ENTOMOPATHOGENIC BACTERIUM *Serratia marcescens* ISOLATED FROM *Episparis tortuosalis* CAUSING A DAMAGE TO *Chukrasia tabularis* IN VIETNAM

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ABSTRACT

Episparis tortuosalis Moore, 1867 is a serious pest of the *Chukrasia tabularis* A. Juss which is widely planted in plantations in Vietnam. A biocontrol option to manage this pest is a priority for the forest sector. The objective of this study was to evaluate whether there are potential biocontrol candidates within the *E. tortuosalis* population. Sixteen bacterial strains were isolated from *E. tortuosalis* larvae that were parasitized in a *C. tabularis* plantattion in Nghe An province. Four isolates (FPRC06, FPRC07, FPRC14 and FPRC16) had strongest pathogenicity on *Galleria mellonella* larvae at 1, 24 and 36 hours after the injection and 72, 96 and 120 hours after the spraying experiments. The mortality rate of *G. mellonella* resulted from these two experimental approaches at the end of experiments was 93.3–100% and 76.7–100%, respectively. In a greenhouse experiment, the four isolates caused 44.3–61.1%, 52.0–68.0%, 54.3–69.1% and 41.4–60.7% mortality of *E. tortuosalis* larvae after 3, 5 and 7 days of spraying, respectively. These results were similar to applying a commercial *Bacillus thuringiensis*. The two isolates (FPRC07, FPRC14) were identified as *Serratia marcescens* based on phylogenetic analysis of 16S rRNA. Thus, this finding reveals the potential opportunities for the development of a natural insecticide of *E. tortuosalis* in Vietnam.

Keywords: *Chukrasia tabularis*, *Episparis tortuosalis*, natural insecticides, *Serratia marcescens*.

1. INTRODUCTION

Episparis tortuosalis is distributed naturally in South and Southeast (SE) Asia [1]. This insect has caused severe damage to *Chukrasia tabularis* (Meliaceae) [2] and *Michelia champaca* (Magnoliaceae) [3] in India and SE Asia, and *Azadirachta indica* (Meliaceae) in China [4].

Due to high-quality wood, competitive price, and great market demand, *C. tabularis* has been widely domesticated in Vietnam since the 1960s [5]. The rate of planting has risen from about 120 ha per year [6] over a decade ago to 3,000 ha per year, and the total area was 35,000 ha in 2020 [7]. However, the rapid expansion of *C. tabularis* plantations has faced the increasing risk of damage from *E. tortuosalis*. A recent study has reported that from 2013 to 2021 the geographical range of the pest has increased from one to nine provinces with the damage incidence ranging from 28.5 to 100% and the damage index was from 0.82 to 3.66 [8].

The use of chemical insecticides for pest population management is proving to be counter-productive since its adverse consequence to human health [9]. In addition, many insect herbivores and their offsprings have developed resistance to a wide range of insecticides [10]. In the face of the escalating perils from insecticides, strategies of integrated pest control incorporating biological control agents are interests of the Vietnam Ministry of Agriculture and Rural Development [11]. However, no research has been undertaken on natural enemies of *E. tortuosalis* in Vietnam and this has limited the deployment of biocontrol in the field. Recent field surveys have found larvae of *E. tortuosalis* are often incapacitated or dead

by entomopathogenic bacteria with a parasitism rate of 21% [8]. These natural enemies are expected to be one of the effective biological agent candidates for the control of *E. tortuosalis*. Therefore, the aim of this paper was to explore entomopathogenic bacteria presenting in larvae of *E. tortuosalis* and assess their potential biocontrol ability.

2. RESEARCH METHODOLOGY

Sixteen parasitized *E. tortuosalis* larvae (Fig. 1) were collected in November 2021 from a *Chukrasia tabularis* plantation in Nghia Hung commune, Nghia Dan district, Nghe An province (19°20'433"N; 105°20'668"E). Each sample was washed three times in distilled water, and then transferred into 20 mL of distilled water. After one hour, the bathing water was diluted to 10^{-4} and 10^{-5} with distilled water and aliquots were placed on King's B medium [12]. Petri dish was incubated at 27-28°C in the dark for 3 days. Bacterial colonies were sub-cultured onto PDA medium in new Petri dishes and incubated at 27-28°C. A total of 16 bacterial isolates were obtained from the 16 parasitized *E. tortuosalis* larval samples.

