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Biological control of the small leafhopper, *Empoasca flavescens* F. (Homoptera: Cicadellidae) using the entomopathogenic fungus, *Verticillium lecanii*

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Abstract

Background Nowadays, instead of chemicals, using microbial pesticides is very important and significant to protect the environment. *Verticillium lecanii* (Z), the entomopathogenic fungus, is widely used for management of many pests. The aim of this work is to determine whether the small leafhopper, *Empoasca flavescens* F. (Homoptera: Cicadellidae), can be exterminated only by biopesticide—*V. lecanii* or not.

Results After spraying with diluted suspension of *V. lecanii* spores on the small leafhopper, *E. flavescens*, the effects of infection and reinfection in accordance with the time, spore density and growth stage of the small leafhoppers were studied. When *E. flavescens* was sprayed with over 1.25×10^8 spores/ml of diluted suspension of fungal spores indoors, the reduction % was over 80% after 5 days and the decrease of imagoes was higher than that of larvae. Outdoors, *E. flavescens* was reduced by 50% for 3 years after treatment, while the reduction % was about 90% by reinfection year by year.

Conclusion Ninety percentages of *E. flavescens* on peach trees can be effectively exterminated in 2 years by spraying the diluted suspension of *V. lecanii* spore, and no chemical pesticides are needed for the control of *E. flavescens* in practice.

Keywords *Empoasca flavescens*, Small leafhopper, *Verticillium lecanii*, Reinfection

Background

Nowadays, the effort of human beings to protect the environment is being enhanced, thus, to protect the environment is essential for welfare of human beings and social development. The recently discovered additional roles of entomopathogenic fungi (EPF) provide opportunities to use these fungi in the integrated pest management (IPM) strategies.

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Recently more breeding demands in the field become into reality, thus, incorporation of EPF-oriented biopesticides maximizes management effect against pests within IPM program. Although there are almost 700 species in about 100 genera of EPF, the majority of the commercially produced fungi are only based on a few species in *Beauveria*, *Metarhizium*, *Isaria*, and *Lecanicillium* (Lara et al. 2017). *Verticillium lecanii* (Z) Viegas is EPF of the order Hypocreales that has already been used successfully as a biocontrol agent against many plant-damaging insects (Butt et al. 2001). *Verticillium lecanii* had a greater ability to infect eggs of Heterodera and this significantly reduced the number of oocytes and eggs (Ryoji Shinya et al. 2007). The results clearly indicated that EPF such as *Beauveria bassiana* (Bals.-Criv.) Vuill. 1912 and

V. lecanii had a strong potential for microbial control of whitefly larvae infesting tomato crops at moderate ambient humidity in Mediterranean glasshouses (Fargues et al. 2003). *Verticillium lecanii* had a strong insecticidal effect on *Azteca instabilis* Smith 1862 and *Coccus viridis* Green 1889 (Vandermeer et al. 2009) while *V. lecanii* and *Metarhizium anisopliae* (Metschn.) Sorokīn 1883 had selectively pathogenicity for first instar larvae of *Ceraeochrysa cincta* Schneider 1851 (Eliane et al. 2007). In general, engorged *Rhipicephalus microplus* Canestrini, 1888 females treated with 1×10^8 conidia ml^{-1} of oil suspension of *V. lecanii* died over 97.6% before laying eggs (Isabele et al. 2010).

Larvae of *Galleria mellonella* Linnaeus, 1758 were infective by *V. lecanii* but only more than lethal amount (Fariba et al. 2019). The recent study of Ravindran et al, (2018) reported a novel approach for identification of insecticidal compounds produced by *V. lecanii* and toxic to the diamondback moth, *Plutella xylostella* Linnaeus 1767 Different concentrations of the *V. lecanii* were successively applied against *Toxoptera citricida* Kirkaldy and the pathogenic effects were analyzed, in addition, bioassays on the major parasitoid, *Lysiphlebus testaceipes* Cresson 1880 to test the pathogenic effects of *V. lecanii* on mortality and the percent emergence from mummies were conducted (Balfour and Khan 2012). EPF and botanical extracts from neem or eucalyptus reduced survival and fecund rates of the wheat aphid, *Sitobion avenae* (Fab.) (Hemiptera: Aphididae) significantly (Sajjad et al. 2018). The corn leafhopper, *Dalbulus maidis* (Hemiptera: Cicadellidae), killed by *Metarhizium brasiliense* in irrigated maize fields located in Northeast Brazil (Daniela et al. 2021). The aim of this work was to evaluate if *V. lecanii* could manage *E. flavescens* on peach on a short-term and long-term basis.

Methods

Culturing for *V. lecanii*

The EPF used in this study was *Verticillium lecanii* (Z) Viegas 1939 which had been separated from the dead bodies of a small leafhopper in the peach field and identified in Microbiology Research Institute of Academy of Sciences of DPR Korea in 2016.

