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RESEARCH PLAN: EFFECTS OF CHEMICAL HERBICIDES AND GENE FLOW ON NON-TARGET PLANTS

Pesticides Research Project
National Health and Environmental Effects Research Laboratory
Western Ecology Division
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Corvallis, Oregon 97333

To address the needs of OPP and OPPT, EPA's Office of Research and Development (ORD) is conducting research and fostering the sound use of science and technology to provide scientific information to facilitate health and ecological risk assessments. ORD has developed a multi-year plan (MYP) to establish long-term research goals and to coordinate research among different research laboratories and centers concerning health and ecological effects from pesticides and genetically engineered plants. Under the ORD MYP, the National Health and Environmental Effects Research Laboratory, Western Ecology Division (WED) in Corvallis, will identify and understand the specific terrestrial effects of pesticides (focusing on herbicides) and genetically engineered plants.

Within WED, the Pesticides Project has been established to develop tools to improve EPA ecological risk assessments for use chemical herbicides and genetically engineered plants. With these tools, ecological risk assessments will be better equipped to predict potential effects of chemical herbicides and engineered plants upon important agricultural and ecological endpoints, i.e., for agricultural ecosystems, crop quality and yield; and for non-agricultural or native plant ecosystems, ecosystem structure and function, especially as they relate to wildlife habitat and the viability of threatened and/or endangered species. The Pesticides Project has developed this Research Plan *"Effects of Chemical Herbicides and Gene Flow on Non-Target Plants"* which identifies three research goals relating to terrestrial ecosystems to address ORD's long-term goals and OPP and OPPT's ecological research needs. These goals are to: *1) determine ecological effects of gene flow from transgenic crops, 2) develop regional analysis and interpretation tools, and 3) determine effects of chemical herbicides on non-target crops and native plants.* The research to address these goals is described in three strategic components of the Research Plan: *Regional Analysis and Interpretation, Effects of Chemical Herbicides on Terrestrial Plants, and Ecological Effects of Gene Flow from Transgenic Crops.*

The **Regional Analysis and Interpretation Research** will develop a system to collect, analyze and interpret data for use in the Problem Formulation and Risk Characterization phases of assessing risks from chemical herbicides and GM crops. Data and model components will be obtained through collaborative efforts with federal, state and local agencies, as well as industry as feasible. The analysis will use Geographic Information System (GIS) to carry out the assessments on a regional basis. The GIS research will provide tools for spatially locating plant species potentially at risk from use of a new product, as well as phenology (e.g., timing of occurrence of developmental events during plant life-cycle, such as flowering) of non-target plants relative to timing of pesticide application. The system will provide a basis for selecting appropriate test species and response endpoints for risk assessments. The GIS platform will then be used to characterize risk, by combining exposure models and relevant plant response data in a probabilistic framework. As part of this regional analysis effort, plant responses relevant to ecosystems (e.g., species composition, productivity) will be recorded as possible input parameters for wildlife habitat models that are being constructed by the WED Terrestrial Habitat Project.

The **Effects of Chemical Herbicides on Terrestrial Plants Research** will develop methodologies to test effects of chemical herbicides on individual terrestrial plant species and communities. It will use outputs from the regional analysis research to identify crop and native plant species, herbicides, and herbicide treatment (e.g., timing, concentrations) conditions important for specific areas of the United States. Experimental protocols for terrestrial plant tests will be refined for application to nontraditional species (e.g., perennials or woody species) and response endpoints (e.g., seed yield or other reproductive or developmental parameters). Research will also be conducted to develop molecular or cellular tools to extrapolate responses to non-tested species or to verify field exposures. The plant test research will focus on low-dose, high-potency herbicides, but also use some high-volume compounds. The protocols developed for chemical herbicide testing will also be adapted for use with other industrial chemicals.

The **Ecological Effects of Gene Flow from Transgenic Crops Research** will develop molecular methods to detect the presence of transgenic or other marker genes, evaluation of gene flow from engineered to non-engineered plants, measurement of potential ecological effects of gene flow on plant community structure and function, and definition of inputs for a prototype model for gene flow. Experiments will be conducted at various scales, ranging from contained laboratory, to growth chamber or greenhouse, to field. Current advances in genomics and proteomics will be evaluated for their ability to identify potential adverse effects of gene flow in agronomic and non-agronomic ecosystems.

Overall, this research project will provide tools to assist EPA in its regulatory role in registration of chemical herbicides and genetically engineered crops that produce chemical pesticides; thereby promoting sustained productivity of agricultural crops while maintaining the ability of ecosystems to support wildlife and to carry out other essential services. The tools also will aid post-registration monitoring to determine the success of registration restrictions in protecting non-target crops or native plants. Furthermore, the will be useful for determining ecological effects of other chemical pesticides and industrial chemicals.

Disclaimer

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Cover Photos: Upper left- greenhouse experiments with crops for herbicide toxicity test development, upper right- Geographic Information Systems analysis of counties with risk from non-target herbicide drift, lower left- contig assembly showing the automated sequencing chromatogram trace DNA data, lower right- wild mustard growing adjacent to agricultural fields

EXECUTIVE SUMMARY

This project supports EPA's mission to protect human health and to safeguard the natural environment — air, water, and land — upon which life depends. Specifically, we address EPA's responsibility to prevent pollution and reduce the impacts from pollution to communities and ecosystems (Government Performance and Results Act (GPRA) Goal 4, "Safe Communities"). To achieve this goal, EPA's Office of Prevention, Pesticides, and Toxic Substances (OPPTS) requires scientifically credible information and methods for use in assessing health and ecological risks from products used in commerce, including chemical pesticides and genetically engineered plants. OPPT regulates chemical and biological pesticides primarily under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) administered through the Office of Pesticide Programs (OPP). Other acts and programs, especially the Toxic Substances Control Act (TSCA), and the Federal Food, Drug and Cosmetic Act (FFDCA) are administered by OPPTS's Office of Pollution Prevention and Toxics (OPPT) to provide for protection of the environment from chemicals and biological pesticides. In the past, protection of ecological resources has received minimal attention under these regulations compared to concerns regarding impacts on human health. Recently, however, awareness of adverse effects from drift of new low-dose high-toxicity herbicides to non-target crops and native vegetation has heightened awareness of the need to improve tests for effects of chemical herbicides to plants. Similarly, public concern regarding the release of genetically engineered plants and the adoption of the "Final Rules and Proposed Rules for Plant-Incorporated Protectants" (40CFR Parts 152 and 174) have increased the need for tools to evaluate the risks from engineered plants and gene flow from engineered crops to other plant species. Thus, OPP and OPPT need tools to assess ecological risks from transgenic crops, improved methods for spatially explicit ecological risk assessments, new methods to provide for efficient and effective gathering and interpretation of herbicide hazard identification and dose-response data, and investigations of the potential effects of high priority hazards.

To address the needs of OPP and OPPT, EPA's Office of Research and Development (ORD) is conducting research and fostering the sound use of science and technology to provide scientific information to facilitate health and ecological risk assessments. ORD has developed a multi-year

plan (MYP) to establish long-term research goals and to coordinate research among different research laboratories and centers concerning health and ecological effects from pesticides and genetically engineered plants. Under the ORD MYP, the National Health and Environmental Effects Research Laboratory, Western Ecology Division (WED) in Corvallis, will identify and understand the specific terrestrial effects of pesticides (focusing on herbicides) and genetically engineered plants.

Within WED, the Pesticides Project has been established to develop tools to improve EPA ecological risk assessments for use chemical herbicides and genetically engineered plants. With these tools, ecological risk assessments will be better equipped to predict potential effects of chemical herbicides and engineered plants upon important agricultural and ecological endpoints, i.e., for agricultural ecosystems, crop quality and yield; and for non-agricultural or native plant ecosystems, ecosystem structure and function, especially as they relate to wildlife habitat and the viability of threatened and/or endangered species. The Pesticides Project has developed this Research Plan *"Effects of Chemical Herbicides and Gene Flow on Non-Target Plants"* which identifies three research goals relating to terrestrial ecosystems to address ORD's long-term goals and OPP and OPPT's ecological research needs. These goals are to: *1) determine ecological effects of gene flow from transgenic crops, 2) develop regional analysis and interpretation tools, and 3) determine effects of chemical herbicides on non-target crops and native plants.* The research to address these goals is described in three strategic components of the Research Plan: *Regional Analysis and Interpretation, Effects of Chemical Herbicides on Terrestrial Plants, and Ecological Effects of Gene Flow from Transgenic Crops.*

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and response endpoints for risk assessments. The GIS platform will then be used to characterize risk, by combining exposure models and relevant plant response data in a probabilistic framework. As part of this regional analysis effort, plant responses relevant to ecosystems (e.g., species composition, productivity) will be recorded as possible input parameters for wildlife habitat models that are being constructed by the WED Terrestrial Habitat Project.

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Overall, this research project will provide tools to assist EPA in its regulatory role in registration of chemical herbicides and genetically engineered crops that produce chemical pesticides; thereby promoting sustained productivity of agricultural crops while maintaining the ability of ecosystems to support wildlife and to carry out other essential services. The tools also will aid post-registration monitoring to determine the success of registration restrictions in

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ACKNOWLEDGMENTS

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1 PROJECT GOAL AND OBJECTIVES

The Pesticides Research Project at the Western Ecology Division (WED) supports EPA in its responsibility to prevent and reduce the impacts from pollution to communities and ecosystems (Government Performance and Results Act (GPRA) Goal 4, "Safe Communities"). To accomplish this, we will meet critical research needs identified by two offices within EPA's Office of Prevention, Pesticides and Toxic Substances (OPPTS), specifically the Office of Pesticide Programs (OPP) and Office of Pollution Prevention and Toxics (OPPT), as necessary to provide scientific support for ecological risk assessments for proposed or existing plant pesticidal products. The four research needs as shown in the Project Critical Path (Figure 1.1) are to:

1. assess ecological risks from transgenic crops,

2. improve methods for spatially explicit ecological risk assessments,

3. develop new methods to provide for efficient and effective gathering and interpretation of herbicide hazard identification and dose-response data, and

4. investigate potential effects of high priority hazards.

Research to address these needs is important as there are limited methods or approaches available for OPP and OPPT to assess ecological risks associated with movement and expression of novel genetic material from genetically engineered crops. Furthermore, there is a need to evaluate potential risks for herbicides and genetically engineered crops in spatially explicit and probabilistic modeling frameworks that go beyond the more traditional deterministic framework for risk assessments. Both OPP and OPPTs need targeted test development for improved hazard identification and to obtain hazard dose and plant response data. Even though there are standard methods available to OPP and OPPT to test the effects of most herbicides on crops, OPP and OPPT need additional tests to evaluate herbicide effects on non-target, non-crop plants, and to determine the effects of high-priority hazards to both non-target crops and non-crop plants such as low-dose, high-potency herbicides.

