ASSAY KIT FOR SPECIES AND INSECTICIDE RESISTANCE DIAGNOSIS FOR TOBACCO BUDWORM AND BOLLWORM IN COTTON

R. M. Roe, W. D. Bailey, G. Zhao,
H. P. Young, L. M. Carter, F. Gould,
C. E. Sorenson, G. G. Kennedy and J. S. Bacheler
North Carolina State University
Raleigh, NC

Abstract

Bioassays based on feeding disruption have been developed which accurately distinguish larvae of the tobacco budworm (*Heliothis virescens)* from the bollworm (*Helicoverpa zea*) and diagnose larval resistance to *Bacillus thuringiensis* and chemical insecticides in the budworm. This assay technology should be applicable to other pest species.

Introduction

Recombinant cotton expressing a CryIAc toxin derived from the bacterium, *Bacillus thuringiensis* (*B.t.*), provides an effective control strategy for lepidopteran pests (Gill et al., 1992). This technology has potential for extensive environmental and economic benefits resulting from reduced applications of broad-spectrum insecticides (Gasser and Fraley, 1989). In order to preserve the effectiveness of *B.t.* cotton for the future, careful management will be required to forestall the evolution of genetically-based resistance in field populations (Gould, 1988; McGaughey and Whalon, 1992; Tabashnik, 1994). New techniques are needed for the early detection of resistance in time for corrective action to be taken. Ideally, a method for resistance detection should rapidly (in 1-2 days) detect most mechanisms of resistance simultaneously, detect resistance at the stage of insect development where control is aimed, assay resistance on either individuals or groups of insects and be applicable to the field at minimal cost. The assay technology described here meets these criteria.

An additional concern is the need to differentiate between the budworm and bollworm during economically important early life stages (egg and young larvae) when species identification based on appearance is either impossible or impractical. *B.t.* cotton exhibits differential toxicity to the budworm (Jenkins et al., 1993; Mascarenhas et al., 1994) versus the bollworm (Lambert et al., 1996), with surviving bollworm larvae potentially inflicting significant damage to cotton when populations are high. Determination of the species composition present during overlapping flights is therefore critical for making appropriate IPM control decisions.

We propose the use of a novel bioassay based on feeding disruption measured as decreased fecal production, for species differentiation and the practical diagnosis of resistance to *B.t.* and chemical insecticides in the budworm/bollworm complex in cotton (Bailey et al., 1998; Roe et al., 1998).

Materials and Methods

Insects

All insects were routinely reared at 27$\pm$1 degrees C with a 14:10 (light:dark) cycle on a standard artificial diet (Gould et al., 1995; Rose et al., 1995). Mortality data were analyzed using probit analysis (PROC PROBIT, SAS 1991). A Wake (*B.t.* susceptible) strain of the tobacco budworm was originally collected in 1986 from tobacco in Wake Co., NC. The LC_{50} of CryIAc (MVP, Mycogen Corp., San Diego, CA) was 0.0017 *u*g (95% CI, 0.0002-0.0038; slope 1.03) per ml diet. A *B.t.* resistant (YHD2) strain was originally collected in 1988 from tobacco in Yadkin Co., NC. Larvae were reared each generation ~7 days from egg hatch on 1000 *ug* of CryIAc per ml of diet prior to transfer to insecticide-free diet for the remainder of development. The LC_{50} of CryIAc was 2952 *u*g (2247-3604; 3.23) per ml diet. The F1 larvae of YHD2 (female) X Wake (male) crosses had an LC_{50} of 0.129 *u*g (0.091-0.178; 3.19) CryIAc/ml diet. Wake and YHD2 larvae were also reared from egg hatch to the third stadium on cotton, *Gossypium barbardense* (var. Delta Pine Nutty) in the greenhouse at 25 degrees C (day) and 18 (night).

A laboratory strain of the bollworm (Hz lab) was obtained from cotton in Plymouth (Washington Co.), NC in August and September, 1996 and reared in the lab on artificial diet as described earlier for *H. virescens*. A field strain of the bollworm was collected in July 1998 from Alachua Co., FL (Hz Alachua) and a field strain of the tobacco budworm was collected in July 1998 from Quitman Co., GA (Hv Quitman). These insects were reared in the laboratory on artifical diet for only 4 generations prior to assay. The thiodicarb (Larvin) resistant strain of the tobacco budworm (Hv LarvinR) is described by Zhao et al. (1996) with an LC_{50} of 1.435 *u*g (0.799-2.870; 0.9) thiodicarb/ml diet. The Wake strain LC_{50} for thiodicarb was 0.0083 *u*g (0.0059-0.0111; 1.7)/ml diet.

