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LARVAL FEEDING DISRUPTION TESTS (FDT) FOR MONITORING INSECT RESISTANCE TO CRY1AC, CRY1F AND C RY1AB

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Abstract

Feeding disruption tests (FDTs) were developed for monitoring insect resistance to three Bt proteins--Cry1Ab, Cry1Ac and Cry1F. The tests were designed to minimize false positives and negatives for resistance. The assays rely on a diagnostic dose of Bt toxin in a hydrateable meal pad containing a blue indicator dye. Insects resistant to Bt are able to consume the artificial diet and produce blue feces on a white background. Fecal production was inversely correlated with Bt concentration in the hydrated meal pad. The diagnostic dose for resistance determined by FDT for Cry1Ab against *Heliothis virescens* (Hv) was 15 micrograms a.i./ml hydration solution and for *Helicoverpa zea* (Hz) was 500 micrograms per ml; Cry1Ac: Hv=10, Hz=40; and Cry1F: Hv=20, Hz=200. The assays were successfully tested against Bt resistant tobacco budworms (YHD2 strain) and on *H. virescens* collected as eggs from tobacco in North Carolina. The kits were also evaluated by cooperators (results reported). FDT kits provide an easy-to-use (off-the-shelf) standard assay method for monitoring insect resistance to Bt and chemical insecticides. The technology has other applications in high-throughput insecticide screening, routine insect rearing and the evaluation of the insecticidal activity of Bt transgenic crops.

Introduction

Transgenic plant technology is becoming a widely adopted method for the control of lepidopteran pests in cotton and other crops (Wrona, 1998). On-going development of this technology has been promoted by the need to improve crop production while minimizing any possible adverse effects of insect control on the environment. Cooperation between industry and academic institutions has yielded an increasing number of commercially available plants expressing Bt toxins coupled with herbicide resistance. The improved efficacy of the next generation of insect resistant cotton plants has been achieved in part by the simultaneous expression of at least two Bt toxins. Bt toxins are highly active against caterpillars but have no animal toxicity (Roe et al., 1985).

The use of transgenic cotton producing Bt toxin for insect control has been one of the first test cases for the application of biotechnology for the improvement of crop production. Transgenic cotton is also the first example of the widespread use of a protein as an insecticide. The Bt proteins are expressed at high levels in the cotton plant throughout most of its life. As a consequence, caterpillar mortality on cotton in the field is high along with the selective pressure for the evolution of insect resistance to Bt. The concern of many scientists (e.g. Gould, 1988; McGaughey and Whalon, 1992; Tabashnik, 1994) is the risk that natural insect pest populations will evolve genetically-based resistance to Bt making this strategy of control ineffective. There now exist significant laboratory and field data documenting that pests are capable of evolving high levels of resistance to the Bt toxins (Tabashnik et al., 1990; Tabashnik, 1994; Bauer, 1995; Gould et al., 1997). Resistance to Bt in the field is currently not a problem.

Management of insect resistance requires cost effective, user-friendly methods of resistance monitoring. The most common method for resistance monitoring for traditional chemical insecticides has been the adult vial test (Plapp et al., 1987). This assay method is not applicable to Bt, which must be ingested by the insect. Bt resistance is monitored using a feeding bioassay where the end point is mortality or growth inhibition; the assay typically takes 4-7 d to perform (Sims et al., 1996; Mascarenhas et al., 1998). In addition to the long assay time, Bt feeding bioassays are complicated by the difficulty of incorporating Bt proteins into artificial insect diet, the need for relatively large amounts of toxin for diet incorporation and diet storage until the assay is needed.

