AN ABSTRACT OF THE THESIS OF

Lian Yu for the degree of Master of Science in Entomology presented on May 5, 1995. Title: Effects of *Bacillus thuringiensis* Toxins in Transgenic Plants on the Biology of the Non-Target, Soil Inhabiting Arthropods, *Folsomia candida* (Collembola: Isotomidae) and *Oppia nitens* (Acari: Orbatidae).

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Abstract approved: _____ _____
Brian A. Croft Ralph E. Berry

Genetic engineering makes it possible to express foreign genes whose products are toxic to pests in the target tissues of major crops, for the purpose of sprayless of pest control. However, the large scale field production of toxins has provoked serious concern regarding their possible effects on non-target organisms in the agroecosystem. In this study, two bioassay methods were established to test the effects of *Bacillus thuringiensis* subsp. *kurstaki cry*IA(b) or *cry*IA(c) toxin expressed in cotton and *Bacillus thuringiensis* subsp. *tenebrionis cry*IIIA toxin expressed in potato leaves on two soil non-target organisms, *F. candida* and *O. nitens*. Three biological indices, egg production, the final body length and the time of oviposition, were investigated. Animals fed on the leaves

of transgenic plants did not show significant difference in these indices compared with animals fed on non-transgenic plants. Test animals fed on bean leaves contaminated by heavy metal, cadmium, showed a dose-dependent increase in mortality in the same assay, demonstrating the validity of the assay methods.

Overall, these results suggest that *B.t.* toxin expressed in transgenic plants do not have significant impact on these two testing animals and the bioassay methods established in this study can be applied for the purpose of general environmental monitoring.

Effects of Bacillus thuringiensis Toxins in Transgenic Plants on the Biology of the Non-Target, Soil Inhabiting Arthropods, Folsomia candida (Collembola: Isotomidae) and Oppia nitens (Acari: Orbatidae)

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Lian Yu, Student, Author

DEDICATION

To: Jianqing Yu and Yun Liang

My wonderful parents and best friends

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Chapter 1

Introduction and Literature Review

Modern agriculture uses many insecticides to control pest damage. Most are chemically synthesized. There are many advantages of using synthetic chemical pesticides in agriculture, such as ease of use, quick and consistent effects, etc. However, pest control with pesticides has resulted in problems, including contamination of the environment and pest resistance to pesticides. To achieve pest control in the field, higher doses have to be used to kill resistant pests and this, in turn, further accelerates the development of resistance. As pesticide safety for humans and the environment has increased, other pest control methods have drawn more attention. One is biological control, using *Bacillus thuringiensis* (*B.t.*) Berliner.

B. thuringiensis is a free-living soil bacterium that accumulates high levels of insecticidal proteins during sporulation (Hofte and Whiteley, 1989). Various *B. thuringiensis* isolates have been shown to be selectively

lethal to many lepidopteran, coleopteran, and dipteran insects. Lepidoptera and Coleoptera include the majority of the 10,000 recognized insect pests of agricultural crops (Barton and Miller, 1993).

Bacterial formulations of *B. thuringiensis* have been used for several decades as biological insecticides. World-wide annual production of *B.t.* was estimated at 2.3 million kg in 1987 (Rowe and Margaritis, 1987) and it is believed that production has been increasing ever since. Commercially available bacterial formulations have some disadvantages, such as the relatively high costs incurred by multiple applications and poor persistence due to the instability of the crystal protein.

Advances in molecular biology have identified *B. thuringiensis* genes whose products are toxic to pests and that can be expressed in plants. Cloning insecticidal crystal protein genes of *B. thuringiensis* (Whiteley and Schnepf, 1986) and expressing them in plant-associated microorganisms (Obukowicz et al., 1986) or transgenic plants (Barton et al., 1987; Fischhoff et al., 1987; Vaeck et al., 1987; Adang et al., 1987) has provided alternative methods of pest control.

Several major crops have been genetically engineered to express *B. thuringiensis* insecticidal proteins including tomato, cotton and potato. They have shown promise for providing pest control in the field (Delannay et al., 1989). However, these toxins in crop plants may be released into

agricultural fields in the open environment. Also, they may be expressed in every part of the crop in high amounts, up to 0.1% soluble protein (Perlak et al., 1990), Thus, extraordinary amounts of toxins may be released into the environment which may cause adverse effects such as mortality or reductions in non-target organisms. One group of non-target organisms that may be particularly affected are those living in soil. Because most toxic proteins often end up in soil (except possibly where field-burning is practiced), their impact on living organisms may be substantial. This impact may be direct or indirect. With *B. thuringiensis* toxins, the direct effect against non-target soil organisms may be on primary phytophages and detritivores. In addition, decomposition of residues by bacteria, fungi and other organisms may spread through food chains and cause indirect effects.

The major objective of this research was to quantify the effects of *B. thuringiensis* delta-endotoxin expressed in cotton and potato leaves on two non-target, soil inhabiting arthropods: *Folsomia candida* Willem (Collembola: Isotomidae) and *Oppia nitens* C. L. Koch (Acari: Orbatidae) and to establish a laboratory bioassay system to monitor such effects.

Collembola are an abundant, cosmopolitan group of soil arthropods, which are exceeded in importance only by the Acari (Wallwork, 1970). Collembola are important primary communitors/detritivores in many ecosystems, and play a

major role in dissemination of fungi and in breakdown of feces of larger soil arthropods. They are generally more active than most other soil arthropods (Hale, 1967), and are believed to be an important contributor to metabolic activity in soils. They are found in forests and grasslands worldwide. Collembola have been used in laboratory studies to investigate the effects of different factors on fecundity and population growth (Butcher and Snider, 1975; Van Anelsvoort and Usher, 1989).

F. candida (Willem) has been shown by Goto (1960), Milne (1960), Marshall and Kevan (1962) and Green (1964) to be a parthenogenetic species. Green (1964) found that *F. candida* did not mature until the fifth or sixth instar, about 15 days after hatching. Snider (1973) found that *F. candida* oviposited with great regularity, with alternating productive and non-productive instars. The majority of the intervals between ovipositions encompassed two instars. At 21^0C eggs were laid predominantly in the 6th, 8th, 10th ...instars, and 100% of all females followed this pattern. The number of eggs produced during the first oviposition period is usually small, but the number increases during later periods of oviposition. Adult *F. candida*, fed yeast, produced about 17 eggs during the first oviposition period, and up to 54 during later oviposition periods (Snider, 1973). Its ease of culture and high reproduction rate make *F. candida* ideal for study. The colony used in this study was collected from a local lumber yard near Corvallis,

Oregon.

Oribatoid mites, once thought to be harmless plant feeders, are now known to be of economic significance. They play a key role in adding fertility to forest soils (Sellnick, 1928). An oribatoid, *Oppia nitens* was chosen as a test animal in this study because they play an important role in soil ecosystems. Like *F. candida*, they have multiple stages of development, which can be tested for effects of toxins. Also, their small size, and fast growth made them ideal test animals for laboratory use.

O. nitens, like other oribatoid mites, hatches from the egg into a six-legged larva. They pass through four molts before reaching the adult stage. After the first molt, they enter the first eight-legged nymphal stage, called a protonymph, and subsequently pass through two nymphal stages, the deutonymph and the tritonymph. Each molt is initiated by a quiescent period of variable length. The developmental cycle is strongly dependent on the food it consumes. Stefaniak et al., (1981) cultured *O. nitens* on *Penicillium viridicatum* (Westling) and *Penicillium spinulosum* (Thom) and reported that the females laid eggs 14 days after hatching. *O. nitens* used in these experiments were from a laboratory-adapted colony, colonized from the College of Agriculture, MacDonald College of McGill University, Montreal, Province Quebec, Canada.

Chapter 2

Effects of *Bacillus thuringiensis* subsp. *kurstaki* Toxins Expressed in Cotton Leaves on the Biology of *Folsomia candida* and *Oppia nitens*

ABSTRACT

The effects of *Bacillus thuringiensis* subsp. *kurstaki* delta-endotoxins HD-1 *Cry*IA(b) or HD-73 *Cry*IA(c) expressed in two transgenic cotton varieties (#81 and #249) were evaluated on two non-target soil organisms: *Folsomia candida* Willem and *Oppia nitens* C. L. Koch. Laboratory bioassay methods were developed for both arthropods using a "wet-pouch" technique to maintain high humidity during experiments. Three biological indices: final body length, oviposition period and egg production of *F. candida* and *O. nitens*, were not significantly affected when fed leaves of two transgenic cotton varieties.

INTRODUCTION

Lepidopteran insects are major pests on cotton and are responsible for substantial economic damage. Cotton bollworm, *Heliothis zea* (Boddie), tobacco budworm, *H. virescens* (Fabricius) and pink bollworm *Pectinophora*

gossypiella (Saunders) are three major pests (Perlak et al., 1990; Pendergrass, 1989). Chemical control of these pests is a major expense and problem. In the United States, the average cotton field is sprayed with 5 to 8 insecticide treatments per growing season. Rates of application are escalating because of decreasing effectiveness due to the development of pest resistance to pesticides and emergence of secondary pests.

In response to the problems of insecticide use, cotton growers in the United States and other countries began to devise strategies of integrated pest management (IPM) in the 1970s. IPM involves limited use of insecticides, altering cultural practices to reduce pest populations, planting rapid-fruiting short-season varieties of cotton that are harvested before insect feeding reaches a maximum, and biological control of pests using natural insect pathogens (Adkisson et al., 1982; Frisbie et al., 1989; Green and Lyon, 1989).