Feeding Disruption Bioassay

The feeding disruption bioassay is conducted with artificial diet containing 20 mg of Trypan Blue (Direct Blue 14; Matheson Coleman and Bell, Norwoood, OH) per 100 ml of diet. The blue indicator is used to differentiate feces resulting from the ingestion of assay diet from feces derived from other sources. Assays were conducted with blue indicator diet in either 1-ounce clear plastic cups or 24-well microtiter plates for late instars and in 96-well microtiter plates for neonates.

Reprinted from the *Proceedings of the Beltwide Cotton Conference*
Volume 2:926-930 (1999)
National Cotton Council, Memphis TN

The principle of the resistance and species bioassays is based on disruption of feeding in individuals or populations susceptible to *B.t.* or a chemical insecticide incorporated into artificial diet. The end point being measured is the production of blue feces. For example, *B.t.*- or thiodicarb-resistant budworm larvae will feed and produce blue feces at a diagnostic dose of the insecticide, while the susceptible budworm larvae will not. The species diagnosis is based on the same principle, i.e., the bollworm on a diagnostic dose of CryIAc will produce blue feces while tobacco budworms on the same diet do not produce feces.

The studies that follow examine the utility of this approach for resistance diagnosis in cotton for the biological insecticide *B.t.* and the chemical insecticide thiodicarb and for distinguishing the tobacco budworm from the bollworm. These studies also include a preliminary field validation of this assay technique. Thiodicarb (as Larvin) was provided by Rhone Poulenc Agricultural Chemical Company, Research Triangle Park, NC.

Results and Discussion

Blue Indicator Diet for Feeding Disruption Assay

An essential component of the feeding disruption assay technology is the ability to differentiate feces generated from insects feeding on the assay diet with feces derived from other sources, such as brown feces produced from normal artificial diet (important in laboratory experiments) or green feces from cotton. Neonates may also produce feces resulting from ingestion of egg chorion and larvae can generate feces as a result of cannibalism. Trypan blue was chosen as a marker because of its unique color not normally associated with feeding and feces production in the Lepidoptera.

In order to examine the effects of Trypan Blue on lepidopteran feeding, 3rd instars of the Wake and YHD2 strains of the tobacco budworm were placed on regular and Trypan Blue diet for 24 hours (Fig. 1). The rate of fecal production by the YHD2 strain did not differ between the regular and blue diet (no diet main effect; $F = 0.13$; df = 1,18; $P = 0.7248$ and no diet X time interaction effect; $F = 0.08$; df = 1,18; $P = 0.7834$). For the Wake strain, a small difference in fecal production was observed between diet treatments (diet X time interaction; $F = 6.05$; df = 1,24; $P = 0.0215$). The YHD2 strain also had a significantly higher feces production rate on blue diet than the Wake strain (strain X time interaction, $F = 19.62$; df = 1,24; $P = 0.002$). However, the differences that were found were small relative to the inhibitory effects on fecal production produced by insecticides (data follows) and did not preclude the use of Trypan Blue as a feeding indicator in the feeding disruption bioassay. The appearance of feces originating from diet containing the blue indicator is easily distinguished from those resulting from other sources.

Feeding Disruption Assay for *B.t.* Resistance Detection in Tobacco Budworm Populations

The rate of fecal production for *B.t.* susceptible (Wake), resistant (YHD2), and hybrid (YHD2 female X Wake male) budworms on blue diet containing different concentrations of CryIAc is shown in Fig. 2. Recall (see Materials and Methods) that the YHD2 stain is highly resistant and the YHD2 X Wake cross less resistant to *B.t.* Each test was conducted in triplicate on 15 3rd instars per replicate. Relatively few feces are produced by Wake budworms as compared to the YHD2 strain in these studies. For example, after 24 h at 0.032 *u*g of CryIAc per ml of diet, Wake budworms produced a total of 12 fecal pellets/15 larvae (0.8/larva) as compared with 470 (31/larva) for YHD2 larvae. The resistant 3rd instar budworm population could be separated from the susceptible population in as little as 4 h at doses between 0.008-0.064 *u*g CryIAc per ml diet. A dose of 0.032 *u*g/ml was considered optimum in these laboratory studies due to the sharply diminished fecal production by the Wake strain at this dose.

