Effects of Serratia marcescens (SM1) and its interaction with common biocontrol agents on the termite, Odontotermes formosanus (Shiraki)

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Abstract Odontotermes formosanus (Shiraki), a black-winged subterranean termite, is a common forest pest. A red pigment-producing bacterial strain isolated from the termite was identified as Serratia marcescens and named SM1. A bioassay of SM1 on O. formosanus show that the LD50 ranged from 1.77 × 104 to 10.82 × 10 4 cells/termite over 21–39 h. Three biological control agents, Beauveria bassiana (2 × 1010 cells/mL), Metarhizium anisopliae (1 × 1010 cells/mL) and Bacillus thuringiensis (1.6 × 108 IU/mL), were used for an O. formosanus bioassay. The results show that the insecticidal effect of B. bassiana was stronger than that of M. anisopliae. In addition, two mixtures were obtained by combining B. bassiana (2 × 1010 cells/mL) with SM1 (1.5 × 1010 cells/mL), and M. anisopliae (1 × 1010 cells/mL) with S. marcescens (SM1) (1.5 × 1010 cells/mL) in equal volumes. The results show that B. bassiana and SM1 was less effective than SM1 alone. However, the insecticidal effect of M. anisopliae and SM1 was better than that of M. anisopliae or SM1 individually. These studies provide an important contribution for termite biocontrol.

Keywords Odontotermes formosanus (Shiraki) · Serratia marcescens strain SM1 · LD50 · Biological control agents

Introduction

Current control methods for Odontotermes formosanus (Shiraki) rely mainly on chemical treatments, including spraying liquid chemicals, feeding bait, dusting and grouting. The active ingredients in these chemicals are synthetic organic pesticides that present different levels of threat to soil, water and the human body. Therefore, alternative, environmentally friendly termite control agents such as biological control agents need to be developed (Liang et al. 2012). An active bacteria strain isolated from O. formosanus, was identified as Serratia marcescens (SM1) (Fu et al. 2019).

S. marcescens, is a Gram-negative bacterium and an important member of the Enterobacteriaceae. It can effectively control a number of pests and pathogenic bacteria in the field of biological control (Babashpour et al. 2012; Wang et al. 2013) and can also enhance plant resistance to pathogenic bacteria (Nobutaka et al. 2005; Lavania et al. 2006; Chakraborty et al. 2010; Ting et al. 2010). In recent years, it has been found that S. marcescens has insecticidal properties. Liu et al. (1988) found it in diseased planthoppers. Wang et al. (2010) isolated a strain of S. marcescens, KI3, which showed an obvious toxic effect to aphids. In addition, a strain of S. marcescens isolated from the cotton bollworm, Helicoverpa armigera Hübner, had a strong pathogenicity and control on H. armigera, on the cabbage white worm, Pieris rapae L. and beet armyworm,
Spodoptera exigua (Hübner) (Chen et al. 2001). The development and utilization of S. marcescens bacterial strains and their mechanism of action on plant pests has attracted increasing attention from researchers worldwide. However, the application of S. marcescens to O. formosanus has not yet been studied.

Mixing pesticides may increase their toxicity, and the combined use of different bacteria may also increase the preventive effects of pesticide treatment. Yin et al. (2004) found that chitinase enzymes produced by S. marcescens hydrolyze chitin in the midgut peritrophic membranes of locusts and perforate intestinal membranes, which makes insecticidal proteins of S. marcescens more likely to penetrate into the insect body and destroy the digestive tract, thereby greatly improving the insecticidal effect on the locusts. At the same time, chitinases produced by S. marcescens also cause significant damage to allow other pathogens to invade locusts and increase the infection and fatality rates. Chitinases that is beneficial for Bt infection have a synergistic effect on Bacillus thuringiensis (Bt) (Sampson and Gooday 1998). In addition, Liu et al. (1988) reported a synergizing effect from the mixed use of S. marcescens and B. thuringiensis strain HD-1. However, the toxicity of S. marcescens to O. formosanus has not been reported. Therefore, the SM1 strain of S. marcescens, which we isolated and identified, was used to

