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NEW COMMERCIAL FEEDING DISRUPTION BIOASSAY KIT FOR SPECIES AND INSECTICIDE RESISTANCE DIAGNOSIS IN THE TOBACCO BUDWORM AND COTTON BOLLWORM IN COTTON

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Abstract

The feeding disruption bioassay kit for insect resistance detection and species diagnosis is in the commercialization phase of development. The kit consists of a specially designed white plastic 16-well plate with recessed, hydrateable meal pads containing a diagnostic dose of insecticide and a blue indicator dye to monitor larval feeding. The appearance of blue feces easily seen on the background of the white plate is a measure of feeding rate. Neonates that produce blue feces after a 24 h incubation are diagnosed as being resistant. The assay kit has an extended shelf-life at room temperature and is ready to use off the shelf for any pesticide. Kits for monitoring resistance were developed and tested for the tobacco budworm and/or the cotton bollworm for the following insecticides: Bt, spinosyn, permethrin, indoxacarb and emamectin benzoate. A species diagnostic kit based on feeding disruption was also developed and field tested for distinguishing the budworm from the bollworm. This kit uses the same principle as that of the resistance kit and with a diagnostic dose of Bt that detects the natural resistance of the bollworm to this pesticide. Neonates that produce blue feces are diagnosed as cotton bollworms. The species diagnosis assay was successfully tested and validated using insects from NC and LA. The utility of the feeding disruption assay was further demonstrated by developing a method for monitoring for spinosyn resistance in the cabbage looper. The commercial manufacturer has developed state-of-the-art robotics technology for the mass production of these resistance and species diagnostic kits.

Introduction

One aspect of pesticide resistance management of lepidopteran pests in agriculture is rapid and reliable methods for resistance diagnosis. The need to monitor for resistance has become even more apparent with the expanded use of the Bt toxins in plant protection. The primary concern of many scientists (e.g. Gould, 1988; McGaughey and Whalon, 1992; Tabashnik, 1994) concerning the use of the Bt genes in transgenic crops, is the risk that natural insect populations will evolve genetically-based resistance. Although there may have been some skepticism about the potential of resistance development to Bt (Briese, 1981; Krieg and Langenbruch, 1981), there now exists significant laboratory (Tabashnik, 1994; Bauer, 1995) and field data (Tabashnik et al., 1990; Gould et al., 1997) documenting that pests are capable of evolving high levels of resistance to commonly used Bt toxins. Resistance development is not limited to Bt. For example, Bailey et al. (1999) found that the laboratory selection of tobacco budworms with Tracer produced high levels of resistance to this insecticide in less than 10 generations. This was unexpected, since insects had not been previously exposed to this insecticide. Resistance development is a wide-spread problem for agriculture that threatens the efficacy of all insecticide classes.

The most common method for resistance monitoring is the adult vial test (Plapp et al., 1987), and this technique is still useful today. The problem is that the assay is only applicable to contact insecticides and therefore can not be used for Bt. The assay also is conducted on the adult stage while insecticides for lepidopteran pests are used to control larvae; for some of the newer contact insecticide technologies, the compounds are not active on the adult stage, and it is at least theoretically possible that larval resistance will not always be expressed in the adults. Finally, adults may not be resistant, but the next generation of larvae could produce significant crop damage.

This laboratory has been developing a novel feeding disruption assay for monitoring resistance to the tobacco budworm and cotton bollworm (Bailey et al. 1998, 1999, 2000, 2001; Roe et al. 1999, 2000a,b). The feeding disruption bioassay kit for insect resistance detection and species diagnosis is now in the commercialization phase. The kit consists of a specially designed white plastic 16-well plate with recessed, hydrateable meal pads containing a diagnostic dose of insecticide and a blue indicator dye to monitor larval feeding. The appearance of blue feces easily seen on the background of the white plate is a measure of feeding rate. Neonates that produce blue feces after a 24 h incubation are diagnosed as being resistant. This kit can also be used for species diagnosis using the same principle as that of the resistance kit. A diagnostic dose of Bt is used to detect the natural resistance of the cotton bollworm to this pesticide. Neonates that produce blue feces are diagnosed as

bollworms. In this paper, we demonstrate that the commercial feeding disruption assay can be used for species diagnosis and for the detection of resistance to Bt and other chemical insecticides.