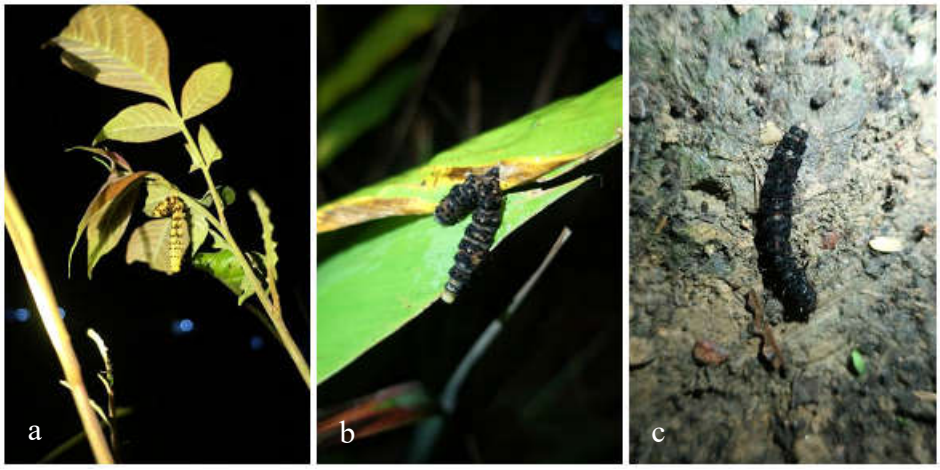

Figure 1. Larvae of *Episparis tortuosalis* parasitized by entomopathogenic bacteria in the field. a. leaves of *Chukrasia tabularis*. b. leaves of shrub. c. ground.

Two bioassay experiments were conducted to evaluate the efficacy of 16 bacterial isolates against the 4th larval instars of *Galleria mellonella* in the laboratory. The model insect fed on an artificial diet as described by Campbell et al. (1975) [13]. The direct inoculation experiment followed the method of Walters and Ratcliffe (1983) [14]. Bacteria were collected from the five-day-old colonies grown in 400 ml King's B medium in one-liter Erlenmeyer flasks on a rotary incubator at 200 rpm, at 28°C. The bacterial suspension was centrifuged at 400 g/min for 30 min (Avanti J-E, Beckman Coulter), the supernatant was removed and the bacteria were resuspended in distilled water to a dosage of 2×10^6 CFU/mL. A commercial pesticide containing *Bacillus thuringiensis* (Delfin 32WG, SDS Biothech K.K., Tokyo, Japan, dosage of 2.0 g/L) was applied in the assay as a positive control. Distilled water was included as the negative control. The larvae were inoculated by the injection using a glass-micro needle (Harvard Apparatus, 300038, 1 mm outside diameter, 0.78 mm inner diameter) into the haemocoel of the last pro-leg [14] with 10 µL of the bacterial suspensions and distilled water. In the second experiment, the treatments were sprayed onto the larvae at a dosage of 0.1 mL/larva using a mini spray bottle (5 mL). The treatments were mixed with 1% Tween 80 prior to spraying and the final dosage was the same as in the injection

method. Each of the 18 treatments had 10 larvae and there were 5 replicates of each treatment. In total, 50 larvae were used for each treatment. After treatment, the larvae were placed in sterilized moist chambers consisting of plastic boxes (9 cm in diameter, 3 cm in depth) with wet filter paper at room temperature of 27–28^{o}C. A 15 g portion of artificial diet was supplied to each plastic box. The number of parasitized larvae was recorded at 1, 24 and 36 hours after the injection experiment, and at 72, 96 and 120 hours after the spraying experiment. The *G. mellonella* larvae were assessed based on their external symptoms consisting of the appearance of gray or black spots along the body, the entire body turning gray or black, larvae becoming rot and death (Fig. 2).

