

US EPA ARCHIVE DOCUMENT

November 6, 2002

MEMORANDUM

SUBJECT: Transmittal of Meeting Minutes of the FIFRA Scientific Advisory Panel Meeting
Held August 27-29, 2002

TO: Marcia E. Mulkey, Director
Office of Pesticide Programs

FROM: Paul I. Lewis, Designated Federal Official
FIFRA Scientific Advisory Panel
Office of Science Coordination and Policy

THRU: Larry C. Dorsey, Executive Secretary
FIFRA Scientific Advisory Panel
Office of Science Coordination and Policy

Joseph J. Merenda, Jr., Director
Office of Science Coordination and Policy

Please find attached the meeting minutes of the FIFRA Scientific Advisory Panel open meeting held in Arlington, Virginia from August 27-29, 2002. This report addresses a set of scientific issues being considered by the Environmental Protection Agency regarding corn rootworm plant-incorporated protectant non-target insect and insect resistance management issues.

Attachment

cc:

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SAP Meeting Minutes No. 2002-05

August 27-29, 2002

**FIFRA Scientific Advisory Panel Meeting,
held at the Sheraton Crystal City Hotel, Arlington,
Virginia**

**A Set of Scientific Issues Being Considered by the
Environmental Protection Agency Regarding:**

**Corn Rootworm Plant-incorporated Protectant Non-
target Insect and Insect Resistance Management Issues**

**Part A: Corn Rootworm Plant-incorporated Protectant
Non-target Insect and Insect Resistance Management
Issues:**

Non-target Insect Issues

**Part B: Corn Rootworm Plant-incorporated Protectant
Non-target Insect and Insect Resistance Management
Issues:**

Insect Resistance Management Issues

NOTICE

This report has been written as part of the activities of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Scientific Advisory Panel (SAP). This report has not been reviewed for approval by the United States Environmental Protection Agency (Agency) and, hence, the contents of this report do not necessarily represent the views and policies of the Agency, nor of other agencies in the Executive Branch of the Federal government, nor does mention of trade names or

commercial products constitute a recommendation for use.

The FIFRA SAP was established under the provisions of FIFRA, as amended by the Food Quality Protection Act (FQPA) of 1996, to provide advice, information, and recommendations to the Agency Administrator on pesticides and pesticide-related issues regarding the impact of regulatory actions on health and the environment. The Panel serves as the primary scientific peer review mechanism of the EPA, Office of Pesticide Programs (OPP), and is structured to provide balanced expert assessment of pesticide and pesticide-related matters facing the Agency. Food Quality Protection Act Science Review Board members serve the FIFRA SAP on an ad-hoc basis to assist in reviews conducted by the FIFRA SAP. Further information about FIFRA SAP reports and activities can be obtained from its website at <http://www.epa.gov/scipoly/sap/> or the OPP Docket at (703) 305-5805. Interested persons are invited to contact Larry Dorsey, SAP Executive Secretary, via e-mail at dorsey.larry@epa.gov.

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SAP Meeting Minutes No. 2002-05A

**FIFRA Scientific Advisory Panel Meeting,
August 27, 2002, held at the Sheraton Crystal City Hotel,
Arlington, Virginia**

*A Set of Scientific Issues Being Considered by the
Environmental Protection Agency Regarding:*

**Part A: Corn Rootworm Plant-incorporated
Protectant Non-target Insect and Insect
Resistance Management Issues: Non-target**

Insect Issues

Mr. Paul Lewis
Designated Federal Official
FIFRA Scientific Advisory Panel
Date: 11/6/02

Christopher Portier, Ph.D.
FIFRA SAP Session Chair
FIFRA Scientific Advisory Panel
Date: 11/6/02

**Federal Insecticide, Fungicide, and Rodenticide Act
Scientific Advisory Panel Meeting
August 27, 2002**

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INTRODUCTION

The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Scientific Advisory Panel (SAP) has completed its review of the set of scientific issues being considered by the Agency pertaining to corn rootworm plant-incorporated protectant non-target insect and insect resistance management issues.

Advance notice of the meeting was published in the *Federal Register* on July 24, 2002. The review was conducted in an open Panel meeting held in Arlington, Virginia, on August 27, 2002. The meeting was chaired by Christopher Portier, Ph.D. Mr. Paul Lewis served as the Designated Federal Official.

Janet Andersen, Ph.D. (Office of Pesticide Programs, EPA) opened the session providing an overview of the topics to be discussed. Ms. Robyn Rose (Office of Pesticide

Programs, EPA) provided a review of ecological non-target insect studies for *Bacillus thuringiensis* Cry3Bb1 protein. In preparing these meeting minutes, the Panel carefully considered all information provided and presented by the Agency, as well as information presented by public commenters. These meeting minutes address the information provided and presented at the meeting, especially the response to the charge by the Agency.

PUBLIC COMMENTERS

Oral statements were made by:

Clifford Habig, Ph.D., on behalf of Exponent, Inc.

Jane Rissler, Ph.D., on behalf of the Union of Concerned Scientists

Mr. Robert Maddrey, on behalf of the National Wild Turkey Federation

John Foster, Ph.D., private citizen

Mike McKee, Ph.D., and Graham Head, Ph.D., on behalf of Monsanto Company

Written statements were received by:

Center for Science in the Public Interest

Exponent, Inc.

Monsanto Company

Union of Concerned Scientists

CHARGE

Monsanto Company has applied to EPA for registration of their corn rootworm plant-incorporated protectant (PIP) product. As part of their application, Monsanto has submitted studies on effects of the PIP to non-target invertebrates and soil fate studies. Some of these studies are ones typically required for PIPs and some are unique to this product, which is intended to control a soil rather than foliar insect pest. EPA has evaluated 13 studies as part of its assessment of potential impact on non-target invertebrates and soil fate. These studies, along with EPA's reviews and preliminary risk assessment, have been provided to the Panel members and made available to the public through the Office of Pesticide Programs Public Docket. EPA requests the Scientific Advisory Panel to provide guidance to the Agency on the following questions related to its preliminary risk assessment for non-target invertebrates and soil fate.

Question 1: Single Species Testing vs Field Data Approach

In October 2000, the FIFRA Scientific Advisory Panel recommended that non-target testing be focused on species exposed to the crop being registered. The Agency has determined that the non-target organisms most likely to be exposed to the protein in transgenic corn fields are beneficial insects feeding on corn pollen and soil invertebrates, particularly Coleoptera. In lieu of extensive and difficult single species soil coleopteran toxicity testing followed by an extrapolation from the results to a community risk assessment, direct field data on coleopteran insect effects and abundance were received and evaluated.

A) Please comment on the relative strengths and weaknesses of such field data vs. laboratory feeding studies performed on a limited number indicator organisms, for purposes of hazard assessment.

The Agency believes that a complete census of the invertebrate community would be costly and unlikely to be useful for Bt proteins which are usually target specific groups of invertebrates.

B) The Panel is requested to comment on the logistics, validity, cost and expected scientific gain, if any, of conducting a census of the invertebrate community vs concentrating the studies on specific indicator organisms. In addition, please comment on suggested indicator groups such as Carabids and Staphylinids in the case of Cry3Bb1, that would be most likely to provide the Agency with meaningful data for assessing the potential hazards to non-target invertebrates from corn rootworm PIPs.

Question 2: Duration of Field Abundance Studies

A two-season field invertebrate abundance study indicates that MON 863 corn does not have a negative impact on the abundance of non-target invertebrates. Data also indicated that planting event MON 863 results in less impact on non-target invertebrate than conventional pest management practices.

Please comment on the adequacy of the 2 year field abundance study for making a determination of the potential risks from commercial use of event MON 863.

Question 3: Green Lacewing Larva Test

The Agency accepts data on lacewing larvae fed on a Cry protein-coated moth egg diet. The testing is performed with a concurrent positive control which incorporates arsenate into the moth egg diet. However, there are published comments that this protocol does not expose the larvae to the test substance because the larvae pierce the eggs and feed on the egg fluids, thus not getting exposure to the Cry protein which coats the outside of the eggs. Tritrophic studies using a diet of aphids fed on Bt corn plants have been suggested as a more valid approach. This may not be a solution to the problem, because the lacewing larvae are also said to feed on the aphid body fluids which do not contain the Cry proteins. The Cry proteins are confined to the digestive tract of the aphid.

The Agency solicits the Panel=s comments on an appropriate design for evaluating the toxicity of Cry3Bb1 proteins to lacewing larvae.

Question 4: Soil Degradation/Accumulation of Cry3Bb1

The reviewed data indicate that Cry3Bb1 protein in plant tissue degrades rapidly in sandy loam soil. However, corn is not necessarily grown in sandy loam soil in all regions. Corn is grown in other soil types such as clay loam and silt loam soils in various regions of the U.S. Cry protein has also been shown to bind to clay soils. Therefore, it may be desirable that soil

degradation and persistence studies be conducted in other common agricultural soils, perhaps for 3 years.

A) The Panel is requested to comment on the advisability of testing additional soil types and for having soil persistence studies for up to 3 years.

B) What soil types would need to be tested and what duration is needed for soil persistence studies?

The soil fate studies submitted to EPA describe DT₅₀ (time to 50% degradation of the Bt protein in soil) and DT₉₀ (time to 90% degradation of the Bt protein in soil) for Cry3Bb1 protein in sandy loam soil based on ELISA test are 2.76 and 9.16 days. However, the value of these results are not necessarily correlated with activity in insect guts because it is unknown if the extractable protein in the ELISA test was functional or non-functional. The DT₅₀ and DT₉₀ determined by insect bioassays with CPB were 2.37 and 7.87 days respectively.

C) Are these studies truly expressing the time to 50% or 90% degradation of Bt protein in the soil or whether they are only determining the level of detection of Cry3Bb1 protein in the soil. Discuss the acceptability of these studies for a preliminary risk assessment to evaluate the fate of Cry3Bb1 in soil.

D) What if any difference would it make in the values of these ELISA based studies if clay particles to which the Cry3Bb1 protein might bind are present in the soil being tested? What measures should be taken to ensure that the test is not measuring inactive protein fragments?

Question 5: Preliminary Risk Assessment for Non-target Invertebrates and Soil Fate

The Agency's preliminary risk assessment based on single species laboratory toxicity studies on adult and larval lady beetles, green lacewing larvae, a parasitic hymenopteran, adult and larval honey bees, Collembola, earthworm, the monarch butterfly, field invertebrate census evaluations, and a soil persistence study indicates no unreasonable adverse effects on the invertebrate fauna of the corn field.

Please comment on the Agency's non-target invertebrate and soil fate assessment.

PANEL RECOMMENDATIONS

- The Panel did not agree with the Agency that single species testing was necessarily difficult, or that field-based testing can be seen as a substitute for tier one laboratory tests. The Panel also noted that laboratory-obtained test data may not provide a basis for extrapolation to a community risk assessment, but that they are best viewed as the initial stage of a risk assessment procedure that determines the possibility of harm occurring within representative taxonomic groups. The Panel concluded that a complete

census of invertebrates was not feasible, given limitations of sampling methodologies and realistic expectations for taxonomic resolution of invertebrates.

- The Panel found that it may be likely, but cannot be assured, that future data would suggest that MON 863 would not have a negative impact on non-target invertebrates. However, the Panel did not support the Agency's statement that "MON 863 results in less impact on non-target invertebrates than conventional pest management practices." The Panel found that a two-year field study would not be sufficient to reach a decision on whether MON 863 would have a negative impact on the abundance of non-target invertebrates. Most of the Panel thought it was important to ensure that rigorous studies be carried out under operational field conditions over a period of at least three to four years to determine the impact, or lack thereof, of transgenic crops on non-target organisms. However, the Panel is aware that the state-of-the-science to conduct such long-term studies needs to improve in order for the research to be conducted successfully with meaningful results.
- The Panel concluded that the protocol used to test the impact of the Cry3Bb1 protein on *Chrysoperla carnea* was inadequate and/or inappropriate. Further, most Panel members recommended that a better subject for this test would be *Orius insidiosus*. A series of recommendations were made by several Panel members regarding acceptable standards for the design and conduct of laboratory studies.
- The Panel concluded that there is a need for the use of several different soils in the study of persistence since this parameter is likely to be the least in the sandy loam soil studied. While it is difficult to select an arbitrary length of time (e.g. 3 years) for soil persistence studies, the duration should be significantly long enough to provide a meaningful assessment of the protein's degradation and persistence.

DETAILED RESPONSE TO THE AGENCY'S CHARGE

The specific issues to be addressed by the Panel are keyed to the Agency's background document, dated July 26, 2002, and are presented as follows:

Monsanto Company has applied to EPA for registration of their corn rootworm plant-incorporated protectant (PIP) product. As part of their application, Monsanto has submitted studies on effects of the PIP to non-target invertebrates and soil fate studies. Some of these studies are ones typically required for PIPs and some are unique to this product which is intended to control a soil rather than foliar insect pest. EPA has evaluated 13 studies as part of its assessment of potential impact on non-target invertebrates and soil fate. These studies, along with EPA's reviews and preliminary risk assessment, have been provided to the Panel members and made available to the public through the Office of Pesticide Programs Public Docket. EPA requests the Scientific Advisory Panel to provide guidance to the Agency on the following questions related to its preliminary risk assessment for non-target invertebrates and soil fate.

Question 1: Single Species Testing vs Field Data Approach

In October 2000, the FIFRA Scientific Advisory Panel recommended that non-target testing be focused on species exposed to the crop being registered. The Agency has determined that the non-target organisms most likely to be exposed to the protein in transgenic corn fields are beneficial insects feeding on corn pollen and soil invertebrates, particularly Coleoptera. In lieu of extensive and difficult single species soil coleopteran toxicity testing followed by an extrapolation from the results to a community risk assessment, direct field data on coleopteran insect effects and abundance were received and evaluated.

- A) Please comment on the relative strengths and weaknesses of such field data vs. laboratory feeding studies performed on a limited number indicator organisms, for purposes of hazard assessment.

Question 1A:

The Panel first addressed assertions by the EPA in the preamble concerning the charge for Question 1, wherein the Agency stated that *'in lieu of extensive and difficult single species soil coleopteran toxicity testing followed by an extrapolation from the results to a community risk assessment, direct field data on coleopteran insect effects and abundance were received and evaluated'*. The Panel did not agree with the Agency that single species testing was necessarily difficult, or that field-based testing can be seen as a substitute for tier one laboratory tests. This supports comments on non-target organism data requirements in SAP Report No. 99-06 (February 4th, 2000), where it was stated that *'field scouting is an important tool to risk assessment, but should not replace Tier 1 testing'*.

The Panel noted that laboratory test methods for non-target invertebrates are widely available, and had been reviewed previously at an EPA meeting in 1992, prior to the initial registration of plant-incorporated protectants (PIPs) (e.g. Jepson *et al.*, 1994). Additionally, GLP-compatible, and readily adaptable laboratory test protocols for non-target Coleoptera are used by industry to meet regulatory requirements for conventional pesticides in the EU and other regulatory jurisdictions (Jepson, 1993; Barrett, 1992; Barrett *et al.*, 1994). For example, an adult *Poeicillus* (= *Pterostichus*) *cupreus* (Coleoptera: Carabidae) standardized laboratory test method is used for regulatory testing in the EU (Heimbach, 1992; Candolfi *et al.*, 2000), and a larval *P. cupreus* test method is currently being tested by the internationally respected IOBC/WPRS Working Group 'Pesticides and Beneficial Invertebrates' (IOBC Profile, 2002), including method development for seed applied and granular pesticides. GLP-compatible test protocols are also available for other coleopteran families, including Staphylinidae (e.g. *Philonthus cognatus* (Coleoptera: Staphylinidae); Metge & Heimbach, 1998).

The Panel also noted that laboratory-obtained test data may not provide a basis for extrapolation to a community risk assessment, but that they are best viewed as the initial stage of a risk assessment procedure that determines the possibility of harm occurring within

representative taxonomic groups. Where no potential for harm is detected, further testing may be deemed unnecessary. However, where harm is detected, a further stage of testing may be recommended, rather than an extrapolation exercise or recommendation of a full field inventory .

In its charge to the Panel to comment on the relative strengths and weaknesses of field data versus laboratory feeding studies on a limited number of species, the Agency asserted that it believed that a complete census of the invertebrate community would be costly and unlikely to be useful for *Bt* proteins, which are target specific. The Panel also concluded that a complete census of invertebrates was not feasible, given limitations of sampling methodologies and realistic expectations for taxonomic resolution of invertebrates in the U.S. Furthermore, it also concluded that the extremes of field inventory versus laboratory-based tests under current guidelines did not constitute appropriate alternatives, and that extended laboratory, semi-field methods plus more focused and targeted field studies would add substantially to the scientific quality of the data input to risk assessment if they were applied properly.

Strengths and weaknesses of laboratory-derived data: Laboratory testing can be conducted efficiently which allows for timely decision-making on economically important problems. The conditions of tests and administration of the test material doses are controlled much more rigorously than in the field. Further, the health of test organisms can be controlled and measured. Laboratory approaches eliminate most, if not all, potentially confounding factors, which is not possible in field surveys. Laboratory data provide information on cause and effect relationships. Field surveys are not controlled experimental evaluations and, thus, only provide correlational relationships. Although the quality of test organisms may be high, the organisms may not represent a field genotype, but rather one produced as a result of rearing selection in lab conditions and may lack genetic variation found in field individuals. It is unclear however, that a consistent prediction can be made concerning the influence of these factors (if they exist) on test results.

