 1). All strains of *B. bassiana* and *V. lecanii* caused significant bean aphid mortality at all different concentrations. The maximum effect of the fungal strain (85.3%) was collected on the eighth day after treatment with the concentration of 1×10^8 conidia mL^{-1} from strain B76, whereas the minimum effect (71.4%) was collected at the lowest concentration of B76 (i.e., 1×10^6 conidia mL^{-1}, Figure 1). The virulence from fungal strain B252 against *M. japonica* showed a similar tendency to the condition of strain B76. The highest mean effect (66.0%) of strain B252 was collected with a concentration of 1×10^8 conidia mL^{-1} at 8 dpi. The minimum mean effect of strain B252 (49.9%) was collected at the lowest concentration (1×10^6 conidia mL^{-1}). Similarly, the effect of *V. lecanii* strains, L2 and L5, on *M. japonica* presented that corrected mortality of bean aphid improved with the exposure time and concentration of fungal strains (Figure 1).

Table 1. Analysis of variance (ANOVA) for the mortality of *M. japonica* by three different concentrations from four entomopathogenic fungi strains at different times against bean aphids.

Source of Variation	DF	Sums of Squares	Mean Squares	F Ratio	Prob
Treatment	4	39,204.6	9801.15	******	0.000
Concentration	2	3151.77	1575.88	296.72	0.000
Time	3	57,007.1	19002.4	******	0.000
Treatment × Concentration	8	849.592	106.199	20.00	0.000
Treatment × Time	12	12,258.7	1021.56	192.34	0.000
Concentration × Time	6	625.468	104.245	19.63	0.000
Treatment × Concentration × Time	24	454.597	18.9415	3.57	0.000
RESIDUAL	120	637.332	5.31110		
Total	179	114,189.	637.928		
CV%	7.0				

CV%: Coefficient of variation.

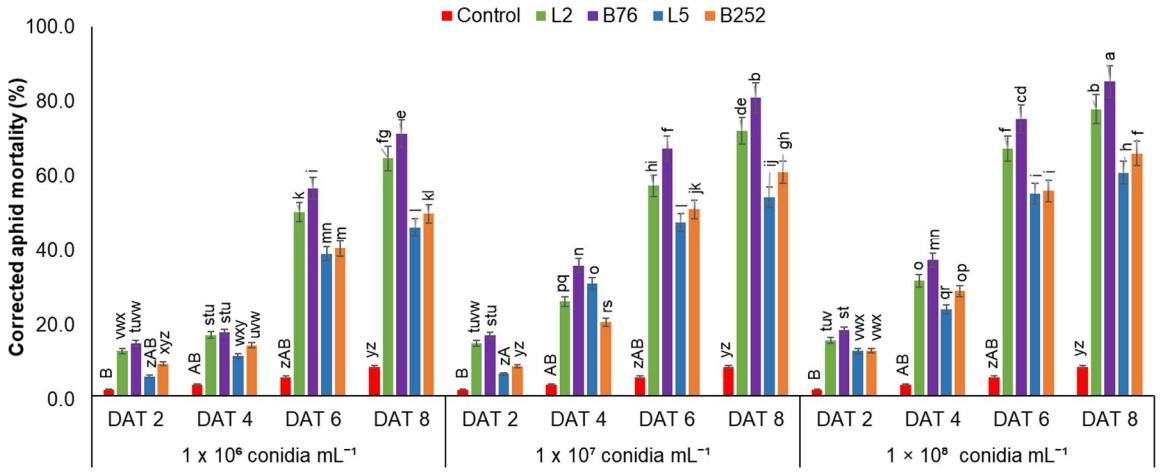

Figure 1. The mortality of *Megoura japonica* by different concentrations of *Verticillium lecanii* (L2 and L5) and *Beauveria bassiana* (B76 and B252) spores at different time intervals. Columns show the mortality of aphids with different fungal strains ± SE (n = 50). The different letters express significant differences between different treatments (three-way factorial analysis of variance (ANOVA); least significant difference (LSD) test at α = 0.05). DAT: Days after treatment.

