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1 INTRODUCTION

Agricultural products are attacked by a large number of arthropod pests that belong to different orders, such as Acari, Diptera, Thysanoptera, Homoptera, Coleoptera and Lepidoptera causing at least 10-30% losses (BIGGS et al. 2000, LIU and SENGONCA 2004). In order to control the pests, broad-spectrum insecticides, e.g. many pyrethroids, carbamates and organophosphates, have been widely used in agricultural ecosystem (OMAR and TIMIN 1983, SHEPARD et al. 2001). However, even its intensive and continued application could not fully suppress pests, and also has disrupted the pest-enemy balance by killing predators and parasitoids as well as led to serious pest resistance to the insecticides (VIKRAM et al. 2000, VALLES 2004).

Due to increasing problems on insect resistance and impacts on non-target organisms, during the past years the research has been shifted towards using biological pesticides based on bacteria, viruses and their toxins that have had striking successes as IPM components (ASOKAN 1996). Unfortunately, field failures and resistances to biocides, e.g. *Bacillus thuringiensis* (B.t.) and Abamectin, a natural product from *Streptomyces avermitilis*, have been documented most recently (WU et al. 2002, TABASHNIK et al. 2003). Thus, there is an urgent need for biocides with different modes of action for rotation program to reduce selection pressure and prolong the usefulness of all products (ZHU et al. 2005b).

GCSC-BtA (**G**ermany-**C**hina **S**cientific **C**ooperation-***B**acillus **t**huringiensis*-**A**bamectin), a new type of biocide, was developed by bioconjugating δ-endotoxin of B.t. with Abamectin through conjugator EDC [1-Ethyl-3-(3-dimethyl-amino-propyl carbodiimide hydrochloride)] for controlling agricultural pests (LIU and SENGONCA 2003a). Results of UV-photo absorption analysis, the death symptom of 3rd instar of *Plutella xylostella* L. (Lep., Plutellidae), the toxicity and LT_{50} against agricultural pests have proved the bioconjugation possibility of GCSC-BtA, which had broader host spectrum and faster killing speed than B.t. or Abamectin did. The potential of GCSC-BtA to be a useful biocide in IPM program has also been recorded with high efficiency against agricultural pests and relative safety to natural enemies (SENGONCA and LIU 2001, 2002, 2003, SENGONCA et al. 2001, LIU and SENGONCA 2002, 2003a, b).

Once a new promising biocide has been formulated, the success of a biological control program of a biocide depends on high efficiency against agricultural pests and low side-effect on natural enemies (ABDEL MEGEED et al. 1998). Moreover, the insecticide toxicity to insects is subject to a number of environmental and insect factors, therefore, to determent optimum application rate for a good efficacy in the field, where a pesticide will achieve maximum effect with minimum active ingredient, it is important to investigate the mode of action of the biocide and the factors affecting its efficiency to pests (ROBERTSON and WORNER 1990). Furthermore, the application of the biocide under more field conditions is necessary to evaluate its efficiency to control a given pest species. However, such sufficient knowledge of GCSC-BtA is still lacking in the literature.

HPLC (High Pressure Liquid Chromatography) is an advanced technology commonly for substance analysis of insecticide, medicine and other organic compounds (NOZAL et al. 2003, LIU et al. 2004). To further study the characteristic of GCSC-BtA, it is necessary to study the linkage process and identify the characteristic of bioconjugated GCSC-BtA by using HPLC method.

The present work aimed, therefore, to study in the laboratory the characteristic of biocide GCSC-BtA by using HPLC, as well as its efficiency on different agricultural pests and their natural enemies in comparison with B.t. toxin crystal, Abamectin and a pyrethroid Cypermethrin. The modes of action of GCSC-BtA on insecticidal activity of stomach, contact, systemic and fumigant toxicities, antifeedant effect of feeding inhibition and deterrency as well as sublethal effect of suppressing subsequent progeny formation against pests were also determined. Further experiments were devoted to investigate the factors affecting its efficiency to pests, such as arthropod species, developmental stage, temperature, exposure time and persistence. Finally, field experiments were carried out to estimate the efficiency of GCSC-BtA against pests and its side-effect on natural enemies in alfalfa pasture, tea garden and broccoli field, comparing with the commercial insecticide Cypermethrin. All these experiments have the goals to enrich application characteristics of this new biocide and so its use in biological control programs against agricultural arthropod pests.