Laboratory evaluations are extraordinarily narrow in their focus and by their nature, unrealistic. For example, the level and route of exposure to the administered material compared with the field is often uncertain. Further, assessments of mortality are made after only partial exposure of organisms, not lifetime exposures as might occur in the field. Organisms in the field are also subject to supplementary stresses that have additive effects, including the physical influences of sub-optimal temperature and humidity, and starvation and parasitism, that amplify impacts that occur under the optimal physical and biological conditions of laboratory tests. Many other variables (both physiological and behavioral) that influence fitness can theoretically be measured in laboratory testing. They are rarely considered, however, and the only response variable for almost all tests is mortality. Finally, laboratory tests may evaluate an appropriate category of organism, yet fail to evaluate an appropriate species. For example, although it is clearly important to evaluate the effects of new control modality against insect parasitoids, it is less than ideal to use a species that has little or no relevance to the agroecosystem in which pest and crop are found.

In the regulatory process, tier one laboratory testing data are intended to determine the potential for harm arising under conditions of high dose exposure. They are used within the

regulatory process to screen out those materials that pose very limited risks to test organisms and the taxa that they represent. It is widely accepted that they cannot be used to determine the likely level of harm that will arise in the field if effects are detected, for the reasons listed above. However, tier one tests can be used as a trigger for further testing, to resolve whether or not an impact detected in a high exposure bioassay could occur in more realistic conditions. It is important to note that a targeted field experiment may also trigger a subsequent controlled laboratory study. The Panel did not agree with the Agency that a full field census was the appropriate next stage in the testing regime, following tier one laboratory testing. Panel members cited widely used intermediate testing methods, including extended laboratory tests (use of more realistic substrates and exposure pathways within the laboratory) or semi-field tests (confinement of individual or multiple species of test organisms within microcosms, mesocosms, field cages or barriered arenas). There is extensive literature on these methods for conventional pesticides and the risk assessment regimes within which they fit (Jepson, 1993; IOBC Working Group 'Pesticides and Beneficial Invertebrates' publications (<http://www.iobc.wprs.org/pubs/>)). Government and university researchers, the chemical industry and regulators have all been involved in test method development (e.g. Barrett, 1992; Campbell et al., 2000). In addition, international working groups and professional scientific societies have convened a number of meetings to refine test methodology (Barrett *et al.*, 2000; Candolfi *et al.*, 2001). Although these test methods have been developed for conventional pesticides, the Panel believes that they can be readily adapted for PIPs, because they are already regularly adapted for seed-delivered or granular pesticides where dietary exposures are often incorporated into tests as deviations of Standard Operating Procedures within GLP guidelines. This is consistent with the recommendations concerning non-target insect testing within the SAP Report No. 99-06 (February 4th, 2000).

Do the submitted laboratory tests exhibit these strengths? The Panel noted that with respect to laboratory testing, the consensus of the Panel reporting in SAP Report No. 99-06 (February 4th, 2000) was that the Agency should provide applicants with detailed recommendations regarding experimental design, criteria for the desired level of detection of the experiment, data analysis, and how the Agency would consider such data in order to establish an acceptable level of statistical power. The Panel recommended that guidelines for experimental design should be developed to provide registrants with clear guidance concerning the minimum standard required for test data to be deemed acceptable. In addition to the areas focused upon in the SAP Report 99-06, several Panel members also recommended that the Agency consider the following:

- (1) Verification of exposure levels of test organisms to proteins throughout the bioassay.
- (2) Detailed quantification of expected environmental concentrations of the protein in the field.
- (3) Stated endpoints attained within the test.
- (4) A clear statement that tests which fail to reach the designated endpoint are not eligible for consideration.

- (5) Consistency in the way in which control treatment mortality data are evaluated.
- (6) Foods used by test species in their relevant habitat should be used in laboratory tests.
- (7) Quantitative analysis of field expression levels to determine appropriate laboratory exposure levels.
- (8) Verification that the food offered to the species actually contained the administered material, at the intended dose, throughout the investigation.
- (9) Verification that all life stages of the species are exposed appropriately to the transgene product (i.e. actually contact the toxin in relevant ways).
- (10) Use of intact plants or plant parts in the experimental system and verify that the chosen plant parts contain the transgene product.
- (11) Have a proper scientific control.
- (12) Have sufficient replication and numbers of insects screened based on statistical power and desired criteria.

Most Panel members noted flaws (some serious) in the submitted test data to address the Panel recommendations as noted above. The levels of exposure of *Chrysoperla* and *Nasonia* to active protein were not, for example, determined throughout their respective tests. The test protein was held for a week within a diet broth in the *Chrysoperla* test chamber, and could have degraded considerably. The procedures for validating the concentration and bioactivity of test protein reported in the *Apis* adult test contrasts greatly with the procedures used in the *Chrysoperla* and *Nasonia* studies. These Panel members noted the paucity of data concerning protein expression levels in the field, and were concerned that this did not provide an adequate basis for determining maximum hazard doses. With respect to the use of control mortality to trigger cessation of the test, Panel members noted inconsistency in the registrant's submitted test reports. For example, the *Chrysoperla* test was stopped before the designated endpoint of pupation was reached because control mortality exceeded 20% mortality at 10 days, whereas the *Apis* adult test continued beyond 20% control mortality for a more comprehensive comparison between treatment and control.

Strengths and weaknesses of field-derived data: Field data can provide a measure of ecological impact if the design of the experiment is appropriate. Field tests can, in theory, be used as the ultimate approach to determine whether a specific hazard that has become apparent from laboratory or intermediate tests can arise under realistic conditions similar to those under commercial implementation of the technology. Field data can be used to measure not only the level, but also the duration of perturbations caused by the tested material. However, broad field surveys do not test cause and effect relationships. Decisions based on types of organisms and particular species and/or appropriate types of tests based on protocols for the evaluation of other "older" chemical pesticide modalities, may not be relevant to the "new" type of approach

that is represented by PIPs. For example, many conventional pesticides are non-persistent and exhibit acute and often broad-spectrum effects, which are much easier to evaluate in replicated experiments than materials, including PIPs, that have potentially more subtle impacts on fewer taxa. Although trials in many locations provide useful information, our lack of understanding of how locales differ among each other makes interpretation of results difficult. Finally, the sheer volume of data, lack of taxonomic support, costs in generating basic data, and numbers of years required to conduct adequate field trials may prove prohibitive.

The Panel also noted that although field data can be used to establish the existence of a hazard, these data are often ineffective for establishing the absence of hazard. One reason is because field experiments often have large sources of environmental variance, which can obscure differences caused by a particular toxic effect. Depending on the magnitude of variation within and among experimental units, a typical field experiment may need an unrealistic number of replications (about 100) to detect a desired 10-20% response to a treatment. Thus, small effects will normally be undetectable using field experiments. A second reason is that the abundance of species is often too small to be able to measure accurately, let alone estimate the differences between two treatments. Third, if the plot size is too small, differences among treatments might be masked by movement of arthropods among the plots. Fourth, the sampling effort may be insufficient to estimate plot means precisely enough to allow the determination of statistically significant differences, especially for soil sampling. Finally, if the field layout is in the form of a split plot design, there is in general greater statistical power associated with the subplot treatments than the whole plot treatments. Thus, a split-plot experiment with +/- *Bt* as the main plots and +/- insecticides as the subplots, generally will be able to detect smaller insecticide effects than *B.t.* effects.

Sampling regime, scale and layout must be adjusted accordingly to accommodate the repeated expression and season-long persistence of PIPs and the target specific nature of the toxin. The Panel identified several essential requirements for field tests to provide a rigorous test of the technology under review:

- (1) Evaluation of sites from a number of candidates, possibly in the previous season, to determine whether the organisms of concern are present and sufficiently abundant, to provide a basis for statistical discrimination of small but significant effects.
- (2) Use of sampling methods of known efficiency and precision with consideration of within-plot variability when determining intensity and frequency of sampling.
- (3) A scale and layout of the experiment that minimizes the risks of edge effects and reinvasion from untreated control plots, and which takes into account the dispersal rates and phenologies of the organisms of concern.

Scale is of considerable importance for evaluations of impacts upon Carabidae. Carabidae have been found to disperse between experimental plots (Jepson and Thacker, 1990; Duffield and Aebischer, 1994), and the form of pesticide impacts against Carabidae have been found to be scale-dependent (Sherratt and Jepson, 1993; Jepson, 2002). In addition, the

dynamics of the prey species of Carabidae, (including Collembola) within experimental plots is also scale dependent (Duffield *et al.*, 1996) and small scale experimental plots exhibit eccentric and unrealistic invertebrate population dynamics that do not apply to the dynamics that occur in the whole fields characteristic of commercial agriculture. Thus, the transitory effects of pesticides noted for within-field experimental designs can amplify to local extirpation when treatments are applied to whole fields (Burn, 1992). These limitations do not necessarily imply that all experiments should be conducted with treatments assigned to whole fields, but they do limit the ability of tests within field to discriminate small but significant effects, and the degree to which test data can be used in risk assessment procedures.

Census of invertebrate communities in the field can reveal potential hazards, but this approach answers different questions than focused laboratory experiments for establishing potential hazards. A census could give a false positive if the statistical methods do not protect for Type I experimental errors. However, any effect detected in such a field experiment should probably be confirmed in a follow-up experiment.

The Agency believes that a complete census of the invertebrate community would be costly and unlikely to be useful for Bt proteins which are usually target specific groups of invertebrates.

A) The Panel is requested to comment on the logistics, validity, cost and expected scientific gain, if any, of conducting a census of the invertebrate community vs concentrating the studies on specific indicator organisms. In addition, please comment on suggested indicator groups such as Carabids and Staphylinids in the case of Cry3Bb1, that would be most likely to provide the Agency with meaningful data for assessing the potential hazards to non-target invertebrates from corn rootworm PIPs.

The Panel was asked to comment on the logistics, validity, cost and expected scientific gain, if any, of conducting a census of the invertebrate community versus concentrating the studies on specific indicator organisms. They were also asked to comment on suggested indicator groups such as Carabidae and Staphylinidae that would be most likely to provide the Agency with meaningful data for assessing the potential hazards to non-target invertebrates from corn rootworm PIPs.

In addition to the responses to question 1A, which are of direct relevance to question 1B, the Panel discussed species selection as a key component in the development of laboratory-based testing and field screening programs. Non-target invertebrates can be subdivided among a number of functional groups (Table 1) and the Panel recommended that a more comprehensive analysis of potential test taxa be developed for the designation of appropriate organisms for testing and evaluation (see also table in Jepson *et al.*, 1994).

Table 1. Example of a functional classification for terrestrial non-target organisms, in or near agricultural systems, for pre-release testing of transgenic plants.

| Functional Group | Examples |
|-------------------------------------|--|
| Anthropocentric Functions | |
| Secondary pests | -Sporadic pests, induced pests |
| Natural enemies | -Predators, parasitoids, parasites, competitors, ants, and weed-eating herbivores |
| Rare or endangered species | -Red list species or species of value for biodiversity conservation |
| Species that generate income | -Honey bees, silk moths |
| Species of social or cultural value | -Monarch butterflies or honey bees |
| Ecological Functional Groups | |
| Non-target herbivores | -Plant eating species that are not the target of the transgene |
| Secondary consumers | -Species that eat herbivores; predators, parasitoids, parasites |
| Pollinators | -Bees, selected Diptera (e.g. Syrphidae) and Coleoptera, etc. |
| Decomposers | -Scavengers, ants, Collembola, micro- organisms, earthworms, mites, nematodes. |
| Seed dispersers | -Birds, small mammals, ants |

The Panel agreed with the Agency that non-target Coleoptera should form a part of the risk assessment for the PIP under evaluation. Carabidae and Staphylinidae are diverse beetle families, fulfilling important ecological and economic roles within agroecosystems. Long-term monitoring of carabids and staphylinids in European agroecosystems had shown a clear negative relationship between the diversity and abundance of these polyphagous predators and pest population densities. Long-term monitoring data are lacking from the U.S. but the Panel believes there is little doubt that these organisms contribute to pest limitation and to ecological processes. The Panel noted international efforts to develop standardized testing methods against representative Carabidae and Staphylinidae cited in the response to question 1A. The benefit of using these standardized test organisms lies in the detailed development process for the test that ensures reliability, repeatability and cost effectiveness. There is also, however, a case to be made for developing tests that are specific to particular crops and regions that expose species in the areas where the crop is to be grown. This is particularly relevant to crops such as corn, which is grown on a large geographic scale. The use of relevant taxa in tier one is consistent with the recommendations of SAP Report No. 99-06 (February 4th, 2000).

Relevant Carabidae that could be screened against the PIP under review include: *Bembidion quadrimaculatum* (numerically abundant in corn fields, primarily predaceous, and probably exhibiting a high reproductive rate), *Pterostichus melanarius* (large species, primarily predaceous, abundant and primarily predaceous in corn) and *Amara* sp. or one of the smaller *Pterostichus* spp. (these are medium sized species that are omnivorous and could feed on decomposing vegetation, such as corn residue). One staphylinid that could be screened is *Stenus flavicornis*.

Other Coleoptera can play important roles within agroecosystems, and the Panel noted that Chrysomelidae may play a role in weed suppression (some species are used as weed biological control agents), and also act as food for farmland birds.

The Panel reviewed the respective roles of laboratory-based tests and field census methods in its answers to question 1A and question 2, but again drew attention to the fact that these represent extremes from a spectrum of test methods that also include extended laboratory and semi-field procedures. The Panel consensus was that these intermediate tests may offer logistical, economic and scientific benefits, and that greater scientific validity would result from increased control within semi-field tests, combined with greater realism, particularly where organisms are caged within experimental plots of the crop under evaluation. Cage and barrier methods can also be employed within existing field census studies to facilitate more mechanistic analyses of effects that emerge from field sampling. The Panel noted that this approach can be used to compensate for unrealistically small plot sizes.

Finally, the Panel noted a wider role for appropriately scaled and designed field census studies and also for monitoring on a larger scale. These investigations offer the prospect of revealing indirect effects, and also the benefits of PIPs, particularly in areas that are released from the broad spectrum suppressive effects of conventional pesticides to non-target invertebrates.

Question 2: Duration of Field Abundance Studies

A two-season field invertebrate abundance study indicates that MON 863 corn does not have a negative impact on the abundance of non-target invertebrates. Data also indicated that planting event MON 863 results in less impact on non-target invertebrate than conventional pest management practices. Please comment on the adequacy of the 2 year field abundance study for making a determination of the potential risks from commercial use of event MON 863.

Before commenting specifically on the field abundance study (455382-06), the Panel made the following points about the statements in the Agency's opening paragraph that precedes the question. First, the data provided to the Panel consisted only of a one-year interim report for the year 2000 and not a two year study. Second, although the Panel found that it may be likely, but cannot be assured, that future data would suggest that MON 863 would not have a negative impact on non-target invertebrates, the Panel did not support the Agency's statement that "MON 863 results in less impact on non-target invertebrates than conventional pest management practices." In fact, in the Conclusions section of its report, Monsanto states that "The data indicate that the prominent beneficial non-target invertebrates such as soil dwelling Araneae (spiders) and Carabidae (ground beetle) and foliage-dwelling beneficial insects like *C. maculata*, *O. insidiosus* and *M. grandis* were equally abundant in the test and control plots."

From both theoretical and practical perspectives, the Panel found that a two-year field

study would be insufficient to reach a decision on whether MON 863 would have a negative impact on the abundance of non-target invertebrates (as noted in the Panel's response to question 1). The principal reason for this is that there are natural annual variations in invertebrate populations making it difficult to draw conclusions about non-target effects based on relatively short-term studies. Most Panel members thought that studies of the impact of *Bt* crops on non-target abundance would generally take from three to four years, and require large-scale field trials. Results of studies such as those submitted by Monsanto, which used relatively small plots with limited replication, would still only be considered preliminary, even if conducted for three or four years. PIPs represent a new class of pest control products that have raised questions concerning their potential negative effects on non-target organisms such as monarch butterfly and chrysopids. Thus, most of the Panel thought it was important to ensure that rigorous studies be conducted under operational field conditions over a period of at least three to four years to determine the impact, or lack thereof, of transgenic crops on non-target organisms. This goal is rather different from the ecological risk assessment goal addressed in the Panel response to question 1, and related more to the general ecological attributes of transgenic plants, rather to any particular risk. Overall, the Panel concluded that the state-of-the-science required to conduct such long-term studies with these broader goals needs to improve if the research to be conducted successfully and with meaningful results.

The high specificity of Cry3Bb1 in MON 863 makes it possible that long-term field abundance studies will benefit non-target populations, in comparison with broad spectrum synthetic chemical insecticides, which are used extensively in many regions of the Corn Belt. But to develop data that show the effects of crops like MON863 on non-target organisms, it is clear that the types of studies being performed in the field can be improved, especially with respect to statistical power (avoiding Type II experimental error). Toward this end, several Panel members recommended that the following methodological improvements be considered in conducting studies of the effects of transgenic crops such as MON 863 on the abundance of non-target invertebrates, if field testing is required in the future:

- (1) Add additional +Bt and -Bt hybrids with a clear statement of the number of back cross generations that separate MON 863 hybrids from RX670.
- (2) Attempt first to identify non-targets invertebrates that might be at risk of toxicological impacts through laboratory studies that focus on representative species. For example, with MON 863, suitable candidates would be Carabidae, Staphylinidae and Coccinellidae, in addition to the standard test taxa.
- (3) Undertake barrier/cage studies in the field as an intermediate choice between laboratory and full-scale field studies where effects are detected in the tier one tests.
- (4) Include a highly toxic, gut-active insecticide to serve as a positive control (in the specific case of the MON 863 study, this would be used instead of tefluthrin).
- (5) Better synchronization of sampling by increasing the sampling, but shortening the sampling time. For example, samples should be taken on the day prior to the application of

a foliar insecticide, and then for several days immediately after, for example, on days one, three, five, and seven. However, the sampling period should be only one day as opposed to three days to prevent over-trapping.

