UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF PREVENTION,
PESTICIDES AND TOXIC
SUBSTANCES

September 26, 2006

MEMORANDUM

SUBJECT: Technical Review of Materials Supporting Arizona's 24(c) Applications [AZ05009 and AZ050010] to Replace Structured Non-*Bt* Cotton Refuges with Sterile Insect Technology and 100% Bollgard and Bollgard II Cotton (EPA Reg. No. 524-478 and 524-522) to meet their Special Need for their Pink Bollworm Eradication Program [Submission dated August 3, 2006, MRID#s 469048-00 to -05]

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ACTION REQUESTED: Provide a technical review of materials supporting Arizona's 24(c) Applications [AZ05009 and AZ050010] to replace the required, structured, non-*Bt* cotton refuges with Sterile Insect Technology and 100% Bollgard® and Bollgard II® cotton (EPA Reg. No. 524-478 and 524-522) to meet their Special Local Need for their Pink Bollworm Eradication Program [submission dated August 3, 2006].

CONCLUSIONS AND RECOMMENDATIONS

The state of Arizona and the Arizona Cotton Research and Protection Council submitted information to support continuation of the two Special Local Need registrations (24(c) registrations [AZ-050009 and AZ-050010] in place for the 2006 growing season. A major concern with eliminating the structured non-*Bt* cotton refuges and the possible use of 100% *Bt* cotton is the possible increased risk of pink bollworm resistance to the *Bt* proteins expressed in Bollgard and Bollgard II cotton varieties. This is the important issue for EPA. Data from June 25 through July 22, 2006 were available for this submission due to the timing needed by EPA to review the data and make a decision about the

continuation of the 24(c) registrations.

Based on the review of the preliminary pheromone trapping data, spatial analysis, and simulation modeling, eliminating the structured non-*Bt* cotton refuges, use of 100% *Bt* cotton, in combination with the release of PBW sterile moths and pheromones with limited use of insecticides during the PBW eradication program in Arizona, will not result in increased risk of pink bollworm resistance to the *Bt* proteins expressed in Bollgard and Bollgard II cotton varieties.

The Kriging maps of native and sterile PBW populations in Arizona's eradication program from June 25 through July 22, 2006 are found in **Figures 6A-H** (attached). This analysis indicates that the sterile PBW adult populations were more abundant, consistent and more widely distributed than the native population. The native populations were limited to 1-5 moths per trap with 3-5 areas as "hot spots" (PBW captures > 25) during this four week sampling period. The sterile PBW populations were more abundant with captures > 50 in many areas. The sterile populations were maintained at a steady level through daily releases from aircraft during this program.

Early results from the eradication program indicate that the sterile releases have been quite successful in reducing native PBW populations. It is recommended that the spatial analysis be conducted on all of the trapping data collected during the 2006 growing season and these results be submitted to the Agency for review as a follow-up submission. The current analysis uses the centroid of the field to spatially locate the pheromone trap for the Kriging analysis rather than the exact location of the trap within the field. It is also recommended that in future years of the eradication program that the exact GPS coordinates of each trap be provided for the spatial analysis to allow for greater precision in the analysis. This would allow a more precise examination of the within field distribution of PBW. For example, one would be able to identify "hot spots" on one side of the field vs. another.

The pheromone traps give a relative estimate of the population using only male captures. Other sampling methods, such as boll sampling, will complement the pheromone trapping method to estimate PBW populations and increase the precision of the spatial analysis.

Preliminary modeling using "pessimistic" (i.e., "worst-case") parameter assumptions predict that the four-year eradication program in Arizona will suppress pink bollworm without creating a problem with Cry1Ac resistance to *Bt* cotton. The current simulations suggest that the release of sterile moths in *Bt* cotton fields is important for driving PBW population densities to extremely low levels. However, these are preliminary simulations and certain parameter values are best estimates rather than actual field measurements. Based on the summary of actual sterile release rates (thru August 25, 2006; see **Table 4**), and the actual percentage of *Bt* and non-*Bt* cotton fields see **Table 1**), the range of input parameters used in the modeling simulations were accurate (**Table 8**). Additional modeling simulations should be done using actual field values based on the data collected

in 2006 and submitted to the Agency as a supplemental submission.

PBW resistance to the Cry2Ab2 toxin was not considered in either the simulation modeling or DNA screening analyses. Additional consideration of PBW resistance to the Cry2Ab2 toxin would only be important if the selection pressure dramatically increases in the next two years, i.e., much more Bollgard II planted in the eradication zone. If some or all of Arizona's *Bt* cotton had two toxins, Cry1Ac + Cry2Ab, evolution of resistance would be much less likely than it is with only Cry1Ac. Modeling resistance to cotton that produces only Cry1Ac is the most pessimistic. The modeling predictions (using only Cry1Ac resistance), therefore, are conservative, i.e., they tend to overestimate resistance risk. Based on simulation models examining the likelihood of insect resistance to pyramided toxins in *Bt* crops (e.g., Roush, 1998; Zhao *et al.*, 2005), even if Bollgard II acreage substantially increases, the likelihood of PBW resistance to both the Cry1Ac and Cry2Ab2 toxins would remain low during the four-year PBW eradication program in Arizona.

It is recommended that the full season boll sampling and trapping data (with the spatial analysis), DNA screening (molecular analysis) data, and larval resistance monitoring data be submitted to the Agency for review to confirm that there is a low likelihood of PBW resistance to the Cry1Ac and Cry2Ab2 toxins expressed in Bollgard and Bollgard II,. Additional modeling simulations should be done with actual field data to refine the model parameters as a way to partially field validate the model and increase the accuracy of predictions that PBW resistance to *Bt* cotton will be low during the planned PBW eradication program in Arizona. Follow-up resistance monitoring should also be done during the eradication program and its aftermath.

BACKGROUND AND SUMMARY OF THE SUBMISSION

The use of 100% Bollgard® and Bollgard II® cotton, in conjunction with the use of pheromones, sterile insect technology, and limited conventional insecticides for the purposes of pink bollworm (*Pectinophora gossypiella* (Saunders), PBW) eradication, is a significant change to the insect resistance management program. The state of Arizona (Department of Agriculture) and the Arizona Cotton Research and Protection Council provided additional information in support of two existing special local needs (SLN) registrations, AZ-050009 and AZ-050010. This information was submitted to address the conditions as outlined in the Agency's March 27, 2006 letter. The specific data needed were the result of the June 22, 2006 teleconference between representatives of the EPA and representatives of the ACRPC, the state of Arizona's Department of Agriculture, and the National Cotton Council. As a result of this teleconference, the following data were submitted to address the uncertainty of the effectiveness of the PBW eradication program using sterile insect technology, 100% *Bt* cotton, pheromones, and limited insecticide use.

1. Data from GPS mapping of locations of all *Bt* cotton and all non-*Bt* cotton plantings. The identity of individual grower fields is protected. (Volume 1)
2. Data from systematic monitoring (weekly) of pink bollworm population in eradication zones using pheromone traps and sampling of bolls were provided.

(Volume 2)
3. Data from systematic monitoring of resistance in moths collected in pheromone traps within eradication zones (molecular analysis). (Volume 3)
4. Data from the systematic resistance monitoring program for 2005/2006 larval populations. (Volume 4)
5. Output from simulation modeling comparing population suppression vs. resistance risk for the duration of the eradication program. (Volume 5)

The Agency's technical assessment of these data is the subject of this review.

BI-NATIONAL PINK BOLLWORM ERADICATION PROGRAM BACKGROUND

The National Cotton Council estimates PBW costs western cotton producers an estimated $21.6 million annually for prevention, control, and yield losses. An extensive review of PBW biology, ecology, and population dynamics and integrated pest management options and approaches in the southwestern United States is provided in Henneberry and Naranjo (1998). Pink bollworm eradication is possible in the southwestern United States due to the following factors:

1. Limited hosts of PBW: cotton and okra (non-preferred and extremely limited in distribution)

2. Availability of a very specific, highly efficient survey tool for population detection and monitoring through trapping, i.e., Gossyplure baited survey traps

3. Diverse and effective control mechanisms are available: *Bt* cotton, pheromone mating disruption, sterile insect technology, insecticides, mandated plow down to lower over-wintering populations.

4. Economic feasibility.

Pink bollworm eradication efforts were begun in 2001 in the Trans-Pecos/El Paso, TX area as part of a dual boll weevil and pink bollworm eradication program. A bi-national organization coordinates the U.S. and Mexican bi-national pink bollworm eradication program. This program is a three-phase program. Phase I was begun in 2001 in the Trans-Pecos and El Paso areas of Texas with pheromone applications followed by sterile moth releases in 2002. Phase I was expanded to include parts of New Mexico, and Chihuahua, Mexico in 2002 for a total of approximately 160,000 acres of cotton. Phase II was initiated in 2006 and, in its entirety, will include western New Mexico and south-eastern and central Arizona (240,000 acres). Phase III is scheduled to begin in 2007 and will include western Arizona, southern California, and northwestern Mexico (276,000 acres). After five years, Phase I has achieved a 99.4% reduction in both pink bollworm adults trap catches and larval infestation percentages in the Trans-Pecos/El Paso area and virtually a 99.9% reduction in both adult captures and larval infestation percentages in South Central New Mexico and Chihuahua, Mexico (USDA/APHIS presentation by Dr.

Osama El-Lissy given at the January 4-7, 2006 Beltwide Cotton Conferences, San Antonio, Texas). These results were achieved using Bollgard or Bollgard II cotton under the current, structured refuge requirements.

USDA-APHIS is responsible for technical support and coordination of the bi-national program and for the administration of the sterile pink bollworm moth component through a centralized management system. The program technologies are applied area-wide over a four-to-five (or more) year period. Local grower communities comprised of committees and/or foundations are responsible for the daily implementation of program operations, including mapping all cotton fields, tracking the distribution of all transgenic *Bt* cotton fields, detection surveys, and timely applications of pheromones on non-*Bt* cotton fields.

The San Joaquin Valley, California prevention program has 700,000 acres. Sterile insect technology has been used for close to 40 years in the San Joaquin Valley program and has successfully prevented the pink bollworm from becoming established in that key cotton-producing area.

Field data exist for the use of 100% Bollgard II in Hudspeth County, Texas on approximately 2,100 acres in 2004. The Agency approved this amendment in April, 2004. The Agency concluded in its technical review of this amendment (Reynolds, 2004a) that the amendment was acceptable and should not lead to pink bollworm resistance during the duration of the eradication program because it was: 1) limited to less than 3,000 acres, 2) for one growing season, 3) the area is isolated, at least 30 miles, from any overwintering populations of tobacco budworm and cotton bollworm, 4) Bollgard II expresses two high dose proteins (Cry1Ac and Cry2Ab2) for pink bollworm control, thus reducing the likelihood of resistance, 5) the eradication program included the release of sterile pink bollworm males that will mate with any rare resistant survivors, and finally, 6) intensive resistance monitoring was to be conducted in this area. The results of this field test were provided to the Agency by the State of Arizona in December, 2005. Based on the review of the data, this field test was 100% effective based on the use of both bloom and boll surveys (data collected in July and September, respectively by Staten, Jenkins, and Walters, USDA/APHIS/PPQ). Sterile pink bollworm were released throughout the growing season after the first bloom at a 60: 1 sterile to native ratio to mate with an potential resistant females emerging from Bollgard II cotton fields. There was no evidence of a significant change in susceptibility to either Cry1Ac or Cry2Ab2 based on the resistance bioassays conducted by the University of Arizona. The Agency received no requests for any other amendments to the Bollgard or Bollgard II cotton registrations for the use of sterile moths and 100% Bollgard or Bollgard II cotton in 2005.

Under the current terms and conditions for Bollgard and Bollgard II cotton registrations (amended September 30, 2004), there are specific structured refuge requirements to mitigate the likelihood of tobacco budworm (*Heliothis virescens*), cotton bollworm (*Helicoverpa zea*), and pink bollworm resistance to the Cry1Ac and Cry2Ab2 toxins: 1) 5% external, unsprayed structured refuge (must be within ½ mile of the *Bt* fields, but ¼ mile or immediately adjacent is preferred), 2) 5% embedded refuge (must be a least 150

feet wide, but preferably 300 feet wide), 3) for pink bollworm, an in-field refuge strip refuge may be used, with one row of non-*Bt* cotton planted for every six to ten rows of *Bt* cotton, and 4) 20% external, sprayed structured refuge (must be within one mile of the *Bt* fields, but ½ mile or closer is preferred). A community refuge option is also allowed for multiple growers within a one-mile square area to use a combination of either the 5% external, unsprayed refuge option and/or 20% external, sprayed refuge option. The embedded or in-field refuge options are not allowed under the community refuge program. Tobacco budworm and cotton bollworm are not insects of concern in Arizona.

SUMMARY OF ARIZONA'S SPECIAL LOCAL NEED REGISTRATIONS

The state of Arizona's Special Local Need (FIFRA section 24(c)) Registrations (AZ-050009 and AZ-050010) permit the use of 100% Bollgard and Bollgard II cotton varieties in a sanctioned pink bollworm eradication program. The eradication program (Phase 2) in Arizona was initiated during the 2006 cotton growing season. In May of 2004, Arizona cotton growers passed by a 79% majority, a statewide referendum that authorized the implementation of a pink bollworm eradication program in Arizona. The program is directed and executed by the Arizona Cotton Research and Protection Council (ACRPC). The program in Arizona has been approved to run for a maximum of four years in each region where it will be undertaken. If eradication is not achieved after four years, the effort will be discontinued. If eradication is achieved, some level of program surveillance and maintenance, comparable to that currently in place for boll weevil, will be continued. Support letters for this program were provided by the University of Arizona scientists involved in pink bollworm resistance management supporting the proposed use of sterile insect technology refuges and 100% Bollgard and Bollgard II cotton and by Monsanto who stated that they neither support nor object to the program as presented.

In 2004, Arizona growers planted 73% Bollgard and Bollgard II cotton varieties. The ratio of adoption between Bollgard and Bollgard II cotton varieties is approximately 95:5 based on review of Monsanto's annual sales information for the 2004 growing season (Matten, 2005). Bollgard II cotton varieties became available in the 2003 growing season and are still fairly limited. Arizona growers have experienced an increase in returns of $5 million per year (± $2 million) as adopter grains have outweighed non-adopter losses. Insecticide use in Arizona cotton has declined since 1996 (the first year that Bollgard cotton was available) from an average if six applications to an average of two treatments in 2000. Dramatic reductions in pink bollworm populations have been shown to occur over large areas of Arizona in which *Bt* cotton was high as noted in Carrière et al. (2003). The state of Arizona, therefore, believes that use of *Bt* cotton provides the potential to eradicate pink bollworm during the four-years of the statutorily approved program beginning with the 2006 growing season.

A major concern with eliminating the refuge is the possible development of pink bollworm resistance to the *Bt* proteins expressed in Bollgard and Bollgard II cotton varieties. This is the important issue for EPA. The University of Arizona researchers

have conducted statewide monitoring of *Bt* resistance since 1997. After nine years of use of *Bt* cotton, *Bt* resistance alleles have been shown to be extremely rare in Arizona (Tabashnik *et al.*, 2006). In addition, the University of Arizona researchers have shown that Cry1Ac resistance to *Bt* cotton is recessive.

The ACRPC will release sterile moths in all *Bt* and non-*Bt* fields within active eradication zones. These fields will be determined by ACRPC personnel using GIS mapping techniques. Cotton variety planting information will be gathered from all growers and physically verified by ACRPC supervisors as to the presence or absence of Bollgard and Bollgard II cotton. The ACRPC will maintain the theoretical 1:500 ratio of susceptible (i.e., sterile moths) to potentially resistant PBW (this ratio was recommended by the FIFRA SAP Panel in 1998) by releasing sterile moths up to three times per week in *Bt* fields from first bloom until defoliation in order to establish a rate of 20 sterile moths/acre/day. The sterile moths will be released directly over or adjacent to the *Bt* field thus, putting them in the direct vicinity of any developing resistant populations.

The ACRPC, in consultation with USDA and University experts, has devised the following plan that replaces the biological function served by non-*Bt* cotton refuges with artificially raised sterile pink bollworm moths.

1. Planting of 100% Bollgard and Bollgard II cotton varieties will be encouraged by eradication personnel, but not required.
2. Annual identification and GPS mapping of the locations of Bollgard and Bollgard II and non-*Bt* cotton plantings to provide precise information with which to determine fields that do not satisfy the current refuge requirements and to respond to future problems, should they arise.
3. Non-Bollgard fields will be treated with a combination of pheromone and insecticides.
4. When PBW populations in non-*Bt* fields will have declined sufficiently for establishing suitable sterile/native moth ratios, sterile moths will be released on non-Bollgard fields. Sterile moths may not be used in non-*Bt* cotton until year two of the program in most areas of the state.
5. The State of Arizona proposes to release 20 sterile moths/acre three times per week (49.4 sterile moths/hectare) in 100% *Bt* cotton fields to satisfy the 500:1 susceptible/resistant moth ratio currently targeted by the conventional refuge strategy. The release in *Bt* cotton would start at first bloom in year one in each zone of the eradication effort and continue for the duration of the cotton growing season through the length of the four-year program. The sterile moth release rate over non-*Bt* cotton fields is at a rate equivalent to 100 moths per acre per day (three times per week) or higher (247 moths per hectare per day).
6. Systematic monitoring of resistance in moths detected in pheromone traps within eradication zones, using pheromone traps and sampling of bolls, will be conducted.
7. Systematic monitoring of resistance in moths detected in pheromone traps within eradication zones, using molecular biological methods to detect the mutations that have been shown to be associated with pink bollworm resistance to *Bt* cotton in

Arizona.

8. Statutory limitation of the duration of the eradication efforts to a maximum of four years in each zone.
9. Monitoring of resistance using established methods based on collection, culturing and bioassaying of larval populations of suitable density, if and when they are found in any *Bt* cotton within eradication zones.
10. Continuation of a multi-agency pink bollworm group composed of the U.S. Environmental Protection Agency, ACRPC, USDA, and University of Arizona personnel, as well as grower and industry representatives, who will closely monitor all scientific and regulatory aspects of the eradication program and formulate case specific contingency plans for responding to resistance development in eradication zones.
11. Primary emphasis on the use of environmentally benign pheromone methods for control of pink bollworm in non-*Bt* fields within eradication zones and secondary emphasis on limited use of conventional chemical insecticides.
12. Lastly, an emergency response team that has been in place for nine years to respond immediately to field developments will be used. It is a collaboration of ACRPC personnel under the consultation of University of Arizona resistance monitoring experts. Their task would be to verify, document, and respond with remedial action to resistance problems. The plan for remedial action proposed by the Arizona multi-agency working group and EPA is attached **(Appendix 1)**.

