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NOTE

Evaluation of Insecticidal Activity of a Bacterial Strain, *Serratia* sp. EML-SE1 against Diamondback Moth

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To identify novel bioinsecticidal agents, a bacterial strain, *Serratia* sp. EML-SE1, was isolated from a dead larva of the lepidopteran diamondback moth (*Plutella xylostella*) collected from a cabbage field in Korea. In this study, the insecticidal activity of liquid cultures in Luria-Bertani broth (LBB) and nutrient broth (NB) of a bacterial strain, *Serratia* sp. EML-SE1 against thirty 3rd and 4th instar larvae of the diamondback moth was investigated on a Chinese cabbage leaf housed in a round plastic cage (Ø 10×6 cm). 72 h after spraying the cabbage leaf with LBB and NB cultures containing the bacterial strain, the mortalities of the larvae were determined to be 91.7% and 88.3%, respectively. In addition, the insecticidal activity on potted cabbage containing 14 leaves in a growth cage (165×83×124 cm) was found to be similar to that of the plastic cage experiment. The results of this study provided valuable information on the insecticidal activity of the liquid culture of a *Serratia* species against the diamondback moth.

Keywords: diamondback moth (*Plutella xylostella*), insecticidal activity, *Serratia* sp., storage period

The diamondback moth (*Plutella xylostella* L.), which belongs to the insect order Lepidoptera, is a notorious pest of cruciferous crops worldwide, causing annual losses on the order of $1 billion (Verkerk and Wright, 1996). It has been reported that the most damage is caused by larval feeding and there are no effective natural enemies against the larvae of the diamondback moth. Continued long-term use of insecticides to control the diamondback moth has resulted in the development of insecticide resistance (Magaro and Edelson, 1990). Interest in biological control of plant insects has increased in recent years due to the agriculture industry's interest in greater sustainability and public concerns over the use of hazardous pesticides in the environment. A number of methods have been developed for the biocontrol of the diamondback moth. An exotic parasitoid, *Diadegma semiclausum*, was developed at the Asian Vegetable Research and Development Centre in Taiwan to control the diamondback moth. In addition, an entomopathogenic nematode, *Steinernema carpocapsae*, was shown to control the larvae (Schroer *et al*., 2005). *Bacillus thuringiensis* has often been successfully used as a biological agent against Lepidoptera, Diptera, Coleoptera, and Hymenoptera (Bravo *et al*., 2007).

Several *Serratia* strains have been shown to be lethal to insect pests when ingested in high doses; however, in some cases the strains can be highly virulent at low doses, killing the larvae in 2-3 days with symptoms similar to a virus infection (Steinhaus, 1959; Lysyk *et al*., 2002). Especially, red-colored *Serratia* biotypes are more often associated with insects than non-pigmented biotypes (Grimont *et al*., 1977). In a previous study, treatment of the tobacco budworm (Lepidoptera: Noctuidae), *Heliothis virescens*, with *S. marcescens* (Bizio) was shown to produce sublethal effects, such as reduced adult longevity and a decreased ratio of oviposition and egg hatching (Sikorowski and Lawrence, 1998; Inglis and Lawrence, 2001). Also, entomopathogenic strains of *S. entomophila* have been used to control various insect genera including *Anomala*, *Costelytra*, and *Phyllophaga* (Nunez-Valdez *et al*., 2008). In addition, strains of *S. entomophila* and *S. proteamaculans* were shown to kill grass grub, *C. zealandica* (Jackson *et al*., 1993, 2001; Sikorowski *et al*., 2001; Nunez-Valdez *et al*., 2008). Furthermore, *Serratia marcescens* which produces chitinase, has been used to control plant diseases (Ordentlich *et al*., 1998; Someya *et al*., 2005).

To identify novel insecticidal agents, a bacterial strain, *Serratia* sp. EML-SE1, was isolated from a dead larva of the diamondback moth collected from a cabbage field in Korea. The objective of this study was to evaluate the *in vitro* and *in vivo* insecticidal activity of *Serratia* sp. EML-SE1 against the diamondback moth both on a sliced cabbage leaf in a round plastic cage and on whole leaves of potted cabbage.

The lepidopteran diamondback moths used in the bioassay were maintained in the laboratory for about 5 days after they were collected from a cabbage field at Chonnam National University located in Gwangju, Korea. The diamondback

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moths were reared in the laboratory at a temperature of 25±1°C and relative humidity (RH) of 60±5%, with a photoperiod of 16:8 h (Light:Dark).