Materials and Methods

Materials and Insects

The commercial feeding disruption assay kit is a (4×4) 16-well plastic plate accompanied with transparent cover strips manufactured by Agdia Inc., Elkhart, IN. Recessed into the center of each well is a hydrateable meal pad containing a blue indicator dye used to monitor feeding. The following pesticides were used for our studies: Bt (MVP®II, 20% Delta endotoxin of *Bacillus thuringiensis* aqueous flowable based on the Cellcap® encapsulation system, Mycogen Corporation, San Diego, CA), spinosyn (Tracer, 44.2%, Dow AgroSciences, Indianapolis, IN), permethrin (Pounce, 38.4%, 3.2EC, FMC, Philadelphia, PA), and indoxacarb (Steward, DPX-MP062, 1.25LB/GAL A. I., Dupont, Wilmington, DE). The insects used were as follows: HvS (the Hv97 susceptible tobacco budworm, *Heliothis virescens*, insectary strain from the Department of Entomology, North Carolina State University), HvR-Bt (the YHD2 Bt resistant tobacco budworm strain from Dr. F. Gould, Department of Entomology, North Carolina State University), HvR-spinosyn (spinosyn resistant tobacco budworms from Dr. R. M. Roe), HzS (susceptible cotton bollworms, *Helicoverpa zea*, from the Department of Entomology, North Carolina State University), and a cabbage looper, *Trichoplusia ni*, susceptible strain (Entopath Incorporated, Easton, PA).

Field Collections

Cotton or tobacco leaves, stems, flowers or parts thereof containing one or more eggs were removed from the whole plant in the field, transferred into a paper bag, and then transported to a work bench in a cool place either in the field or laboratory. Then the eggs along with a small strip of plant tissue were removed using needle pointed dissecting forceps and transferred to a clear plastic cup. The container was then sealed and incubated at 27±3 °C, 50% relative humidity, and a 14/10 light/dark cycle until the eggs hatch, usually in 1-2 days. Eggs from tobacco were collected from Clayton, Scotland (County) and Lumberton, NC in June and July 2001. Eggs from cotton were collected from Rocky Mount, NC and Macon Ridge, LA in July or August 2001.

Feeding Disruption Assay Protocol

Step 1. Add five to six ml of distilled water to a paper towel in the bottom of a Glad Ware® Entrée plastic container (739ml volume), and then seal the container with the matching lid provided by the manufacturer.

Step 2. Make-up a pesticide solution in distilled water (for example, Bt at 0.8 µg active ingredient/ml solution).

Step 3. Remove the feeding disruption assay plate from its aluminum foil wrapper and appropriately label the plate or the outside of each well, indicating the test pesticide and insect to be added to each well.

Step 4. Place the 16-well plate on top of an aluminum foil sheet. Hydrate each blue meal pad from the top for 10-15 min with 100-120 µL aliquots of the test pesticide solution (eg., Bt 0.8µg/ml). Excess pesticide solution (not absorbed by the blue meal pad) is removed from the wells either by tapping the plate upside down on a paper towel and/or by wiping each well with the Q-tip cotton swab provided with the assay plate. Control wells are hydrated by the same method with distilled water.

Step 5. Transfer a single newly hatched neonate (larva) to each well using a camel hair brush and then seal the well with a "Peel and Seal" strip, one strip covering four wells in a line.

Step 6. After neonates are added to each well, transfer the plate(s) to the surface of the wet paper towel (see Step 1), close the lid of the container, and incubate 24 h ideally at the conditions described above. We usually use two plates or more per treatment.

Step 7. After 24 h, count the number of blue fecal pellets produced per insect/well using a hand lens or low powered microscope.

The mean and standard error of the mean was determined for each sample population tested. After counting fecal pellets from field collected insects, they were transferred from the Agdia test kit to standard 1 oz. rearing cups containing artificial diet (Burton, 1970) and incubated (as described earlier) until adulthood in order to definitively determine the species. A dose of pesticide that produced 0-2 or 0-5 fecal pellets (depending on the application) was considered discriminatory.

Results and Discussion

Species Diagnosis

Typical 'no feeding' and 'feeding' responses are shown in Figure 1. For species diagnosis, we examined the effect of the concentration of Bt in the hydration solution on fecal production for the tobacco budworm (HvS) and the cotton bollworm (HzS)(Figure 2). The number of fecal pellets decreased as the Bt concentration increased for both strains. However, at 0.8 μg/ml, tobacco budworm feeding was reduced on the average to only 1.3 fecal pellets per larva while bollworm feeding continued at an average rate of 17 fecal pellets/larva. This dose of 0.8 μg/ml was used as our discriminatory dose for distinguishing the budworm from the bollworm.