The detection of resistance in heterozygotes with a much lower LC_{50} than that of the YHD2 strain was also possible (Fig. 2), although differences between the F1 hybrids and Wake larvae were not as distinct as those between YHD2 and Wake. A rate of fecal production by the hybrids intermediate to that of YHD2 and Wake strains exists at all concentrations of CryIAc tested. The 0.032 *u*g/ml dose at 24 h resulted in 470 fecal pellets (31/larva) for the YHD2 strain, 68 (5/larva) for the hybrid and 12 (0.8.larva) for the Wake susceptible strain. A single diagnostic concentration of 0.032 *u*g/ml can distinguish the Wake strain from the highly resistant YHD2 strain after a minimum of 4 h and the YHD2 X Wake hybrid strain with lower resistance at 24 h.

Since the previous experiments were conducted on budworms reared on artificial diet, the efficacy of this resistance assay for plant-reared YHD2 and Wake larvae was examined. Neonates from both strains were reared to the 3rd stadium on cotton and assayed at 0.032 *u*g CryIAc per ml blue diet (Fig. 3). The results are similar to those of insects reared on artificial diet (Fig. 2) with essentially no feces produced by the Wake strain as compared to >140 fecal pellets produced per 15 larvae for YHD2 in 24 h (Fig. 3).

It is clear from these laboratory studies, that the feeding disruption assay is able to diagnose different levels of *B.t.* resistance in homogenous populations of the tobacco budworm in 4-24 h, even when the insects were transferred from cotton. As long as the LC_{50} of the population to be tested is significantly higher than the reference population, a diagnosis should be possible with this technique. The most appropriate diagnostic dose must be determined from field populations and will be dependent on the choice of the susceptible reference strain.

Feeding Disruption Assay for Resistance Detection to Chemical Insecticides in Tobacco Budworm Populations

The feeding disruption assay can also detect resistance to chemical insecticides (Fig.4). Thiodicarb (Larvin) resistant tobacco budworms (Hv LarvinR) continued to produce feces at a diagnostic concentration of 1 *u*g/ml blue diet while the Wake susceptible insects produced no feces at this concentration.

Diagnosis of *B.t.* Resistance on Individual Neonates

Although we used 3rd instars in the above studies to demonstrate the feasibility of using feeding disruption as an indicator of resistance, the most practical application of this bioassay in cotton is to assay budworms collected as eggs or neonates. Eggs are the easiest stage to collect from the field, and a resistance diagnosis in newly hatched larvae would provide the grower with timely information for making pest management decisions. The common endpoint for assessing *B.t.* susceptibility is mortality at 7-10 d after treatment.

The feeding disruption assay should work for any instar; however, the diagnostic dose could change with the age of the larva. It might also be advantageous to diagnose resistance in individual insects. To address both of these questions, the feeding disruption assay at 0.032 *u*g of CryIAc per ml blue diet was used to diagnose resistance in individual insects for the Wake and YHD2 strain (Fig. 5, top). At 24 h only a small percentage of the YHD2 (<4%) could not be distinguished from the susceptible Wake strain. The reliability of the diagnosis can be increased further by increasing the observation period an additional 12-24 h. The same diagnostic dose was used for resistance detection for both 3rd instars and neonates.

Feeding Disruption Assay for Distinguishing the Tobacco Budworm from the Bollworm

Natural pest populations in US cotton today include the *B.t.*-susceptible tobacco budworm and *B.t.*-susceptible bollworm. Using the discriminating dose of 0.032 *u*g CryIAc per ml blue diet at 24 h, our assay clearly distinguished neonates of the susceptible (Wake) budworm from the bollworm (Fig. 5, compare top and bottom). Larvae producing ≤6 fecal pellets were budworm and those producing ≥7 blue fecal pellets were bollworm. Only 2% of *H. virescens* produced 6 blue fecal pellets and 99% of the bollworms produced >15 fecal pellets.

Two commercial immunodiagnostic assays are available which differentiate eggs of the budworm from the bollworm, the LepTon Kit from Abbott Laboratories (Cibulski and Ng, 1996) and the *Hel*-ID Kit from Agdia, Inc. (Zeng et al., 1998). One of the major advantages of the feeding disruption assay is that it not only detects the presence of the bollworm but it simultaneously assays for *B.t.* resistance in the tobacco budworm. No current immunoassay technique has this capability. In addition, the feeding disruption assay does not destroy the insect as is the case for the immunoassays for species diagnosis. This provides the grower with the ability to run multiple diagnostic tests on the same collection of insects. For example, species diagnosis, resistance to *B.t.* or chemical insecticides, a mortality assay or immunodiagnostics could be run in various combinations. Finally the feeding disruption assay can be constructed by an individual grower or consultant as needed or could be produced commercially (Roe et al., 1998).