This laboratory has been examining possible improvements to the Bt feeding bioassay and has developed a feeding disruption test (FDT) for monitoring larval resistance to Bt for the common lepidopteran pests found on cotton and other crops (Bailey et al. 1998, 1999, 2000, 2001; Roe et al. 1999, 2000 a,b, 2002, 2003). The core of the assay kit is a 16-well plate with recessed, hydrateable meal pads containing a diagnostic dose of the Bt insecticide and a blue indicator dye to monitor larval feeding. The appearance of blue feces on the background of the white plate is the indicator of resistance. If the insects are susceptible, the background in each well of the plate remains white; if the insects are resistant, blue fecal pellets are observed. The assay kit can be stored for months at room temperature and is ready to use off-the-shelf, requiring only that two drops of water be added to each meal pad in order to re-hydrate the diet prior to the addition of neonate larvae. The assay is conducted in 24 h, and the end-point is detected simply by observation unaided by a microscope or hand lens. In addition to Bt, FDT can be used for traditional chemical insecticides and provides a standardized, easy-to-use format for insecticide bioassays. The technology also has applications in routine insect rearing, high throughput insecticide screening, and the quantitation of the Bt "killing power" of transgenic plants (useful in plant breeding and routine field monitoring of transgenic plants). The kits are available commercially and are custom produced to the specific needs of the application.

The objectives of the current research project were to develop FDT kits for monitoring tobacco budworm (*Heliothis virescens*) and cotton bollworm (*Helicoverpa zea*) resistance to three Bt toxins--Cry1Ab, Cry1Ac and Cry1F; to test the kits with a laboratory, Bt-resistant strain of tobacco budworm; to test the kits with field-collected Bt-susceptible insects; and to evaluate the performance of the kits in the field by first-time users.

Materials and Methods

Development of Diagnostic Doses for Cry1Ab, Cry1Ac and Cry1F

An FDT diagnostic dose (micrograms Bt per ml aqueous hydration solution) was determined for three insecticidal Bt proteins (Cry1Ab, Cry1Ac and Cry1F) against susceptible strains of the tobacco budworm, *Heliothis virescens*, and the cotton bollworm, *Helicoverpa zea*. The proteins were provided by Dow AgroSciences (Indianapolis, IN). The Cry1Ab was a 14% water-soluble dry solid, Cry1Ac was a 14% water-suspendible dry solid and Cry1F was a 23% water-suspendible dry solid. Dilutions of each protein were prepared in sterile distilled water. Susceptible tobacco budworms (strain Hv02) and cotton bollworms (strain Hz02) were obtained from the Department of Entomology, North Carolina State University, Raleigh, NC. FDT plates containing hydrateable meal pads (Figure 1) were used in all studies that follow and were constructed by the Roe laboratory. For dose response studies, 80 microliters of varying concentrations of Cry1Ab, Cry1Ac or Cry1F in sterile, distilled water were added to individual meal pads. The meal pads were allowed to hydrate for at least 30 min prior to use. Control wells contained meal pads hydrated with 80 microliters of sterile, distilled water without Bt. After hydration, a single neonate was transferred with a fine-tipped brush to each well of the FDT plate, the wells sealed with a clear plastic adhesive cover, the plates transferred into a plastic container (Glad Ware® Entree plastic container, 25 oz/739 ml; Figure 1) containing a wet paper towel on the bottom, and the container sealed with a plastic top. The plates were

incubated for 24 h at 27±1°C (60% relative humidity, 14:10 L:D). The number of blue fecal pellets produced per larva in each well was determined after 24 h using a 10X Bausch and Lomb dissecting microscope. The mean number of fecal pellets produced per larva ± 1 standard error of the mean was calculated for each Bt dose (expressed in micrograms of active ingredient (a.i.) per ml hydration solution). The Bt toxin concentration at which individual larvae produced two or fewer fecal pellets was considered the diagnostic dose for distinguishing susceptible from resistant neonates for each species. After the fecal pellets were counted, larvae were transferred from the control wells to artificial diet without Bt and reared to mature larvae to confirm the species identity as determined by examination of larval pinaculae (Sparks and Liu, 2001).