One of the most commonly used insect pathogens is *B. thuringiensis*. *B. thuringiensis* is a gram-positive soil bacterium characterized by its production of crystalline inclusion bodies during sporulation. These inclusions consist of proteins exhibiting a highly specific insecticidal activity (reviewed by Aronson et al., 1986 and Whiteley and Schnepf, 1986). When the *B. thuringiensis* toxins are ingested by a susceptible insect, the crystalline inclusion bodies dissolve in the highly alkaline insect

midgut (Broussean and Masson, 1988), releasing one or more insecticidal crystal proteins. Most crystal proteins are protoxins which are activated by proteolytic cleavage and converted into smaller toxic polypeptides. The activated toxin affects midgut epithelium cells of susceptible insects (Fast and Donaghue, 1971; Harvey and Wolfersberger, 1979). Electrophysiological (Harvey et al., 1983) and biochemical (Knowles et al., 1987) evidence suggests that the toxin binds to sites on the microvillar membrane, and generates pores in the cell membrane, thus disturbing the osmotic balance. Consequently, cells within the midgut epithelium swell or collapse, with disintegration of the microvilli and associated tissues (Luthy and Ebersold, 1981). Infected insects stop feeding and eventually die.

There are four major classes of *B. thuringiensis* protein toxins which have been characterized based on structural similarities and insecticidal properties, *Cry*I proteins are toxic to Lepidoptera, *Cry*II proteins are toxic to both Lepidoptera and Diptera, *Cry*III proteins are toxic to Coleoptera, and *Cry*IV proteins are toxic to Diptera (Hofte and Whiteley, 1989). The Lepidoptera-specific crystal proteins are the best-studied. The *Cry*I genes encode 130- to 140-kilodaltons (kDa) proteins, which accumulate in bipyramidal crystalline inclusion bodies during the sporulation of *B. thuringiensis*. These proteins are protoxins. When ingested by a susceptible insect, the protein is solubilized in the alkaline midgut and activated

through cleavage by midgut proteases, releasing a toxic core peptide of about 65 Kda (Whiteley and Schnepf, 1986). The HD-1 isolate of *B. thuringiensis* subsp. *kurstaki* was the first successful commercial product for insect control. Later studies showed that this isolate contained five cry genes and encode five insecticidal proteins, *cry*IA(b) is one of these proteins. Another isolate, HD-73, has only one cry gene, *cry*IA(c). Although *B. thuringiensis* spore/crystal mixtures are toxic to some major pest on cotton, problems associated with field stability and narrow host ranges have limited their use.

Cloning of insecticidal crystal protein genes (Whiteley and Schnepf, 1986) and use of Agrobacterium-mediated T-DNA to transfer *B. thuringiensis* subsp. *kurstaki* toxin genes into cotton plants has provided a new method of pest control for lepidopteran pests. Transgenic plants expressing *B. thuringiensis* subsp. *kurstaki* proteins may cause either feeding avoidance or acute mortality if feeding persists. Expression of *B. thuringiensis*-derived toxin in crops like tobacco and tomato has been achieved. The effectiveness of these plants in controlling susceptible insect pests has been demonstrated (Vaeck et al., 1987; Fischhoff et al., 1987). However, the typical expression level of *B.t.k.*-derived toxin in transgenic plants is low, only about 0.001% of the total soluble proteins. At this expression level, some protection from relatively sensitive insect species was achieved in tomato. But these tomato plants also showed

incomplete protection from less sensitive species, such as tomato fruitworm (Delannay et al., 1989). In contrast, the expression at a low level of *B.t.k.* protein in cotton was insufficient to protect cotton from insects which were relatively sensitive to *B.t.k.* such as cabbage looper. To obtain stronger, more consistent field control, a higher level of expression was required. Wild type *B. thuringiensis* toxin genes have been modified and transferred to cotton plants by Monsanto Co, the Agricultural Group (Chesterfield, MO 63198). Cotton leaves #81 and #249 that express the modified *B.t.k. cry*IA(b) or *cry*IA(c) genes produced a much higher level of toxin protein, up to about 0.05-0.1% of the total soluble proteins, as shown by Western blot analysis. Biological assay results also have shown that at this level of expression, cotton plants can be protected from attack from the least susceptible insects, such as cotton bollworm and beet armyworm (Perlak et al., 1990). These results have demonstrated the technical feasibility of obtaining cotton with insect tolerance to agroeconomically important lepidopteran pests, and may offer farmers an additional weapon to use in an integrated pest management system to reduce insect damage to crops in an environmentally safe way. However, the large scale field production of toxins has provoked serious concern regarding possible effects on non-target organisms in the agroecosystem. The concern is even greater in the case of plants expressing *B. thuringiensis* toxins, because it has already been

demonstrated that the mortality of several other organisms is increased by the bacterial form of *B. thuringiensis* endotoxin, including beneficial and non-target Arthropoda and Nematoda (Flexner et al., 1986). Pratt et al. (1993) showed that cotton leaves expressing *B. thuringiensis* toxins had biological activity even after 30 days of decomposition in soil, suggesting the unexpected persistence of these toxins in the environment. The risks to soil ecosystems are probably greatest under conservation tillage regimes, which represent a substantial and growing sector of United State agriculture (Hendrix et al., 1986).

In this study, an experimental bioassay system was developed and used to investigate the impact of *B. thuringiensis* subsp. *kurstaki* HD-1 *cry*IA(b) and HD-73 *cry*IA(c) expressed in cotton leaves on two soil arthropods: *Folsomia candida* and *Oppia nitens*. Three biological indices were measured during this study: time of oviposition, final body length and egg production.

MATERIALS AND METHODS

Folsomia candida

Preparation of culture container and experimental apparatus

Cultures of *F. candida* were maintained in plastic containers (3.14 cm^2 x 3.0 cm) with snap-on lids. Plaster-of-Paris was used as the substrate to provide moisture since these soil arthropods require nearly 100% relative humidity (RH) to survive and reproduce (Searls, 1928). Activated charcoal was mixed with the Plaster-of-Paris at a ratio of 1:10 to facilitate removal of waste gases, and to provide a background color against which the white bodies of *F. candida* could easily be detected (Wharton, 1946). Distilled water was added to the mixture (10%, W/W) of Plaster-of-Paris and activated charcoal to make a slurry. This mixture was poured into containers to about 5 mm in depth. The mixture was allowed to air dry to form a solid base. Distilled water then was added to near saturation. To keep the container moist, a small amount of distilled water was added twice a week.

The experimental apparatus, a nanocosm, consisted of 24 chambers (3.4 ml/chamber) in a plastic box. A 1.2 cm diameter hole was cut in the bottom of each chamber and filled with the Plaster-of-Paris and charcoal mixture as

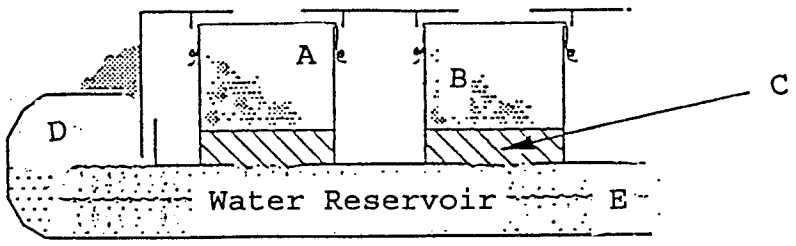


Figure 1. Experimental apparatus for testing *F. candida*. A: nanocosm. B: Mixture of soil and leaves. C: Permeable adsorptive base. D: wet pouch. E: towel.

described above. A towel was placed on the bottom of the plastic container. The nanocosms sat above the towel which was saturated with water and provided constant water supply to the nanocosms (Fig. 1). Each chamber was covered with a plastic film which was perforated with six 1 mm diameter holes. This structure maintained moisture and provided adequate gas exchange. To prevent water loss, edges of the nanocosm and plastic container were sealed with playdough.

Preparation of milled cotton leaves

Six-week-old transgenic cotton, #81 and #249 which has an expected relative expression rate of 0.05-0.1% soluble protein (Perlak et al., 1990), and non-transgenic cotton, coker, were obtained from the United States Environmental Protection Agency (US-EPA) at Corvallis, Oregon (provided by Monsanto Co, Chesterfield, Mo 63198). Leaves from plants

were cut and put in a plastic bag and placed in an -80^{0}C refrigerator until they were frozen. After freezing, leaves were compressed and broken into sizes appropriate for milling and placed on dry ice. Leaves were milled in a hardened steel Micro-Mill^R Grinder at 14,000 revolutions per minute for 60 seconds, then stopped for 2 minutes. The grinder was pre-cooled and cooled continuously during use to a temperature of between -20^{0}C to -35^{0}C, using an external circulating methanol bath fueled with solid dry ice. This process was repeated 3 times. Processed leaves were stored in a plastic box at 80^{0}C until they were used.

