Method Article

A method for evaluating the toxicity interaction of binary mixtures

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A B S T R A C T

Improving the formulation of biological insecticides for greater efficiency and competitiveness is of particular importance with respect to the successful application of these agents in the field. In this regard, mixing different agents is known to be an effective strategy for enhancing practical pest control. However, traditional chemical-based control strategies have significant limitations that compromise control effectiveness, as effects tend to become increasingly dose-dependent over time. To overcome such limitations, and thereby ensure the continuous effective control of insect pests, we herein assessed the efficacy of binary mixtures of biological agents, with the aim of establishing an optimal ratio. The optimized mixture showed a significantly higher insecticidal effect, whereby biological pest control was considerably enhanced.

- We combined Beauveria bassiana spores with azadirachtin, a chemical pesticide of botanical origin, and by assessing the efficacy of these two elements combined in different ratios, obtained an optimal formula. Additionally, we evaluated the compatibility between the two elements, and then assessed potential synergistic effects, as determined by the co-toxicity coefficient.
- This protocol is dependent on the results of bioassays. It is not only suitable for combining fungal and chemical agents for the purposes of biocontrol but is also applicable to a variety of biological pesticides.
- The evaluated binary mixture showed enhanced effective insect control. This approach will contribute to reducing applied dosages, thereby promoting efficient pest control and reducing application costs.

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A R T I C L E  I N F O

Method name: A dual agent mixing method using microbial and chemical agents
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Specifications Table

| Subject Area | Agricultural and Biological Sciences |
|--------------|--------------------------------------|
| Method name  | Biological pest control |
| Name and reference of original method | Analysis of mixtures containing two insecticides based on co-toxicity coefficient determinations |

Background

The use of microbial agents for insect pest control has received increasing attention in recent years, as a consequence of increased public awareness regarding residue, resistance, and resurgence (3R) problems, particularly with respect to the resistance of pests to chemical insecticides, and the potential of microbial agents to provide effective environmentally friendly pest control [1]. Accordingly, a variety of biological control organisms are now commercially applied in the field, including: predatory arthropods (or mites), parasitic insects, and insect-pathogenic microorganisms, among which, entomogenous fungi often have the advantage of ease of application and low cost. Therefore, the use of microbial agents has become a preferential strategy for agricultural pest management. As the beneficial effects of microbial insecticidal agents generally develop slowly, mixtures of such microbial agents with botanical insecticides are predicted to be a more effective solution [2]. In this regard, the nymphs of whiteflies (*Trialeurodes vaporariorum*), which do not crawl or move, are particularly susceptible upon exposure to pathogenic microbes, whereas in contrast, once they reach the adult stage, whiteflies can easily avoid microbial infestation by flying. Therefore, entomopathogenic fungi are most effective for the control of whitefly at the nymphal stage.

Method details

**Reagents and equipment**

- Azadirachtin EC 0.5% (*Yunnan Guangming Neem Industry Development Co., Ltd)*
- *Beauveria bassiana* spores (isolate Bb252)
- Tween-80 (*Macklin Co., Ltd)*
- Potato dextrose agar (medium) (*NEST Biotechnology Co., Ltd)*
- Beaker
- Graduated cylinder
- Volumetric flask
- Petri dish
- Absorbent cotton
- Artificial climate box (*RDN-260A; Ningbo Dongnan)*
- Biochemical incubator (*RSZ-10; Ningbo Dongnan)*
- Microscope (*Primotech; ZEISS)*
- Haemocytometer
Preparation

Insects and plants

Trialeurodes vaporariorum individuals were collected from the leaves of tobacco growing in the Fengjie area of Chongqing, China. Identification of the species was based on cytochrome c oxidase (COI) gene barcoding, which confirmed T. vaporariorum (GenBank submission: KX024590) identity. Whiteflies were bred for more than 30 generations in the laboratory on tobacco cv. ‘Yunyan 87’, cultured at 26 °C ± 1 °C and RH = 75% ± 7% under a 14:10 h light/dark cycle. Tobacco plants were used in trials when they had reached a height of 25 cm. Prior to the beginning of the trials, 50 adult whiteflies were released onto a tobacco seedling and allowed to lay eggs for 24 h, after which the adults were removed, and trials commenced when the eggs had hatched and the nymphs had reached third-instar stage.