To meet OPP and OPPT's needs EPA's Office of Research and Development (ORD) has prepared a multi-year plan (MYP) to address GPRA Goal 4. This plan identifies Long Term Goals (LTG) for ORD research, and explains how research will be coordinated among ORDs laboratories and centers. WED has developed this research plan to assist ORD in achieving two LTG (Figure 1.1). We address LTG # 3, "To provide OPPTS with the scientific underpinnings for guidance to prevent or reduce risks of human environments within communities, homes, workplaces and to assess risks of biotechnology to ecological systems," which will improve risk assessments for transgenic crops (OPP and OPPT needs 1 and 2). We also address LTG 4 #, "To provide OPPTS with strategic information and advice concerning novel or newly discovered hazards," which will improve risk assessments for chemical herbicides and potentially other chemicals (OPP and OPPT needs 2, 3 and 4).

WEDs Pesticides Research Project has developed this Research Plan *"Effects of Chemical Herbicides and Gene Flow on Non-Target Plants"* which identifies three unique research goals relating to terrestrial ecosystems which will address ORD's LTGs and OPP and OPPTs ecological research needs (Figure 1.1). These goals are to:

1. determine the effects of gene flow from transgenic crops,

2 develop regional analysis and interpretation tools, and

3. determine effects of chemical herbicides on non-target crops and native plants.

For Goal 1, we will develop molecular biology and genetic methods to measure gene flow and ecological consequences to determine risk in a spatially explicit landscape construct. For Goal 2 we will improve models to determine ecological risks of herbicide use and gene flow from transgeneic plants to other plants in a spatially explicit landscape. For Goal 3 we will produce comprehensive and efficient *in vivo* assays to evaluate adverse effects of chemical herbicides at critical plant life stages, and will develop new approaches for tier testing, including methods for native plant species

WED Pesticides Project also will support EPAs GPRA Goal 4 to provide "Safe Communities", not only by providing tools for initial ecological risk assessments for registration

of chemical herbicides and transgenic crops, but also by providing tools useful in post-registration monitoring. The pesticide and gene flow research will advance other research at WED which supports GPRA Goal 8 "Sound Science," by increasing EPAs ability to assess, improve, and restore the integrity and sustainability of ecosystems over time. At WED, the Pesticides Project complements the Terrestrial Habitat Project, which is developing spatially explicit models to evaluate the risks to wildlife populations resulting from changes in landscape structure and habitat quality, including the use of herbicides.

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Pesticides Project Critical Path

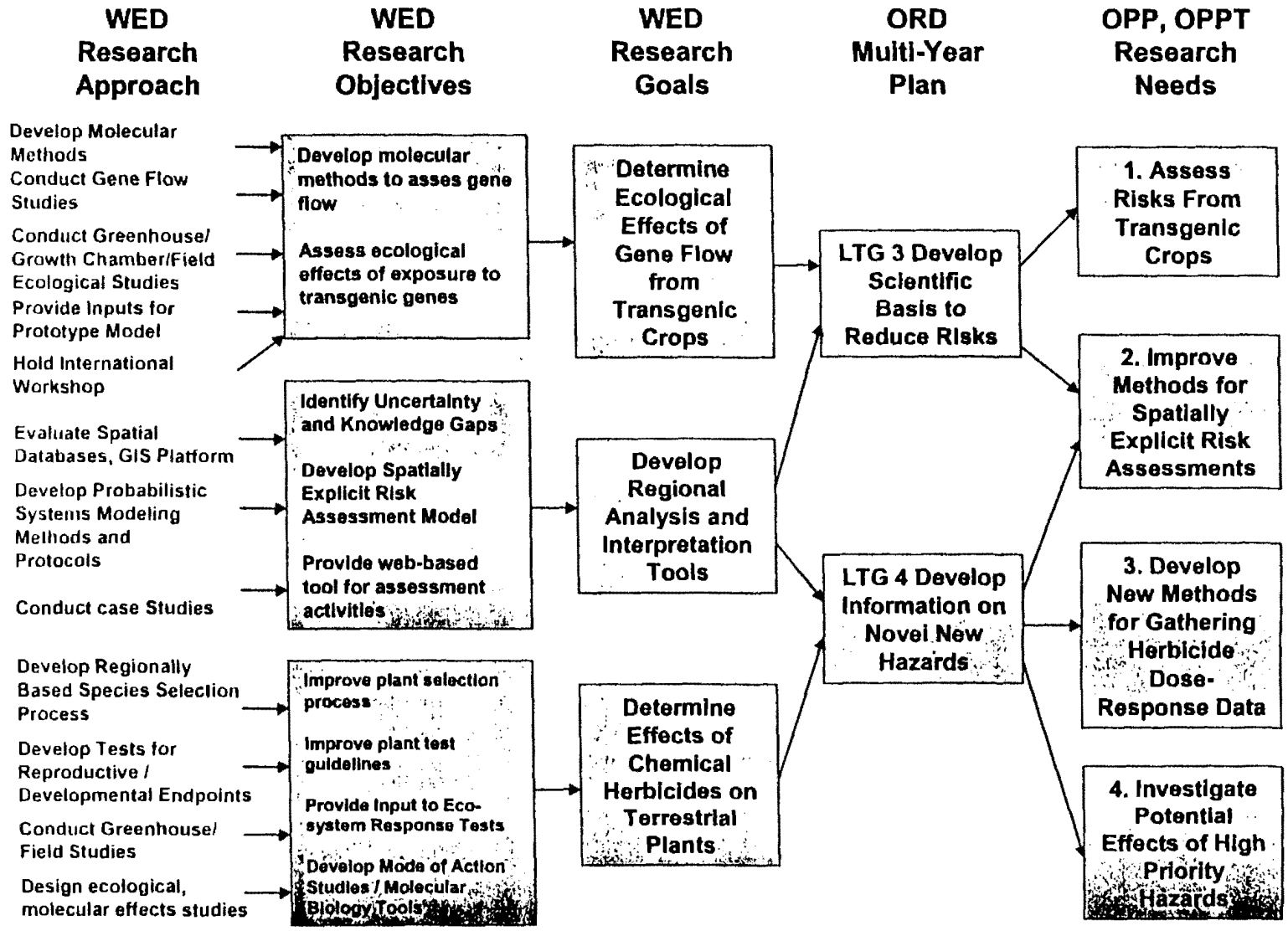

Figure 1-1 Critical path of Pesticides Project to meet research needs of Office of Pesticide Programs (OPP) and Office of Pollution Prevention and Toxics (OPPT). It describes Office of Research and Development (ORD) multi-year plan goals, Western Ecology Division (WED) research goals, and the objectives and approach for WED to address the goals and meet OPP and OPPT needs. The project has three components corresponding to WED goals.

2 GENERAL PROJECT OVERVIEW

2.1 Regulatory Authority and Responsibilities for Control of Chemical and Biological Pesticides

EPA's mission to protect human health and to safeguard the natural environment — air, water, and land — upon which life depends. EPA is responsible for protecting human health and ecosystems, by enforcing government laws which limit the release of pollutants produced by human activities into the environment. EPA's Office of Prevention, Pesticides, and Toxic Substances (OPPTS) regulates chemical and biological pesticides under the authority of the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), administered by the Office of Pesticide Programs (OPP). Other chemicals and biological materials are regulated under the authority of the Toxic Substances Control Act (TSCA), Federal Food, Drug and Cosmetic Act (FFDCA), Pollution Prevention Act (PPA), and the Residential Lead-Based Paint Hazard Reduction Act administered by the Office of Pollution Prevention and Toxics (OPPT). The OPPT also manages programs concerning new and existing chemicals in the marketplace, asbestos, lead, PCB's, and other areas.

Within OPP, it is responsibility of the Ecological Fate and Effects Division (EFED) to evaluate the potential ecological risks of pesticides (including those produced by genetically engineered crops) during the product registration process, and the responsibility of the Biopesticides and Pollution Prevention Division (BPPD) to evaluate ecological risks of biological pesticides. Within OPPT, the Risk Assessment Division (RAD) evaluates the risks of toxic substances.

In the past, OPP and OPPT had relatively fewer tools to protect ecological resources compared with those available to protect human health. However, awareness of adverse effects from drift of new low-dose high-toxicity herbicides to non-target crop and native vegetation has heightened awareness of the need to improve tests for effects of chemical herbicides (and other industrial chemicals) to plants. These tests would be useful not only in the pesticide or chemical registration processes, but also for post -registration monitoring. At the same time, public concern regarding the release of genetically engineered plants and the adoption of the "Final Rules and Proposed Rules for Plant-Incorporated Protectants" (PIPs) (40CFR Parts 152 and

174) have emphasized the need for new tools to evaluate the risks from chemical compounds and genetically engineered plants in terrestrial ecosystems.

Development of the appropriate test methods and risk models for use by program offices historically has been done by EPA's Office of Research and Development (ORD). The National Health and Environmental Effects Research Laboratory (NHEERL) is ORD's focal point for scientific research on the effects of chemical compounds and genetically engineered plants on human health and ecosystems. NHEERL also is ORD's lead laboratory for development of a multi-year plan under GPRA Goal 4 to formulate and conduct research to address key concerns regarding chemical compounds and genetically engineered plants, including both ecological and health effects research. Other components of ORD, such as the National Center for National Exposure Research Laboratory (NERL), Environmental Assessment (NCEA) and National Risk Management Research Laboratory (NRML) also contribute to the Goal 4 research in areas of fate and transport of chemicals, environmental assessment and environmental remediation, respectively. The Western Ecology Division (WED) in Corvallis is the component of NHEERL charged with identifying and studying potential effects of chemical and gene flow from engineered crops to other plant species, through its Pesticides Research Project.

2.2 Risk Assessment Needs

To determine potential environmental impacts of regulated pollutants (including chemical and biological pesticides), EPA developed The Ecological Risk Assessment Framework (US EPA, 1992, 1998) (Figure 2.1). The framework has three main components:

Problem Formulation Phase. During this phase, EPA managers meet with interested parties including risk assessors, risk managers, scientists, industry and the public to articulate the problem, define the scope of the problem, and develop a plan to characterize and manage the potential risk of the pollutant (or other environmental stress).

Analysis Phase. During this phase, aspects of the pollutant exposure and the resulting effects on target organisms and ecosystems are evaluated. Both exposures and effects are characterized quantitatively and the complex relationships between exposure and effects are determined.

Risk Characterization Phase. In this phase, the exposure and stressor response (effects) profiles are integrated to estimate the risk from different levels of exposure. Models are used to integrate exposure and effects data. The final product is a description of the likely risk along with a description of key assumptions, scientific uncertainties, and strengths and limitations of the analyses and characterization activities.

EPA's risk assessment process is iterative: as new data are acquired they are used to strengthen the science to inform decisions, and as analysis and risk characterization occurs new hypothesis and experiments become evident. The assessment results are communicated to risk managers who develop a plan to manage the risk and communicate the results to interested parties. A few examples of locations where the risk assessment approach was used to understand potential impacts to ecosystems from a range of stressors such as sediments and organic compounds include the Cordorus Creek Watershed in Pennsylvania (Obery and Landis, 2002), Darby Creek Watershed in Ohio (Cormier et al., 2000), Clinch River/Poplar Creek System in Tennessee (Cook et al., 1999) and Fjord of Port Valdez in Alaska (Wiegers et al., 1998).