Experimental animals, food supply, and climatic conditions

F. candida of similar ages were obtained as follows: 10-16 adults were randomly assigned to each plastic container. More adults per container resulted in a reduction in the number of eggs produced per *F. candida*. After 2 days, all adult *F. candida* were removed, and the eggs which had been oviposited were allowed to develop. Eggs hatched in about 2 week and 6-day-old juvenile *F. candida* were used in all experiments. For food, granulated dry yeast was added to stock containers once or twice a week. To avoid microbial contamination, only a small amount of food was supplied each time. All *F. candida* were maintained in the laboratory at 21^{0}C (range 19-23^{0}C), in a photophase of 12:12 hr.

Experimental design

An important part of the experiments was the environment in which test animals were exposed to toxin. A good compromise was might between uncontrolled ecological conditions in the field, and simplistic conditions which are typical of classical toxicology. The former are difficult to reproduce and interpret, and require many months to stabilize the mesocosm before testing can begin. The latter offer advantages of direct interactions between toxin and test organism, but may not reliably indicate whether such interactions would occur in the field, especially in the case of soil microfauna which only thrive in physically complex microenvironments. We devised appropriately small microcosms with sterile soil which permitted the natural interactions between leaf exudes (containing toxin, at least initially), soil solids but which minimized variable microbial attack on the decaying leaf material. The presence of a physically complex but , microscopically porous substratum, greatly promoted the viability and reproduction of these soil arthropods, This substratum was an important condition necessary to reveal the possibly harmful effects in the added material.

To test the bioassay method and to provide a positive control to document toxic effects, *F. candida* were exposed to the heavy metal, cadmium, and the resulting effects on growth and development were measured. Bean leaf discs (13 mm

diameter) were soaked in different concentrations of cadmium nitrate, then added to a nanocosm chamber which contained 0.7 g autoclaved Hermiston soil (collected from the Hermiston Agricultural Research and Extension Center, Hermiston, Oregon) and a single adult *F. candida*. After 5 weeks, the total number of *F. candida* in each chamber was counted.

To test the effects of *B.t.* toxin expressed in cotton, 0.3 g of milled cotton leaves were added to each container containing 6-day-old juveniles at the beginning of the experiment. One week later, 24 juvenile *F. candida* were transferred to separate nanocosm chambers, one in each chamber. Before adding the juvenile to each chamber, 0.7 g of autoclaved Hermiston soil and 0.3 g of milled cotton leaves were added. Fresh cotton leaves were cut from six-week-old transgenic cotton plants of two varieties: 1) transgenic cotton #81 which expresses the modified *cry*IA(b) gene of *B. thuringiensis* subsp. *kurstaki* HD-1, and 2) transgenic cotton #249 which expresses the modified *cry*IA(c) gene of *B. thuringiensis* subsp. *kurstaki* HD-73. Leaves from six-week-old non-transgenic cotton plants were used as controls. Leaves in nanocosm chambers were changed every week. All juvenile *F. candida,* produced by a single adult, were counted and removed during the experiment. After 7 weeks, experiments were terminated. The body lengths of adult *F. candida* were measured at the end of the experiment. *F. candida* were collected from chambers by injecting a

mixture of printing ink that was saturated with diethyl ether into each chamber and gently stirring it into soil with a small needle. The *F. candida* floated quickly to the surface. Because of the black ink background, *F. candida* could be seen easily and picked up using a small brush. Individuals were then transferred to adhesive coated slides for body length measurement under a 10x microscope. There were three replicates, each treatment was represented eight times. The results were analyzed by One-Way Analysis of Variance using StatGraphics 5.0 (STSC inc, 1991).

Oppia nitens

Preparation of rearing container

Soil organisms vary in their requirements for humidity and a surface water film that supports maximum growth and reproduction. The method used to culture *O. nitens* must provide high humidity along with convenience for microscopic examination and ease of manipulation. Traditional methods for providing the appropriate water tension have been labor intensive and demanded *ad hoc* provisioning. In this experiment, a system which automatically provided different water tensions through a continuous supply of water-saturated air was developed to rear and test *O. nitens*.

The apparatus for rearing the mites consisted of eight small plastic containers and a "wet-pouch" (Fig. 2).

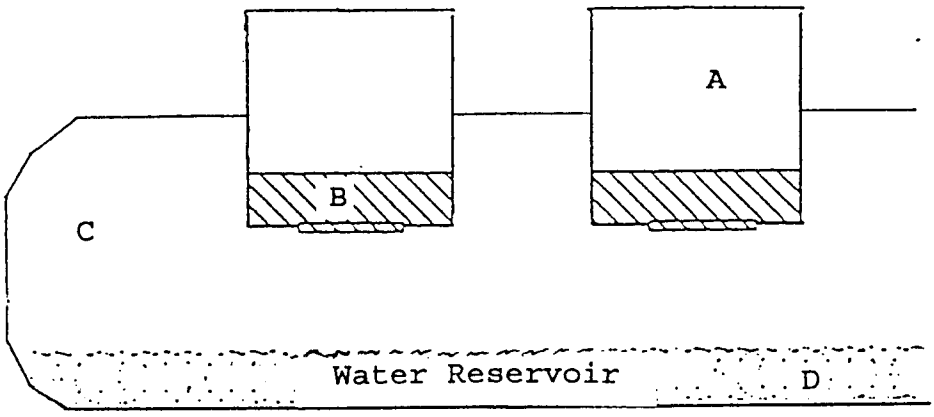


Figure 2. Experimental apparatus for rearing *O. nitens*. A: plastic container (3.14 cm^2 x 3.0 cm). B: permeable base. C: wet pouch. D. towel

The small plastic containers (3.14 cm^2 x 3.0 cm) with snap-on lids were used as rearing containers. A 1.2 mm diameter hole was cut in the bottom of the container. The mixture of Plaster-of-Paris and charcoal, mixed at a ratio of 10:1 provided the base for the culture, as described previously. The wet-pouch was a 1.5 liter plastic container covered with a snap-on lid. Six 3.5 cm diameter holes were cut in the lid. A towel was placed on the bottom of the wet-pouch, and distilled water was added just above the towel. The small rearing containers were placed on the wet-pouch above the water reservoir.

The experimental apparatus consisted of a nanocosm, a

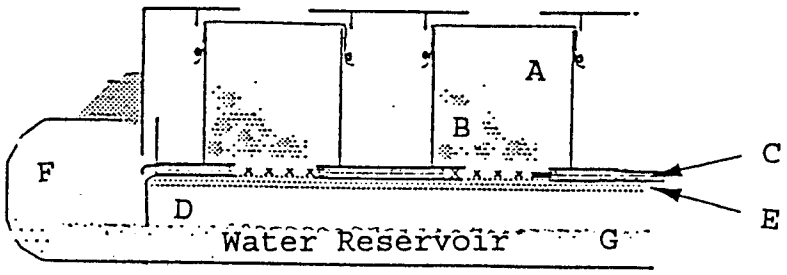


Figure 3. Experimental apparatus for testing *O. nitens*. A: nanocosm. B: mixture of soil and leaves. C: 0.1 mm pore nylon screened foramina. D: platform. E: agar. F: wet pouch. G: towel.

nylon screened foramina, a platform, and a wet pouch as shown in Fig. 3. The wet pouch was prepared by putting a towel on the bottom of a 1.5 liter plastic container. The lid of the container was cut to a size which just fit the nanocosm. The wet pouch was filled with water and this kept the towel water saturated but without free water on it. Each nanocosm consisted of 24 chambers (3.4 ml/chamber). A 1.2 mm diameter hole was cut at the bottom of each chamber. A gasket with 0.1 mm pore nylon screened foramina was placed under the nanocosm and a platform made from the lid of the nanocosm was placed beneath the gasket. A 1.2 mm diameter hole was cut in the lid of each chamber which was then filled with 2.5% agar to about 3 mm deep. When the agar was solidified, the lid was turned upside down. The nanocosm,

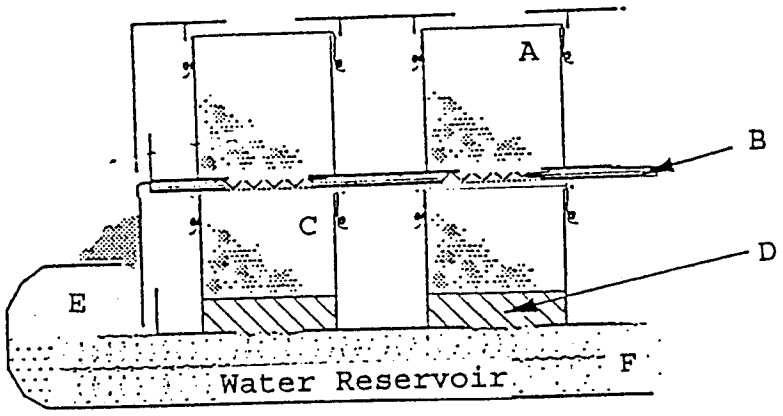


Figure 4. Experimental apparatus for collecting *O. nitens*. A: testing nanocosm. B: 1.0 mm pore screened foramina. C: trap chamber. D: permeable adsorptive base. E: wet pouch. F: towel.

filled with 2.5% agar to about 3 mm deep. When the agar was solidified, the lid was turned upside down. The nanocosm, gasket and platform were held together with a rubber band. reservoir. When the experiment was completed, the gasket was replaced with a 1.0 mm pore nylon screened foramina which allowed the mites to pass through very easily. The platform was replaced with a trap chamber. The trap chamber was a nanocosm with 10:1 mixture of Plaster-of-Paris and charcoal. Also a 1.2 mm diameter hole was cut in the bottom of each chamber. A small amount of brewers yeast was added as a food

source. By increasing the rate of water vapor loss through the roof of the chamber, all the mites migrated slowly out of the soil and through the nylon screened foramina into the trap nanocosm below, where they were easily recovered for counting (Fig. 4).

