ARIZONA'S MULTI-AGENCY RESISTANCE MANAGEMENT PROGRAM FOR BT COTTON: SUSTAINING THE SUSEPTIBILITY OF PINK BOLLWORM

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Abstract

Bt cotton has been used in Arizona since 1996 with exceptionally positive results in terms of economic returns to growers and reductions in insecticide use in cotton. Yet, the isolation of pink bollworm highly resistant to Bt cotton from collections made in Arizona in 1997 demonstrated the seriousness of the threat that resistance poses to transgenic Bt technology. For this reason unparalleled measures have been taken to detect and manage resistance of pink bollworm to Bt cotton in Arizona. This paper presents results of statewide monitoring of pink bollworm susceptibility to the Bt toxin, Cry1Ac, conducted from 1997 to 1999. Mean susceptibility of Arizona pink bollworm to Cry1Ac increased from 1997 to 1999. Mean corrected mortality in 1µg/ml Cry1Ac assays was 52.3% in 1997, 90.6% in 1998, and 97.9% in 1999. Mean corrected mortality in bioassays of 10 µg/ml was 94.5% in 1997, 99.8% in 1998, and 100% in 1999. Selection with Cry1Ac in the laboratory has produced from 1997 field collections a strain possessing 200 to 900-fold resistance to Cry1Ac. This resistant strain is capable of surviving on Bt cotton. We provide an overview of other components of the multi-agency collaboration to sustain efficacy of Bt cotton in Arizona. These include: 1) evaluation of the field performance of Bt cotton; 2) mapping and analysis of use of Bt and non-Bt cotton and compliance with refuge requirements; 3) effectiveness of internal versus external refuges and movement of pink bollworm moths from refuges; and 4) activities of the Arizona Bt Cotton Working Group to formulate and implement effective resistance management strategies.

Introduction

The registration of Bt cotton in the US in 1996 marked the beginning of a revolution in agricultural pest management. The major insect pest of Arizona cotton, pink bollworm (*Pectinophora gossypiella*) is highly susceptible to the toxin expressed in Bt cotton, Cry1Ac. Producer gains from use of Bt cotton in Arizona, averaging $15,000 per farm (Frisvold et al. 2000), have promoted rapid adoption of this new technology (Table 1). Additionally, the environment and integrated pest management are beneficiaries of the associated decline in use of conventional insecticides. In 1995, the year preceding registration of Bt cotton, an average of 6.3 insecticide applications were made per acre of cotton in Arizona (Figure 1). Insecticide use in Arizona cotton has declined each year since 1995, reaching a low of 2.2 treatments per acre in 1999. While these dramatic reductions in insecticide use are not solely attributable to Bt cotton, it is clear that Bt cotton has played a major role in this outcome.

Industry, academics, governmental regulators, and environmental groups have given unprecedented attention to insect resistance to transgenic Bt crops (Mellon and Rissler 1998). The management strategy currently in place requires planting refuges of cotton that do not produce Bt toxins. While resistance continues to be viewed as a major threat to the future of this technology, it is now clear that the worst fears of academics and environmentalists in this regard have not materialized. Now in its fifth year of use, no failures of Bt cotton have been observed in Arizona, nor have resistance-related failures been reported elsewhere in the US.

Preserving insect susceptibility to Bt crops is considered by many to be the greatest challenge ever addressed by resistance management due to the many months that insecticidal toxins are produced in plants. This challenge seemed all the more daunting for Arizona cotton following the discovery of pink bollworm highly resistant to the Bt toxin produced by Bollgard® cotton, Cry1Ac (Bartlett 1995, Patin et al. 1999). With the benefits and risks of Bt cotton in mind, we established the Extension-based, multi-agency collaboration described herein. In this paper we provide results of three years of monitoring of susceptibility of Arizona pink bollworm to Cry1Ac. We describe pink bollworm selected for resistance in the laboratory and we overview other major components of our multi-agency resistance management program for Bt cotton.