EPA's Review

EPA's issues is whether eliminating the structured non-*Bt* cotton refuges and the use of 100% *Bt* cotton will result in increased risk of pink bollworm resistance to the *Bt* proteins expressed in Bollgard and Bollgard II cotton varieties. EPA's review is divided into five sections corresponding to the five volumes in the state of Arizona's/ACRPC's submission. The data were collected in the certified PBW eradication zones for the 2006 season, June 25 through July 22, 2006, except for the larval resistance monitoring data which were based on 2005 collections.

1. *Bt*/non-*Bt* Mapping and Program Management Data

Geospatial mapping data of the location of all *Bt* cotton and all non-*Bt* cotton plantings were collected to provide precise information as to the location of all *Bt* and non-*Bt* fields. Data were provided on the management of these fields: sterile release rates (per acre), program applied pheromone treatment (per acre), program applied insecticide treatments for PBW (per acre), pheromone trap captures expressed as moths per trap per unit time by field, and boll infestation levels expressed as larvae per 100 bolls in fields selected for program evaluation. The ACRPC supplied four maps illustrating the *Bt*/non-*Bt* field locations in Central and Eastern Arizona (Maricopa, Pinal, Pima, Graham, Cochise, and Greenlee counties) and the sterile moth release zone (**Figures 1-4** from Volume 1 of the submission, MRID# 469048-01) listed below.

Map 1A (**Figure 1**). *Bt*/non-*Bt* field locations - Central Arizona
Map 1B (**Figure 2**). *Bt*/non-*Bt* field locations - Eastern Arizona
Map 2 (**Figure 3A and B**). Expandable format of Map 1A (Fig. 1) and 1B (Fig. 2) (with individual fields numbered to match trapping/treatment data bases
Map 3 (**Figure 4**). Sterile moth release zones with expanded inset maps including flight path data.

The total acreage represented by *Bt* fields and non-*Bt* fields in the 2006 PBW eradication program in each of the six counties in Arizona is shown in **Table 1.** There were a total of 165,632.95 acres in the 2006 PBW eradication program in Arizona. Ninety-two percent of the total cotton acres in the program were in Pinal, Maricopa, and Graham counties with sixty percent of the total cotton acres in Pinal county.

Table 1. 2006 *Bt* and non-*Bt* Cotton Acreage Per County in Arizona Pink Bollworm Eradication Program [Table from Letter L. Antilla, ACRPC to S. Matten, USEPA/OPP/BPPD, dated September 14, 2006]

	COCHISE	GRAHAM	GREENLEE	MARICOPA	PINAL	PIMA	TOTAL
BT	2,126.00	23,435.30	546.10	29,833.06	89,045.83	9,182.10	154,168.39
TOTAL NON-BT	0.00	1,873.60	463.50	1,811.70	6,451.16	864.60	11,464.56
TOTAL	2,126.00	25,308.90	1,009.60	31,644.76	95,496.99	10,046.70	165,632.95

There were 4,626 total fields in the eradication zone: 334 non-*Bt* fields (6.92%) and 4,292 *Bt* fields (93.08%) **(Table 2).** Each field is numbered. These fields are the target areas for the sterile moth releases, pheromone, and insecticide treatments. There were a total of 4,541 pheromone traps placed in all fields with 3,541 pheromone traps placed in *Bt* fields and 1,000 pheromone traps place in non-*Bt* fields. The number of traps per field ranged from 0 to 14. The scheme for using the trapping and map data is shown in **Figure 5**.

Table 2. 2006 *Bt* and non-*Bt* Cotton Total Acres, Percent, Total Fields, and Total Traps in Arizona Pink Bollworm Eradication Program [Modified Table from Letter L. Antilla, ACRPC to S. Matten, USEPA/OPP/BPPD, dated September 14, 2006]

	TOTAL ACRES	PERCENT	TOTAL FIELDS	TOTAL TRAPS
BT	154,168.39	93.08%	4,292	3,541
TOTAL NON-BT	11,464.56	6.92%	334	1,000
TOTAL	165,632.95	100.00%	4,626	4,541

The program applied pheromone and insecticide treatment data (June 25 through July 22, 2006) are summarized in **Table 3**. A total of 806.8 total acres (10 fields, some more than once) were treated. This represents only a small fraction, 0.2%, of the total cotton fields in the program. The PBW pheromone rope (PB-Rope L® ***((Z,Z)-7,11-Hexadecadien-1-yl Acetate 46.7% (Z,E)-7,11-Hexadecadien-1-yl Acetate 44.1%)***, Gossyplure, Pheromone) was applied to all non-*Bt* cotton at the sixth to seventh true leaf stage at a rate of 200 dispensers per acre (approx. 11,500 acres were treated). The dispensers are applied several different ways. Dispensers are either hand tied to the plants, wrapped on

a bamboo stick and placed in the planted row by hand or, wrapped on a bamboo stick and mechanically inserted into the planted row. PB-Rope L is a 60-90 pheromone mating disruption treatment. The following other insecticides were used: Dual® and Lock-On®. Dual indicates a dual treatment including a mating disruption pheromone constituent such as NoMate PBW fiber ***((Z,Z)-7,11 -Hexadecadien-1-01 acetate 3 .80% + (Z,E)-7,1 1-Hexadecadien-1-01 acetate 3.80%)*** (Gossyplure, Pheromone) or NoMate PBW **MEC** ***((Z,Z)-7,11- Hexadecadien-1- yl Acetate 10.0% + (Z,E)-7,11- Hexadecadien-1 -yl Acetate 10.0%)*** (Gossyplure, Pheromone) (Microencapsulated concentrate) and a chemical component such as Lock-On. Lock-On is a formulation of microencapsulated chlorpyrifos.

Table 3. ARIZONA COTTON RESEARCH AND PROTECTION COUNCIL PROGRAM APPLIED PHEROMONE AND INSECTICIDE TREATMENT DATA

JUNE 25 THROUGH JULY 22, 2006

Field#	Type	Pheremone	Insecticide	Acres Sprayed
2367	DUAL	MEC	LOCK-ON	41
2367	DUAL	MEC	LOCK-ON	146.5
2367	DUAL	MEC	LOCK-ON	146.5
2366	DUAL	MEC	LOCK-ON	27.1
2366	DUAL	MEC	LOCK-ON	27.1
2365	DUAL	MEC	LOCK-ON	27.9
2365	DUAL	MEC	LOCK-ON	27.9
2364	DUAL	MEC	LOCK-ON	28.3
2364	DUAL	MEC	LOCK-ON	28.3
2363	DUAL	MEC	LOCK-ON	28.2
2363	DUAL	MEC	LOCK-ON	28.2
2362	DUAL	MEC	LOCK-ON	28.1
2362	DUAL	MEC	LOCK-ON	28.1
2359	DUAL	MEC	LOCK-ON	39.9
2359	DUAL	MEC	LOCK-ON	39.9
2358	DUAL	MEC	LOCK-ON	33
2358	DUAL	MEC	LOCK-ON	33
2187	DUAL	MEC	LOCK-ON	24
2186	DUAL	MEC	LOCK-ON	23.8
GRAND TOTAL ACRES SPRAYED				**806.8**

Pheromone rope or "PBROPE" was applied to all NON-BT cotton at the sixth to seventh true leaf stage. The rope was applied at the rate of two hundred dispensers per acre. 11,537.96 acres were treated with PBROPE from May 7, 2006 to July 10, 2006.

The success of using sterile moths in the PBW eradication program is dependent on whether the sterile moths are as competitive as the native moths. Miller *et al.* (1994) in their research found that both male and female sterile PBW moths are comparable to native moths in their mating responses. Studies by Tabashnik *et al.* (1999) indicate that both native and sterile male PBW can move up to 400 meters (approx. ¼ mile) from non-*Bt* and *Bt* cotton. This means that sterile releases within 400 meters of *Bt* cotton field should provide a sufficient PBW populations of both males and females within the boundary for effective interaction with native moths whether they are *Bt*-susceptible or resistant. Further research conducted by the California Department of Food and Agriculture (Keaveny *et al.*, 2006 in Vol. 2, MRID# 469048-02) indicates that sterile moths release in one mile (approx. 1600 meters) corridors move effectively at least one mile offsite for potential encounters with native moths. The movement information is important for use in the Kriging analysis and for determining the sterile release flight corridors.

The protocol for the sterile moth releases is as follows:

1. Non-*Bt* Cotton: Sterile moth (sterile insect technology, SIT) releases are made three times per week directly over non-*Bt* fields at a rate equivalent to 100 moths per acre per day or higher (247 moths per hectare per day or higher).

2. *Bt* Cotton: SIT releases are made 2-3 times per week along one mile corridors over *Bt* fields at a rate equivalent to 20 moths per acre per day or higher (49.4 moths per hectare per day or higher). This release rate represents a minimum of two times the USDA/APHIS release rate for Bollgard and Bollgard II referenced in Arizona's 24(c) labels. Corridors are offset by one half mile (approx. 800 meters) on alternate release days to ensure that sterile moth populations are maintained within one quarter mile (approx. 400 meters) of *Bt* fields at all times throughout the season.

3. All SIT releases are monitored through GPS assisted guidance systems which produce flight recordings which are downloaded, printed and reviewed daily. These include flight paths and color coded designations of all release operations.

4. Arizona: Allocation of sterile moths is 70 million moths per week for an overall average of 10 million moths per day. Releases are made seven days a week and cover the period of May 1st through October 15, 2006.

5. Pheromone traps on all *Bt* and non-*Bt* fields are serviced weekly and counts of native vs. sterile moths are recorded by trained ID personnel at each field office. Because sterile moths are reared on a diet containing red dye, either visual or simple chemical assays separate sterile moths from native moths.

Information on Actual Sterile Release Rates (personal communication from B.Tabashnik, U of Arizona to S. Matten, USEPA/OPP/BPPD, dated September 14, 2006)

Table 4. Sterile Release Rates through 8/25/06

Non-Bt cotton, 3 releases per week (1 release per 2.3 days) **Mean to date = 251 moths per acre per release (621 per ha per release)** 251 moths per acre per release X 3 releases per week = 753 moths per acre per week 753 moths per acre per week divided by 7 days per week = **108 moths per acre per day**
Bt cotton, 3 releases per week (1 release per 3 days) **Mean to date = 53.1 moths per acre per release (131 per ha per release)** 53.1 moths per acre per release X 3 releases per week = 159.3 moths per acre per week 159.3 moths per acre per week divided by 7 days per week = **22.8 moths per acre per day**
If non-Bt = 7% of acreage, Bt = 93% of acreage, 108 X 0.07 + 22.8 X 0.93 = 7.56 + 21.2 = **actual mean release rate = 28.8 moths per acre (71.1 per ha) per day** **Note: Production of 70 million moths per week /165,000 acres of cotton = 420 moths per acre per week = potential mean release rate = 60 moths per acre per day (estimated mean is half of this)**

Discussion

The *Bt* and non-*Bt* cotton field maps and the sterile moth release protocol are "acceptable." Actual sterile release rates in *Bt* and non-*Bt* fields are as expected (see Table 4 above).

2. Pink Bollworm Trapping and Boll Sampling Data

Data from systematic weekly monitoring of PBW populations in eradication zones using pheromone traps and sampling of bolls was provided for the period June 25-July 22, 2006. Conclusions based on these data will be preliminary given the short period in which the trapping and boll sampling data represented. Weekly monitoring will be continued through October 15, 2006. Cumulative trapping, treatment and sterile release data should be provided to the Agency at the end of the 2006 cotton growing season as a follow-up submission.

Adult PBW populations were monitored using a program-wide standard PBW delta trap baited with PBW sex (male) pheromone (Gossyplure, Pheromone, ***Hexadecadienyl acetate***). Traps were placed in each cotton (*Bt* and non-*Bt***)** in the eradication zone (six Arizona counties noted above). The number of traps per field varied based on the size of the field, and the type of cotton grown. The number of traps ranged from 0 to 14. A field may have no trap located in it because there was one central trap that covered several

small fields. The average number of moths per trap was calculated for each cotton field by dividing the total capture (steriles or natives) by the number of traps. A total of 4,541 traps were placed in 4,626 cotton fields in 2006 (see Table 2 above). Traps were checked weekly by program personnel and numbers of male moths (native and sterile) were recorded. A detailed description of the trapping and sampling methodology is found in **Appendix 2.**

Raw data listing weekly program trapping for all fields, the number of traps per field and the total number of moths counted each week, for the period of June 25 through July 22, 2006 are provided in Volume 2, Table 1 (146 pages) of the submission (MRID# 469048-02). Some trapping dates have missing information (i.e., null data). No information as to why trap counts are missing for these dates is provided. The average number of native and sterile moths per field for the four week combined date range of June 25 to July 22, 2006 are provided in Volume 2, Table 2 (100 pages) of the submission (MRID# 469048-02). The weekly PBW eradication program averages by cotton type during the period of June 25 to July 22, 2006 are provided below in **Table 5**.

Table 5. Weekly PBW (Natives and Steriles) Eradication Program Averages by Cotton Type 6/25-7/22/2006. (Dates correspond to the start date of the trapping week)

Cotton Type	**Nat/Trp 6-25**	**Nat/Trp 7-02**	**Nat/Trp 7-09**	**Nat/Trp 7-16**	**Str/Trp 6-25**	**Str/Trp 7-02**	**Str/Trp 7-09**	**Str/Trp 7-16**
Bt	3.20	1.92	2.75	1.83	20.50	29.77	46.48	29.94
Non-Bt	0.18	0.05	0.11	0.10	0.73	1.09	1.53	1.16

The average weekly sterile to native moth ratio for the *Bt* fields for each trapping week varied from 6.4 to 16.9. The average weekly sterile to native moth ratio for the non-*Bt* fields for each trapping week varied from 4.1 to 21.8. Captures (steriles and natives) were always greater in *Bt* fields than non-*Bt* fields.

Spatial Analysis

The spatial analysis of the trapping data was conducted by David Bartels, USDA-APHIS-PPQ-CPHST (Edinburg, TX) and Michelle Walters, USDA-APHIS-PPQ-CPHST (Phoenix, AZ) (see details of the method described in Bartels and Walters, 2006 in Volume 2 of the submission, MRID# 469048-02). To present the trapping data as a predicted surface of PBW numbers (i.e., trap counts at a particular point), the Kriging method was used to calculate a predicted value for areas between the known values of each field. Kriging is a validated geostatistical method used to estimate the optimal interpolation of these points across the spatial domain. This method handles spatial autocorrelation and is not sensitive to uneven sampling in specific areas, such as the distribution of cotton fields in the eradication program. Ordinary Kriging using a spherical model was applied to trap counts for each week (see Volume 2, Table 1 of the submission, MRID# 469048-02) to develop a predictive surface model encompassing the cotton fields. Kriging constructs a weighted moving average that estimates the value of a spatially distributed variable from adjacent values while considering the interdependence. Kriging results in a smoothing effect in which high original values are underestimated

and low original values are overestimated. It is a best linear unbiased estimator because it minimizes the variance of the estimation errors.

To create a point for the trap captures, the center of each cotton field containing a trap was calculated using its geographic boundary. Krigid surfaces were generated from a total of 3,472 center points from the cotton fields (used weekly trapping data from June 25 to July 22, 2006, Vol. 2, Table 1, MRID# 469048-02). Ideally, one would use the exact GPS coordinates for the specific traps, but this information was not available for the preliminary spatial analysis. A two kilometer range of influence from the center point of the field was used so that each field's data is only affected by other fields within approximately one kilometer of the outside border. This one kilometer limit reflected the perceived day to day movement of PBW adults in *Bt* cotton fields and limits the mathematical influence of a "hot" field on a large area. Trap values were truncated to 100 moths/field/week as this indicates a "hot" field biologically and also, because a weighted average is used in Kriging, capping the high value limits the undue graphical influence of a single field. Traps with greater than 100 PBW moths are potentially more unreliable because the efficiency of the trap declines once it fills up with moths.

Results and Discussion

The Kriging maps of native and sterile PBW populations in Arizona's eradication program from June 25 through July 22, 2006 are found in **Figures 6A-H** (attached). This analysis indicates that the sterile PBW adult populations were more abundant, consistent and more widely distributed than the native population. The native populations were limited to 1-5 moths per trap with 3-5 areas as "hot spots" (PBW captures > 25) during this four week sampling period. The sterile PBW populations were more abundant with captures > 50 in many areas. The sterile populations were maintained at a steady level through daily releases from aircraft during this program.

Early results from the eradication program indicate that the sterile releases have been quite successful in reducing native PBW populations. It is recommended that the spatial analysis be conducted on all of the trapping data collected during the 2006 growing season and these results be submitted to the Agency for review. The current analysis uses the centroid of the field to spatially locate the pheromone trap for the Kriging analysis rather than the exact location of the trap within the field. It is also recommended that in future years of the eradication program that the exact GPS coordinates of each trap be provided for the spatial analysis to allow for greater precision in the analysis. This would allow a more precise examination of the within field distribution of PBW. For example, one would be able to identify "hot spots" on one side of the field vs. another.

The pheromone traps give a relative estimate of the population using only male captures. Other sampling methods, such as boll sampling, will complement the pheromone trapping method to estimate PBW populations and increase the precision of the spatial analysis.

Boll Sampling

Details of the boll sampling methodology are found in **Appendix 2.** Preliminary boll sampling data from July 16 to July 22, 2006 were provided to the Agency (see Volume 2, Table 4 in MRID# 469048-02). A total of 43 fields were selected at random (23 non-*Bt*/20 *Bt*). Attempts were made wherever possible to select *Bt*/non-*Bt* pairs that were a mile apart or less. A total of 100 bolls from each of four field quadrants comprised the sample. These were then examined under magnification for signs of PBW. Only 26 fields were able to be sampled at this early date. Only 1 boll from one non-*Bt* field was found to be infested with 1 larva. The total infestation rate for all bolls analyzed was 0.04%.

Discussion

Boll sampling this early in the season is preliminary. It is recommended that the boll sampling data collected during the entire 2006 growing season be provided to the Agency for review as a supplemental submission.