Preparation of milled cotton leaves

Six-week-old transgenic cotton, #81 and #249 and non-transgenic cotton, Coker, were obtained from the US-EPA Laboratory at Corvallis, Oregon (provided by Monsanto Co) and cotton leaves well milled as described previously and stored at -80^{0}C until used.

Experimental animals, food supply, and climatic conditions

Oppia nitens was reared in stock culture. In this experiment, 10 mites were placed in each chamber of the nanocosm. Brewers yeast was added to the stock containers twice a week. To avoid microbial contamination, only a small amount of food was supplied each time. The experimental apparatus was put in a large plastic box. The box was covered with aluminous foil to darkened the chamber. The box was kept in an incubator at a constant temperature of 23^{0}C (range 22-24^{0}C), 80-90% humidity.

Experimental design

The exposure of *O. nitens* to transgenic leaf substrate was very similar to that of *F. candida* suggesting that normal growth and development of *O. nitens* occurred under experimental conditions. No positive assay was used to assess the toxic response of *O. nitens* to a toxin such as cadmium as was done with *F. candida*.

In this experiment, 0.7 g of autoclaved Hermiston soil and cotton leaf discs 13 mm diameter or 0.3 g of milled leaves were placed in each chamber of the experimental nanocosm in such a way that the soil surface was covered with a leaf disc or milled leaves. Ten newly emerged adults were transferred to each chamber of the experimental nanocosm. Because of the high humidity, fungi grew very rapidly on the milled cotton leaves, and were very easily detected by the naked eye. After about 7 weeks, the total number of offspring was counted. There were three replicates, each treatment was represented eight times.

In this experiment, three different cotton plants were tested: 1) transgenic cotton #81; 2) transgenic cotton #249; and 3) non-transgenic cotton. The results were analyzed by One-Way Analysis of Variance using StatGraphics 5.0 (STSC inc, 1991).

RESULTS AND DISCUSSION

Folsomia candida

The experiment with Cadmium indicated that there was a reduction in animal numbers in each chamber due to decreased survival and reproduction (F= 224, P< 0.001. Fig. 5). The response was dose-dependent. This positive control indicated that the assay method could serve as a general bioassay to monitor effects of toxins on soil inhabiting insect species.

Feeding on cotton leaves expressing *B. thuringiensis* subsp. *kurstaki* toxins did not have a significant effect on the time of the first three oviposition periods of *F. candida* (Fig. 6) (first oviposition: F= 0.83, P= 0.44; second oviposition: F= 1.63, P= 0.204; third oviposition: F= 1.16, P= 0.32). Also the number of eggs produced per female was not affected by feeding on transgenic cotton leaves (Fig. 7) (first batch of eggs, F= 1.637, P= 0.20; second, F= 2.14, P= 0.13; third, F= 0.89. P= 0.42). Final body length of *F. candida* after feeding on leaves of normal verses transgenic plants do not differ significantly at 5% level (F= 0.651 P= 0.5247) (Fig. 8).

Overall, results showed that *B. thuringiensis* subsp. *Kurstaki* toxins *cry*IA(b) and *cry*IA(c) expressed in cotton did not significantly affect egg production, time of oviposition and final body length of *F. candida*.

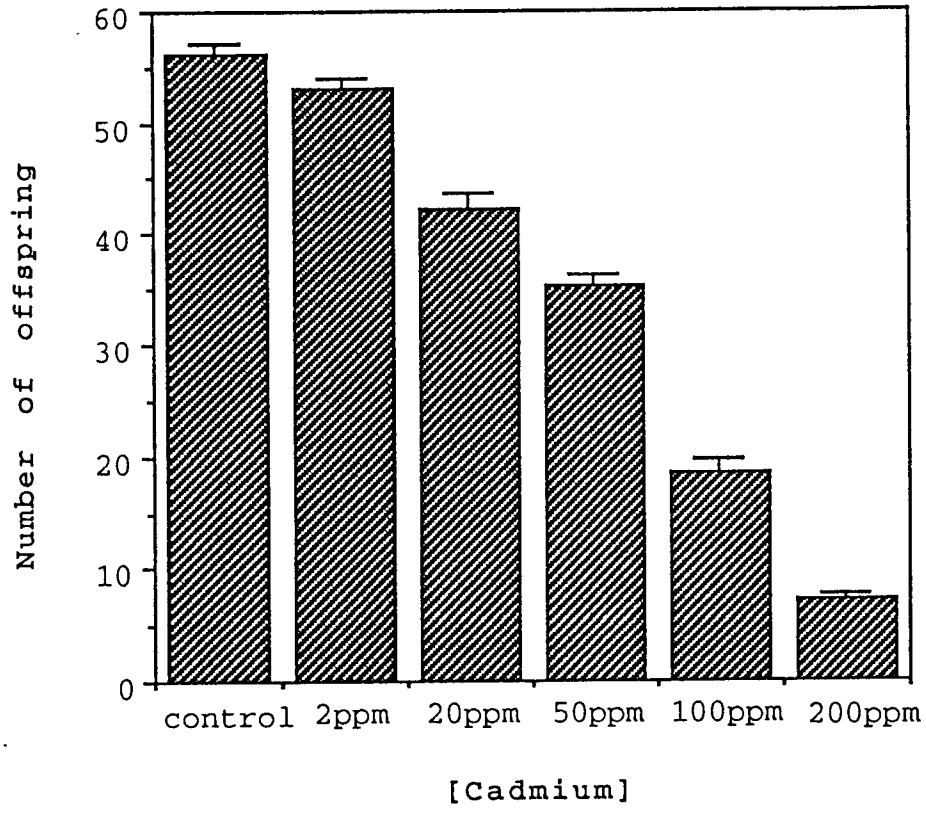


Figure 5. Effects of feeding on different concentrations of Cadmium-treated bean leaves on *F. candida*. Data represent mean ± S.E. (n= 144).

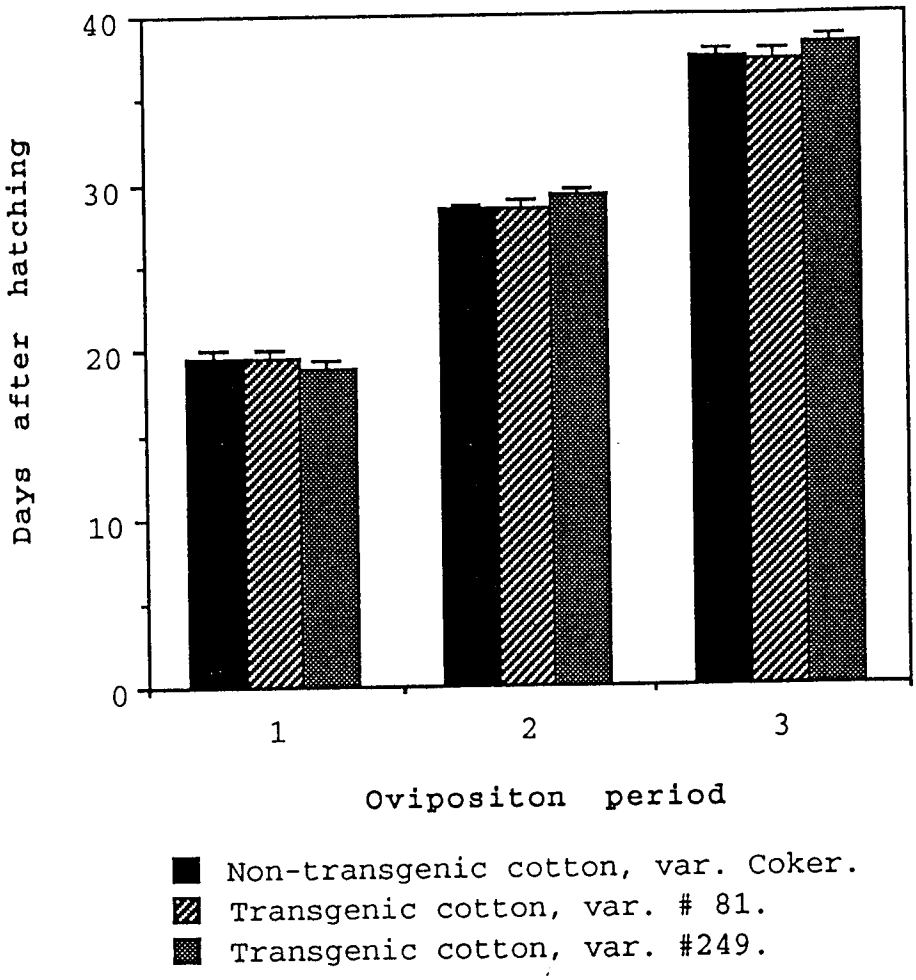


Figure 6. Effects of feeding on transgenic cotton leaves expressing *B. thuringiensis* toxins on *F. candida* reproductive maturity. The beginning of the first three oviposition periods (days after hatching) were compared for each treatment. Results represent mean ± S.E. (n= 72). Number 1, 2 and 3 represents the results of the 1st, 2nd and 3rd oviposition periods.