Field Validation of the Feeding Disruption Assay

The determination of discriminating doses for species and resistance diagnosis for field populations of the tobacco budworm and bollworm from different regions of the SE US is in progress. Fig. 6 shows that a discriminating dose of 0.032 *u*g of CryIAc per ml blue diet was able to distinguish the field strains, Hv Quitman from Hz Alachua with minimal overlap.

Summary

A feeding disruption assay is described for the detection of insect resistance to *B.t.* and chemical insecticides and for species diagnosis of the tobacco budworm and the bollworm in cotton. The assay does not destroy the insect allowing for multiple diagnostic tests to be performed on the same collection. Detection of *H. zea* and *B.t.* resistant *H. virescens*, both of which can be destructive to *B.t* cotton, are achieved simultaneously. Immunoassays for species diagnosis cannot detect resistance. The feeding disruption assay detects most mechanisms of resistance simultaneously in little as 4 h, assays for resistance in larvae (the insecticide target), detects resistance on either individuals or groups of insects and should be applicable to field use at a minimal cost per assay.

Acknowledgments

This project was supported by grants from the North Carolina State University/NSF Research Center for Integrated Pest Management (IPM94-007), the North Carolina Agricultural Research Service, Cotton Incorporated (98-632) and the Southern Region IPM Program (98-34103-6269). For providing assistance in procuring strains for these studies, we wish to thank Dr. R. Smith of Auburn University, the cotton entomology staff at the Wiregrass Experiment Station in Headland, AL and the Department of Entomology Insectary at North Carolina State University. The technical assistance of Vann Covington is greatly appreciated.

References

Bailey, W. D., G. Zhao, L. M. Carter, F. Gould, G. G. Kennedy and R. M. Roe. 1998. Feeding disruption bioassay for species and *Bacillus thuringiensis* resistance diagnosis for *Heliothis virescens* and

Helicoverpa zea in cotton (Lepidoptera: Noctuidae). Crop Protection. 17(7): 591-598.

Cibulsky, R. J. and S. S. Ng. 1996. Lepton HTK: a diagnostic test kit to improve cotton insect control. Proceedings Beltwide Cotton Conferences. 889-891.

Gasser, C. S. and R. T. Fraley. 1989. Genetically engineering plants for crop improvement. Science 244: 1293-1299.

Gill, S. S., E. A. Cowles and P. V. Pietrantonio. 1992. The mode of action of *Bacillus thuringiensis* endotoxins. Annu. Rev. Entomol. 37: 615-636.

Gould, F. 1988. Genetic engineering, integrated pest management and the evolution of pests. Trends Ecol. Evol. 3: S15-S18.

Gould, F., A. Anderson, A. Reynolds, L. Bumgarner and W. Moar. 1995. Selection and genetic analysis of a *Heliothis virescens* (Lepidoptera: Noctuidae) strain with high levels of resistance to *Bacillus thuringiensis* toxins. J. Econ. Entomol. 88: 1545-1559.

Jenkins, J. N., W. L. Parrott, J. C. McCarty Jr., F. E. Callahan, S. A. Berberich and W. R. Deaton. 1993. Growth and survival of *Heliothis virescens* (Lepidoptera: Noctuidae) on transgenic cotton containing a truncated form of the delta endotoxin gene from *Bacillus thuringiensis.* J. Econ. Entomol. 86: 181-185.

Lambert, A. L., J. R. Bradley Jr. and J. W. Van Duyn. 1996. Effects of natural enemy conversation and planting date on the susceptibility of Bt cotton to Helicoverpa zea in North Carolina. Proceedings Beltwide Cotton Conferences. 931-935.

Mascarenhas, V. J., R. G. Luttrell and J. C. Schneider. 1994. Activity of transgenic cotton expressing delta-endotoxin against tobacco budworm. Proceedings Beltwide Cotton Conferences. 1064-1067.

McGaughey, W. H. and M. E. Whalon. 1992. Managing insect resistance to *Bacillus thuringiensis* toxins. Science 258: 1451-1455.

Roe, R. M., W. D. Bailey, F. Gould, G. G. Kennedy and C. L. Sutula. 1998. Insecticide resistance assay. Patent pending.

