# STATUS OF PINK BOLLWORM SUSCEPTIBILITY TO BT IN ARIZONA

**A.L. Patin, T.J. Dennehy, M.A. Sims, B.E. Tabashnik and Yong-Biao Liu**
**University of Arizona**
**Tucson, AZ**
**L. Antilla**
**Arizona Cotton Research and Protection Council**
**Tempe, AZ**
**D. Gouge and T.J. Henneberry**
**USDA, ARS, WCRL**
**Phoenix, AZ**
**R. Staten**
**USDA, APHIS, PPQ**
**Phoenix, AZ**

## Abstract

Statewide monitoring of pink bollworm susceptibility to Bt toxin was conducted in Arizona in 1997 and 1998. In bioassays in which Bt toxin was incorporated into insect diet, a concentration of 10 $\mu$g/ml Cry1Ac caused >80% mortality of all field populations. LC50s of Arizona populations ranged from 0.35 to 1.7$\mu$g Cry1Ac/ml of insect diet. A laboratory strain of pink bollworm had an LC50 of 0.53 $\mu$g/ml. A selection experiment was conducted in which survivors of bioassays of 3.2 and 10 $\mu$g/ml of Cry1Ac from all Arizona populations tested in 1997 were pooled and reared for one generation on diet containing 10 $\mu$g Cry1Ac/ml. Selection produced a strain of pink bollworm that was 100- to 460-fold less susceptible to Cry1Ac, than were the field populations from which it was derived. Preliminary results from greenhouse evaluations concerning survival of this resistant strain on Bt cotton showed that larvae were able to complete development in bolls of Bt cotton, pupate, and successfully reproduce. We conclude from these preliminary results that the type of resistance we have isolated in the laboratory is likely to negatively impact field performance of Bt cotton in Arizona in the future. However, we found no evidence that field performance of Bt cotton has yet been compromised. In large field trials, in-field refuges of non-Bt cotton were contrasted with the standard, external refuges. During both 1997 and 1998, in-field refuge plots yielded comparable to, or better than, plots of 100% Bt cotton, yet they produced substantial numbers of pink bollworm late in the season on non-Bt plants.

## Introduction

Transgenic cotton derived from *Bacillus thuringiensis* represents the biggest technological breakthrough in cotton insect management in many decades. In Arizona, it has been extremely effective at controlling pink bollworm (PBW), *Pectinophora gossypiella*. Control of this pest was estimated to cost cotton producers in Central and Northwestern Arizona \$18.49-\$83.74 per acre in 1997. Control costs for Arizona and Southern California were estimated to exceed 1.2 billion dollars over the past 30 years (Robberson *et al.* 1998). The benefits of using Bt cotton include reduced environmental and worker exposure to conventional insecticides, reduced selection for resistance to conventional insecticides, and improved conservation of natural enemies. Bt cotton in Arizona was planted to 60%-70% of Upland cotton acreage in 1997 (Silvertooth, 1998) and 70% in 1998 (Monsanto).

Many debates regarding deployment of Bt transgenic crops have focused on combating what is widely agreed to be a very high risk of resistance development in target pests. In cotton, this conclusion stems from the 5-7 months that pests are exposed to Bt toxins in the plants, coupled with irrefutable evidence that insects can evolve physiological mechanisms of resistance to Bt. Tabashnik *et al.* (1990 and 1997), characterized diamondback moth resistance to foliar treatments of Bt. McGaughey and Johnson (1987) described Indianmeal moth resistance to Bt. More recently, Gould *et al.* (1997) found that one in 350 adults of the tobacco budworm, *Heliothis virescens,* carry an allele for resistance to Bt. In some cases, cross-resistance has reduced insect susceptibility to a number of Bt toxins (Gould *et al.* 1992 and Tabashnik 1994).

In this report we present the second year of studies conducted in Arizona to detect and manage PBW resistance to Bt. We describe: 1) baseline monitoring of populations collected throughout the State in 1997; 2) selection with Bt of a composite of Arizona populations; 3) evaluations of efficacy of Bt cotton in 1998; and, 4) field evaluations of contrasting strategies for deploying Bt cotton in Arizona.