Statewide Monitoring of Resistance Materials and Methods

Susceptibility of Arizona PBW to the Bt Endotoxin, Cry1Ac

Collection. Collections from Arizona cotton fields (Figure 2) commenced as early as August and continued through as late as December. In 1997, collections were made from 9 sites: Coolidge, Eloy (2 samples), Marana, Mohave Valley, Paloma, Parker, Safford, Solomon, and Stanfield. In 1998, boll collections were made at 12 sites. These sites were in the vicinities of Buckeye, Casa Grande, Coolidge, Cotton Center (North), Cotton Center (South), Eloy, Hopeville, Marana, Mohave Valley, Parker, and Safford. Additionally, there was one collection from the Palo Verde Valley in California. Fourteen boll collections were made in the 1999 season in proximity of Buckeye, Coolidge (2 samples), Cotton Center South, Eloy, Harquahala Valley, Marana, Maricopa, Mohave Valley (2 samples), North Gila Valley, Parker, Safford, and Stanfield.

At each location 300 to 2,000 bolls were collected, mainly from non-Bt cotton fields in areas adjacent to Bt fields. In three instances it was possible to collect pink bollworm from the upper bolls (second fruiting cycle) of Bt cotton: Eloy Bt in 1997, and Collidge Bt and Mohave Valley Bt in 1999. Bolls were taken to the University of Arizona Extension Arthropod Resistance Management Laboratory (EARML) in Tucson and put in boll boxes (17.6 cm x 50.4 cm x 35.2 cm). Boll boxes suspended infested bolls on wire racks approximately 3 cm above sheets of paper toweling on the floor of the boxes. Fourth instar larvae cut out of infested bolls and dropped onto the paper toweling on the bottom of the boxes. Larvae were transferred to pupation boxes, consisting of tightly sealed, 1.7 liter rectangular Rubbermaid® containers enclosing sheets of paper towel. 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 being held in boxes for 30 days, bolls were opened to recover any larvae that had diapaused within.

Rearing. We reared PBW using a modified version of the method of Bartlett and Wolf (1985). F1 offspring of field-collected PBW were reared singly in 1 oz cups containing approximately 9 g diet. Subsequent generations were reared in 16 oz cups containing approximately 158 g of diet, as described by Patin et al. (1999).

Bioassaying PBW Susceptibility to Cry1Ac. Susceptibility of each collection of pink bollworm to Cry1Ac was determined using 21-day diet-incorporation bioassays (Patin et al. 1999). MVP-II® Bioinsecticide (Mycogen, San Diego, CA) was mixed into sterilized distilled water to produce a stock solution of Cry1Ac toxin. The stock was then added to liquid wheat germ diet (Adkinson et al. 1960) in amounts necessary to create final concentrations of 0 (control), 1.0, 10, and 100 µg Cry1Ac/ml diet solution. In 1997, concentrations of 0.1 and 3.2 µg/ml was were also evaluated but no bioassays of 100 µg/ml were conducted. Solutions were blended thoroughly into liquid diet at 50-60°C. Diet was made in 1 liter

Reprinted from the *Proceedings of the Beltwide Cotton Conference*
Volume 2:1175-1179 (2001)
National Cotton Council, Memphis TN

batches of each concentration. It was then cooled, shredded and ca. 9 g of diet per cup dispensed into 1 oz medicine cups with tight fitting lids. Neonate larvae were placed individually in each 1 oz cups and the tops were affixed. Subjects were assigned to replicates consisting of 10 bioassay cups for each concentration. Bioassay cups were placed in egg cartons and incubated in darkness at 29±2 °C for 21 days, after which mortality and developmental stage of survivors (Watson and Johnson 1974) were recorded. Live fourth instar larvae and pupae were scored as alive. Corrected mortality was computed using Abbott's formula (Abbott 1925).

For each population our goal was to complete 8 replications of 10 larvae tested at each bioassay concentration. In 1997, a limited number of bioassays of F1 larvae were included in analyses. Thereafter, bioassays commenced in the F2 generation and, if necessary to complete the desired number of replicates, continued through the F7 generation, contingent on the numbers of eggs produced per generation. Results obtained from each population were pooled to obtain a single estimate of mortality for each concentration. The total subjects bioassayed were 1540, 3140, and 3406 in 1997, 1998, and 1999, respectively. These comprised an average of 59.2 larvae tested per concentration in 1997 (range 20-90, *SD*=18.7), 92.3 larvae per concentration in 1998 (range 10-240, *SD*=55.6), and 66.8 (range 10-200, *SD*=37.9) larvae per concentration in 1999.