Eggs were collected from tobacco in North Carolina and from cotton in North Carolina and Louisiana. Using the diagnostic concentration of 0.8 μg of Bt/ml hydration solution, fecal pellet production per neonate was measured for these insect collected from the field as eggs. The results are shown in Figure 3. Insects that produced 0-2 fecal pellets were classified as Hv, and neonates producing >3 fecal pellets were classified as Hz. Insects collected from tobacco by this classification were 90 to 100% tobacco budworms (Figure 3). This was in excellent agreement with the actual species identification using adult morphology which ranged from 91-100%. For cotton, 97-99% of the insects were bollworm as determined by adult morphology as compared to >97% as determined by the feeding disruption assay. These studies suggest that the feeding disruption assay can effectively distinguish the tobacco budworm from the cotton bollworm under the conditions of our study.

Bt Resistance Assay for the Tobacco Budworm

Since feeding on the average is only 1.3 fecal pellets per neonate for susceptible budworms (HvS) at 0.8 μg of Bt/ml hydration solution (Figure 2), this diagnostic dose was used to discriminate Bt susceptible from Bt resistant neonates. Insects that are resistant to Bt like the HvR-Bt strain should be able to continue feeding at the diagnostic dose. All of the Bt susceptible budworms produced 0-5 fecal pellets while 100% of the Bt resistant insects produce >5 fecal pellets in 24 h (Figure 4). It appears from these studies that 0.8 μg of Bt/ml hydration solution is an appropriate diagnostic dose for monitoring Bt resistance using the commercial feeding disruption assay.

Bt Resistance Assay for the Cotton Bollworm

In Figure 5, we examined different concentrations of Bt in the hydration solution and its effect on fecal production for Bt susceptible bollworms (HzS) in order to find a diagnostic dose which would stop feeding. From our studies shown earlier (Figure 2), we predicted that much higher concentrations of Bt would be needed to achieve this goal. As expected, fecal production declined as the concentration of Bt was increased (Figure 5). At 500 μg/ml, fecal production averaged only 1.4 fecal pellets per neonate. This dose should be an appropriate discriminatory concentration for resistance monitoring for the cotton bollworm. Bt resistant bollworms were not available to test this diagnostic dose further.

Spinosyn Resistance Assay for the Tobacco Budworm

Using the same methodology to obtain a diagnostic dose as that used for Bt, we found that 0.6 μg of spinosyn/ml hydration solution reduced fecal production down to an average of 0.6 fecal pellets per neonate for the HvS strain (Figure 6). For the resistant strain, concentrations of 80 and 100 μg/ml were needed to reduce production to 1.1 and 0.2 pellets per HvR-spinosyn neonate, respectively (Figure 7). Using the diagnostic dose of 0.6 μg of spinosyn/ml hydration solution, we were able to distinguish spinosyn susceptible from the resistant strain (Figure 8). All of HvS produced 0-5 fecal pellets with 95% producing 0-2 fecal pellets while 100% of the spinosyn resistant budworms produced >5 pellets. These studies show that the feeding disruption assay can also be used for chemical insecticides like spinosyn which act both by contact and feeding and which act directly on the nervous system. Sayed et al. (2002) also was successful in developing a feeding disruption assay for emamectin benzoate in the tobacco budworm where the difference in susceptibility between the resistant and parental laboratory strains was only 5-10 fold.

Permethrin and Indoxacarb Resistance Assay for the Tobacco Budworm

Dose response relationships were also established for permethrin (Figure 9) and indoxacarb (Figure 10). For permethrin, the diagnostic dose was 10 μg/ml with an average fecal production of 1.1/neonate in 24 h for the tobacco budworm. For indoxacarb, the diagnostic dose was 0.5 μg/ml with an average fecal production of 1 pellet per neonate.

Spinosyn Resistance Assay in the Cabbage Looper

The feeding disruption assay method should be applicable to any insect that can feed on an artificial diet and as demonstrated in the studies discussed earlier, can be used to monitor resistance to different insecticides including gut toxins and nerve poisons. To further validate the utility of this technology, we examine the use of the commercial feeding disruption assay designed for the tobacco budworm/cotton bollworm complex on the cabbage looper, *Trichoplusia ni.* Again, we found a dose response between the concentration of spinosyn in the hydration solution and the level of production of blue feces.

From these studies, the diagnostic dose for resistance monitoring would be 0.8 μg/ml. At this concentration, the average fecal production was 0.3 pellets/neonate.