The grain materials for the solid culture were corn flour, bean flour and rice chaff which was widely used. Culture and production was conducted in the microbiology laboratory of Bioengineering Institute, Wonsan University of Agriculture. The solid culture products were made as the following: EPF, *V. lecanii* was inoculated to the PDA medium and cultured for 7 days at the temperature of 22 °C, while the spores were turbid with 0.05% Tween-80 and inoculated to the liquid medium. The inoculation was conducted with the amount of 10% (about 5 ml) and 1.875×10^7spores/ml of diluted suspension of fungal spores and it was shake-cultured in the liquid medium at the temperature of 24 °C at the speed of 180 rpm/min for 4 days. To extensively culture the spores, the shake-cultured fluid was mixed with corn flour, bean flour and rice chaff and filled 50% in the culture bottles of 500 ml each. After autoclaving, they were inoculated with 10 ml of liquid culture each and cultured for 8–10 days and dried to use. To measure the number of spores, 1 g of the culture was soaked in the 40 ml of sterile water for 2 h, filtered through the sterilized gauze, diluted 100 times and examined by Olympus microscope (400 ×) with the cytometer.

Indoor experiment

Indoors, diluted suspension of fungal spores was sprayed onto the larvae of the small leafhoppers on the fresh leaves of one-year-old peach tree grown in the pot (10 cm of diameter). Each tree was separated with gauze so that the insects could not escape. After the sprayed leaves were completely dried, the room temperature and humidity were insured as the infective conditions (Temperature 23 ± 1 °C, Relative humidity (RH) 85 ± 5%, L:D 14:10 h). The movement and color change of *E. flavescens* (imago) after infection was investigated.

Outdoor experiment

The diluted suspension of solid culture product was sprayed two times on the peach trees in the field in 7 days from mid-June, and after 10 days, dead imagoes and larvae were separately counted to investigate the infective effect of *V. lecanii* at different developmental stages of *E. flavescens*. The investigation was repeatedly conducted at 5 different areas.

To examine the reinfection, the diluted suspension of fungal spores (1.25×10^8 spores/ml) was sprayed on the peach trees where the small leafhoppers had appeared on June 20 and the infective states were investigated on June 30 and August 30 every year (for 3 years). When estimating the dead insects in the field, those with white mycelia on the surface of the body were regarded as dead.

The ANOVA and FISHER LSD test were used to analyze the data of spore density.

Results

The solid culture product of *V. lecanii* made in this work contained 2.5×10^{10} spores per gram. The results of the research on the biological control of the small leafhoppers using *V. lecanii* were conducted indoors by spraying different densities of diluted suspension of fungal spores on the larvae of *E. flavescens* under the suitable conditions and pathogenic effect was analyzed. Statistical analysis of the obtained data in Table 1 shows that the

Table 1 2-way ANOVA Analysis result

Factor	SS	MS	df	*F*-value	*P*-value
F1	34,790.8	6958.16	5	1862.46**	< 0.001
F2	63,857.8	12,771.6	5	3418.51**	< 0.001
F1 × F2	7226.60	289.060	25	77.37**	< 0.001
E	268.994	3.7360	72		

F1: spore density, F2: day, F1 × F2: interaction between F1 and F2, E: error

*indicates the difference between values, in a word, the values have significant to be measured and used for research

morality rate is relevant to both spore concentration and day.

As shown in Table 1, the *P*-values were smaller than 0.001, when the measurements were treated by Fisher LSD Test.

As shown in Table 2, the lethality rate was increased by increasing the spore density. When the density was more than 1.25×10^8 spores/ml, the lethality rate was over 80% after 5 days and it was clear that the optimum density was 1.25×10^8 spores/ml.

The infection effects at larval and imago stages are shown in Table 3.

As shown in Table 3, *V. lecanii* was more effective (about 1.5 times) to imago stage than to larvae. The number of dead imagoes was 59.4% of total corpses, while the number of dead larvae was 40.6%.

After spraying the diluted suspension of solid culture product in the peach field on June 20th, the insecticidal effects by the infection and reinfection (Table 4) were investigated for 3 years.

The diluted suspension of *V. lecanii* spores was sprayed over 50 peach trees once every time. The number of insects on the sample leaves was about 10–12 prior to the spray. The corpses investigated on June 30th were regarded as dead by the first infection, while those investigated on August 30th were regarded as the death was by reinfection. As shown in Table 4, *E. flavescens* was dead about 50% by infection until June 30th for 3 years by the spore diluted suspension of *V. lecanii* spore, while the lethality rate by reinfection increased year by year.