Materials and methods

Isolation and culture of the S. marcescens strain, SM1

S. marcescens strain SM1 was isolated from infected O. formosanus and was deposited at Nanjing Forestry University in Nanjing, China. Solid bacterial medium (1 L) consists of peptone 10 g, beef extract 20 g, NaCl 2 g, K2HPO4 2 g, agar 18 g and H2O 1 L; pH 7.2–7.4.; Seed medium (1 L) consists of peptone 10 g, yeast extract 20 g, NaCl 2 g, K2HPO4 2 g, agar 18 g and H2O 1 L, pH 7.2–7.4.; Seed medium (1 L) consists of peptone 10 g, beef extract 20 g, NaCl 2 g, K2HPO4 2 g, agar 18 g and H2O 1 L; Zymotic medium (1 L) consists of peptone 10 g, soybean oil 30 g, NaCl 2 g, K2HPO4 2 g and H2O 1 L. The isolated SM1 strain was cultured on solid media without light at 27 °C. A single colony was isolated, placed in a 250 mL Erlenmeyer flask containing 50 mL sterilized seed medium and incubated for 12 h at 30 °C and 200 r/min. The 70 mL seed solution was added to 250 mL zymotic medium and cultured in a shaking incubator at a rotational speed of 200 r/min at 30 °C for 36 h (Zhang et al. 2015).

Determination and dilution of the concentration of SM1

The zymotic medium was used as stock liquid and diluted and quantified. A drop of the bacterial liquid was used to fill the counting area made up of 25 squares, and the number of bacteria in five squares (upper left, lower left, upper right, lower right and centre) was counted according to their diagonal position.

Calculation formula:

\[ \text{SM1 cells/L} = \frac{\text{number of bacteria in the five squares}}{5} \times 25 \times 10 \times 10^6 \times \text{dilution factor} \]

where, (The number of bacteria in the five squares)/5 is the average number of bacteria in the five middle squares (pink), and N/5 × 25 the total number of bacteria in the center (i.e., the total number of bacteria in 0.1 mm3), N/5 × 25 × 10 is the total number of bacteria in 1 mm3, and N/5 × 25 × 10 × 106 the total number of bacteria in 1 L.

The concentration of the stock liquor was 1.5 × 10¹⁰ cells/mL, diluted to 10, 10², 10³ and 10⁴ to obtain 1.5 × 10², 1.5 × 10³, 1.5 × 10⁴ and 1.5 × 10⁶ cells/mL.

Source and concentration of three biological agents

Beauveria bassiana and Metarhizium anisopliae were obtained from Yancheng Shenwei Microbiological Strain Technology Co. Ltd., and B. thuringiensis from Hubei Kangxin Agricultural Pharmaceutical Co., Ltd. The three were diluted as suspensions so that B. bassiana was 2 × 10¹⁰ cells/mL, M. anisopliae 1 × 10¹⁰ cells/mL and B. thuringiensis 1.6 × 10⁸ IU/mL.

Bioassay of O. formosanus with SM1 and three biological agents

Ten O. formosanus workers of similar size were placed in a 7-cm petri dish on a wet filter paper. A 0.12 μL quantity of the different concentrations of biocontrol agents was titrated on the pronotum of each worker; 0.12 μL water was titrated as the control. Each experiment was repeated three times and mortality was observed and recorded every hour in the dark at 27 °C. When the mortality rate of the
control group was approximately 20\%, the experiment was concluded.

To study the combination of the two biological agents and SM1, another ten \textit{O. formosanus} workers of similar size were placed in a 7-cm petri dish, on wet filter paper, and \textit{B. bassiana} (2×10^{10} cells/mL) or \textit{M. anisopliae} (1×10^{10} cells/mL) mixed with SM1 (1.5×10^{10} cells/mL) (volume ratio = 1:1). The same method was used for the bioassay experiments. Each experiment was repeated three times and mortality recorded hourly in the dark at 27 °C. When the mortality rate of the control group was approximately 20\%, the experiment was concluded.

**Statistical analysis**

Data were processed by DPS 4.5 software and LD_{50} with a 95\% confidence interval was obtained. The data were subjected to analysis of variance using InStat software (GraphPad, San Diego, CA) with significance defined as \(P<0.05\).

**Results**

**Determination of SM1 toxicity to \textit{O. formosanus}**

The bioassay results show that SM1 clearly affected \textit{O. formosanus}, and the higher the concentration, the greater the toxicity. In addition, the longer the processing time of SM1, the stronger the toxic effect. The LD_{50} of SM1 at 24 h was 6.66×10^{4} cells/termite, and at 39 h 1.77×10^{4} cells/termite (Table 1).