There is a critical need for information with which to conduct similar risk assessments of ecological effects from chemical pesticides and genetically engineered organisms (Fairbrother and Kapustka, 2001; Taylor, 2001; Peterson et al., 2000; Landis et al., 2000). Through the research described in this plan we will contribute information and develop tools to improve all phases of the ecological risk assessment project (Figure 2.1):

Problem Formulation Phase. The project will develop tools for spatially explicit models and methods (Geographic Information System or GIS-based) for determining which non-target crops and native plants might be exposed to off-site drift of a proposed pesticide. The spatial analysis will be in a regional context, so that species to be considered in the risk assessment are pertinent to the geographic location where the crop/pesticide combination is likely to occur. It also will develop the phenological relationships between timing of pesticide application(s) and life-history patterns of non-target plants. These relationships will provide a rational basis for requesting dose-response information on particular species and endpoints. Similar concepts

apply to gene flow; i.e., geographic areas in which genetically engineered crops are likely to outcross to compatible native plants need to be identified.

Analysis Phase. The project will determine whether existing plant test protocols are suitable for non-target annual herbaceous and also for perennial or woody species; and non-traditional endpoints such as tuber formation, fruit set, and yield also will be evaluated. Where existing test protocols are inadequate, new protocols will be developed and tested to provide the capacity to obtain dose-response information on regionally important non-target species. On a broader level, chemical herbicide effects need to be extrapolated from the small number of crop plants usually tested, to species that have not been tested. Given the large number of species in the plant kingdom, it is not possible to test all directly. Species-to-species extrapolations can be improved based on cellular and/or molecular mechanisms of action for representative species of most plant groups. Similarly, few tools exist to measure, quantify and determine non-target ecological effects of gene flow from crops to native plant species. We will conduct research to assist in development of such tools to study ecological effects of gene flow.

Risk Characterization Phase. The spatially explicit, probabilistic modeling framework also will be used to characterize risks to non-target plants from off-site drift of pesticides and movement of genetically modified genes. This modeling framework will incorporate measures of drift (e.g., AgDRIFT model for chemicals and new models for GM crops) with the dose-response information developed in the Analysis Phase work. It will include stochasticity in exposure parameters (including input variable such as wind speed) and variability in response functions to develop probability bounds on risk outputs. Probability distribution functions will be assigned to each source of uncertainty and variability in the data and knowledge bases in order to fully assess the sensitivity of the output response to perturbations in the input data.

Similarly, potential movement of novel transgenes from genetically engineered crops to adjacent crops or native vegetation depends upon close phylogenetic, geographic and phenological relationships (e.g., timing of occurrence of developmental events during plant life-cycle such as flowering) between crop and native plants. Gene flow can occur when pollen is disseminated by wind or by pollinators to compatible recipient plant species. The GIS platform will be useful for determination of co-location of related plant species and phenological

relationships such as the timing of flowering of sympatric populations of weedy, native or crop species that may be compatible with genetically engineered crops.

All of the tools developed in this project will help give risk managers the data they need to determine the level of protection which they want to put in place for chemical herbicides or genetically engineered plants.

2.3 Research Plan Organization

For each of the three WED Pesticide Research Project goals described in the Critical Path, there is a corresponding component in this Research Plan: *Regional Analysis and Interpretation (Section 3.0), Effects of Chemical Herbicides on Terrestrial Plants (Section 4.0), and Ecological Effects of Gene Flow from Transgenic Crops (Section 5.0).* In each Section, we provide an introduction including the regulatory and scientific rationale for the research, objectives and scientific approach (Figure 1.1), and detailed time-line. The Critical Path and specific details for each research component are intended to be a dynamic set of guiding principles and not inflexible requirements for the direction of the Project. We will be in regular contact with EPA staff and other scientists in the regional analysis, chemical pesticide and gene flow research communities to further define the research objectives and approach. In addition, the International Workshop in 2005 will not only aid in development of studies on ecological effects of gene flow, but also be an opportunity to evaluate the progress and prospects for the regional analysis and chemical herbicide research.

While each of the three research components in the plan is described independently, they are intimately related. For example, the regional analysis and interpretation research provides the basis for selecting species and exposure conditions to develop testable hypotheses for research on effects of chemical herbicides on terrestrial plants. The regional analysis research also provides information for selecting native and weedy compatible plant species which could be affected by gene flow from genetically modified crops. The chemical herbicide and gene flow research will contribute inputs for development of probabilistic risk assessment methods. Applications of the molecular methods developed in the gene flow research may be useful in the development of new protocols for rapid screening of the sensitivity of a wide range of plant

species to chemical herbicides and/or to be used as markers indicating that plants have been affected by herbicides

In addition to the three current terrestrial ecosystem research components, an additional area could be added to the Pesticides Research Project to address non-target effects of chemical herbicides on plants in aquatic ecosystems. Any aquatic plant research would have to have 1) specific objectives address OPP and/or OPPT research needs, 2) a well thought out series of experiments using appropriate and well described methodology, and 3) defined outputs for use by agency offices. Any plan for aquatic plant research would have to be peer reviewed, and meet project management and QA requirements. An example of possible aquatic plant research is shown in Appendix A.

Finally, specific outputs from the research will be the Annual Performance Measures (APMs) which indicate the success of the project in meeting the EPA GPRA goals (Section 6.0 of this plan). Over time, these outputs will help provide the EPA with the broad outcome of reducing non-target effects from chemical herbicides and gene flow to vegetation and improving the health of ecosystems. In addition, a management plan is needed to assure that the project follows the Critical Path and that the outputs produced by this project are reliable. Thus, Section 7.0 of this plan describes Project Management and Quality Assurance (QA) aspects of the project, including the responsibilities of project participants, efforts to promote communications within and to those outside the project, and QA requirements.

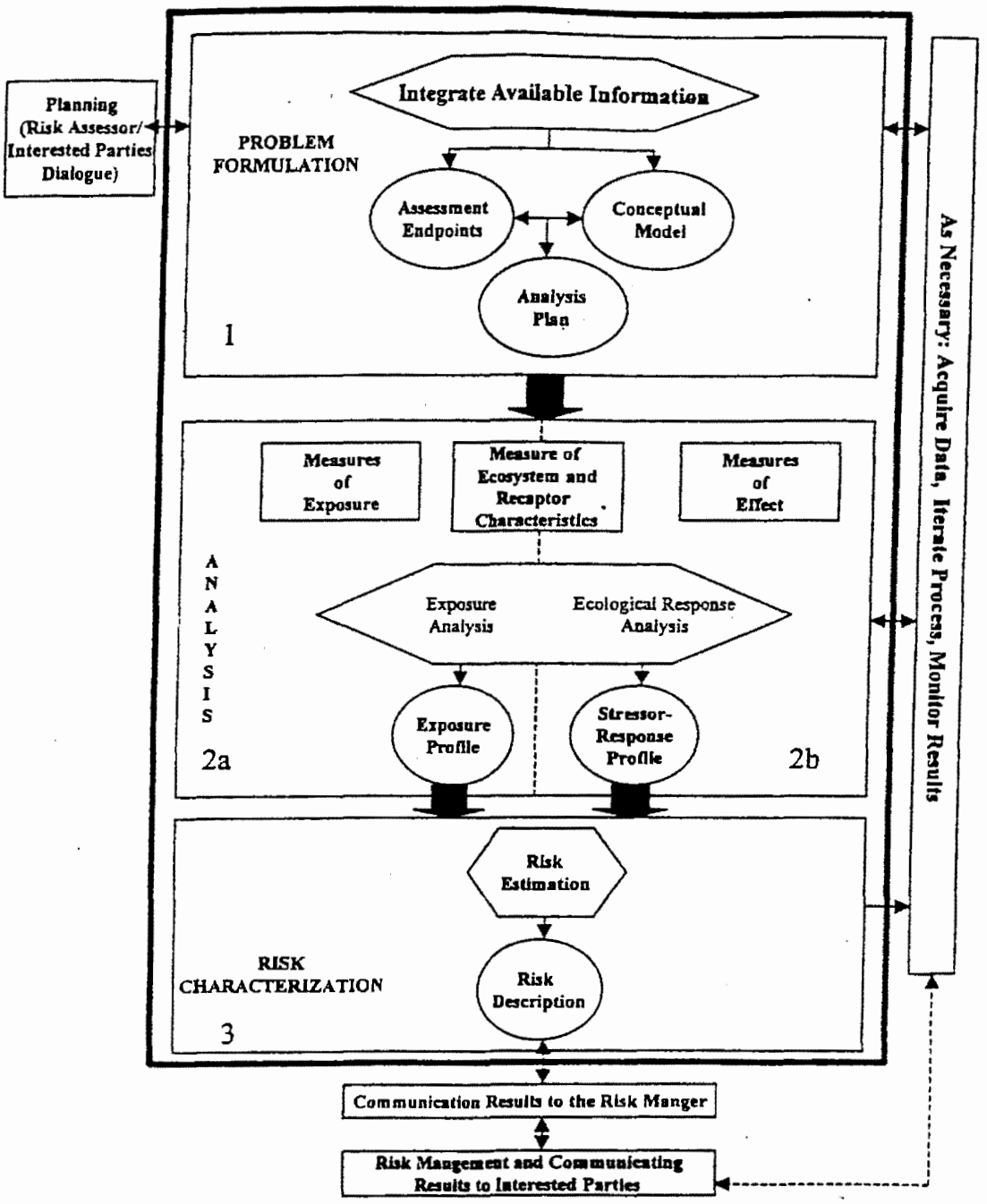

Figure 2.1. Expanded risk assessment framework with expanded views of problem formulation, analysis and risk characterization phases (U.S. EPA 1998). Rectangles indicate inputs, hexagons indicate actions and circles represent outputs. This project will make contributions to all components in the problem formulation and risk characterizations phases, and the items to the right of the dashed line in the analysis phase.

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3 REGIONAL ANALYSIS AND INTERPRETATION

3.1 Introduction

Pesticide drift to unintended fields is inevitable and the magnitude of potential and the effects to non-target plants are highly variable over time and space. The OPP is mandated under FIFRA to evaluate the potential ecological risks of crop pesticide drift to non-target plants. Specific research is needed to assess the potential impact of pesticide drift and to understand the effects on individual plants and higher biological assemblages across the landscape. A probabilistic systems modeling approach will be used to deal with variation in plant responses (spatial, temporal), and to quantify uncertainty in modeled exposure/effect relationships for individual plant species and communities. Probabilistic systems modeling offers the advantage of incorporating the uncertainty and variability in the existing databases from multiple sources as well as the uncertainty in the knowledge gap when no data are available. The existing databases represent potentially large sources of variation because the data were not specifically designed for regional risk assessment and, consequently, the data supports had low spatial and/or temporal resolution. Though the basic probabilistic modeling approach used in this project will be developed to assess the magnitude of the risk from chemical herbicides to non-target crops and native species, it also will be applicable to evaluate risks from other classes of pesticides as well as other chemicals. Aspects of the probabilistic modeling approach also will be applicable to questions concerning impacts of gene flow from target crops to native species.