The medium used for isolation of *Serratia* sp. EML-SE1 was nutrient agar (NA) containing 3 g beef extract, 5 g peptone, 15 g agar (Difco, USA) per 1 L distilled water. The sample was maintained on an agar slant and Petri plate at 4°C. The isolate was also maintained as a cell suspension in 20% glycerol at -80°C.

Previously described procedures were used for DNA extraction, PCR and sequencing of 16S rRNA gene (Kim *et al*., 1998). The primer set used to amplify the 16S rRNA gene was 27f (5′-AGA GTT TGA TCM GGC TCA G-3′) and 1492r (5′-GGT TAC CTT GTT ACG ACT T-3′). The sequence data were aligned with those of representative members of the selected genera of the family *Enterobacteriaceae* using PHYDIT version 3.2 (http://plaza.snu.ac.kr/~jchun/phydit/). The nucleotide length that was used to identify the strain was 1,378 bp. Phylogenetic trees were inferred using suitable programs in the PHYLIP package (Felsenstein, 1993). Phylogenetic distances were calculated from the Jukes-Cantor model (Jukes and Cantor, 1969), and trees were constructed on the basis of the neighbor-joining (Kimura, 1980; Saitou and Nei, 1987) and maximum-likelihood algorithms (Felsenstein, 1981). Bootstrap analysis was performed with 1,000 resampled datasets using the SEQBOOT and CONSENSE programs of the PHYLIP package.

The EML-SE1 strain was identified as a *Serratia* species based on 16S rRNA gene sequence analysis (Fig. 1). Phylogenetic analysis of almost all the 16S rRNA gene sequences of related species revealed that the strain formed a distinct lineage within the genus (Fig. 1). The two subspecies of *Serratia marcescens* were found to be the nearest neighbors, which was supported by the high bootstrap value and the maximum-likelihood tree. Physiological and cultural characteristics also indicated close affiliation of the strain to *S. marcescens*. The sequence similarity between the strain and each of the subspecies of *S. marcescens* was 99.8% (corresponding to a 3 nucleotide difference), whereas this value was 98.4% with the next closest species, *S. ureilytica* (corresponding to a 22 nucleotide difference).

The three liquid media that were used for seed culture and preculture included nutrient broth (NB, pH 6.8, Difco) medium containing 3 g beef extract, 5 g peptone, per 1 L, Luria-Bertani broth (LBB, pH 7.0, Difco) medium containing 10 g tryptone, 5 g yeast extract, 10 g sodium chloride, per 1 L; and Bushnell-Haas broth (BHB, pH 7.0, Junsei) medium containing 1 g K_2HPO_4, 1 g KH_2PO_4, 1 g NH_4NO_3, 0.02 g $CaCl_2$, 0.2 g $MgSO_4$, 0.05 g $FeCl_3$, per 1 L distilled water (Grant and Betts, 2004). A culture was initially started in a 10 ml test tube (the so-called seed culture). The culture size was then scaled up to 1 L in Erlenmeyer flasks. For culture of the strain, 1 L of each liquid medium was inoculated with 1 ml of stock solution (LBB culture). The flask was incubated on a