3. Resistance Monitoring Data from Moth Collected in Pheromone Traps (Molecular Analysis)

It has been shown in previous analyses that in laboratory-selected strains of pink bollworm and at least two other major lepidopteran pests of cotton, mutations in a cadherin gene are tightly linked with recessive resistance to Cry1Ac (Gahan et al. 2001, Morin et al. 2003, Xu et al. 2005). Previous work conducted at the University of Arizona has identified three mutant alleles (*r1, r2,* and *r3*) of a cadherin gene (*BtR*) are associated with resistance to Cry1Ac and survival on *Bt* cotton (Morin *et al*., 2003, 2004; Tabashnik *et al.* 2004, 2005b). Each *r* allele has a deletion predicted to eliminate at least eight amino acids upstream of the putative Cry1Ac-binding region of the cadherin protein (Morin *et al.*, 2003). A PCR-method was developed to detect the *r1*, *r2*, and *r3* alleles in PBW (Morin *et al.*, 2004). This PCR-method was used to screen for the three *r* alleles in PBW sampled from 59 cotton fields in Arizona, California, and Texas during 2001-2005 (Tabashnik *et al.*, 2006). No *r* alleles were detected in 5,571 field-derived insects.

Methods for detecting false negatives, false positives, and non-detection are described in detail in Tabashnik *et al.* (2006). These methods are also detailed in **Appendix 3.**

In brief, false negatives are possible from three causes: i) The PCR reaction is not working properly, ii) The cadherin DNA of field-sampled insects is not amplified, iii) The PCR is working and cadherin DNA is amplified from field samples, yet *r* alleles are present and are not detected.

To detect false positives, all tests of field samples include blanks, which are gel lanes containing all of the PCR reagents, but no DNA. If a blank yields a positive result, this indicates contamination (i.e., a false positive). In this case, PCR reaction conditions are corrected and the field samples are retested. Results are included in the data analysis only if the blanks do not yield positive results.

Of the 5,571 field-sampled insects tested in Tabashnik et al. (2006), none yielded positive results. Thus, the problem of false positives is minimal to nil. When a pool of field-sampled insects yields a positive result for an *r* allele (e.g., *r2*), each individual in the pool will be tested separately to verify the positive result and to more precisely estimate the frequency of resistance in the pool.

As described in Tabashnik et al. (2006), the likelihood of non-detection is estimated as follows:

> *"The probability of detecting no r alleles in a sample of N individuals was calculated as (1-[F X D])$^{2N X A}$, where F is the frequency of resistance alleles, D is the probability of detecting an r allele present in screened individuals (0.97, based on the data from blind controls), 2N is the number of alleles screened, and A is the probability of amplifiable cadherin DNA occurring in field-sample insects (estimated as 0.986, based on the proportion of positive results for amplification of a conserved sequence in 835 insects tested individually). We assumed that the probability of an r allele occurring was an independent event at each cadherin allele screened. For example, with an r allele frequency of 0.001, the probability of detecting no r alleles in the sample of 5,571 individuals (11,142 alleles) is 0.000023 = (1-[0.001 X 0.97])$^{11,142 X 0.986}$. Analogously, with an r allele frequency of 0.0003, the probability of detecting no r alleles in the sample of 5,571 individuals is 0.041 = (1 – [0.0003 X 0.97])$^{11,142 X 0.986}$."*

<u>*Results*</u>

As stated above, a series of positive and negative controls were used to make sure the screening method was performing as expected. Testing was on insects in pools of 11 samples or fewer to minimize the chances of missing *r* alleles. Ninety-seven percent of *r* alleles were detected in the blind positive control samples in these fields. Statistical analysis was used to calculate the probability of detecting no *r* alleles in a sample of *N* individuals. Based on the 98.6% amplification of cadherin DNA and a detection rate of 97% for *r* alleles, the estimated probability is <0.0001 that the frequency of *r* alleles in the field was equal to or greater than 0.001 (Tabashnik *et al*, 2006). The estimated probability is <0.05 that the frequency of *r* alleles in the field was equal to or greater than 0.0003. Using this estimate and assuming Hardy-Weinberg equilibrium, the estimated frequency of rr is less than 1 in 10 million (0.0003 X 0.0003). Results from these analyses indicate that *r* alleles for Cry1Ac resistance are rare.

The resistance allele frequency estimated from bioassays conducted on collections from 2001 to 2005 is somewhat higher than the estimate based on DNA screening during the same time period. The bioassay data for 2001 to 2004 were summarized in Tabashnik *et al.* (2005a) and have been reviewed by the Agency (Reynolds, 2004b, c, 2005, 2006). The mean yearly estimate resistance allele frequency from 2001-2004 bioassays is 0.024 (range = 0 to 0.075, 95% confidence interval = 0 to 0.062). Tabashnik *et al.* (2006) suggest that the difference in the estimates between bioassays and DNA screening in some years might be due to an overestimation of the estimates using bioassays and underestimation of the estimates by DNA screening, or possibly both. It should be noted, however, that there may be other mutant alleles of the cadherin gene or other genes

associated with resistance to Cry1Ac and survival on *Bt* cotton that have not yet been identified.

Plans for DNA Screening of Pink Bollworm in Arizona 2006

A DNA screening plan was developed by Dr. Tim Dennehy and Dr. Bruce Tabashnik, University of Arizona to screen 500 field-sampled insects (50 or more per site from 10 sites) in Arizona for the three known cadherin resistance alleles (*r1, r2,* and *r3*) using the PCR method described by Tabashnik *et al*. (2006). A brief description was found in Volume 3, p.1, MRID# 469048-03). Details of this screening plan are provided below (personal communication, T. Dennehy, U. of Arizona to S. Matten, USEPA/OPP/BPPD, dated September 14, 2006). Low numbers may limit the number of insects that can be collected and screened from the eradication zone. If possible, at least 300 field-sampled insects from at least 6 sites in the eradication zone will be screened. Special effort will be made to collect insects for DNA screening from any areas in which trapping data show unexpectedly high numbers of native moths in the eradication zone. Large numbers of native moths (50-100) needed for these analyses are not normally available until September or October when late season moth flights occur.

Sampling methods: As described by Tabashnik et al. (2006), insects for DNA screening will be sampled from bolls and from traps baited with sex pheromone:

> *"Cotton bolls were sampled from 19 cotton fields (18 in Arizona and 1 in California) from 2001 to 2005 as described by Dennehy et al. (2004). At each site, 300 to 2,000 bolls were collected from non-Bt cotton fields near Bt cotton fields. Bolls were taken to the University of Arizona Extension Arthropod Resistance Management Laboratory in Tucson. We obtained pink bollworm by collecting fourth instars that exited bolls and by opening bolls and removing larvae found inside."*
>
> *"Pink bollworm males were collected in sticky traps baited with female sex pheromone (Tabashnik et al. 1999) in 40 cotton fields (36 in Arizona, 3 in California, and 1 in Texas) from 2003 to 2005. At each site, several traps were placed around the perimeter of a cotton field, collected after 1 to 2 days, and brought to the laboratory. Live males that showed normal movement of appendages were removed from traps using wooden toothpicks. A new toothpick was used for each male to avoid cross-contamination."*

Progress and sampling plans:

1. In 2006, ACRPC proposed to identify up to five sample areas in which elevated native *Bt* fields may suggest a resistance threat. As of July 22, 2006, only one group of fields gave any indication of increased native moth levels (field #s 4335-4338). Approximately 80 male moths were caught. These moths will be screened using the three DNA markers and PCR.

2. To produce strains for bioassay testing, bolls have been sampled from four sites (two in the eradication zone, two outside the eradication zone). If numbers are sufficient, subsamples of 50 insects per site collected directly from bolls will be screened for *r* alleles.

3. Trapping at 10 sites in the eradication zone will be done to obtain males for PCR screening in late September. If this sampling does not yield enough males, trapping will be repeated in mid- to late October.

Discussion

DNA screening analyses of insects sampled from the field in 2006 could not be performed prior to the August submission required by EPA to support the two 24(c) registrations. No information can yet be gained about the presence or absence of the three mutations associated with Cry1Ac resistance and survival on *Bt* cotton until the molecular analyses are performed later this year. However, based on previous DNA screening analyses of insects sampled from 2001 to 2005 (Tabashnik *et al.*, 2006), no individuals were identified as having these specific *r* allelic mutations for Cry1Ac resistance. It is recommended that the results of these analyses be provided to the Agency. The methods for conducting the molecular analyses for detecting the three mutations associated with Cry1Ac resistance and survival on *Bt* cotton are "acceptable." If alleles other than cadherin mutants *r1, r2,* and *r3* confer pink bollworm resistance to Bt cotton, the results using these three DNA markers DNA could underestimate the frequency of resistance. However, Tabashnik *et al.* (2006) concluded that additional resistance alleles at the cadherin locus or other loci are rarer than the three known resistance alleles because such additional alleles have not been discovered in extensive testing of several laboratory-selected resistant strains. DNA screening based solely on males caught in pheromone traps could underestimate resistance allele frequency if the probability of capture in traps was lower for *rr* or *rs* males than for *ss* males. However, experiments conducted in large cages (64 m^3) in the field refuted this argument (Carrière *et al.*, 2006). Furthermore, DNA screening of pink bollworm of both sexes from bolls also detected no *r* alleles. It is recommended that the results of the 2006 DNA screening be submitted to the Agency for review as a supplemental submission.

No information is available about the nature of potential PBW resistance to the Cry2Ab2 toxin. Assuming adoption of Bollgard II will continue to increase; understanding the genetics and possible mechanisms of resistance to the Cry2Ab2 toxin will become more important. It is recommended that the genetics and potential mechanisms of PBW resistance to the Cry2Ab2 toxin be studied. Specific DNA markers would need to be developed based on PBW resistance to Cry2Ab2. These specific markers would then be used in the DNA screening program. One caveat is that the adoption of Bollgard II has been low in Arizona (i.e., 95:5 ratio of Bollgard:Bollgard II adoption) and the PBW eradication program is limited to four-years so the need for such information may not be crucial unless the adoption of Bollgard II, and consequently, Cry2Ab2 selection pressure, increases dramatically in the next couple of years. However, as noted in the modeling section below, PBW resistance to both Cry2Ab2 and Cry1Ac would be unlikely during the four-year eradication program. No information on the adoption of the relative adoption of Bollgard and Bollgard II in the eradication zone for the 2006 growing season is yet available.

4. Resistance Monitoring Data for 2005/2006 Larval Population

Annual *Bt* cotton resistance monitoring data for pink bollworm is a requirement of the Bollgard and Bollgard II registrations. All of the Cry1Ac and Cry2Ab2 resistance monitoring data for pink bollworm larval populations through the 2004 growing season have been previously reviewed by the Agency (see EPA 2001; Reynolds 2004b (review of 2001/2002 growing season collections), 2005 (review of 2003 growing season collections), 2006 (review of 2004 growing season collections)). In these reviews, it was concluded that through the 2004 season, there was no evidence of pink bollworm resistance to the Cry1Ac or Cry2Ab2 delta-endotoxins produced by Bollgard or Bollgard II cotton cultivars under field situations. The state of Arizona submitted a preliminary monitoring report of the bioassays for the 2005 collections of pink bollworm (Dennehy *et al.*, 2006; in Vol. 4, MRID# 469048-04). These data are discussed below.

The 2005 monitoring work for pink bollworm was conducted in Arizona by researchers at the University of Arizona and the ACRPC, who have been conducting the work since 1997 (see Volume 4, MRID# 469048-04). The methodology for the 2005 PBW assays was largely the same as in previous years and utilized artificial diet tests with a 21-day observation period. A discriminating dose type approach was used in which PBW mortality was assessed to two test concentrations for both Cry1Ac and Cry2Ab2. Baseline susceptibility (i.e. a LC_{50} or similar measure) was not determined. The two test concentrations of Cry1Ac and Cry2Ab2 used were 1.0 µg/ml and 10 µg/ml. Negative controls (no toxin) were also tested. The Cry1Ac toxin used in the assays was obtained from Dow AgroSciences (MVP-II Bioinsecticide) while the Cry2Ab2 toxin was obtained from freeze dried corn powder provided by Monsanto. These toxin sources were also used for the 2005 monitoring. In addition to the laboratory bioassays, field efficacy was assessed in 2005 using adjacent pairs of *Bt* and non-*Bt* fields at 44 Arizona locations.

2005 Sampling and Assays

PBW were collected as larvae (from bolls brought to the laboratory) from Arizona (12 sites) and California (4 sites). No samples were collected in New Mexico and Texas due to ongoing PBW eradication efforts in those areas. At each location, 300 to 2,000 bolls were collected from non-*Bt* cotton fields. Laboratory cultures were established with ≥100 PBW from each collection site. A susceptible laboratory strain was also used as an internal standard for the experiments. Additionally, for the Cry2Ab2 tests, a Cry1Ac-resistant PBW laboratory colony was included. Fourth instar larvae emerging from bolls were reared to adulthood to produce progeny for testing (F_2 - F_8 progeny were used in the tests). The bioassays were conducted with artificial diet incorporated with the two test concentrations (an untreated control was also used). Neonate larvae were placed in one ounce cups with diet and observed for 21 days. Larvae that failed to develop past the third instar by the end of the test were considered "dead" and Abbott's formula was used to obtain corrected mortality scores (i.e. to justify mortality in the control groups).

2005 Cry1Ac Results

No larvae from any tests of 2005 strains survived treatments of 10 µg/ml Cry1Ac (100% corrected mortality). Only one Arizona population was tested at the 1.0 µg Cry1Ac/ml dose and had a corrected mortality of 66.9%. The susceptible laboratory colony used as a control group showed 100% mortality to the test concentration of 10 µg Cry1Ac/ml. Overall, the authors concluded that PBW remains susceptible to Cry1Ac and that are no indications of resistance in the field. The 2005 results from Arizona are summarized and compared with historical data in **Table 6** below.

2005 Cry2Ab2 Results

No larvae from any tests of 2005 strains survived treatments of 1 µg/ml and 10 µg/ml Cry2Ab2. The susceptible laboratory colony used as control had 100% mortality at the 10.0 µg Cry2Ab2/ml concentration. As with Cry1Ac, the authors concluded that the sampled PBW populations remained highly susceptible to Cry2Ab2. The 2005 Arizona data are summarized in **Table 7** below.

2005 Field Efficacy Studies

In addition to the susceptibility bioassays, the Arizona monitoring group sampled large numbers of *Bt* and non-*Bt* cotton bolls throughout the state (obtained from 40 pairs of *Bt* and non-*Bt* fields). The procedures were similar to the boll sampling that was also conducted during 2004. Infestation rates in the 2005 field efficacy study were 0.37% average infested *Bt* bolls (range not reported in Dennehy *et. al,* 2006 as this was a preliminary report). This rate is slightly higher than that reports for the 2004 growing season. The average infestation *Bt* cotton rate for 2004 was 0.34% bolls and for 2003 was 0.21% (range 0 to 1.40%) bolls. For non-*Bt* cotton, 8,100 non-Bt bolls were examined with an average infestation rate of 24.0% (range not reported) compared with 21.7% (range from 0 to 100%) in 2004 and 29.0% (range from 0 to 100%) in 2003). In 2004, subsequent analysis of the *Bt* bolls determined that many were non-expressing off-types (Dennehy *et al.*, 2005). No analysis was provided for the 2005 data (Dennehy *et al.*, 2006 is a preliminary report). It is likely that many of the collected *Bt* bolls with PBW larvae will be non-Bt expressing off-types rather than the result of adaptation to *Bt* toxins (see Dennehy *et al.*, 2005 for discussion of 2004 results). Attention should still be given to any increase in boll infestation rates in *Bt* fields. Results of the field efficacy studies conducted in Arizona from 1995 to 2004 are shown in **Figure 7** (at the end of this review).

Table 6. Field-Collected PBW Mortality to Discriminating Concentrations of Cry1Ac from 1997 to 2005 (Reynolds, 2006; updated with Dennehy et al. 2006)

Year	Average Mortality of Field Collected PBW (%)[1]	
	1.0 μg Cry1Ac/ml concentration	10 μg Cry1Ac/ml concentration
1997	57.4	94.1
1998	90.6	99.9
1999	97.9	100
2000	97.4	100
2001	94.8	99.4
2002	85.7	99.8
2003	68.3	99.8
2004	95.4	99.9
2005	66.9[2]	100.0

[1] Mortality values are corrected for mortality observed in control groups.
[2] Only one location was tested at this test concentration.

Table 7. Field-Collected PBW Mortality to Discriminating Concentrations of Cry2Ab2 from 2003 to 2005 (Reynolds, 2006; updated with Dennehy *et al.*, 2006)

Year	Average Mortality of Field Collected PBW (%)[1]	
	1.0 μg Cry2Ab2/ml dose	10 μg Cry2Ab2/ml dose
2003	97.3	99.9
2004	99.1	100
2005	100	100

[1] Mortality values are corrected for mortality observed in control groups.

Discussion

Since the PBW monitoring methodology has remained consistent throughout the resistance monitoring program, the data can be placed in a historical context to evaluate long-term shifts in susceptibility. The 10.0 μg/ml concentration for both Cry1Ac and Cry2Ab2 is essentially a true discriminating dose, i.e. a PBW LC_{99} that can be used to distinguish potentially resistant insects from susceptible ones.

Through 2005, ten years of monitoring data have now been tabulated for Cry1Ac and three years for Cry2Ab2. Based on BPPD's analysis of the larval susceptibility data and boll infestation data, PBW susceptibility to both toxins remains high **(see Tables 6 and 7**

above) and there are low boll infestation rates in *Bt* fields as observed in the field efficacy trials. Monitoring data and field efficacy data from 1997 to 2005 indicate there hasn't been an increase in resistance to Cry1Ac and to *Bt* cotton (see Dennehy *et al.*, 2002, 2003, 2004, 2005, 2006; Unnithan *et al.*, 2004). Tabashnik *et al.* (2005a) confirm that the resistance allele frequency did not increase over the period from 1997 to 2004 based on bioassays.