The isolates exhibiting the strongest pathogenicity in the *G. mellonella* experiments were evaluated for their effectiveness in killing *E. tortuosalis* in a nursery experiment. The experimental design was a completely randomized block with 10 treatments (30 plants/treatment) and 5 replications. The Delfin 32WG and clean water were used as positive and negative controls, respectively. Two-year-old *C. tabularis* saplings (120–130 cm in height) planted in a netted house of Forest Protection Research Centre, Hanoi were used for the experiments.

Episparis tortuosalis larvae were mass-reared in a nursery on *C. tabularis* from eggs collected in the field. Fifty 2nd larval instars were placed on the foliage of each *C. tabularis* sapling covered by netted cage (1.0 × 1.0 × 1.5 m). One day later, approximately 2 × 10^6 CFU/mL and the water control were sprayed (0.2 L/plant) to the foliage and body of *E. tortuosalis* using a hand sprayer. All treatments were mixed with 1% Tween 80 prior to application. The treatments were applied in the late afternoon. The number of parasitized *E. tortuosalis* larvae was recorded 72, 150, and 250 days after the spraying.

Genomic bacterial DNA was extracted by mechanical cell disruption followed by phenol/chloroform extraction and silica-based DNA purification as described in Thanh et al. (2018) [15]. Partial 16s rRNA gene sequences were amplified by polymerase chain reaction (PCR) using bacteria-universal primers offered by 1st BASE (27F 5' AGAGTTTGATCMTGGCTCAG 3' and 1492R 5' TACGGYTACCTTGTTACGACTT 3') [16]. The PCR amplification was carried out in a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, USA) using the following thermal program: 94°C for 3 min; 30 cycles of 94°C for 30 s, 52°C for 30 s and 72°C for 1.5 min; 72°C for 10 min. Amplification products were separated in 1% agarose gels in 0.5 TAE (20 mM Tris, 10 mM acetic acid, 0.5 mM EDTA). Amplicons were sequenced using the service provided by First BASE Laboratories Sdn Bhd (Selangor, Malaysia). The sequences were compared with the available National Center for Biotechnology Information (NCBI) database using Basic Local Alignment Search Tool (BLAST) to determine the identity of the bacterial isolates. A phylogenetic tree was constructed using the Maximum Likelihood (ML) method based on the Hasegawa-Kishino-Yano model [17] in MEGA v. 7.0 [18]. Bootstrap analyses were performed from 1000 random re-samplings [19]. Numbers at branch points indicate bootstrap percentages from Maximum Likelihood analyses. Only values greater than 50% are shown. The bar represents an expected sequence variation of 1%.

From the parasitized larvae data, the parasitized rate was calculated as follows:

$$P\% = (n/N) \times 100$$

where:

n is the number of parasitized larvae;

N is total number of larvae assessed.

Data were analyzed using GenStat Release 12.1 software package (VSN International Ltd.,

Hemel Hempstead, UK). One-way analysis of variance (ANOVA) was used to test the signiicant difference in treatment effect (parasitized rate – *P%*) between bacterial isolates, followed by Duncan's Multiple Range Test for comparisons of means at $\alpha = 0.05$.

3. RESULTS

There were numerous bacterial colonies in plates from all *E. tortuosalis* larvae parasitized in the field, but most of the colonies had similar characteristics. Therefore, one bacterial isolate was selected from each group of colonies in each sample giving a total of 16 bacterial isolates from 32 parasitized larvae.

There was a significant difference ($P < 0.001$) in the percentage of parasitized *G. mellonella* larvae in the injection and spray experiments among the 16 bacterial isolates (Table 1). Overall, the mortality rate of *G. mellonella* larvae after 36 hours of the injection was highest, the parasitism range with these isolates were 10.0–100% (Table 1).