Summary

We described a commercial feeding disruption assay for monitoring insect resistance to insecticides that act only through feeding like Bt and to contact insecticides like spinosyn, permethrin, indoxacarb, and emamectin benzoate. The assay was optimized for neonates but we have previously shown that the technology is applicable to third instars and should work for any larval stage. The assay time used in the current study was 24 h for individual neonates but the assay can be conducted in as little as 2-4 h on homogenous populations of third instars (Bailey et al., 1998). A probit model was established using insect populations throughout the SE US to validate the detection methodology for species diagnosis (Bailey et al., 2001). The commercial assay combines three technologies, the feeding disruption detection method for measuring toxicity, hydrateable meal pads and a novel device. The advantages of this assay approach is that it targets the insect developmental stage against which the insecticide is used, targets specific field locations where the insects are collected, and is applicable to contact and oral insecticides. Most resistance mechanisms will be detected by this method, and the assay is non-destructive to the insect. In respect to the latter, resistant insects can be transferred to artificial diet and reared to the adult stage. The assay kit can be stored on the shelf for months and is ready to use when needed. Finally, the commercial feeding disruption assay can be used to distinguish the tobacco budworm from the cotton bollworm, which can be important in cotton pest management.

Acknowledgments

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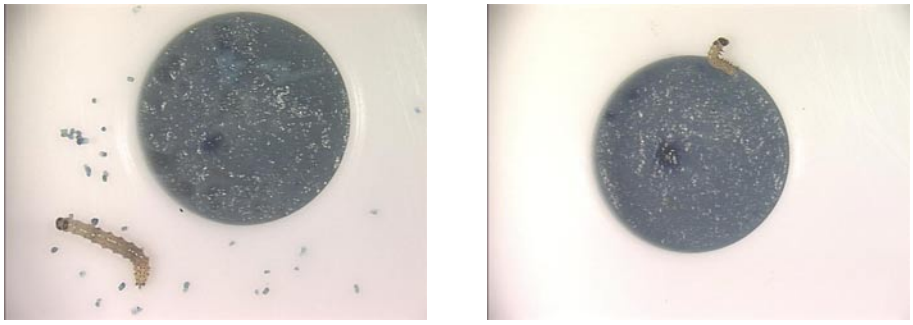

Figure 1. Top view of a single well in the 16-well commercial feeding disruption assay kit. The dark circle is a hydrated meal pad with a blue indicator dye used to monitor feeding. The meal pad extends below the well and is opened below the well to the outside. Hydration is maintained via a wetted surface below the wells. Left, a typical response with no feeding. Right, a feeding response. The presence of blue feces at a diagnostic dose of insecticide is a marker for insect resistance.

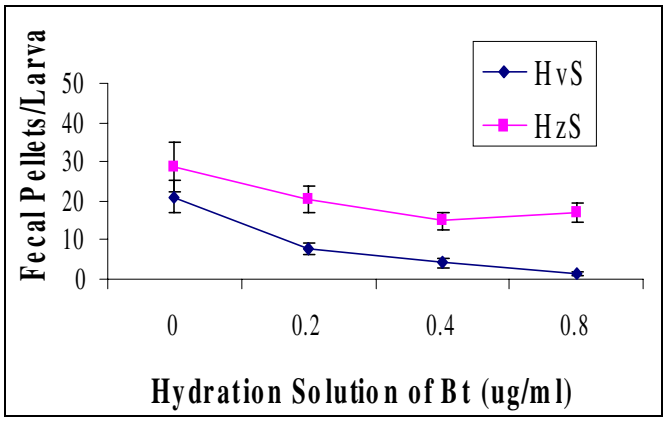

Figure 2. Dose response for fecal production from Bt susceptible tobacco budworms (HvS) and cotton bollworms (HzS). The error bars are ±1 standard error of the mean.

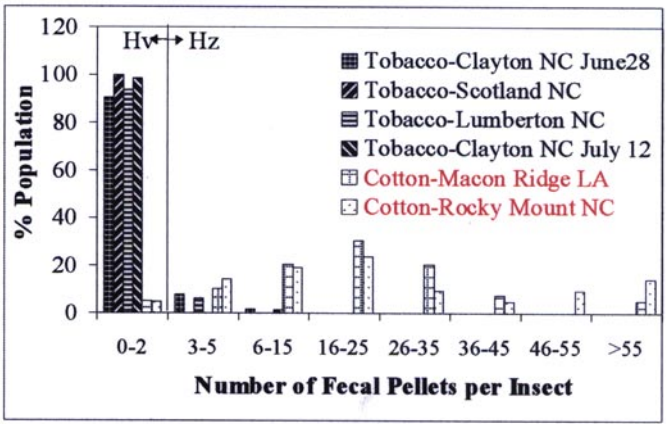

Figure 3. Field validation of the feeding disruption assay for tobacco budworm and cotton bollworm species diagnosis. The diagnostic dose for Bt in the hydration solution was 0.8 µg/ml.