2.2. Fungal Strain Combinations Against Bean Aphid

In the combination bioassays test, the results showed a high significant effect of all treatments (F = 618.56; $p < 0.01$), the different time intervals (F = 775.30, $p < 0.01$), and the interaction between different times and different treatments on fungal strains (F = 40.60, $p < 0.01$, Table 2). The highest inhibition effect of the fungal strain (90.5%) was observed for L2 + B76, while the effect of the combination of B76 + L5 was 81.1%. Binary combination of L2 + B252 caused 71.6% corrected bean aphid mortality (Figure 2). Nevertheless, for the combination of four different fungal strains (L2 + L5 + B76 + B252), the bean aphid mortality rate was recorded as 69.4%. This phenomenon was most probably due to the reduced number of spores of the high virulence strain (B76 and L2) in the combination (B76 + L2 + B252 + L4). So, its effect was reduced to be significantly lower against *M. japonica* than other combinations (Figure 2).

Table 2. Analysis of variance (ANOVA) for the mortality of *M. japonica* by the binary combinations from four fungal strains of *V. lecanii* and *B. bassiana* at different time intervals.

Source of Variation	DF	Sums of Squares	Mean Squares	F Ratio	Prob
Treatment	4	20,964.9	5241.22	618.56	0.000
Time	3	19,708.0	6569.34	775.30	0.000
Treatment × Time	12	4127.90	343.992	40.60	0.000
RESIDUAL	40	338.932	8.47329		
Total	59	45,139.7	765.080		
CV%	7.2				

CV%: Coefficient of variation.

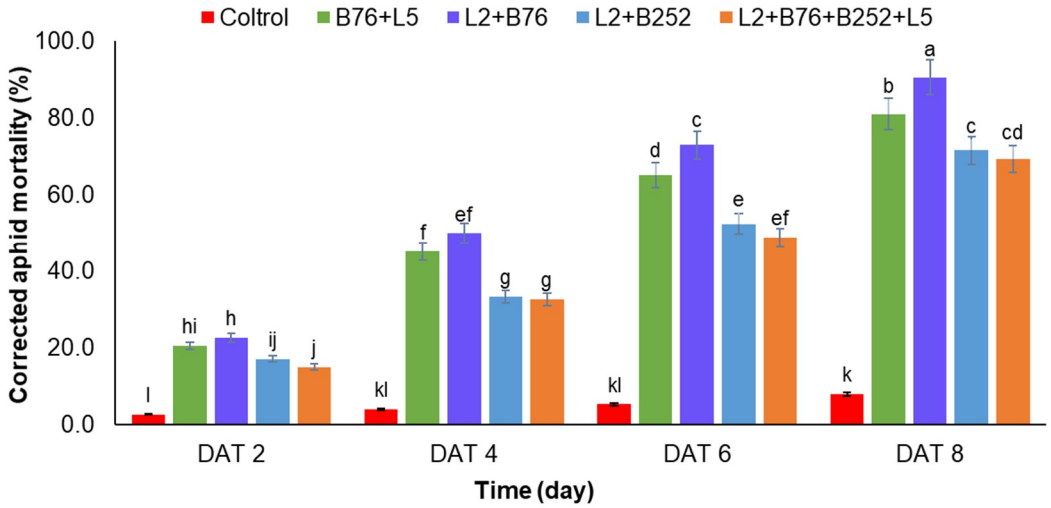

Figure 2. The mortality of *Megoura japonica* by the binary combinations from strains of *Verticillium lecanii* and *Beauveria bassiana* (L2 + B76, B76 + L5, L2 + B252, L2 + B76 + B252 + L5, and control) recorded at different time intervals. Columns show the bean aphid mortality with different fungal strains ± SE (n = 50). The different letters express the significant difference between different treatments (two-way factorial analysis of variance (ANOVA); least significant difference (LSD) test at $\alpha = 0.05$). DAT: Days after treatment.