## Materials and Methods

### Susceptibility of Arizona PBW to the Bt Endotoxin, Cry1Ac

***Collection and Rearing.*** Collections commenced in August and continued through November 1997 at one location each in the vicinity of Parker, Safford, Mohave, Coolidge, Paloma, Stanfield, and Eloy, Arizona. Preliminary findings representing a subset of these monitoring data were reported by Simmons *et al.* (1998). At each location 1,000 to 2,000 bolls were collected from non-Bt cotton fields, in areas adjacent to Bt fields, and transported to The University of Arizona Extension Arthropod Resistance Management Laboratory (EARML) in Tucson. There they where placed in boll boxes (17.6 cm x 50.4 cm x 35.2 cm). Boll boxes suspended infested bolls approximately 3 cm above sheets of paper toweling. Fourth instar larvae cut out of infested bolls and dropped onto the paper toweling on the bottom of the boxes. These larvae were then transferred to pupation boxes, consisting of tightly sealed, 1.7 l rectangular Rubbermaid® containers enclosing sheets of paper towel.

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To prevent or disrupt diapause, larvae that had cut out of bolls and webbed up were disturbed, twice per week, by pulling the paper toweling apart and spraying it lightly with water. After a sample of bolls had been in boxes for 30 days, bolls were opened to recover any larvae that had diapaused within.

We reared PBW using a modified version of the rearing method detailed by Bartlett and Wolf (1985). F1 offspring of field-collected PBW had low survival on diet in 16 oz cups, but survival of F2 larvae was much higher. F1 survival was enhanced by rearing larvae singly in 1 oz medicine cups. F2 and subsequent generations were reared in the 16 oz containers as described above.

***Bioassaying PBW Susceptibility to Bt.*** For bioassays, we put neonate larvae on a wheat germ diet (Adkinson *et al.*), into which Cry1Ac toxin had been incorporated, and evaluated mortality after 21 days (Bartlett, 1995). MVP-II® Bioinsecticide (Mycogen, San Diego, CA) was mixed into sterilized distilled water to produce a stock solution of toxin. The stock was then added to liquid wheat germ diet in amounts necessary to create final concentrations ranging from 0.001 to 320 $\mu$g/ml Cry1Ac. Solutions were blended thoroughly into liquid diet at 50-60°C. Concentrations of toxin used in 1997 for routine monitoring were 0, 0.1, 1, 3.2 and 10 $\mu$g Cry1Ac/ml of diet. Diet was made in 1 liter batches of each concentration evaluated. It was then cooled, shredded and dispensed into 1 oz cups. One neonate larva was transferred with a fine brush into each bioassay cup. Using this procedure we tested four to nine replicates of ten larvae for each concentration. Bioassays were incubated in darkness at 29±2 °C for 21 days, after which survivorship and developmental stage (Watson and Johnson 1974) were recorded.

**Selection of the Arizona PBW Populations for Resistance to Bt in the Laboratory**

Pink bollworm from the 1997 collections that survived bioassay concentrations of 3.2 and 10 $\mu$g/ml were pooled into a composite strain designated AZP-R. This strain was then reared for one generation on diet containing 10 $\mu$g/ml of Cry1Ac and tested for susceptibility to Cry1Ac.

**Preliminary Greenhouse Evaluations of Survivorship of AZP-R**

After selecting the AZP-R population for one generation as described above, survivorship was evaluated on greenhouse-grown Bt cotton. Three to four Bt (Delta Pine 50B) and non-Bt (Delta Pine 50) plants, 80-100 days old were used. Bolls 2-3 weeks post-bloom were infested with PBW by placing under the brackets of the bolls small pieces (10 x 10 mm) of gauze on which 40-50 PBW eggs had been laid. Eggs on the gauze squares hatched and neonates bored into bolls. Bolls thus infested were enclosed in cages constructed from 5 oz plastic cups fitted with a screened lid. Counts of neonate entrance holes were made seven days after infestation. Plants were examined for emergence of fourth instar larvae twice per week beginning at 14 days after infestation. After 35 days all bolls were dissected and the number and developmental stages of surviving larvae were recorded.

**Evaluation of Field Performance of Bt Cotton Throughout Arizona**

At thirty-five locations throughout Arizona, paired non-Bt and Bt fields were evaluated from September through December, 1998, for survival of large PBW larvae (=3rd instar) in bolls. A minimum of 50 bolls were sampled from non-Bt plants and 500 bolls from adjacent areas in the paired Bt fields.

**Evaluation of Contrasting Bt Deployment Strategies**

A 200-acre field trial was established in 1997 (Simmons et al 1998) in Eloy, Arizona, and was increased in 1998 to 296 acres. Three strategies for deploying Bt were evaluated: 1) external refuges (Monsanto strategy), 2) in-field refuges, and 3) biointensive treatments. These were contrasted with control groups comprising 100% non-Bt and 100% Bt cotton. The size of the treatment blocks, numbers of replicates and varieties of cotton grown in the various treatments are detailed in Table 1. Selection of varieties and placement of control blocks were based on exigencies of commercial production and restrictions imposed by seed contracts.