The rationale underlying the proposed research on herbicide drift effects is that non-target crops and native plants in fields in close proximity to target crops are likely to be exposed, and to respond to, chemical pesticides. The potential for exposure that can cause effects is further determined by local wind conditions. In our regional analysis studies we will use existing data and not conducting exposure research as such, i.e., we will not develop new ways to determine, quantify or model exposure.

Herbicide drift generally occurs within 300 m of the crop field margin during and shortly after application (SDTF, 1997b). Drift amount and location depends upon the application rate and method (ground or aerial), environmental conditions, droplet spectrum,

application height, and distance from the field boundary (Bird et al., 2002). Vegetation located at the immediate boundaries of agricultural crop fields is at greatest risk from drift (Teske et al., 2002). The magnitude of herbicide drift effects on plant productivity or community composition depends upon the amount and type of herbicide and plant sensitivity at time of exposure, which varies with plant species and its phenological stage.

3.2 Objectives

The overall goal of this component of the research plan is to develop tools to improve regional analysis and interpretation aspects of ecological risk assessments (Figure 1.1). The primary objective to address this goal is to provide a spatially explicit (using a GIS framework) probabilistic risk assessment model to examine ecological risks associated with pesticide (i.e., herbicide in our studies) drift (Figure 1.1). A secondary objective is to examine the variability and uncertainty in data on herbicide exposure and on the effects of exposure on non-target plant species and population communities over time and space, and to identify the gaps in existing knowledge. Uncertainties in modeling deposition of herbicide spray drift on unintended crop and native plant species are distinctly different than those in modeling pesticide effects on non-target vegetation than exist for target species. Different databases and models need to be developed for estimating pesticide exposures and effects to non-target species. Another objective is to provide a web-based tool for access and extraction of issue-specific databases and maps for use by OPP and OPPT in their risk assessment activities. Though this research is on effects of chemical herbicides, meeting our objectives will also provide a regionally-specific framework with which to evaluate the effects of other pesticides, industrial chemicals and gene flow from genetically modified crops to native terrestrial plant species

Depending upon availability of data sources and models, WED will develop:

- **A spatial database of potential herbicide exposure to non-target plants**: This will require the linkage through a GIS platform of existing herbicide-specific data (e.g., toxicity, application rates and usage), climate (e.g., wind speed and direction, temperature, relative humidity), general data (e.g., crop land cover, native vegetation cover, soil type, hydrology, agricultural practices, field boundaries) with a spray drift

model (Figure 3.1). The spatial database will be used to: 1) estimate potential crop pesticide drift deposition to adjacent vegetation; 2) determine dominant crop, native and endangered species at greatest risk from herbicide exposure for plant testing; and 3) select areas of highest risk of gene flow associated with wind-pollinated genetically engineered crops which include PIPs. We will use existing data that are national in scope. For particular case studies, more site specific data may be used if available.

- **A database of herbicide effects on plants**: This will require the compilation of scientific literature relating to the ecological effects of herbicides on non-target terrestrial plants over wide geographic and taxonomic ranges, including stages of the plant life-cycle that are not covered by standard phytotoxicity testing protocols. Ecological effects may be direct, such as reduction in reproductive output or change in plant community composition, structure or function. No similar information exists for risks associated with gene flow. Non-target effects of low-dose, high-potency herbicides (e.g., ALSase inhibitors) and broad-spectrum herbicides (i.e., glyphosate) are a primary concern due to their increasing use and widespread distribution (Maxwell and Weed, 2001). If feasible, manufacturer's databases on herbicide effects will be obtained for our analysis.

- **A database of crop planting dates, pesticide use dates and weed emergence dates**: This will reveal the time and location of greatest potential for substantial impact of herbicide drift to non-target species. Due to the scarcity of herbicide use data, scenarios for herbicide usage will be generated based on local knowledge of the target crop and the presence of weeds.

- **Several case studies**: These will identify and prioritize potentially important uncertainties, and identify the actions needed to address the gaps in the knowledge base. Several regions will be selected for intensive study and development of the probabilistic risk assessment of the impact of herbicide drift to individual species and plant communities at the landscape level.

- **Web-based tool**: This will provide on-line access to databases on herbicide exposure and plant effects and generate issue-specific databases and maps for use by OPP and OPPT in their risk assessment activities.

3.3 Approach

A. Spatial Databases

General aspects of the regional analysis and interpretation research approach are shown in Figure 1.1. In terms of spatial databases, spatial information on land use, field boundaries and ownership, crop and non-crop coverage, pesticide use, climate, soil, and hydrology will be obtained and compiled in a GIS. For detailed, probabilistic analyses, determination of agricultural field boundaries is especially critical for evaluation of vegetation at risk. Downwind deposition decreases with distance from the edge of the field and approaches zero at 300 meters in a typical aerial application (SDTF, 1997b). This defines the spatial resolution needed to estimate pesticide exposure due to drift. Thus, databases for delimiting agricultural fields where pesticides have been applied are the most important spatial data required to adequately assess the potential drift impact on non-target species. The USDA National Agricultural Statistics Service cropland data coverage based on Landsat thematic mapper (TM) scenes at 30 m^2 resolution is available for eight states (Arkansas, Illinois, Indiana, Iowa, Mississippi, Missouri, Nebraska and North Dakota). This database will be critical in determining the spray drift loadings to vegetation downwind for those states.

For California, cropland data for several counties at 30 m^2 resolution are available from the California Department of Water Resources. The California Department of Pesticide Regulation (2000) has improved the resolution of the pesticide use data from 1 square-mile to an actual field site for several counties (Neal, 2002). Field border databases and parcel boundaries for reported agricultural field sites are currently available or are under development for counties from the San Joaquin and Sacramento valleys, the coastal region, the Sierra foothills, and the San Diego-Imperial area in California

Crop, native vegetation, pesticide use and effects data will be obtained through collaborative efforts with federal, state and local agencies as well as industry as feasible.

Existing databases including the USGS National Land Cover, GAP state land use coverage, the California PUR and the USDA databases based on Landsat satellite imagery will be used to identify the crops and native vegetation growing within and adjacent to the field boundaries, especially for those states without detailed data. This will be used to identify the non-target crops and native vegetation at risk from drift within a 300-m zone adjacent to the field boundaries of specified target crop species. Pesticide deposition within the 300-m zone will be determined based on pesticide use information reported to the field level as required by state law in California or from state agricultural extension services for other states. Spatial data on wind speed and direction and other factors will be used to refine the drift zone of herbicides on unintended vegetation in later stages of model development.

B. GIS Platform and Analysis

Spatial information will be compiled in a GIS platform using ARCINFO (or other GIS software). The GIS will be adapted and documented to provide maximum usefulness as a web-based tool for OPP and OPPT staff and other interested individuals.

Figure 3.2 is a simplified example of how the GIS platform and databases might be used to determine counties in the United States which are at risk for herbicide drift based on the intensity and diversity of agriculture, the amount of herbicide usage, and wind speed data. It includes representative steps for species selection and potential herbicide exposure. The example used agricultural statistics (crop acreage per county, number of crops per county) were from the 1997 Census of Agriculture (USDA-NASS, 1999). Herbicide use data were from the National Center for Agricultural Policy (NCFAP), which has released summaries of agricultural pesticide use for 1997. The example uses the total amount of all herbicides applied in a county. Consideration of acres sprayed with all herbicides, or a particular herbicide, could be considered in the future, when selecting species to be tested for specific purposes. Wind speed data were from the National Climate Data Center (NCDC) at the National Oceanic and Atmospheric Administration (NOAA). Hourly wind speed based on NCDC's TD-3280 and TD-3281 databases were obtained from EarthInfo's Surface Airways database.

The geographic areas with greatest risk for non-target herbicide effects as identified by the type of analysis shown in Figure 3.2, will be candidates for more detailed probabilistic analysis of herbicide impacts. This type of GIS-based analysis also will be used to determine areas to be evaluated for selection of candidate crop and non-crop test species for plant tests to determine the risks from herbicides to non-target vegetation on a regional basis in the US (see Section 4.3 A below). Crop and non-crop-related information could be used in such an analysis to determine areas of the US of interest for more intensive gene flow research (see Section 5.4 below).

C. Probabilistic Systems Modeling

The proposed probabilistic approach will define the probability and magnitude of the risk, and uncertainty that spray drift effects will occur, on non-target species using a GIS-based framework. Following the ECOFRAM approach (US EPA 1992, 1998), a tiered approach for regional assessment will be used. A simple deterministic model will be developed first, followed by a probabilistic systems approach to deal explicitly with spatial and temporal variation in exposure, plant response and sensitivity, and uncertainty in input parameters. Level 1 is a screening step and is based on existing data required to identify the regions at greatest risk to pesticide drift. In case studies at WED over the next 2-3 years, published generic exposure-response functions based on controlled experiments will be used to infer pesticide effects on crop and non-crop species. If feasible, efficacy (dose-response) data also will be obtained from herbicide manufacturers.

The risk assessment-related research is concerned with aerial applications of particular pesticides because, in a typical ground hydraulic application, more than 99.9% of the applied active ingredient stays on the field and less than 0.1% drifts, whereas about 8% of the applied active ingredient drifts off field in a typical aerial application (Spray Drift Task Force, 1997a,b). Scenarios of pray drift loading to non-target areas will be developed using AgDRIFT 2.0.05, a spray drift model developed under a cooperative research agreement with EPA, USDA and the Spray Drift Task Force (Teske et al., 2002; Bird et al., 2002). The AgDRIFT model will be used to estimate the fraction of pesticide drift and downwind deposition based on climate information and default settings for method of application. For each pesticide, information on the date,

location of application, maximum application rate, and timing of application in relation to non-target plant phenology will be used in conjunction with the AgDRIFT output to estimate pesticide exposures to non-target areas.

An initial screening (Level 1) will be used to identify the pesticides, geographic locations and non-target species of greatest concern as case studies to illustrate the probabilistic approach for assessing risk. A "worst-case" scenario of potential non-target herbicide effects will be used to identify those pesticides and regions of greatest concern for more intensive study.

Level 2 and Level 3 assessments will be stochastic in nature, using progressively finer level data to identify areas with low, medium and high impacts from chemical herbicides. Level 2 assessments will refine the earlier calculations using more detailed GIS layers on land use, pesticide use, field boundaries and climate as well as more specific crop profiles and exposure-response functions. Level 2 estimates will still rely on point estimates for most input parameters for estimating pesticide exposure and effect whenever published data are available. For other parameters, expert judgment will be used to set the parameter values or establish hypothetical probability distributions. A sensitivity analysis will be performed at this level to identify those parameters that contribute the highest variability to the risk assessment. For Level 2 assessments, a spatial database of expected pesticide use will be developed assuming recommended application rate, timing and method for each chemical and crop type. Any more specific herbicide exposure-crop (or native plant) response functions available from the plant testing component of this project (see Section 4.3,A,B) will be incorporated into these assessments.