2.3. Comparison of the Mortality of Bean Aphid by Fungi Filtrate and Conidia

The filtrate bioassay results revealed that the overall mean effect of different fungi filtrate against bean aphid was higher in comparison to their conidial treatments. Statistical processing results showed that there was a significant effect of fungal conidia and filtrates on corrected bean aphid mortality ($F = 117.71$, $p < 0.01$, Table 3). The highest effect of the filtrate solution was strain B76, with 91% recorded on the eighth day of treatment, while the effect of strain B76 conidia was recorded as 84% on the eighth day of treatment ($p < 0.01$). Other fungal strains had similar results. However, the lowest effect of filtrates and conidia was in fungal strain L5, recorded as 63.8% and 55.1%, respectively (Figure 3).

Table 3. Analysis of variance (ANOVA) for the comparison of the mortality of *M. japonica* by filtrate and the conidia from different fungal strains (*V. lecanii* and *B. bassiana*).

Source of Variation	DF	Sums of Squares	Mean Squares	F Ratio	Prob
Treatment	8	15,365.3	1920.66	117.71	0.000
RESIDUAL	18	293.692	16.3162		
Total	26	15,659.0	602.268		
CV%	6.0				

CV%: Coefficient of variation.

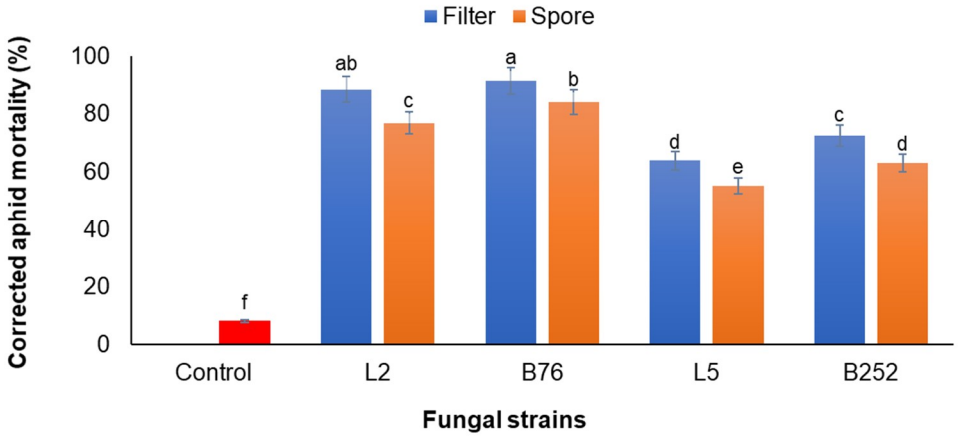

Figure 3. Comparison of the mortality of *M. japonica* by filtrate and the conidia from different fungal strains (*V. lecanii* and *B. bassiana*). Columns show the mortality of aphids by different fungal strains ± SE (n = 50). The different letters express the significant difference between different treatments (one-way factorial analysis of variance (ANOVA); least significant difference (LSD) test at α = 0.05).

3. Discussion

Developing biological control methods, based on entomopathogenic fungi with resistance to pathogens and insect pests, is one of the core areas of current biological control research. Many virulent fungal strains were found to be effective against different insect pests [31,32]. This study determined the effect of different entomopathogenic fungi against bean aphid. The results of this study presented that four strains, *V. lecanii* (L2 and L5) and *B. bassiana* (B76 and B252), could be effective biocontrol agents against bean aphids. This study result is also similar to previous research showing the effect of different strains of *V. lecanii* and *B. bassiana* against aphids [26,33].