A resistance allele frequency of 0.16 was estimated for recessive resistance to Cry1Ac based on bioassay results from collections of pink bollworm at 10 sites in Arizona in 1997 (Tabashnik *et al.,* 2000). Subsequent work at the University of Arizona showed that resistant larvae from at least four different laboratory-selected strains survived on *Bt* cotton, resistance was recessive and tightly linked with mutations in a cadherin gene that encodes a Cry1Ac-binding protein. Three cadherin resistance alleles were identified (*r1, r2,* and, *r3*). University of Arizona researchers do not know why the frequency of resistance was so high in 1997. Setting aside this unusually high frequency of pink bollworm resistance to Cry1Ac detected in 1997, the bioassay results since 1998 provide evidence that pink bollworm resistance to *Bt* cotton did not increase substantially from 1998 to 2005. Indeed, no resistant individuals were detected in bioassays of 5,358 individuals sampled from Arizona and California in 2005. Further, no alleles for resistance were detected in 5,571 individuals sampled from Arizona, California, and Texas from 2001 to 2005 (Tabashnik *et al.*, 2006). Therefore, the resistance allele frequency of 0.01 used as one of the parameter values in the simulation model is quite conservative and is much higher than the gene frequency that was detected in 2005 (see modeling section below).

Field efficacy data indicates that there has been a numerical, but not a statistically significant, increase in percent infested bolls in *Bt* cotton from 2003 to 2005 (data from 1995 to 2004 shown in **Figure 7;** Dennehy *et al.*, 2006 for 2005 data). Tests of infested bolls (n=35) collected from *Bt* fields in 2004 revealed that a large portion of infested bolls (over 90%) did not have Cry1Ac protein in the seeds. Similar analyses have net yet been performed for *Bt* bolls collected in 2005 (Dennehy *et al.*, 2006). Infestation levels in *Bt* fields have averaged ≤0.370% over the past ten years; an amount of less than four pink bollworms per 1000 bolls (**Figure 7**).

The ACRPC has indicated that follow-up susceptibility testing will be conducted with PBW larvae recovered from *Bt* bolls (verified expressing the *Bt* toxin). This is important because Bollgard and Bollgard II are considered high dose for the control of PBW; therefore, larvae recovered from *Bt* bolls may be heterozygous or homozygous for *Bt* toxin resistance. The determination that these larvae are carrying heritable resistance traits could provide an early indication of a resistance problem. Under the PBW eradication program, in the unlikely event that widespread field resistance be a concern, additional actions as prescribed by the Remedial Action Plan for PBW Resistance to *Bt* Cotton (see **Appendix 1**) would be implemented. Follow-up testing will also be conducted on survivors of the 10 µg/ml Cry1Ac and Cry2Ab2 discriminating concentrations. These larvae may be homozygous for Cry1Ac or Cry2Ab2 resistance

alleles and warrant additional scrutiny.

There have been pink bollworm survivors at the 10 µg Cry1Ac/ml discriminating concentration in 2001, 2002, 2003, and 2004 (**Figure 8)**. Given that some PBW have survived the 10 µg Cry1Ac/ml discriminating concentration in previous years, it is possible that resistance alleles are relatively common in PBW populations in western cotton growing regions. However, selection data and PCR data don't support this interpretation (see Tabashnik *et al.*, 2006). Survivors may not be genetically-resistant. For example, in 2001 there were 31 survivors from Arizona at the diagnostic concentration in bioassays (Tabashnik *et al.,* 2005a), yet selection with Cry1Ac in diet did not yield a resistant strain. This lack of response to selection suggests that the survival was not heritable and thus the 2001 resistance allele frequency was overestimated from bioassays. In contrast, with strains derived from Arizona cotton fields in 1997, as few as three generations of selection with Cry1Ac produced strains capable of surviving on *Bt* cotton (Tabashnik et al. 2000, 2005b). Contamination of field-derived strains by resistant laboratory strains may have been possible for bioassays conducted during 2001 to 2004, although unlikely, according to the explanation provided in Tabashnik *et al.* (2006). There were no resistant strains available to contaminate the bioassays in 1997. Also, no such contamination affected bioassay results in 1999, 2000 and 2005 when no resistant individuals were detected in field-derived strains (total n = 11,400 larvae tested at the diagnostic concentration). One final piece of information that should be considered is that researchers in Arizona have not been able to select for a resistant strain from field collections from Arizona despite trying every year. Only in 1997, were resistance colonies established from field collections (see Tabashnik *et al.*, 2005a).

Considering the high use of *Bt* cotton (93% in the eradication zone in 2006; 100% was allowable for 2006) under the four-year PBW eradication program, it will be important to closely monitor PBW and *Bt* cotton for resistance to Cry1Ac and Cry2Ab2 and possible unexpected field damage.

5. Simulation Modeling Comparing Population Suppression Vs. Resistance Risk for the Duration of Eradication Program

The Agency required that simulation modeling be used to compare the impact of population suppression vs. resistance risk over the period of the eradication program. The simulation model used was a revised version of the spatially explicit, stochastic model discussed in Sisterson *et al.* (2004). The simulations examined population suppression (number of PBW per ha) and risk of resistance to *Bt* cotton (rate of increase of resistance allele frequency). This model considers resistance controlled by a single, recessive gene. This model is based on PBW resistance to Cry1Ac. Resistance to Cry2Ab2 is not considered in the model. **Table 8** below provides the parameter values for the simulation model. Default values are based on best estimates available in the public literature and recent field data. Recent PBW data have been collected in Arizona regarding the genetic basis of resistance to *Bt* cotton that produces Cry1Ac, the frequency of resistance alleles in field populations, population sizes, population dynamics, and

movement. Modifications to the model include the release of sterile moths. Default values are based on best estimates available in the public literature. Alternatives to the default values were systematically evaluated for parameters such as resistance allele frequency and movement. A variety of scenarios were simulated using the best estimates of the parameter values as well as more optimistic and more pessimistic scenarios. Relative to simulations using best estimates for parameters, more optimistic scenarios might underestimate the risk of resistance, while more pessimistic scenarios might overestimate the risk of resistance. Modeling will also explore the impact on population suppression and resistance risk of alternative management options (e.g., variable refuge size and release rates of sterile moths). A region with 4096 cotton fields was modeled for four years (the extent of the intended PBW eradication program in Arizona). Overwintering larvae from the fourth year are checked to assess the following criteria.

- Resistance will occur if the resistance allele frequency exceeds 0.50.
- Population recovery will occur if the final population is equal to or greater than the initial population size (29,000 overwintering larvae per field is the default value).
- Population suppression will occur if the mean PBW density in the region is equal to or less than 0.1 overwintering larvae per 15 ha (=0.0067 larvae per ha).
- Regional loss will occur if all fields in the region modeled have 0 PBW.

Results

In the analyses to date, 12 sets of assumptions, each of them simulated 5-16 times, for a total of 128 simulation runs were examined. In all cases, except for the default case, the initial Cry1Ac resistance allele frequency (r) was 0.01, which is ten times higher than the default value of 0.001. This is a realistic estimate based on the bioassay data discussed in Tabashnik *et al.,* 2005a (and above in this review) as well as DNA screening (Tabashnik et al., 2006). In all cases, 500 sterile moths per ha were released in non-*Bt* cotton fields, with one release per three days. Results of the simulation outcomes are summarized in **Table 9.** In 11 of 12 sets of assumptions examined, the simulated eradication program eliminated the PBW from the 4096 fields modeled in two years or less without the development of resistance. These included "worst-case or pessimistic" simulations in which resistance was inherited as a dominant trait, 90% *Bt* cotton and 10% non-*Bt* cotton refuges (rater than 100% *Bt* cotton), no fitness cost of resistance (rather than a 10% fitness cost in homozygous resistant insects, and release rates of 1, 2, 3, 4, 5, 10, or 15 sterile moths per ha in *Bt* cotton fields (rather than 75 sterile moths per ha). Tabashnik (personal communication, B. Tabashnik, U. of Arizona to S. Matten, USEPA/OPP/BPPD, dated September 14, 2006) noted that more recent simulations show regional loss (i.e., 0 PBW) with 2 per ha per release in Bt cotton and 10 per ha per release in non-Bt cotton.

In the one exception, PBW was not removed from the region when there was no release of sterile moths in *Bt* fields, 90% *Bt* cotton, and r = 0.01 in all five replications. In this case, the population density declined by 98% (460 final overwintering larvae per

field/29,000 starting overwintering larvae per field) and the resistance allele frequency increased from 0.01 to 0.02 after four years. While the resistance allele frequency doubled after four years, it is still far below the 0.50 value typically used as a criterion for a resistance problem.

Table 8. Parameter values for eradication model (revised from Sisterson *et al.*, 2004). Default values, which are used unless noted otherwise for parameters with more than one value, are indicated by an asterisk. **[Taken from Volume 5, MRID# 469048-05]**

Parameters	Values
Adults	
Mean % of adults that leave their natal field	10, 55*, 75
Number of eggs per female per day in Bt cotton fields	10
Number of eggs per female per day in non-Bt cotton fields	10
Mean % of adults that die each day	10
Egg-pupae	
Mutation rate (from S to R per allele)	5×10^{-5}
Mean % of SS and RS killed in non-Bt cotton fields	79.2
Mean % of RR killed in non-Bt cotton fields	79.2, **81.3*(10% fitness cost)**
Mean % of SS and RS killed in Bt cotton fields	99.8[a], **100***
Mean % of RR killed in Bt cotton fields	79.2, 83.2***(incomplete R=0.9)**
Development time (degree days)	433
Mean % of larvae that die during overwintering	95
Region	
Initial R allele frequency	0.0001, **0.001***, 0.01
Number of fields	4096 (64 X 64 square)
Size of fields	15 hectares
Percentage of Bt fields	80, 85, 90, 95, **100***
Percentage of Bt plants in Bt fields	99[a], **100***
Distribution of fields	Random
Carrying capacity per field	4,200,000
Initial overwintering larvae per field	2900, **29,000***, 290,000

[a] 99.8% mortality of RS and SS simulates 100% Bt fields that have 99% Bt cotton plants and 1% non-*Bt* cotton plants (contaminants); 100% die on the Bt plants, 79.2% die on the non-*Bt* plants (0.99 X 100% + 0.01 X 79.2% = 99.8%)

Steriles	
Release period	May 1-Oct 15 (1st bloom to defoliation)
Frequency of releases in each field	1 per 3 days per field
Sex ratio of steriles	1 female: 1 male
Steriles per ha per release in Bt cotton fields	0, 1, 2, 3, 4, 5, 10, 15, **75***
Steriles per ha per release in non-Bt cotton fields	0, 100, **500***, 1000

Pheromone ropes only in non-Bt cotton fields	
All non-Bt fields treated once early in season	May 17-June 20 (6-leaf stage)
Daily % reduction in fecundity caused by pheromone ropes	20, **40***, 60 for 30 days

Insecticide & pheromone sprays only in non-Bt cotton fields	
Spray threshold	≥60 no spray
(check sterile male:native male ratio weekly)	30-59 spray pheromone
	0-29 spray pheromone + insecticide
Daily reduction in fecundity caused by pheromone sprays	20, 40*, 60 for 14 days
Mean % of adults killed daily by insecticide	37 per day for 5 days
Larvae are not killed by sprays	95 per day for 5 days

Table 9. Simulation outcomes: effects of pessimistic assumptions on simulated outcomes of the pink bollworm eradication program in Arizona. In all cases except the default case, the initial resistance allele frequency (r) was 0.01, which is ten times higher than the default value of 0.001. In all cases, 500 sterile moths per ha were released in non-Bt cotton fields, with one release per three days. **[Taken from Volume 5, MRID# 469048-05]**

Parameter values different from default values	**Outcome***
None (all parameters at default values)	Loss in 1 year
Initial resistance allele frequency (r) = 0.01	Loss in 2 years
No fitness cost, r = 0.01	Loss in 2 years
Dominant resistance to Bt cotton, r = 0.01	Loss in 2 years
90% Bt cotton, r = 0.01 Steriles released in Bt cotton fields at 1, 2, 3, 4, 5, 10, or 15 steriles per ha	Loss in 2 years
90% Bt cotton, r = 0.01 No steriles released in Bt cotton fields	averages after 4 years r = 0.02 460 larvae per field (overwintering survivors)

*loss means that no pink bollworm were present in any of the 4096 cotton fields modeled

Discussion

Even with pessimistic parameter assumptions, simulations of the four-year eradication program in Arizona yielded suppression of pink bollworm without creating a severe problem with resistance developing to *Bt* cotton. The current simulations suggest that the release of sterile moths in *Bt* cotton fields is important for driving PBW population densities to extremely low levels. However, these are preliminary simulations and certain parameter values are best estimates rather than actual field measurements. Additional modeling simulations should be done using field values. This will allow partial field-validation of the model. Dr. Bruce Tabashnik (University of Arizona) has indicated that the data from the field will be used to test the model's predictions about the ratios of sterile:native moths and resistance allele frequency (e-mail from B. Tabashnik, University of Arizona to S. Matten, USEPA/OPP/BPPD, dated September 14, 2006). If the model's predictions are wrong, Tabashnik notes that any incorrect assumptions will be changed to improve the accuracy of the model. The results of the additional modeling simulations should be submitted to the Agency for review as a supplemental submission.

Tabashnik (personal communication, B. Tabashnik, U. of Arizona to S. Matten, USEPA/OPP/BPPD, dated September 14, 2006) writes that additional pessimistic assumptions

will be investigated to determine which conditions might cause severe resistance problems. Some of these simulations are currently being performed. Some of assumptions were modified and another set of scenarios using sensitivity analysis varying emigration (10, 55, or 75%), dominance (recessive or dominant), number of fields in the region (400 or 4096), percentage of *Bt* fields (80, 95, 100), carrying capacity per field (2,885, 28,885, 288,850), and numbers of steriles per ha per release in *Bt* and non-*Bt* cotton fields (0,0; 2,10; 100, 500; and 200, 1000) were run. As in the first 12 scenarios run (i.e., the results in Vol. 5, MRID# 469048-05), all of the additional scenarios yielded regional loss (i.e., no PBW) in 2 years except for one case. The only case in which loss did not occur was with no steriles released in *Bt* or non-*Bt* cotton (0, 0). The lowest non-zero release rate tested was 2 per ha per release in *Bt* cotton and 10 per ha per release in non-*Bt* cotton. This low release rate scenario yielded regional loss in 2 years. Based on the actual sterile release rates (thru August 25, 2006; see **Table 4** above), and the actual percentage of *Bt* and non-*Bt* cotton fields see **Table 1** above), the range of input parameters used in the modeling simulations was reasonable (see **Table 8**). New simulations using the field data collected in 2006 should be used to confirm the outcomes of the preliminary modeling.

Pink bollworm resistance to the Cry2Ab2 toxin (present in Bollgard II) is not considered in the modeling. The use of 100% Bollgard and Bollgard II both have been authorized for use under the two 24(c) registrations. Based on sales information through the 2005 growing season, the ratio of Bollgard to Bollgard II cotton sales in Arizona is approximately 95:5. This means that there has been very little selection pressure to the Cry2Ab2 toxin, to date, in Arizona. The greatest selection pressure for resistance is to the Cry1Ac toxin present in Bollgard and Bollgard II cotton. Sales information for the 2006 growing season will be provided to the Agency by Monsanto in January 2006 (requirement of the terms and conditions of the Bollgard and Bollgard II registrations). No information was provided by the state of Arizona as to the breakdown of Bollgard and Bollgard II cotton use in the eradication zone (4626 fields mapped) during the 2006 growing season. What is known is that there were approximately 93% *Bt* cotton fields and 7% non-*Bt* cotton fields in the eradication zone for 2006.

If some or all of Arizona's *Bt* cotton had two toxins, Cry1Ac + Cry2Ab, evolution of resistance would be much less likely than it is with only Cry1Ac. Modeling resistance to cotton that produces only Cry1Ac is the most pessimistic. The modeling predictions (using only Cry1Ac resistance), therefore, are conservative, i.e., they tend to overestimate resistance risk. Several researchers have modeled the benefit of managing resistance evolution to two toxins with dissimilar modes of action using a pyramided approach (e.g., Zhao et al. 2005; Roush 1998).

References

Pink Bollworm Monitoring Data Submissions

Dennehy, T., L. Shriver, M. Sims, D. Holley, Y. Carrière, and B. Tabashnik, 2002. Monitoring for Cry1Ac protein susceptibility among field populations of pink bollworm during the 2001 cotton growing season. Cooperative Extension. The University of Arizona. Extension Arthropod Resistance Management Laboratory. Unpublished study submitted to EPA. MRID # 457065-01.

Dennehy, T., S. Brink, B. Wood, D. Holley, G. Unnithan, Y. Carrière, and B. Tabashnik, 2003. Susceptibility of pink bollworm to Cry1Ac: final results of 2002 season studies. Cooperative Extension. The University of Arizona. Extension Arthropod Resistance Management Laboratory. MRID#

Dennehy, T., G. Unnithan, S. Brink, B. Wood, Y. Carrière, B. Tabashnik, L. Antilla, and M. Whitlow, 2004. Susceptibility of pink bollworm to Cry1Ac and Cry2Ab2: final results of 2003 season studies. Cooperative Extension. The University of Arizona. Extension Arthropod Resistance Management Laboratory. Unpublished study submitted to EPA. (Vol. 4, MRID# 469048-04)

Dennehy, T., G. Unnithan, S. Brink, B. Wood, Y. Carrière, and B. Tabashnik, 2005. Susceptibility of Bt toxins Cry1Ac and Cry2Ab2 to Southwestern pink bollworm in 2004. Cooperative Extension. The University of Arizona. Extension Arthropod Resistance Management Laboratory. Unpublished study submitted to EPA. (Vol. 4, MRID# 469048-04)

Dennehy, T. J., G. C. Unnithan, S. Brink, and B. Wood. 2006. Preliminary Report of susceptibility to *Bt* toxins Cry1Ac and Cry2Ab2 of Southwestern pink bollworm in 2005. Cooperative Extension. The University of Arizona. Extension Arthropod Resistance Management Laboratory. Unpublished study submitted to EPA. (Vol. 4, MRID# 469048-04)

Unnithan, G.C., S. Brink, B. Wood, Y. Carrière, B. Tabashnik, L. Antilla, and M. Whitlow. 2004. Susceptibility of Southwestern pink bollworm to Cry2Ab2: Baseline Responses in 2001 and 2002. Cooperative Extension. The University of Arizona. Extension Arthropod Resistance Management Laboratory. Unpublished study submitted to EPA. (Vol. 4, MRID# 469048-04)

EPA Reviews of the Resistance Monitoring Data

U.S. Environmental Protection Agency (U.S. EPA). 2001. Bt plant-incorporated protectants October 15, 2001 biopesticides registration action document. Available at http://www.epa.gov/pesticides/biopesticides/pips/bt_htm.