2 MATERIAL AND METHODS

2.1 Laboratory experiments

2.1.1 Rearing of arthropod pests and natural enemies

2.1.1.1 Arthropod pests

For the experiments on efficiency of biocide GCSC-BtA against agricultural pests, stock cultures of pest mites *Tetranychus cinnabarinus* (Boisd.) (Acari, Tetranychidae) and *Tetranychus urticae* Koch (Acari, Tetranychidae) as well as insects *Frankliniella occidentalis* Pergande (Thys., Thripidae), *Aphis fabae* Scopoli (Hom., Aphididae), *P. xylostella* and *Cameraria ohridella* Deschka et Dimić (Lep., Gracillariidae) were established.

The stock cultures of *T. cinnabarinus*, *T. urticae* and *F. occidentalis* were maintained on bean plants *Phaseolus vulgaris* cv. Marona, while *A. fabae* on bean plants *Vicia fabae* cv. Scirocco, under 25 ± 2 °C temperature, 70 ± 10% RH and a 16:8 h (L:D) photoperiod at the Institute of Phytopathology, University of Bonn. Two stock cultures of *P. xylostella* originated from broccoli fields were established by rearing on 5-6 weeks old cabbage plants (*Brassica oleraceae* L. var. *capitata*) in pots (Fig. 1) under the same climate condition above mentioned at the institute and the Biotechnology Center, Fujian Academy of Agricultural Sciences (FAAS), PR China, respectively. The host plants were grown under greenhouse conditions without foliar insecticide application before transferring to climate room. Fresh plants were added weekly, and the old plants were removed when the insects had moved to new plants. For chestnut leaf-miner *C. ohridella*, a colony was established on horse chestnut plants (*Aesculus hippocastanum* L.) under natural condition at the garden of the institute. To obtain uniformly aged individuals for the experiments, the pest adults were collected and allowed to lay eggs or 1st nymph on host plant leaves for 24 h and then removed. Afterwards the leaves were kept in climate chambers at 25 ± 1°C temperature, 70 ± 10% RH and a 16:8 h (L:D) photoperiod for further rearing till the desired stage in the next generation. The required stage of *C. ohridella* was identified under a binocular microscope in the laboratory on leaves obtained form the host plants.

Fig.1: Cabbage plants for the stock culture of *Plutella xylostella* at the Biotechnology Center, Fujian Academy of Agricultural Sciences (FAAS), PR China

2.1.1.2 Natural enemies

For the experiments of side-effect of biocide GCSC-BtA on natural enemies, the stock cultures of three predators *Erigonidium graminicola* (Sundevall) (Araneide, Linyphiidae), *Orius strigicollis* Poppius (Het., Anthocoridae) and *Stethorus cantonensis* Pang (Col., Coccinellidae) as well as a parasitoid *Diadegma semiclausum* Hellen (Hym., Ichneumonidae) were established at the Biotechnology Center, Fujian Academy of Agricultural Sciences (FAAS), PR China.

The stock cultures initiated with few individuals of *E. graminicola* collected from tea garden, *O. strigicollis* from eggplant field, *S. cantonensis* from citrus orchard and *D. semiclausum* from broccoli field in Fuzhou area, Fujian province of southeastern China. The predators were reared in meshed cages with immature stages of *A. fabae* as prey, while the parasitoid was maintained on *P. xylostella* reared on potted cabbage plants in cloth-covered sleeve cage in climatic rooms at 25 ± 2 °C temperature, 75 ± 10% RH and a 16:8 h (L:D) photoperiod. The particular stage or age of the natural enemies used in bioassays was obtained directly from the stock culture.

