July 20, 2006

MEMORANDUM

SUBJECT: Review of 2004 pink bollworm monitoring data and revised monitoring protocol submitted by Monsanto for Bt cotton (Bollgard and Bollgard II). EPA Reg. No. 524-478, 524-522. DP Barcode: No barcode assigned. Decision: 363972. MRID#: 467350-01.

TO: Leonard Cole, Regulatory Action Leader
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division (7511C)

FROM: Alan Reynolds, Entomologist /s/
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division (7511C)

PEER REVIEW: Sharlene Matten, Ph.D., Biologist /s/
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division (7511C)

Action Requested

BPPD has been asked to review pink bollworm resistance monitoring data submitted by Monsanto for the Bollgard (Cry1Ac) and Bollgard II (Cry2Ab2) Bt corn registrations (EPA Reg No. 524-478 and 524-522). The submitted report (MRID# 467350-01) includes the monitoring results collected from the 2004 growing season for the Cry1Ac and Cry2Ab2 toxins.

Background

The major target pests of Bollgard Bt cotton, tobacco budworm (TBW), cotton bollworm (CBW), and pink bollworm (PBW) have been monitored for susceptibility to Cry1Ac since the product was first registered in 1996. Monitoring for PBW susceptibility to Cry1Ac has been conducted in the state of Arizona from 1996 by the University of Arizona/Extension Arthropod Resistance Management Laboratory.

All of the Cry1Ac monitoring data through the 2000 growing season have been previously reviewed by the Agency during the 2001 Bt crops reassessment (EPA 2001). In this review, it was concluded that through the 2000 season, there was no evidence of TBW, CBW, or PBW

resistance to the Cry1Ac delta endotoxin produced by Bollgard cotton cultivars under field situations. Since the 2001 reassessment was completed, BPPD monitoring reviews have been completed for Cry1Ac data through the 2003 growing season (see BPPD 2004a, 2005a). These reviews also concluded that no field resistance has been detected for the three major target pests.

Bollgard II, which expresses Cry2Ab2 and Cry1Ac, was registered for the 2003 growing season with similar monitoring requirements for Cry2Ab2. Data for PBW and Cry2Ab2 were initially received with the 2003 report and have been previously reviewed (see BPPD 2005a). The 2003 data showed that PBW were highly susceptible to the toxin at the two test doses. The higher test dose used, 10 µg/mL, appeared to be functional as a "discriminating dose" for PBW.

For the 2004 growing season, Cry1Ac and Cry2Ab2 monitoring data were submitted for TBW and CBW (reviewed in BPPD 2005b). However, PBW data were not submitted with TBW/CBW report because PBW are typically collected late in the season and must be reared for several months to generate progeny for the bioassays. As such, a supplemental report was submitted for PBW and is reviewed in this memorandum.

Conclusions and Recommendations

1) Monsanto has submitted an acceptable report for the 2004 Cry1Ac and Cry2Ab2 monitoring of PBW. No further information is needed for the 2004 PBW monitoring efforts. However, BPPD has a number of comments, detailed below, that should be addressed in future monitoring assays and study reports.

2) The 2004 data showed that PBW remains highly susceptible to both Cry1Ac and Cry2Ab2. At the discriminating test concentration used (10 µg/ml), only one larva survived on Cry1Ac and no larvae survived on Cry2Ab2. At the lower test concentration (1 µg/ml), mortality to both toxins exceeded 95%.

3) A separate boll sampling program revealed low PBW infestation among collected Bt bolls. Of the sampled Bt bolls, 0.34% were infested with PBW, although many of the putative Bt bolls were later determined to be non-expressing off types. The rate of boll infestation observed in 2004 is comparable to rates seen in previous years.

4) It is recommended that in future monitoring efforts, follow-up susceptibility testing be conducted with PBW larvae recovered from Bt bolls (verified expressing the Bt toxin), if historical infestation averages are exceeded for Bt cotton. Also, follow-up testing should be conducted on survivors of the discriminating test concentrations. It is noted that such PBW larvae may contain resistance genes (either heterozygous or homozygous) that should be further analyzed. For 2004, such testing was not needed because: a) the rate of boll infestation in Bt cotton was within historical averages, and b) only one PBW larvae survived the discriminating test concentrations.