Fungus and azadirachtin

Beauveria bassiana (isolate Bb252), which has been isolated from a range of herbivorous insect host species (including Tetranychus urticae, Chilo suppressalis, Sitophilus granarius, and Myzus persicae), was used in these experiments and stored at the Biotechnology Centre of Southwest University at −80 °C. B. bassiana was initially isolated from C. suppressalis feeding on maize in the Yongchuan District of Chongqing, China, and was separated into single spores. For all trials, Bb252 was grown in the dark on potato dextrose agar (PDA) medium (pH = 6.5–6.7) at 28 °C ± 1 °C. We applied 0.1% Tween-80 to emulsify the blastospores and determined the concentration using a haemocytometer.

The plant-based pesticide used in the bioassay contained 0.5% azadirachtin EC (Yunnan Guangming Neem Industry Development Co., Ltd).

Experimental procedure

Bioassay of B. bassiana and azadirachtin

A normalized dose-response method was used for evaluating the efficacy of B. bassiana Bb252 and azadirachtin. Determination of fungal pathogenicity was based on a gradient comprising five concentrations of spores: $1 \times 10^4$, $1 \times 10^5$, $1 \times 10^6$, $1 \times 10^7$, and $1 \times 10^8$ conidia/mL. Similarly, the lethality (median lethal concentration, i.e., LC$_{50}$) of azadirachtin was evaluated over a range of concentrations (0.5, 1.0, 5.0, 50, and 500 mg/L), with sterile water being used as the control treatment. Separate batches of 30 third-instar nymphs were immersed three times in the treatment solutions, each for 10 s. Thereafter, the treated nymphs were placed in culture dishes (9 cm diameter) and stored at 27 °C ± 1 °C and RH = 75% ± 7% under a 14:10 h light/dark cycle. Nymph mortality was assessed after 7 days.

Effect of azadirachtin on the growth rate of B. bassiana

An experiment was performed to assess the effects of the aforementioned concentrations of azadirachtin on the growth of B. bassiana. Petri dishes (10 mL) containing PDA medium supplemented with azadirachtin at each of the five concentrations were inoculated with a plug of fungal mycelium, which was cut using a 0.5-cm-diameter) puncher, with five replicate plates being prepared for each insecticide concentration. The dishes were then placed in a dark biochemical incubator (28 °C ± 1 °C, RH = 75% ± 7%). After 9 days, the diameter of the fungal colonies was measured, and inhibition rates were calculated according to the following formula: inhibition rate (%) = (control test)/control × 100. Furthermore, spores on the medium were collected and used to prepare spore suspensions ($1 \times 10^4$ conidia/mL). After a 3-day period of culture (28 °C ± 1 °C, RH = 75% ± 7%), a drop of the conidial suspension was placed in a haemocytometer and the percentage rate of conidial germination was calculated using the following formula: spore germination rate (%) = (number of germinated spores/number of observed spores) × 100 [3].

Toxicity interaction of binary mixtures of azadirachtin and B. bassiana against whiteflies

All toxicity interaction studies were based on LC$_{50}$ values for third-instar nymphs exposed to a 7-day treatment. As binary combinations, azadirachtin and B. bassiana were mixed in the following
Table 1
The virulence of mixtures of different ratios of azadirachtin and Beauveria bassiana against whiteflies and the corresponding co-toxicity coefficients.

| Mixture ratios (A: B) | Regression equation | \( r^2 \) | \( LC_{50} \) (mg/L) | Co-toxicity coefficient (CTC) |
|-----------------------|---------------------|---------|----------------------|-----------------------------|
| 9:1                   | \( y = -0.35x + 50.5 \) | 0.94    | 7.42                 | 54.41                       |
| 4:1                   | \( y = -0.12x + 51.3 \) | 0.99    | 50.63                | 86.60                       |
| 1:1                   | \( y = -3.77x + 54.1 \) | 0.96    | 3.32                 | 104.18                      |
| 1:4                   | \( y = -3.92x + 50.6 \) | 0.93    | 0.22                 | 294.23                      |
| 1:9                   | \( y = -3.90x + 61.1 \) | 0.94    | 2.66                 | 41.03                       |