Level 3 assessments will use the best available data on the potential hazards of herbicides to non-target plant species to address the uncertainty and variability in the impact of pesticide drift on crop and non-crop species in adjoining fields. Level 3 will focus on exposure and effects parameters identified as important contributors to risk in the Level 2 assessment, as well as on specific case studies of the pesticides and species of greatest concern. This level will address highly specific pesticide use scenarios and incorporate additional data to establish the temporal and spatial pattern of exposure and effect on individual populations and communities, including estimates of uncertainty in the data. Given the geographically focused nature of databases for

location of plant species and identification of cropping practices, it is possible to estimate pesticide deposition to unintended fields using a distribution of potential deposition rates. There is considerably less information on the time of pesticide application in relation to the developmental stage and sensitivity of the non-target species at the time of exposure and the ensuing plant response for each plant species and chemical. Dose-response distributions (with uncertainty bounds) will be retrieved from the literature, or PHYTOTOX database, or developed by WED researchers for input to the model. Due to limited information of pesticide effects on reproductive endpoints (fruit, seed, tuber production), it is likely that the uncertainty in pesticide effect will contribute as much, or more, to the probabilistic impact of spray drift to non-target crop and non-crop species.

3.4 Time Line

The research on regional assessment of pesticide drift effects on non-target plants has three distinct phases: 1) development of a GIS database for estimating pesticide exposure to non-target plants across time and space; 2) development of a phytotoxicity database for estimating pesticide effects on crops and native vegetation; 3) synthesis and integration whereby information on pesticide exposure and effects are used to develop a probabilistic risk assessment model (Table 3.1). During FY2002-2003 the focus has been on development of the GIS databases and procedures necessary for risk assessment, identifying crop and native plant species suitable for tests on regional bases, and gene flow research. The GIS databases will be used for initial screening to select crop and native species that are most likely to be exposed to pesticide drift or gene flow.

The time line for development of the GIS platform is pursuant to the availability of high spatial and temporal resolution data for crop use and native vegetation, pesticide use, and wind speed and direction. Currently, cropland data coverage at 30 m^2 resolution is available for eight states and several counties in California. Over the next six years, high resolution crop land coverage based on Landsat images are expected to become available for more counties in California and other states. In California, all agricultural pesticide use must be reported to the Department of Pesticide Regulation via the county agricultural commissioners; the reports must include information on the date and location of application, kind and amount of pesticide, and

type of commodity if applied to a crop. Oregon is the second state that has a law that requires detailed reporting on pesticide use, similar to that for California, but uncertainties in funding and the extent of pesticide use reporting have hampered progress towards implementing this legislation.

From FY 2004 onward, the research will focus on the states for which crop land data coverage is available at sufficient resolution to determine crop field boundaries in order to estimate pesticide drift. The initial case study will be California where pesticide use data have been used to identify the agricultural field boundaries and to provide detail needed to estimate the amount and timing of pesticide drift to non-target species from aerial and ground applications. Data from California will be used to determine whether pesticide exposures can be generalized to other states where crop use data at 30 m^2 resolution and county-level pesticide use data are available. During FY2005-2007 research will extend the modeling efforts to infer pesticide drift impacts on non-target species for states with low spatial and temporal resolution data on crop use and pesticide use. Research is needed to understand how uncertainty of risk predictions increases with decreased knowledge of field boundaries and pesticide use.

The research will link the pesticide exposure data with the plant effects database using a probabilistic risk assessment model to estimate the potential impact of pesticide drift on crop and native plant species. The products of the five-year research plan are probabilistic risk assessment tools for evaluating potential ecological risks from pesticide products based on data at different spatial and temporal scales. These products will range from databases, to models, to a web-based tool for on-line access to databases on herbicide exposure and plant effects for use by OPP and OPPT in their risk assessment activities.

Table 3.1 Time line for regional assessment and GIS platform

FY 2003	FY 2004	FY 2005	FY 2006	FY 2007
Level 1 - screening ▪ Obtain GIS databases ▪ Crop coverage ▪ Noncrop coverage ▪ Pesticide use ▪ Climate ▪ Soil properties ▪ Hydrology ▪ AgDRIFT model ▪ AGDISP model ▪ Field boundaries ▪ Weed management practices ▪ Weed profiles ▪ Crop profiles ▪ Exposure-response functions ▪ Literature review Contribute to APM on Strategy for Updated Test Guidelines: Finalized Research Plan	Level 1 - screening ▪ Pesticide exposure - estimate drift deposition to non-target areas ▪ Pesticide effect - estimate plant response to pesticide exposure on regional scale ▪ Select region for case studies based on potential exposure and effect Contribute to APM on evaluation of risk assessment methods for herbicides	Level 2 - case studies ▪ Refine pesticide exposure estimates- more detailed info on land use, pesticide use, field boundaries, climate, etc. ▪ Refine pesticide effect estimates - crop-specific exposure-response functions, spatially explicit coverage of target and non-target areas ▪ Sensitivity analysis (iterative process continuing in 2006 and 2007) Contribute to APM on regional approach to risk assessment	Level 3 - probabilistic risk assessment ▪ Refine pesticide exposure and effects estimates ▪ Focus on parameters identified by sensitivity analysis ▪ Determine probability distributions for model inputs and parameters ▪ GIS to develop high-resolution maps of target and non-target species ▪ Time of pesticide application in relation to developmental stage of non-target species	Level 3 ▪ Develop regional assessment tool based on GIS framework and probabilistic risk assessment ▪ Regional case studies

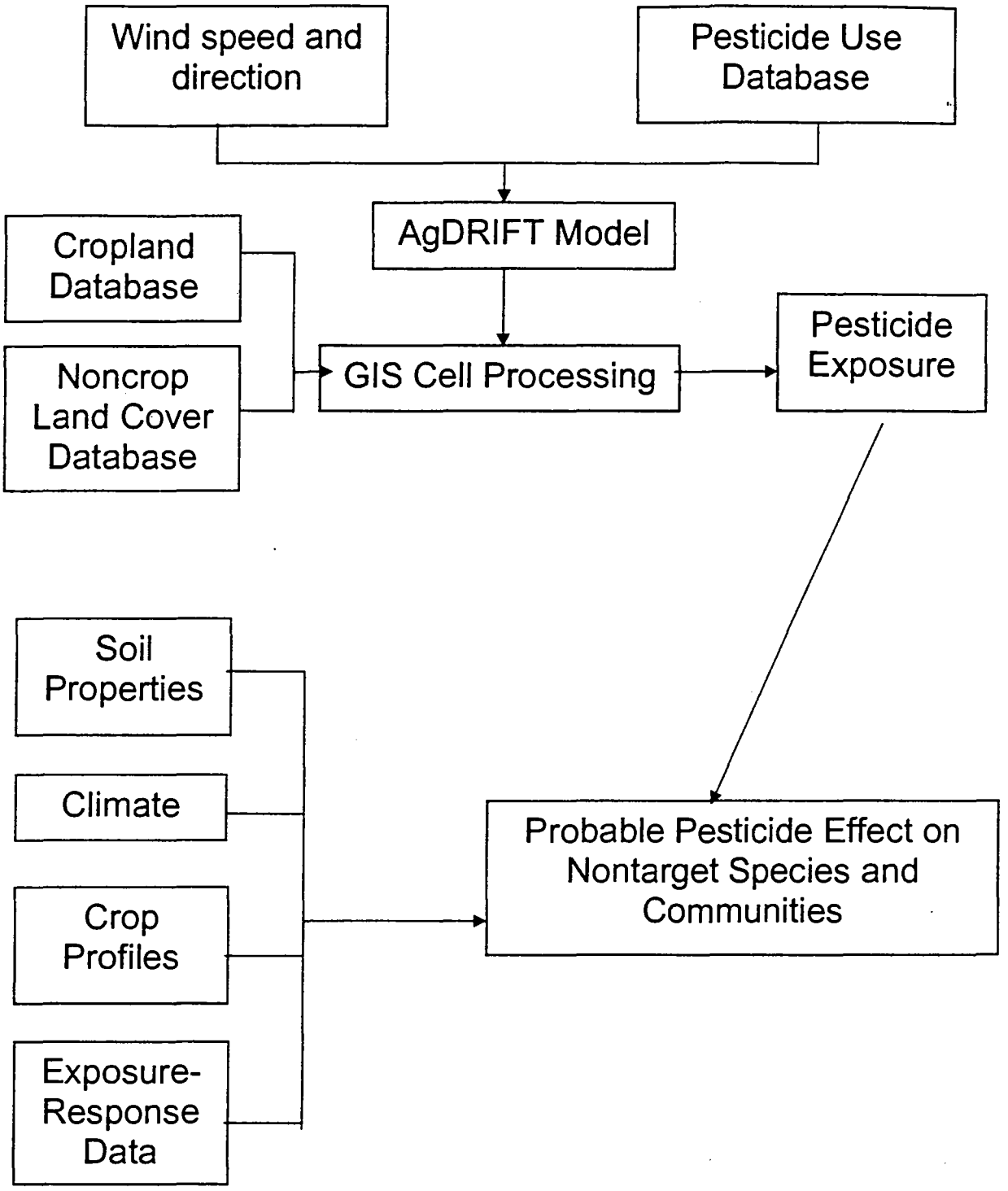

Figure 3-1 Databases for Probabilistic Pesticide Risk Assessment. The focus in this project will be risks from herbicides.

