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Establishment of a dietary exposure assay for evaluating the toxicity of insecticidal compounds to *Apolygus lucorum* (Hemiptera: Miridae)☆

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ABSTRACT

With the commercialization of transgenic cotton that expresses Bt (*Bacillus thuringiensis*) insecticidal proteins, mirid bugs have become key pests in cotton and maize fields in China. Genetically engineered (GE) crops for controlling mirids are unavailable owing to a lack of suitable insecticidal genes. In this study, we developed and validated a dietary exposure assay for screening insecticidal compounds and for assessing the potential effects of insecticidal proteins produced by GE plants on *Apolygus lucorum*, one of the main mirid pests of Bt cotton and Bt maize. Diets containing potassium arsenate (PA) or the cysteine protease inhibitor E-64 were used as positive controls for validating the efficacy of the dietary exposure assay. The results showed that with increasing concentrations of PA or E-64, *A. lucorum* larval development time was prolonged and adult weight and fecundity were decreased, suggesting that the dietary exposure assay was useful for detecting the toxicity of insecticidal compounds to *A. lucorum*. This assay was then used to assess the toxicity of Cry1Ab, Cry1Ac, Cry1F, Cry2Aa, and Cry2Ab proteins, which have been transformed into several crops, against *A. lucorum*. The results showed that *A. lucorum* did not show a negative effect by feeding on an artificial diet containing any of the purified Cry proteins. No significant changes in the activities of digestive, detoxifying, or antioxidant enzymes were detected in *A. lucorum* that fed on a diet containing Cry proteins, but *A. lucorum* fitness was reduced when the insect fed on a diet containing E-64 or PA. These results demonstrate that *A. lucorum* is not sensitive to the tested Cry proteins and that the dietary exposure assay is useful for evaluating the toxicity of insecticidal compounds to this species.

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1. Introduction

Undoubtedly, the development and worldwide cultivation of insect-resistant genetically engineered (IRGE) crops that express insecticidal proteins derived from the soil bacterium *Bacillus thuringiensis* (Bt) has revolutionized agriculture (James, 2016). The most widely expressed insecticidal crystal (Cry) proteins in IRGE crops are Cry1 and Cry2 toxins including Cry1Ab and Cry1F in Bt maize (Clark and Coats, 2006; Perry and Tebbe, 2012), Cry1Ac and Cry2Ab in Bt cotton (Sivasupramaniam et al., 2008), and Cry2Aa in Bt rice (Wang et al., 2014a). These crops provide excellent control of economically important lepidopteran pests (Siebert et al., 2008; Tabashnik et al., 2013; Tindall et al., 2009; Xie et al., 2016) but fail to control hemipteran pests.

In China, mirid bugs (Hemiptera: Miridae) have been historically considered as secondary pests in most crops such as cotton, cereals, fruits, and vegetables (Lu et al., 2010a). Mirids, however, have become important pests in China with the use of Bt cotton to control cotton bollworm. The planting of Bt cotton has resulted in a reduced application of insecticides (Wu et al., 2008), which has allowed some mirids, especially *Apolygus lucorum*, to become dominant economic pests of cotton, maize, and other crops (Lu et al., 2010a, 2010b). To date, no IRGE plants conferring resistance to mirids bugs have been commercialized owing to the lack of suitable insecticidal compounds, and the main tactic for the control of mirids is the application of synthetic pesticides. The application of synthetic pesticides, however, causes serious health and environmental problems and also leads to mirid resistance to widely

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used pesticides (Tan et al., 2012a, 2012b). It follows that new strategies for control of mirids are needed, and one strategy involves the development and use of new IRGE crops.

A recently developed cotton variety expresses a Bt crystal protein, Cry51Aa2, that is toxic to *Lygus hesperus* Knight and *Lygus lineolaris* (Anilkumar et al., 2016). Although the commercial use of this variety is still unknown, the development and application of transgenic crops with resistance to mirids would greatly benefit agriculture worldwide. To facilitate the development of additional IRGE crops with activity against mirid pests, researchers must first identify insecticidal compounds with high toxicity to mirids. The identification of such compounds requires the development of a bioassay method that could be used to screen orally active proteins for toxicity to mirids. Although several bioassay methods have been developed for screening and testing the toxicity of insecticidal compounds to mirids, such as the topical exposure bioassay method, the leaf-dip bioassay method, and the glass-vial bioassay method, none of them selected positive controls for validating the sensitivity of the bioassay method and suitable method for monitoring the stability and bioactivity of insecticidal compounds during the experimental process, which is important to accurately evaluate the toxicity of insecticidal compounds to mirids (Liu et al., 2015; Pan et al., 2014).

In the current research, we developed and validated a dietary exposure assay for screening insecticidal compounds against *A. lucorum*. In addition to being useful for screening new insecticidal compounds, this assay system could also be used in the environmental risk assessment of transgenic crops to evaluate the toxicity of insecticidal proteins that are currently expressed by IRGE crops. Although studies on the potential effects of IRGE crops on their nontarget organisms mainly focus on nontarget predatory and parasitic enemies (Azimi et al., 2014; Tian et al., 2015), the effects of IRGE crops on nontarget pests such as mirids and other hemipterans should also be noticed; this will enhance our knowledge on how transgenic crops affect nontarget pests and will provide the theoretical base for the application of IRGE crops in China. In addition to developing and validating a dietary exposure assay, we also used the new assay method to test the effects of the following Cry proteins on *A. lucorum*, which are currently expressed in several transgenic crops: Cry1Ab, Cry1Ac, Cry1F, Cry2Aa, and Cry2Ab.