Validation of FDT Kit for Bt-Resistance Detection in the Tobacco Budworm

Bt-resistant budworms (neonates from the YHD2 strain) were provided by Dr. Fred Gould, Department of Entomology, North Carolina State University, Raleigh, NC. The meal pads of FDT plates were individually hydrated in the laboratory with 80 microliters of each Bt toxin at the diagnostic doses determined in the experiments described earlier (for Cry1Ab at 15, Cry1Ac at 10, and Cry1F at 20 micrograms per ml hydration solution). Control wells were hydrated with 80 microliters of sterile, distilled water without Bt protein. The feeding disruption test and analysis of the results was conducted as previously described.

Validation of FDT Kits for Field-Collected Susceptible Tobacco Budworms

Tobacco budworm eggs were collected from tobacco, which had not been treated with insecticides grown on the research farm at North Carolina State University (Clayton (Johnston County), NC). Tobacco flowers and leaves (or parts thereof) containing apparent lepidopteran eggs were collected on 24 June and 3 August 2004. The collected plant material was transported to the Dearstyne Entomology Building at North Carolina State University, Raleigh, NC where a small amount of plant tissue containing an egg was removed from the bulk of the plant material collected from the field. These eggs were placed into plastic containers, sealed with lids and incubated at 27±1°C (60% relative humidity, 14:10 L:D). Neonates were collected twice daily and transferred to meal pads containing diagnostic doses of Cry1Ac or Cry1F protein. At the time of the field collections, the determination of the diagnostic doses for Cry1Ac and Cry1F (described earlier) had not been completed. Therefore, for the June field collection from tobacco, the Cry1Ac doses used were 0.05 and 0.1 micrograms of Bt protein per ml hydration solution. The Cry1F doses were 0.4 and 0.8 micrograms per ml. For the August collection, the Cry1Ac solution was 0.1 micrograms per ml, and the Cry1F solution was 0.8 micrograms per ml. The plates were infested by transferring a single neonate larva to each well via a fine-tipped brush. The feeding disruption test and analysis of the results was conducted as previously described.

Testing of FDT Kits by Field Cooperators

FDT kits (shown in Figure 1) containing 10 assay plates (per kit) were sent to cooperators in the U.S. cotton belt for evaluation during the 2004-growing season. On each 16 well plate, 12 of the hydrateable meal pads were pre-loaded with 100 micrograms of Cry1Ac protein (active ingredient); the remaining 4 control wells per plate did not contain any Bt. Each plate was vacuum sealed in a re-sealable foil pouch (Figure 1) along with a small amount of desiccant. Pre-loading of the meal pads with the Bt toxin minimizes end-user error and should produce a reliable, standardized assay. When the assay is needed, the user removes the plate from the foil pouch and adds a small amount of water to each meal pad as described below.

In addition to the assay plates, the kits contained:

- a vial of distilled water for hydration of the meal pads
- a small plastic transfer pipette for meal pad hydration
- a fine-tipped brush for transferring a neonate to each well
- clear plastic adhesive covers for confining neonates to individual wells
- a plastic Glad Ware® container (with paper towel on bottom) for plate incubation at high humidity
- cups of dehydrated insect diet to be hydrated at the end of the FDT assay and used for rearing larvae for species confirmation
- kit instructions

The kit instructions included the following eight-step protocol:

Step 1 Remove 16-well plate from foil pouch and place on top of pouch.

Step 2 Add two drops (about 80 microliters) of distilled water to the surface of the blue, dehydrated meal pad in each well.

Step 3 Wait approximately 30 min for meal pads to hydrate.

Step 4 Transfer 1 larva to each well and then seal with a clear plastic adhesive cover. In the case of very mobile larvae, the operator may find it useful to cut the adhesive cover into four strips in order to infest and seal the plate four wells at a time.

Step 5 Saturate a paper towel with water and place in the bottom of the Glad Ware® container. Transfer plate(s) into the Glad Ware® box. Install the lid and incubate for 24-48 h at 68-86° Fahrenheit (20-30° Centigrade).

