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Use of an artificial diet system to study the toxicity of gut-active insecticidal compounds on larvae of the green lacewing *Chrysoperla sinica*

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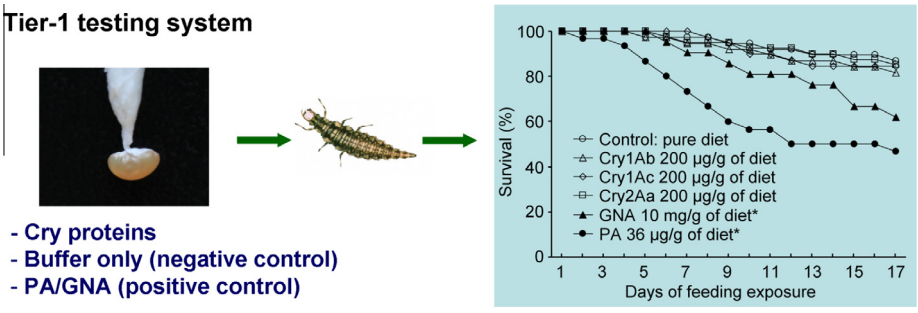
HIGHLIGHTS

- A semi-liquid artificial diet was established for *Chrysoperla sinica* larvae.
- A dietary assay for assessing toxicity of insecticidal compound on lacewing larvae.
- The toxicity of Cry1Ab, Cry1Ac and Cry2Aa proteins to lacewing larvae was assessed.
- *C. sinica* larvae are not sensitive to test *Bt* proteins at high levels.

GRAPHICAL ABSTRACT



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ABSTRACT

A semi-liquid artificial diet was established and found to be a suitable food source for *Chrysoperla sinica* larvae, comparable to aphid prey. Using the artificial diet, we established and validated a dietary exposure assay by using the insecticidal potassium arsenate (PA) as positive control. Dose-dependent responses were documented for all observed life-table parameters of *C. sinica* larvae such as survival rate, pupation rate, larval weight, and larval development time. Thus, the dietary assay can detect the effects of insecticidal compounds on the survival and development of *C. sinica* larvae. Using the established dietary assay, we subsequently tested the toxicity of Cry1Ab, Cry1Ac, and Cry2Aa proteins (which are produced by transgenic maize, cotton or rice plants) to *C. sinica* larvae. Artificial diets containing *Galanthus nivalis* agglutinin (GNA) or PA were included as positive controls. Survival and development of *C. sinica* larvae were not affected when the artificial diet contained purified Cry1Ab, Cry1Ac, or Cry2Aa at 200 µg/g diet. In contrast, *C. sinica* larvae were adversely affected when the diet contained PA and GNA. The stability and bioactivity of the Cry proteins in the diet and Cry protein uptake by the lacewing larvae were confirmed by bioassay with a Cry-sensitive insect species and by ELISA. The current study describes a suitable experimental system for assessing the potential effects of gut-active insecticidal compounds on green lacewing larvae. The experiments with the Cry proteins demonstrate that *C. sinica* larvae are not sensitive to Cry1Ab, Cry1Ac, and Cry2Aa.

1. Introduction

Green lacewings (Neuroptera: Chrysopidae), such as *Chrysoperla carnea* (Stephens) and *Chrysoperla sinica* (Tjeder), contribute to the biological control of crop pests. They are important insect predators, have a wide geographic distribution, and occur in many different crop systems including maize, cotton, and rice (New, 1975; Brooks, 1994; Bai et al., 2005; Jiang and Xiao, 2010). In addition, both species can be easily reared and manipulated in the laboratory and are regarded as useful for laboratory studies that contribute to the environmental risk assessment of plant protection

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products (Romeis et al., 2013b). Green lacewings have been used to assess the non-target effects of insecticides (Nasreen et al., 2007; Sabry and Ei-sayed, 2011), fungicides (Nasreen et al., 2005), and genetically engineered (GE) plants (Hilbeck et al.,1998a,b; Dutton et al., 2002; Romeis et al., 2004; Obrist et al., 2006; Lawo et al., 2010; Li et al., 2008, 2013; Wang et al., 2012; Tian et al., 2013).

For the assessment of the potential effects of GE plants on a non-target organism, the primary step is to evaluate the potential hazard (toxicity) of the plant-expressed insecticidal proteins, such as Cry proteins from the bacterium *Bacillus thuringiensis* (*Bt*), to the selected species (Romeis et al., 2008; Garcia-Alonso et al., 2006; Andow and Zwahlen, 2006). For that assessment, an appropriate experimental system is needed in which defined concentrations of the test compound can be directly fed to the test species (Romeis et al., 2011).

Although artificial diet systems are available and have been used to test the effect of gut-active compounds on the longevity, fecundity, and fertility of green lacewing adults (Li et al., 2008; Wang et al., 2012), no reliable test system has been established for larvae. Lacewing larvae have been commonly tested in tri-trophic experiments in which the tested Cry toxins were indirectly transferred to the larvae through their prey (Hilbeck et al., 1998a; Dutton et al., 2002; Lawo et al., 2010; Li et al., 2013; Tian et al., 2013). Adverse effects were observed in cases where lepidopteran larvae, that were themselves sensitive to the ingested toxins and sublethally affected, were used as prey (Hilbeck et al., 1998a; Dutton et al., 2002; Obrist et al., 2006; Lawo et al., 2010; Li et al., 2013). Thus, effects of the *Bt* toxin on the lacewing larvae were likely indirect, caused by the reduced prey quality as has been reported from tri-trophic studies with other predators and parasitoids (Romeis et al., 2006; Naranjo, 2009). When non-susceptible herbivores were used as prey, no such adverse effects on green lacewing larvae were observed. This includes two studies with *C. carnea*, one using the spider mite *Tetranychus urticae* (Koch) as prey and Cry1Ab as the toxin (Dutton et al., 2002; Obrist et al., 2006), and another using *Bt*-resistant larvae of *Helicoverpa armigera* (Hübner) as prey and Cry1Ac as the toxin (Lawo et al., 2010). In a recent study, Tian et al. (2013) used different strains of Lepidoptera with resistance to Cry1Ac, Cry1Ac/Cry2Ab or Cry1F to expose larvae of *Chrysoperla rufilabris* (Burmeister) to the Cry toxins. Again, no adverse effects on the predator were detected. Two of the studies provide evidence that the prey herbivores contained the Cry proteins in a bioactive form (Obrist et al., 2006; Tian et al., 2013), and thus provide evidence for a lack of direct toxin effects by the tested Cry proteins.