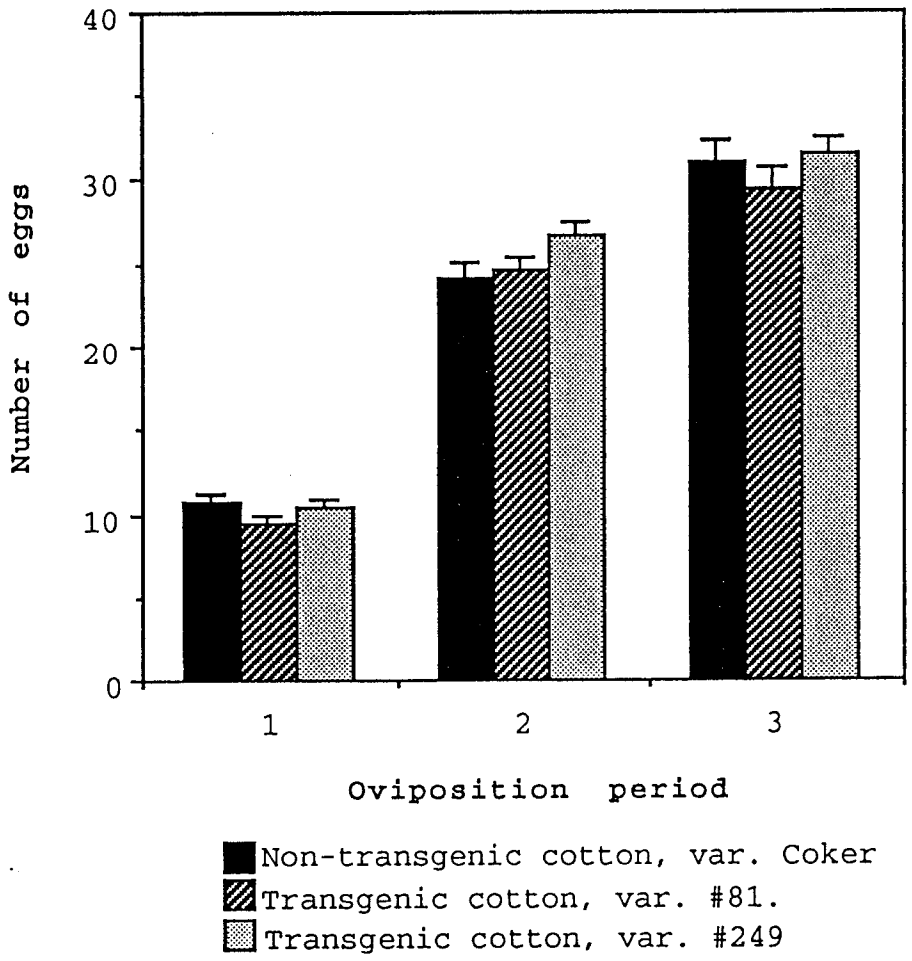
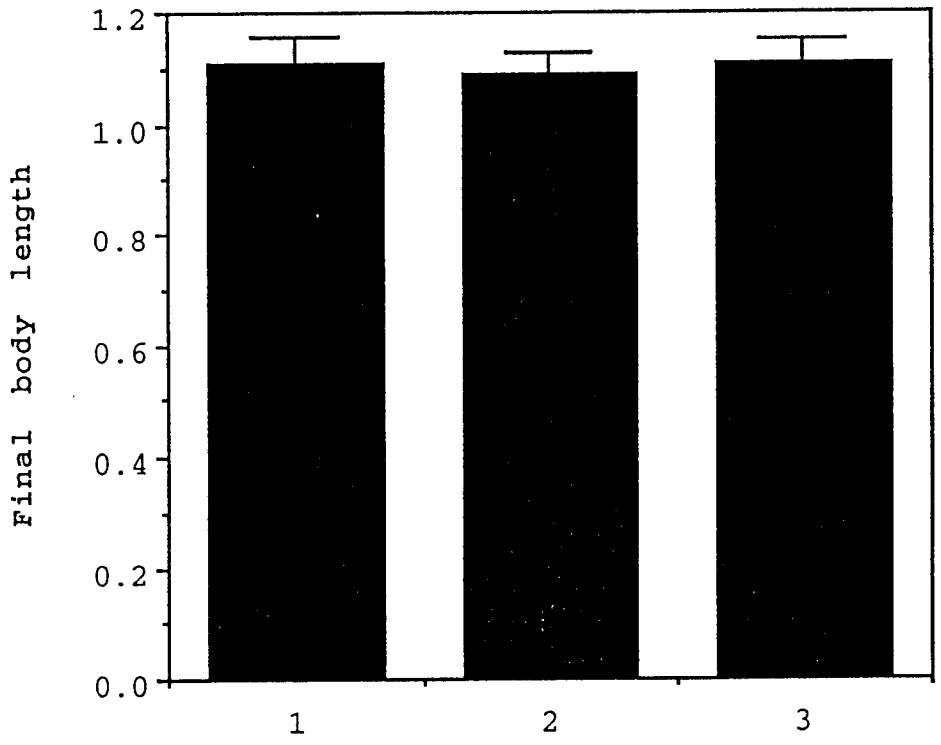


Figure 7. Effects of feeding on transgenic cotton leaves expressing *B. thuringiensis* toxins on *F. candida* egg production. The number of eggs produced through the first three oviposition periods were counted. Results represent mean ± S.E. (n= 72). Number 1, 2 and 3 represents the results of the 1st, 2nd and 3rd oviposition period.



1. Non-transgenic cotton var. Coker
2. Transgenic cotton var. #81
3. Transgenic cotton var. #249

Figure 8. Final body length of *F. candida* after feeding on non-transgenic and transgenic cotton (#81 and #249) leaves. Data represent mean ± S.E. (n= 72).

Collembola have a wide variety of diets, but most soil forms feed on decayed vegetation and/or microflora. In these experiments, cotton leaves were provided as the only food source. Direct consumption of leaf powder by the *F. candida* was observed as the transparent animal guts became green after they were fed on cotton leaves while their guts remained yellowish when yeast was the only food source. The number of eggs produced during the first three oviposition periods by sixth, eighth, and tenth instars in the control group were relatively low, compared with previously published data on the biology of *F. candida* when fed yeast (Snider, 1973). This may be explained by the different food provided in these studies. Fresh cotton leaves are probably not a preferred food, compared with yeast. However, I observed that eggs were first laid in the fourth week immediately after *F. candida* molted to the sixth instar, which was similar to that reported by Snider (1973).

It has been reported that some strains of *B. thuringiensis* are lethal to eggs and larvae of several non-target zooparasitic nematodes, *Trichostrongylus colubriformis* and the free-living worm, *Caenorhabditis briggsae* (Bottjer et al., 1985; Ciordia and Bizzell 1961; Bone et al., 1985). Toxins from *B. thuringiensis* subsp. *kurstaki* also reduce population growth of the free-living nematode *Turbatrix aceti* (Meadows et al., 1990). The lack of negative side-effects observed in this study may be explained as follows: first, the *B. thuringiensis* toxin may

not be very stable and may decay rapidly after leaves are cut, but this explanation does not agree with Pratt et al., (1993) who found that transgenic cotton leaves still had biological activity 30 days after decomposition in soil. Second, *F. candida* may not be susceptible to *B. thuringiensis* derived toxins. Additional experiments are needed to directly bioassay the toxicity of *B. thuringiensis* toxins on *F. candida* using purified toxin proteins at higher levels. Finally, other non-target species may be more susceptible to *B. thuringiensis* than *F. candida*. Should this be the case, similar bioassay with other soil organisms should be assessed in the future.

Oppia nitens

Oppia nitens is mycophagous and different food sources have different effects on its development and growth. Farahat (1966) reported that fungal spores are commonly found packed in the gut of *O. nitens*. When *O. nitens* was fed *Penicillium viridicatum* and *Penicillium spinulosum,* adults became pigmented after two days, they reached sexual maturity comparatively early, and laid large numbers of eggs. Here, *O. nitens* was first fed only on non-transgenic Coker (control) cotton leaf discs. Although *O. nitens* fed on the discs, most died or did not lay any eggs. Therefore, it was concluded that cotton leaves alone were not a suitable

food.

Fungi are the preferred food of *O. nitens* (Stefaniak et al., 1981). Because of the high humidity that was maintained in experimental chambers, fungi grew rapidly on milled cotton leaves. Mites also grew very well. Milled cotton leaves were subsequently used as a standard food source. When feeding on this food, it took about 5 weeks from egg to adult, and females began laying eggs after 2 to 3 weeks.

After establishing standard rearing conditions, the effects of mites feeding on transgenic cotton leaves and fungi were tested (Fig. 9). After two months, reproductive rates for mites fed on transgenic cotton leaves and associated fungi were slightly lower than that for the control, but the differences were not significant (final adult: $F= 1.298$, $P= 0.28$; final nymph: $F= 0.853$, $P= 0.43$).

Base on the results, it was concluded that *B. thuringiensis* toxins expressed in cotton do not affect normal growth of *F. candida* and *O. nitens*. It is important to recognize that *O. nitens* is involved in both direct and indirect uptake of toxins. In this study secondary conversion of *B. thuringiensis* toxins, by fungi did not generate any adverse effect on *O. nitens*. In this experiments, test animals were exposed to *B. thuringiensis* toxins for 2 months. While these studies show safety to two key non-target arthropod in soils, the ecological effects of transgenic plants on more species, with longer exposure, need to be studied before we can be confident that they are

safe to the environment.