The external refuge treatment was designed to reflect the 20% managed refuge option of the so-called ‘Monsanto strategy’. In this case 14.7 acre blocks of Bt cotton had adjacent 4.34 acre blocks of non-Bt cotton. The in-field refuge treatment, an alternative to current practice, placed refuge plants uniformly within Bt fields. One center hopper of a six-row planter dispensed non-Bt cotton seed in one row for every five rows of Bt cotton. The biointensive treatment comprised the combined action of Bt cotton and an application of parasitic nematodes to kill PBW surviving in the soil. The parasitic nematode, *Steinernema carpocapsae* is a natural enemy of PBW (Gouge et. al, 1997). It attacks the larvae once they cut out from bolls and enter the soil to pupate.

***Monitoring PBW Infestations.*** Infestations of PBW were monitored in all treatments using pheromone traps, counts of rosetted blooms, and sampling of bolls. Delta pheromone traps monitored adult PBW populations in all plots throughout the season. Traps were serviced and pheromone septa replaced weekly. Throughout July, rosetted blooms were scouted, once per week, in the center 20% of each plot. Between 10 AM and 2 PM blooms were observed while walking along a row in the middle of each treatment. Fifty bolls approximately 2.5 cm in diameter were sampled from each replicate of non-Bt cotton on 28 July, 1 September, and 29 September, 1998. All boll collections were sampled from the central 20% of each replicate. The bolls were taken to the laboratory where they were refrigerated for up to two weeks, dissected (cracked) and the number and instar of the PBW recorded. Sampling of Bt plots commenced once bolls from non-Bt plots indicated that PBW populations were increasing. On 6 October, 17 October and 1 November, samples of 100 bolls were collected from all Bt plots and 50 bolls each from non-Bt plots. On 1 November, 1000 bolls were collected from each Bt plot and 100 bolls from each non-Bt plot.

For in-field refuge plots, independent samples of bolls were collected from the Bt and non-Bt cotton rows. Similarly, Bt and non-Bt portions of the external refuge plots were sampled independently. All plots were harvested during the second week of November. Yields were obtained by summing the bales of lint harvested for: 1) the in-field refuge treatment (combined Bt and non-Bt portions); 2) the non-Bt portion of the external refuge treatment; 3) the 100% Bt plots (the Bt portion of the external refuge treatment, the 100% Bt control plots, and the 100% Bt biointensive plots) and; 4) the 100% non-Bt control plots. The total number of bales produced was divided by the number of acres in each treatment to derive yield in bales per acre.

Nematodes for the biointensive treatment were obtained as the formulated product, Millennium (NEMA 1), from Thermo Trilogy (Columbia, MD). This water dispersible granule was applied through routine furrow irrigation. A single application of 526 million nematodes per acre was made to each replicate of the biointensive treatment on 17-19 June, 1998. A nurse tank containing 800 gallons of water and ten billion nematodes was emptied into the irrigation canal to treat 19 acres. Continuous agitation of the nurse tank maintained the nematodes in suspension. To evaluate efficacy of nematodes, fifty cassettes containing fourth instar PBW larvae were buried a few inches below the surface, just prior to applying nematode treatments. These were arranged approximately 30 furrows apart in five rows of five cassettes each on the top of the furrows and five rows of five cassettes each in the bottom of the furrows. Within these rows the cassettes were approximately 100 meters apart. Forty-eight hours after irrigation, the cassettes were collected and percent parasitism was scored by dissecting larvae under a compound microscope.

***Other Treatments Made to Plots in 1998.*** An application of the insecticide, Penncap-M® (methyl parathion) was applied at 3.05 pints per acre on 8 August for control of lygus bugs. A second application for lygus was made on 30 August and consisted of 1.63 pints per acre of Lorsban® 4E (chlorpyrifos). Both of these treatments were made only to non-Bt cotton control plots. A third and final insecticide application of 16.0 oz Orthene® 90WSP (acephate) was applied on 16 August, across all treatments for lygus control.