Monitoring Results - PBW (2004)

The 2004 monitoring work for PBW was conducted in Arizona by researchers at the University of Arizona and the Arizona Cotton Research and Protection Council, who have been conducting the work since 1997. The methodology for the 2004 PBW assays was largely the same as in previous years and utilized artificial diet tests with a 21-day observation period. The monitoring also included the continuation of Cry2Ab2 testing initiated in 2003 (applicable to the Bollgard II registration). To conduct the bioassays, a discriminating dose type approach 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 2003 monitoring. In addition to the laboratory bioassays, field efficacy was assessed in 2004.

2004 Sampling and Assays

PBW were collected as larvae (from bolls brought to the laboratory) from three western cotton-growing states: Arizona (15 sites), California (2 sites), and New Mexico (1 site). Sampling was limited in New Mexico and Texas (no collections) due to ongoing PBW eradication efforts in those areas. All collection sites were represented in the Cry2Ab2 testing, though two sample sites from Arizona were not used in the Cry1Ac testing. 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).

2004 Cry1Ac Results

The results of the Cry1Ac assays from Arizona-collected PBW at the 1.0 µg Cry1Ac/ml dose were variable and revealed an average corrected mortality of 96.5% (range 78.7 - 100%). At the 10 µg Cry1Ac/ml dose, the average mortality for the Arizona collections was 99.9% (range 99.7 - 100%), with only one population exhibiting less than 100% mortality to the test concentration (only one larva survived to the fourth instar). Similar results were observed for the California and New Mexico collections, with a mortality range at the 1.0 µg Cry1Ac/ml dose of 88.2 - 100% and 100% mortality for all three tested populations at the 10.0 µg Cry1Ac/ml concentration. The susceptible laboratory colony used as a control group showed 58.5% mortality at the 1.0 µg Cry1Ac/ml

concentration and 100% mortality to the 10 µg Cry1Ac/ml In comparison to previous years, the 2004 results at the higher 10.0 µg/ml dose (99.9% overall mortality) were similar to the results from 2002 and 2003 (99.8% average mortality was observed in both years). However, the overall PBW mortality observed in 2004 at the 1.0 µg Cry1Ac/ml concentration (95.4) was higher than in previous years (68.3% in 2003, 85.7% in 2002) (also see table 1 below). In the report, the authors pointed out that PBW mortality to the higher discriminating concentration (10 µg Cry1Ac/ml) has been higher in recent years (>99.4%) than was observed in the first year of Cry1Ac monitoring (94.1% mortality in 1997). Overall, the authors concluded that PBW remains susceptible to Cry1Ac and that are no indications of resistance in the field. The 2004 results from Arizona are summarized and compared with historical data in table 1 below.

2004 Cry2Ab2 Results

As with Cry1Ac, PBW were sensitive to Cry2Ab2 during the 2004 testing. At the 1.0 µg/ml test concentration, mortality (corrected) for Cry2Ab2 ranged from 97.9 to 100% for Arizona collections (average 99.8%) and 95.2 to 100% for California and New Mexico collections (average 97.2%). At the 10.0 µg Cry2Ab2/ml dose, no survivors were observed from any of the Arizona, California, or New Mexico samples (100% overall mortality). The susceptible laboratory colony used as control had average mortalities of 82.7% to the 1.0 µg Cry2Ab2/ml concentration and 100% to the 10.0 µg Cry2Ab2/ml concentration. The Cry1Ac-resistant colony showed somewhat less mortality to the lower 1.0 µg concentration (70.7%), but like the other groups, had 100% mortality to the higher 10.0 µg concentration. The 2004 assays represented the second full year of monitoring for resistance to the Cry2Ab2 toxin and had similar results to the 2003 monitoring (see table 2) and also to previous baseline work with Cry2Ab2 done in 2001 and 2002 (see BPPD 2004b). As with Cry1Ac, the authors concluded that the sampled PBW populations remained highly susceptible to Cry2Ab2. The 2004 Arizona data are summarized in table 2 below.

2004 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 2003. Bolls were examined for PBW larvae – of 39,500 Bt boll sampled, 133 PBW larvae were found (for an infestation rate of 0.34%). By comparison, the infestation rate for 2003 was 0.21%. For non-Bt cotton, 2082 larvae were found in 10,375 non-Bt bolls, an infestation rate of 21.7% (compared with 27.4% in 2003). As was the case in 2003, subsequent analysis of the Bt bolls determined that many were non-expressing off-types (33 out of 35 infested Bt bolls were found to be off-types). The authors noted that the infestation rate observed in Bt fields has historically averaged less than 0.35%.