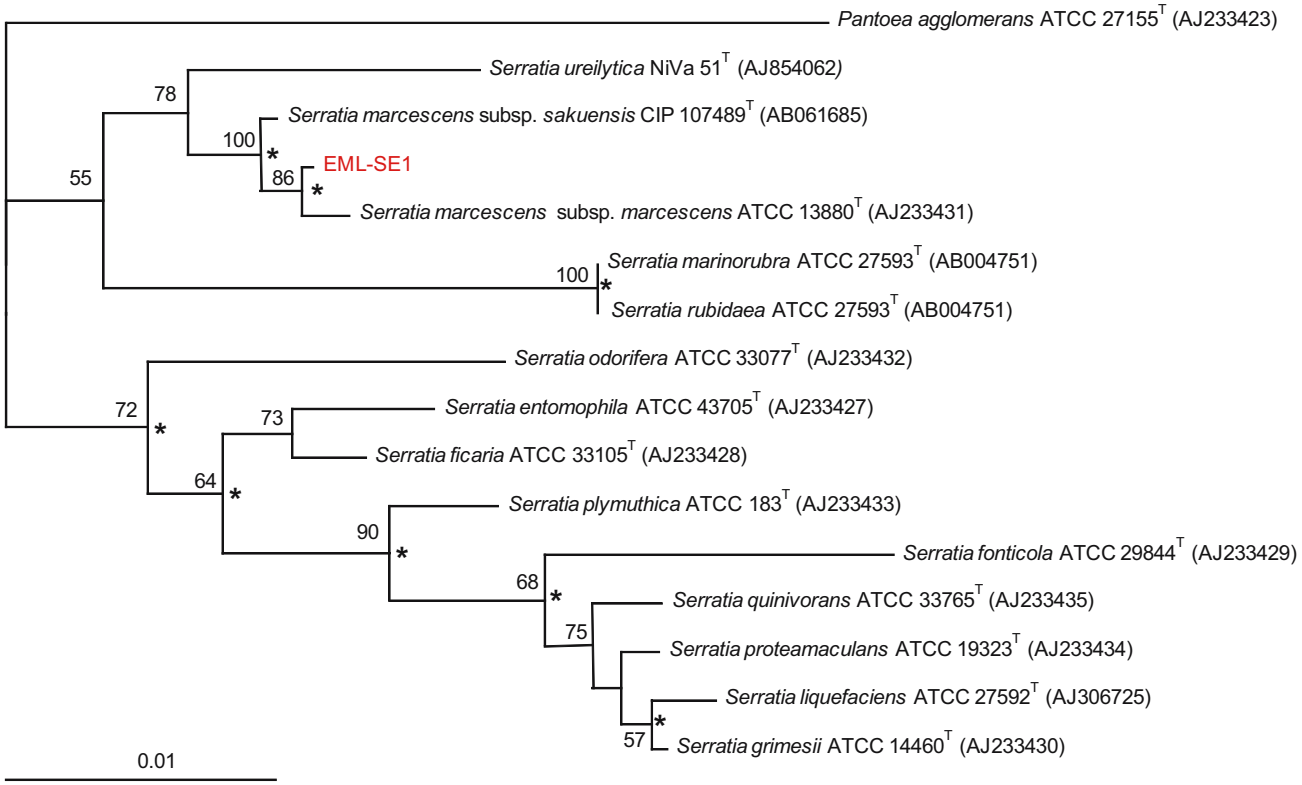

Fig. 1. NJ tree showing the phylogenetic status of *Serratia* sp. EML-SE1 and its relationships among 15 Enterobacteriae based on analysis of aligned completed 16S rRNA sequences. *Pantoea agglomerans* was used as an outgroup. Asterisks indicate branches that were recovered in the maximum-likelihood tree. The scale bar represents 0.01 substitutions per nucleotide position. Bootstrap support values greater than 50% for 1,000 replications are shown at the nodes.

reciprocal shaker (120 rpm) at 32°C for 24 h. The cell concentration in the liquid culture was determined by the indirect method of limited logarithmic dilutions. 0.1 ml was plated on nutrition agar medium in petri dishes (ø 100 mm). The sample was quantified after 48 h of cultivation at 32°C. Red colonies of *Serratia* sp. EML-SE1 were enumerated using a standard plate counter and the results were expressed as means (± standard deviation). The bacterial growth curves in the three liquid media described above were the average sums from the number of the colony forming units (CFU) from three dilutions in three repetitions (data not shown).

The insecticidal activity of the liquid culture of *Serratia* sp. EML-SE1 on a cabbage leaf was evaluated using the spraying method described by Lee *et al.* (1993). A cabbage leaf was cut to the size of Ø10 cm and inoculated with the 3rd and 4th instar larva of *P. xylostella*. Three different liquid cultures of *Serratia* sp. EML-SE1 containing 0.001% Tween 80 were sprayed on both leaf surfaces, which were housed in a plastic cage (Ø 10×6 cm), using a plastic handheld sprayer and incubated in an incubator at a temperature of 25±1°C and RH of 60±5%, with a photoperiod of 16:8 h (Light:Dark). Every 12 h, the percent dead larvae was recorded for up to 48 h. Larvae were considered dead when no movement occurred for at least one min or almost the whole larval body turned a red color. A commercial Bt (*Bacillus thuringiensis*) product, Tobagi (Dongbu Co., Korea) was diluted 1:500 in distilled water according to the instructions provided by the company and was used as a positive control. The active ingredient in this product was derived from *B. thuringiensis* subsp. *aizawai* NT0423. LBB medium and H_2O were used as a negative control. All tests were conducted in an incubator where the temperature and RH were set at 24°C and 50-60%, respectively, with a photoperiod of 16:8 h (L:D). The number of larva per treatment was 30. All experiments were carried out in triplicate and repeated two times.

Of the three media tested (LBB, BHB, and NB), maximal *Serratia* sp. EML-SE1 growth was observed in the LBB culture 24 h after inoculation (data not shown). However, the total CFU and curve pattern in the three liquid media were similar. 36 h after inoculation, a rapid reduction in the cell population was observed (data not shown).