## Results and Discussion

### Field Performance of Bt Cotton in Arizona

The performance of Bt cotton continued to be excellent in 1998, based on evaluations of a total of 35 pairs of Bt and adjacent non-Bt fields sampled throughout Arizona (Table 2). Twenty pink bollworm (=3rd instar) were found in 29,300 bolls collected by the ARCPC from 33 Bt fields, yielding a statewide average of 0.068% infested bolls. This value was consistent with reports by Flint and Parks (1997) and Flint *et al.* (1996) of overall PBW occurrence in Bt cotton ranging from 0.034 to 0.042% for five pairs of fields monitored in 1995 and 1996. In 1998, non-Bt fields averaged 30% of bolls infested with =3rd instar pink bollworm (Table 2). Over half of all the infested Bt bolls collected by the ARCPC in 1998 came from one field in La Paz county (Tables 2). Of the 1000 Bt bolls collected at this site, 13 bolls were infested with large larvae of pink bollworm.

A second large boll sample was collected from La Paz County by EARML staff (Table 2). This was done without knowledge of the aforementioned ARCPC collection and from a cotton field approximately ten miles from the ARCPC collection site. Whereas the first La Paz County sample yielded 1.3% infested bolls, the second sample had a very similar, 1.2%, infestation rate. Thirteen large pink bollworm larvae were recovered from 1,103 Bt bolls (Table 2). We cannot conclude at this time whether these elevated infestation rates, =20-times the overall state average, were the result of normal survival of susceptible PBW on rogue, non-Bt plants, or whether they reflect the early onset of resistance. To distinguish between these two hypotheses, we are testing for the presence of Bt endotoxin in the bolls from which surviving large larvae were recovered. Bt cotton can legally contain as much as 2% non-Bt seed.

In the 1997 season, we reported (Simmons *et al.* 1998) infestation rates as high as 4.1% large larvae at the site of our field trial in Eloy, Arizona. This was an isolated instance and was not observed elsewhere in Arizona in 1997. In 1998, at this same Eloy location, Bt cotton had and average of only 0.11% infested bolls. Out of a total of 14,300 bolls evaluated, 16 large larvae were recovered (Table 2). From these results we concluded that the prior year's higher infestation rates were likely the result of a seed lot that had an unusually high proportion of non-Bt contaminants.

### Susceptibility of Arizona PBW to the Bt Endotoxin, Cry1Ac

Arizona PBW populations were very similar in susceptibility to Cry1Ac toxin in 1997 (Fig. 1). LC50 values differed <5-fold between the 7 populations evaluated and ranged from 0.35 to 1.7 $\mu$g Cry1Ac/ml. The susceptible reference population, APHIS-S, maintained in the laboratory for over two decades, had an LC50 of 0.53$\mu$g Cry1Ac. The similarity of response to Bt of this laboratory population versus field populations suggests that major decreases in susceptibility of field populations to Bt had not occurred in 1997.

**Selection of Arizona PBW Populations for Resistance in the Laboratory**

Selection of the pooled Arizona strain in the laboratory, by rearing on diet containing 10 $\mu$g/ml Cry1Ac, yielded a strain 100 to 460-fold less susceptible than the individual populations from which it was derived, based on LC50s. We designated this strain AZP-R. The LC50 of the AZP-R strain was 162 $\mu$g/ml. The LC50 derived from bulking all Arizona populations evaluated in 1997 was 0.914 $\mu$g/ml. Therefore, an overall estimate of the intensity of resistance in the selected AZP-R strain, relative to field populations statewide, would be a 177-fold reduction in toxicity of Cry1Ac, in diet bioassays.

Though we found that field performance of Bt cotton against PBW continued to be excellent in Arizona in 1998, the results with AZP-R show that PBW has the genetic potential for resistance to Cry1Ac. Finding this, we then undertook greenhouse experiments to determine the degree to which resistance in the AZP-R strain increased survival in bolls of Bt cotton. Preliminary findings show that the resistance of AZP-R conferred the ability for PBW to complete larval development in bolls of greenhouse-grown Bt cotton, pupate, and successfully reproduce.

**Evaluation of Contrasting Bt Deployment Strategies**

***Lint Yields.*** For the second consecutive year, very promising results were obtained from in-field refuges (Fig. 2). Yield of the 5:1 in-field refuge treatment averaged 2.08 bales/acre. This was somewhat higher than all other treatments. The external refuge treatments yielded 1.86 bales/ acre. Figures reported for in-field and external refuge treatments combined the yield of Bt and non-Bt portions of the trial into one total value. Treatments of 100% Bt yielded 1.86 bales/acre and non-Bt (control) plots yielded 1.85 bales/acre (Fig. 2).