Table 1. Mortality rate of *Galleria mellonella* larvae at different times after 2-way infection with entomopathogenic bacteria isolated from *Episparis tortuosalis* larvae. Different letters within a column indicate statistical significance among isolates

Bacterial isolates	Innoculation				Spraying		
	Larval number	1 hour	24 hours	36 hours	72 hours	96 hours	120 hours
FPRC01	30	0.0^{a}	0.0^{a}	76.7^{e}	0.0	0.0^{a}	23.3^{g}
FPRC02	30	0.0^{a}	0.0^{a}	10.0^{b}	0.0	0.0^{a}	10.0^{bc}
FPRC03	30	0.0^{a}	36.7^{c}	36.7^{c}	0.0	0.0^{ab}	10.0^{bc}
FPRC04	30	16.7^{b}	40.0^{c}	60.0^{d}	0.0	16.7^{c}	16.7^{cd}
FPRC05	30	0.0^{a}	30.0^{c}	40.0^{c}	0.0	0.0^{ab}	0.0^{a}
FPRC06	30	73.3^{e}	73.3^{fg}	93.3^{f}	0.0	0.0^{ab}	76.7^{h}
FPRC07	30	73.3^{e}	73.3^{fg}	93.3^{f}	0.0	13.3^{c}	93.3^{h}
FPRC08	30	73.3^{e}	83.3^{gh}	76.7^{e}	0.0	6.7^{a}	73.3^{g}
FPRC09	30	43.3^{d}	60.0^{de}	70.0^{de}	0.0	0.0^{ab}	16.7^{cd}
FPRC10	30	0.0^{a}	60.0^{de}	70.0^{e}	0.0	0.0^{a}	3.3^{ab}
FPRC11	30	40.0^{cd}	76.7^{fg}	76.7^{e}	0.0	0.0^{a}	0.0^{a}
FPRC12	30	0.0^{a}	13.3^{b}	43.3^{c}	0.0	0.0^{a}	36.7^{e}
FPRC13	30	16.7^{b}	53.3^{d}	70.0^{e}	0.0	0.0^{a}	3.3^{ab}
FPRC14	30	36.7^{cd}	90.0^{h}	100.0^{f}	0.0	56.7^{d}	100.0^{h}
FPRC15	30	33.3^{c}	70.0^{ef}	76.7^{e}	0.0	0.0^{a}	56.7^{f}
FPRC16	30	16.7^{b}	83.3^{gh}	100.0^{f}	0.0	0.0^{a}	76.7^{g}
Delfin	30	76.7^{e}	93.3^{h}	100.0^{f}	16.7	60.0^{d}	100.0^{h}
Water control	30	0.0^{a}	0.0^{a}	0.0^{a}	0.0	0.0^{ab}	0.0^{a}
Pvalue		<0.001	<0.001	<0.001	*	<0.001	<0.001

The most toxic isolates were FPRC06, FPRC07, FPRC14 and FPRC16 with their respective mortality was 93.3%, 93.3%, 100% and 100%. The toxicity of these 4 isolates was equal to the Delfin control (Table 1, Fig. 2).