- (6) In studies of epigeal fauna, the alleyways between plots should be seeded with dense vegetation to reduce inter-plot movement.
- (7) Maintain alleyways of at least 20 feet between all plots (not just between replicates).
- (8) Edge effects should be minimized by using the same variety as in the plots.
- (9) Eliminate root ball samples, or increase the number per plot to about 10.
- (10) Increase pitfall traps to at least 10 per plot and concentrate these toward the center of the plot. Confirm the precision of the population estimates so obtained.
- (11) Consider adding whole plant visual samples (> 50 per plot).
- (12) Eliminate the drop cloth method. This is a good preliminary method but less suitable for quantitative analysis.
- (13) Analyze and interpret data only for those species that are sufficiently abundant that sampling precision is much less than mean density.

In addition to these specific recommendations, the principles and methods discussed in response to Question 1 should be used as a background for designing field studies aimed at determining the effects of transgenic crops on non-target abundance.

Finally, whereas the Panel agreed that the MON 863 study (455382-06) on the abundance of non-target organisms was of limited utility, a few Panel members concluded that it should not inhibit the ability of the Agency to complete a risk assessment. These Panel members noted that despite any inadequacies in the data submitted by Monsanto, that a considerable body of data exists and is being published in the refereed literature that Cry proteins appear to have minimal, if any, negative impact on non-target organisms under field conditions. There is no evidence that crops producing Cry proteins have a significant negative impact on non-target organisms, and the data available indicate that they are unlikely to have greater environmental impacts than synthetic chemical insecticides. The rationale behind this position is that (1) MON 863 is much more specific in its target spectrum than synthetic chemical insecticides, and thus could have no effect or may actually benefit (i.e. higher survival and species abundance) non-target populations and (2) published and on-going large-scale studies of Cry1Ac cotton indicate few or no significant effects on non-target abundance, especially when compared to plots in which conventional chemical insecticides are used. Thus, while longer-term studies are needed, emerging results suggest that most non-target populations are at low risk from exposure to PIPs that produce Cry proteins.

Question 3: Green Lacewing Larva Test

The Agency accepts data on lacewing larvae fed on a Cry protein-coated moth egg diet. The testing is performed with a concurrent positive control which incorporates arsenate into the moth egg diet. However, there are published comments that this protocol does not expose the larvae to the test substance because the larvae pierce the eggs and feed on the egg fluids, thus not getting exposure to the Cry protein which coats the outside of the eggs. Tritrophic studies using a diet of aphids fed on Bt corn plants have been suggested as more valid approach. This may not be a solution to the problem, because the lacewing larvae are also said to feed on the aphid body fluids which do not contain the Cry proteins. The Cry proteins are confined to the digestive tract of the aphid.

The Agency solicits the Panel's comments on an appropriate design for evaluating the toxicity of Cry3Bb1 proteins to lacewing larvae.

The Agency has expressed concern that the impact of the Cry3Bb1 protein on *Chrysoperla carnea* was not properly determined in the tests submitted. The Panel concluded that the protocol used to test the impact of the Cry3Bb1 protein on *Chrysoperla carnea* was inadequate and/or inappropriate. Further, most Panel members recommended that a better subject for this test would be *Orius insidiosus*. Panel members provided details of flaws in the current protocol that can be addressed in a revised experimental protocol and other experimental options that could be incorporated into a new protocol for the evaluation of Cry3Bb1 protein on natural enemies.

Comments on Experimental Design

Chrysoperla were presented either with eggs suspended in water or eggs suspended in water with the Cry3Bb1 protein added. The primary response variables were mortality and pupation. Based on the current experimental design, the actual availability of the Cry3Bb1 in appropriate doses is uncertain. First, the Cry3Bb1 protein likely adsorbs to the egg so that larvae actually contact only a small fraction of the protein added, while arsenic (the positive control) is consumed readily because it may stay in solution. In addition, there is a strong possibility that the protein in the solution given to the lacewing may degrade through time. Thus, an additional issue which was not addressed by the experimental design was the documentation of the persistence, at appropriate doses, of the Cry3Bb1 protein. Even if persistence of the protein at appropriate doses had been established, Panel members concluded it is unclear whether the MON 859 transgene product is an adequate mimic of the MON 863 transgene product to allow it to represent (or serve as a surrogate) for non-target hazard identification.

An additional concern is the lack of an appropriate control in the lacewing experiments. The text in the document states that the controls consisted of water prepared by reverse osmosis. Clearly the appropriate control would have been a moth egg (*Sitotroga* sp.) and water meal diet without Cry3Bb1 protein. Further, replication number and sample size in the experiments are inadequate. There was only one replication of the experiment (with 30 larvae

per treatment). Associated concerns are that eggs appear to have been from a single generation (or single source), the *Bt* toxin was from a single extraction, and the larvae were apparently kept in the same environmental chamber.

Another element of concern is the duration of the test given the response variables that were to be measured. It is impossible to measure pupation if the criterion for termination in the experiment is clearly a set level of mortality in the control that precludes the determination of pupation. An issue that is not addressed by the experimental protocol is the impact of pollen (*Bt* vs. non-*Bt*) on *Chrysoperla*. Although *C. carnea* apparently does not consume much pollen, the availability of pollen in the field and a protocol to test for pollen effects suggest that it might be advisable to evaluate the potential impact of transgenic pollen.

Based on these analyses, the Panel concluded that the study is inadequate to indicate the lack of an effect of Cry3Bb on chrysopid larvae or that the NOEC is less than the MEEC. However, one Panel member suggested that the experiment might be irrelevant because chrysopid larval exposure to pollen is negligible.

Alternative Approaches

Based on deficiencies with the study, Panel members proposed several alternative options with regard to the experimental protocol. First, the ability to detect and determine the nature of the effects of the Cry3Bb1 protein would be accomplished more effectively using a synthetic diet for lacewings. There are chemically defined diets available for *Chrysoperla carnea*. Hasegawa *et al.*, (1989) reported rearing larvae to the adult stage on four diets, and other diets have been used earlier by other researchers. Adults of *Chrysoperla* were reared on one of Hasegawa *et al.* diets that were capable of producing more than 1000 eggs in 2 months. Thus, with Hasegawa *et al.*-s, or other diets that had been reported in the literature, testing different concentrations of the Cry3Bb1 protein would be straightforward and more rigorous than the protocol which had been used. Further, these diets and test compounds can be delivered using several approaches developed by researchers working with egg parasitoid and predators. Various methods have been used to create artificial eggs that may be suitable for incorporation of diet with and without Cry3Bb1 protein for toxicity evaluations. Thus, one can create surrogate wax eggs, including paraxylene egg shells and egg cards (heat-sealed polyethylene membranes) (Nettles *et al.* 1983; Voegelé *et al.* 1988; Grenier, 1994). Techniques such as the use of artificial eggs have been used for *Chrysoperla* since the 1960's (Hagen and Tassan. 1965) and continue to be used today (Hilbeck *et al.* 1998).

Lastly, the choice of *Chrysoperla carnea* is perhaps not the most appropriate natural enemy to have selected for these tests. Other natural enemies are much more important in corn, such as *Orius insidiosus*. Not only is this species important and known to feed on pollen, but first instars usually feed exclusively on plant tissue (enhancing the potential for contact with the Cry3Bb1 protein). Thus, an evaluation of the impact of Cry3Bb1 on a non-target natural enemy of a corn agroecosystem may be better served by a focus on *Orius insidiosus*.

Question 4: Soil Degradation/Accumulation of Cry3Bb1

The reviewed data indicate that Cry3Bb1 protein in plant tissue degrades rapidly in sandy loam soil. However, corn is not necessarily grown in sandy loam soil in all regions. Corn is grown in other soil types such as clay loam and silt loam soils in various regions of the U.S. Cry protein has also been shown to bind to clay soils. Therefore, it may be desirable that soil degradation and persistence studies be conducted in other common agricultural soils, perhaps for 3 years.

A) The Panel is requested to comment on the advisability of testing additional soil types and for having soil persistence studies for up to 3 years.

The Panel concluded that there is a need for the use of several different soils in the study of persistence since this parameter is likely to be the least in the sandy loam soil studied. While not intentional, the registrant's use of a sandy loam soil maintained under optimum conditions for degradation would most likely show relatively fast rates of degradation.

There was general agreement that monitoring for persistence of the Cry3Bb1 protein for a full three years was excessive, especially given the fact that the protein appears to degrade within a matter of days to weeks. This conclusion is supported by the fact that the vast majority of all proteins degrade in soil within a matter of days or a few weeks, not months or years. There is nothing unique about the Cry3Bb1 protein that would cause extended persistence in soil. However, given the possibility of longer persistence of single repeated doses throughout a growing season, the duration of degradation tests should be increased. While it is difficult to select an arbitrary length of time (e.g., 3 years) for soil persistence studies, the duration should be sufficiently long enough to provide a meaningful assessment of the protein's degradation and persistence. The Panel recommended that persistence be monitored for a minimum of one growing season after harvest and until such time that the protein can no longer be detected. Generally, it is also important to continue monitoring for one or two additional sampling times to assure that the first lack of detection was not simply an analytical error.

B) What soil types would need to be tested and what duration is needed for soil persistence studies?

The Agency's concern with different textural classes is well placed. However, published evidence demonstrates the importance of the type of clay in protein decomposition (e.g., expanding vs. nonexpanding-lattice clays). Organic matter (not humic acid per se) is also important because it too may render proteins less available for biodegradation. Consideration, therefore, needs to be given to the type and abundance of clay type and percentage organic matter because they pertain to the soil types important in the major corn-growing regions.

At a minimum, it is recommended that two additional soils be examined for persistence. In particular, soils with higher organic matter content and soils with a higher concentration of expanding clay should be evaluated. Expanding clays can potentially entrap proteins inside their lattice, thus extending persistence. However, the Panel would prefer the use of a number of dissimilar soils that would show the full range of persistence.

In addition to alternative soils, it is desirable to examine persistence under less than optimum conditions. This might include low or high temperatures or low or high soil moisture content. Incubation conditions should be guided by the prevailing conditions of those areas where the corn will be cultivated. Furthermore, attention should also be given to the possibility of sequestration. Although no data exist confirming that proteins become sequestered (or “aged”), there is *a priori* basis to suggest that sequestration does occur. Sequestration would increase persistence, but it would also decrease the bioavailability of the residual compound.

Because corn roots extend to deep sites in soil and those sites typically have low microbial activity and possibly lesser rates of biodegradation, the rates of *Bt* protein disappearance from deep sites should be examined. The rapid initial biodegradation suggests little or no toxicity to microorganisms, although this does not necessarily apply to protozoa and nematodes.

Further, some Panel members were concerned with the method used for addition of the protein to soil. Leaf, not root tissue, was used as the source of the *Bt* protein. The degradation of leaf tissue may be slower than root material. Also, ground tissue was used, not intact plant material. Grinding artificially increases the surface area exposed to microorganisms and thus, increases the biodegradation rate. For future studies, it is recommended that plant material be used that more closely represents that which might be incorporated into soil.

If a protein is freely available, its degradation in soil would probably follow the growth pattern of bacteria (i.e., 1, 2, 4, 8, 16, 32, etc.) in each unit of time. In terms of disappearance of a protein, this would be reflected, on a linear plot, by an apparently increasing rate of disappearance with time (i.e., 100, 99, 97, 93, 85, 69, 37, and 0) in each time unit. This is not first order kinetics. The *Bt* protein degradation reported by Monsanto appears to follow such kinetics initially, but then the rate is less than expected from simple growth kinetics. The protein appears to become, with time, less available to microbial attack, as measured by simple growth kinetics.

In contrast, when the ELISA data are examined in a logarithmic-disappearance plot (first-order kinetics), the results appear to suggest first-order kinetics. However, as stated above, proteins that are freely available are not degraded by first-order kinetics. Degradation would be first-order if availability is governed not by the intrinsic growth rate of microorganisms but by some abiotic rate-limiting factor that leads to the release of the compound for microbial utilization.

In calculating the disappearance rate from the beetle bioassays, the zero numbers of larvae dead at 7 and 14 days are accepted, but the values at 21, 28 and 42 days are ignored. There is no basis for this because, for example, the value at 21 days is almost the same as the 7-day value. If one considers all the data from the beetle assays, the kinetics of disappearance suggest the existence of a residual, persistent, poorly bioavailable fraction, which remains after the initial, rapid biodegradation. This is a similar pattern to the linear plot of the ELISA data.

The soil fate studies submitted to EPA describe DT₅₀ (time to 50% degradation

of the Bt protein in soil) and DT₉₀ (time to 90% degradation of the Bt protein in soil) for Cry3Bb1 protein in sandy loam soil based on ELISA test are 2.76 and 9.16 days. However, the value of these results are not necessarily correlated with activity in insect guts because it is unknown if the extractable protein in the ELISA test was functional or non-functional. The DT₅₀ and DT₉₀ determined by insect bioassays with CPB were 2.37 and 7.87 days respectively.

C) Are these studies truly expressing the time to 50% or 90% degradation of Bt protein in the soil or whether they are only determining the level of detection of Cry3Bb1 protein in the soil. Discuss the acceptability of these studies for a preliminary risk assessment to evaluate the fate of Cry3Bb1 in soil.

Clearly, one needs to monitor degradation rates as one of the very first measures of risk. The Panel noted that the degradation sometimes is rapid. While this may be true for most proteins, there will be some exceptions. Panel members suggested diligence in searching for these rare exceptions since the consequences might be great. Another factor to be considered is related to repeated doses of protein added to soil throughout the growing season. This could cause the protein to bioaccumulate over a number of seasons. Longevity of repeated doses is likely to be greater than single doses suggesting the need for multi-year or extended testing. However, if the protein-degrading microorganisms become more abundant because of the previous addition of protein to soil, its biodegradation could be rapid.

Given the similarity in the kinetic patterns shown by the ELISA and beetle-bioassay data, it is likely that both assays are detecting the same *Bt* protein. Nevertheless, more of the protein may be present than that released by the mild extraction used for the ELISA assay, a likelihood since the extraction method was reported by its developers (Pahm *et al.*, 1994) to give only 27% recovery in a soil rich in clay and 60% recovery in a clay-poor soil. That extractable fraction recognized in the ELISA assay may also be the available fraction detected by mortality of beetle larvae. If the total concentration is not immediately available to affect the insects or to be extracted for the ELISA determination, the existence of a sequestered or strongly sorbed protein is likely. The question should then be raised whether that fraction would, in reasonable periods of time, become bioavailable by wetting/drying or freezing and thawing of the soil.

Critical to this question is an understanding of recovery efficiency of the protein from the soil(s) tested by ELISA. Recovery of added protein should be determined, especially with the passage of time, since data on other compounds indicate that recovery from soil declines with time. This process should be conducted with sterilized soil (preferably gamma irradiated). Declining recovery with time means that conventional chemical assays (e.g., ELISA) that start with mild extractions may give values that are increasingly different from the actual concentrations. Conversely, the concentration available for toxicity may be the concentration that is readily available, but the fraction not readily extractable could become available as a result of freezing/ thawing, wetting /drying, cultivation or destruction of the soil structure soiling the protein.

D) What if any difference would it make in the values of these ELISA based studies if clay particles to which the Cry3Bb1 protein might bind are present in the soil being tested? What measures should be taken to ensure that the test is not measuring inactive protein fragments?

The Panel noted that there was binding of the *Bt* protein to constituents in the soil tested. Such an effect is known for proteins in general (Ensminger and Gieseck, 1942; Greenland, 1965). The effect will be greater in soils with higher clay content.

ELISA measures all fractions of the protein, whether bound or free, and whole or partially degraded. Thus, while we do not know the extent that the measure takes into account inactive fractions, it is clear that the method will overestimate persistence. Real life or true persistence is likely to be equal to or less than that measured with ELISA.

To determine whether the ELISA results reflect active protein fragments, the results should be carefully compared to the insect bioassays. Care should be taken in extrapolating results of studies with humic acid to actual soil because humic acid reflects only a portion of soil organic matter. Humic acid does not have the nanoporosity characteristics of soil, its physical state differs from that of soil and it does not have the properties of the clay-organic complexes important in soil.

Question 5: Preliminary Risk Assessment for Non-target Invertebrates and Soil Fate

The Agency=s preliminary risk assessment based on single species laboratory toxicity studies on adult and larval lady beetles, green lacewing larvae, a parasitic hymenopteran, adult and larval honey bees, Collembola, earthworm, the monarch butterfly, field invertebrate census evaluations, and a soil persistence study indicates no unreasonable adverse effects on the invertebrate fauna of the corn field.

Please comment on the Agency=s non-target invertebrate and soil fate assessment.

In general, the Panel noted that the LC50 to MEEC (maximum expected environmental concentration) ratio was reported to be greater than 10 for all species except the adult honey bee. Some Panel members felt that the MEEC had not been adequately established from field-derived expression data. The Panel also noted that the level of exposure could not be determined from some of the study protocols, including those for the lacewing test and the hymenopteran parasitoid test.