Differences between years in susceptibility of pink bollworm collected from Arizona cotton were analyzed with Wilcoxon/Kurskal-Wallis tests using the JMPin statistical software (SAS Institute Inc., Cary, NC). To do this corrected mean mortality in bioassays of 0.1 and 10 µg/ml Cry1Ac was computed for each collection made in 1997, 1998, and 1999. Wilcoxon/Kurskal-Wallis tests were then used to contrast mean mortality of 1997 versus 1998, and 1997 versus 1999 collections for concentration of 0.1 and 10 µg/ml Cry1Ac. Response to Cry1Ac of the APHIS-S laboratory culture was used as an internal standard (control) each year. Concentration-response to Cry1Ac of the 1997 field collections and the F6 and F14 generations of pink bollworm selected in the laboratory for resistance to Cry1Ac were estimated using probit analysis (POLO-PC, LeOra Software, Berkeley, Calif.).

Results and Discussion

1997

Mean mortality of the 10 pink bollworm populations collected in 1997 was 30.2%, 70.2% and 95.9% at concentrations of 0, 1.0 and 10 µg/ml, respectively (Table 2). Corrected mortality for these collections was 52.3% at 1.0 µg/ml and 94.5% at 10 µg/ml. Thus, when corrected for mortality in controls, ca. 50% of 1997 collections survived exposure to 1.0 µg/ml. Five of the nine collections had survivors of 10 µg/ml bioassays. These survivors were subsequently bulked and selected with 10 µg/ml Cry1Ac incorporated into diet.

1998

A total of twelve populations collected in 1998 were successfully reared and evaluated, including one from California (Table 3). These had mean mortality of 19.8%, 92.5%, 99.9%, and 99.9% in bioassays of 0, 1.0, 10 and 100 µg/ml, respectively. Corrected mortality was 90.6% at 1.0 µg/ml, 99.8% at 10 µg/ml, and 99.9% at 100 µg/ml. Thus, when corrected for mortality in controls, ca. 9% of 1998 collections survived exposure to 1.0 µg/ml Cry1Ac, and 0.2% and 0.1% survived the 10 and 100 µg/ml assays, respectively (Table 3).

1999

Fourteen collections of Arizona pink bollworm were successfully reared and evaluated in 1999 (Table 4). Mean mortality was 22.3%, 98.4%, 100% and 100% in bioassays of 0, 1.0, 10, and 100 µg/ml, respectively. Thus, when corrected for mortality in controls, ca. 2% of the 1999 collections survived exposure to 1.0 µg/ml Cry1Ac and there were no survivors of 10 or 100 µg/ml assays (Table 4).

Changes 1997-99

Arizona pink bollworm were significantly less susceptible to Cry1Ac in 1997 than 1998 (*chi-square* = 10.1, *df* = 1, $p = .0015$) or 1999 (*chi-square* = 16.1, *df* = 1, $p < .0001$) in bioassays of 1.0 µg Cry1Ac/ml (Figure 3). Mean mortality (corrected) in bioassays of 1.0 µg/ml increased from 52.3% in 1997 to 90.6% and 97.9% in 1998 and 1999, respectively. Mortality in bioassays of 1.0 µg/ml of our internal standard, the APHIS-S population, was relatively unchanged from 1997 (66.1%) to 1998 (61.3%) but increased to 92.6% in 1999 (Figure 3). We cannot be certain why the APHIS-S strain was apparently more susceptible to Cry1Ac in 1999. It is possible that this strain was affected by inbreeding depression. Nonetheless, the response of the APHIS-S laboratory standard was quite consistent from 1997 to 1998, during which time Arizona field populations became significantly more susceptible to Cry1Ac. Survivorship of pink bollworm was also significantly greater in bioassays of 10 µg/ml in 1997 versus 1998 (*chi-square* = 3.97, *df* = 1, $p = .0462$) or 1999 (*chi-square* = 8.32, *df* = 1, $p = .00390$).

Our results show that the Arizona pink bollworm evaluated had not become resistant to Cry1Ac after four years of use of Bollgard cotton. Indeed, 1999 collections were significantly more susceptible to Cyr1Ac than were 1997 collections. Analysis of the 1997 data by Tabashnik et al. (2000) yielded an average proportion of 0.16 (95% confidence interval = 0.05-0.26) for a recessive resistance allele conferring resistance to Cry1Ac.