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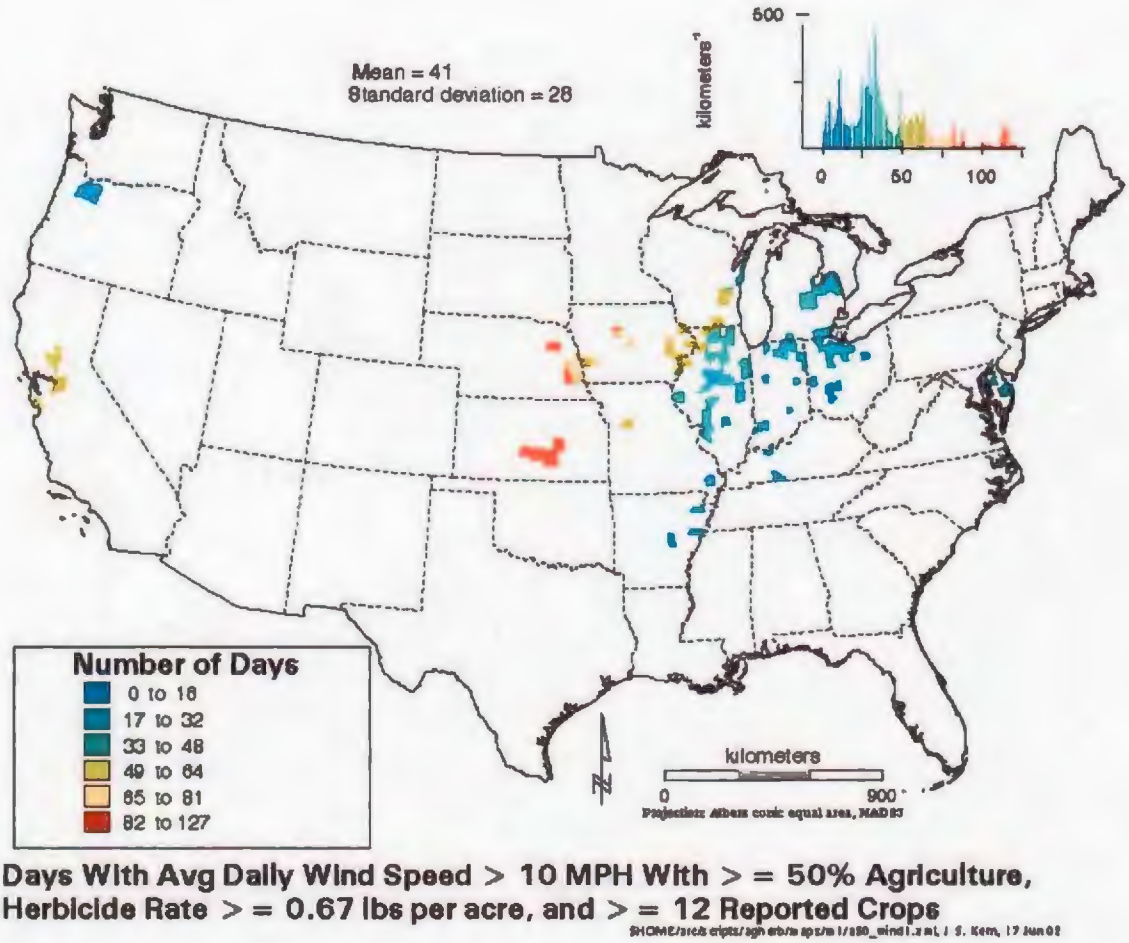

Figure 3-2. Results from preliminary analysis of U.S. counties with vegetation at highest risk from drift of agricultural herbicides. Counties depicted here in different colors had variable numbers of days with wind speeds averaging > 10 mph. These counties also had ≥50% of total acreage in agriculture, ≥0.67 pounds/acre of herbicides applied to the agricultural acreage, and ≥12 different crops grow.

4 EFFECTS OF CHEMICAL HERBICIDES ON TERRESTRIAL PLANTS

4.1 Introduction

A. Regulatory Basis

Since the end of World War II, American agriculture has become dependent on synthetic chemical pesticides. According to the most recent EPA estimates (1997, EPA OPP website), approximately 778 million pounds of conventional pesticide active ingredients (herbicides, insecticides, fungicides) are used in the United States each year. Of this amount approximately 73%, or 568 million pounds, are herbicides, 83% of which are used in agriculture. Greater than 90% of all corn and soybean acreage in the U.S. are treated with one or more herbicides annually. Because herbicides are designed to kill certain plant species (i.e., weeds), they have a high potential for impacting individual non-target plants, plant communities, and function and structure of ecosystems if they migrate off the intended use area. Depending on the mode of action and spectrum of pest control, herbicides can cause visible damage to plants within hours, days, or weeks following exposure. Furthermore, persistent herbicides can remain in plants, sediments and/or soil and affect plants in subsequent growing seasons.

Chemical compounds are regulated under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) and the Toxic Substances Control Act (TSCA) by EPA's Office of Pesticides Prevention and Toxic Substances (OPPTS) to protect human health and the environment. However, in the past the protection of plant resources has received minimal attention under these regulations (OPP Environmental Fate and Effects Division), and there have been questions whether non-target plants have been affected by herbicides. Some states have restricted the use of certain pesticides (e.g., 2,4-D) for the protection of non-target plants since the 1950s and 1960s. The registration in 1986 of clomazone for use on soybeans changed the regulatory picture because movement of clomazone resulted in bleached non-target vegetation and started the first serious actions by OPP to review plant test data. Incidences of clomazone impacts were followed by cases of off-target plant damage from the low-dose, high-potency herbicides, rising public concerns that could not be satisfactorily addressed with the scientific data available in the early 1990s. These concerns suggested that plant resources outside the bounds of treated areas are at risk from the movement of

herbicides from targeted lands. At potential risk are non-targeted crops, rare and endangered plant species, native plant communities and organisms that are dependent on natural plant communities for food and shelter.

EPA required tests for plant effects are the Pesticide Assessment Guidelines Subdivision J, which have been refined as the Series 850, Ecological Effects Test Guidelines. Guideline 850.4000 provides general information on conducting plant tests with pesticides and industrial chemicals for both OPP and OPPT. In addition there are OPP-specific methods (850.4025 Target Area Phytotoxicity, 850.4100 Seedling Emergence, 850.4150 Vegetative Vigor, and 850.4300 Terrestrial Plants Field Study), and OPPT-specific methods (850.4230 Early Seedling Growth Toxicity Test, 850.4600 *Rhizobium*-Legume Toxicity, and 4800 Plant Uptake and Translocation). These tests are usually required in a "Tier" sequence, i.e., for "Tier I" or maximum challenge tests, a single concentration of a pesticide is required to determine the general phytotoxicity of a chemical. In "Tier II" tests, multiple concentrations of a pesticide are required to establish pesticide dose-response functions when the chemical is known to have phytotoxic effects. The Tier II chemical dose (concentration)-plant response data are used to establish EC_{25} (effective pesticide concentration for a 25% reduction in plant response) values for the different species. In a "Tier III" test (OPPTS 850.4300) plants are grown under conditions similar to those where they would be exposed to a pesticide in the field. The field test is a long-term test where plants are grown and evaluated for pesticide responses over their entire life cycle. This enables determination of reproductive, biomass, richness of species, population density, and other parameters as indicated at time by Agency. Tier III studies are requested on case by case basis with protocols determined for a particular situation of decreasing risk uncertainty to non-target plants. If there is no standard EPA test for a particular pesticide or chemical application, other standard plant tests can be used, such as those approved by ASTM International or the Organization for Economic Co-operation and Development (OECD).

Table 4.1 summarizes the general protocol for growing conditions for EPA's Tier I and II vegetative vigor test and other compatible tests of other agencies and organizations for plant effects from pesticides (Stavely 2002a). In brief, plants are grown under standardized, controlled-environment climatic conditions (or possibly small field plots). The conditions (e.g., 25/20 C day/night temperature, 350 $\mu mol/m^2/s$ light intensity in wavelengths between 400-700 nm) can produce generally healthy vegetative plant growth in most greenhouses and growth chambers. A

natural mineral soil with ≤3% organic matter is used with bottom watering preferred. Only a small number (3) of the experimental units (pots) are required for the test, with data averaged for several plants (10) per pot. Pesticides are applied either to the leaves or incorporated into the soil. Ten species of plants normally are suggested for the OPP Tier I vegetative vigor test. Corn and soybean are required, and a root crop (often carrot), plus tomato, cucumber, lettuce, cabbage, oat, ryegrass and onion usually are used. Plant response measurement endpoints generally are injury, height and shoot dry biomass. The vegetative vigor test involves pesticide application at an early growth stage (14 days after seedling emergence) and harvest for analysis a short time later (generally 7 to 14 days).

However, since the establishment of the OPP Subdivision J test protocols in the 1980's and OPPTS Series 850 tests in the 1990's, EPA has sought to improve and provide scientific justification for these tests to better evaluate the toxicity of chemicals to terrestrial plants and plant communities. Similar efforts have occurred in Canada (Boutin and Rogers, 2000), and in OECD countries. Recently, there has been a movement to harmonize all regulatory test protocols including plant tests across agencies and countries. Table 4.2 summaries a series of meetings and events over the past 12 years which have helped to crystallize the need for a new program focusing on revising tests for plant effects from chemical pesticides. Recommendations regarding those tests have been stated in the report from the 1991 workshop on non-target plant testing (Fletcher and Ratsch, 1991), expanded upon by subsequent meetings and documents, culminating in findings from the International Workshop on Plant Tests held in Alexandria, Virginia in January 2002. Some important findings from the Alexandria meeting are summarized as follows (Stavely, 2002b) :

> "Some of the most important research needs were: tests for terrestrial plant development and reproduction; ...field tests, including multi-species approaches; monitoring tools; ...research on alternative test species, on relative sensitivity, and on approaches to selecting test species; methods to evaluate recovery; and greenhouse-to-field extrapolations."

B. Scientific Rationale

Non-target Plant Test Protocols. Plant research addressing ecological effects of herbicides traditionally has fallen under two headings: target and non-target. Target herbicide research is confined to studying the influence of herbicides on approximately 100 crop and weed species (Fletcher, 1997) that are intended recipients of particular herbicides applied within the borders of cultivated fields. Non-target herbicide research deals with the influence of herbicides on all plant species growing outside the borders of fields treated with herbicides.

The goals of target plant herbicide research are to identify suitable herbicides, application concentrations, and methods which will eradicate weeds in cultivated fields without damaging and/or reducing the yield of crop plants of interest. These herbicides must not have human health effects, must be compatible with other cultural practices for crops of interest, and must be economically viable to develop, produce and market.

Target plant herbicide research has been primarily interested in managing the development of herbicide resistance in weeds because resistance reduces the usefulness of the specific herbicide for future use. Unfortunately, this research is not useful for addressing the risks of herbicides to non-target plants, risks that include species and exposure conditions (herbicide concentrations, time during plant life cycle, environmental condition) not typical of target plants.

The existing Tier I, II and III test protocols used to determine non-target plant effects were established using the best available consensus scientific information at the time, but do not reflect subsequent methodological questions or advances in scientific methodology. For example, concerns have arisen regarding both aquatic and terrestrial test species used by EPA to collect preregistration data for protecting plants and other photosynthetic organisms. EPA-required tests for terrestrial plants currently use ten angiosperm species, six dicotyledonous species from four families including soybean and a root crop, and four monocotyledonous species from at least two families including corn. All other species are only recommended, and substitution of other test species (other crops, weeds controlled native plants, perennials, woody species) especially is encouraged when species sensitivity to the test compound is known ahead of time. However, in practice, all ten species are annual agricultural species as indicated earlier.

In any event, the ten species are considered to be surrogates for all potential non-target native plant species (16,000 in the U.S., Heywood, 1978), in addition to non-target crops. However, the narrow taxonomic and life form range of the ten required test species raises the question of whether the majority of plant species will be protected, including all the rest of the angiosperms (both herbaceous and woody), gymnosperms, and ferns. Species also vary greatly by ecological regions and taxonomy yet there is no provision for including this diversity in non-target plant risk assessments.

In addition to species concerns, the time frame for current tests may not be appropriate for non-target plants. The Tier I and II vegetative vigor tests last a maximum of 28 days. This time period is insufficient to capture the reproductive phase of the plant's life cycle. Not only is this important for the individual plant's ability to pass along its traits, but reproductive yield is one of the most important economic aspects of agriculture. Furthermore, many wildlife species depend upon seed production of noncrop plants for their food source. The limited data available suggest that exposure during the vegetative versus reproductive phases may not have equal influences on reproduction and crop yield (Fletcher et al., 1996). Current Tier I and Tier II tests include only measurements of injury, height and biomass that do not correlate well with yield. There is some argument that early seedling growth parameters are protective of reduction in yield responses, but this may lead to over regulation of some herbicides. Table 4.4 indicates the limitations of different current and possible assessment indicators for non-target effects of herbicides (Maxwell and Weed, 2001; Obrigawitch et al., 1998).