Rose, R. L., L. Barbhaiya, R. M. Roe, G. C. Rock and E. Hodgson. 1995. Cytocrome P450-associated insecticide resistance and the development of biochemical diagnostic assays in *Heliothis virescens*. Pestic. Biochem. Physiol. 51: 178-191.

Tabashnik, B. E. 1994. Evolution of resistance to *Bacillus thuringiensis*. Annu. Rev. Entomol. 39: 47-79.

Zeng, F., S. B. Ramaswamy and S. Pruett. 1998. Monoclonal antibodies specific to tobacco budworm and bollworm eggs. Ann. Entomol. Soc. Am. 91(5): 677-684.

Zhao, G., R. L. Rose, E. Hodgson and R. M. Roe. 1996. Biochemical mechanisms and diagnostic microassays for pyrethroid, carbamate, and organophosphate insecticide resistance/cross-resistance in the tobacco budworm, *Heliothis virescens*. Pestic. Biochem. Physiol. 56: 183-195.

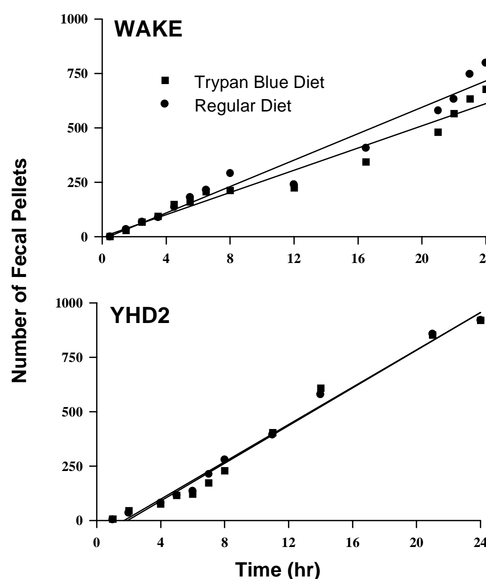


Figure 1. Effect of Trypan Blue in artificial diet on fecal production in two strains of the tobacco budworm (Bailey et al., 1998; Roe et al.,1998).

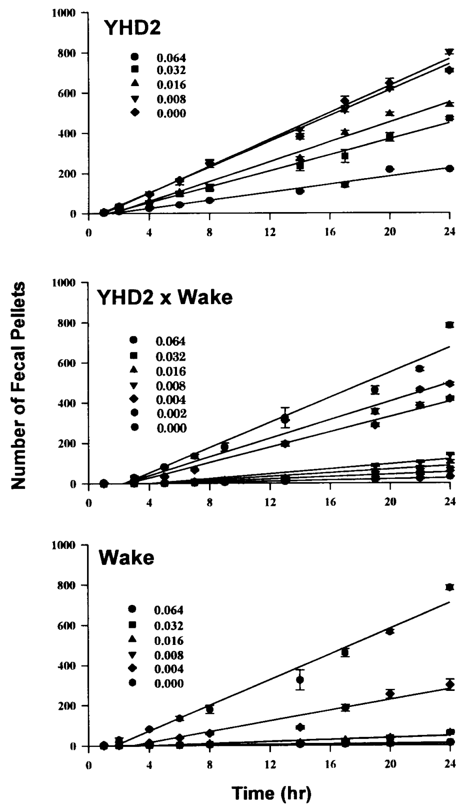


Figure 2. Effect of different concentrations of CryIAc (*u*g/ml blue diet) on fecal production in three strains of the tobacco budworm (Bailey et al., 1998; Roe et al., 1998). The error bars are $\pm$1 SE.

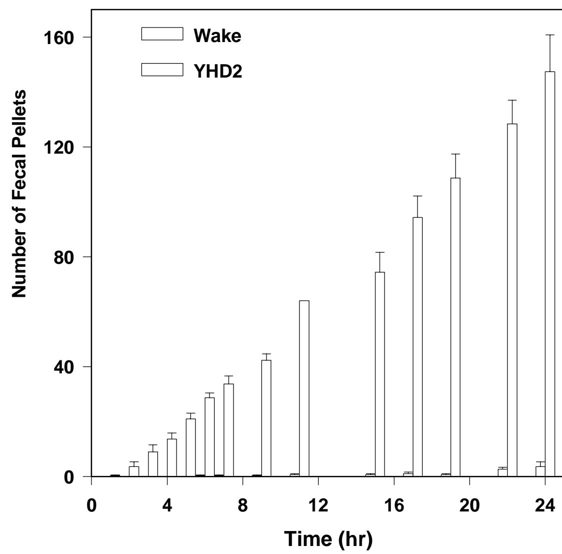


Figure 3. Effect of 0.032 *u*g CryIAc per ml blue diet on fecal production in *B.t.* resistant (YHD2) and susceptible (Wake) tobacco budworms reared on cotton. The error bars are $\pm$1 SE.