2.1.2 Using biocides and insecticides

2.1.2.1 Preparation of biocide GCSC-BtA

The preparation of GCSC-BtA was according to the method by LIU and SENGONCA (2003a). B.t. strain LSZ9408 belonging to *Bacillus thuringiensis* var. *kurstaki* was isolated from fields in Fujian province, PR China. The two-phase extract liquid for purification of B.t. toxin crystal consisted of B.t. fermentation liquid:1%Na_2SO_4:CCl_4 = 7:6:7 (vol/vol). Dithiothreitol (DTT) (CAS 27565-41-9) with molecular weight of 78.1 was used to solubilize B.t. toxin crystal. Pure Abamectin powder with average molecular weight of 871.8 was obtained from the standard sample of Zhejiang Biok Biological Co. Ltd, PR China, which contained 95.36% Abamectin B_{1a} ($C_{48}H_{72}O_{14}$) with molecular weight of 873.1 and 3.94% Abamectin B_{1b} ($C_{47}H_{70}O_{14}$) with molecular weight of 860.1. Sodium hydride (NaH) (CAS 7646-69-7) was used to activate Abamectin. Butyric anhydride [$(CH_3CH_2CH_2CO)_2O$] (CAS 106-31-0) with molecular weight of 158.2 was used for carboxylation of Abamectin. 1-Ethyl-3-(3-dimethylaminopropyl carbodiimide hydrochloride) (EDC) (CAS 25952-53-8) with molecular weight of 191.7 was used for biochemical linkage between B.t. protoxin and Abamectin.

To prepare B.t. protoxin (δ-endotoxin), the B.t. toxin crystal was purified from the fermentation liquid by the two-phase extract liquid method modified after BURGERJON (1977) (Fig. 2). One gram purified crystal (Fig. 3) was added to 100 ml of 0.1 mol/l $NaCO_3$-HCl buffer (pH 9.5). Fresh DTT was added to get a final concentration of 25 mM, kept at 37°C for 2 h, which was followed by centrifugating at 16000 rpm for 10 min at 4°C. The upper extract protein liquid was identified by the presence of typical protein bands by sodium dodecyl sulfate-polyacrylamode gel electrophoresis (SDS-PAGE) after the method described by SUN and YU (1996). The final protein concentration was determined by Lowry's method (LOWRY et al. 1951) and adjusted to contain 10 mg/ml B.t. protoxin as a standard solution for GCSC-BtA bioconjugation.

During formation of carboxylated Abamectin (Abamectin-COONa), 2 g of pure Abamectin was solubilized in 100 ml of toluene and then 2 g NaH was added (Fig. 4). The complex was activated immediately by heating at 35°C for 10 min, following by filtrating off the residue of unreacted NaH and vaporizing the residual toluene in the system to get the pure reactive product (expected Abamectin natrium salt). Afterwards, 1 g butyric anhydride was put into the system with the

addition of fresh toluene again to obtain a total volume of 100 ml for carboxylation. The complex added with zeolites (small boiling stone balls) was installed into the water circumfluent condensation device that was attached with a $CaCl_2$ pipe for drying (Fig. 5), and then heated at 111°C for 2 h to produce expected Abamectin-COONa. The reactive product was obtained by filtering off the zeolite and vaporizing the residual toluene. Finally, it was diluted in 100 ml alcohol to make a final concentration of 20 mg/ml equivalent, as a standard solution for GCSC-BtA bioconjugation.

Fig. 2: Two-phase liquid method for B.t. toxin crystal purification (The upper layer was B.t. toxin crystal)

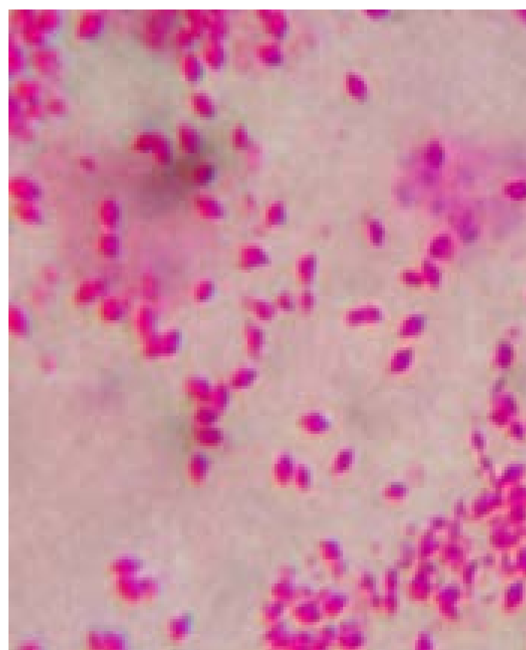

Fig. 3: B.t. toxin crystal (B.t. var. *kurstaki* strain LSZ9408) under light microscope (×1600 times)