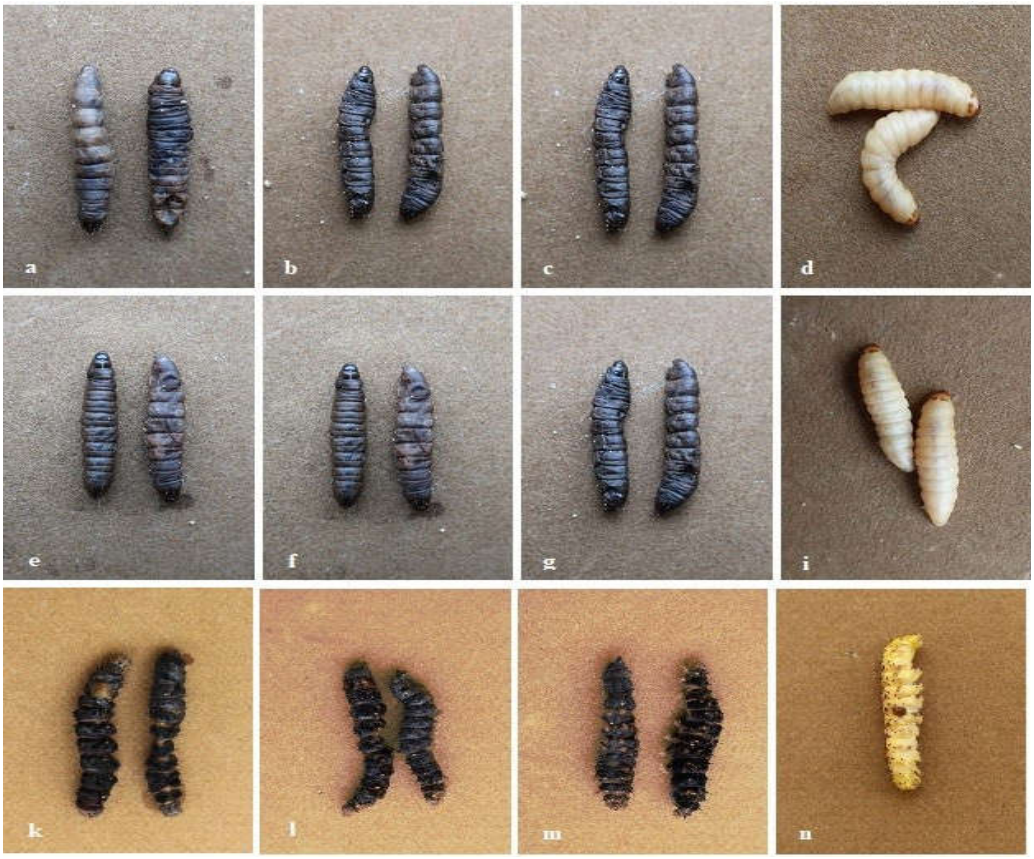

Figure 2. Larval *Galleria mellonella* and *Episparis tortuosalis* parasitized by entomopathogenic bacteria in the laboratory and nursery: a, b, c, d 36 hours after injection; e, f, g, h 120 hours after spraying. a, e, k isolate FPRC07; b, f, l isolate FPRC14; c, g, m Delfin; d, i, n water control.

The remaining 14 *E. tortuosalis* isolates had very weak and moderate toxicity with the parasitism range being 10.0–76.7%, respectively (Table 2). The mortality rate of *G. mellonella* larvae was highest after 120 hours of the spraying treatments, the 16 bacterial isolates showed virulence against larvae with the parasitism range was 0.0–100% (Table 1). Isolates FPRC06, FPRC07, FPRC14 and FPRC16 had the highest toxicity against *G. mellonella*, their respective mortality was 76.7%, 93.3%, 100% and 76.7%, equivalent to the Delfin treatment. Strains with very weak toxicity in the injection experiment did not cause larva death in the spray experiment (Table 2).

Table 2. Mortality rate of *Galleria mellonella* larvae at different times after treatment with entomopathogenic bacteria in a greenhouse. Different letters within a colum indicate statistical significance among isolates.

Isolate	Mortality rate (%)			
	Larval number	3 days	5 days	7 days
FPRC06	90	44.3^{b}	50.1^{b}	61.1^{b}
FPRC07	90	52.0^{c}	54.7^{b}	68.0^{bc}
FPRC14	90	54.3^{c}	53.8^{b}	69.1^{bc}
FPRC16	90	41.1^{bc}	50.3^{b}	60.7^{bc}
Delfin	90	46.3^{bc}	58.0^{b}	70.3^{c}
Water control	90	0.0^{a}	0.0^{a}	0.0^{a}
Pvalue		<0.001	<0.001	<0.001

There was a significant effect (P <0.001) of spraying the isolates (FPRC06, FPRC07, FPRC14 and FPRC16) on the mortality of *E. tortuosalis* larvae in the nursery experiments after 3, 6, and 9 days in comparison with the water control. The percentage of dead larvae with these isolates after 3, 5, and 7 days were 44.3–61.1%, 52.0–68.0, 54.3–69.1% and 41.1–60.7%, respectively (Table 2, Figure 2). These results were equal to the Delfin control treatment (Table 2).