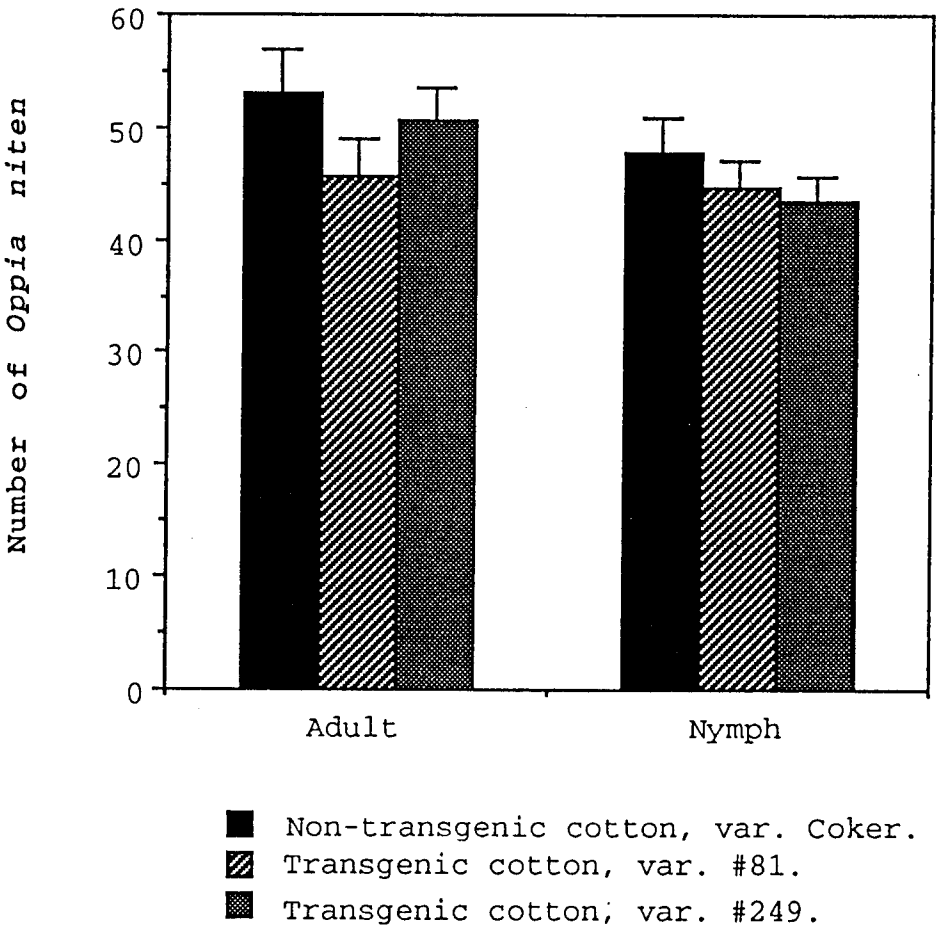


Figure 9. Effects of feeding on transgenic cotton leaves and fungi growing on them on reproduction rate of *O. nitens*. After two months feeding on cotton leaves or fungi,the number of adults and nymphs produced during the experimental period were counted separately. The results are mean ± S.E. (n= 72).

Chapter 3

Effects of *Bacillus thuringiensis* subsp. *tenebrionis* Toxin Expressed in Potato Leaves on the Biology of *Folsomia candida*

ABSTRACT

Experiments were conducted to investigate the effects of *Bacillus thuringiensis* subsp. *tenebrionis*, *cry*IIIA expressed in transgenic potato leaves, on the soil arthropod, *Folsomia candida* (Collembola: Isotomidae). The "wet-pouch" technique was used as the bioassay method. No significant differences were observed in the biological indices: egg production, final body length and the time of oviposition between *F. candida* fed non-transgenic potato leaves and transgenic potato leaves.

INTRODUCTION

The potato ecosystem is attacked by many different species of insects and mites. Radcliffe et al. (1991) listed 170 arthropod species as potato pests in North America alone. In high input potato production systems, acceptable control of these pests usually can be obtained only with use of insecticides (Raman et al., 1992). The Colorado potato

beetle (CPB), *Leptinotarsa decemlineata* (Say), is one of the major defoliating pests of potatoes in the northeastern United States (Ferro, 1985).

Genetic engineering provides the opportunity to express foreign genes whose products are toxic to pests in the target tissues of major crops, for the purpose of pest control without the use of chemical insecticides. It has been known that *B. thuringiensis* subsp. *tenebrionis* produces rhomboidal crystal protein, *cry*IIIA, that is characterized by its high unit activity and specificity for certain coleopteran insects. The coleopteran-specific *cry*IIIA genes encode proteinaceous protoxins of 72-kDa. It is processed to an approximately 66-kDa toxin by a proteolytic cleavage of 57 amino-terminal residues (Mcpherson et al., 1988). It has been reported that *cry*IIIA forms cation-selective channels in lipid bi-layers, and disturbs insect ion balance and causes larval death. The wild type *cry*IIIA gene was first transferred and expressed in potato leaves. Although potatoes expressing this wild-type *B.t.t. cry*IIIA gene showed protection from insect damage to some degree, higher toxin levels in potato leaves are obviously needed to achieve good and consistent field protective effects. Recently genetic modifications of wild type *B.t.t. cry*IIIA genes was made by Monsanto Co to achieve higher expression. Russet Burbank potato plants which have been genetically engineered to express the modified *cry*IIIA gene are nearing commercial availability. The expression level of the

modified *Cry*IIIA protein in Russet Burbank potato is about 0.1% of the total protein, as estimated by Western blot immunoassays analysis. Results from laboratory tests have demonstrated that this transgenic potato can provide protection from damage by the Colorado potato beetle (*Leptinotarsa decemlineata*). Field tests also have shown dramatic levels of protection at multiple field locations. Analysis of these genetically modified potatoes indicated that they conform to the standards for Russet Burbank potatoes in terms of agronomic and quality characteristics, including flavor (Perlak, et. al., 1993).

It is generally believed that when transgenic plant gene products are introduced into the soil, they are likely to degrade so quickly that they will have little impact on the ecosystem. However, some products will be more recalcitrant and thus may exhibit potentially adverse effects on the ecosystem. Actually, it has been reported that even after 30 days decomposition in soil, *B.t* toxins expressed in plant leaves still have biological activity (Pratt et al., 1993). Therefore, before transgenic potatoes are introduced into the field on a large scale, their effects on soil organisms, should be evaluated because some of the toxins may eventually end up into soil.

In this study, the effects of *B. thuringiensis* subsp. *tenebrionis cry*IIIA insect control protein expressed in Russet Burbank potato on the biology of soil arthropod, *F. candida*, were investigated.

MATERIALS AND METHODS

Preparation of culture container

Cultures of *F. candida* were maintained in plastic containers (3.14 cm^2 x 3.0 cm) with snap-on lids. Containers were filled 5 mm deep with a 10:1 (weight/weight) Plaster-of-Paris and charcoal mixture. The mixture was prepared by adding distilled water to make a pouring slurry which was allowed to air dry to form a solid base in the bottom of the plastic containers. Distilled water was added to the dry base to near saturation and added twice a week to maintain the moisture level. The experimental nanocosms were prepared as described in Chapter 2.

Preparation of milled potato leaves

Transgenic potato and non-transgenic potato were planted and grown in the laboratory (provided by Monsanto Co). The transgenic *B.t.t. cry*IIIA potato leaf has an expected relative expression rate of 0.1% soluble protein (Perlak et al., 1993). In this experiment, six-week-old plants were used. The leaves were cut from the plant and milled to leaf powder as described in chapter 2. The leaf powder was stored at -80^{0}C until it was fed to the test organisms

Experimental animals, food supply, and climatic conditions

F. candida of the same age were obtained by allowing adults to lay eggs in plastic containers. After 2 days, all adults were removed from containers and eggs were left in the container. Six-day-old juveniles which hatched from these eggs were used for experiments.

Granulated dry yeast was added to the stock culture once or twice a week. To avoid microbial contamination, only a small amount of food was supplied at each feeding. All *F. candida* in the stock culture and those used for experiments were maintained in the laboratory at 21^0C (range 19-23^0C), in a photophase of 12:12 hr.

Experimental design

To begin the experiment, 0.3 g milled potato leaves were added to each container containing six-day-old juveniles. After one week, 24 juvenile *F. candida* were transferred to the nanocosm chambers, one juvenile in each chamber. Before placing *F. candida* in the chambers, 0.7 g of autoclaved Hermiston soil (collected from the Hermiston Agricultural Research and Extension Center, Hermiston, Oregon) and one 13 mm diameter potato leaf disc was added. In each test, leaves from both transgenic Russet Burbank potato expressing the modified *cry*IIIA gene from *B. thuringiensis* subsp. *tenebrionis*, and non-transformed Russet

Burbank potatoes (control) were used. After 2 months, the number of eggs produced by a single adult were counted and the body length of each adult was measured. *F. candida* were collected by injecting a mixture of diethyl ether-saturated diluted printing ink to each chamber. After gently stirring the mixture with a small needle, the white body of *F. candida* floated to the surface. Because of the black background, *F. candida* was easily seen and picked up with a small brush. They were transferred to adhesive coated slides to measure body length. There were two replicates, each treatment was represented twelve times. The mean values were analyzed by Two-Sample Student t-test using StatGraphics 5.0 (STSC, 1991).