Step 6 Read plate:

RESPONSE

Positive Control Wells (No Bt in meal pad)--Should observe more than 2 blue fecal pellets per well per larva (blue pellets on white background in bottom of well).

Bt Treated Wells

Bt Susceptible Insects--Should observe two blue fecal pellets or less (white, no blue pellets).

Bt Resistant Insects--Should observe greater than two fecal pellets (blue pellets on white background in bottom of well).

Step 7 Record plate observations.

Step 8 Hydrate diet in rearing cups with water, transfer larvae from the control wells to these cups (1 larva per cup), cap, rear to a mature larva and determine species.

After completing the assays, cooperators were asked to send their observations and comments to R.M. Roe.

Results and Discussion

FDT Kits for Cry1Ab, Cry1Ac and Cry1F

The basic components of the FDT kit are shown in Figure 1. For the laboratory studies in this report to determine

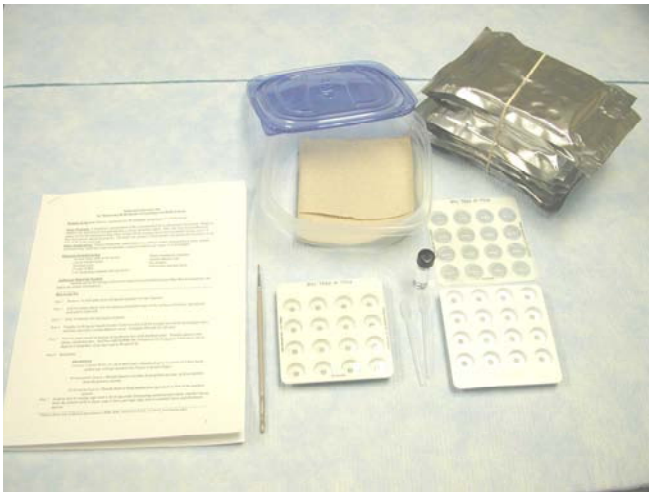

Figure 1. Feeding disruption test (FDT) kit.

the diagnostic dose for each Bt toxin (assay development) and to test the assays on laboratory, Bt resistant and field

collected, susceptible caterpillars, the plates were hydrated using a Gilson pipetman; we did not used the eye droppers as illustrated in Figure 1. The assay method relies on a diagnostic dose of Bt toxin in the hydrated artificial diet that will inhibit feeding of Bt-susceptible lepidopteran neonates. In this study, the research focused on two common cotton pests, the tobacco budworm and cotton bollworm; however, diagnostic doses for other pest species can also be developed by the same method. Our hydrateable meal pads should be acceptable to many larval Lepidoptera. Feeding inhibition in our studies was defined as the production of two or fewer blue fecal pellets after neonates were allowed to feed for 24 h on the test diet at 27±1° C. The feeding of Bt resistant budworms is not inhibited by the diagnostic dose, and the larvae produce greater than two blue fecal pellets per larva.

Diagnostic doses for Cry1Ac resistance detection in the tobacco budworm and cotton bollworm were developed previously by this laboratory using MVP II, Mycogen Corporation, San Diego, CA (Bailey et al. 1998, 1999, 2000, 2001; Roe et al. 1999, 2000 a,b, 2002, 2003). In the current study, Cry1Ac along with Cry1Ab and Cry1F were provided by Dow AgroSciences. The proteins from this source were synthesized by heterologous expression in *Pseudomonas fluorescens*. We used the toxins from the bacterial source as our standard instead of that from the plant to obtain individual proteins with the highest possible purity.