The insecticidal activity of the liquid cultures in controlling the *P. xylostella* larva varied with the media tested (Fig. 2). After 24 h, the insecticidal activities of LBB and NB were 0.0% and 8.3%, respectively. After 48 h, the activities increased up to approximately 50% (LBB: 45.0%, NB: 53.3%), although the control values were low in comparison with the commercial product, Tobagi, which had an activity of 100%. After 72 h, the insecticidal activities of LBB and NB cultures of *Serratia* sp. EML-SE1 against the diamondback moth larva in a round plastic cage were similar (91.7% and 88.3%) and not significantly different (Fig. 2). In contrast, the insecticidal activity of BHB was low (data not shown), although the strain grew to a similar CFU in this medium.

Pot tests were also conducted to evaluate the *in vivo* insecticidal activity of *Serratia* sp. against the larva of *P. xylostella*. The seeds of Chinese cabbage were sowed in a plastic cage (6 cm×6 cm×10 cm) and used in tests 30 days after being grown in a growth cage (165 cm×83 cm×124 cm) with a photoperiod of 16:8 h (L:D). After 30 days of growth, the samples were inoculated with twenty 3rd and 4th instar larvae, where *Serratia* sp. was applied using the spray method. The mortality of the 3rd and 4th instar larvae of *P. xylostella* was investigated after inoculation of a liquid culture containing 0.001% Tween 80 and *Serratia* sp. EML-SE1, which had been cultured for 24 h. To investigate the effect of the storage period on insecticidal activity, the liquid cultures of *Serratia* sp. EML-SE1 were kept in a refrigerator (5±2°C) for 2 days (2Ds) and three months (3Ms). Control larvae were treated with medium (LBB) and sterile distilled water as a negative control using the same technique. All treated samples were maintained at 25°C and 50-60% RH with a photoperiod of 16:8 h (L:D). Every 12 h, the mortality was determined for up to 72 h after the larvae were placed on the cabbage. The

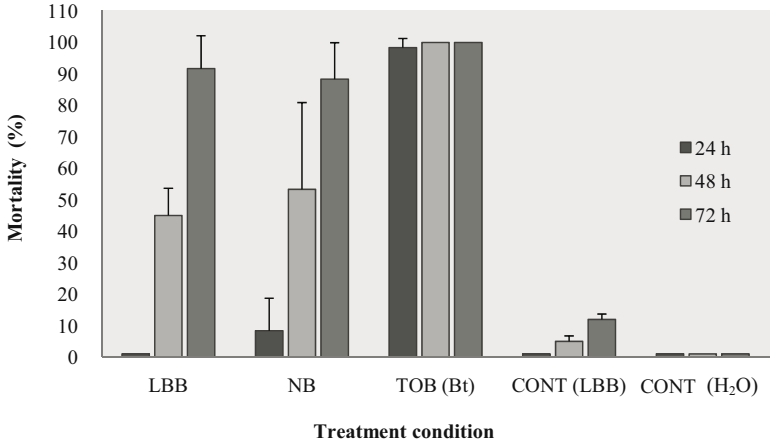

Fig. 2. Insecticidal activity of cultures of *Serratia* sp. EML-SE1 against the larva of *P. xylostella*. The mortality of the 3rd and 4th instar larvae of *P. xylostella* after inoculation with *Serratia* sp. EML-SE1 is shown after 24 (A), 48 (B), and 72 (C) h. LBB, culture grown in Luria-Bertani broth; NB, culture grown in nutrient broth; TOB (Bt), Tobagi (a Bt agent) as a positive control; CONT (LBB), control (Luria-Bertani broth medium not cultured); CONT (H_2O), control (distilled water). The total number of larva per treatment was 30. All experiments were carried out in triplicate and repeated two times. Bars represent standard errors.