To make bioconjugation, 10 ml of the B.t. protoxin (10 mg/ml) was mixed with 10 ml of Abamectin-COONa (20 mg/ml), and then 383.4 mg fresh conjugator EDC was added to obtain a final concentration of 0.1 M. The bioconjugation system was kept at about 25°C room temperature for 24 h (HERMANSON 1996). The bioconjugated substance from the final reaction was named as GCSC-BtA, which consisted of 15 mg/ml active ingredient with equivalents to 5 mg/ml B.t. protoxin and 10 mg/ml Abamectin, as the standard solution for the following experiments. The mechanism of GCSC-BtA bioconjugation is illustrated in Fig. 6.

Fig.4: Removement of unreacted NaH by filter paper to stop Abamectin activation reaction

Fig.5: Water circumfluent condensation device for the formation of Abamectin-COONa

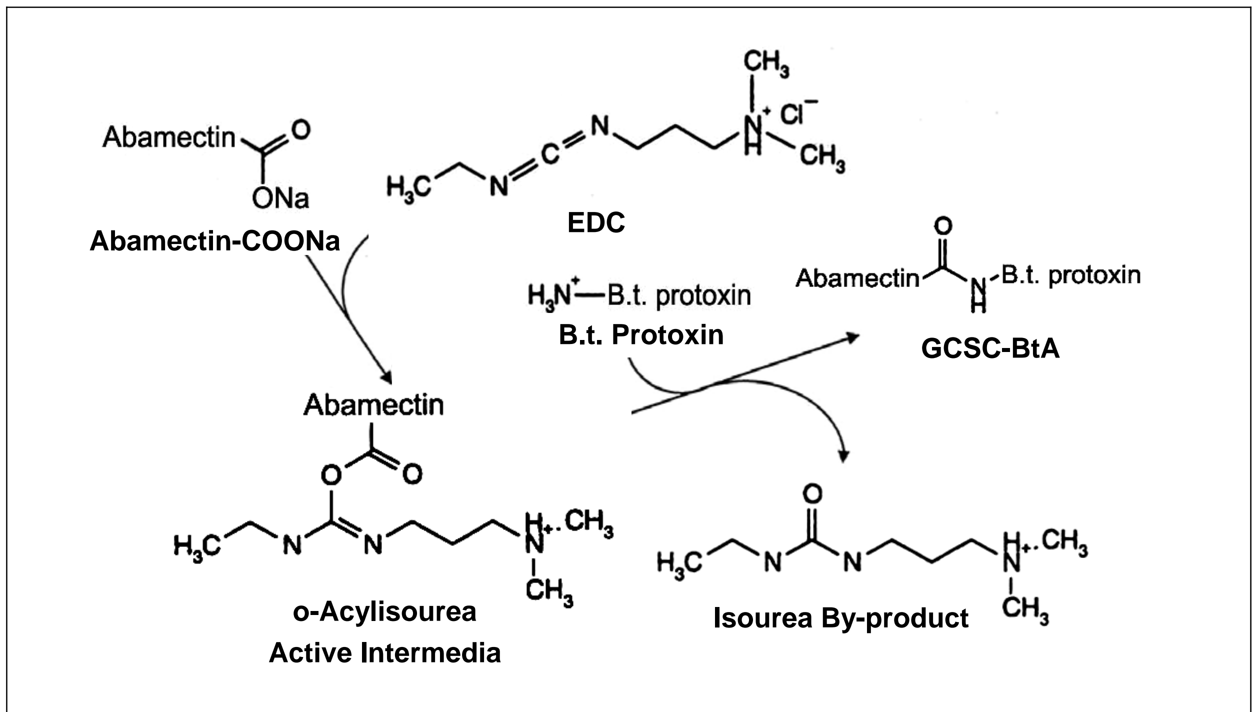

Fig. 6: Bioconjugation mechanism of GCSC-BtA, forming a biochemical linkage between B.t. protoxin and Abamectin through conjugator EDC