As with past reports, the PBW reports for 2004 was thorough and well-organized. Since the methodology has remained consistent throughout the PBW monitoring efforts, the data can be placed in a historical context to evaluate long-term shifts in susceptibility. Through 2004, eight years of monitoring data have now been tabulated for Cry1Ac and two years for Cry2Ab2. Overall, the authors note that PBW susceptibility to both toxins remains high. BPPD agrees with this assessment, based on the susceptibility data compiled to date (see tables 1 and 2 below) and the low infestation rates observed in the field efficacy trials. PBW mortality at the 10.0 µg/ml dose was comparable to previous years (close to 100% mortality). The 10.0 µg/ml concentration is essentially a true discriminating dose, i.e. an LC_{99} that can be used to distinguish potentially resistant insects from susceptible ones. Infestation rates in the field efficacy study were 0.34% infested Bt bolls, which is higher than has been observed in other years. However, many of the collected Bt bolls with PBW larvae were later determined to be non-Bt expressing off-types and the increased infestation rate is unlikely to be the result of adaptation to Bt toxins. PBW susceptibility to Cry1Ac at the lower 1.0 µg/ml concentration increased in 2004 after two years in which lower mortalities were observed (see table 1). The researchers had attributed the reduced susceptibility in 2002 and 2003 to a potential decrease in the potency of the Cry1Ac toxin used in the tests (similar reductions in susceptibility were noted with the unselected laboratory colony). It is unclear from the report if the researchers obtained new, more potent toxin or addressed the issue through other means, although the 2004 results were comparable to the data obtained prior to 2002.

The 2004 PBW report contained the second year of monitoring with the Cry2Ab2 toxin (expressed in Bollgard II cotton). PBW were found to be highly susceptible to the toxin at both of the chosen concentrations: 1.0 µg/ml and 10.0 µg/ml. The 10.0 µg/ml dose appears to be a functional discriminating dose, with 100% mortality to all sampled PBW populations.

It is recommended that in future field monitoring efforts, follow-up susceptibility testing be conducted with PBW larvae recovered from Bt bolls (verified expressing the Bt toxin) if historical infestation averages are exceeded for Bollgard. Past reports have noted that 0.300% infested bolls (3 per 1000) is the average PBW infestation rate for Bt cotton. To date, PBW sampling has not revealed infestations in Bt cotton bolls exceeding this historical rate after non-expressing off types are discounted. Since Bollgard and Bollgard II are considered high dose for PBW, 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. It is also noted that if widespread infestation is observed in Bt fields (i.e. above historical averages), additional action may be necessary, as prescribed by remedial action plans for the registrations. It is also recommended that follow-up testing be conducted on survivors of the 10 µg/ml Cry1Ac and Cry2Ab2 discriminating concentrations. These larvae may also be homozygous for Cry1Ac or Cry2Ab2 resistance alleles and warrant additional scrutiny. On the other hand, survivors of the 1 µg Cry1Ab/ml dose may be heterozygotes with one resistance allele. For the 2004 data, only one larva was observed to survive the discriminating 10 µg/ml Cry1Ac concentration (no larvae survived the Cry2Ab2 discriminating concentration). As such, no follow-up testing was needed for the 2004 monitoring work. However, given that some PBW have survived both test concentrations in in

previous years, it is possible that resistance alleles are relatively common in PBW populations in western cotton growing regions. Considering the high Bt cotton adoption in these regions, it will be imperative to closely monitor PBW and Bt cotton for increases in tolerance to Cry1Ac and possible unexpected field damage.

Table 1. Field-Collected PBW Mortality to Discriminating Concentrations of Cry1Ac from 1997 to 2004 (taken from Dennehy et al. 2002 - 2005)

Year	Average Mortality of Field Collected PBW (%)[1]	
	1.0 µg Cry1Ac/ml dose	10 µg Cry1Ac/ml dose
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

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

Table 2. Field-Collected PBW Mortality to Discriminating Concentrations of Cry2Ab2 from 2003 to 2004 (taken from Dennehy et al. 2004, 2005)

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

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

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Pink Bollworm Monitoring

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