Additional uncertainty about current non-target tests concern the extrapolation of greenhouse tests to field conditions where plants exist in complex relationships with other organisms, typically are in competition for water, nutrients, space and light, and where they are threatened by herbivores and pathogens. Fletcher et al. (1990) compared results from studies included in the PHYTOTOX (Fletcher et al., 1985) database that were conducted on similar plant species with the same chemical in both greenhouse and field. Out of 20 combinations, 55% showed plants in the field to be more sensitive, 30% were more sensitive under greenhouse conditions, and 15% were equal in sensitivity in the field and greenhouse. Sensitivity differences were less than 2-fold in all cases. In contrast, McKelvey et al. (2002) reported that the crop species currently used in vegetative vigor tests usually were more sensitive than non-crop plants tested. However, the results from McKelvey et al. (2002)

only considered "weed" species (primarily annuals) as non-target plants and only used high (several x the field application rate) herbicide concentrations.

Current plant tests do not fully address risk assessment needs, as they were developed before the risk assessment paradigm came into common use, and before development of GIS technology for spatial and temporal analysis of data sets. EPA's Science Advisory Panel (SAP, 2001) endorsed an approach to incorporate both these technologies into the risk assessment process for herbicides. Specifically, an initial problem formulation process should be conducted to determine the plant species growing in proposed areas of herbicide use, and their stage of development at time(s) of application. This information provides a rational basis for selection of species and endpoints to test in the Tier I and II assessments. GIS systems also can be used to collate post-registration incident monitoring data, which will provide further information about types of plants and adverse effects most frequently associated with herbicide use.

ALS Inhibitors. Much of the recent interest in herbicide testing has been associated with low dose, high potency herbicides such as acetolatacte synthase (ALSase) inhibitor herbicides which where initially introduced into U.S. agriculture in the mid '80s (Fairbrother and Kapustka, 2001). Use of these chemicals potentially addressed major environmental concerns regarding herbicide toxicity in that they have a relatively narrow spectrum of susceptible organisms, are relatively short-lived in the environment, nonbioaccumulative and used in low volume. The first class of these herbicides used was the sulfonylureas (SU). They were quickly followed by the imidazolinones and more recently by the triazolopyrimidine sulfonanilides and pyrimidinyl oxybenzoates. The primary mode of action of these herbicides is by disruption of the synthesis of the branched chain amino acids leucine, isoleucine and valine. However, there may be secondary modes of action within plants leading to the accumulation of toxic metabolites, disruption of assimilate transport and inhibition of reproduction (Fairbrother and Kapustka, 2001; Taylor, 2001). These herbicides are generally not considered to be toxic to animal systems due to animals' inability to synthesize branched chain amino acids. However, the ALS inhibitors can affect bacteria and fungi which play key ecological roles in nutrient cycling, soil fertility, and plant nutrition and health.

One of the most striking features of the ALSase herbicides is their exceptionally low field application rates (g Ha^{-1} or oz Ac^{-1}). Such low rates make chemical detection of these herbicides on

plant material impossible with present technology. Thus, millions of pounds (est. 2.1 million pounds in 1996, Fairbrother and Kapustka, 2001) of these toxic materials are released into the environment without practical means of tracking their fate and influence in the environment. However, from past experience with other pesticides, from information presented at a recent workshop on low-dosage-herbicides in December, 1999 (Ferenc, 2001), and based on findings from the FIFRA Scientific Advisory Panel Meeting in June, 2001, there is general agreement that they are moving off site in water, on soil particles and as spray drift. For example, the use of Oust® (sulfometuron) on rangeland may have resulted in damage to potatoes at least 8 km distant (personal communication, Dr. Pamela Hutchinson, University of Idaho, Aberdeen Research and Extension Center).

Maxwell and Weed (2001), Obrigawitch et al., (1998) and Ratsch and Fletcher (1991) summarized recent reports of non-target impacts from herbicides, many of which were ALSase herbicides. Obrigawitch et al. (1998) specifically assessed the effects of sulfonylurea herbicides using field approaches. They concluded that the risks to non-target plants from sulfonylureas were similar to those from other herbicides used at higher application rates. Obrigawitch et al. (1998) also stressed the need for standardized protocols to assess the effects of herbicides, in general, on non-target plants. The review by Ratsch and Fletcher (1991) and other papers in the report by Fletcher and Ratsch (1991) indicated pesticide effects occurred at various stages of plant development, including reproduction.

Based on the reviews by Maxwell and Weed (2001), Obrigawitch et al., (1998) and Ratsch and Fletcher (1991), and additional literature; Table 4.3 summarizes some of the reproductive or other developmental effects of herbicides, especially ALSase inhibitors. These effects were found primarily in controlled herbicide exposures. There is additional literature on non-target effects of herbicides on leaf injury (e.g., AL-Khatib et al., 1993). However, the relationship between leaf injury and reproductive effects is not clear. Some of these studies showed reproductive effects at herbicide concentrations that did not produce visible leaf injury (Fletcher et al., 1996; Fletcher et al., 1993). In contrast, other studies showed that while reproductive effects from herbicides were always associated with leaf injury (Al-Khatib and Peterson, 1999), the amount of leaf injury was directly related to the amount of yield loss. The nature of the injury-yield loss relationship may depend on when plants come in contact with the herbicide during their life cycle (Fletcher et al., 1996). Especially important may be periods when rapidly growing sinks such as seeds or tubers are at risk

due to the effects of ALSase inhibitors on cell division, meristematic activity, phloem loading and photosynthate transport (Taylor et al., 2001).

Overall, there are a number of reasons why low-dose, high-potency herbicides such as the ALSase inhibitors may be of greater interest than conventional herbicides. As summarized by Maxwell and Weed (2001), compared with conventional herbicides, the low-dose, high-potency herbicides may: 1) have increased total amount applied on crops if current use trends continue, 2) have greater aerial drift because of their application methods, 3) be used more at the boundaries of agricultural regions as they become used more for roadside maintenance, 4) be used more to suppress forest understory plants in forest ecosystems, and 5) have more potential for reproductive effects due to exposure amounts and timing for different crops. In his preliminary ecological risk assessment and characterization for ALSase inhibitors, Taylor (2001) concluded that "...the uncertainty of the data is significant in terms of breadth and depth..." He recommended areas of needed research to provide peer-reviewed literature addressing the uncertainties including...

> "...analytical methodologies to quantify the concentration of low-dose, high potency herbicides in multiple media..." (including biosphere, e.g. plants)
>
> "...effects...on plant structure and function..."
>
> "...role of temperature, light, precipitation, pH, etc....with respect to behavior as well as effects on non-target species."
>
> "The development of a system-level model to predict the behavior of low-dose, high-potency herbicides is needed, with a special interest in simulating exposure of at-risk processes in the biosphere."

Ecosystem Responses to Herbicides. The movement of herbicides from targeted land has the potential to adversely affect both agricultural and natural ecosystems. The ecological effects may be direct, such as the elimination or reduced reproductive output of certain plant species in a community, which leads to the alteration of the community's species composition, structure and function. Effects may also be indirect, such as changes in microbial communities, controlling plant pathogens, or diminished insect populations causing wildlife populations to increase. These changes

can lead to numerous negative impacts on wildlife habitat, nutrient cycling, control of soil erosion, recreation, timber or pulp production, livestock grazing, control of noxious plant species, and aesthetics (Obrigawitch et al., 1998). In 1991 the SAP recommended that:

> "Community response measures need to be developed that have the potential to identify significant structure and functional changes in exposed communities. Many more invaded natural communities will be targeted for herbicide use, given the increased recognition of invasive plant problems. Community response metrics are available in the ecological literature. However, the specific value of these responses with regard to characterizing responses due to chemical exposures need to be determined and possible modifications of designs identified."

Direct effects. Observational data suggests that plant assemblages at field margins experienced substantial change in species frequency and distribution due to differential susceptibility to herbicides (Kleijn and Snoeijing, 1997). Others (Jobin et al., 1997) have found lower species diversity in the herbaceous layers of hedgerows and woodland edges of cultivated fields with a history of herbicide use as compared with those near fields without herbicide use. In controlled experiments with plant communities, Pfleeger and Zobel (1995) demonstrated that variable species responses to herbicide exposure alter the competitive interactions within a community.

The high selectivity of the low dose, high potency herbicides could accentuate the differential stresses and subsequent shifts in dominance in a plant community. Such shifts in a community can result in changes in frequency and production and even extinction of desired species (Tillman, 1988). In addition, Boutin and Jobin (1998) demonstrated that herbicides can contribute to shifts in plant communities adjacent to intensively cropped fields from native species toward more weedy species, and, thus, these adjacent communities can promote the spread of weed species. Additionally, crops are being genetically engineered to be tolerant to the highly active herbicides, which will stimulate more widespread use and subsequent potential for non-target effects (Maxwell and Weed, 2001).

Besides undesirable changes in plant communities, threatened and endangered plant species are at risk. The federal government has listed over 500 plant species and the Nature Conservancy considers 5000 of the 16,000 native species in the U.S. to be at risk. Almost 50% of these species are annuals that are dependant on seed production or the seed bank for survival. The highest percentage of these plants is located in Southeast wetlands and Southwest deserts.

Because the data available are very limited and highly speculative, there is a need for controlled experiments in different regions of the country to determine the effects of herbicide exposure on plant community dynamics. Considerable work will be required to identify an effective methodology for determining meaningful endpoints.

Indirect effects. The direct effects of herbicide drift to plants and plant communities is straightforward compared with the complexity of food web dynamics and habitat alteration effects on wildlife populations. The vast majority of the reproductive output of plants is used by animal species as sources of energy. Therefore, changes in plant community dynamics will affect wildlife populations. For example, mammal populations in eastern deciduous forests are controlled by the abundance of acorns, years with high acorn production mouse populations increase (Ostfeld et al., 1996). Increased mouse populations lead to more effective gypsy moth control through increased mouse predation of moth larvae. Abundant acorn crops also provide deer with sufficient food, and as a result, they browse less on tree samplings. More saplings grow into the tree canopy, thus determining future forest composition. In another example, the reproductive output of the tree *Casearia corymbosa* in Costa Rica is responsible for the survival of at least seven bird species during the dry season (Howe, 1977). Many granivorous invertebrates depend on the reproductive output of plants for survival and do not have the luxury that most birds have of moving to a new habitat when food resources are scarce. Besides performing ecological functions, many phytophageous invertebrates are the basis of many webs that support vertebrate species that are popular with the public (Greig-Smith, 1991).