**Toxicity of \textit{B. bassiana}, \textit{M. anisopliae} and \textit{B. thuringiensis} to \textit{O. formosanus}**

Comparing the titration results for these three biological agents, toxicity levels of the \textit{B. bassiana} (2×10^{10} cells/mL) and \textit{M. anisopliae} (1×10^{10} cells/mL) were significantly different from the control. \textit{B. bassiana} and \textit{M. anisopliae} were more toxic to \textit{O. formosanus}; \textit{B. thuringiensis} (1.6×10^{8} IU/mL) was not different from the controls, i.e., there was no toxicity (Table 2).

Comparing the effects of \textit{B. bassiana} and \textit{M. anisopliae} over 48 to 84 h, the mortality rate with \textit{B. bassiana} reached 50\% about 48 h, while it required 84 h for the mortality rate with \textit{M. anisopliae} to reach 50\%. \textit{B. bassiana} needed approximately 72 h and \textit{M. anisopliae} approximately 96 h to achieve an 80\% mortality rate. Therefore, \textit{B. bassiana} had a stronger insecticidal effect on \textit{O. formosanus} than \textit{M. anisopliae} (Table 2).

**Toxicity of combined biological agents to \textit{O. formosanus}**

The results of the bioassay of the combined biological agents showed that the mixture of \textit{B. bassiana} (2×10^{10} cells/mL) and SM1 (1.5×10^{10} cells/mL) was less effective than using SM1 alone. The effect of the mixture of \textit{M. anisopliae} (1×10^{10} cells/mL) and SM1 (1.5×10^{10} cells/mL) on \textit{O. formosanus} was better than the components used separately. It required 30 h to kill 80\% of the \textit{O. formosanus} using only SM1, but only 26 h with the mixture of SM1 and \textit{M. anisopliae} (Table 3).

**Discussion**

Control of \textit{O. formosanus} is mainly through chemical means and the effective components of pesticides are synthetic organic compounds. These compounds present different degrees of toxicity levels to soils, water and to humans. Although development of the practical application of biological controls is progressing slowly, its environmental and

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Table 1  Toxicity of \textit{S. marcescens} strain SM1 to \textit{O. formosanus}

| Time (h) | Toxicity regression line | LD_{50} (95\% confidence interval) (×10^{4} cells/termite) |
|----------|--------------------------|----------------------------------------------------------|
| 21       | \(y=4.0066+0.9607x\)     | 10.82 (6.04–19.35)                                        |
| 24       | \(y=3.9949+1.2203x\)     | 6.66 (4.23–10.49)                                         |
| 27       | \(y=4.3135+1.3613x\)     | 3.19 (1.64–6.20)                                          |
| 29       | \(y=4.3081+1.3940x\)     | 3.14 (1.62–6.07)                                          |
| 39       | \(y=4.7059+1.1914x\)     | 1.77 (0.58–5.39)                                          |

Table 2  Bioassay of three biological agents on \textit{O. formosanus}

| Time (h) | Control group | \textit{B. bassiana} | \textit{M. anisopliae} | \textit{B. thuringiensis} |
|----------|---------------|----------------------|------------------------|--------------------------|
| 12       | 0.0±0.0 b     | 23.3±5.8 a           | 0.0±0.0 b              | 0.0±0.0 b                |
| 24       | 3.3±2.9 b     | 23.3±5.8 a           | 16.7±5.8 a             | 0.0±0.0 b                |
| 36       | 8.3±2.9 ab    | 26.7±11.6 a          | 16.7±5.8 ab            | 6.7±5.8 b                |
| 48       | 8.3±2.9 c     | 46.7±5.8 a           | 26.7±11.6 b            | 10.0±0.0 bc              |
| 60       | 13.3±2.9 b    | 56.7±5.8 a           | 26.7±11.6 b            | 23.3±5.8 b               |
| 72       | 21.7±2.9 b    | 80.0±0.0 a           | 36.7±20.8 b            | 23.3±5.8 b               |
| 84       | 21.7±2.9 c    | 80.0±0.0 a           | 50.0±10.0 b            | 30.0±0.0 c               |
| 96       | 21.7±2.9 b    | 86.7±5.8 a           | 76.8±5.8 a             | 30.0±0.0 b               |