RESULTS AND DISCUSSION

Effects of *B. thuringiensis* subsp. *tenebrionis* delta-endotoxin *cry*IIIA expressed in transgenic Russet Burbank potato on *F. candida* were assessed for three parameters: oviposition time, egg production and final body length. There were no significant differences in any of the three indices measured when *F. candida* was fed non-transgenic or transgenic Russet Burbank potatoes. In three oviposition timing tests, *F. candida* began laying eggs at the same time (first oviposition: P= 0.86; second oviposition: P= 0.18; third oviposition: P= 0.18) (Fig. 10). Although *F. candida* that were fed non-transgenic potato leaves produced more eggs than those fed on transgenic leaves, the difference was not statistically significant (P= 0.089, 0.138, and 0.259 for three oviposition tests, respectively) (Fig. 11). After the 2 month experimental period of development, the final body length was about the same between the two groups of *F. candida* fed non-transgenic and transgenic potato leaves (P= 0.1) (Fig. 12).

Studies on the effects of insecticides on collembola to date have emphasized their usefulness as a bioassay tool. A number of investigators have used collembola to assess the toxicity of insecticide residues in soils (Way and Scopes 1965; Griffiths et al., 1967). Thompson and Gore (1972) concluded that *F. candida* was a useful test organism for

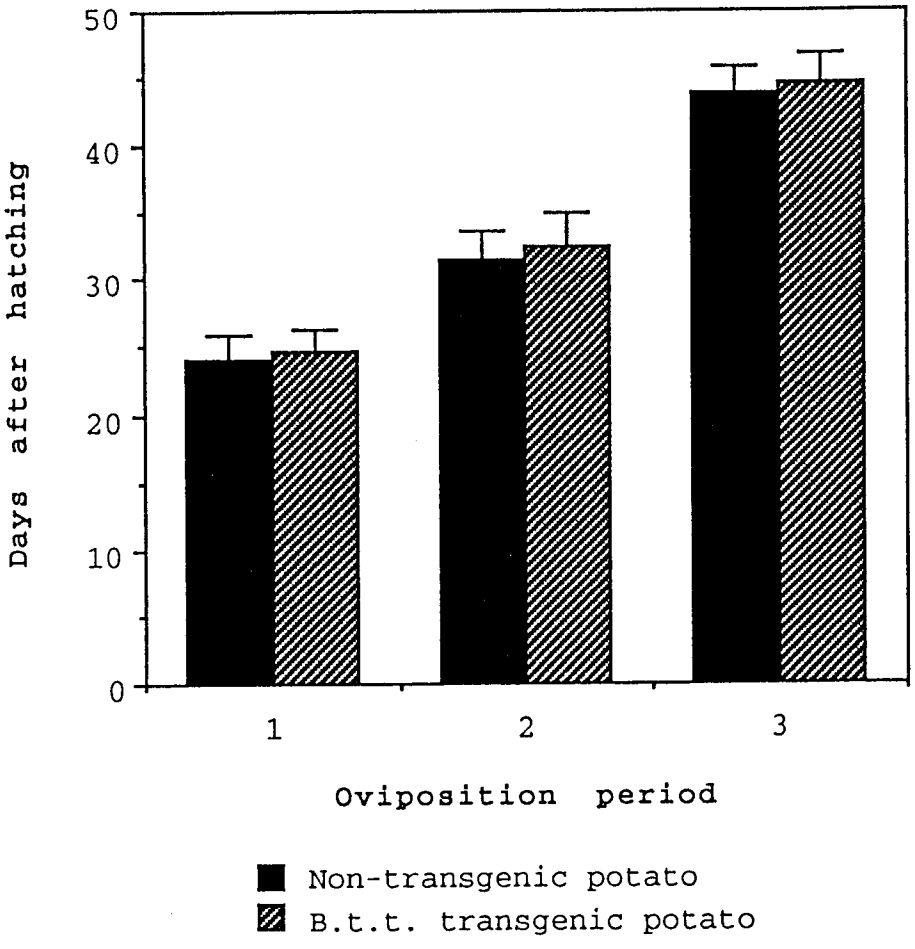


Figure 10. Effects of feeding on potato leaves expressing modified cryIIIA toxin gene on *F. candida* reproductive maturity. Newly hatched juveniles were fed leaves of non-transgenic potatoes and transgenic *B.t.t* potatoes. The time of the first three oviposition periods was recorded. Results are expressed as mean ± S.D. (n= 48). Numbers 1, 2 and 3 represents the results of the 1st, 2nd and 3rd oviposition periods.

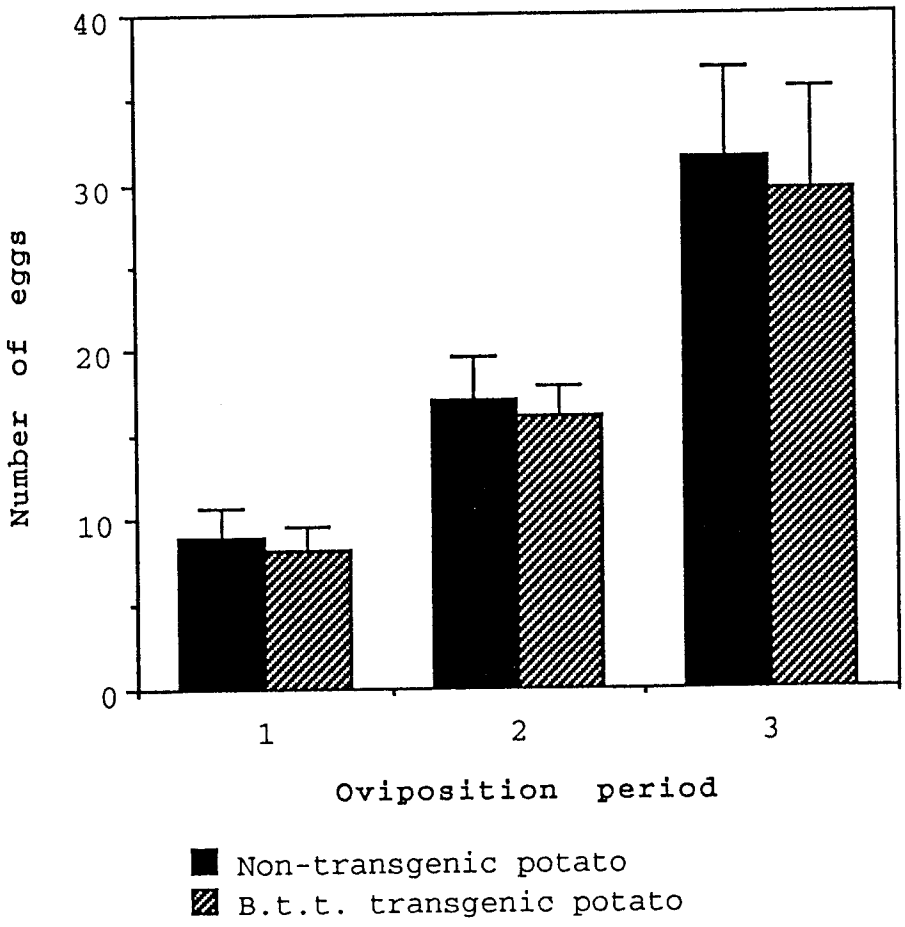


Figure 11. Effects of feeding on transgenic potato leaves expressing modified *B.t.t. cry*IIIA toxin gene on *F. candida* egg production. Six days old juveniles were fed on potato leaves from control and transgenic plants (*B.t.t* potato). The number of eggs produced through the first three oviposition periods were counted. Results are expressed as mean ± S.D. (n= 48). Numbers 1, 2 and 3 represents the results of the 1st, 2nd and 3rd oviposition periods.

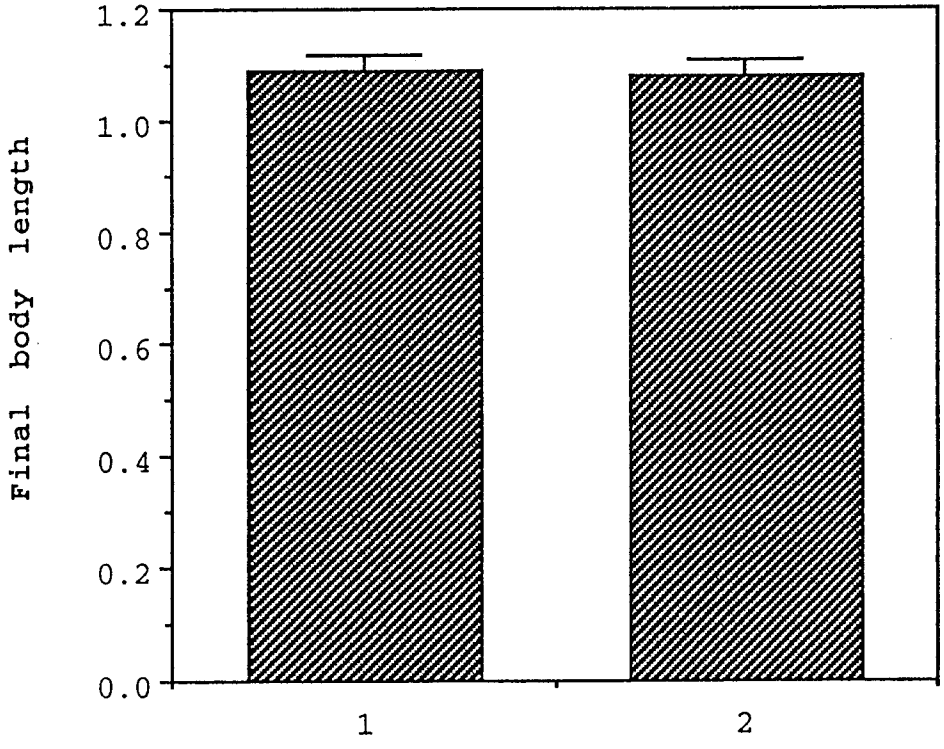


Figure 12. Effects of feeding on transgenic potato leaves expressing modified *cry*IIIA toxi gene on *F. candida* final body length. Six days-old juveniles were fed on leaves from non-transgenic and transgenic *B.t.t.* potatoes. Final body length increase through the seven weeks experimental period was measured. The results are expressed as mean ± S.D. (n= 48).