Note: A: azadirachtin; B: Beauveria bassiana.

ratios: 9:1, 4:1, 1:1, 1:4, and 1:9 [3]. The prepared stock solution was serially diluted 10-fold to give a concentration gradient of 1 \( \times \), 10 \( \times \), 100 \( \times \), 1000 \( \times \), and 10000 \( \times \), and mixtures at these five concentrations were applied in dose–mortality testing, with each binary combination being assessed in triplicate. All samples were incubated at 26 \( ^\circ \)C \( \pm 1 \) \( ^\circ \)C and assessed after 7 days. A linear fitting equation was obtained based on assessments of multiple dilutions, and using this, \( LC_{50} \) values were estimated [4]. Values for the co-toxicity coefficient (CTC), which were based on the 7-day \( LC_{50} \) value for each binary mixture, were used to determine interaction efficiency. A CTC value higher than 120 indicated marked synergism, whereas a value between 80 and 120 was considered cumulative, and a value of less than 80 was taken to be indicative of antagonism [5,6]. As each mixture (M) was formulated with parts A and B, the CTC value for M was calculated as follows:

Toxicity index (TI) of A = 100;

\[ TI \text{ of } B = LC_{50} \text{ of } A/LC_{50} \text{ of } B \times 100; \]

Actual TI of M = \( LC_{50} \) of A/ \( LC_{50} \) of M \( \times \) 100;

Theoretical TI of M = TI of A \( \times \% \) (W) of A in M + TI of B \( \times \% \) (W) of B in M;

CTC = Actual TI of M/Theoretical TI of M \( \times \) 100.

Note: (W) represents the agent ratio in mixture.

Statistical analysis

Average of insect mortality was calculated by linear regression fitting and all the values were corrected with respect to the control using Abbott’s formula (percentage corrected control = (% alive in the control – % alive in the treatment/percentage alive in the treatment) \( \times \) 100) [7,8].

Method validation

Comparison with pesticide control effect

By fitting the data to the virulence equation, the \( LC_{50} \) for the mixed formulations was found to range from 0.22 to 50.63 mg/L (Table 1), whereas CTC values ranged between 41.03 and 294.23. Maximum values for both indices (CTC: 294.23 and \( LC_{50} \): 0.22 mg/L) were obtained for the binary mixture prepared using an azadirachtin to B. bassiana mixing ratio of 1:4, thereby indicating a synergistic effect. However, the \( LC_{50} \) for the azadirachtin treatment alone was 5.76 mg/L (R-sqr=0.998, Chi-sqr = 1.51349). Pesticide used alone required higher doses than mixed formulas to obtain an optimal response.

Observation of fungal infection cycle

To assess the effects of the plant-derived compound azadirachtin on fungal spore germination, scanning electron microscopy (SEM, Hitachi S-3000N10100) was used to observe the conidia that had adhered to the whitefly nymphs, which became infected following spore germination. For tissue fixation, all nymphs were fixed with 2.5% glutaraldehyde. Prior to observation, the samples were dried by vacuum freeze-drying (SJA-10N-60A; Ningbo Shuangjia). Observations revealed that spores which were mixed in the optimal 1:4 ratio had a significant infection rate. The complete spore infection cycle could be observed more clearly in the mixed formula samples (Fig. 1). These results indicate
Fig. 1. Proliferation of *Beauveria bassiana* conidia on whiteflies. 1. *B. bassiana* conidia only. 2. A mixture of azadirachtin and *B. bassiana* at a ratio of 1:4. 1–1 and 2–1: conidia on the cuticle of whitefly nymphs (24 h); 1–2 and 2–2: penetration of germinated conidia (48 h); 1–3 and 2–3: growth of hyphae (72 h); 1–4 and 2–4: hyphae colonising the entire host insect (96 h); 1–5 and 2–5: development of fungal hyphae into a dense network (120 h).
that the combination of azadirachtin and B. bassiana have great potential for application in insect pest control.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2020.101029.

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