In terms of selection of appropriate taxa for testing, based upon the spectrum of activity of the test material, non-target Coleoptera may be most at risk from direct toxicological impacts, if they are susceptible, and if exposure pathways, via foliage, pollen or secondary consumption of prey exists. A focus of concern on coleopteran taxa that have an ecological association with corn was therefore considered to be important by the Panel. The applicants conducted laboratory studies with Coccinellidae, and the target Chrysomelidae, but relied upon field-

derived

census data to determine the potential for impacts on other non-target Coleoptera. The Panel drew attention to the fact that laboratory test methodologies for Carabidae and Staphylinidae in particular, may be sufficiently well established in regulatory testing elsewhere, to be eligible for testing with this *Bt* toxin. This also drew attention to the possibility of using test procedures that are intermediate between laboratory-based and fully field-based evaluations where the first tier of laboratory tests reveal the potential for effects to occur. The Panel acknowledged that these test procedures are not currently part of the recommended test procedures published by the Agency, and that they had not previously been referred to in other SAP consultations.

The Panel was divided on the adequacy of the tier one laboratory tests. Although several tier one tests (e.g., honey bee larvae, collembolans, monarch butterflies) suggest there will be no adverse effects on some non-target taxa, concerns were expressed regarding statistical power, design duration and controls involved in experimental designs of these tests. In most cases, the presented information did not allow the Panel to adequately scrutinize the experimental design or to determine the statistical power, and thus to conclude no unreasonable adverse effect. One Panel member argued that some of the laboratory tests could be improved, but also commented that the overall tier one evidence was sufficient to suggest Cry3Bb1 would have no unreasonable adverse effects on invertebrate fauna in cornfields.

Most of the Panel agreed that the green lacewing larval tests were inadequate (see Panel response to Question 3). One Panel member questioned the relevancy of such a test, when it is unlikely green lacewing larvae would be exposed to *Bt* protein. *Bt* protein is not found in plant phloem and, even if it was, it would be confined to the crop or midgut of the feeding insect. Green lacewing larvae typically pierce their prey and feed on fluids, and it is unlikely that they would be exposed to the contents of the insect's digestive system.

The Panel noted that the applicant performed supplemental tests on non-target invertebrates beyond those required by EPA guidelines. Among these were a laboratory test on the effects of soil leachates and root extracts on survival of three nematode species, a root-pathogen (*Meloidogyne incognita*), a bacterial-feeder (*Caenorhabditis elegans*), and an insect parasite (*Steinernema carpocapsae*). Further recommendations regarding evaluation of impacts on nematodes are reported below.

The Panel agreed that the submitted field data were insufficient to make any general statements on effects of Cry3Bb1 on non-target species and that they were preliminary at best. As a result of the issues addressed in the Panel's response to Question 2 with respect to field studies (e.g., scale, community complexity, environmental variance), it may not be possible to employ short-term field studies to determine with adequate statistical power whether Cry3Bb1 has little or no effect on communities of non-target organisms. The Panel's conclusion is based on the current state-of-the-science with regard to evaluating non-target effects of transgenic and conventional crops in the field.

In general, the Panel agreed that field evaluation (of a more focused and targeted kind) should be used as the ultimate higher tier response to specific hazards identified by intermediate

scales of testing and which are a cause for concern. Some Panel members suggested that field evaluations are being used inappropriately by EPA as a substitute for laboratory testing. In that context, they can not be used to verify a lack of effect on any specific taxon, for the reasons the Panel provided in response to questions 1 and 2. In addition to being the ultimate higher tier test procedure, field studies fulfill other purposes when carried out rigorously, particularly that of incorporating indirect effects and that of enabling functional endpoints to be analyzed.

In the split-plot experimental design employed under field conditions, it will have been more difficult to detect treatment effects associated with the MON 863 hybrids as main plots, than it would be to detect the effects of insecticide treatments to subplots. If field investigations are to be recommended, guidelines concerning design, layout, and statistical analysis are needed. Although the results from the field studies are flawed for the reasons described here, it would be appropriate to conduct a meta-analysis of the multiple studies that are being undertaken to determine what kind of overall conclusions can be drawn. The Panel agreed that many of the field reports should be considered preliminary because they are based on data from only one field season. The Panel recommended that the Agency review completed reports that contain data from multiple years and full statistical analysis when they are available. A few Panel members noted that due to the high specificity of Cry3Bb1 in MON863, they would expect long-term field studies to demonstrate very limited impacts to non-target populations, especially when compared with the effects of conventional chemical insecticides.

The Panel agreed with the Agency assessment that “the reviewed data indicate that Cry3Bb1 protein in plant tissue degrades rapidly in sandy loam soil”; however, the Panel did make recommendations (in response to Question 4) that should improve the robustness of these tests. The Panel was split regarding impact of Cry3Bb1 protein on soil invertebrates. Most of the Panel members believed the tests were inadequate to conclude that there were no unreasonable adverse effects to soil invertebrates. The minority opinion was that unreasonable adverse effects were unlikely since Cry3Bb1 protein appears to degrade rapidly.

Additional recommendations and comments that arose during the discussion of this question are outlined below. The responses were typically raised by one or a few panel members, and did not represent a consensus, largely because of differing expertise of Panel members:

- Testing *Tetraopes* beetles would have been a more logical choice compared to conducting monarch tests.
- The relevancy of the hymenopteran parasitoid *Nasonia vitripennis* is questionable because it is a gregarious pupal endoparasitoid of dipteran hosts and it rarely, if ever, occurs in corn. A different species of hymenopteran might have been appropriate.
- Minute pirate bugs would have been an appropriate choice for testing, perhaps even better than lacewings (see Panel response to Question 3).
- Repeat green lacewing tests with specific modifications as noted in response to question 3.

- One ladybird beetle species that might be most likely to be affected by Cry3Bb1 is *Coleomegilla maculata*. Within the present data package, this species was tested more frequently than any other species or species group. This beetle can complete development on corn pollen, which for MON 863 contains 62 ± 18 (sd) $\mu\text{g/g}$ fwt Cry3Bb1 protein (454240-01), a level as high as that in the other corn tissues, and at least two orders of magnitude greater than the concentration of Cry1Ab toxin in MON 810 pollen. Several studies address this issue: MRIDs 453613-01, 455382-04, 455382-06, and 456530-03 (in appendices A, E and G). These represent three laboratory studies and four field studies. The studies on *C. maculata* are insufficient to come to a preliminary assessment of the potential hazard of Cry3Bb1 toxin to *C. maculata* for the several reasons. Most significantly, and contrary to the claims of the registrant and the work of Pilcher *et al.*, 1997., it is not difficult to rear *C. maculata* from egg to adult with <20% mortality on a pure pollen diet. Indeed, it is not difficult to rear them from egg to 40-day-old adult with <20% mortality on a pure pollen diet. One Panel member also suggested that the laboratory studies could be aimed better at establishing the existence of a potential hazard, by using pure pollen diet rather than a mixture of lyophilized tephritid eggs and pollen (MRIDs 453613-01 and 455382-04). Food mixtures can have different effects on the growth, development and survival of generalist feeders than single foods. In addition, from the perspective of hazard identification, a 100% pollen diet represents a reasonable worst-case scenario, and needs to be evaluated. During the latter part of anthesis (pollen shed) in the corn plant, pollen is about the only food available for *C. maculata*. The lab results in Appendix E of 456530-03 are based on 100% pollen diets, but are flawed because of high control mortality, probably because availability of water was not controlled sufficiently. Moreover, it is also possible to rear this species on an artificial diet to which very high concentrations of Cry3Bb can be added so that MEEC for the pure toxin can be estimated.
- Although studies of nematode species were not required for registration, preliminary greenhouse and laboratory tests suggested that abundance of the root-pathogen and bacterial-feeding nematode were reduced significantly when exposed to MON 863. Methods employed for *Melioidogyne incognita* appear standard. Although farmers may welcome an added benefit of control of a secondary pest or pathogen, it would be reassuring to have data to demonstrate that the protein does not tax expression of another plant defense (e.g., increasing susceptibility to nematode parasites). However, not all nematodes are pathogens. Nematodes are prolific soil fauna, playing essential and beneficial roles in soil. Nematode species vary in their sensitivity or tolerance to different types of environmental disturbance or stress. Therefore, if nematode studies are to be conducted adequately, it is important to include representatives from a broad range of trophic groups and relative sensitivity to stress. The experimental design of laboratory tests should report the concentration of protein in soil leachates and extend the survival tests through at least one full generation, which would range from 3 to 14 days for bacterial-feeding nematodes. Løkke and Van Gestel (1998) provided standard protocols for ecotoxicological tests on two bacterivorous nematodes, *Plectus acuminatus* and *Heterocephalobus pauciannulatus*, that would be more appropriate and relevant for testing than *C. elegans*. *C. elegans* is not an ecologically relevant species for corn systems. A Panel member also

suggested that root extracts would be more realistic than soil extracts for testing effects on non-target nematodes; nematodes are more likely to encounter the protein directly from roots or microbes feeding in the rhizosphere than soil solution.

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**FIFRA Scientific Advisory Panel Meeting,
August 28-29, 2002 held at the Sheraton Crystal City Hotel,
Arlington, Virginia**

*A Set of Scientific Issues Being Considered by the
Environmental Protection Agency Regarding:*

**Part B: Corn Rootworm Plant-incorporated
Protectant Non-target Insect and Insect
Resistance Management Issues: Insect Resistance
Management Issues**

**Mr. Paul Lewis
Designated Federal Official
FIFRA Scientific Advisory Panel
Date: 11/6/02**

**Christopher Portier, Ph.D.
FIFRA SAP Session Chair
FIFRA Scientific Advisory Panel
Date: 11/6/02**

**Federal Insecticide, Fungicide, and Rodenticide Act
Scientific Advisory Panel Meeting
August 28-29, 2002**

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INTRODUCTION

The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Scientific Advisory Panel (SAP) has completed its review of the set of scientific issues being considered by the Agency pertaining to corn rootworm plant-incorporated protectant non-target insect and

insect resistance management issues.

Advance notice of the meeting was published in the *Federal Register* on July 24, 2002. The review was conducted in an open Panel meeting held in Arlington, Virginia, on August 28-29, 2002. The meeting was chaired by Christopher Portier, Ph.D. Mr. Paul Lewis served as the Designated Federal Official.

Ms. Robyn Rose (Office of Pesticide Programs, EPA) presented a review of Monsanto's interim resistance management plan for *Bacillus thuringiensis* event MON 863 on corn rootworm protected field corn.

In preparing these meeting minutes, the Panel carefully considered all information provided by the Agency, as well as information presented by public commenters. These meeting minutes address the information provided and presented at the meeting, especially the response to the charge by the Agency.

PUBLIC COMMENTERS

Oral statements were made by:

Nicholas P. Storer, Ph.D., on behalf of Dow AgroSciences LLC
Jane Rissler, Ph.D., on behalf of the Union of Concerned Scientists
Mr. Gary Queen, private citizen
Ms. Helen Inman, on behalf of the National Corn Growers Association
Mr. John Beshaler, private citizen
Jon Tollefson, Ph.D., on behalf of Iowa State University
Teresa A. Gruber, Ph.D., J.D., on behalf of the Council for Agricultural Science and Technology
Ty Vaughn, Ph.D. on behalf of Monsanto Company
Doug Gurian-Sherman, Ph.D., on behalf of the Center for Science in the Public Interest

Written statements were received by:

Center for Science in the Public Interest
Exponent, Inc.
Monsanto Company
Union of Concerned Scientists

CHARGE

Monsanto Company submitted an application to EPA for the registration of *Bacillus thuringiensis* (Bt) Cry3Bb1 protein and the genetic material (Vector ZMIR13L) necessary for its production in corn. Corn expressing the Cry3Bb1 protein is intended to provide protection against the corn rootworm (CRW, *Diabrotica* spp.). This product has been designated event MON 863 by Monsanto. EPA has determined that an insect resistance management (IRM) plan is necessary for this product. At EPA's request for a IRM plan, Monsanto designed a plan intended to be both scientifically valid for resistance risk mitigation and feasible for growers to understand and implement. EPA's preliminary assessment of the Monsanto IRM plan for

MON 863 has determined that further data and evaluation is needed to develop a robust, practical, long-term IRM plan. The proposed plan submitted by Monsanto might be used for 3 years while in-field testing and evaluation is conducted to develop a IRM plan which might be used for 10 or more years. In order to develop such a long-term IRM plan, grain growers, and researchers need to be able to grow MON 863 corn for a period of time so that important information can be generated including how an IRM plan can be effective in areas where MON 863 is used alone and in areas where MON 810 (used for control of certain lepidopteran pests such as European corn borer) is combined with MON 863. EPA requests the Scientific Advisory Panel to provide guidance to the Agency on the following questions related the Agency's assessment of the interim IRM plan and information that needs to be generated to develop a long-term IRM plan for corn rootworm plant-incorporated protectant (PIP).

Question 1: Pest Biology Research

Pest biology is important to refuge placement since the goal is to encourage random mating between pests emerging from the transgenic and non-transgenic corn fields. Knowledge of corn rootworm (CRW) biology, dispersal characteristics, host range, feeding habits and history of insecticide resistance is important in developing an IRM strategy. Most information provided to the Agency thus far relates to western corn rootworm (WCRW) and limited information was provided on northern corn rootworm (NCRW). The Mexican corn rootworm (MCRW) is only briefly discussed and the southern corn rootworm (SCRW) is not considered in Monsanto's IRM proposal.

The Panel is requested to comment on the Agency's conclusion that additional information is needed on various aspects of CRW pest biology as it relates to a long-term IRM strategy. Specifically, discuss:

- A) Whether an IRM strategy designed for WCRW (and NCRW) is applicable to other corn rootworm species? How much species-specific data is needed vs. how much can the Agency rely on existing data for WCRW and NCRW to predict what would be an adequate IRM plan for SCRW and MCRW?
- B) Whether, and if so what, additional research regarding male and female adult and larval WCRW and NCRW dispersal potential is needed to determine placement of non-Bt corn refuges?
- C) Whether, and if so what, more information is needed on mating habits, ovipositional patterns, number of times a female can mate and fecundity as it relates to refuge structure and placement?
- D) How should CRW extended diapause and oviposition outside of corn (e.g., soybean rotation) be used to evaluate the effectiveness of IRM plans?

Question 2: Dose.

Determining the level of dose is crucial to size and structure of a refuge needed to delay

CRW resistance to Cry3Bb proteins. In the February 1998 Scientific Advisory Panel meeting, a high dose for lepidopteran-active Bt proteins was defined as 25 times the amount of Bt delta-endotoxin necessary to kill susceptible individuals. Based on Monsanto's modified version of a model by Caprio, a moderate dose is defined as 30% survival of larvae and a low dose as 50% survival. Data provided by Monsanto shows 17% to 62% survival of larvae. EPA believes that a 17% to 62% survival of larval CRW constitutes a low to moderate dose of Cry3Bb1 protein in MON 863 corn.

A) The Panel is requested to comment on EPA's determination that MON 863 expresses a low to moderate dose for CRW. The Panel is requested to provide guidance on definitions of a high, moderate and low dose for a corn rootworm-protected Bt corn product.

B) What techniques should be used to determine dose for Cry3Bb1?

As a part of this discussion the Panel might want to consider the definition of high dose provided by the February 1998 SAP noting that for Bt corn, the pests are above ground feeding lepidopteran insects. The relevant excerpt from the Panel's report is provided below.

The Subpanel discussed ways to define and measure a high dose in plants. It was agreed that the definition of high dose as a 25 times the toxin concentration needed to kill susceptible larvae was reasonable based on current empirical data. However, the Subpanel recognized that it is conceivable that a heterozygote may develop with higher than 25-fold resistance.

The major problem identified by the Subpanel was in determining if the 25-fold level was achieved in a specified cultivar. After much discussion, it was concluded that there were at least 5 imperfect ways to assess this 25-fold level, and that some approaches were more appropriate for specific crop pests. The Subpanel concluded that a cultivar could be considered to provide a high dose if two of the five approaches described here indicated presences of a high dose.

The five approaches are:

- (1) Serial dilution bioassay with artificial diet containing lyophilized tissues of Bt plants (tissue from non-Bt plants serving as controls);
- (2) Bioassays using plant lines with expression levels approximately 25-fold lower than the commercial cultivar (determined by quantitative ELISA or some more reliable technique);
- (3) Survey large numbers of commercial plants on sentinel plots in the field (e.g. sentinel sweet corn method) to make sure that the cultivar is at the LD99.99 or higher to assure that 95% of heterozygotes would probably be killed. With this approach Bt sweet corn hybrids are used to attract high densities of ECB and cotton bollworm (*Helicoverpa zea*) (Boddie)) (CBW/CEW) moths, sampling can be limited to sweet corn ears in the

Bt plot (ca. 1/4-1/2 acre block), and a frequency of resistance phenotypes can be estimated as the ratio of density of larvae/plant in Bt sweet corn to density of larvae/plant in an adjacent planting of non-Bt sweet corn (Andow and Hutchison, 1998; Hutchison, unpublished data).

(4) Similar to (3) above, but would use controlled infestation with a laboratory strain of the pest that had an LD50 value similar to field strains;

(5) Determine if an older instar of the targeted pest could be found with an LD50 that was about 25-fold higher than that of the neonate larvae. If so, that stage could be tested on the crop plants to determine if 95% or more of the older stage larvae were killed.

Question 3: Models.

Simulation models are one of the tools used to evaluate IRM strategies to delay resistance. Assumptions in resistance models are based on aspects of pest biology including CRW survival and fitness. EPA has used predictive models to compare IRM strategies for Bt crops. Because models cannot be validated without actual field resistance, models have limitations and the information gained from the use of models is only a part of the weight of evidence used by EPA in assessing the risks of resistance development. It was the consensus of the October, 2000 FIFRA SAP that models were an important tool in determining appropriate Bt crop IRM strategies. They agreed that models were the only scientifically rigorous way to integrate all of the biological information available, and that without these models, the Agency would have little scientific basis for choosing among alternative resistance management options.