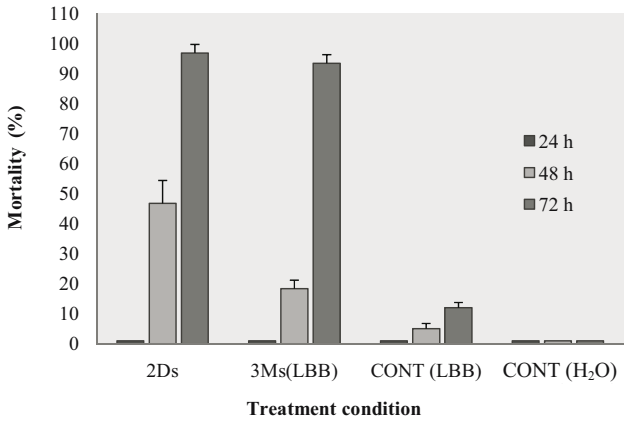

Fig. 3. Effect of storage period on the *in vivo* insecticidal activity of the *Serratia* culture against the larva of *P. xylostella*. The mortality of the 3rd and 4th instar larvae of *P. xylostella* after inoculation with *Serratia* sp. EML-SE1 is shown after 24 (A), 48 (B), and 72 (C) h. LBB, culture grown in Luria-Bertani broth; NB, culture grown in nutrient broth; Control (LBB), control (Luria-Bertani broth medium not cultured); CONT (H_2O), control (distilled water). 2Ds, culture of *Serratia* sp. EML-SE1 stored in a refrigerator for 2 days; 3MS, culture of *Serratia* sp. EML-SE1 stored in a refrigerator for 3 months. The total number of larva per treatment was 20. All the experiments were carried out in triplicate and repeated two times. Bars represent standard errors.

number of larva per treatment was 20. All experiments were carried out in triplicate and repeated two times.

In addition, the insecticidal activity of the culture against the *P. xylostella* larva on potted cabbage was found to be similar to that observed for the plastic cage experiment (Fig. 3). These combined results demonstrate that this species acts as a bioinsecticidal agent. No rapid decrease in the insecticidal activity against the target insect was observed when the cultures of *Serratia* sp. EML-SE1 were stored in a refrigerator for 2 days and three months (Fig. 3). In addition, no prominent differences in the insecticidal activities of liquid cultures stored in a refrigerator for 2 days and 3 months were observed. As shown in Fig. 3, the insecticidal efficacies of the 2Ds culture (storaged in refrigerator for two days) and 3Ms culture (storaged in refrigerator for three months) of *Serratia* sp. EML-SE1 were 96.7%, and 93.3%, respectively, demonstrating that the liquid cultures of the strain were very stable over a long time.

The focus of this paper was to identify an insecticidal agent through *in vitro* and *in vivo* screening. As shown in Fig. 3, the activity of the liquid cultures was maintained even after storage in the refrigerator for several months, although the activity slightly decreased. These results show that the mechanism of action of the cultures depended on the metabolites or enzymes and not on the living cells. Our test showed that filtered supernatants from cultures, which did not contain bacterial cells, was also active against the moth. Because of this continued activity after storage of the cultures, it appears that the *Serratia* sp. EML-SE1 may be a powerful agent for controlling the diamondback moth. It has been shown that the host of the diamondback moth encompasses a wide variety of different plants including virtually all cruciferous vegetable crops, such as broccoli, cabbage, and cauliflower. It has also been well established that the diamondback moth is resistant to many pesticidal agents, including *Bacillus thuringiensis* (Ferre *et al*., 1991; Lee *et al*., 1993). Alternative control methods, particularly organically based pesticides, have been required to eliminate this agricultural pest. This study focused on the bioinsecticidal activity of the bacterial strain, *Serratia* sp. EML-SE1, against some pests of the cruciferous crops, including the aphid and diamondback moth. The activity of this strain varied with media and exposure time as described in previous studies (Visnovsky *et al*., 2008). Out of the three media tested, the insecticidal activity was similar between LBB and NB. However, BHB (24 h culture) was only marginally effective against the larva of the diamondback moth (data not shown). The pupation rate within 60 h ranged from 63.3 to 80%. In contrast, the liquid culture of the strain was slightly active against the nymph of turnip aphid (*Lipaphis erysimi*) (data not shown).

Red- or pink-colored *Serratia* strains are known to be lethal to insect pests when ingested (Grimont *et al*., 1977). After *P. xylostella* was infected with *Serratia* sp., the larva body of the pest changed to a red color, indicating complete infection. In our study, the color varied according to the culture conditions (data not shown). The activity was different and the pink-colored solution was the most active against insects.

Although the bacterial strain, *Serratia* sp. EML-SE1 holds promise for use as a biocontrol agent to control the diamondback moth, its practical use may be very limited since it could be pathogenic to animals. Some previous studies reported that *S. marcescens* and the *S. liquefaciens* complex were routinely associated with human infections as low grade human disease organisms and that some *Serratia* species including *S. ficaria*, *S. fonticola*, *S. odorifera*, *S. plymuthica*, and *S. rubidaea* had different susceptibility to antimicrobial agents (Stock *et al*., 2003). Thus, the active compounds including antibiotics or enzymes produced by this species would be more useful than the bacterial cells (Braunagel and Benedik, 1990; Tao *et al*., 2006). Thus, more studies on the isolation of the active compounds, host spectrum and mode of action are necessary.

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