In all experiments, the effect of fungal strains appeared to be dose and time-dependent and increased by increasing time after application and the conidia concentrations of fungus, [34] which was also demonstrated in our conidial treatment study. They use different concentrations of the entomopathogenic fungus, *B. bassiana*, against *Metopeurum fuscoviride* and *A. fabae*, and reported that the highest concentration (1×10^8 spores mL^{-1}) exhibited the highest mortality percent on seventh day post-treatment. It was also demonstrated that the mortality of aphids increased with time and conidial concentration exposure [35,36]. Similarly, it was described that the mortality percentage was affected by the concentration of conidia, temperature, and exposure time [37]. The mortality percentage was directly proportional to the concentration of conidia using different concentrations of *B. bassiana* (1×10^6, 1×10^7 and 1×10^8 conidia mL^{-1}) *Rhopalosiphum padi, Schizaphis graminum, Lipaphis erysimi,* and *Brevicoryne brassicae*. All concentrations were effective for the control of aphids, but the highest concentration (1×10^8 conidia mL^{-1}) caused the highest percentage mortality [38]. The research was conducted for the determination of the virulence of *V. lecanii* and its result showed that concentration of 1×10^8 conidia mL^{-1} had the best effect, with an 86% mortality rate of nymphal observed after five days of application [39]. It was also reported that at concentrations of 10^7 and 10^8 conidia mL^{-1} of *V. lecanii*, the maximum mortality rate of *M. persicae* was recorded as 100% after 12 days of application [40]. Similarly, the effect of *B. bassiana* on *M. persicae* was also determined under the controlled condition with the concentration of 1×10^7 conidia mL^{-1} and showed that three strains of *B. bassiana* (BAU019, BAU004, and BAU018) showed high effect on aphids, with a mortality of over 75% [34,41–44]. According to filtrate treatment, our results are similar to results which revealed that degradation of the insect body was observed at higher infiltrate treated aphids, and the reduction of aphid population was higher in higher dose filtrate treatment [27]. The maximum efficacy was due to the production of metabolites in the culture filtrate that helped to degrade the cuticle and deform the hemocoel. As compared with the filtrate, conidia need more time to germinate and release the required enzymes for the degradation of the insect body. It was demonstrated that the use of filtrate

application for the control of insects was the best method, and also effective for insects which had a short life cycle because these insects have more chances to shed the conidia from their body by molting, since germination of conidia needs a specific time [45,46]. According to their findings, our results were similar. Filtrate had more ability to control the insect population, because they contained toxic enzymes for the degradation of the insect body. Through filtrate optimization, the production of enzymes could be enhanced easily with fungal genetic manipulation. Filtrate production, storage, and transportation are more suitable than conidia, and it meets the commercialization requirements. In this study, the effect of different fungal strains was found to be time dependent in cases of conidia, filtrate suspension, and combinations. The maximal effect was recorded after eight days, while the minimal effect was recorded after two days. These results are similar to research that showed an increase in the effect with the increase in time and concentration [39].

Regarding the pathogenicity of different fungal combinations, our results showed that the highest synergistic effects were a combination of fungal strains (B76 + L5, and L2 + B76). However, there was no synergistic effect for the combination of fungal strains L2 + B252 and L2 + B76 + B252 + L5. These fungal strains may show synergies if applied in a sequential combination, as demonstrated in the case of nematodes and pathogenic fungi [47]. Therefore, the use of the entomopathogenic fungi is considered safe and environmentally friendly compared to chemical pesticides [48], so they are recommended against harmful pests, such as aphids.

4. Materials and Methods

4.1. Insect Culture

Bean aphids were collected from the Institute of Plant Protection, Beijing, China. Then, they were reared on Chinese cabbage plants (*Brassica rapa*) placed in cages in the growth chamber at 50–60% relative humidity and 25 ± 2 °C, with a 16:8 h light:dark photoperiod. The first instar aphid was used for all bioassays in this study.

4.2. Fungal Isolates

All fungal isolates (Table 4) were collected from fields in China and cultured on potato dextrose agar (PDA) medium (20.0 g agar, 200.0 g potatoes, 20.0 g dextrose, and 1 L distilled water) on Petri-dishes for 20 days in the dark at 25 ± 2 °C.