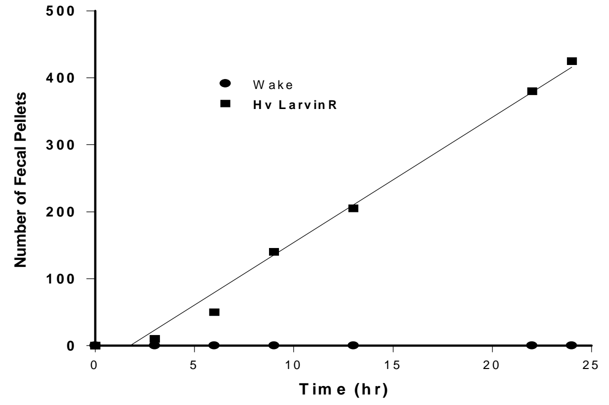
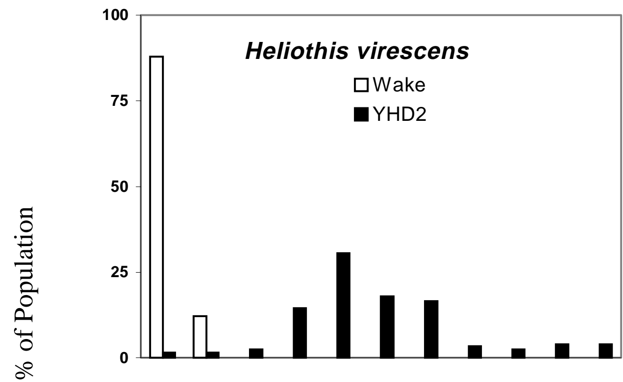


Figure 4. Effect of 1.0 *u*g of thiodicarb (Larvin) per ml blue diet on fecal production in resistant (Hv LarvinR) and susceptible (Wake) tobacco budworms. The numbers of fecal pellets reported are per 15 insects at each time point.



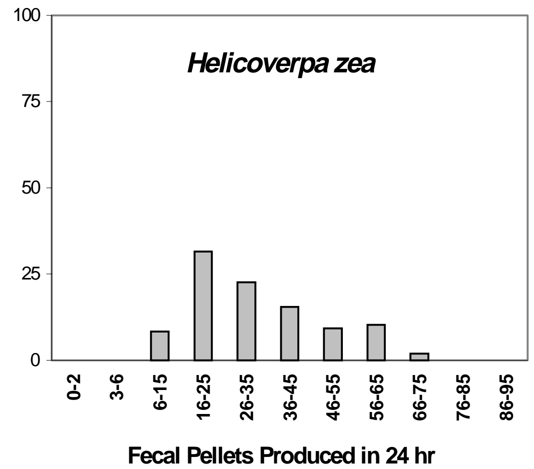


Figure 5. Percentage of population producing blue fecal pellets as neonates in 24 h on 0.032 *u*g of CryIAc per ml blue diet (Bailey et al., 1998; Roe et al., 1998). The results are from two replicates of 25 insects per replicate per species and strain.

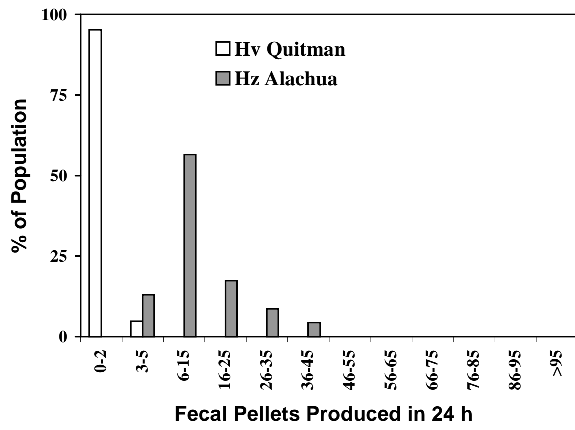


Figure 6. Percentage of population producing blue fecal pellets as neonates in 24 h on 0.032 *u*g of CryIAc per ml blue diet. The results are from a single treatment of 24 insects per strain.