The susceptibility of larval Lepidoptera to Bt in a feeding bioassay can be quite variable, even when using highly inbred laboratory colonies. Probit models to describe this variability for FDT assays were previously developed in this lab for budworm and bollworm collections from the SE US (Bailey et al., 2001). In our current studies to determine the diagnostic dose for Cry1Ab, Cry1Ac and Cry1F, the challenge was to obtain a dose of Bt in artificial diet that would minimize this variability and produce the lowest number of false positives and negatives for resistance. To achieve this goal, the diagnostic dose that was chosen was the concentration of Bt toxin in artificial diet that produce two or fewer blue fecal pellets in 100% of the test population. This was not the optimum for the most sensitive resistance assay since the diagnostic dose must be high enough to prevent fecal production in all members of the susceptible population in the test sample. However, this approach is necessary for a standardized single dose assay that minimizes the insect sample size needed for a diagnosis. Also note that the diagnostic dose does not represent the practical dose needed in the cotton plant to control insects. FDT is a feeding bioassay and all of the standard Probit-based statistical methods normally used to characterize the susceptibility of an insect population are applicable; however, these approaches require large sample sizes and multiple Bt doses. The diagnostic doses and the FDT kits developed in the current study are to be used for screening field populations with the goal of minimizing the number of insects needed for a diagnosis. The assay method developed will diagnose resistance in a single neonate collected as an egg directly from the field. For the analysis of a population of insects, FDT kits with varying concentrations of Bt can be used to obtain dose responses and probit analysis as previously described (Bailey et al., 2001).

Figures 2-5 show the number of blue fecal pellets produced per neonate at different concentrations of Bt in the hydration solution for Cry1Ab, Cry1Ac and Cry1F in FDT assays with the tobacco budworm and cotton bollworm. The graphs show both the average number of blue fecal pellets produced per insect (± 1 standard error of the mean) at each dose tested as well as the maximum number of pellets produced per insect in the test population. It is the latter data that were used to determine the diagnostic dose, i.e., the concentration of Bt toxin that reduces the number of pellets to two or less in the test population. The diagnostic doses are indicated by vertical arrows: Cry1Ab, Hv (Figure 2)=15 micrograms a.i./ml hydration solution and Hz (Figure 3)=500; Cry1Ac (Figure 4), Hv=10; Hz=40; Cry1F (Figure 5), Hv=20; Hz=200. Using these diagnostic doses and assuming the budworm and bollworm populations chosen for our studies were typical of field populations, the number of false positives that would be obtained using this assay is shown in Figure 6. In most cases, no false positives would be expected; the only exception was 1 false positive out of 28 insects tested for Cry1Ab on the cotton bollworm.

In Figures 2-5, feeding disruption was measured by counting the number of blue fecal pellets produced per insect after a 24 h incubation. Counting blue fecal pellets, which is a quantitative measure of feeding activity, was used in these studies for method development. In practice, the user of an FDT kit would be determining resistance on the basis of the appearance of blue color in each test as observed by the unaided eye. Counting the number of fecal pellets is not required.

The susceptibility of larvae to Bt intoxication varies between the different Bt toxins as indicated by the different diagnostic doses that were obtained for FDT in the present study (Figures 2-5). These differences are overcome relative to the control of insects on transgenic cotton by the high level of toxin expression in the plant. However,

varying susceptibility of insects to Bt is problematic in resistance diagnosis for populations of mixed species or when the insect species are unknown. To resolve this problem, assays can be conducted with diagnostic concentrations that will inhibit feeding in the least susceptible species expected in a test population. Alternatively, a species diagnosis can be conducted first followed by the resistance assay with the appropriate diagnostic dose. Often researchers have some knowledge of the species at the time of collection based on light trapping data, time of the year, and experience with the specific agricultural system. In addition, FDT does not kill the insect in the short 24 h incubation; the insects can be transferred to non-Bt diet and reared to mature larvae or adults for identification. In this case multiple diagnostic doses can be used, and the results of the FDT test evaluated once the species is determined. FDT is a rapid, easy-to-use modification of the insect feeding bioassay, which has been the gold standard for resistance detection and characterization of resistance for Bt. Identification of species is critical for any type of feeding bioassay.