Statistical contrasts could not be made between these yields because the producers' harvesting operation bulked treatment replicates within the entire 296 acre trial. Yield of the external refuge plot was computed by weighted average consisting of 0.80 the yield per acre in Bt cotton plus 0.20 the yield in the adjacent non-Bt (refuge) plots (Fig. 2). Yield reported for the internal refuge plots was the actual bales of cotton per acre harvested independently from the Bt and non-Bt portions of this treatment, bulked for the three treatment replicates.

***PBW Infestation Levels.*** Pink bollworm levels throughout Arizona were lower in 1998 than in recent years, presumably owing to relatively cool and wet spring conditions. This fact was demonstrated by pheromone trap catch at the Eloy plot in 1997 versus 1998 (Figure 3). Larval infestation levels in non-Bt cotton at Eloy reflected this delay in the onset and intensity of population development area-wide. No large PBW larvae (=3rd instar) were found before 1 September in any of the non-Bt treatments. However, populations increased in September and intensive sampling of all treatments commenced on 6 October, when low numbers of larvae were detected in non-Bt plots (Table 3). By 1 November, when sampling was discontinued, infestations in the large non-Bt (control) blocks had reached 13.4%. The non-Bt blocks of the external refuge treatment had 4-5% infestation of large larvae, while non-Bt plants of the 5:1 internal refuge plots had 4.7-7.3% infestation.

Infestation levels in Bt plots ranged from undetectable for the external refuge plots to a high of 0.9% for the in-field refuge plots (Table 3). This 0.9% level is 13-fold higher than the aforementioned statewide average of 0.068% infested bolls in Bt cotton, and it is very similar to the two exceptional cases in La Paz county that had 1.2 and 1.3% boll infestation (Table 2). However, these infestation rates continue to be below the legal limits for non-Bt seed in Bt seed bags. There were too few surviving PBW in Bt plots at Eloy to allow establishment of a culture for testing survivors for resistance. We are currently testing for the presence of endotoxin in bolls from which large larvae were collected to allow us to further interpret this outcome.

Infestation levels in the biointensive treatment, in which parasitic nematodes were applied in conjunction with Bt cotton, were indistinguishable from those of the 100% Bt treatments. Field bioassays of efficacy of the nematode treatment showed that they killed an estimated 31.7% of 4th instar larvae buried in cassettes. This mortality was considerably lower than the >80% levels experienced in the previous year (Simmons *et al.* 1998).

***In-field Versus External Refuges.*** We currently do not have precise scientific answers regarding the optimal size and configuration of refuges for thwarting resistance to Bt cotton. We do know, however, that for refuges to be effective, they must produce susceptible adult moths in sufficiently high numbers, and in sufficiently close proximity to areas of Bt cotton, to ensure that resistant survivors of Bt plants mate with susceptible individuals. To this end, in-field refuges of non-Bt cotton offer some distinct advantages over external refuges. They allow susceptible PBW to be generated systematically throughout Bt fields. They also simplify some of the complex decisions that growers currently must make regarding placement of external refuges and, in doing so could potentially reduce problems with non-compliance of the current external refuge strategy.

Mixtures of Bt seed with 10-20% non-Bt seed were evaluated previously in Arizona (Watson, 1995) and were judged to be promising. Our two years of evaluations at Eloy show that yield in the in-field refuge plots was comparable to, or better than, the external refuge plots. It is critical that refuge plants, whether internal or external to the Bt plantings, produce adequate numbers of adult moths. In this regard, it appears that the in-field refuge was successful (Table 3). At the end of the 1998 season the in-field refuge treatments had somewhat higher densities of large larvae than did the external refuge non-Bt plants (Table 3). Long-term evaluations of in-field versus external refuges will be necessary to determine their impact on resistance development.

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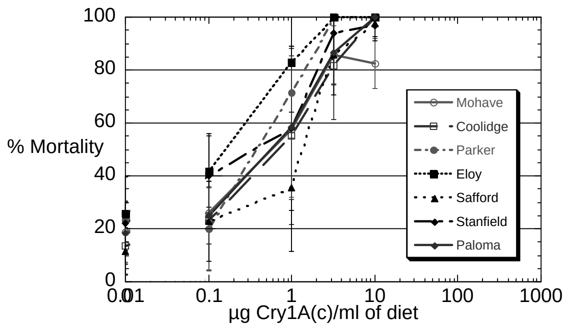


Figure 1: All populations of pink bollworm collected in 1997 responded very similarly to Cry1Ac endotoxin in diet incorporation bioassays. Small but statistically-significant differences in susceptibility between populations were detected.