Matten, S. 2003. Technical review of Monsanto's submissions: resistance monitoring protocols

for monitoring of Cry1Ac susceptibility among field populations of pink bollworm, tobacco budworm, and cotton bollworm. S. Matten memorandum to L. Cole, September, 2, 2003.

Reynolds, A. 2004b. Review of 2001-2 monitoring data submitted by Monsanto for Bt cotton (Bollgard). A. Reynolds memorandum to L. Cole, September 8, 2004.

Reynolds, A. 2004c. Review of preliminary monitoring data submitted by Monsanto for Bollgard II Bt cotton. A. Reynolds memorandum to L. Cole, May 26, 2004.

Reynolds, A. 2005. Review of 2003 monitoring data submitted by Monsanto for Bt cotton (Bollgard and Bollgard II). A. Reynolds memorandum to L. Cole, June 8, 2005.

Reynolds, A. 2006. Review of 2004 pink bollworm monitoring data and revised monitoring protocol submitted by Monsanto for Bt cotton (Bollgard and Bollgard II). A. Reynolds memorandum to L. Cole, July 20, 2006.

Other Citations

Bartels, D., M. Walters. 2006. Spatial analysis of native and sterile pink bollworm in the Arizona PBW eradication program area, June 25-July 22, 2006.

Carrière, Y., C. Ellers-Kirk, M. Sisterson, L. Antilla, M. Whitlow, T.J. Dennehy and B.E. Tabashnik. 2003. Long-term regional suppression of pink bollworm by *Bacillus thuringiensis* cotton. Proc. of the National Academy of Sciences. USA 100: 1519-1523.

Carrière, Y., M. E. Nyboer, C. Ellers-Kirk, J. Sollome, N. Colletto, L. Antilla, T. J. Dennehy, R. T. Staten and B. E. Tabashnik. 2006. Effect of resistance to Bt cotton on pink bollworm (Lepidoptera: Gelechiidae) response to sex pheromone. J. Econ. Enomol. 99: 946-952.

Gahan, L. J., F. Gould, and D. G. Heckel. 2001. Identification of a gene associated with Bt resistance in *Heliothis virescens*. Science 293: 857-860.

Henneberry, T.J. and S.E. Naranjo. 1998. Integrated management approaches for pink bollworm in the southwestern United States. Integrated Pest Management Reviews 3: 31-52.

Keaveny, D., J. Hessler, J. Rudig. 2006. Local movement of sterile pink bollworm moths in the San Joaquin Valley of California. Special Report 2006.

Matten, S.R. 2005. Technical review of Monsanto's sales and acreage data for Bollgard® and Bollgard II® Cotton as well as reports of number of units sold and not returned in 2003 and 2004. Memorandum from S. Matten to L. Cole. (contains Confidential Business Information) November 9, 2005.

Miller, T.A., E. Miller, R. Staten, and K. Middleham. 1994. Mating response behavior of sterile pink bollworms (Lepidoptera: Gelechiidae) compared with natives. J. of Econ. Entomol. 87: 680-686.

Morin, S.R., W. Biggs, M.S. Sisterson, L. Shriver, C. Ellers-Kirk, D. Higginson, D. Holley, L.J. Gahan, D.G. Heckel, Y. Carrière, T.J. Dennehy, J.K. Brown, and B.E. Tabashnik. 2003. Three cadherin alleles associated with resistance to *Bacillus thuringiensis* in pink bollworm. Proc. Natl. Acad. Sci. 100: 5004-5009.

Morin, S., S. Henderson, J. A. Fabrick, Y. Carrière, T. J. Dennehy, J. K. Brown, and B. E. Tabashnik. 2004. DNA-based detection of Bt resistance alleles in pink bollworm. Ins. Biochem. Mol. Biol. 34: 1225-1233.

Reynolds, A.H. 2004a. Review of Monsanto's proposed amendment to revise the refuge requirements for Bollgard II cotton. Memorandum from A. Reynolds to L. Cole. April 12, 2004.

Roush, R.T., 1998. Two-toxin strategies for management of insecticidal transgenic crops: can pyramiding succeed where pesticide mixtures have not? Philos T Roy Soc 353: 1777-1786.

Sisterson, M.S., L. Antilla, Y. Carrière, C. Ellers-Kirk and B.E. Tabashnik. 2004. Effects of insect population size on evolution of resistance to transgenic crops. J. Econ. Entomol. 97: 1413-1424.

Tabashnik, B.E., A.L. Patin, T.J. Dennehy, Y.-B. Liu, E. Miller, and R.T. Staten. 1999. Dispersal of pink bollworm (Lepidoptera: Gelechiidae) males in transgenic cotton that produces a *Bacillus thuringiensis* toxin. J. Econ. Entomol. 92: 772-780.

Tabashnik, B.E., A.L. Patin, T.J. Dennehy, Y.-B. Liu, Y. Carrière, M.A. Sims, L. Antilla. 2000. Frequency of resistance to *Bacillus thuringiensis* in field populations of pink bollworm. PNAS 97: 12980-12984.

Tabashnik, B. E., Y.-B. Liu, T. J. Dennehy, M. A. Sims, M. Sisterson, R. Biggs, and Y. Carrière. 2002. Inheritance of resistance to Bt toxin Cry1Ac in a Þeld-derived strain of pink bollworm (Lepidoptera: Gelechiidae). J. Econ. Entomol. 95: 1018-1026.

Tabashnik, B. E., Y. Carrière, T. J. Dennehy, S. Morin, M. Sisterson, R. T. Roush, A. M. Shelton, and J.-Z. Zhao. 2003. Insect resistance to transgenic Bt crops: lessons from the laboratory and Þeld. J. Econ. Entomol. 96: 1031-1038.

Tabashnik, B. E., Y.-B. Liu, S. Morin, D. Unnithan, Y. Carrière, and T. J. Dennehy. 2004. Shared genetic basis of resistance to Bt toxin Cry1Ac in independent strains of pink bollworm. J. Econ. Entomol. 97: 721-726.

Tabashnik, B. E., R. W. Biggs, D. M. Higginson, S. Henderson, D. Unnithan, C. Ellers-Kirk, M. S. Sisterson, T. J. Dennehy, Y. Carrière, et al. 2005a. Association between resistance to Bt cotton and cadherin genotype in pink bollworm. J. Econ. Entomol. 98: 635-644.

Tabashnik, B. E., T. J. Dennehy, and Y. Carrière. 2005b. Delayed resistance to transgenic cotton in pink bollworm. Proc. Natl. Acad. Sci. U.S.A. 43: 15389-15393.

Tabashnik, B.E., J.A. Fabrick, S. Henderson, R.W. Biggs, C.M. Yafuso, M.E. Nyboer, N.M. Manhardt, L.A. Coughlin, J. Sollome, Y. Carrière, T.J. Dennehy, and S. Morin. 2006. DNA screening reveals pink bollworm resistance to Bt cotton remains rare after a decade of exposure. J. Econ. Entomol. In Press.

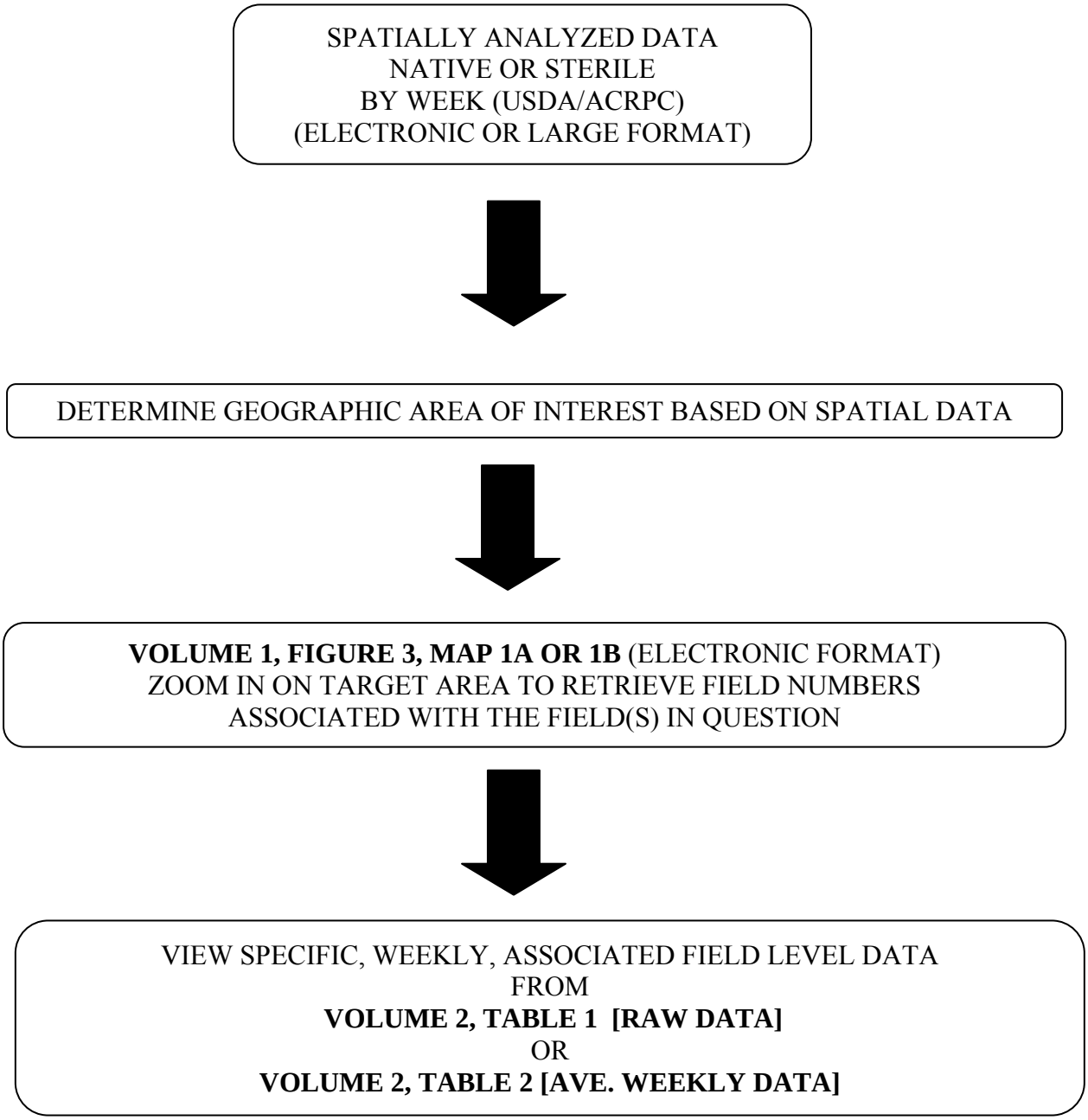
Xu, X., L. Yu and Y. Wu. 2005. Disruption of a cadherin gene associated with resistance to Cry1Ac δ-endotoxin of *Bacillus thuringiensis* in *Helicoverpa armigera*. Appl. Environ. Microbiol. 71: 948-954.

Zhao, J.-Z., J. Cao, H.L. Collins, S.L. Bates, R.T. Roush, E.D. Earle and A.M. Shelton, 2005. Concurrent use of transgenic plants expressing a single and two *Bacillus thuringiensis* genes speeds insect adaptation to pyramided plants. Proc. Natl. Acad. Sci. 102: 8426-8430.

FIGURES

1. **Figures 1-4**. Arizona Cotton Research Protection Council copywrite-protected *Bt*/non-*Bt* field maps for Central and Eastern Arizona and the sterile moth release zone are in a separate attachment (**Appendix 4**).
2. **Figure 5.** Scheme: Suggested Use of Trapping and Map Data
3. **Figures 6A-H.** Kriging Maps of the Sterile and Native Pink Bollworm Trapping Data
4. **Figure 7.** Field efficacy to *Bt* cotton in Arizona: 1995 to 2004
5. **Figure 8**. Changes in pink bollworm susceptibility to Cry1Ac in Arizona from 1997 to 2004

FIGURE 5. SCHEME**:** SUGESSTED USE OF TRAPPING AND MAP DATA SUBMITTED AS SUPPORTING DOCUMENTATION FOR SPECIAL LOCAL NEED REGISTRATION 24C, FOR BT COTTON FOR THE PURPOSE OF PINK BOLLWORM ERADICATION



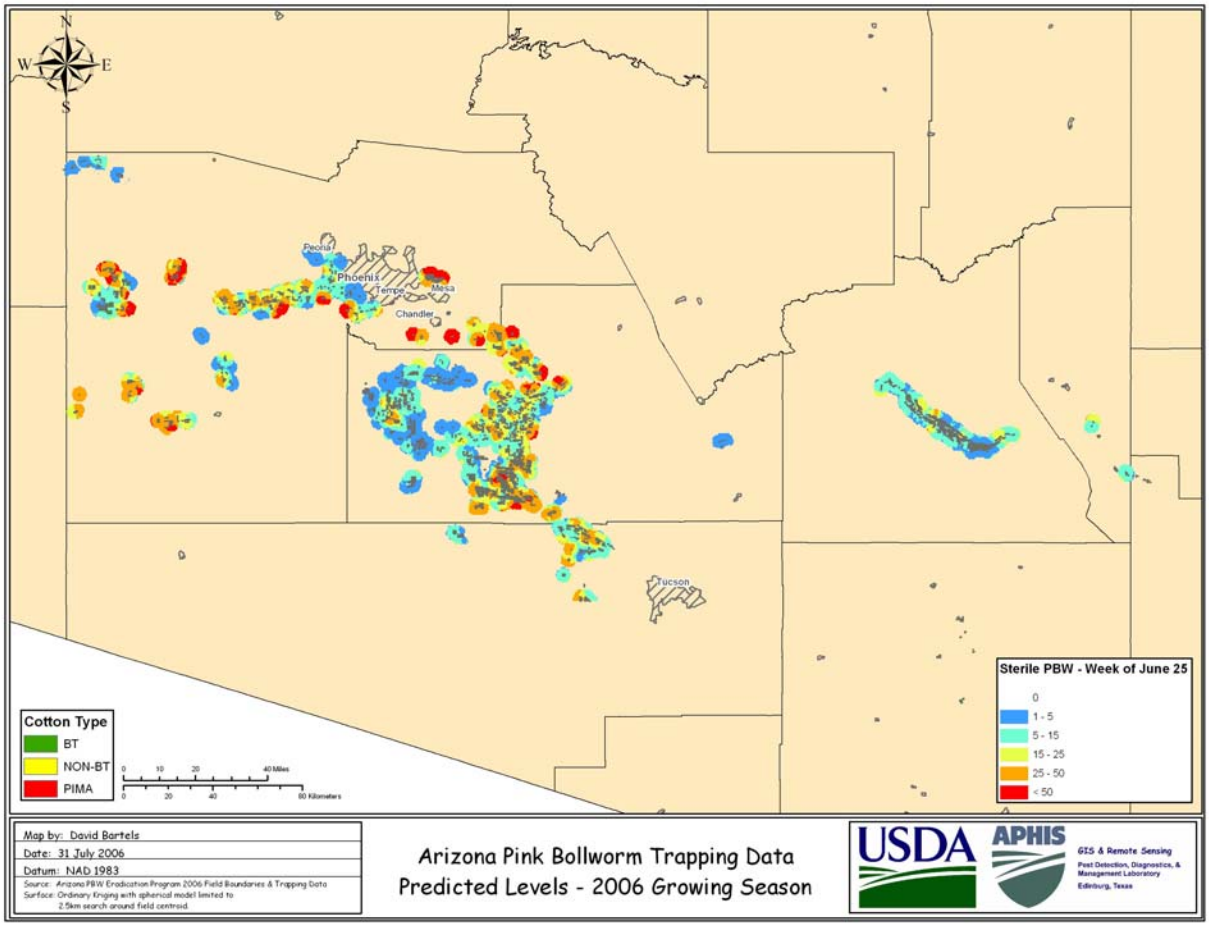


Figure 6A. Kriging map of Arizona Sterile Pink Bollworm Trapping Data - Week of June 25, 2006.

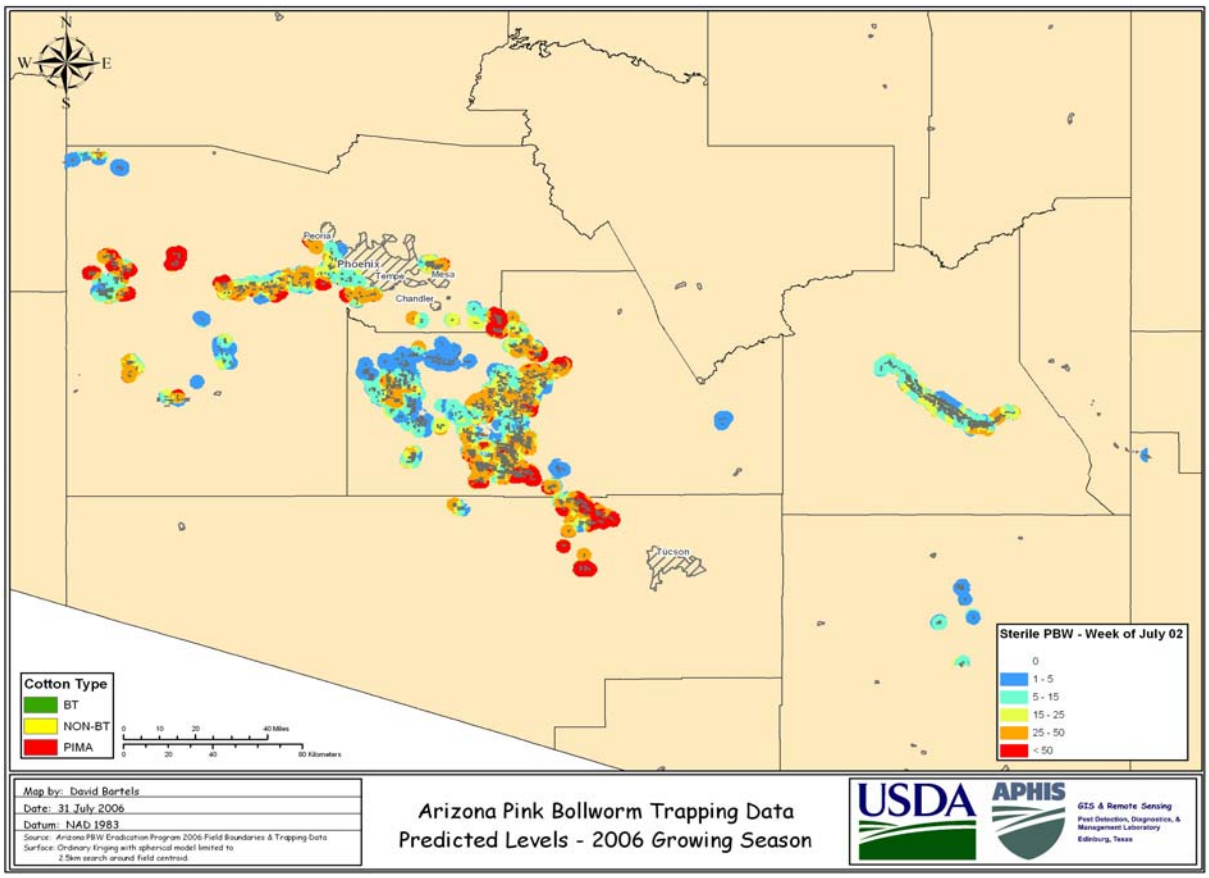


Figure 6B. Kriging map of Arizona Sterile Pink Bollworm Trapping Data - Week of July 2, 2006.