Table 4. Name of fungal strains, hosts and geographical of the entomopathogenic fungi.

Name	Symbols	Geographical Origin
Verticillium lecanii 2	L2	Institute of Plant Protection, Beijing, China
Verticillium lecanii 5	L5	Institute of Plant Protection, Beijing, China
Beauveria bassiana 76	B76	Institute of Plant Protection, Beijing, China
Beauveria bassiana 252	B252	Institute of Plant Protection, Beijing, China

4.3. Conidial Suspension

The conidia were collected from PDA dishes in 0.02% Tween solution after 20 days culture and were filtered using sterile cheesecloth. The spore concentrations of all fungal strains were counted under the microscope using a hemocytometer. The conidia viability was checked before using for the design of the bioassays experiment [49].

4.4. Fungal Filtrate

The primary culture of the four strains (B76, B252, L2, and L5) was prepared by mixing 100 mL of Adamek liquid medium (ALM) with 5 mL of conidial suspension and shaken for 3 days at 150 rpm. The secondary culture (1.0%) was prepared by mixing 250 mL of ALM with 2.5 mL of the primary culture medium and shaken for 6 days at 150 rpm, 25 °C. The mycelium was removed by centrifugation

for 15 min at 12,000 rpm, 4 °C and then the supernatant was filtered through the 0.45 µm pore-size filter (Millipore Corp) to get the filtrate.

4.5. Pathogenicity Bioassays

The effect of the entomopathogenic fungal strains (B76, B252, L2, and L5) against bean aphids was measured by conducting their conidial and filtrate bioassays, and evaluating the binary combinations of different fungal strains. All bioassays were measured using the leaf-dip method [50] with slight modifications. All treatments were performed on 90 mm Petri dishes containing a thin layer of 1.0% agar, and a 60 mm detached leaf disk of Chinese cabbage was placed on the Petri dish. The control leaves were dipped only in 0.02% Tween. Fifty newly molted bean aphids up to 12 h old (first instar) were released on the treated and control leaf on a Petri dish, and then they were stored at 50–60% relative humidity and 25 ± 2 °C with a 16:8 h light:dark photoperiod. All the treatments were replicated 3 times (each treatment has 5 Petri dishes for each time). For the bioassay treatment of binary combination, the uppermost concentration 1×10^8 conidia mL^{-1} of each fungal strain was mixed with the 1 mL conidia of each other fungal strain for all combinations, and then a 2 mL mixed combination sample for each treatment was collected. The combinations, L2 + B76, B76 + L5, L2 + B525, and L2 + B76 + B252 + L5 were designed. For all treatments, the effect was recorded on 2, 4, 6 and 8 days. All dead bean aphids from each experiment were maintained at 25 ± 2 °C and 90% relative humidity in dark to confirm mortality by the pathogens. The effect of all fungal strains was determined using Abbott's formula: Effect (%) = (X − Y)/X) × 100 (X: the percent living in the control; Y: the percent living in the treatment) [51].

4.6. Data Analysis

The data was analyzed using Statistix 8.1 (Analytical Software, Tallahassee, FL, USA). Comparisons of the treatment means were performed, using variances (ANOVA) to determine the significance of individual differences of the least significant difference (LSD) test at $\alpha = 0.05$ level.

5. Conclusions

In brief, this study shows the effect of four different fungal strains of *V. lecanii* (L2 and L5) and *B. bassiana* (B76 and B252) against *M. japonica*. Through a series of bioassays, the results demonstrated that different fungal strains of *V. lecanii* (L2 and L5) and *B. bassiana* (B76 and B252) have different virulence potential. The different application materials and their dosages also affect pathogenicity on bean aphid. Filtrate application is the most suitable material for the control of *M. japonica*. In the combination of bioassays, the binary combination of strains of B76 and L2 exhibited high mortality of bean aphid (90.5%). Thus, the results of this study suggest that these fungal strains may be used as novel biological control agents against bean aphids.

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