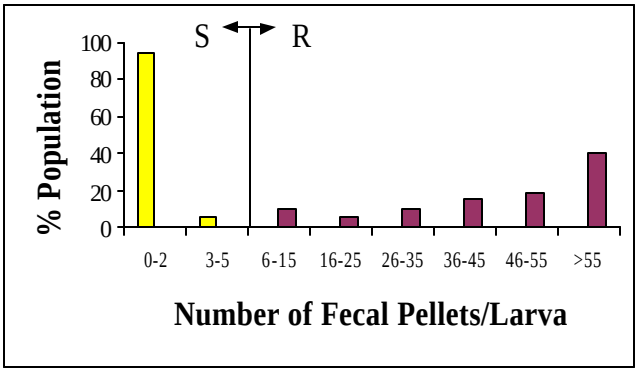

Figure 4. Validation of the feeding disruption assay for Bt resistance in the tobacco budworm. The diagnostic dose was 0.8 µg of Bt/ml hydration solution. S is a Bt susceptible and R a Bt resistant tobacco budworm strain.

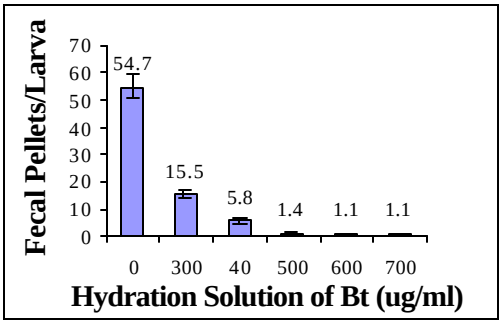

Figure 5. Dose response for fecal production from Bt susceptible cotton bollworms (HzS). The error bars are ±1 standard error of the mean.

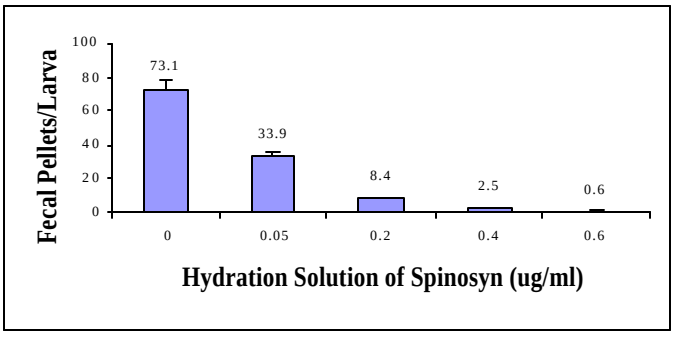

Figure 6. Dose response for fecal production from spinosyn susceptible tobacco budworms (HzS). The error bars are ±1 standard error of the mean.

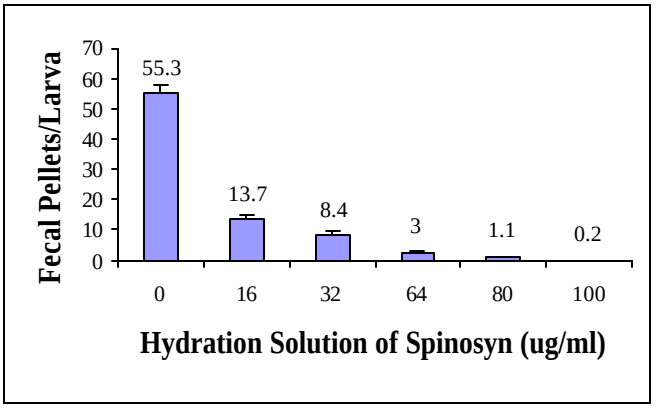

Figure 7. Dose response for fecal production from spinosyn resistant tobacco budworms (HvR-spinosyn). The error bars are ±1 standard error of the mean.