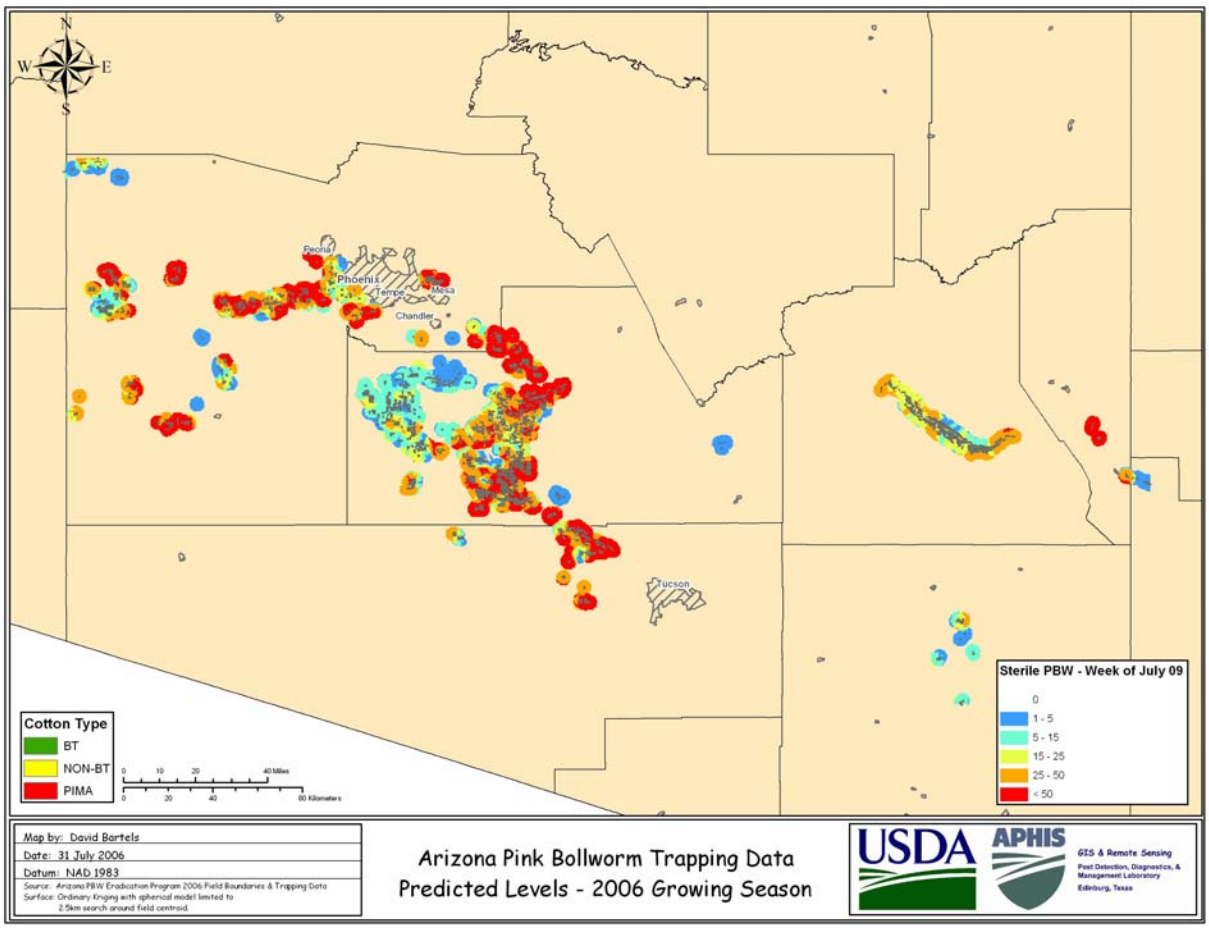


Figure 6C. Kriging map of Arizona Sterile Pink Bollworm Trapping Data - Week of July 9, 2006.

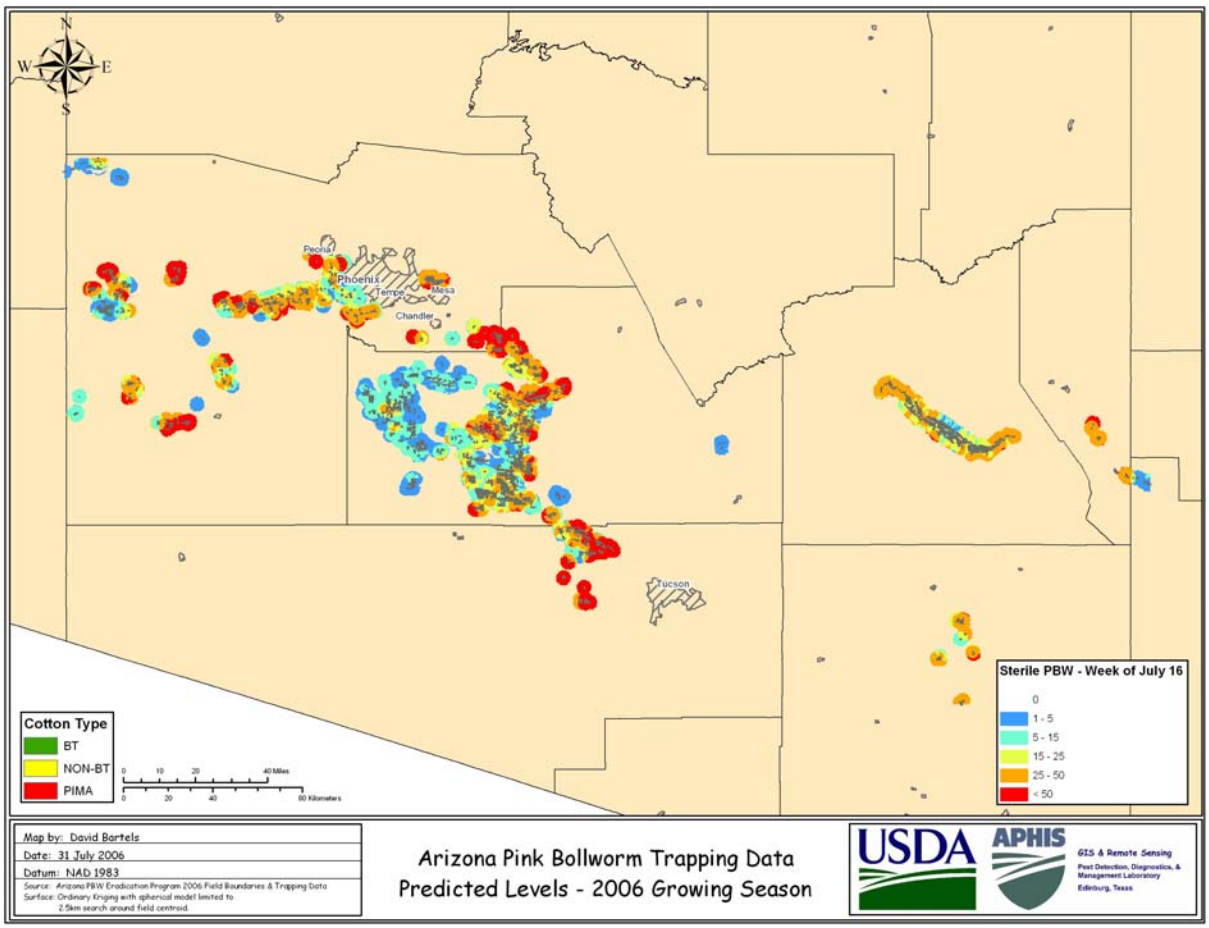


Figure 6D. Kriging map of Arizona Sterile Pink Bollworm Trapping Data - Week of July 16, 2006.

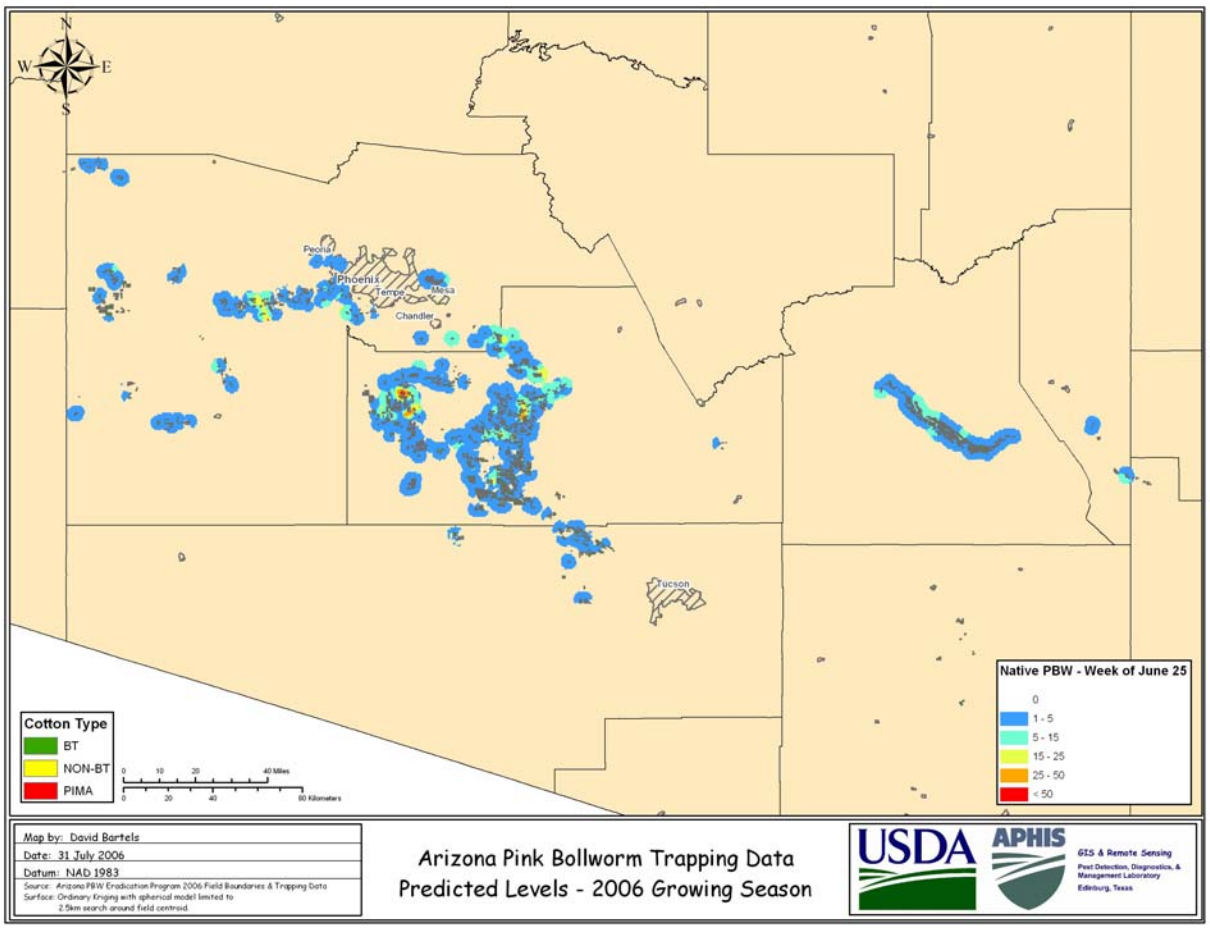


Figure 6E. Kriging map of Arizona Native Pink Bollworm Trapping Data - Week of June 25, 2006.

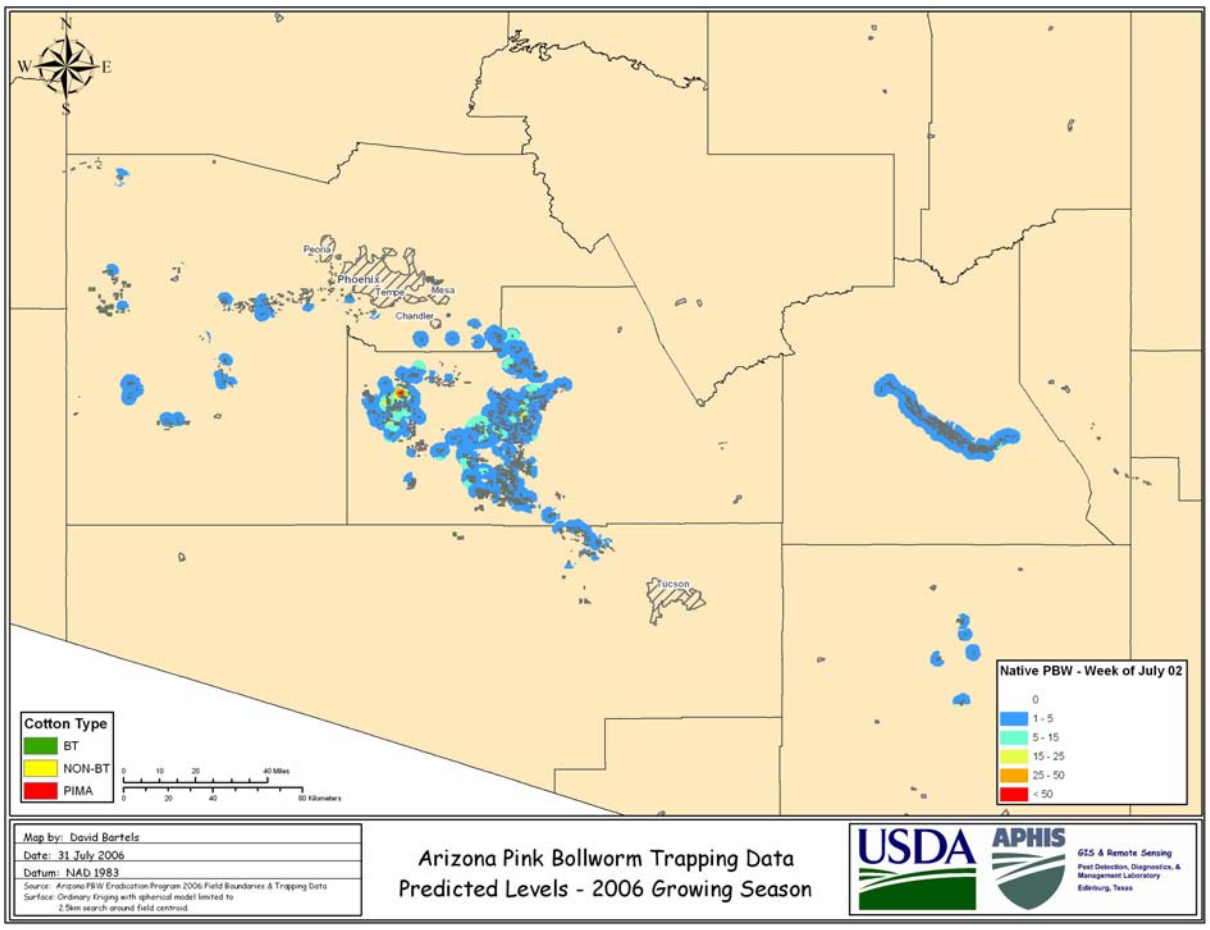


Figure 6F. Kriging map of Arizona Native Pink Bollworm Trapping Data - Week of July 2, 2006.

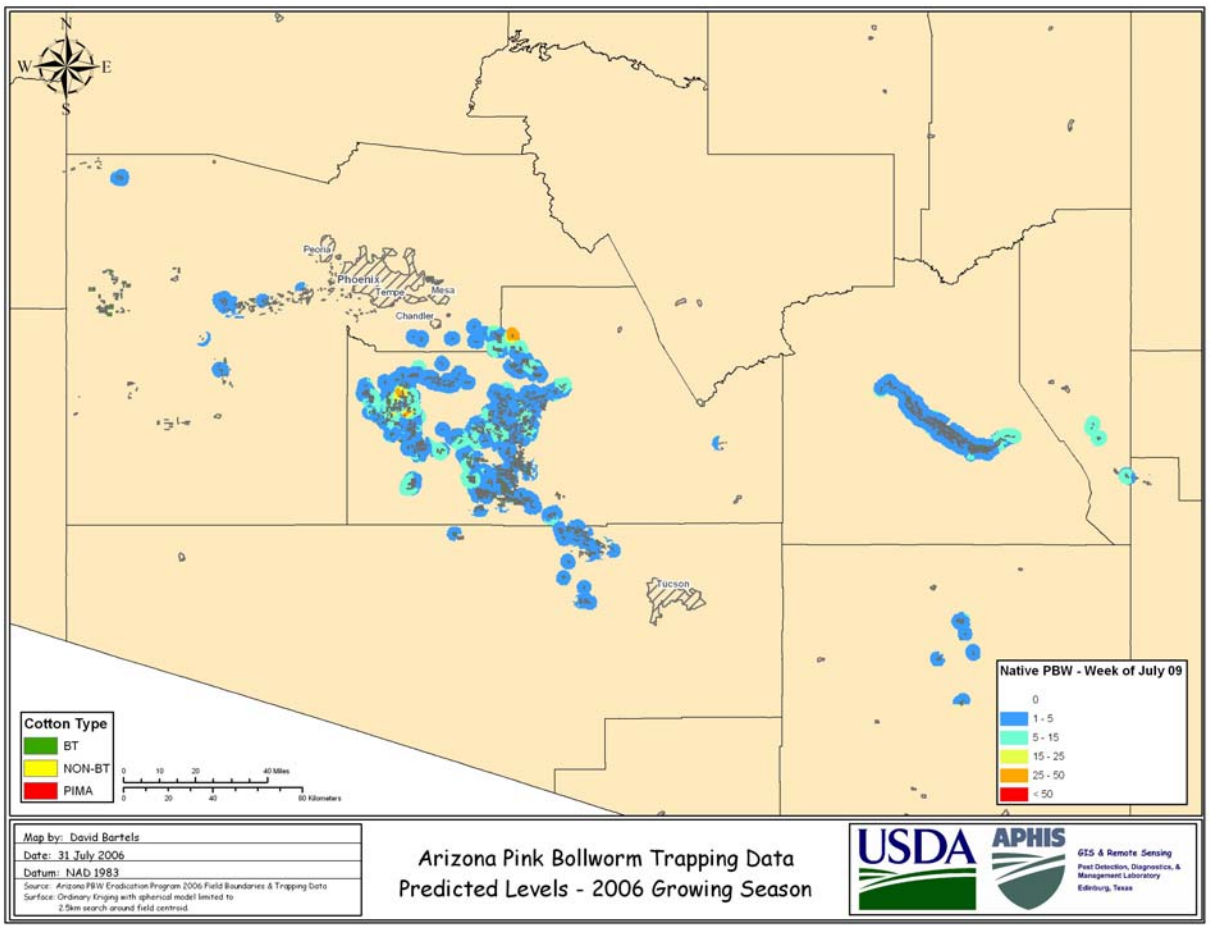


Figure 6G. Kriging map of Arizona Native Pink Bollworm Trapping Data - Week of July 9, 2006.