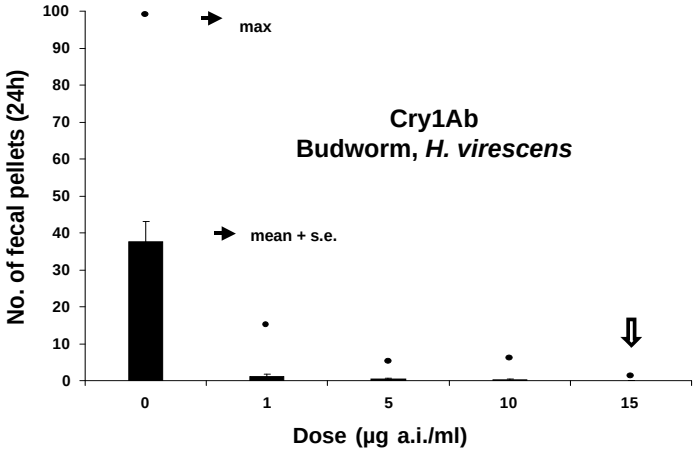

Figure 2. FDT Cry1Ab dose response (laboratory budworms).

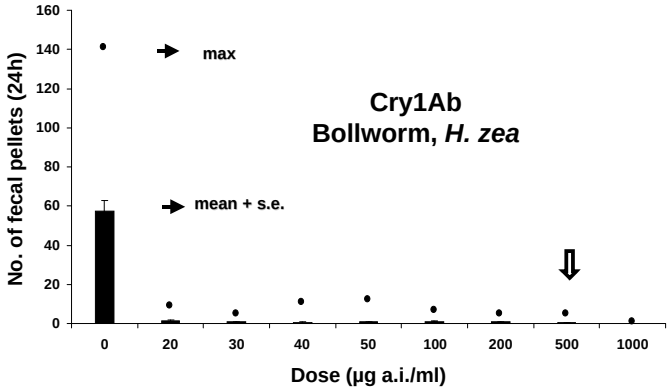

Figure 3. FDT Cry1Ab dose response (laboratory bollworms).

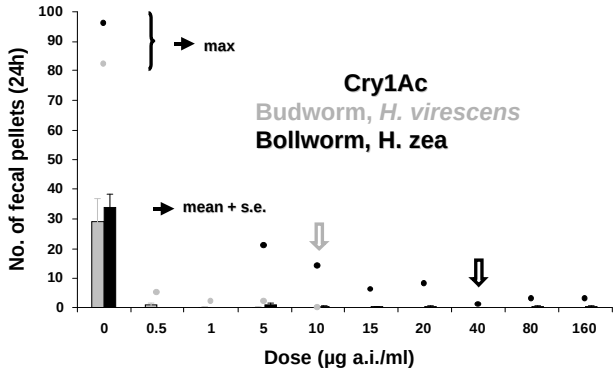

Figure 4. FDT Cry1Ac dose response (laboratory budworms and bollworms).

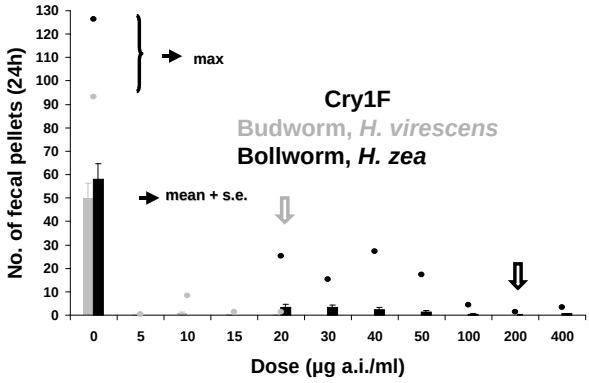

Figure 5. FDT Cry1F dose response (laboratory budworms and bollworms).

