
Genetically Modified Crops and Ground Beetles as Nontarget Organisms: Developing Dietary Toxicity Assays for Larvae of *Poecilus chalcites* (Coleoptera: Carabidae).

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Many crops have been genetically modified (GM) to express genes that encode insecticidal traits for control of selected groups of insect pests. Currently, commercialized GM crops for insect control contain genes that encode insecticidal crystalline (Cry) proteins derived from the common soil-dwelling bacterium, *Bacillus thuringiensis* Berliner (Bt). Plants genetically modified to express other insecticidal protectants, such as plant-derived protease inhibitors (PIs), are also being developed and tested for the control of insects, including lepidopteran and coleopteran pests (Hilder et al. 1987, Leple et al. 1995, Gatehouse and Gatehouse 1999). Several insect-protected Bt crops, such as cotton, corn, and potato, have been commercialized over the past decade; and this technology has the potential to reduce the use of synthetic chemical insecticides for crop pest management (James 2003).

An important step in commercializing GM crops is that the potential toxicity of the plant-incorporated protectants (PIPs) to nontarget organisms must be first evaluated in the laboratory under worst-case exposure scenarios (U.S. EPA 1994). Bt Cry proteins and PIs generally have no contact toxicity and must be ingested by a susceptible organism to be effective. Thus, dietary exposure assays are required to assess the potential for toxicity or hazard of the introduced PIPs against test nontarget organisms. In these dietary exposure assays, known amounts of PIPs are incorporated into a diet substrate, from which test organisms ingest the material. Currently, various Bt Cry proteins and some PIs have been tested against several groups of nontarget insects including honey bees, ladybird beetles, lacewings, springtails, and parasitic wasps via direct dietary assays for nontarget hazard evaluation (O'Callaghan et al. 2005).

One group of nontarget organisms that has been largely overlooked in testing is the ground beetles (Coleoptera: Carabidae). The interspecific diversity of behavior and physiological requirements makes ground beetle communities important bioindicators

of habitat quality and agricultural practices (Lövei and Sunderland 1996, Kromp 1999). Furthermore, ground beetles are biological control agents of numerous insect pests and weed seedbanks, as well as an important source of biodiversity within agroecosystems. This lack of investigations with ground beetles is due in part to a lack of effective rearing protocols to maintain most species of ground beetles in laboratory culture (Tomlin 1975, Goulet 1976).

In this article, we describe some of the first research pertaining to using ground beetle larvae as surrogate nontarget organisms for assessing the nontarget impacts of GM crops expressing PIPs. This research focuses on the abundant predatory species *Poecilus chalcites* (Fig. 1) as an indicator species representative of predaceous ground beetles. This ground beetle is commonly encountered in crop habitats throughout North America east of the Rockies, and it is a predator of several insect pests. *P. chalcites* has been considered for use as the surrogate insect predator species in testing with insecticidal proteins (e.g., Cry3Bb1) expressed in transgenic Bt corn for control of coleopteran pests (e.g., corn rootworms, *Diabrotica* spp.) (U.S. EPA 2003).

Ground Beetle Rearing Protocol

The first steps toward developing a dietary exposure assay for nontarget testing with plant-incorporated novel protectants are to have a reliable supply of insects and to develop effective rearing protocols for maintaining and/or culturing test insects in the laboratory (Lundgren et al. 2005). For our study, we collected a large number of adults of *P. chalcites* with dry pitfall traps from agricultural habitats throughout the growing season. Field-collected adults were maintained in the laboratory (30 °C, 65 ± 5% RH, and a 16:8 [LD] photoperiod) and fed an artificial diet developed by Lundgren et al. (2005). The artificial diet consists of water (60.7%), chicken egg (19.7%), cat food (17.3%), agar (1.1%), vitamin (vitamin B12, niacinimide hydrochloride, calcium pantothenate, riboflavin, thiamine hydrochloride, and pyridoxine hydrochloride).



Fig. 1. *Poecilus chalcites* adult and larva.

ride) (0.6%), sorbic acid (0.4%), and tetracycline (0.2%). For egg production, adults of *P. chalcites* were maintained in glass jars containing moist soil with high levels of clay; female beetles readily laid eggs directly into the soil.

Eggs of *P. chalcites* usually take about 3–7 d to hatch at 30 °C. Larvae need to be reared individually in moist soil substrates because they are cannibalistic and very sensitive to dry environmental conditions. Results from our rearing study (Lundgren et al. 2005) indicated that the larvae can successfully complete their development to pupation and adulthood under these conditions, but the substrate is of particular importance to larval survivorship. *P. chalcites* has three stadia and usually requires 21–35 d to pupate, depending on environmental conditions (Lundgren et al. 2005).