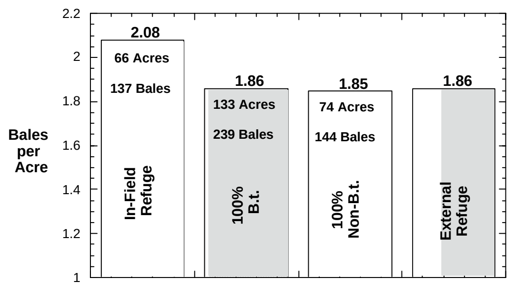


Figure 2: As in 1997, in-field refuges of non-Bt cotton yielded very satisfactorily at Eloy in 1998, relative to non-Bt and 100% Bt plots. In-field refuge plots comprised one row of Sure-Grow 125 for every five rows of Hartz 1560BG. The 100% Bt plots comprised Hartz 1560BG. The non-Bt plots and the non-Bt portion of the external refuge plots were Sure-Grow 125.

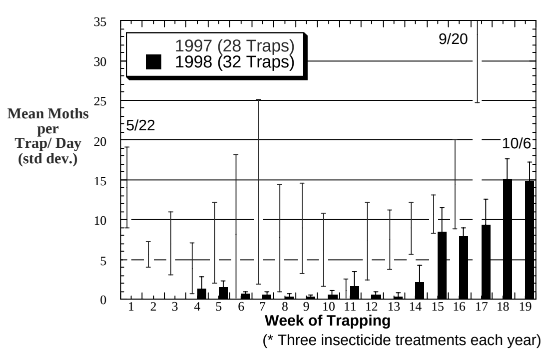


Figure 3: The delayed onset and reduced intensity of pheromone trap catch at the Eloy test site in 1998, relative to 1997, is consistent with the overall reduced intensity of PBW in Arizona in 1998.

Table 1: Details of 1998 Eloy, Arizona, evaluations of Bt deployment strategies.

| Treatment | Variety | Block Size | Reps |
|---|---|---|---|
| **External Refuge (80% Bt/20% Non-Bt)** | | | |
| Bt | Hartz 1560BG | 205 rows | |
| Non-Bt | Hartz 1560 | 41 rows | 2 |
| **In-Field Refuge (5 rows Bt:1 row Non-Bt)** | | | |
| Bt | Hartz 1560BG | 14.7 Acres | |
| Non-Bt | SG 125 | 4.3 Acres | 3 |
| **Biointensive (Bt + Nematodes)** | | | |
| | Hartz 1560BG | 19 Acres | 3 |
| **100% Non-Bt** | | | |
| | SG 125 | 26 Acres | 3 |
| **100% Bt** | | | |
| | Hartz 1560BG | 19 Acres | 3 |

Table 2: Efficacy of Bt cotton in Arizona in 1998.

| PBW Source | Infested Bolls | Total Bolls | % Infested |
|---|---|---|---|
| **I. ARCPC-33 Locations** | | | |
| Statewide Averages | | | |
| Bt Plants | 20 | 29300 | 0.068 |
| Non-Bt Plants | 760 | 2550 | 30 |
| La Paz #1 | | | |
| Bt Plants | 13 | 1000 | 1.3 |
| Non-Bt Plants | 76 | 100 | 76 |
| **II. EARML Locations** | | | |
| Eloy Field Trial | | | |
| Bt Plants | 16 | 14300 | 0.11 |
| Non-Bt Plants | 155 | 2548 | 6.1 |
| La Paz #2 | | | |
| Bt Plants | 13 | 1103 | 1.2 |
| Non-Bt Plants | 67 | 100 | 67 |

***** Large Larvae = = 3rd instar

Table 3: Percentage of bolls infested with large larvae (=3rd instar) of PBW in the Bt deployment trial at Eloy, Arizona, in 1998.

| Collection Date | | 36073 | 36084 | 36099 |
|---|---|---|---|---|
| In-Field Refuge: | Bt Plants | 0.67% | 0.0% | 0.90% |
| | Non- Bt Plants | 7.3% | 7.3% | 4.7% |
| External Refuge: | Bt Plants | 0.0% | 0.0% | 0.0% |
| | Non- Bt Plants | 4.0% | 4.0% | 5.0% |
| 100% Bt Plants | | 0.0% | 0.16% | 0.15% |
| 100% Non-Bt Plants | | 12% | 11% | 13% |