Number of False Positives / Total Larvae Tested
(based on susceptible strains)

	Cry1Ab	Cry1Ac	Cry1F
Budworm	0/28	0/27	0/20
Bollworm	1/28	0/28	0/27

Figure 6. False positives by FDT at the respective diagnostic doses.

Use of Cry1Ab, Cry1Ac and Cry1F FDT Kits to Diagnose Bt Resistance in the YHD2 Strain of the Tobacco Budworm

Figure 7 shows the application of the FDT kits using the diagnostic doses established in Figures 2-5 to the YHD2 Bt resistant strain of the tobacco budworm developed by Dr. Fred Gould (Department of Entomology, NC State University, Raleigh, NC). In the absence of Cry1Ab, Cry1Ac or Cry1F in the meal pad (the untreated controls), blue fecal production was high (greater than 20 fecal pellets per insects). At the diagnostic dose for each toxin, the maximum number of blue fecal pellets produced per insect by the susceptible strain approached zero while that for the YHD2 Bt resistant strain was comparable to the control insects. Figure 8 shows that in the vast majority of cases, we were successful in our diagnosis of Bt resistance; only one assay produced a false negative. Also note that the YHD2 strain developed by selection with Cry1Ac was cross resistant to Cry1Ab and Cry 1F as determined by FDT (Figure 7).

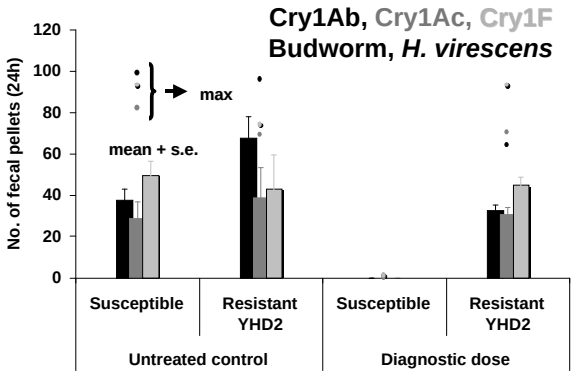

Figure 7. FDT for laboratory susceptible versus resistant budworms.

Number of False Negatives / Total Larvae Tested
(based on resistant strain YHD2)

	Cry1Ab	Cry1Ac	Cry1F
Budworm	0/26	1/27	0/28

Figure 8. False negatives by FDT at the respective diagnostic doses.

Use of Cry1Ac and Cry1F FDT Kits to Diagnose Bt Susceptible Tobacco Budworms Collected Directly from the Field

In addition to the development and testing of FDT kits with susceptible lab strains of the tobacco budworm and cotton bollworm, assays were conducted with budworms collected directly from the field as eggs. The eggs were collected from tobacco leaves and flowers in fields not previously treated with insecticide at the NC State University research station in Clayton (Johnston County) NC in June and August of 2004. The eggs on plant material were

transferred to the laboratory, each egg plus a small amount of plant material to which they were attached transferred to a plastic container, and the eggs allowed to hatch. The neonates were collected within 24 h of hatching and transferred individually to the wells of FDT plates in which the hydrated meal pads contained no Bt toxin, were hydrated with 0.05 or 0.1 micrograms per ml of Cry1Ac, or were hydrated with 0.4 or 0.8 micrograms per ml of Cry1F. Since these experiments were conducted prior to the completion of our finalized diagnostic doses (Figures 2-5), the Bt concentrations chosen for these studies were not optimized and were lower than what later studies showed would be required for susceptible budworms. Nevertheless, it is apparent that the assay was consistent in diagnosing Bt susceptibility in the field collected tobacco budworm population (Figure 9). The untreated controls produced 20 fecal pellets or more per larva on average while the highest dose chosen for Cry1Ac and Cry1F (0.1 and 0.8 micrograms per ml hydration solution, respectively) resulted in a reduction in fecal production approaching zero. With respect to our criterion that susceptible budworms should produce two or fewer fecal pellets, the error rate was small. For Cry1Ac, 4 out of 52 insects tested would have been misdiagnosed as resistant in June and 0 out of 45 in August. For Cry1F, the ratios were 2 out of 46 and 2 out of 39, respectively. It should be noted in these studies that the neonates were reared to mature larvae to verify species and that the assumption was made that there were no Bt resistant budworms in the Clayton budworm population.