Isolation and Characterization of Resistance

Materials and Methods

Selection of 1997 Arizona Populations. In the course of evaluating the 1997 pink bollworm collections we pooled the survivors of the higher bioassays concentrations and placed them on diet containing 10 µg/ml Cry1Ac (Patin et al. 1999, Tabashnik et al. 2000). From the first field collection evaluated, Eloy non-Bt, we pooled survivors of the 1.0, 3.2 and 10 µg/ml bioassays. To this we added survivors of the 3.2 and 10 µg/ml bioassays from all subsequent 1997 collections evaluated. These survivors, totaling 159 individuals, comprised the parents of the resistant AZP-R strain. This strain was again selected in the F5 generation by placing ca. 100,000 larvae on diet containing 10 µg/ml Cry1Ac. Thereafter, the culture was selected in alternating generations by placing at least 100,000 larvae on diet treated with 10 µg/ml Cry1Ac. Susceptibility of the selected strain was bioassayed in 1998 (F6) and 1999 (F14) using the aforementioned methodology and concentrations of ≤ 320 µg/ml Cry1Ac.

Results and Discussion

We rapidly selected for a very strong resistance in Arizona pink bollworm to Cry1Ac (Figure 4). Estimates of the susceptibility to Cry1Ac of the 1997 field collections were reported by Patin et al. (1999). LC50s ranged from 0.352 to 1.69 µg/ml and an LC50 derived from bulking the responses all the 1997 field collections was 0.914 µg/ml. The LC50 of the F6 generation of the selected strain (AZP-R) was 162 µg/ml Cry1Ac (95% F.L. = 138-191). Relative to the field collections from which it was derived, the F6 generation of AZP-R had susceptibility to Cry1Ac that was reduced 100 to 460-fold (Patin et al. 1999). By the F14 generation the LC50 of AZP-R was ≥ 320 µg/ml Cry1Ac (Figure 4). Thus, by 1999 the resistance of AZP-R to Cry1Ac was approximately 200 to 900-fold, based on contrasts of LC50s with the 1997 collections. Greenhouse evaluations (Liu et al. 1999, Tabashnik et al. 2000) showed that this strain could survive on Bollgard cotton.

Our findings eliminate any doubt that Arizona pink bollworm possess the genetic potential to overcome Bollgard cotton. Though resistance has not

yet become a problem in the field, our results show clearly that a gene or genes conferring strong resistance to Cry1Ac exist in field populations. A 100-fold resistance to Cry1Ac was previously reported from a laboratory strain of pink bollworm (Bartlett 1995, Liu et al. 2001). Our rapid selection of resistance from field populations corroborate Bartlet's earlier finding.

Field Performance of Bt Cotton

Documenting the field performance of Bt cotton is an important objective of our multi-agency collaboration. This work is based at the Arizona Cotton Research and Protection Council. Pink bollworm infestations at the interface of adjacent Bt cotton and non-Bt cotton (refuge) fields have been measured at 33 to 36 locations throughout Arizona since 1998. Results have shown that Bollgard cotton continues to perform extremely well in Arizona (see Antilla et al. 2001).

Mapping use of Bt Cotton and Compliance with Refuge Requirements

Since 1998, the locations of Bt and non-Bt cotton fields throughout Arizona have been identified by censuses conducted by the Arizona Cotton Research and Protection Council. Maps, produced using geographic information systems software (Figure 5), allow quantification of the amount of Bt cotton used in specific areas, as well as analysis of the deployment of refuges of non-Bt cotton (Carrière et al., 2001). When resistance to Bt cotton occurs in the field in Arizona, these maps will be valuable for analyzing the conditions under which it developed.

Effectiveness of Refuges

Proper placement and management of refuges of non-Bt cotton are vital for preserving efficacy of Bt cotton. Current efforts are contrasting the benefits of in-field versus external refuges and obtaining improved estimates of the dispersal of pink bollworm from refuges (Carrière et al. 2001, Tabashnik et al. 1999). In-field refuges are created by having one hopper of a cotton planter (6, 8, 10-row planter) dispense non-Bt seed while the remaining hoppers dispense Bt seed. The result is refuges of non-Bt cotton that are systematically placed throughout Bt fields. While not recommended for areas where bollworm or tobacco budworm occur regularly, and unsuitable for seed production situations, in-field refuges have produced favorable yields while sustaining sizeable densities of pink bollworms on non-Bt plants (Patin et al. 1999, Simmons et al. 1998, Antilla et al. 2001).