2. Materials and methods

2.1. Insects

Nymphs and adults of *A. lucorum* were collected from alfalfa fields in 2005 at the Langfang Experimental Station of the Chinese Academy of Agricultural Sciences (CAAS), in Hebei Province, China. A colony was established and maintained in the laboratory on fresh green bean pods (*Phaseolus vulgaris* L.) and fresh maize kernels (*Zea mays* L.) at 25 ± 1 °C and with 70% ± 5% relative humidity (RH) and a 14:10 h light:dark photoperiod. The colony was never exposed to insecticides (Lu et al., 2008).

In 1996, adults of a strain of *Helicoverpa armigera* (96S) were collected from a cotton field in Xinxiang County, Henan Province, China. A colony of this strain, which is sensitive to Bt toxins (Liang et al., 2008), was maintained in the laboratory on an artificial diet (Liang et al., 1999) without exposure to any Bt toxin at 27 ± 1 °C and with 60% ± 10% RH and a 14:10 h light:dark photoperiod.

2.2. Insecticidal compounds and LC_{50} values of Cry proteins to *H. armigera*

The insecticidal compounds used in this study included potassium arsenate (PA, KH_2AsO_4), the protease inhibitor E-64[N-[N-(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl]-agmatine)], and the Bt proteins Cry1Ab, Cry1Ac, Cry1F, Cry2Aa, and Cry2Ab. PA and E-64 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cry1Ab, Cry1Ac, Cry1F, and Cry2Aa were provided by Envirotest-China (an agent for EnviroLogix Inc., Portland, ME, USA; www.envirotest-china.com), and Cry2Ab was supplied by the Biotechnology Research Group, Institute of Plant Protection, CAAS. The production and purification of the Cry proteins were previously described (Li et al., 2015; Wei et al., 2015; Zhang et al., 2014).

To determine the LC_{50} values for use in a sensitive-insect bioassay with *H. armigera* and the five Cry proteins (this bioassay is described later in the Methods section), we added various concentration of each Cry protein or the same volume of phosphate-buffered saline (PBS, 135 mM NaCl, 2 mM KCl, 10 mM Na_2HPO_4, and 1.7 mM KH_2PO_4, pH 7.4) as a control to the *H. armigera* artificial diet. After they were solidified at room temperature, the *H. armigera* diets were cut into pieces, and one piece was placed in each well of 24-well plates (TianJin Xiangyushun Co., TianJin, China) with one neonate larva of *H. armigera*. There were three replicates for each treatment, and each treatment tested 24 neonates. Larval mortality was recorded 7 days later to determine the LC_{50} (toxin concentration causing 50% mortality of larvae) of the Cry toxins. In addition to larvae that were clearly dead, larvae that had not reached the third-instar stage by day 7 were also considered dead. The LC_{50} values of Cry1Ab, Cry1Ac, Cry1F, Cry2Aa, and Cry2Ab toxins were 0.09, 0.06, 1.38, 0.67, and 0.17 μg/g diet, respectively (Supplementary Table S1). These LC_{50} values were used to guide the sensitive-insect bioassay, which is described later.

2.3. Experimental conditions

All experiments described below were conducted in climatic chambers (RXZ-500C, Jiangnan Experimental Equipment, Zhejiang, China) at 25 ± 1 °C and with 70% ± 5% RH and a 14:10 h (light:dark) photoperiod.

2.4. Establishment of a dietary exposure assay for *A. lucorum*

2.4.1. Fitness of *A. lucorum* fed on an artificial diet

The major components of the semi-liquid artificial diet used in the dietary exposure assay included whole chicken eggs, chicken egg yolks, brewer's yeast, toasted soy flour, lima bean meal, and soybean lecithin (Feng et al., 2012; Jin et al., 2013). All diet ingredients were thoroughly blended before the diet was wrapped in pieces of Parafilm (100 μL of diet per piece) that were stretched to 2–3 times their normal size. The packaged diets were stored at −80 °C and thawed at room temperature before they were provided to *A. lucorum*.

The suitability of the exposure assay diet was tested by comparing the survival and development of *A. lucorum* that were fed the diet or a natural food, i.e., fresh green bean pods. One diet capsule or one 2-cm-long section of fresh green bean pod was placed in a glass tube (height 5 cm, diameter, 3 cm) with a folded strip of paper (1 × 5 cm) before one newly emerged *A. lucorum* nymph (less than 12-h old) was placed in the tube, and the tube was covered with a nylon screen (5 × 5 cm). Each diet was replicated four times with 25 tubes and nymphs per replicate. The capsules and green bean sections were replaced every 2 days, and the survival and development of nymphs were assessed daily at 9:00 am.

When the *A. lucorum* nymphs had developed into adults, the newly emerged adults were weighed on an electronic reading balance (AEL-160-11, Shimadzu, Japan, readability = 0.1 mg, repeatability <±0.1 mg). The gender of the adults was determined, and males and females were randomly paired within each dietary

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