A) The Panel is asked to comment on the product duration or longevity of corn rootworm susceptibility considered in CRW IRM models. B) Considering EPA's evaluation of the three models addressed in the Monsanto submission, discuss the applicability of each of the models for assessing the likelihood of CRW developing resistance to Cry3Bb1.

C) Please comment on the appropriateness of the following input parameters of these simulation models for CRW-protected field corn: Resistance allele frequency, dominance of the heterozygote, movement of the males and females, mating and ovipositional behavior, and other genetic and behavioral parameters.

D) How does insecticide use in the refuge and/or Bt fields affect the predictions of time to resistance?

Question 4: Refuges.

Refuges are planted to delay potential pest resistance to a Bt crop. Planting non-Bt corn within or near Bt corn fields will provide CRW offspring that will remain susceptible to the Cry3Bb proteins. The refuge should be structured to provide an adequate number of susceptible individuals that are available to mate with potentially resistant individuals and dilute

resistance alleles in the field. Based on current information on CRW biology, MON 863 dose, simulation models, hybrid availability and adoption rate, a 20% refuge should be adequate on an interim basis to produce enough CRW adults to delay resistance. EPA has concluded that it is acceptable to plant refuges as continuous blocks or in-field row-strips. Based on the only available currently published paper, in-field strips should consist of at least 6 to 12 consecutive rows planted within 9 to 18 m of the center of the transgenic corn field.

EPA has concluded that a 20% refuge is adequate to delay resistance during a three-year period.

A) Please comment on whether this refuge strategy is adequate to delay resistance?

B) Because the current plan being evaluated is based on limited data and is an interim plan, limitations to the total number of acres MON 863 might be considered. If so, should the limitations be on acres planted per state or per county or on another basis during the time an interim IRM plan is in place?

C) The Panel is asked to comment on the adequacy of in-field row-strips and/or immediately adjacent blocks to delay resistance during a three-year period and whether one method or another is preferred.

D) The Panel is requested to comment on the width of the in-field strips. As an example, the Agency is aware that at least 6 to 12 consecutive rows have been discussed in the following paper: Onstad, D. W., C. A. Guse, J. L. Spencer, E. Levine and M. E. Gray. 2001. Modeling the dynamics of adaptation to transgenic corn by western corn rootworm (Coleoptera: Chrysomelidae). *J. Econ. Entomol.* 94(2): 529-540.

E) Please comment on EPA's conclusion that alternate hosts should not be considered and refuges should only consist of non-Bt corn that are similar hybrids to the Bt corn.

F) The Panel is requested to comment on whether, and if so under what conditions, insecticides could be used in the refuge.

Question 5: Monitoring.

A resistance monitoring strategy for Bt corn is needed to test the effectiveness of resistance management programs. Detecting shifts in the frequency of resistance genes (i.e., susceptibility changes) through resistance monitoring can be an aggressive method to detect the onset of resistance before widespread crop failure occurs. As such, the utilization of sensitive and effective resistance monitoring techniques is critical to the success of an IRM plan. EPA believes the mechanism of potential resistance of CRW to MON 863 should be determined to develop an appropriate long-term IRM strategy. EPA has concluded that CRW resistance is necessary to determine the mechanism and genetics of resistance to Cry3Bb1. Therefore, colonies resistant to Bt should be established and evaluated in the laboratory during the initial three years MON 863 is grown commercially.

Please comment on the Agency's conclusions regarding refinements to Monsanto's resistance monitoring program. In your response, please consider the following factors: how should CRW resistance should be monitored; the value of developing resistant colonies of CRW to determine the mechanism and genetics of resistance; insect rearing for CRW spp. and whether one colony in more than one laboratory should be established.

Question 6: Mitigation/Remedial Action.

Remedial action plans are a potential response measure should resistance develop to Bt crops. Since resistance may develop in a localized pest populations, it may be possible to contain the resistance outbreak before it becomes widespread. There is a concern regarding Monsanto's proposed outline of detecting and confirming resistance. Monsanto suggests that they will initiate mitigation measures when unexpected levels of CRW damage occur. However, Monsanto does not describe what is meant by unexpected levels of damage. Some level of damage is expected since there is not a high dose of MON 863 expressed to control the CRW and research has shown that some level of grazing will occur. Monsanto also suggested using a root damage rating scale to determine unexpected levels of damage. However, this method may not be appropriate for CRW protected Bt corn.

A) The Panel is requested to discuss an appropriate method of determining suspected and confirmed resistance for CRW including recommendations as to how suspected resistance or unexpected damage may be identified.

B) Please discuss whether root ratings are an appropriate indicator of suspected resistance. If so, how could a typical farmer use root ratings to identify suspected resistance.

PANEL RECOMMENDATIONS

- It was the consensus of the Panel that since the western corn rootworm and the Mexican corn rootworm are subspecies, many types of data collected with the western corn rootworm will be applicable to the Mexican corn rootworm, but should be verified when possible and practical.
- Although the southern corn rootworm is in the same genus, the biology of this insect differs greatly from the biology of the western corn rootworm, Mexican corn rootworm, and northern corn rootworm. It was the consensus of the Panel that large-scale studies on male movement and fitness from beetles produced from both MON 863 and isolines are of particular importance.
- The Panel did not recommend developing a demarcation line between low and moderate dose. Instead it concluded that determining the impact of each transgenic event on selection intensity is important for determining appropriate refuge size.
- The Panel concluded that the use of *SS* survival rates was sufficient to demonstrate that

MON 863 is not high dose, because *SS* survival is so much higher than that expected at 25X the LC99.

- The Panel concluded that for low/moderate dose plants, the four current models (Onstad et al., Caprio, Andow/Alstad and Storer) were adequate for assessing the longevity of rootworm susceptibility if the initial frequency of major and minor resistance genes was as low as assumed by the models.
- The Panel differed on what percent refuge would be appropriate, conservative, and workable. The majority of the Panel members concluded that an appropriate, conservative, approach for an insect resistance management plan (IRM) plan would involve a refuge size of approximately 50%. Because important data are lacking and because grower adoption rates are likely to be low initially, these members viewed the 20% refuge as premature.

Other Panel members differed with the majority. A few Panel members were supportive of a 20% refuge. Their justification for supporting this figure was that it was compatible with the current refuge recommendation for Bt corn resistant to European corn borer, the 20% refuge amount would set the stage for IRM recommendations that would be compatible for both ECB and western corn rootworm, and it was noted that a simpler IRM strategy would be less confusing to growers, and ultimately would increase compliance.

- It was the consensus of the Panel that any cap in the amount of acreage planted to MON 863 should be at the farm level (i.e. if such a cap were considered, it should be done with the refuge percentage required per farm, not at the county, state, or regional level).
- It was the consensus of the Panel that there was not sufficient data to support in-field strips over immediately adjacent blocks or vice versa to delay resistance during a three-year period.
- The Panel agreed that a resistance monitoring strategy is needed to evaluate the effectiveness of resistance management programs. The Panel agreed that laboratory bioassays were too expensive to use routinely for monitoring populations, and suggested that a tiered monitoring system be developed.
- The Panel agreed that growers were likely to be the first to encounter unexpected rootworm damage manifested as lodged corn plants. When such damage is reported, a registrant representative should: 1) request the grower check planting records; 2) rule out damage from nontarget insects, weather, or other environmental factors (e.g., excessive weediness whereby western corn rootworm could complete partial development on grasses then move to transgenic corn); 3) conduct tests to verify MON 863 was planted and that the correct percentage of plants are expressing and; 4) if plants are MON 863 and damage approaching a 0.5 (node-injury scale) is found on

any expressing plant, evaluate roots from the corresponding refuge.

DETAILED RESPONSE TO THE AGENCY'S CHARGE

The specific issues to be addressed by the Panel are keyed to the Agency's background document, dated July 26, 2002, and are presented as follows:

Monsanto Company submitted an application to EPA for the registration of *Bacillus thuringiensis* (Bt) Cry3Bb1 protein and the genetic material (Vector ZMIR13L) necessary for its production in corn. Corn expressing the Cry3Bb1 protein is intended to provide protection against the corn rootworm (CRW, *Diabrotica* spp.). This product has been designated event MON 863 by Monsanto. EPA has determined that an insect resistance management (IRM) plan is necessary for this product. At EPA's request for a IRM plan, Monsanto designed a plan intended to be both scientifically valid for resistance risk mitigation and feasible for growers to understand and implement. EPA's preliminary assessment of the Monsanto IRM plan for MON 863 has determined that further data and evaluation is needed to develop a robust, practical, long-term IRM plan. The proposed plan submitted by Monsanto might be used for 3 years while in-field testing and evaluation is conducted to develop a IRM plan which might be used for 10 or more years. In order to develop such a long-term IRM plan, grain growers, and researchers need to be able to grow MON 863 corn for a period of time so that important information can be generated including how an IRM plan can be effective in areas where MON 863 is used alone and in areas where MON 810 (used for control of certain lepidopteran pests such as European corn borer) is combined with MON 863. EPA requests the Scientific Advisory Panel to provide guidance to the Agency on the following questions related the Agency's assessment of the interim IRM plan and information that needs to be generated to develop a long-term IRM plan for corn rootworm plant-incorporated protectant (PIP).

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Pest biology is important to refuge placement since the goal is to encourage random mating between pests emerging from the transgenic and non-transgenic corn fields. Knowledge of corn rootworm (CRW) biology, dispersal characteristics, host range, feeding habits and history of insecticide resistance is important in developing an IRM strategy. Most information provided to the Agency thus far relates to western corn rootworm (WCRW) and limited information was provided on northern corn rootworm (NCRW). The Mexican corn rootworm (MCRW) is only briefly discussed and the southern corn rootworm (SCRW) is not considered in Monsanto's IRM proposal.

The Panel is requested to comment on the Agency's conclusion that additional information is needed on various aspects of CRW pest biology as it relates to a long-term IRM strategy. Specifically, discuss:

A) Whether an IRM strategy designed for WCRW (and NCRW) is applicable to other corn rootworm species? How much species-specific data is needed vs. how much can the Agency rely on existing data for WCRW and NCRW to predict what would be an adequate IRM plan for SCRW and MCRW?

In the Corn Belt, the majority of research has been conducted on western corn rootworm, *Diabrotica virgifera virgifera*, in continuous corn systems because that is where high populations develop first. Less information is available on the northern corn rootworm, *D. barberi*, in areas with or without extended diapause, and relatively little is known about the Mexican corn rootworm, *D. virgifera zea*. The southern corn rootworm, *D. undecimpunctata howardi*, is not typically a pest in the Corn Belt, but is occasionally in the south. Pest biology is important to refuge placement since the goal is to encourage sufficient mating between pests emerging from the transgenic and non-transgenic corn fields. Knowledge of corn rootworm biology, dispersal characteristics, host range, feeding habits and history of insecticide resistance is important in developing an IRM strategy. Most information provided to the Agency thus far relates to western corn rootworm and limited information was provided on northern corn rootworm. The Mexican corn rootworm is only briefly discussed and the southern corn rootworm is not considered in Monsanto's IRM proposal.

It was the consensus of the Panel that since the western corn rootworm and the Mexican corn rootworm are subspecies, many types of data collected with the western corn rootworm will be applicable to the Mexican corn rootworm, but should be verified when possible and practical. Since colonies of the Mexican corn rootworm are not currently available, it may not be possible to collect some types of data directly from this subspecies. Behavioral data generated with the western corn rootworm would be less likely to be applicable to the Mexican corn rootworm. Even within the western corn rootworm, biotypes from Nebraska and Illinois are vastly different in adult movement behavior. Data on the biology of the Mexican corn rootworm including such data as adult mating behavior, migration of the males and females, and female reproductive biology and fecundity is needed to verify whether the IRM plan is suitable for this subspecies. In addition, more complete data on transgenic efficacy and adult emergence from transgenic corn is needed.

Although the southern corn rootworm is in the same genus, the biology of this insect differs greatly from the biology of the western corn rootworm, Mexican corn rootworm, and northern corn rootworm. Larvae of the southern corn rootworm are polyphagous, feeding on more than 250 species of plants in many families. Larvae of the other corn rootworm species are oligophagous, feeding only certain species in the grass family. Information on the biology of the western corn rootworm is less likely to be applicable to the southern corn rootworm. In Monsanto's response to this question, they stated that the southern corn rootworm is "not adequately controlled by MON 863 under field situations". If Monsanto removes the southern corn rootworm from the label, data needs on this species could become moot if larval survival is high enough. It is the Panel's understanding that neonate western, Mexican, northern, and southern corn rootworm larvae are all controlled with similar doses of Cry3Bb1. It is the typical life cycle of the southern corn rootworm in corn and the tolerance of later instar larvae to Cry3Bb1 that likely make this species difficult to control in field situations. Unlike the western

corn rootworm, where eggs overwinter in the soil, southern corn rootworm eggs are laid by overwintering adults. Southern corn rootworms rarely overwinter (if ever) in most of the Corn Belt. In early spring, southern corn rootworm adults begin to migrate north, often laying eggs near grasses. Southern corn rootworm eggs often hatch before corn roots are available, and larvae often feed on other host roots before moving onto corn. Later instar southern corn rootworm larvae (and larger larvae of other species) are not controlled by MON 863. As suggested in public comments submitted by Monsanto in response to this question and noted previously, it was the consensus of the Panel to recommend that the southern corn rootworm should be removed from the label.

With regard to the question of “Whether an IRM strategy designed for WCRW (and NCRW) is applicable to other corn rootworm species?” it was the consensus of the Panel that the same strategy might be applicable to the western corn rootworm and the northern corn rootworm, but that more data are required on the northern corn rootworm and on a number of geographic populations of the western corn rootworm before this question could be definitively answered.

B) Whether, and if so what, additional research regarding male and female adult and larval WCRW and NCRW dispersal potential is needed to determine placement of non-Bt corn refuges?

C) Whether, and if so what, more information is needed on mating habits, ovipositional patterns, number of times a female can mate and fecundity as it relates to refuge structure and placement?

The Panel will address questions 1B and 1C together, since they are related. While in the central Corn Belt the major corn complex is composed of the western corn rootworm and the northern corn rootworm, and these species have been studied for well over 50 years, there are key biological parameters that need to be clarified if modeling efforts to simulate adaptation to MON 863 are to produce accurate predictions. Specifically, large-scale field studies with and without MON 863 are needed to examine field movement of adult males and females. It was the consensus of the Panel that large-scale studies on male movement and fitness from beetles produced from both MON 863 and non-Bt isolines are of particular importance. In addition, data are needed that will allow average movement rate to be predicted (e.g., distance/time; leaving rate from natal field) of males and mated females. Other studies mentioned include evaluating the impact of adult density on migration patterns of adults, whether a delay in male emergence from MON 863 affects male fitness and lowers their chances for mating, and whether there are sublethal effects of MON 863 on female fecundity, offspring quality and other fitness parameters. Since there are behavioral differences between western corn rootworm populations from the eastern and western regions of the species range, it would be best for all of the movement studies noted above to be conducted in each region. Lastly, relatively little is known about northern corn rootworm movement and sublethal effects of MON 863 on males or females. If possible, similar studies should also be conducted with the northern corn rootworm and western corn rootworm.

It was the consensus of the Panel that larval movement is unlikely to be important if refuges are planted as blocks and that larval movement, in general, is less important with a low dose event such as MON 863 than a high dose event. The effects of a seed mixture on adult movement, mating, and fecundity deserve investigation. The Panel provided more comments on additional research at the conclusion of this report.

The Panel recognized that in a May 29, 2001, letter to the EPA, the views of NCR-46, a technical committee of research and extension entomologists and selected cooperators that are considered by many researchers to be the national authorities on corn rootworm biology, ecology, and management, wrote the following in answer to a similar question:

“Further research is required on various topics in order to develop a robust IRM plan. These topics include:

- Characterize tissue expression, dose, and the mechanism by which corn rootworms survive on transgenic corn expressing Cry3Bb.
- Continue to quantify movement patterns of corn rootworm larvae when feeding on transgenic (expressing Cry3Bb) and nontransgenic corn.
- Quantify pre- and post-mating dispersal of corn rootworm, movement within and between fields, and its implications for IRM.
- Quantify the relative fitness of rootworm individuals that survive on transgenic corn vs. nontransgenic corn.
- Re-evaluate the host status of major grassy cornfield weeds and other grasses commonly found near corn; estimate the potential impact these alternate hosts may have on corn rootworm population dynamics.
- Continue to develop toxicological bioassays and resistance monitoring techniques.
- Determine the genetic nature of resistance to corn rootworm-active Cry compounds.
- Improve rearing techniques for certain corn rootworm species to facilitate laboratory and greenhouse bioassays, genetic studies, etc.
- Generate more complete data sets on transgenic efficacy, adult emergence from transgenic corn, etc. for all targeted corn rootworm species.
- Evaluate IRM options other than a refuge strategy, especially if an event is not classified as high-dose.
- Examine the impacts of refuge configuration, including seed mixtures, on development of resistance and likelihood of farmer adoption.
- Continue to develop and refine computer simulation models that build on current knowledge to guide development of IRM strategies.

Reconcile corn rootworm and ECB IRM needs into an optimal IRM plan.”

Some Panel members are aware that many studies have been conducted or initiated since May, 2001, to address some of these concerns. However, the members of the NCR-46 and their affiliates are the scientists very familiar with corn rootworms and their thoughts should be considered by the Agency.