assessing insecticide toxicity either by direct contact or when incorporated into soil. But in this experiment, I evaluated the effects of transgenic potato plants on *F. candida* by feeding them on fresh transgenic or non-transgenic potato leaves. When I compared the results from the control group with Snider's study (1973), I observed that the number of eggs produced by the sixth, eighth, and tenth instars fed on control potato leaves was lower than those fed on yeast in Snider' study (1973). But the time of first oviposition of *F. candida* in my experiment was similar to results reported by Snider (1973).

The levels of *B.t.t.* toxin expressed by the transgenic potato was up to 0.1% of total soluble protein, it shows high toxicity to it's target insect. Perlak et al., (1993) has shown that transgenic Russet Burbank potatoes provided dramatic protection from damage by the target insect Colorado potato beetle, *Leptinotarsa decemlineata*. From this experiment, we do not know if the non-target soil arthropod *F. candida* are susceptible to *B.t.t.*-derived toxin or if the *B.t.t.* toxin decays very rapidly after the leaves are cut.

CHAPTER 4

SUMMARY

Two laboratory bioassay systems were developed in this study using *Folsomia candida* and *Oppia nitens* as indicator species to evaluate the effects of feeding on transgenic cotton and transgenic potato leaves on egg production, oviposition period and final body length of these non-target soil arthropods.

Bioassay systems are useful methods, which can be used to monitor fate of toxic proteins like the *B. thuringiensis* toxin in the environment. Several techniques developed during this study made use of the bioassay systems for *F. candida* and *O. nitens* more effective. The "wet-pouch" techniques successfully solved the problem of maintaining moisture control for these soil arthropods by changing the moisture in the nanocosm. We developed a simple technique to collect mites from soil in the nanocosm through changing the moisture in the soil to drive mites to a collection chamber. Injecting a mixture of printing ink and ether to locate and measure *F. candida* and *O. nitens* was an improvement. The bioassay for each of these species can be used for the general purpose of monitoring environmental stress on soil animals.

Three types of *B. thuringiensis* derived toxin proteins expressed in two important agricultural crops, cotton and

potato, were tested for their ecological impact on the two non-target soil arthropods. Three biological indices: oviposition period, fecundity, and body length were assessed. Arthropods feeding on leaves from either of the two transgenic plants directly and/or fungi associated with plant tissue did not show significant difference in the three indices measured. More detailed studies with more species are necessary before the impact of transgenic plants on the environment can be ascertained with certainty.

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APPENDICES

Table 1. The number of offspring produced by *F. candida* when exposed to Cadmium: Mean ± S.E.

	mean	± S.E
control	56.25	1.10
2.0ppm	54.67	1.33
20.0ppm	42.21	1.51
50.0ppm	37.38	0.98
100.0ppm	19.91	1.33
200.0ppm	7.12	0.98

Table 2: The number of eggs produced per oviposition period by *F. candida* when fed on cotton: Mean ± S.E.

Ovipos.	Non-trangenic cotton		Transgenic cotton (#81)		Transgenic cotton (#249)	
	mean	± S.E.	mean	± S.E.	mean	± S.E.
1	10.75	2.45	9.5	2.38	10.38	2.76
2	24.13	5.09	24.58	4.33	26.67	4.14
3	31.08	5.83	29.42	6.60	31.50	4.5

Table 3. Age (in days) of *F. candida* at beginning of oviposition when fed on cotton. Mean ± S.E.

Ovipos.	Non-transgenic cotton		Transgenic cotton #81		Transgenic cotton #249	
	mean	± S.E.	mean	± S.E.	mean	± S.E.
1	19.63	0.40	19.58	0.45	18.92	0.46
2	28.46	0.27	28.54	0.39	29.25	0.35
3	36.43	0.44	36.25	0.60	37.17	0.53

Table 4. Final body length (mm) of *F. candida* when fed on cotton: Mean ± S.E.

Non-transgenic cotton		Transgenic cotton (#81)		Transgenic cotton (#249)	
mean	± S.E.	mean	± S.E.	mean	± S.E.
1.11	0.047	1.09	0.038	1.11	0.042

Table 5. The final total offspring produced by *O. nitens* when fed on cotton: Mean ± S.E.

Non-transgenic cotton		Transgenic cotton (#81)		Transgenic cotton (#249)	
mean	± S.E.	mean	± S.E.	mean	± S.E.
101	5.60	90.79	4.58	93.5	5.11

Table 6. The number of eggs produced per oviposition period by *F. candida* when fed on potato: Mean ± S.D.

Ovipos.	Non-transgenic		Transgenic	
	mean	± S.D.	mean	± S.D.
1	8.91	1.81	8.08	1.50
2	17.04	2.59	16.08	1.72
3	31.54	5.25	29.66	0.26

Table 7. Final body length (mm) of *F. candida* When fed on potato: Mean ± S.D.

Non-transgenic potato		Transgenic *B.t.t.* potato	
mean	± S.D.	mean	± S.D.
1.09	0.03	1.08	0.03

Table 8. Age (in days) of *F. candida* at beginning of oviposition when fed on patota. Mean ± S.E.

Ovipos.	Non-transgenic potato		Transgenic *B.t.t.* potato	
	mean	± S.D.	mean	± S.D.
1	19.13	2.74	19.25	2.38
2	31.50	2.25	32.46	2.60
3	43.83	1.99	43.83	2.14

Table 9. ANOVA for the number of eggs produced at the first oviposition by *F. candida* feeding on transgenic and non-transgenic cotton.

Source of Variation	Sum of Square	Degrees of freedom	Mean Squares	F-ratio	Significant Level
Between group	19.750	2	9.875	1.637	0.202
Within group	416.725	69	0.630		

Table 10. ANOVA for the number of eggs produced at the second oviposition by *F. candida* feeding on transgenic and non-transgenic cotton.

Source of Variation	Sum of Square	Degrees of freedom	Mean Squares	F-ratio	Significant Level
Between group	88.08	2	44.04	2.137	0.1257
Within group	1421.79	69	20.61		

Table 11. ANOVA for the number of eggs produced at the third oviposition by *F. candida* feeding on transgenic and non-transgenic cotton.

Source of Variation	Sum of Square	Degrees of freedom	Mean Squares	F-ratio	Significant Level
Between group	58.33	2	29.17	0.88	0.416
Within group	2267.67	69	32.86		

Table 12. ANOVA for the time of first oviposition of *F. candida* feeding on transgenic and non-transgenic cotton.

Source of Variation	Sum of Square	Degrees of freedom	Mean Squares	F-ratio	Significant Level
Between group	7.5833	2	3.792	0.830	0.440
Within group	315.291	69	4.569		

Table 13. ANOVA for the time of second oviposition of *F. candida* feeding on transgenic and non-transgenic cotton.

Source of Variation	Sum of Square	Degrees of freedom	Mean Squares	F-ratio	Significant Level
Between group	9.0833	2	4.542	1.629	0.204
Within group	192.417	69	2.789		

Table 14. ANOVA for the time of third oviposition of *F. candida* feeding on transgenic and non-transgenic cotton.

Source of Variation	Sum of Square	Degrees of freedom	Mean Squares	F-ratio	Significant Level
Between group	15.528	2	7.764	1.163	0.3185
Within group	460.458	69	6.673		

Table 15. ANOVA for the final number of adult *O. nitens* when feeding on transgenic and non-transgenic cotton.

Source of Variation	Sum of Square	Degrees of freedom	Mean Squares	F-ratio	Significant Level
Between group	691.694	2	345.85	1.298	0.2797
Within group	18389.23	69	266.511		

Table 16. ANOVA for the number of nymph *O. nitens* when feeding on transgenic and non-transgenic cotton.

Source of Variation	Sum of Square	Degrees of freedom	Mean Squares	F-ratio	Significant Level
Between group	257.69	2	128.85	0.853	0.4305
Within group	10420.63	69	151.02		

Table 17. ANOVA for the final body length of *F. candida* feeding on transgenic and non-transgenic cotton.

Source of Variation	Sum of Square	Degrees of freedom	Mean Squares	F-ratio	Significant Level
Between group	0.0025	2	0.0013	0.651	0.5247
Within group	0.1325	69	0.0019		