A dietary exposure technique has been described for *C. carnea* larvae by Hilbeck et al. (1998b) in which the test compounds were incorporated into a liquid diet of unknown composition that was then encapsulated within small paraffin spheres. The encapsulated diet, however, could only be accessed by 2nd and 3rd instars of *C. carnea* and lead to an unacceptable high mortality of about 30% in the control group. Using this dietary exposure technique, Hilbeck et al. (1998b) reported adverse effects of Cry1Ab on *C. carnea* larvae. This result, however, could not be confirmed in subsequent studies in which Cry1Ab was directly fed to the larvae by dissolving it in sucrose solution (Romeis et al., 2004; Lawo and Romeis, 2008) or when provided in form of spider mites as prey that had fed on *Bt* (Cry1Ab) maize (Dutton et al., 2002; Obrist et al., 2006).

In the current study, an experimental system was established for evaluating the effects of gut-active, insecticidal compounds on the predatory larvae of *C. sinica*, a common green lacewing species in China. The new experimental system was used to assess the effects of Cry1Ab, Cry1Ac, and Cry2Aa on *C. sinica* larvae. These proteins have been transformed into several crops including cotton, maize, and rice.

2. Materials and methods

2.1. Insects

Specimens of *C. sinica* were collected at the experimental field station of the Institute of Plant Protection, CAAS, near Langfang city, Hebei Province, China (39.5 °N, 116.7 °E) in 2010, and a colony was subsequently maintained in the laboratory without introduction of field-collected insects for several generations. Larvae of *C. sinica* were reared on soybean seedlings infested with *Aphis glycines* Masumura (Homoptera: Aphididae). Adults were fed an artificial diet containing sucrose powder and brewer's yeast at a ratio of 1:1, and water was supplied via saturated cotton balls. Newly hatched (<24 h after emergence) *C. sinica* larvae were used for the experiments.

A *Bt*-susceptible strain of *Chilo suppressalis* Walker (Lepidoptera: Crambidae) was used to test the bioactivity of the Cry proteins. This strain has been maintained on an artificial diet for over 30 generations in the laboratory (Han et al., 2012).

2.2. Insecticidal compounds

Insecticidal compounds used in this study included lyophilized *Galanthus nivalis* agglutinin (GNA, a lectin isolated from snowdrop bulbs), potassium arsenate (PA, KH_2AsO_4), and the *Bt* proteins Cry1Ab, Cry1Ac, and Cry2Aa. GNA and PA were purchased from Sigma–Aldrich (St. Louis, MO), and the *Bt* proteins were purchased from Envirotest-China (agent for EnviroLogix Inc., Portland, Maine, USA; www.envirotest-china.com). The protoxins from *B. thuringiensis* had been expressed as single-gene products in *Escherichia coli* (Cry1Ab and Cry1Ac) or in a cured *Bt* strain (Cry2Aa) at Case Western Reserve University (USA). The protoxin inclusion bodies were then dissolved and trypsinized (except for Cry2Aa), and isolated and purified by ion exchange HPLC followed by desalting and lyophilizing the pure fractions. Purity is about 94–96% (Marianne P. Carey, Case Western Reserve University, personal communication) .

Bioactivity of the Cry proteins was confirmed in sensitive insect bioassays in our laboratory using neonate larvae of *C. suppressalis* that were fed for 7 days with artificial diet containing a range of Cry protein concentrations (for methods see the following Section 2.4.4). The EC_{50} (toxin concentration resulting in 50% weight reduction compared to the control) was estimated to be 26.1, 52.0, and 1310 ng/ml for Cry1Ab, Cry1Ac, and Cry2Aa, respectively.

2.3. Establishment of a dietary exposure test system

2.3.1. Artificial diet

A semi-liquid diet for *C. sinica* larvae was developed based on three diets that were previously used to rear larvae of *C. rufilabris*, *C. carnea*, and *C. nippoensis*, respectively (Cohen and Smith, 1998; Sattar et al., 2007; Li et al., 2010). The diet ingredients are listed in Table 1, and the diet was prepared in five steps: (i) the vitamin solution was prepared with the ingredients listed in Table 1 and was kept at 4 °C until used; (ii) beef and pig liver were weighed, cut into pieces, and subsequently ground in a food blender together with approximately 15 ml of distilled water; (iii) diet ingredients 4–12 were weighed and mixed thoroughly into the mixture of beef and pig liver; (iv) hen egg yolk was mixed with approximately 6 ml of distilled water and then heated to 80–90 °C for 4 min; and (v) once the egg yolk suspension cooled to room temperature, it was thoroughly blended with the other ingredients. At this stage, the insecticidal compounds were incorporated. The diet was then "encapsulated" in three steps: (i) pieces of Parafilm (1.5 × 1.5 cm) were stretched to 2–3 times their normal size; (ii)