D) How should CRW extended diapause and oviposition outside of corn (e.g., soybean rotation) be used to evaluate the effectiveness of IRM plans?

The Panel discussed whether or not growers in areas with extended diapause or eastern biotype of the western corn rootworm would be expected to use the technology. In these areas, it may be possible that farmers would choose to plant transgenic seed in first-year corn fields. Economic injury level predictions are available based on adult catches in soybeans for first-year corn in Illinois and Indiana. Similar information can be made available for first-year corn in extended diapause areas. For consistency, and to prevent abuse, the Panel concluded that regardless of the region, it is best for the refuge to always have the same crop history the previous year as the MON 863. It is the number of susceptible beetles in a refuge relative to the number of resistant beetles in the Bt crop that directly impacts resistance development. Therefore, the total acreage used as a refuge is not always a good measure of how effective the refuge will be. A farmer who uses first year corn as a refuge in regions with extended diapause or altered oviposition is unlikely to produce as many refuge beetles as a farmer who uses an equal area of second year corn as a refuge.

Question 2: Dose

Determining the level of dose is crucial to size and structure of a refuge needed to delay CRW resistance to Cry3Bb proteins. In the February 1998 Scientific Advisory Panel meeting, a high dose for lepidopteran-active Bt proteins was defined as 25 times the amount of Bt delta-endotoxin necessary to kill susceptible individuals. Based on Monsanto's modified version of a model by Caprio, a moderate dose is defined as 30% survival of larvae and a low dose as 50% survival. Data provided by Monsanto shows 17% to 62% survival of larvae. EPA believes that a 17% to 62% survival of larval CRW constitutes a low to moderate dose of Cry3Bb1 protein in MON 863 corn.

- A) The Panel is requested to comment on EPA's determination that MON 863 expresses a low to moderate dose for CRW. The Panel is requested to provide guidance on definitions of a high, moderate and low dose for a corn rootworm-protected Bt corn product.**

The Panel did not recommend developing a demarcation line between low and moderate dose. Instead it concluded that determining the impact of each transgenic event on selection intensity is important for determining appropriate refuge size for non-high dose events.

The Panel consensus was that MON 863 was definitely not a high dose product. MON 863 is characterized by a variable but high percentage of beetles that can develop from larvae to adult on the plants. Western corn rootworm larvae that survive on MON 863 take longer to develop than larvae that develop on non-transgenic plants. A notable difference between larvae on MON 863 and non-Bt corn is the absence of root tunneling by first instar western corn rootworm larvae on MON 863. Larvae feed at length on non-transgenic corn but feed intermittently on MON 863 and move to new feeding sites between feedings. This results in a characteristic scarring of the roots of MON 863 and prevents the characteristic root clipping damage common to non-transgenic corn. The increased time to emergence resulted from an

extended first instar larval development time. MON 863 does not have documented effects on second and third instar larvae. Mortality is primarily seen among first instar larvae and Monsanto has indicated an LC50 for the trypsinized toxin. LC50 could not be reached for second and third instar larvae.

The Panel concluded that there was no useful demarcation between a low and moderate dose event. For example, considering the data by Storer (in review), there is a clear differentiation between the rate of adaptation to a dose that causes greater than 95% mortality and lower doses, but there is only a gradual slope in the change of “rate of adaptation as dose declines from that which causes 95% mortality to 20% mortality. While differentiating between low and moderate dose did not seem critical to the Panel, it might be important to differentiate between high dose (as defined by the 1998 EPA-SAP) versus almost high dose, as the latter is the case in which resistance is often expected to evolve most rapidly.

Dose, as related to selection intensity, is important in determining appropriate refuge size. A good approximation to selection intensity would be to measure differential fitness of susceptible homozygotes on transgenic versus non-transgenic plants. It was suggested that a measure of product efficacy against larvae would be a good first order approximation of selection intensity. However, product efficacy against larvae could underestimate selection intensity substantially if there were sublethal effects on female reproduction or emerging male adults have low fitness because most females have already mated by the time these males become sexually mature. It will also be important to factor out effects of density dependent mortality when estimating selection intensity from larval survival data because density dependence could lead to an underestimate of selection intensity. Some members of the Panel suggested that the development of resistant colonies would greatly assist in determining selection intensity.

The Panel also suggested that some consideration should be given to the potential of indirect effects of MON 863 plants on rootworm fitness. Direct mortality from ingesting the Cry3Bb1 toxin might not be the only important mortality factor, and the actual cause of larval mortality from MON 863 is unknown. For example, if developmental delays led to increased mortality (i.e. starvation due to reduced feeding, reduction in number of feeding sites due to lack of tunneling, exposure to the environment outside of the root tunnel or damage due to increased movement or other factors that are tied to length of time in the environment, etc.) and resistant individuals had shorter developmental delays, then these other mortality factors would select for the resistance trait (Gould et al. 1991). Understanding how the larvae respond to the MON 863 is critical for investigating the types and mechanisms of resistance that may develop. Generally, the lower the dose, the greater the number of genetic mechanisms that can potentially create resistant populations. The fundamental question for resistance management, however, is the relative fitness of the different genotypes, not the mechanistic cause of resistance.

Due to the large variation in estimates of susceptible survivorship, it was suggested that a mode or mean value could be utilized with appropriate analysis of variance about this value, perhaps using a Bayesian framework. Overall, changes in survivorship estimates, in the range likely for corn rootworm, are unlikely to change the general recommended strategy, but this

estimate could impact the amount of refuge that would be recommended.

B) What techniques should be used to determine dose for Cry3Bb1?

As a part of this discussion the Panel might want to consider the definition of high dose provided by the February 1998 SAP noting that for Bt corn, the pests are above ground feeding lepidopteran insects. The relevant excerpt from the Panel's report is provided below.

The Subpanel discussed ways to define and measure a high dose in plants. It was agreed that the definition of high dose as 25 times the toxin concentration needed to kill susceptible larvae was reasonable based on current empirical data. However, the Subpanel recognized that it is conceivable that a heterozygote may develop with higher than 25-fold resistance.

The major problem identified by the Subpanel was in determining if the 25-fold level was achieved in a specified cultivar. After much discussion, it was concluded that there were at least 5 imperfect ways to assess this 25-fold level, and that some approaches were more appropriate for specific crop pests. The Subpanel concluded that a cultivar could be considered to provide a high dose if two of the five approaches described here indicated presences of a high dose.

The five approaches are:

- (1) Serial dilution bioassay with artificial diet containing lyophilized tissues of Bt plants (tissue from non-Bt plants serving as controls);
- (2) Bioassays using plant lines with expression levels approximately 25-fold lower than the commercial cultivar (determined by quantitative ELISA or some more reliable technique);
- (3) Survey large numbers of commercial plants on sentinel plots in the field (e.g. sentinel sweet corn method) to make sure that the cultivar is at the LD_{99.99} or higher to assure that 95% of heterozygotes would probably be killed. With this approach Bt sweet corn hybrids are used to attract high densities of ECB and cotton bollworm (*Helicoverpa zea*) (Boddie) (CBW/CEW) moths, sampling can be limited to sweet corn ears in the Bt plot (ca. 1/4-1/2 acre block), and a frequency of resistance phenotypes can be estimated as the ratio of density of larvae/plant in Bt sweet corn to density of larvae/plant in an adjacent planting of non-Bt sweet corn (Andow and Hutchison, 1998; Hutchison, unpublished data).
- (4) Similar to (3) above, but would use controlled infestation with a laboratory strain of the pest that had an LD₅₀ value similar to field strains;

(5) Determine if an older instar of the targeted pest could be found with an LD50 that was about 25-fold higher than that of the neonate larvae. If so, that stage could be tested on the crop plants to determine if 95% or more of the older stage larvae were killed.

Overall, the Panel concluded that the use of *SS* survival rates was sufficient to demonstrate that MON 863 is not a high dose, because *SS* survival is so much higher than that expected at 25X the LC99. MON 863 failed both criteria (4) and (5). A few Panel members expressed the opinion that none of the 5 methods listed in the EPA document were satisfactory for definitively identifying a high dose in the corn rootworm. MON 863 is not a borderline case, and clearly is not a high dose.

The artificial diet assay used by Monsanto has many deficiencies but is useful to determine the LC50 for first instar larvae and is adequate to determine dose. For high dose strategies, accurate measurement of dose is an important component of resistance management. However, low to moderate dose strategies eliminate the need for such rigorous determination of dose and, as described in the Panel's response to the previous question, estimation of selection intensity becomes more important for low to moderate dose events. The artificial diet method more accurately mimics the presentation of the toxin to the larvae in the cropping system compared to other methods of direct dosing of larvae (e.g. imbibing the toxin in a solution of sugar water). The artificial diet method should be adequate for testing the resistance level of larvae produced on MON 863 plants. Other expression systems that might allow more concentrated doses to be obtained, as well as use of the most susceptible stages (eggs and first instar), could result in improved determination of the likelihood that other transgenic rootworm products could meet the definition of a high dose product.

Question 3: Models

Simulation models are one of the tools used to evaluate IRM strategies to delay resistance. Assumptions in resistance models are based on aspects of pest biology including CRW survival and fitness. EPA has used predictive models to compare IRM strategies for Bt crops. Because models cannot be validated without actual field resistance, models have limitations and the information gained from the use of models is only a part of the weight of evidence used by EPA in assessing the risks of resistance development. It was the consensus of the October, 2000 FIFRA SAP that models were an important tool in determining appropriate Bt crop IRM strategies. They agreed that models were the only scientifically rigorous way to integrate all of the biological information available, and that without these models, the Agency would have little scientific basis for choosing among alternative resistance management options.

A) The Panel is asked to comment on the product duration or longevity of corn rootworm susceptibility considered in CRW IRM models.

The Panel concluded that for low/moderate dose plants, the 4 current models (Onstad

et al., Caprio, Andow/Alstad and Storer) were adequate for assessing the longevity of rootworm susceptibility if the initial frequency of major and minor resistance genes was as low as assumed by the models. The major limiting factor in predicting resistance is lack of data to initialize the models for the low/moderate dose plants, not the model frameworks themselves.

Except in certain cases, all of the current models predict that field failures due to resistance will not occur in 3 years, regardless of the use of refuges or the adoption rate. The only exceptions where rapid adaptation is expected are cases with: (1) high-dose with no refuge, and (2) any commercially effective dose when resistant genotypes are common in the population.

Under a considerable range of biological parameters for these low/moderate dose events (including 20% refuge, and rare initial resistance allele frequency), all of the models predict that resistance allele frequencies above 0.03 will not evolve in less than 10-25 years. In general, the time to field failure is predicted to be substantially shorter for moderate-dose cultivars than for high-dose events when a refuge is present. Several models identify conditions when resistance to a low/moderate dose event might evolve at a single locus in less than 10 years. These conditions are more common when SS fitness is low compared to RR fitness (e.g., <25%) and inheritance is additive. This may be the case for MON 863.

All of the current models indicate that the time to resistance does not differ substantially when the refuge size was varied from 10-25%. According to one model, only when refuge approaches 50% and higher does the refuge provide good assurance that resistance will be delayed substantially. Thus, a conservative IRM plan would have a refuge of at least 50%. This conclusion is relevant to refuge strategies that are discussed in the Panel's response to question 4A.

The Panel pointed out that while it is important to consider how long it will take for resistance to evolve and cause product failure; this is not the only important consideration in developing an interim resistance management plan. There is a greater concern for how much gene frequency will be increased during the interim period because any increase in resistance gene frequency in the interim period is expected to impact future resistance management options for MON 863 and other transgenic corn where genes for resistance to MON 863 result in cross resistance.

Finally, none of the models presented considered the presence of quantitative genetic variation and the potential presence of resistance alleles at relatively high frequencies. Until now, emphasis in resistance management research has been on examining high dose/refuge strategies. When considering events with high doses, only single genes that confer very high levels of resistance are expected to evolve. Therefore, all models have focused on one or two locus population genetic processes. For low/moderate dose plants, any gene that confers even slight resistance is expected to be favored by natural selection in the field. These genes with small effects are often common in populations and response to selection can be very rapid (Endler 1986, Falconer and Mackay 1996).

A complete risk assessment for low/moderate dose products should, therefore,

consider the possibility of heritable quantitative variation and the prospect of rapid evolution of resistance. The current models assume resistance is controlled by a single allele at a single locus starting at low frequency. For similar additive genetic variances and equal initial resistance allele frequencies, the single locus system generally results in the shortest time to resistance. However, if a population with multigenic resistance architecture that has intermediate allele frequencies is compared to a population with resistance that is controlled at a single locus that has a low initial frequency of resistance allele, resistance could evolve much faster in the polygenic case when the cultivar produces a low/moderate dose.

B) Considering EPA's evaluation of the three models addressed in the Monsanto submission, discuss the applicability of each of the models for assessing the likelihood of CRW developing resistance to Cry3Bb1.

Similar general issues were discussed by the Panel in response to this question as were already discussed in response to previous questions. However, the Panel felt that it was important to highlight certain factors that are important to consider when reviewing these models. The Panel felt that it was important to first describe general properties of resistance management models before discussing specific issues. The Panel then compared a number of model attributes that could have a bearing on the utility of the models in specific cases. The Panel concluded that for low/moderate dose PIPs, the four current models were adequate for assessing the longevity of rootworm susceptibility if the initial frequency of major and minor resistance genes was as low as assumed by the models. The differences in structure of the four models would be of more importance if the MON 863 were a high dose event.

Discrete versus continuous time models. All of the models that are being considered by the Agency are discrete time models, and this is appropriate for insect resistance management.

Individual-based versus frequency-based models. The individual based models are more flexible in dealing with plant genotype by insect genotype effects on development time and mating success. One Panel member differed, commenting that individual-based models are more mechanistic in dealing with stochasticity than frequency models.

Deterministic versus stochastic. The Onstad et al. model, the Monsanto version of Caprio's original model, and the Andow and Alstad models (these three will henceforth be referred to as the 3 models) are deterministic. In contrast, the Storer model is stochastic. Stochasticity can be particularly important when rare events are modeled. What is gained is: (1) occasionally the qualitative result is different (i.e. resistance evolves according to one model but does not evolve according to the other model) and; (2) a stochastic model can always provide an estimate of the variance associated with the output variables. Stochasticity does not add very much when an event is common (i.e. pest population size is large, so demographic stochasticity adds little insight, but adds considerable computer time) because there is very little variance in the expected result. The Panel concluded that given a deterministic resistance management model and one with well-considered use of stochasticity, it would be preferable to use the stochastic model with its variance estimates.

Uncertainty. A topic related to stochasticity is uncertainty. However, where stochasticity reflects the fundamental randomness of nature, uncertainty reflects the variation that can be anticipated because we do not know enough to allow more precise projections. Formally this can be handled in a Bayesian framework, establishing priors, and calculating posteriors mathematically or through MCMC methods. None of the three models or the Storer model do this. Uncertainty can also be addressed through less formal sensitivity analysis. A number of the models use this approach.

Space. The three models are non-spatial, patch models. Some researchers argue that these are spatial models, but in this case space is not explicitly modeled. A spatially explicit model has individual fields identified in a spatial array. The key question is what new questions can we address with an explicit spatial model? Anything related to field location (e.g., keeping the refuge in the same place; investigating the effects of not planting the refuge close enough; identifying effects of non-compliance; identifying potential hot spots for resistance) can be addressed only by a spatially explicit model. Individual-based, spatially explicit models that examine a large spatial region can address inbreeding at both the individual and population level. In some circumstances, explicit spatial models provide results that are qualitatively similar to the results from the non-spatial, patch models.

Space and stochasticity. There is a limit to the rareness of an event that can be modeled effectively in a spatially-explicit stochastic model.

Monogenic versus polygenic. As described earlier, the Panel concluded that consideration should be given to quantitative genetics models when a low/moderate dose product was being considered. Monogenic models are certainly appropriate for simulations of high dose products, and are probably appropriate for low/moderate dose products as well, but estimates of additive genetic variation should be made to test the validity of such an assumption.

C) Please comment on the appropriateness of the following input parameters of these simulation models for CRW-protected field corn: Resistance allele frequency, dominance of the heterozygote, movement of the males and females, mating and ovipositional behavior, and other genetic and behavioral parameters.

The Panel's review of the parameters are provided below.

Resistance allele frequency. The three models use initial resistance allele frequencies of 1×10^{-5} to 1×10^{-3} . For a low dose event, these values may be too low. While it is not clear what additional values should be considered without additional research, it is possible that frequencies up to 0.1 should be modeled. A sensitivity analysis should be weighted towards higher frequencies. Studies could be easily conducted to determine if the frequencies exceeded 10^{-2} . The Panel did note that resistance allele frequency alters the absolute time to resistance, but is less likely to impact the relative differences between different simulations for low/moderate dose plants. For example, all models will give the general result that a 50% refuge will delay resistance compared to a 20% refuge whether the initial allele frequency was 0.00001 or 0.1.

Dominance. The three models investigate different parameter ranges. Onstad et al. examined the highly recessive, additive, and completely dominant cases. Monsanto reported on the dominant case, and Andow and Alstad examined cases ranging from recessive (0.05) to partial dominance (0.80). As has been reported more generally, dominant inheritance gives faster resistance. The full range of dominance values should be examined, with less weight given to the extreme values. In addition, a quantitative model should be examined.

Movement of males and females. Movement of males prior to female mating could be important when considering a moderate dose, if inheritance of resistance was recessive. In general, the models are not particularly sensitive to pre- and post-mating dispersal parameters, probably because there were large numbers of survivors in both patch types and the additional influx of dispersing individuals had little impact on gene frequencies.