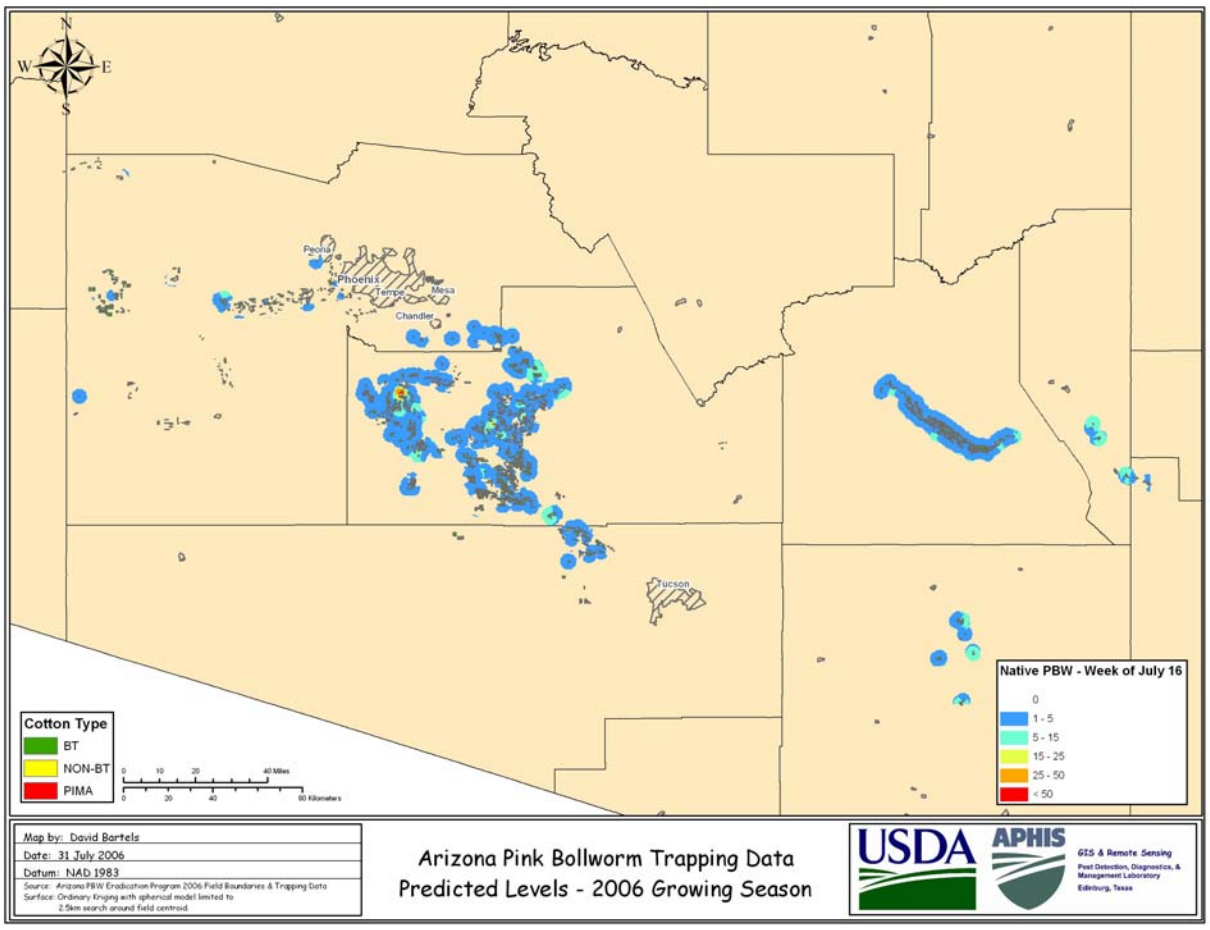


Figure 6H. Kriging map of Arizona Native Pink Bollworm Trapping Data - Week of July 16, 2006.

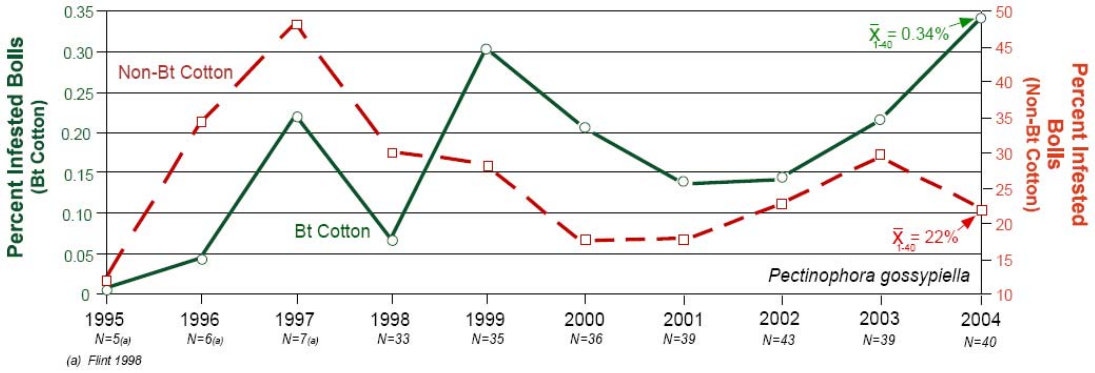


Figure 7. Field efficacy of *Bt* cotton in Arizona: 1995 to 2004. Data from 1995 to 1997 were reported by Flint *et al.*(1995) and Flint and Park (1996). All other data were collected by the Arizona Cotton Research and Protection Council. Shown are means of the percent boll infestation (bolls with ≥ 3rd instar PBW) for pairs of *Bt* cotton (left axis) and non-*Bt* cotton fields (right axis) sampled each year from 1995 to 2004. The number of pairs of *Bt* and non-*Bt* fields (*N*) is indicated for each year. [Figure from Dennehy *et al.*, 2005]

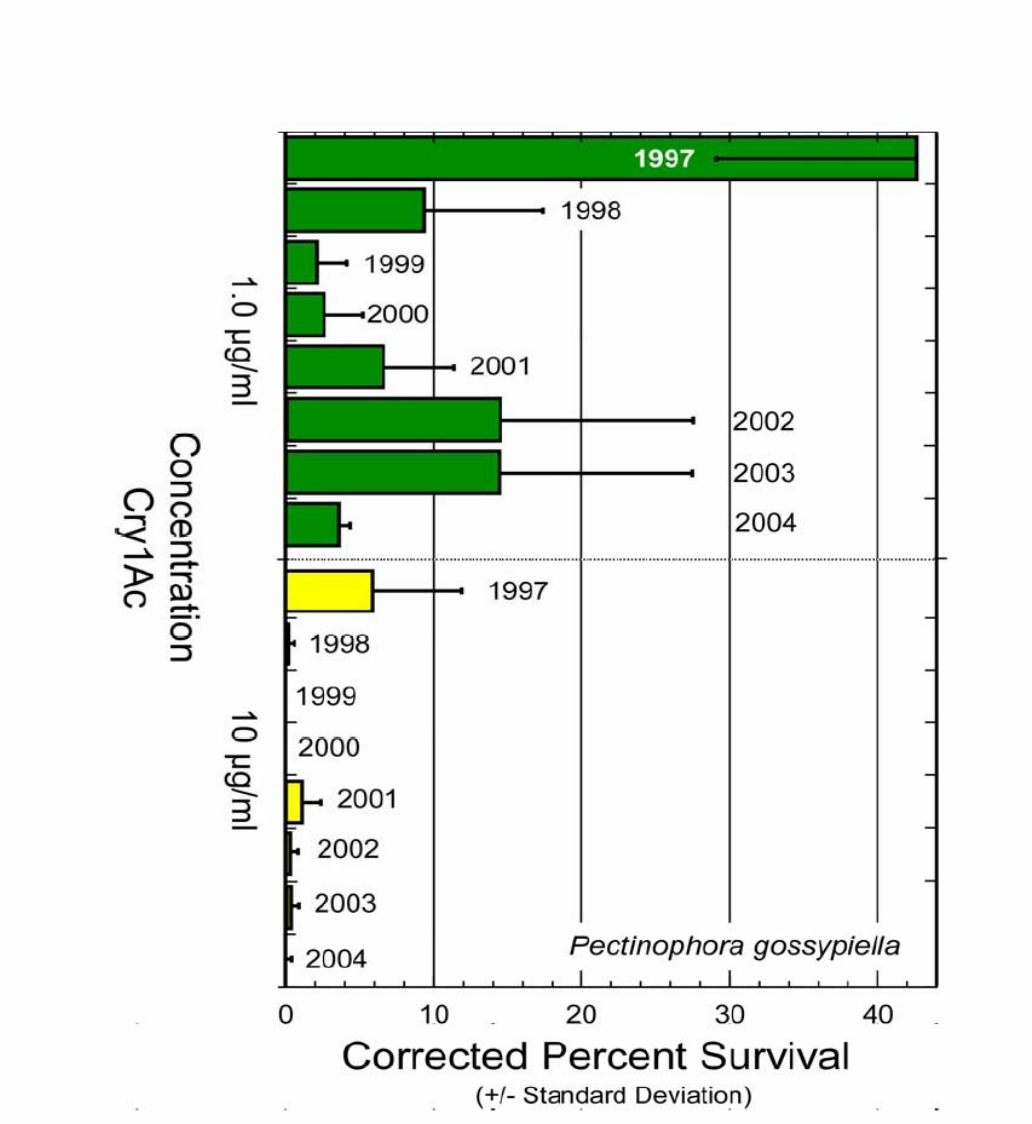


Figure 8. Changes in pink bollworm susceptibility to Cry1Ac in Arizona from 1997 to 2004. Shown are mean values (± standard deviation) of corrected survival observed in replicated bioassays of 1.0 and 10 µg Cry1Ac/ml diet of field collections made throughout Arizona in 1997 (n=9), 1998 (n=12), 1999 (n=14), 2000 (n=17), 2002 (n=13), 2003 (n=16), and 2004 (n=13). No larvae from any tests of 2005 strains survived treatments of 10 µg Cry1Ac/ml diet. [Taken from Dennehy *et al.*, 2006]

APPENDICES

1. Appendix 1: A Remedial Action Plan to Respond to Pink Bollworm Resistance to *Bt* Cotton in Arizona

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Cooperative Extension

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--Reviewed 3 June, 2002, by the AZ Bt Cotton Working Group--

A Remedial Action Plan for Responding to Pink Bollworm Resistance to Bt Cotton in Arizona

Formulated by the Arizona Bt Cotton Working Group
T.J. Dennehy, Chair

I. Definitions

Definition #1. Putative Resistance Event--A Cautionary Alert

A putative resistance event consists of any field of Bt cotton in which collections of 100 bolls yield ≥3% large larvae (≥3rd instar), pupae or PBW exit holes in bolls. This is a cautionary alert and must not be construed to be a verified resistance event until: 1) the plants from which collections were made are confirmed to produce Bt toxin and, 2) bioassays are completed that confirm the reduced susceptibility of the pink bollworm surviving on Bt cotton.

Definition #2. A Verified Resistance Event.

A putative resistance event becomes verified if three conditions are met:

1) A sample of 1000 bolls yields ≥3% containing large larvae (≥3rd instar), pupae, or PBW exit holes.
2) An ELISA test for Bt toxin yields a positive response for Bt toxin in a sample of 25 young bolls collected from plants on which PBW larvae were found in the cotton field of interest.
3) Standardized laboratory bioassays demonstrate that the PBW population of interest is significantly less susceptible to Cry1A(c) toxin than were baseline populations in 1997 (Simmons et al. 1998 and unpublished).

II. Remedial Action

1) Putative Resistance Event: Year of First Detection.

Within one week of confirming that a Bt field has ≥3% of bolls containing large larvae (≥3rd instar), pupae, or PBW exit holes, alternative PBW controls should be implemented in that field. Measures should include one or more of the following:

- Adulticide treatments if crop is in active growing state, followed by additional insecticide applications (2) on a 3-day schedule, or based on adult emergence as predicted by phenological models.

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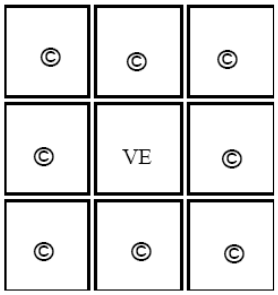
- If crop is senescent, consider chemical termination to reduce squares and bolls less than 10 days old, accelerate harvest, and destroy crop residue by shredding of stalks followed by discing, and deep plowing (6" burial).
- If crop is defoliated, accelerate harvest and destruction of crop residue to further limit survival of resistant pink bollworm. Destroy crop residue as indicated above.

2) Verified Resistance Event: Year of First Detection.

A. If resistance is verified in time to permit it, we strongly recommend that measures be taken to reduce the numbers of resistant pink bollworm that survive to the next season. These could include: adulticide treatments, early termination, and early plowdown, consisting of shredding of stalks followed by discing, and deep plowing (6" burial). Winter irrigation is also recommended to reduce survivorship of overwintering larvae.

B. Bt fields in the immediate vicinity of a verified resistance event should be examined to detect unusual survivorship of PBW. Results should be used to delimit the size of the affected area and to define the 'Bt remedial action zone.' We suggest sampling 300 bolls from all Bt fields located within the 8 sections of land (designated by © in the adjacent figure) that surround the section of land on which the verified event (VE) occurred. Bt cotton fields containing ≥3% bolls infested with PBW should be considered affected by resistance for the purpose of delimiting the remedial action zone.



C. The 'Bt remedial action zone' should be delineated using GPS mapping technology currently in use at the ACRPC. This will ensure accurate records of locations of verified resistance. The remedial action zone should include all sections of land falling within 6 miles of the perimeter of the section(s) of land in which verified resistance events occurred (see figure below).

D. At such time as fields with verified resistance are detected in >3 different townships within a particular cotton growing region, the entire region may be designated as a Bt resistance remedial action zone.

3) Verified Resistance Event: Next Year's Actions.

A. If verified resistance occurred in only Bollgard, then only Bollgard II, or non-Bt cotton should be planted in the remedial action zone in the year(s) immediately following verification of resistance. If verified resistance occurred in Bollgard and/or Bollgard II, then only non-Bt cotton should be planted in the remedial action zone in the year(s) immediately following verification of resistance. These measures should be maintained until such time as bioassays of PBW from the remedial action zone demonstrate that the frequency of resistant individuals has declined to acceptable levels. What will constitute levels of resistance acceptable for allowing resumption of use of Bt cotton will be determined on an *ad hoc* basis by our Working Group, based on research experience that members have obtained from studies of pink bollworm resistance to Cry1Ac.

The ecological fitness of PBW resistant to Cry1Ac is not known at this time and the dynamics of resistance in the field will likely be influenced by factors including overwintering survival of resistant

larvae, intensity of resistance to Cry1Ac, and growth and survival of resistant PBW on Bt and non-Bt plants. Therefore, new information derived from field and laboratory studies currently underway will be pivotal for determining the frequency of resistance (to Cry1Ac or to mixtures of Cry1Ac and Cry2Ab2) at which use of Bt cotton could reasonably be resumed within an area previously designated as a Bt remedial action zone.

B. It is assumed that published University recommendations for monitoring and chemical control of pink bollworm will be followed within remedial action zones in order to limit survival of resistant pink bollworm. Additionally, timely crop termination (no top-crop) and early and thorough crop destruction, as detailed above, is strongly encouraged. Releases of sterile pink bollworm should also be considered.

C. The recommendations of our working group regarding 1) Bt refuge management and 2) remedial action for responding to PBW resistance in Arizona should be re-evaluated annually and modified to account for new findings. Educational programs and regulatory measures should be devised to promote a high level of producer compliance with recommendations.

III. Organizational Roles

1) The Arizona Department of Agriculture should serve a central role in implementing this plan, compiling statistics on use of Bt cotton, and promoting compliance with remedial action.

2) Consideration should be given on a case-by-case basis for making funds available to compensate producers for costs associated with implementing the remedial action measures recommended herein.

3) A sampling team comprising personnel from relevant organizations (ACRPC, UA, USDA) will be formed. This team will be ready in August of every year to conduct the sampling required to delineate resistance problems (as detailed above). Similarly, facilities and personnel at EARML will be prepared to conduct bioassays or molecular tests of up to 40 different populations of PBW per season. Funding for these efforts must be sustained.

4) Monsanto should agree to suspend Bt cotton sales in remedial action zones until such time as either the frequency of resistant individuals is shown to have declined to levels deemed acceptable by our Working Group, or new Bt products free of cross-resistance are introduced, and the Arizona Bt Cotton Working Group has concluded that a modified resistance management strategy has been adopted that will adequately reduce the rate of development of further resistance to Bt cotton products.