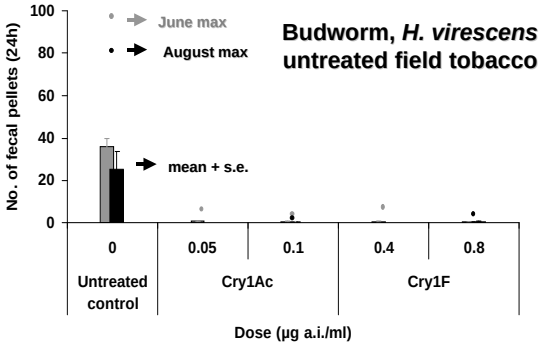

Figure 9. FDT Cry1Ac and Cry1F dose response on field collected budworms.

End User Experiences with FDT

Collaborators with no previous experience with FDT conducted assays of their choosing during the summer of 2004. They were provided a complete kit as shown in Figure 1. The kit also included the written instructions that were developed based on our experience in using the assays at NCSU. Collaborators reported their test experiences directly to Dr. R.M. Roe at NC State University by email or through Dr. G.D. Thompson at Dow AgroSciences (Indianapolis, IN). Even though the assay is reliable in the hands of researchers at NCSU who have been involved in assay development, first-time users noted some problems. The most common problems reported were larvae not eating the artificial diet, and the diet not maintaining hydration during the assay incubation period. FDT is a larval bioassay. Therefore, it is essential that the larvae used in the assay, whether from the laboratory or field, are healthy when transferred to the FDT plates. It was apparent that in some cases the larvae being transferred were not in good condition and for this reason died in the control wells. However, at least in one case the reason for larvae not feeding on the control hydrated meal pads was not apparent and could not be explained based on our prior experience at NCSU with meals pads (Bailey et al., 2001). Further studies are planned to address this question in 2005. The other major problem appeared to be associated with hydration of the paper towel in the bottom of the plastic incubation container (Figure 1). When additional instructions were provided to hydrate the paper towel to saturation, this appeared to resolve the problem.

There are a number of advantages for the use of FDT over current methods for Bt resistance monitoring as follows:

- The assay is a ready-to-use, off-the-shelf assay (just add two drops of water to each meal pad)
- FDT kits can be stored at room temperature for months
- Bt resistance is detected in 24 h from insects collected directly from the field
- The assay is easy-to-read, no special equipment needed

- Assay requires only microgram amounts of insecticide per assay
- FDT can be a standardized, industry method for resistance monitoring
- The assay detects all possible mechanisms of resistance simultaneously
- FDT is applicable to chemical and biological insecticides
- The technology can be used for high throughput insecticide screening and to bioassay the Bt insecticidal activity of plants

Summary

Feeding disruption test kits were developed for monitoring larval lepidopteran resistance to Cry1Ab, Cry1Ac and Cry1F. The assay technique uses a diagnostic dose of Bt toxin in a hydrateable meal pad. The kits are ready-to-use off-the-shelf. Two drops of water are added to hydrate each meal pad before use. Hydration is followed by the addition of a larva to each well. The wells are sealed to prevent the escape of the caterpillar, and the plates incubated for 24 h. The appearance of blue feces on the white background of the plate indicates that the insect is resistant to Bt; a white background indicates the larva is susceptible. The assay kits were successfully tested against laboratory Bt resistant tobacco budworms and on susceptible insects collected directly from the field. The kits were also field tested by cooperators.

Acknowledgments

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