Test of the Protease Inhibitor E-64

After developing the rearing protocol (Lundgren et al. 2005), we evaluated the dietary effect of a synthetic cysteine protease inhibitor E64 on larvae of *P. chalcites* by using direct dietary assay. E64, N-[N-(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl]-agmatine, is a thiol protease inhibitor that specifically inhibits papain and other cysteine proteases and has a level of inhibition of cysteine proteases comparable to transgene-derived oryzacystatin in genetically modified rice, oil rape, and potato plants (Fabrick et al. 2002, Ferry et al. 2003). Previous studies showed that coleopteran insects including adult

carabid beetles use predominantly cysteine proteases for protein digestion (Leple et al. 1995, Fabrick et al. 2002). However, potential effects of the cysteine protease inhibitor on larvae of ground beetles have not been evaluated via direct dietary assays.

For testing, we incorporated the protease inhibitor into the ground beetle artificial diet at concentrations of 0, 60, 150, and 600 µg/g of diet. Test diet for each dose treatment was supplied to each larva hosted in the test arena (35 mL plastic cup) and replenished every 48–72 h. Each cup was filled with ≈15 mL of moist soil (≈45% water by weight) to provide the larva with the critical habitat for surviving. Ten to 15 carabid larvae (<3 d old after hatching) were tested for each of the dose treatments, and the experiment was replicated three times. Larvae were monitored weekly for survival and developmental stages (larval instars, pupae, and adults), and biweekly for biomass (weight), until the test ended after 28 d of continuous exposure to the diet treatments.

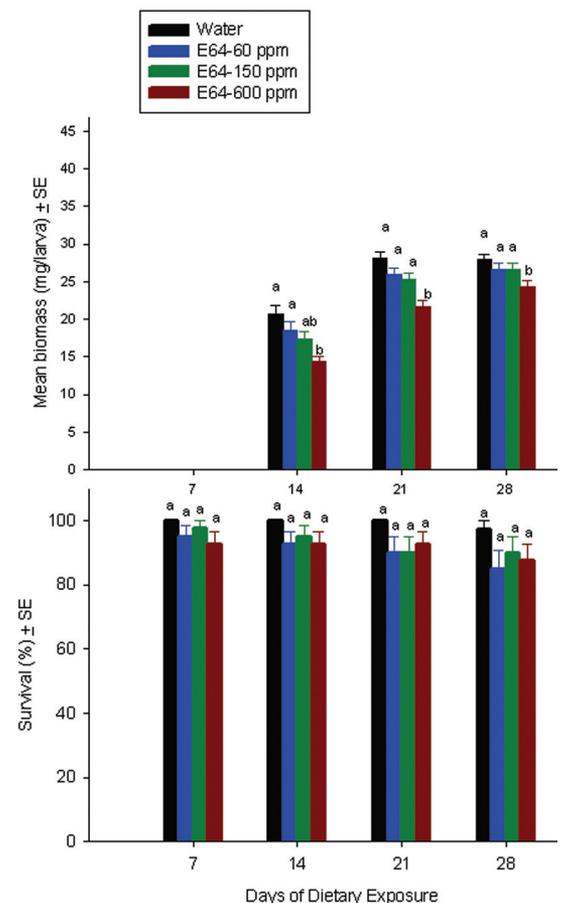


Fig. 2. Mean (\pm SE) survival (%) and biomass (mg/larva) of *Poecilus chalcites* larvae exposed for 7, 14, 21 or 28 d to diets treated with different doses of E-64 (including water control, i.e., diet only). In each assay, 30–45 larvae were tested for each treatment. Bars labeled with the same letter at each observation day within each figure are not significantly different according to likelihood ratio Chi-square tests for percent survival, and multiple mean comparison procedures based on a repeated measure ANOVA for biomass ($\alpha = 0.05$).