The rRNA gene sequences of two entomopathogenic bacteria in this study were compared to the reference sequences downloaded from the Genbank database (NCBI). The consensus tree of the 16S gene showed that the isolates FPRC07, FPRC14 were clustered with *Serratia marcescens* with ≥99% similarity (Figure 3).

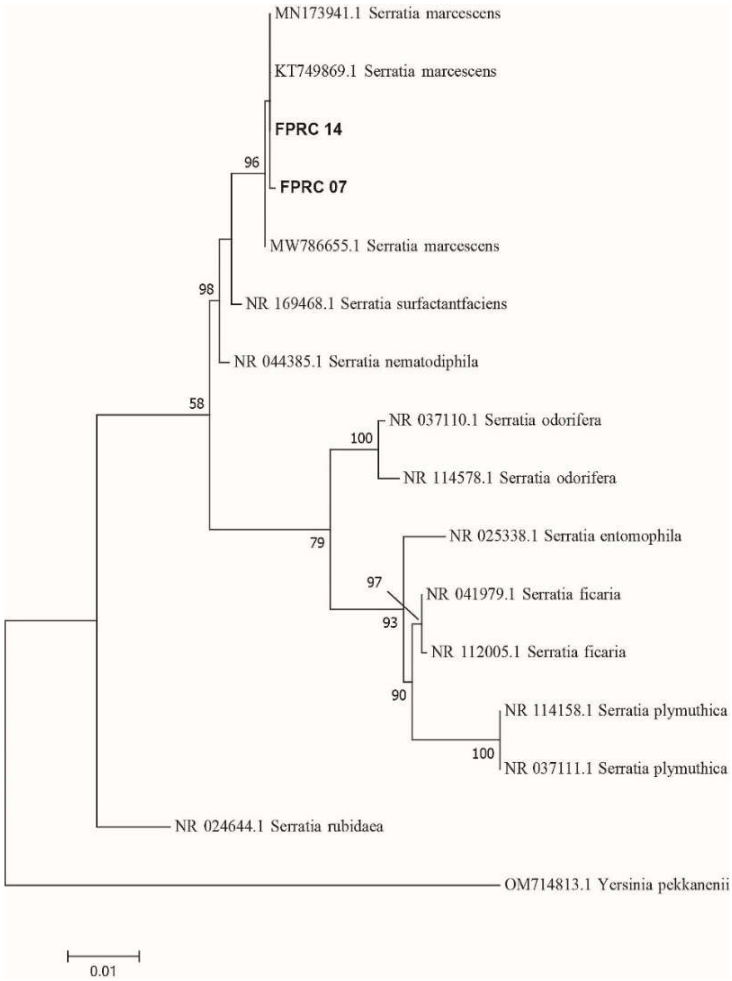

Figure 3. Consensus tree of 16S rRNA from different species of the genus *Serratia.*

4. DISCUSSION

This is apparently the first record of *S. marcescens* bacterial disease in *E. tortuosalis* larvae in *C. tabularis* plantations in Vietnam. The characteristic symptoms due to *S. marcescens* was the head and appendages of the dead *E. tortuosalis* to turn red. This entomopathogenic bacteria has been shown to be pathogenic to different insects such as silkworm (*Bombyx mori*), termite (*Zootermopsis angusticollis*), gipsy moth (*Lymantria dispar*), European corn borer (*Pyrausta nubialis*) [20].

Galleria mellonella has been used as a model host for screening for pathogenicity of different parasitic microbes [14, 21]. Zernhoff (1931) [22] has reported that *S. marcescens* is very pathogenic to the larvae of *G. mellonella* by the

method of inoculation. Therefore, in the present study, we initially did our screenings with this model insect and identified four toxic isolates (FPRC06, FPRC07, FPRC14, and FPRC16). Coincidently, these four isolates were also the most pathogenic to *G. mellonella* in the spraying experiments. We then established that the two most pathogenic isolates (FPRC07, FPRC14) caused death of *E. tortuosalis* larvae in the greenhouse experiments. Similar mortality rate has been observed with *Serratia marcescens* infecting *E. tortuosalis*.