The average fatalities (%) of \textit{B. bassiana} (2×10^{10} cells/mL), \textit{M. anisopliae} (1×10^{10} cells/mL) and \textit{B. thuringiensis} (1.6×10^{8} IU/mL) to \textit{O. formosanus} were compared using sterile water as the control. Means±SD within rows and followed by different letters are significantly different (Tukey’s test, \(P<0.05\)).
Table 3 Bioassays of the combination of two biological agents and S. marcescens strain SM1 on O. formosanus

| Time (h) | SM1 | Mixture of SM1 and B. bassiana | Mixture of SM1 and M. anisopliae |
|---------|-----|--------------------------------|---------------------------------|
| 25      | 46.7 ± 5.8 a | 23.3 ± 5.8 b | 46.7 ± 5.8 a |
| 26      | 50.0 ± 0.0 b | 26.7 ± 11.6 c | 76.8 ± 5.8 a |
| 27      | 56.7 ± 5.8 b | 30.0 ± 0.0 c | 76.8 ± 5.8 a |
| 28      | 63.3 ± 5.8 b | 30.0 ± 0.0 c | 83.3 ± 5.8 a |
| 29      | 70.0 ± 0.0 b | 46.7 ± 5.8 a | 93.3 ± 5.8 a |
| 30      | 83.3 ± 5.8 b | 63.3 ± 5.8 c | 100.0 ± 0.0 a |
| 31      | 83.3 ± 5.8 b | 70.0 ± 0.0 c | 100.0 ± 0.0 a |
| 32      | 100.0 ± 0.0 a | 76.8 ± 5.8 b | 100.0 ± 0.0 a |

Means ± SD within rows followed by different letters are significantly different (Tukey’s test, P < 0.05)

pollution-free characteristics suggest that it could be the principal method for controlling O. formosanus in the future. S. marcescens is a naturally occurring ubiquitous bacteria and pathogenic to insects. In recent years, numerous studies have been carried out on S. marcescens from various infected insects (e.g. H. armigera, P. rapae, and S. exigua) and significant progress has been made (Liu et al. 1988; Chen et al. 2001; Wang et al. 2010). However, the study of S. marcescens for O. formosanus control has not been reported previously. The strain SM1 was extracted from O. formosanus infected by S. marcescens, and toxicity assays with SM1 were carried out. The results show that the SM1 has an obvious toxic effect.

Of the current biological pesticides for O. formosanus control, B. bassiana, M. anisopliae and B. thuringiensis were selected to treat O. formosanus with 2 × 10^10 cells/mL B. bassiana, 1 × 10^10 cells/mL M. anisopliae and 1.6 × 10^8 IU/mL B. thuringiensis. B. bassiana and M. anisopliae were more toxic. B. bassiana achieved 80% mortality after 72 h, and M. anisopliae 80% after 96 h. These biological control agents can be used in the prevention and control of O. formosanus at the early stage of infestation.

The effects of combined biological agents on O. formosanus have also not been reported before. In this study, B. bassiana (2 × 10^10 cells/mL) and M. anisopliae (1 × 10^10 cells/mL), which had good toxic effects to O. formosanus, were combined with SM1 (1.5 × 10^10 cells/mL). The toxicity of the combination of B. bassiana and SM1 was lower than that of SM1 alone, but the toxicity of the combination of M. anisopliae and SM1 was higher than that of SM1 alone. These results show that different biological agents, when combined into different formulations, will produce different results. Therefore, laboratory experiments must be undertaken to choose suitable combinations of biological agents to avoid antagonism between agents.

This study showed that the S. marcescens strain SM1 was significantly toxic to O. formosanus, but the specific components and mechanisms of this effect need to be further examined. Research has shown that the pathogenesis of S. marcescens is mainly due to chitinase enzymes (Regev et al. 1996; Zhang et al. 2000; Xu and Peng 2004; Yin et al. 2004; Jin et al. 2005), and Tao (2006) has shown that the insecticidal protein of S. marcescens is a metallic protein that exists in the supernatant of the bacteria. At the same time, both the living bacteria of S. marcescens and its secretions are toxic to insects (Yang et al. 2012, 2015). Therefore, a titration experiment on O. formosanus using the different constituents of S. marcescens fermentation will be carried out in a follow-up study.

Based on this study of the S. marcescens strain SM1, this bacterium has considerable potential for the biological control of O. formosanus and needs further study. In addition, this experiment includes only an indoor bioassay of the S. marcescens strain SM1, while the control effects of SM1 on an entire colony have not been studied and should be the subject of future research.

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