2.1.2.2 Other insecticides

B.t. toxin crystal (B.t. var. *kurstaki* LSZ9408) was self-prepared from B.t. fementation liquid by using the same procedure as seen in Fig. 2. Abamectin (a.i. 95.36% Abamectin B_{1a} and 3.94% Abamectin B_{1b}, Zhejiang Biok Biological Co. Ltd, PR China) and the pyrethroid Cypermethrin (2.5% EC, Monsanto, USA) were commercially purchased.

2.1.3 Characteristic of GCSC-BtA

As GCSC-BtA is a new type of biocide created by forming a biochemical linkage between two different toxins through conjugator EDC, it is important to use advance technique (such as HPLC) to study the bioconjugation process, prove the existence of the bioconjugated product and determine its characteristics, leading to better understanding of this biocide and then the application of bioconjugation technique on development of new biocides. Therefore, These experiments were conducted to study the linkage process and identify the characteristic of bioconjugated GCSC-BtA by using HPLC method.

The HPLC used to carry out all analyses was HP1100 (Agilent Technologies, USA) with a G1314A variable-wavelength UV detector. The UV detection was set at 254 nm. Liquid chromatography retention data were obtained from HP ChemStation. The volume of sample injection was 10 µl at each time. The separation was carried out on a C18 column (250×4.0 mm, i.d. 5 µm, Agilent Technologies) using gradient elution with a solvent mixture of water (mobile phase A) and acetonitrile (>99.5%) (mobile phase B) (Merck KGaA, Darmstadt, Germany) at ambient temperature (~25ºC). In all cases, water processed with a Millipore water purifying system was used. The gradient elution of the sample started with 20% B at 1 ml/min flow rate, and the percentage of mobile phase B was increased to 80% within 20 min.

2.1.3.1 Detection of GCSC-BtA bioconjugating process by using HPLC

To demonstrate the formation of reactive product during the bioconjugating process, the GCSC-BtA bioconjugation system consisting of 5 mg/ml B.t. protoxin, 10 mg/ml Abamectin-COONa and 19.2 mg/ml EDC was freshly prepared and allowed to react for 120 h, while the samples with HPLC detection was conducted at each 24 h to analyze the substance transformation at different reactive times. To distinguish the bioconjugation product GCSC-BtA and identify its characteristic, the HPLC analysis of 120 h GCSC-BtA bioconjugation system was carried out in comparison with the three reactants and three reactant combinations prepared for 120 h, which had the same concentrations and prepared synchronously as the bioconjugation system, i.e. separate 5 mg/ml B.t. protoxin, 10 mg/ml Abamectin-COONa and 19.2 mg/ml EDC, as well as the mixtures of 5 mg/ml B.t. protoxin and 10 mg/ml Abamectin-COONa, 5 mg/ml B.t. protoxin and 19.2 mg/ml EDC, and 10 mg/ml Abamectin-COONa and 19.2 mg/ml EDC, respectively. Peak number as well as retention time and retention height of the peaks at each sample was recorded.

2.1.3.2 Qualitative analysis of GCSC-BtA characteristic by using HPLC

To further confirm characteristics of GCSC-BtA, qualitative analysis was conducted as following: 4 ml GCSC-BtA bioconjugation system was prepared and divided into 4 parts of 1 ml. 1 ml of 5 mg/ml B.t. protoxin, 1 ml of 10 mg/ml Abamectin-COONa and 1 ml of 19.2 mg/ml EDC were added into the three 1 ml bioconjugation system respectively, while the untreated one was used as control. HPLC detection of each sample was set out immediately after preparation, and the changes of peak number as well as retention time and retention height of the peaks were observed.

2.1.4 Efficiency of GCSC-BtA on different agricultural arthropod pests and their natural enemies

The success of a biological control program of a biocide depends on high toxicity to target pests