Arizona Bt Cotton Working Group

The Arizona Bt Cotton Working Group meets twice per year to formulate regional management recommendations for Bt cotton. This group includes representatives from the cotton industry, producers of Bt products, pesticide regulatory officials and university and government researchers. Recommendations are forwarded to the Environmental Protection Agency. The group established a Rapid Response Team to which growers are encouraged to report problems with unusual survival of pink bollworm in Bt cotton fields. A Remedial Action Plan for responding to resistance to Bt cotton has been formulated by this group (Carrière et al. *accepted*). Lastly, the group provides guidance to Cooperative Extension regarding the need for and content of educational programs and publications dealing with management of Bt cotton.

Summary

The multi-agency collaboration described herein strives to preserve the effectiveness of Bt cotton in Arizona. Statewide monitoring of resistance and evaluations of the efficacy of Bt cotton in the field have shown that it continues to perform exceptionally well against pink bollworm in Arizona. However, pink bollworm capable of surviving on Bollgard cotton have been isolated by exposing larvae to Bt toxin in the laboratory. Our current research efforts aim to learn as much as possible about this resistance and strategies to manage it before it impacts performance of Bt cotton in Arizona fields. Improved knowledge of pink bollworm movement from refuges and the benefits of external versus internal refuges of non-Bt cotton will be important in this regard. Mapping of the placement of Bt and non-Bt cotton fields throughout the state will provide needed information on refuge deployment and will permit analysis of events leading to resistance, once it occurs. And lastly, communication is a critical component of this collaboration. Our regional working group is convened twice per year to assess the status of resistance, recommend needed changes in regulations and management guidelines for Bt cotton, and to formulate goals of relevant research and education programs.

Acknowledgments

We thank the Arizona Cotton Research and Protection Council, Cotton Incorporated, and Monsanto for funding for this project. For assistance with field work we thank R. Webb, and the staff of the Arizona Cotton Research and Protection Council. We thank S. Meyer, C. Ellers-Kirk, D. Holley and S. Anderson for assistance with laboratory and greenhouse components of the project. For assistance in the field and laboratory we also thank J. Holley, J. Sortelli, C. Merrell, M. Wigert, and the staff of the UA Extension Arthropod Resistance Management Laboratory. Facilities for the EARML pink bollworm laboratory are provided by the University of Arizona and maintained by Peter Else and the staff of the UA Campus Agricultural Center for which we are grateful.

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Table 1. Estimated use of Bt cotton in Arizona 1996-99.

Year	Percent Bt Cotton	Total Cotton Acres
1996	<20%	355900
1997	50%	341000
1998	61%	253688
1999	55%	278745

Table 2. Mortality (±SEM) of pink bollworm collected from Arizona cotton in 1997 and tested in diet bioassays incorporating Cry1Ac toxin.

	Concentration Cry1Ac µg/ml diet		
Collection Site	**0**	**1**	**10**
Coolidge	35.5(8.5)	74.5(12)	100(0.0)
Eloy	41.7(4.4)	82.8(4.1)	100(0.0)
Eloy Bt	32.0(6.0)	67.4(8.6)	92.0(5.8)
Marana	46.3(5.6)	96.8(3.7)	100(0.0)
Mohave Valley	22.0(4.7)	59.0(7.8)	82.5(4.8)
Paloma	22.9(6.0)	55.7(9.7)	100(0.0)
Parker	24.5(6.0)	82.1(5.6)	100(0.0)
Safford	22.7(5.6)	58.2(11)	98.0(2.0)
Solomon	17.3(3.6)	59.6(8.4)	90.0(10)

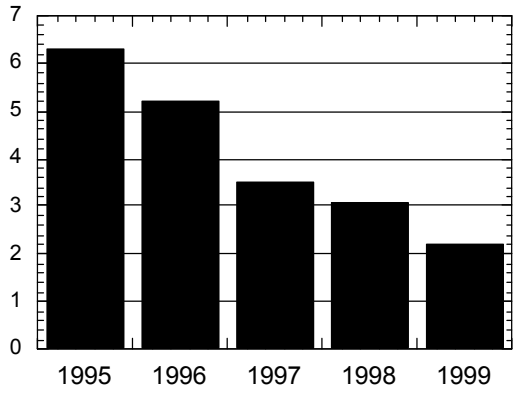

Figure 1. Estimated numbers of insecticide treatments applied to Arizona cotton, 1995-99. Adapted from: Agnew et al. 2000 and Ken Agnew personal communication.