There appeared to be indications of differences between dispersal values for eastern versus western populations of corn rootworm, and research should address these differences.

Inbreeding, whether a result of individual non-random mating or non-random mating among populations, could be important for this species. Such inbreeding would tend to hasten resistance, if inheritance was recessive, especially at local levels. Estimation of inbreeding coefficients would be a valuable research project if resistance has a reasonable probability of being recessively inherited at the phenotypic level.

D) How does insecticide use in the refuge and/or Bt fields affect the predictions of time to resistance?

The Panel concluded that the use of adulticides in the refuge alone was clearly problematic. More research was needed on specific products in specific regions to determine if: 1) treatment of just the refuge with a larvicide, and 2) if treatment of both refuges and *Bt* fields simultaneously with adulticides or larvicides would have an impact on the rate of resistance development.

In general it was recognized that foliar insecticide use in refuges was likely to reduce the number of individuals emerging in these patches and hasten the evolution of resistance. It is also possible that the timing of insecticide application to a *Bt* field or a refuge could interact with the timing of emergence of susceptible and resistant beetles if the susceptible and resistant beetles emerge at different times. For example, if resistant beetles emerged later than susceptible ones, and an insecticide with adulticidal activity were applied early in emergence (e.g. for mite control), then the insecticide would select for resistance to *Bt*. Conversely, if the insecticide were applied late in the emergence period (e.g. for corn borer control), it would select against resistance. It was also emphasized that oversprays, applications applied to both *Bt* fields and refuges, could differentially impact populations in these two areas, which in turn could speed up or slow down resistance depending upon the interaction. The use of current soil insecticides in the *Bt* fields could either delay or hasten the evolution of resistant populations depending on how the *Bt* toxin and insecticide interacted in their effects on susceptible and resistant individuals. While this position is specific to the application of soil insecticides in fields with the corn rootworm PIP, the Panel provided more detailed comments on the use of soil insecticides

in the refuge in their response to question 4f.

The use of soil insecticides over long periods without the evolution of resistance was discussed as a potential model of the evolution of resistance to MON 863. Soil insecticides applied in 7 inch bands, in furrow, or a combination thereof have been used for more than 30 years without an outside structured refuge and without the development of resistance. It is assumed the mechanism to delay resistance to soil insecticides may be the large numbers of susceptible adults that emerge from insecticide treated fields. Under certain environmental conditions, beetle production has even been greater from insecticide treated corn than from the untreated control.

In other studies, adult production from insecticide treated fields is as low as 27% of the untreated control. The question then is whether adults produced in fields treated with soil insecticides have been exposed to a sublethal dose of insecticide or not. In an environment without insecticides, we know that older larvae move to newly emerged nodes of roots as they become available. This would bring them into the insecticide treated zone if the larvae were not repelled. While western corn rootworm larvae are able to detect and avoid organophosphates, no repellency was observed with pyrethroids or a carbamate (Hibbard and Bjostad, 1989; Woodson et al. 1999). Even in fields treated with organophosphate soil insecticides, root systems with significant root pruning are observed, but the level of insecticide found near the roots when the damage occurs is not known. Based on these data, one Panel member commented that many or most adults coming from fields treated with soil insecticides may have experienced a low dose of insecticide. This system may be particularly applicable to the low dose system of MON 863. If modeling efforts and empirical research could demonstrate the similarity between these two systems, the insecticide system might become a useful model for understanding adaptation to MON 863.

Question 4: Refuges

Refuges are planted to delay potential pest resistance to a Bt crop. Planting non-Bt corn within or near Bt corn fields will provide CRW offspring that will remain susceptible to the Cry3Bb proteins. The refuge should be structured to provide an adequate number of susceptible individuals that are available to mate with potentially resistant individuals and dilute resistance alleles in the field. Based on current information on CRW biology, MON 863 dose, simulation models, hybrid availability and adoption rate, a 20% refuge should be adequate on an interim basis to produce enough CRW adults to delay resistance. EPA has concluded that it is acceptable to plant refuges as continuous blocks or in-field row-strips. Based on the only available currently published paper, in-field strips should consist of at least 6 to 12 consecutive rows planted within 9 to 18 m of the center of the transgenic corn field.

EPA has concluded that a 20% refuge is adequate to delay resistance during a three-year period.

A) Please comment on whether this refuge strategy is adequate to delay resistance?

While the Panel concluded problems could exist with the proposed interim IRM plan, the majority of the Panel agreed a 50% refuge should be conservative enough to deal with these problems. In practice it may be tempting for growers to plant a refuge on fields previously planted to soybean because of reduced corn rootworm control costs. A mechanism should be in place to document prior crop history so that the refuge indeed produces adult beetles. The refuge should always be planted in ground that had a crop history in the past year similar to that of the *Bt* field.

The Panel agreed that simulation results from the presented models indicate that under conceivable parameter estimates, product failure due to resistance will not occur with MON 863 hybrids within three years unless the initial frequency of resistance alleles is higher than typically observed with Lepidoptera. The Panel further agreed that for the parameter estimates used in these models, field problems due to resistance development may take 10 to 15 years or even longer. Panel members agreed that there is a lack of empirical data needed to recommend a specific optimal refuge percentage. One model, which assumes completely dominant inheritance of resistance indicated the response curve for refuge is shallow for a low to moderate dose event. Other models found that increasing the refuge from 20% to 50% would at least double the time until resistance became common. Because the model structures and the strategies examined by the presented models were initially designed to examine high dose strategies, the Panel agreed that for low/moderate dose cultivars such as MON 863 there was a need to reexamine model assumptions of single locus control, and the biological parameter estimates used in the models. For example, there is currently significant survival on MON 863, so the Panel was in agreement that it is crucial to verify an assumption of all of the models that the vast majority of beetles currently produced from MON 863 are of a susceptible genotype.

The Panel differed on what percent refuge would be appropriate, conservative, and workable. The majority of the Panel members concluded that an appropriate, conservative, approach for an IRM plan would involve a refuge size of approximately 50%. Because important data are lacking and because grower adoption rates are likely to be low initially, these members viewed the 20% refuge as premature. While the 20% refuge is unlikely to result in field failures due to resistance within the interim period, it could, particularly in local areas, lead to a significant increase in resistance allele frequencies over this time. This increased frequency would limit future options for resistance management relative to Cry3Bb1 and any other toxin for which there was cross-resistance with Cry3Bb1. Panel members indicated that the choice of a 20% refuge for the interim plan was likely to limit choices of refuge size in the future because farmers and companies would not desire to increase refuge size. In addition, they concluded that there was no practical or scientific justification for establishing a precedent for a 20% refuge at this time.

A couple of Panel members of this group felt it would be prudent and feasible to follow the Australian approach in dealing with introduction of moderate dose Bt cotton. In this case, the Australians set the initial refuge size at 85% non-Bt cotton and decreased the refuge size by 5% per year until they came to a refuge size of 70%. The Australians now have a two toxin cotton plant to commercialize and are considering a substantially smaller refuge. There was

disagreement among Panel members about whether a conservative year- to-year phase in approach could work with MON 863. Most Panel members agreed that a 20% refuge will delay resistance compared to a 0% refuge, but more could be gained by going to 50% refuge. In addition, the modeling suggested that a 50% refuge would net at least twice the time to resistance as the proposed 20% refuge.

Other Panel members differed with the majority. A few Panel members were supportive of a 20% refuge. Their justification for supporting this figure was that it was compatible with the current refuge recommendation for Bt corn resistant to European corn borer, the 20% refuge amount would set the stage for IRM recommendations that would be compatible for both ECB and western corn rootworm, and it was noted that a simpler IRM strategy would be less confusing to growers, and ultimately would increase compliance. Supporting these Panel members was the view of the NCR-46 committee that “the proposed 20% refuge either within or adjacent to the transgenic field as acceptable during an interim registration period. Although there are no field data available to support a 20% refuge for a rootworm-protected transgenic product (or any other percentage), results of several resistance model simulations indicate that a 20% refuge can provide adequate product durability (i.e., about 15 years) if effective dose is something less than a high dose.” These Panel members noted that since the exact percentage of refuge is not crucial in a low dose event, an option that is likely to be preferred and adopted by growers may be best.

One Panel member emphasized data are not available to support a science-based recommendation to the EPA on this topic. Most of the empirical and theoretical work done on resistance management has focused on the high dose approach. Indeed the 1998 FIFRA SAP made a strong recommendation to the EPA that a high-dose/refuge strategy must be used for Bt crops. Any introduction of a low/moderate dose crop must be scientifically defended by data demonstrating that the reasoning of the 1998 Panel was incorrect. This Panel member noted absences of answers to the following questions about MON 863 that were needed for a basic scientific assessment:

- (1) What is the selection intensity on corn rootworm larvae from MON 863 in different regions/soils/moistures and at different densities?
- (2) What is the selection intensity on corn rootworm male and female adults from MON 863?
- (3) What is the selection on progeny through maternal effects?
- (4) What is the impact of using whole fields versus rows within fields as refuges on population dynamics and on percent of refuge beetles mating with resistant beetles from the Bt fields?
- (5) How would use of a seed mix impact selection intensity?
- (6) Are some of the surviving larvae on MON 863 more genetically tolerant of the Bt toxin than the general population?

- (7) What could we learn from a quantitative genetic model?
- (8) Is male/female movement different in different areas?
- (9) Can we develop appropriate monitoring strategies?
- (10) Can we develop appropriate mitigation strategies?

This Panel member believed that these questions could be answered by experiments conducted within a two-year time frame. Because the scientific community has no experience with low/moderate dose transgenic cultivars targeted at Coleoptera, this Panel member indicated that arguments could be made based on the current lack of data that no refuge at all was needed or alternatively that a 20% refuge would lead to field failures within 3 years. Thus, this Panel member stated that any decision made to accept the current plan could not be considered a science-based policy decision.

Overall, the majority of the Panel felt that even though there are limitations with the IRM plan, the experiments to address these questions should be conducted after commercialization. It was also pointed out that some of these experiments are already underway by members of NCR-46 and their associates.

The reason for commercializing MON 863 before conducting the above experiments is that significant benefits of the MON 863 technology over currently available options for growers would be lost if MON 863 were not commercialized. These benefits include:

- (1) Equivalent to or better than soil insecticides in terms of plant damage.
- (2) Reduced applicator, handler, and farm worker exposure to insecticides.
- (3) A narrow spectrum of activity could possibly eliminate or greatly reduce the environmental concerns generated by broader spectrum insecticides.
- (4) The technology is easy to use and does not delay planting.
- (5) The technology does not require special application equipment, the need for calibration, or the disposal/return of containers.
- (6) Performance consistency is improved since each plant is protected and this protection is relatively unaffected by weather.

B) Because the current plan being evaluated is based on limited data and is an interim plan, limitations to the total number of acres MON 863 might be considered. If so, should the limitations be on acres planted per state or per county or on another basis during the time an interim IRM plan is in place?

This question presumes that some cap or total amount that would be used. In question 4A, the majority of the Panel suggested that a higher refuge requirement would be preferred. It was the consensus of the Panel that any cap in the amount of acreage planted to MON 863 should be at the farm level (i.e. if such a cap were considered, it should be done with the refuge percentage required per farm, not at the county, state, or regional level). The Panel provided an example of crop rotation resistance to support their position. Crop rotation resistance originally occurred in a localized area of intensive crop rotation and then spread throughout the Corn Belt. This experience suggests that the local level is important for resistance development and that intensive local selection should be avoided.

One Panel member suggested that capping refuge at any level above the farm level would be difficult to document and regulate. Ultimately such regulation could alienate growers and could lead to increased levels of noncompliance. This Panel member suggested trusting the growers to be good stewards of the product would be a better approach in the interim. The level of grower compliance could be measured over a three year period after which a decision on limited acres planted could be made.

C) The Panel is asked to comment on the adequacy of in-field row-strips and/or immediately adjacent blocks to delay resistance during a three-year period and whether one method or another is preferred.

It was the consensus of the Panel that there was not sufficient data to support in-field strips over immediately adjacent blocks or vice versa to delay resistance during a three-year period. The use of in-field strips is strongly affected by source-sink dynamics and considerable knowledge regarding ovipositional behavior is required to design optimal widths. Certain modeling efforts seemed to indicate that strips were not preferred at certain refuge levels. However, the relatively small amount of movement by adults of these insects in all but the eastern Corn Belt might support the use of strips. It should be pointed out that the strips were randomly assigned in Onstad's model and the blocks were a fixed refuge. This is an important point in that the Storer model (Storer, in review) predicted that fixed refuges were better than randomly assigned refuges. The prediction of the Onstad model may be that strips are worse only because they are confounded with a randomly assigned refuge and that blocks are better only because they are confounded with a fixed refuge. The results of both of these models may therefore actually support a fixed refuge.

The Panel considered the inverse of the question as well; is there any scientific evidence to suggest that either in-field row-strips or adjacent blocks should be considered inadequate. The Panel agreed that the evidence suggested that neither could be considered an inadequate refuge at this time.

Overall, refuge design should include consideration of the amount and spatial distribution of the refuge plus the potential effect management practices may have on the abundance and relative phenology of susceptible beetles compared to resistant beetles. If phenology of resistant and susceptible beetles is not well synchronized (12-24 hrs), selected beetles are more likely to intermate than to wait for SS mate. This could effect resistance development if

resistance was phenotypically very recessive.

D) The Panel is requested to comment on the width of the in-field strips. As an example, the Agency is aware that at least 6 to 12 consecutive rows have been discussed in the following paper: Onstad, D. W., C. A. Guse, J. L. Spencer, E. Levine and M. E. Gray. 2001. Modeling the dynamics of adaptation to transgenic corn by western corn rootworm (Coleoptera: Chrysomelidae). J. Econ. Entomol. 94(2): 529-540.

It was the consensus of the Panel that data are currently not available to specify the number of rows that should be used in a strip. As noted above, in Onstad et al. (2001), blocks were set in fixed locations and strips were not. Storer (in review) also had fixed refuges working better than nonfixed refuges. It may not be that blocks are better than strips, but that fixed refuges are better than randomly located refuges. Strips may also have an advantage by increased mixing of susceptible and resistant adults.

The Panel clarified that in Onstad et al. (2001), the studied strips were 6-12 rows wide (rows more than 0.5 m apart). The strips are not 9-18 m from the center of the corn field as the EPA question #4 indicates. This is the distance from each Bt corn strip to refuge rows. One Panel member suggested four-row strips may be necessary for growers with small planters.

E) Please comment on EPA=s conclusion that alternate hosts should not be considered and refuges should only consist of non-Bt corn that are similar hybrids to the Bt corn.

It was the consensus of the Panel that alternate hosts should not be considered refuges at this time. The Panel noted that in one model (Storer, in review), if as few as 0.5% of the adults come from spatially, well distributed non-corn hosts, the onset of resistance would be significantly delayed in a system with a poorly distributed 5% fixed location refuge. This delay is not significant under more conservative refuge deployment scenarios. One Panel member agreed that at this time there are no data to indicate what percentage, if any, of the corn rootworm adults found in corn fields came from non-corn hosts as larvae. It was noted that northern corn rootworm may be more likely to come from non-corn hosts than the western corn rootworm.

Considerable discussion took place on whether the non-Bt corn in the refuge needed to be a similar hybrid or not. The majority on the Panel agreed that the refuge corn should be a similar hybrid. At least one Panel member thought that it could be helpful to have different maturity hybrids in the refuge in order to match the timing of production of beetles from the refuge with beetles from MON 863. The Panel agreed that, when possible, in order to encourage egg laying in the refuge for the subsequent year when blocks are chosen for a refuge, it should be encouraged that the refuge be planted at a later date than the MON 863 and in the same location as the current year. Two simulation models support keeping the refuge in the same place, and delayed planting encourages egg laying.

Finally, the Panel was in agreement that the issue of alternate hosts would need to be

revisited should MON 863 be stacked with herbicide resistance.

F) The Panel is requested to comment on whether, and if so under what conditions, insecticides could be used in the refuge.

It was the consensus of the Panel that soil insecticides and seed treatments targeted toward corn rootworms could be used in the refuge if significant numbers of adult beetles are still produced. This is the case with currently registered soil insecticides. However, if a highly efficacious insecticide that prevented significant adult emergence were to be used, this could have a major detrimental effect on IRM. Although the Panel noted that occasionally more adults are produced from fields treated with soil insecticides than the untreated controls, it was the opinion of the Panel that this likely occurred more in trap-crop situations with researchers than most grower situations. With successful trap crops, root damage can be extremely high and density dependent mortality increases. In other trials, adult production from insecticide treated fields is as low as 27% of the untreated control. It was noted that if, on average, half as many adults are produced from fields treated with soil insecticides, the effectiveness of the refuge in delaying resistance is reduced. An IPM approach of scouting the refuge fields for adults the previous summer should be strongly encouraged to minimize insecticide use to only when it is economically beneficial for the grower to do so. Finally, current in furrow applications of soil insecticides at the time of planting adequately protect the corn from damage while generating large numbers of adult beetles.

Insecticides should not be used for adult corn rootworm beetle control, whether intentional (targeted to reduce oviposition) or fortuitous (targeted at other foliar pests of corn such as spider mites, ECB, southwestern corn borer, etc.) unless it is applied to both refuge and transgenic areas equally. One Panel member noted that it might be helpful to spray only the MON 863. Most of the Panel agreed that this would be a viable option with adequate grower education, but under no circumstances should an adulticide be applied to only the refuge. In answering an earlier question, the Panel pointed out that it is not possible to determine the impacts on resistance development of most spray options until more research is conducted.