Discussion

This experiment was carried out to confirm whether *V. lecanii* could be used as one of biopesticides for management of *E. flavescens*. By the diluted suspension of *V. lecanii* spore, the most of *E. flavescens* could be exterminated in 7 days. This result was similar to that of exterminating

Table 2 Mortality of *Empoasca flavescens* imagos and immature stages in response to varying *Verticillium lecanii* spore densities

Spore density (conidia ml^{-1})	Days after treatments					
	2	3	4	5	6	7
1.25×10^4	0	0	3.55^e	27.50^e	30.87^e	33.13^e
1.25×10^5	0	0	14.63^d	40.67^d	45.50^d	48.93^b
1.25×10^6	0	11.67^c	37.97^c	51.33^c	58.07^c	63.17^c
1.25×10^7	0	22.40^b	42.53^b	61.47^b	66.10^b	78.00^b
1.25×10^8	1.24^b	37.83^a	63.53^a	81.17^a	90.47^a	94.43^a
1.25×10^9	5.41^a	42.23^a	70.30^a	82.87^a	94.97^a	99.97^a
Control	0	0	0.40	0.50	1.50	3.20

Fisher LSD Test, $P < 0.01$, Different letter indicates significant difference

Table 3 Infection effect of the fungal entomopathogen on the growth stages

Investigation no	Number of total corpses (Mean ± SE)	Number of dead imagoes (Mean ± SE)	Number of dead larvae (Mean ± SE)
1	123	67	56
2	114	71	43
3	124	65	59
4	125	69	56
5	118	69	49
Mean	120.8 ± 1.749	71.8 ± 0.97*	49 ± 1.58*

Temperature 23 ± 1 °C, Relative humidity (RH) 85 ± 5%, *V. lecanii*-spore density: 1.25×10^8 spores/ml, $P < 0.05$

*indicates the difference between values, in a word, the values have significant to be measured and used for research

Table 4 Infection and reinfection effects of *Empoasca flavescens* in 3 years (outdoors)

Date of investigation (per one leaf)	**30.06.2017**	**30.06.2018**	**30.06. 2019**
Control			
Number of dead imagoes	0	0	0
Lethality rate by infection	0	0	0
Test (sprayed by *V. lecanii*)			
Number of dead imagoes	6.43 ± 0.31	6.76 ± 0.42	6.82 ± 0.44
Lethality rate by infection	53.6	56.4	56.8
Date of investigation (per one leaf)	**30.08.2017**	**30.08.2018**	**30.08.2019**
Control			
Number of dead imagoes	0.04 ± 0.003	0.06 ± 0.004	0.07 ± 0.006
Lethality rate by infection	0.3	4.6	5.3
Test (sprayed by *V. lecanii*)			
Number of dead imagoes	1.45 ± 0.12*	8.06 ± 0.32*	10.7 ± 0.92*
Lethality rate by infection	12.1*	67.2*	89.5*

Temperature 23 ± 1 °C, Relative humidity (RH) 85 ± 5%, Spore density of *V. lecanii*: 1.25×10^8 spores/ml, $P < 0.05$

*indicates the difference between values, in a word, the values have significant to be measured and used for research

the green peach aphids, *Myzus persicae* by *Lecanicillium longisporum* in 6 days (Emmanouil et al. 2008).

Empoasca flavescens could not move and the color of its body was changed after 36 h, thus, suitable infective condition should be maintained for at least 36 h.

The lethality rate of adults was higher than larvae (about 1.5 times)-This maybe because pathogenicity was conserved due to reinfection by mutual contacts and spread of infective area by flying imagoes with spores. This result was similar to the research of Daniela et al. (2021). Daniela (2021) indicated that *D. maidis* adults infected by *M. brasiliense* were discovered in different geographic area even over 550 km apart. This showed that EPF spores travelled on the bodies of flying imagoes. However, our result was different from the case of the citrus mealybug, *Planococcus citri* Risso (Hemiptera: Pseudococcidae): Larval *P. citri* were more susceptible than imagoes to fungal infection (Sepideh et al. 2017).

Verticillium lecanii spores spent the winter on peach trees and the density of the EPF increased every year. In this test, the lethality rates by first infection for 3 years were about 50% without non-significant difference. This is because the total amounts of small leafhoppers were not still in large scale until June 30, from July to August, the number of small leafhoppers increased dramatically, thus, in mid-August, interactions and physical contacts between imagoes of small leafhoppers also increased. As a result, reinfection process by *V. lecanii* was actively conducted and the lethality rate by reinfection increased significantly every year. In the test, the lethality rate reaches at 90% after 2 years of spraying the diluted suspension of *V. lecanii* spore, which means that any chemical pesticide is needless for the control of *E. flavescens*.

Conclusion

According to metrological and climate conditions of DPR Korea, from mid-June to mid-September, *V. lecanii* (Z) Viegas 1939 are under favorable infective condition in the field (outdoors), thus, about 90% of *E. flavescens* being parasitizing on peach trees can be exterminated in 2 years after spraying with the diluted suspension of *V. lecanii* spore, which means that no chemical pesticides are needed for management of *E. flavescens*. The effect of *V. lecanii* on the beneficial insects such as bees is under research.

Abbreviations

EPF Entomopathogenic fungus
IPM Integrated pest management

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Author contributions

The authors KSY and JHH carried out the study and wrote the manuscript. SHJ and HSK edited the manuscript. The final version of the manuscript was read and approved by all authors.

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