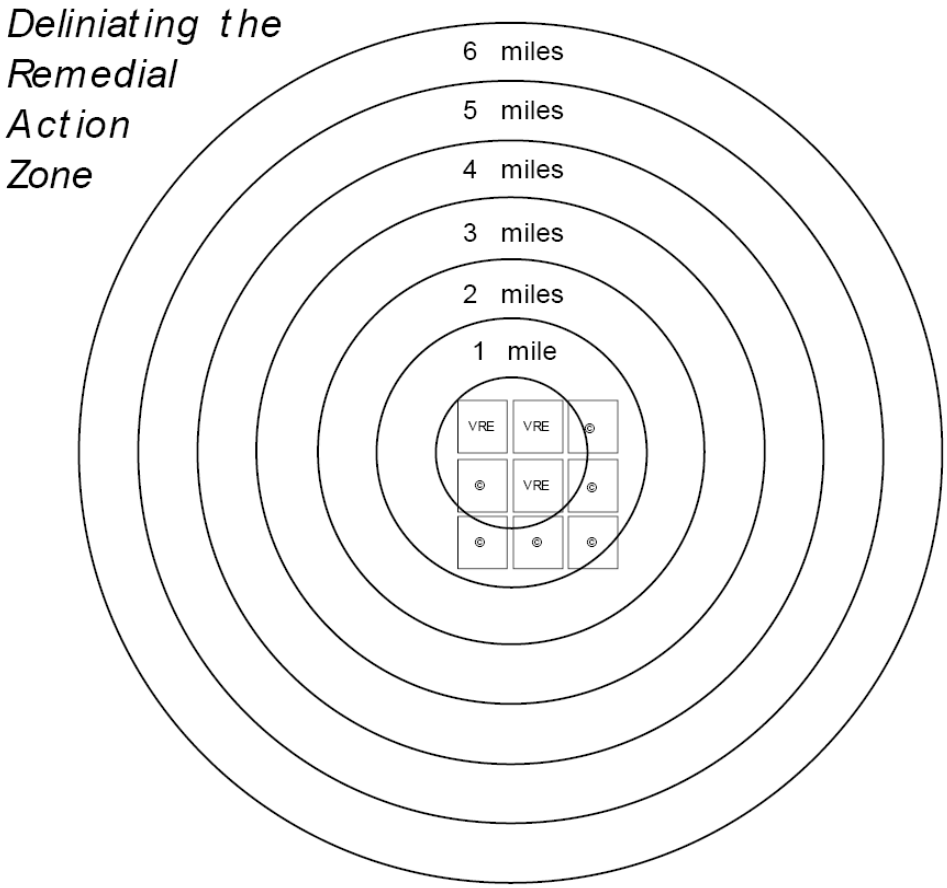
References

a. Simmons, A.L., T.J. Dennehy, B.E. Tabashnik, L. Antilla, A. Bartlett, D. Gouge and R. Staten. 1998. Evaluation of B.t. cotton deployment strategies and efficacy against pink bollworm in Arizona. Proc. 1998 Beltwide Cotton Conferences. *1025-1030.*

b. Liu, Y.-B., Tabashnik, B.E., Dennehy, T.J., Patin, A.L., and Bartlett, A.C. 1999. Development time and resistance to Bt crops. Nature 400:519.

c. Patin, A.L., Dennehy, T.J., Sims, M.A., Tabashnik, B.E., Liu, Y.B., Antilla, L., Gouge, D., Henneberry, T.J., and R. Staten. 1999. Status of pink bollworm susceptibility to Bt in Arizona. Proc. Beltwide Cotton Conferences. National Cotton Council. Pp. 991-996.

d. Tabashnik, B.E., A.L. Patin, T.J. Dennehy, Y.-B. Liu, E. Miller and R.T. Staten. 1999. Dispersal of pink bollworm (Lepidoptera Gelechiidae) males in transgenic cotton that producer a *Bacillus thuringiensis* toxin. J. Econ. Entomol. 92:772-780.



APPENDIX 2 Pink Bollworm Trapping and Sampling Methodology [Taken from letter dated September 14, 2006 from L. Antilla, Arizona Cotton Research and Protection Council to S. Matten, USEPA/OPP/BPPD]

TRAP DENSITY

NON-BT COTTON

- 1 trap per ten acres or 1 trap per field on fields less than ten acres.

BT

- 1 trap per forty acres on small contiguous blocks of fields with out biological separation.
- 1 trap per eighty acres on large contiguous blocks of fields with out biological separation.

TRAP PLACEMENT

Protocol calls for traps to be placed at or near the northeast corner of the field in a protected location (near a permanent structure such as a telephone pole). If a field has more than one trap, the traps are evenly spaced and numbered in a counter clockwise manner. Traps are placed as near to the field edge as possible while not obstructing the movement of equipment in and out of the field. Traps are attached to a wooden survey stake in order to maintain traps at canopy height.

TRAP SERVICE

Traps are regularly serviced once each week unless environmental conditions are prohibitive i.e. moisture soaked terrain inaccessible by 4 wheel drive. When a trap is serviced the entire trap is removed and a new trap with a new Pink Bollworm pheromone lure is placed in the trap. The "old" trap is labeled with the service date and the crop stage. Trap locations are bar-coded and each "new" trap is labeled with the field number and trap number. Traps are baited a with rubber dispenser impregnated with a 4 mg dose of Shinitsu Corporation ***Hexadecadienyl acetate*** (Gossyplure, Pheromone) that has been subjected to field bioassay for field activity by USDA personnel.

The traps removed from the field are transported to the field offices in a protected manor for identification.

MOTH IDENTIFICATION

Traps are brought in from the field each week; identifiers stationed at each field office inspect each trap. All Pink Bollworm moths are counted and recorded as either "native" or "sterile". The sterile pink bollworms are dyed red through the media they are fed in the rearing facility. The trapping date (date the trap was removed from the field), field number, trap number and crop stage are all recorded along the "native" and "sterile" counts for entry into the database. Any questionable determinations regarding the identity of moths are forwarded to the principle identifier.

At the discretion of program management and as readily available samples for Dr. Tim Dennehy's Extension Arthropod Resistance Management Laboratory (EARML), moths are saved for testing related to BT resistance or genetic identity. The moths collected for these functions are placed in alcohol and kept in a freezer to protect from deterioration until testing can be completed.

BOLL SAMPLING

Boll sampling is conducted on randomly selected fields or pairs of fields throughout the program area to determine representative infestation levels. When possible, a BT and a NON-BT field in close proximity at the randomly selected location are included. The purpose of this exercise is to approximate the approach taken in Arizona since 1998 wherein, randomly selected paired BT and NON-BT fields were intensively sampled late season for comparative infestation levels and resistance monitoring.

Based on trapping information, history and targeted field inspection, other fields are checked for boll infestation as needed. Program personnel make these decisions by dedicating the majority of our resources on field based activities whereby anomalies are isolated and investigated to the benefit of the producers in the program. Due to the targeted approach in this instance any findings are neither representative nor random and therefore statistically not indicative of program wide infestation levels.

Sampling each of the four thousand six hundred and twenty six fields each week is not logistically or economically feasible and would certainly be undesirable to producers in the program.

The initial boll survey sampling was performed for 4 sampling cycles (1 sample every other week). Additional sampling will be conducted using boll boxes. Boll boxes data is much more reliable as bolls are picked and then placed in boxes (cages) and stored at controlled temperatures until any organisms inside the bolls have emerged and can be counted.

STERILE/NATIVE TRAPPING DATA

By statute, all growers within the program area must report NON-BT cotton in a timely manner. BT cotton reporting is not legally required and therefore must be ground proofed by program personnel. Circumstances do exist wherein late planted or unreported cotton results in data beginning the week after traps are deployed.

Trapping data is not a self contained gauge of populations. Pheromone traps are subject to hindrance from many biological, environmental and seasonal influences. Inherent variability in trapping information from specific trap location data must be evaluated as an aggregate from multiple data points to be meaningful per the maps provided by Dave Bartel and the statistical data model. Moth numbers found in traps are not directly proportionate to release levels on that

field or the surrounding area. Native populations in the program area are still very high. Native and Sterile females produce pheromone which competes with the traps. As the native populations become less significant the pheromone traps will become more consistent population indicators as individual data points. As discussed in the trapping section above, there are periodic instance where traps cannot be accessed, in these instances no data is available.

As indicated in the trapping section of this document, BT fields are grouped together in regards to trap allocation. Not every BT field has a trap directly assigned to it however; traps are distributed through the region to produce a representative sample of the region and all of the fields therein. Trapping each BT cotton field individually is cost prohibitive and logistically impossible within the constraints of the Pink Bollworm Eradication Program. This approach is in strict contrast to non eradication program grower practices where BT cotton is not monitored with pheromone traps.

All fields have received sterile release every week on timetable with minor variations due to weather and or chemical treatment. Chemical treatment only affects sterile release on NON-BT cotton as BT cotton is not treated with chemical or pheromone treatments. Sterile release has been unremitting once sterile release began. No release days have been compromised due to mechanical failure, moth supply or due diligence. Sterile moths in excess of **1,137,012,553** have been released over Arizona BT and NON-BT cotton as of September 5, 2006 within the Pink Bollworm Eradication Program."

APPENDIX 3: Tabashnik's Answers, dated 9/14/06, to Matten's Questions, dated 9/8/06, Re: PCR [Taken from e-mail, B. Tabashnik, U. of Arizona to S. Matten, USEPA/OPP/BPPD, dated September 14, 2006]

1. Is the Molecular analysis method in the submission to EPA, the one published in Tabashnik et al. 2006? If so, I need an e-mail back to me identifying that this is indeed the method used.
Yes. The molecular analysis method in the submission to EPA is the one published in Tabashnik et al. 2006. Key portions of the paper describing the method are provided below. Please note that initial tests use only a small portion of each field-sampled individual (Tabashnik et al. 2005b), so that re-testing of individuals is possible if desired.

INTRODUCTION
"In laboratory-selected strains of pink bollworm and at least two other major lepidopteran pests of cotton, mutations in a cadherin gene are tightly linked with recessive resistance to Cry1Ac (Gahan et al. 2001, Morin et al. 2003, Xu et al. 2005). In several laboratory-selected strains of pink bollworm, three mutant alleles (*r1*, *r2*, and *r3*) of a cadherin gene (*BtR)* are associated with resistance to Cry1Ac and survival on Bt cotton (Morin et al. 2003, 2004; Tabashnik et al. 2004, 2005b). Each *r* allele has a deletion predicted to eliminate at least eight amino acids upstream of the putative Cry1Ac-binding region of cadherin protein (Morin et al. 2003). We previously developed a PCR-based method for detecting the *r1*, *r2* and *r3* alleles in pink bollworm (Morin et al. 2004). We isolated, cloned and sequenced the genomic region spanning the mutation in each *r* allele and designed allele-specific PCR primers for each region. The method can detect any of the three *r* alleles in a single heterozygote (*r1s*, *r2s*, or *r3s*) pooled with DNA from the equivalent of 19 susceptible (*ss*) individuals (Morin et al. 2004)."

METHODS: "DNA Preparation and PCR. Insects collected from bolls and traps were stored in ethanol at –20°C. DNA was extracted using DNAzol (Tabashnik et al. 2005b) and PCR was done as described by Morin et al. (2004). The maximum number of individuals tested per pool was 5 for samples from 2001-2003 and 11 for samples from 2004-2005."

2. The late season sampling method developed by Dennehy and Tabashnik-- I don't have the specific protocol. Please send it or perhaps it is in the Tabashnik et al. 2006 manuscript. I see a couple of sentences describing the plan, is this it? Similar to Tabashnik et al. (2006), details coming soon.

3. What is the method for estimating false negatives? false positives? What is the likelihood of non-detection? Please clarify.
The methods for estimating false negatives, false positives, and the likelihood of non-detection are detailed in Morin et al. (2004) and Tabashnik et al. (2006), as well as below:

A. False negatives.
False negatives are possible from three causes: i) The PCR reaction is not working properly, ii) The cadherin DNA of field-sampled insects is not amplified, iii) The PCR is working and cadherin DNA is amplified from field samples, yet *r* alleles are present and are not detected.

i) To determine if the PCR reaction is working properly, we use known positive controls in every set of samples tested. This is a standard method. Known positive controls are samples of DNA from our laboratory-reared strains that contain *r* alleles, which are known to the person running the PCR reaction. For example, every test of field-sampled insects for the presence of an *r1* allele includes a gel lane in which DNA from one or more laboratory-reared individuals with the *r1* allele is run simultaneously with the field samples.

If the known sample of *r1* DNA does not yield a positive result for *r1*, the test of the field sample is not valid and must be repeated. In this case, PCR reaction conditions are corrected until the known controls yield positive results with the simultaneously tested field samples. Such corrections usually involve systematic replacement of reagents (primers, Taq, etc.) to ensure all are working properly. Because only tests yielding positive results for known positive controls are included in our analysis of the data, this source of false positives has an effective rate of 0% in the data analysis.

ii) To determine if the cadherin DNA of field-sampled insects is amplified, we test for amplification of a conserved region of the cadherin gene that occurs in all known susceptible and resistant alleles (Morin et al. 2004). As described in Tabashnik et al. (2006), "We checked all pools using this approach and >99% tested positive. Because as few as one amplifiable allele from a pool of insects could yield a positive result for this control reaction, we also tested a subset of insects individually from each of the 59 field samples. Of the 835 individuals tested, 98.6% were positive."

The 98.6% amplification rate of the conserved region of the cadherin gene indicates that DNA was not amplified from 1.4% of field-sampled insects. We take this into account in estimating the likelihood of non-detection by adjusting the sample size accordingly. For example, if 1000 alleles are screened from 500 individuals and the amplification rate of the conserved region is 98.6%, the corrected sample size is 986 (see C below).

iii) As described in Tabashnik et al. (2006): "In addition to standard positive controls for each of the three *r* alleles in all tests, we included "blind" positive controls as follows: Two researchers analyzed each field sample. One researcher prepared DNA and added individuals with one or two *r* alleles from laboratory-selected resistant strains in zero to three (usually one) of the pools tested from each field site. The other researcher performed PCR and did not know which, if any, of the pools contained these blind positive controls. The detection rate for blind positive controls was 97% (97/100)." The rate of false negatives (3%) caused by failure to detect *r* alleles present in pools is incorporated in the estimate of the likelihood of non-detection, as described below (C).

B. False positives. To detect false positives, we use a standard technique. All tests of field samples include blanks, which are gel lanes containing all of the PCR reagents, but no DNA. If a blank yields a positive result, this indicates contamination (i.e., a false positive). In this case, PCR reaction conditions are corrected and the field samples are retested. Results are included in the data analysis only if the blanks do not yield positive results.

Of the 5,571 field-sampled insects tested in Tabashnik et al. (2006), none yielded positive results. Thus, the problem of false positives is minimal to nil. When a pool of field-sampled insects yields a positive

result for an *r* allele (e.g., *r2*), each individual in the pool will be tested separately to verify the positive result and to more precisely estimate the frequency of resistance in the pool.

C. Non-detection. As described in Tabashnik et al. (2006), the likelihood of non-detection is estimated as follows:

"The probability of detecting no *r* alleles in a sample of *N* individuals was calculated as $(1-[F \text{ X } D])^{2N \text{ X } A}$, where *F* is the frequency of resistance alleles, *D* is the probability of detecting an *r* allele present in screened individuals (0.97, based on the data from blind controls), 2*N* is the number of alleles screened, and A is the probability of amplifiable cadherin DNA occurring in field-sample insects (estimated as 0.986, based on the proportion of positive results for amplification of a conserved sequence in 835 insects tested individually). We assumed that the probability of an *r* allele occurring was an independent event at each cadherin allele screened. For example, with an *r* allele frequency of 0.001, the probability of detecting no *r* alleles in the sample of 5,571 individuals (11,142 alleles) is $0.000023 = (1- [0.001 \text{ X } 0.97])^{11,142 \text{ X } 0.986}$. Analogously, with an *r* allele frequency of 0.0003, the probability of detecting no *r* alleles in the sample of 5,571 individuals is $0.041 = (1 – [0.0003 \text{ X } 0.97])^{11,142 \text{ X } 0.986}$."

The goal in 2006 is to screen 500 field-sampled individuals with PCR (i.e, *N*=500). Assuming no *r* alleles are detected and values for *D* and *A* similar to those above, the probability (P) of non-detection is estimated as:
i) for true *r* allele frequency of 0.00316 (frequency of *rr* = 0.00001),
$P = (1- [0.00316 \text{ X } 0.97])^{1000 \text{ X } 0.986} = 0.048$

ii) for true *r* allele frequency of 0.01 (frequency of *rr* = 0.0001),
$P = (1- [0.01 \text{ X } 0.97])^{1000 \text{ X } 0.986} = 0.000067$

iii) for true *r* allele frequency of 0.001 (frequency of *rr* = 0.000001),
$P = (1- [0.001 \text{ X } 0.97])^{1000 \text{ X } 0.986} = 0.38$

Below please find additional discussion of the potential for non-detection from Tabashnik et al. (2006): "It is important to consider potential underestimation of *r* allele frequency based on DNA screening. DNA screening based solely on males caught in pheromone traps could cause underestimation if the probability of capture in traps was lower for *rr* or *rs* males than for *ss* males. However, tests conducted in large cages (64 m^3) in the field refuted this hypothesis for pink bollworm (Carrière et al. 2006). Furthermore, DNA screening of pink bollworm from bolls, which was independent of males caught in traps, also detected no *r* alleles (n = 1,344; Table 1).

If alleles other than cadherin mutants *r1, r2,* and *r3* confer pink bollworm resistance to Bt cotton, the results of our DNA screening could underestimate the frequency of resistance. For example, resistance to Cry1Ac in some strains of diamondback moth is not linked with cadherin (Baxter et al. 2005). However, in four laboratory-selected Cry1Ac-resistant strains of pink bollworm tested so far, all resistant individuals screened have two copies of the known *r* alleles (i.e., *r1r1*, *r2r2*, *r3r3, r1r2, r1r3* or *r2r3)* and no other resistant alleles have been detected (Morin et al. 2003, Tabashnik et al. 2004, 2005b).

Although the presence of additional resistance alleles at the cadherin locus or other loci cannot be excluded, such alleles appear to be more rare than the three known resistance alleles."