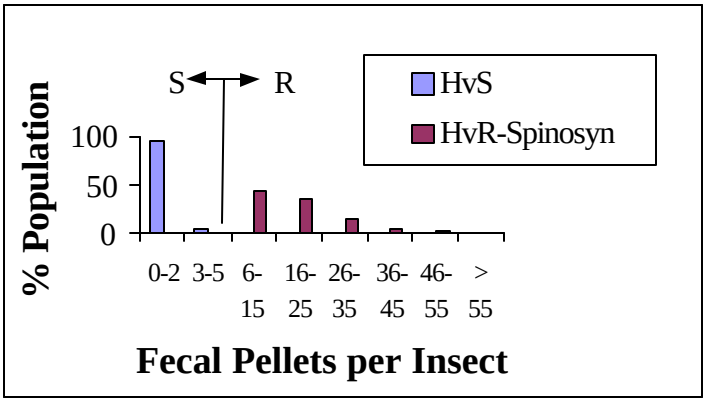

Figure 8. Validation of the feeding disruption assay for spinosyn resistance in the tobacco budworm. The diagnostic dose was 0.6 µg of spinosyn/ml hydration solution. S is a spinosyn susceptible (HvS) and R a spinsoyn resistant tobacco budworm strain.

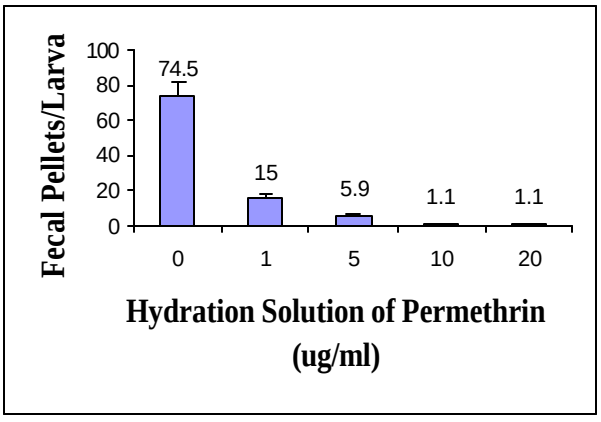

Figure 9. Dose response for fecal production from permethrin susceptible tobacco budworms (HzS). The error bars are ±1 standard error of the mean.

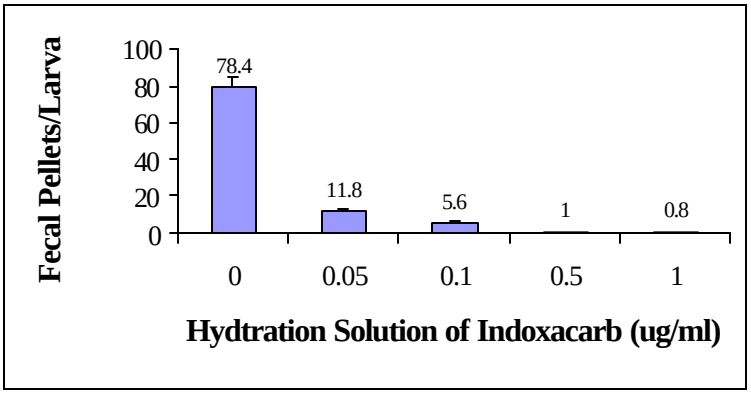

Figure 10. Dose response for fecal production from indoxacarb susceptible tobacco budworms (HzS). The error bars are ±1 standard error of the mean.

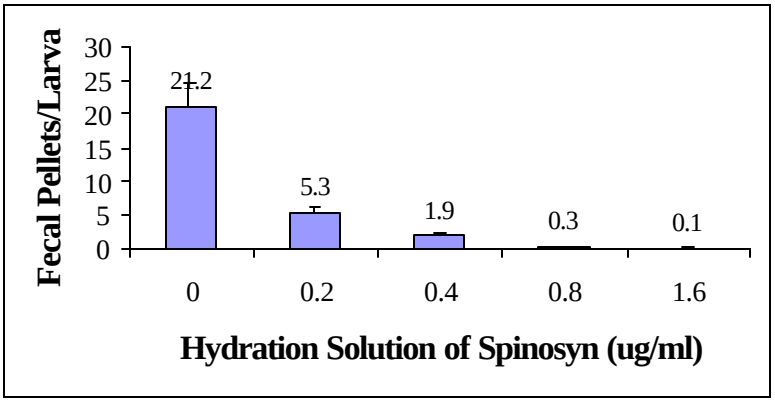

Figure 11. Dose response for fecal production from spinosyn susceptible cabbage loopers. The error bars are ±1 standard error of the mean.