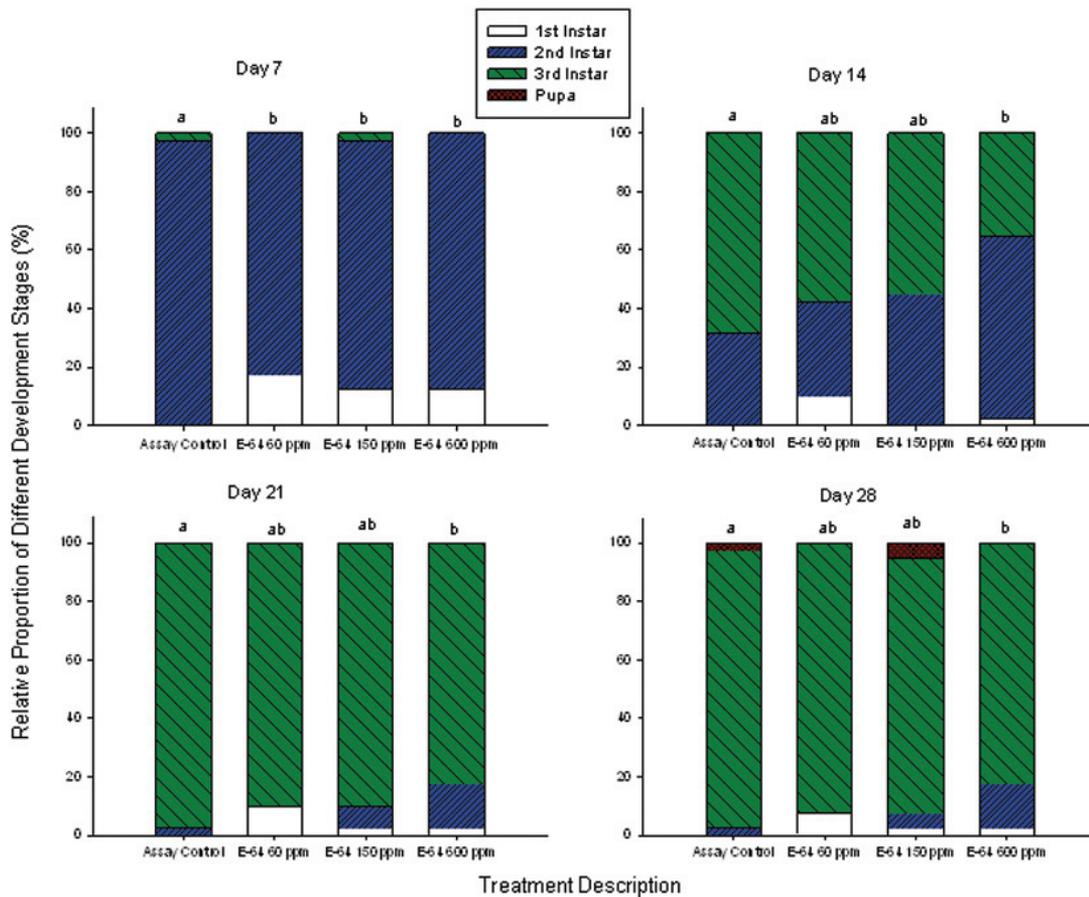


Fig. 3. Developmental stages of *Poecilus chalcites* larvae after 7, 14, 21 and 28 d of exposure to diets treated with different doses of E-64, including diet-only control. Bars labeled with the same letter at each observation day within each figure are not significantly different according to likelihood ratio Chi-square tests ($\alpha = 0.05$). Thirty to 45 larvae were tested for each dose treatment.

Our results revealed that significant adverse effects on larval growth and developmental rates were detected at the highest test dose (600 $\mu\text{g/g}$ of diet) after 14, 21, and 28 d of dietary exposure. However, no significant differences in larval growth and development were observed among the lower test doses (60 and 150 $\mu\text{g/g}$ of diet) and the diet-only control at any of the observation dates (Fig. 2 and 3). Despite causing significant reduction in size and developmental rates, E64 had no significant effect on the survival of *P. chalcites* larvae at the dietary concentrations ranging from 0 to 600 $\mu\text{g/g}$ of diet (Fig. 2).

Summary and Future Directions

This is the first step in developing a framework for assessing the ecological impacts of GM crops on ground beetles: the development of a diet-incorporated assay involving insecticidal proteins and the larvae of an ecologically relevant ground beetle species, *P. chalcites*. This assay involved 28 d of continuous dietary exposure of larvae of *P. chalcites* to an artificial diet treated with known doses of the test substance (E-64). Results from testing with E-64 indicated that the cysteine protease inhibitor adversely affected the growth and development of *P. chalcites* larvae at the high dose

(600 $\mu\text{g/g}$ of diet), but doses <150 $\mu\text{g/g}$ of diet did not measurably reduce the growth and development of *P. chalcites* larvae.

A next step in developing the presented dietary assay protocol is to evaluate the toxicity of the Bt proteins that confer corn-rootworm resistance to corn, including Cry3Bb1 expressed in MON863. In future dietary toxicity assays with novel plant-incorporated protectants, E-64 at the dose of >600 $\mu\text{g/g}$ may be used as a positive control or reference standard to confirm the proper function of the test system.

This research is a critical step toward developing a test that may be useful for evaluating potential hazard or toxicity of insecticidal products such as PIPs to nontarget ground beetles. *P. chalcites* is an important and abundant representative of strictly predaceous ground beetles, but ground beetles are a behaviorally heterogeneous group. It will be interesting to know whether other feeding guilds (e. g., granivores and phytophagous species) respond to the same insecticidal product as do the predaceous ground beetle, *P. chalcites*. Nevertheless, the results of this method development has shown that this important group of insects can be used as a nontarget indicator species of ecological impacts of GM crops expressing insecticidal traits that particularly target beetle pests.

Acknowledgments

We thank Joseph Huesing, Steve Levine, Tom Nickson, John Purcell, Bill Heydens, and Sheila Schuette (Monsanto Company), and Eric Riddick and Wade French (USDA-ARS) for providing comments on earlier drafts of the manuscript.

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