Several measures have been formulated for controlling *E. tortuosalis*. The pesticides Trichlorfon and 666 (Hexacloran) were used to kill *E. tortuosalis* damaging *Azadirachta indica* in China [4], but these chemical substances have been banned from use in Vietnam. Various natural enemies of *Episparis* spp. such as nematode *Hexamermis* sp. in India [23] and arborial ant *Oecophylla smaragdina* in Malaysia [11]. Recently, the entomopathogenic fungi (*Cordyceps* spp.) was found in *C. tabularis* plantations in Nghe An province where *S. marcescens* was isolated from *E. tortuosalis* in this study [24]. Therefore, this finding reveals the potential opportunities for the development of a natural insecticide for effective management of *C. tabularis* in Vietnam. However, further studies are essential to examine the prevention effectiveness of *S. marcescens* on *E. tortuosalis* in *C. tabularis* plantations. Their efficacy can then be compared with other parasitic microrganisms that have been promoted as commercial biocontrol agents for the management of various insect pests in Vietnam such as the bacteria *B. thuringiensis*, and the fungi *Metarhizium anisopliae* and *M. flavoviride* [25].

5. CONCLUSION

Episparis tortuosalis is a major threat to *Chukrasia tabularis* plantations across provinces of Vietnam. The identification of a biological control agent *Serratia marcescens* to this pest species would contribute to its integrated pest management approach in the future.

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VI KHUẨN KÝ SINH (*Serratia marcescens*) PHÂN LẬP TỪ SÂU ĂN LÁ (*Episparis tortuosalis*) GÂY HẠI CÂY LÁT HOA (*Chukrasia tabularis*) Ở VIỆT NAM

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[4]Chi cục Kiểm lâm Hà Nội

TÓM TẮT

Sâu ăn lá (*Episparis tortuosalis* Moore, 1867) là loài gây hại nguy hiểm trên cây Lát hoa ở Việt Nam. Biện pháp phòng trừ sinh học cho loài sâu này đang được quan tâm nghiên cứu. Nghiên cứu này nhằm đánh giá khả năng ký sinh gây bệnh của các chủng vi khuẩn được phân lập từ sâu non của sâu ăn lá bị ký sinh tự nhiên. Nghiên cứu đã phân lập 16 chủng vi khuẩn ký sinh sâu non của sâu ăn lá ở ngoài tự nhiên. Trong số đó, 4 chủng (FPRC6, FPRC7, FPRC14 và FPRC16) có hiệu lực gây bệnh cao nhất cho sâu non của sâu sáp (*Galleria mellonella*) ở thời điểm 1 giờ, 24 giờ và 36 giờ bằng cách tiêm và 72 giờ, 96 giờ và 12 giờ bằng cách phun dung dịch có chứa vi khuẩn lên cơ thể sâu non. Tỉ lệ sâu non của sâu sáp bị chết do hai phương pháp này lần lượt là 93,3%, 76,7–93,3%, 100% và 76,7–100%. Tại nhà lưới, tỉ lệ sâu non của sâu ăn lá (*E. tortuosalis*) bị chết sau 3 ngày, 5 ngày và 7 ngày phun lần lượt là 44,3–61,1%, 52,0–68,0%, 54,3–69,1% và 41,4–60,7%. Các kết quả này tương đương với thuốc trừ sâu sinh học thương mại có chứa vi khuẩn *Bacillus thuringiensis*. Dựa trên trình tự 16S rRNA, hai chủng vi khuẩn ký sinh (FPRC7, FPRC14) được giám định là loài *Serratia marcescens*. Kết quả nghiên cứu này có ý nghĩa quan trọng để phát triển thuốc trừ sâu sinh học phòng trừ hiệu quả sâu ăn lá cây Lát hoa ở Việt Nam.

Từ khoá: Lát hoa, sâu ăn lá, thuốc trừ sâu sinh học, vi khuẩn ký sinh.

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