Figure 2. Locations where pink bollworm collections were made from 1997 through 1999 for assessing susceptibility to the Bt toxin, Cry1Ac.

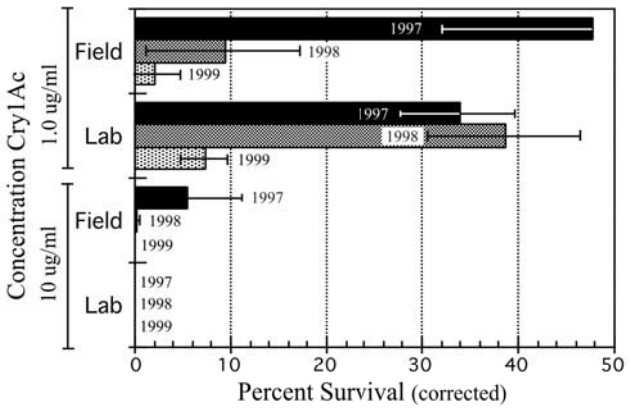

Figure 3. Increase in mean (±SEM) susceptibility of Arizona pink bollworm to the Bt toxin, Cry1Ac, from 1997 to 1999. Shown are mean values of corrected survival observed in replicated bioassays of 1.0 and 10 µg Cry1Ac/ml diet of field collections made throughout Arizona in 1997 (n=9), 1998 (n=12), and 1999 (n=14) and a laboratory reference population (APHIS-S) tested each year.

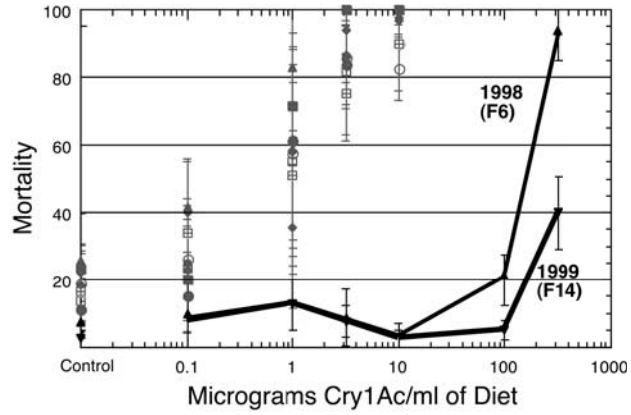

Figure 4. Susceptibility (mean ±SEM) to Cry1Ac of field strains of Arizona pink bollworm collected in 1997 (data points on left) and the F6 and F14 generations of a resistant strain (AZP-R) created in the laboratory by exposing the 1997 collections to Cry1Ac. The resistant strain can survive on Bt cotton.

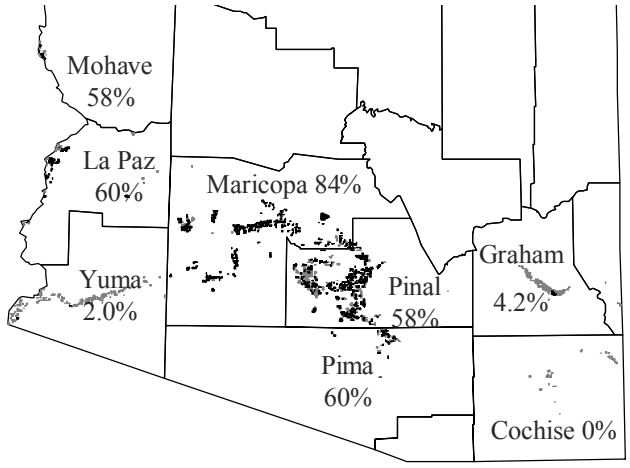

Figure 5. Census information collected by the Arizona Cotton Research & Protection Council identifies Bt cotton (black) and non-Bt cotton (gray) throughout Arizona in 1999. Analysis of use patterns will permit characterization of conditions that give rise to resistance and evaluation of compliance with refuge requirements. Note the large differences between counties in use of Bt county (values shown).