Question 5: Monitoring

A resistance monitoring strategy for Bt corn is needed to test the effectiveness of resistance management programs. Detecting shifts in the frequency of resistance genes (i.e., susceptibility changes) through resistance monitoring can be an aggressive method to detect the onset of resistance before widespread crop failure occurs. As such, the utilization of sensitive and effective resistance monitoring techniques is critical to the success of an IRM plan. EPA believes the mechanism of potential resistance of CRW to MON 863 should be determined to develop an appropriate long-term IRM strategy. EPA has concluded that CRW resistance is necessary to determine the mechanism and genetics of resistance to Cry3Bb1. Therefore, colonies resistant to Bt should be established and evaluated in the laboratory during the initial three years MON 863 is grown commercially.

Please comment on the Agency=s conclusions regarding refinements to Monsanto=s resistance monitoring program. In your response, please consider the following factors:

how should CRW resistance should be monitored; the value of developing resistant colonies of CRW to determine the mechanism and genetics of resistance; insect rearing for CRW spp. and whether one colony in more than one laboratory should be established.

The Panel agreed that a resistance monitoring strategy is needed to evaluate the effectiveness of resistance management programs. The Panel agreed that laboratory bioassays were too expensive to use routinely for monitoring populations, and suggested that a tiered monitoring system be developed. This tiered system would rely on less expensive, tier one monitoring methods to identify locations that would merit testing with the tier 2 laboratory bioassays. As discussed in Question 6, farm-based observations will play a crucial role in monitoring, but these must be supplemented by more sensitive tier two methods.

Establishing an initial baseline susceptibility to the active ingredient in question is needed to evaluate changes in susceptibility over time due to selection. It is now possible to evaluate susceptibility of neonate western corn rootworm larvae using a bioassay that measures death and growth of western corn rootworm larvae on artificial diets with varying doses of Cry3Bb1. This has been done with fourteen feral populations of the western corn rootworm. Thus, this may be the only method that will be available to document whether susceptibility is changing over time.

Although the Panel agreed that early detection of resistance is desired, they also agreed that current methods are not likely to have the sensitivity required. A number of factors complicate this issue:

- (1) MON 863 is a low dose event and some damage is expected.
- (2) Since corn roots support multiple individuals, the effects of resistant individuals on the overall root structure will not be easy to detect unless the resistant individuals are a significant percentage of the population.
- (3) The damage caused by corn rootworms is underground and not visible without destroying plants and washing roots.
- (4) Environmental factors play such a large role in the amount of damage done by these particular insects.
- (5) Above ground symptoms of damage, such as lodging, often have causes other than rootworm.
- (6) Because resistance usually emerges locally, it will be logistically difficult for one organization to sample in enough locations for early resistance detection.

(7) Even within fields sampled, the probability that plants damaged by resistant individuals would be selected for evaluation would be low at low populations of resistant individuals.

(8) Working with northern corn rootworms and Mexican corn rootworms is difficult and there are also difficulties with training and standardizing a subjective evaluation.

One alternative to the feeding bioassay with artificial diets for measuring susceptibility of neonate larvae to Cry3Bb1 would be evaluating corn lines that express varying levels of Cry3Bb1. For instance, MON 862 likely produces the endotoxin at higher levels than MON 863. MON 853 and MON 854 almost certainly express Cry3Bb1 at lower levels. Other events have also been tested, and a whole range of expression likely exists. Access to events with a range of Cry3Bb1 expression would allow mortality and growth data to be produced without some of the other mortality factors and mold problems seen in the bioassay with artificial diets. Because of the difficulties in feeding artificial diet to corn rootworm larvae, measuring mortality and growth from plant lines would also be much easier than measuring these responses from artificial diet and would allow a number of labs to collect these data rather than just one or two. In addition, susceptibility data could be collected with the northern corn rootworm and the Mexican corn rootworm, not just the western corn rootworm. No matter which method is used, it will also be important to direct early detection monitoring efforts to those locations where resistance risk is highest (i.e. the areas with the highest adoption rates of MON 863).

Another alternative is to observe root damage. Observations of rootworm damage to MON 863 reported to the Panel indicated that MON 863 damage is typically limited to root scarring and an absence of significant tunneling or pruning, which occurs on normal corn. This suggests that a method to evaluate root pruning might potentially be used as a monitoring tool. However, this methodology is only potentially useful and has not been tested or validated as a methodology that could be adopted.

Yet another possibility is to observe emergence time in MON 863. Reversion of the delayed emergence of beetles from MON 863 plots to an emergence similar to the refuge may indicate resistance. Emergence time could easily be evaluated in selected plots.

Finally, percentage of males emerging may be correlated with resistance. Susceptible populations emerge from MON 863 with a female biased sex ratio, while in non-Bt corn, the sex ratio is approximately half male. Emergence from MON 863 that is not female biased may indicate resistance. Research would need to be conducted to evaluate the reliability of any of these or other approaches. A few Panel members added that the ideas of NCR-46 would be applicable to determine what type of data should trigger expensive larval feeding bioassays.

Question 6: Mitigation/Remedial Action

Remedial action plans are a potential response measure should resistance develop to Bt crops. Since resistance may develop in Alocalized@ pest populations, it may be possible to contain the resistance outbreak before it becomes widespread.

There is a concern regarding Monsanto=s proposed outline of detecting and confirming resistance. Monsanto suggests that they will initiate mitigation measures when unexpected levels of CRW damage occur. However, Monsanto does not describe what is meant by unexpected levels of damage. Some level of damage is expected since there is not a high dose of MON 863 expressed to control the CRW and research has shown that some level of Agrazing@ will occur. Monsanto also suggested using a root damage rating scale to determine unexpected levels of damage. However, this method may not be appropriate for CRW protected Bt corn.

A) The Panel is requested to discuss an appropriate method of determining suspected and confirmed resistance for CRW including recommendations as to how suspected resistance or unexpected damage may be identified.

The Panel agreed that growers were likely to be the first to encounter unexpected rootworm damage manifested as lodged corn plants. When such damage is reported, a registrant representative should: 1) request the grower check planting records; 2) rule out damage from nontarget insects, weather, or other environmental factors (e.g., excessive weediness whereby western corn rootworm could complete partial development on grasses then move to transgenic corn); 3) conduct tests to verify MON 863 was planted and that the correct percentage of plants are expressing and; 4) if plants are MON 863 and damage approaching a 0.5 (node-injury scale) is found on any expressing plant, evaluate roots from the corresponding refuge. Damage to plants should equal or exceed damage to MON 863 plants even if the refuge was treated with a soil insecticide. If possible, larvae should be collected to verify that the damage is caused by the western corn rootworm, Mexican corn rootworm and northern corn rootworm. Only the species present at the time of sampling can be verified. In some cases, rootworm assisted lodging will occur after larvae have emerged as adults. Experienced workers can distinguish second and third instar southern corn rootworm from the other rootworm species under high magnification by the presence of morphological characteristics (i.e. two urigomphi). In any event, larvae should be identified using appropriate morphological or genetic characters. As an example, genetic markers are now available that can distinguish corn rootworm species. If the larvae causing the damage are greater than 0.5 on the node injury scale and determined to be western corn rootworm, northern corn rootworm or Mexican corn rootworm, and the field has had a history of MON 863 use, it would be identified as a suspected resistance, especially if the field has had a history of MON 863 use. Following these procedures, if resistance is still suspect, the registrant should confirm resistance.

The proposed definition of confirmed resistance is:

Progeny for the sampled pest population will be considered resistant if they exhibit both of the following characteristics in bioassays initiated with neonates:

- *An LC50 in a standard diet bioassay (incorporating the Cry3Bb protein) that exceeds the upper limit of the 95% confidence interval of the mean historical LC50 for susceptible pest populations, as established by the baseline measurements.*

- *Over 50% of Cry3Bb-expressing plants with one or more root nodes destroyed under controlled laboratory conditions.*
- *When available, a discriminating concentration bioassay will be employed to define confirmed resistance.*

The Panel agreed that the discriminating concentration bioassay might take a long time to develop, if it is developed at all. The Panel also agreed that rather than require both clauses, either clause would be sufficient to demonstrate confirmed resistance.

The Panel discussed an alternative criterion based on survival of the sampled population on *Bt* corn. A resistant population would have a survival rate on *Bt* corn not statistically different from the survival of an unselected population on non-*Bt* corn. The statistical resolution of the test should be set so that it is possible to separate survival of the susceptible population on *Bt* and non-*Bt* corn. Note this is a population rather than an individual approach to identify resistance. This definition should be revisited when field resistance is recovered.

The Panel discussed unexpected damage in the form of lodging and root tunneling. Low levels of plant lodging that growers would typically ignore could be the first visible sign of resistance development. In these cases, it would be necessary to rule out lodging due to other sources by comparing lodging levels found in transgenic fields with that of nearby nontransgenic fields. Preliminary research indicates that first instars usually do not tunnel into roots so such damage could be an indicator of resistance. The Panel recognized root tunnels may be difficult to detect, especially after feeding from later instars and tissue senescence alters the tunnels, but this should be explored further.

The Panel also considered time frame and sex ratios of adults emerging from MON 863 as an indicator of resistance, but generally agreed that many environmental factors would influence the emergence of adults and lessen the value of such information. Hence, it was not yet scientifically justifiable to use these measures to define resistance.

B) Please discuss whether root ratings are an appropriate indicator of suspected resistance. If so, how could a typical farmer use root ratings to identify suspected resistance.

Few growers will use root ratings to identify suspected resistance because digging corn roots is very laborious and this method requires training and experience to standardize evaluations. Crop consultants and extension personnel, however, are expected to use the root rating method. In these cases, some Panel members recommended that the node root (0-3) scale should be used.

GENERAL COMMENTS

Panel members provided additional comments that further refine responses noted above or were not addressed in the Agency's charge.

Critical research on Western Corn Rootworm for developing a scientifically based resistance management plan

Estimate selection intensity

A measure of selection intensity will require estimation of the fitness of susceptible individuals exposed to *Bt* corn compared to the fitness of such individuals when exposed to an isolate that does not produce the *Bt* toxin. There are a number of ways to experimentally assess this selection intensity. In-field assessments are preferred. Experiments must be conducted in a number of locations with differing soil and growing conditions, with at least one eastern and one western location given the observation that there are behavioral, and potentially physiological, differences between beetles in these two areas. Overall fitness includes a number of fitness components such as larval survival and growth rate, pupal survival, male mating success, female mating success and lifetime fecundity, and offspring quality as influenced by maternal effects. It may be appropriate to design separate experiments to determine selection intensity on the various components of fitness because the optimal spatial scale of the field experiments may differ. For example, larval survival and growth rate are measurable on a small plot scale. However, to measure the impact of delayed larval development on the mating fitness of males could require carefully designed experiments in which marked males of varied emergence times would be individually monitored for mating success in corn fields with a natural population of males and females.

Without preliminary experiments, it is difficult to predict the level of accuracy and precision that can be attained in estimating selection intensity. It would be advisable to conduct such preliminary experiments. Error around the expected final estimate would negatively affect the accuracy of model predictions of time until a population becomes resistant.

Evaluation of the present status of resistance

When assessing the present status of pest resistance to crops that have a high dose relative to the tolerance of the target pest species, it is appropriate to estimate the initial frequency of alleles of major genes that confer high levels of resistance to a pest individual. In dealing with a low/moderate dose crop cultivar such as MON 863, assessing the present status of resistance requires a different approach because both major and minor genes can have an impact on the current and future status of resistance.

An approach that can be used for this assessment has been used by plant and animal breeders over the past 80 years. This involves estimating the additive genetic variation in the pest population for resistance to the toxic cultivar. One method for approximately estimating this additive genetic variance that should be adequate and feasible with the western corn rootworm involves experiments to measure the selective intensity (S) and the subsequent response (R) to selection. Specifically, $V_A \propto R/S$.

The design of these experiments would basically involve paired plantings of *Bt* corn and non-*Bt* isolines in block designs, with replication over sites as in experiments aimed at estimating

selection intensity. Each site should be selected to have a generally uniform distribution of egg clumps, but with varied densities among sites. This will give added precision but will also estimate affects of environment/density on the estimates. Adults emerging from the *Bt* and non-*Bt* plots must be collected and mated to other adults from the same treatment (but optimally not the same replicate). Offspring of these adults must then be evaluated to determine if there is a difference between treatments in the fitness of this next generation. The experimental setup is in part similar to that for evaluating selection intensity. Indeed, experiments to determine certain components of selection intensity can be combined with work to evaluate response to selection.

In addition to providing an estimate of current additive genetic variance, the measurement of fitness of the offspring could reveal whether major or minor genes were contributing to resistance status.

Impacts of spatial scale of refuges

When dealing with high dose crops it is clear that the spatial scale at which the *Bt* and non-*Bt* crops are planted could influence the rate of adaptation. It is typically important to have the scale large enough so feeding can occur on one type of crop, and small enough so that there is mating of insects from *Bt* and non-*Bt* areas. For low/moderate dose cultivars it is not clear that these findings are applicable. With moderate dose crops, the best spatial scale is likely to be pest specific and cultivar specific. We therefore have no way to judge the most useful refuge scale for MON 863 without experimental evidence.

Appropriate experiments will differ depending on the scales being compared. A comparison of seed mixtures and the larger refuge patches that do not allow larval movement between *Bt* and non-*Bt* plants would require estimation of selection intensity on pest individuals in plantings at the two types of spatial scales. (Narrow row strips may be an intermediate scale, but it would be best to first determine differences between the more extreme spatial scales). These experiments could be worked into a larger field design that broadly examines selection intensity to larval host.

For high dose cultivars, experiments have examined the impacts of spatial scales that could differentially influence adult movement between crop types. These experiments have focused on both premating and postmating movement. For low/moderate dose crops such as MON 863, the Panel has indicated that post mating (preoviposition) movement is likely to be the most important parameter that could influence choice of spatial scale. The experimental design for determining the optimal scale would involve monitoring movements and behaviors of beetles to determine the maximum spatial scale that would still allow sufficient mixing of refuge produced beetles with beetles selected by MON 863. The data on movement and distribution of progeny by female adults should be modeled to determine the degree of mixing between selected and unselected individuals that contribute to the following year's population.

Experiments on movement should definitely be conducted in the eastern and western regions because adult movement is thought to differ in the two areas.

Monitoring methods

There are two factors that require the development and implementation of monitoring programs for the MON 863 system that differs from other *Bt* resistance monitoring programs: (1) the lack of a high dose and (2) the difficulty of conducting bioassays with these soil dwelling insects.

There is a critical need for research to develop monitoring approaches that will efficiently estimate any changes in the resistance status of this pest. A number of potential approaches to monitoring were discussed by the Panel. However without substantial research, it is impossible to know which if any of these approaches would be efficient and feasible.

One of the methods that should be examined is the conventional approach of determining changes in LC50 values. Other more novel approaches include: 1) estimation of the number of *Bt* plants in an infested stand that show root boring activity; and 2) estimation of the degree of difference in sex ratio between beetles emerging from paired *Bt* and non-*Bt* plantings. It is difficult to give guidance on details of how to develop and test these and other approaches. However, it is important to provide evidence that a monitoring method will detect resistance in time to alter a resistance management plan and preserve the efficacy of the *Bt* cultivar.

Mitigation strategy

It is expected that resistance will first be detected locally. The present mitigation strategy does not specify the localness of the mitigation response. Development of a mitigation strategy will require research on approaches that could eliminate beetles within a localized area where resistance is detected before such beetles migrated away from such areas and infested a wider area. In some ways western corn rootworm biology that includes limited movement of adults, monophagy, and an immobile overwintering stage may make mitigation plans more feasible than for other pests (ceasing the planting of any corn in the affected area could cause the death of all newly hatched larvae.) However, research is needed to demonstrate that such a plan would be both biologically and socially feasible.

Other research areas for consideration

(1) There is a need to evaluate long-term response to selection for polygenic resistance. Adults should be collected from MON 863 plots in emergence cages from several geographically distant sites and allowed to mate and be used to establish lab colonies. Similar colonies should be started from nearby non-transgenic fields. MON 863 and a similar non-transgenic corn plant grown in greenhouses should be used as hosts of larvae from MON 863 and non-transgenic colonies. Every generation of the MON 863 and non-transgenic strains should be evaluated for performance on both MON 863 and a similar non-transgenic corn. At a minimum, growth rate, development, mortality and fecundity should be measured. Several generations should be reared on MON 863 to determine if continuous exposure to MON 863 will select for polygenic resistance in the MON 863 strains.

- (2) Determine the interaction between rotation resistance and *Bt* resistance management strategies.
- (3) Study IPM approaches for achieving the maximum benefits from MON 863.
- (4) Assess if there is differential expression of the *Bt* toxin in specific types of root tissues and cell types because this could affect selection intensity on the rootworm.
- (5) Investigate novel low/moderate dose resistance management approaches that do not involve use of refuges.
- (6) Research to determine resistance management strategies for use of corn cultivars with both rootworm and corn borer toxicity.
- (7) Study if the optimal resistance management plan for MON 863 is affected by introduction of corn cultivars with other rootworm specific toxins.
- (8) Research on biological attributes of the northern corn rootworm and Mexican corn rootworm that are relevant to resistance management of MON 863.
- (9) Investigate if the use of MON 863 could cause a shift in the insect pest community on corn.
- (10) Research to determine if the availability of a stacked corn cultivar with ECB and rootworm toxicity will lead to overuse of either of the proteins toxic to the noted insects.

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