Changes in habitat quality caused by herbicides can affect wildlife populations. For example, populations of the gray partridge in the United Kingdom have been affected by herbicide use (Greig-Smith, 1991). Plant species composition within hedgerows between herbicide sprayed fields was altered, resulting in a 50% loss in populations of arthropods, which were a high-protein

food source for partridge chicks. Fewer arthropods resulted in more frequent partridge brood movements leading to greater predation of chicks. Other studies also have shown effects of agricultural pesticides on wildlife (Freemark and Boutin, 1995, 1994; O'Conner, 1992; Mineau et al., 1987; Sheehan et al., 1987; Hill, 1985).

Various effects of herbicides on metabolic activities and on overall growth of a host and/or a pathogen can cause an increase in soil borne diseases, resulting in greater pathogen damage to plants from a variety of organisms including fungi and nematodes (Altman and Rovira, 1989). Though there is limited understanding of effects of herbicides on plant pathogens, even less is known about mycorrhizal associations (Altman and Campbell, 1977). Most plant species require some form of symbiotic relationship with mycorrhizal fungi, and herbicide effects to fungi may have a significant effect on plant health and possibly on ecosystem structure.

Herbicide application can lead to changes in insect herbivory. Increases in herbivory have been attributed to higher concentrations of nitrogenous compounds including amino acids and proteins in exposed plants (Chaboussou 1986). Stanley and Hardy (1984) suggested that bare land after nonselective herbicide treatment provides a suitable environment for invasive species of plants and insects to colonize. Bare soil is receptive to numerous and widely distributed seeds from weed plants and the monoculture crop is an easy target for plant feeding insects such as aphids. Invading insects are preyed upon by similarly invasive predatory species of insects such as ants. The non-crop plant species and invasive insects have several features in common including rapid multiplication and dispersal mechanisms that allow rapid colonization. Herbicide use in and around cultivated fields has also resulted in a decline in the abundance of certain plant species, which in turn has resulted in the decline of certain insect populations (Hume 1987).

Freemark and Boutin (1994) summarized the impacts of herbicides on biotic communities by stating:

> "Different taxonomic groups...play important roles in agroecoystems in soil fertilization and aeration, the recycling of organic material and nutrients and the degradation of contaminants....The use of agricultural herbicides (and other pesticides) can interfere with these functions by altering plant

biochemistry, developmental processes and morphology, changing population dynamics, species composition and diversity, interrupting energy and nutrient flows, degrading water quality and changing the composition, heterogeneity and interspersion of habitats for wildlife."

4.2 Objectives

The overall goal of WED's chemical pesticide research is to assist OPP and OPPT in development of methodologies to determine the effects of chemical herbicides on non-target terrestrial crops and native plants ecological risk assessments (Figure 1.1). Current plant tests, while adequate for determination of basic toxicity of pesticides or other chemicals to plants (i.e., by measuring occurrence of death for young plants) cannot supply OPP and OPPT with the necessary information needed for ecological risk assessments. Test species currently used generally are not the non-target crop species at risk from herbicide exposure, but rather are common agricultural species. In terms of regulatory and scientific needs, recommendations for research made at various meetings over the past 12 years (Table 4.2) illustrate the breadth of new information needed for improved plant tests. Tests are needed at a range of scales of responses from molecular, to individual plant seedling life-cycle responses, to multispecies ecological responses. Despite these needs, there presently is very limited methodology available to determine the risks of herbicides to terrestrial plants, both native and cultivated, plant communities, and the organisms associated with those plants. Methodology must be developed to allow realistic ecological risk assessments to be made prior to the registration of new chemicals or reregistration of existing chemicals

The types of tests needed for risk assessments range from specific, designed for assessing the risk of particular chemicals on particular plants, to general designed to supplement the information required for vegetative vigor and ecosystem response tests in the current tiered approach. There are four specific objectives for research at WED to improve plant tests to evaluate the effects of chemical herbicides (and potentially other chemicals): (A) improve the process for selection of test species, (B) improve species test guidelines, (C) provide input for ecosystem response tests, and (D) develop mode of action studies / molecular biology tools (Figures 1.1, 4.1). In Figure 4.1, the two most important immediate objectives, (A) and (B), are indicated in bold; while objectives (C) and

(D), though very important over the longer term, are indicated with dashed lines to indicate that they are highly dependent on resources in the future.

A. Improved process for selection of test species

Improving the process for selection of test species is our highest priority because of questions regarding the scientific justification for test species selected by EPA for use in collecting registration and re-registration data for protecting green plants (both terrestrial and aquatic test species), (A in Figure 4.1). The development of an improved GIS-based protocol to select plant species for plant testing purposes is central to all other aspects of our plant test research. A GIS-based methodology is being developed as part of the regional analysis and interpretation research described in Section 3.0 above. It will be used to select a range of crop and native plant species for improved vegetative vigor tests to provide the initial screening information indicating whether or not a chemical is toxic to terrestrial plants (current Tier I and II tests). The GIS would be used to select species from regions of greatest herbicide use, and, thus where potential non-target drift problems from the chemical would be expected to be greatest. A larger range of species would allow consideration of diversity in possible plant responses. Inclusion of information on species phenology would allow EPA to better suggest which species should be subject to reproductive or developmental tests as well (current Tier III test). The methodology would also be used to select species assemblages for ecological tests and species of interest for molecular tests.

B. Improved plant test guidelines

Our second-highest priority is development of improved test guidelines that will include endpoints reflecting the entire life-cycle of plants (i.e., developmental responses especially reproduction), and include nontraditional test species (B in Figure 4.1). For life-cycle endpoints, there are limitations in terms of relating currently used growth assessment endpoints, such as shoot height and shoot dry weight, to crop yield (Table 4.4) (Maxwell and Weed, 2001; Obrigawitch et al., 1998). Early growth responses measured two weeks after exposure to an herbicide or chemical may not correlate well with responses when plants are exposed to herbicides at critical developmental stages later in their life-cycle (e.g., at flowering or fruit set). Figure 4.2 illustrates different times of possible herbicide application compared with the life cycle of a plant. Life-cycle responses

especially may be important for perennial crops and native plants. For native plants, life-cycle responses such as reproduction, in terms of seed production, will be critical for determining ecosystem-level responses to chemicals such as changes in plant interspecies competitiveness or changes in suitability as wildlife habitat.

Both traditional seedling-oriented (e.g. vegetative vigor) tests and proposed life-cycle (reproductive/developmental) tests also will be evaluated to verify applicability to nonstandard test species. These may include biennial or perennial crops (herbaceous or woody), native plants (both annual and perennial) and specific threatened or endangered species. The test methodology considered will include standardized cultural procedures for plants and specifics on herbicide application procedures. In terms of cultural procedures, current plant tests are conducted under a minimal set of climatic conditions geared towards production of uniform, vegetative, seedling plants. These likely are not adequate to provide the resources (e.g., soil volume, fertilizer, water, space per pot per plant) or conditions (e.g., photoperiod, air temperature) to carry different species through their full life cycles as required for determination of reproductive and developmental responses. Current plant growth protocols also likely are not adequate for native plants growing under a variety of different conditions. For example, there as been little emphasis in the protocols in terms of growing media other than the requirement for a sandy loam soil. Soil types and soil properties (pH, % organic matter, and cation exchange capacity) differ widely, and affect both plant health and herbicide chemistry. In terms of herbicide exposures, the exposures protocol must represent field application methods, concentrations and timing to realistically assess the impacts of the herbicide on the endpoints of greatest interest.

C. Input for ecosystem response tests

The primary focus of the plant test species selection (A) and test guideline (B) objectives above is to provide information to improve the plant testing protocols where single species are grown independently, either in pots or small plots. However, in reality, ecological risk assessments need ecosystem-based tests to determine the effects of chemicals on multi-species ecosystems and not just individual species. Thus, we will provide input to develop protocols for ecosystem response tests (C in Figure 4.1). The protocols will be based on information from the plant species and response endpoint objectives, and they will take into account the modeling needs for risk

assessments. Such tests will provide guidelines more realistic than those in the current Tier III Field Effects test. The tests will be field-based and include the measures of plant productivity and community structure that are vital for defining quality of wildlife habitat and the persistence of plant communities. An important emphasis of any ecosystem response protocols is the provision for links to wildlife population models, because such models are being developed in other projects such as the WED Terrestrial Habitat Project.

D. Develop mode of action studies and molecular biology tools.

For the long term, research on herbicide modes of action and molecular biology-level responses to herbicides is a critical objective for the plant effects studies (D in Figure 4.1). Research into the application of genomics and proteomics for the prediction of the effects of herbicides on plants was strongly recommended by the SAP in 2001.

Both the current and proposed plant and ecosystem tests (A-C above) focus on whole plant growth and biomass or yield responses of plant species. Such tests are based on known mechanisms of action of herbicides. These tests require growth of whole plants for full life cycles, which takes considerable time, space, and money. If the physiological, biochemical mechanisms, and molecular modes of action were better known for some herbicides, especially in terms of the molecular basis for how herbicides cause reproductive effects, extrapolation and prediction of responses across species may be facilitated. If so, studies with multiple plant species for long periods of time would not be required. Thus, molecular biology offers the promise to develop tests to screen plants susceptibility to herbicides *in vitro* to possibly supplement or replace whole-plant tests in some cases.

Molecular biology tools may also have the potential to the effects of detect low doses of herbicides in field ecosystem studies. Development of molecular biology tools based on chemical herbicide effects needs would be intimately related to the development of molecular biology tools for gene flow studies (genomics and proteomics) as they would use similar molecular biology laboratory methodology, equipment and scientific expertise (See Figure 5-5).

4.3 Approach

Research to address objectives 4.2.1 through 4.2.2 will be through controlled experiments based on the issues and recommendations from scientists and agency policy makers (Table 4.2). Every effort will be made to design the experiments to gain the information needed to meet several objectives with the same study (e.g., by including new possible non-target species, reproductive responses, and different environmental conditions). The research conducted to meet each objective will include measures of the variability in the data in order to more fully characterize the risk and facilitate probabilistic modeling. The major characteristics of our approach to address the four objectives are shown in Figure 1.1 and described below.

A. Plant species

Information on both crop and native plant species to chemical herbicides and other chemicals will be obtained for different types of test endpoints (vegetative vigor, reproductive/ developmental, ecological), and to characterize candidate species for mode of action and molecular research. We will use a regional GIS approach to species selection (see Section 3.3). For major agricultural regions of the U.S., a list of plant species and plant communities expected to be exposed will be generated. The list will include crops ranked by the number of acres occupied, dominant and sub-dominant native plants, important plant species for wildlife habitats or forage and threatened and endangered plants. Plant species of interest will be obtained and cultivated to determine their suitability as test species. Specific steps in the species selection process are:

1. Select species on a regional basis, defined in terms of US agroecoregions of interest. The agroecoregions will be based on:

   a). *Intensity of agriculture*, by identifying relative amount of farmland (or other type of land use) *vs.* total land area in a given spatial unit (e.g., county)

   b). *Herbicide usage*, using statistics to identify crops receiving the most herbicides in an area (pounds/acre, total pounds, total pounds of active ingredient, acres treated)

c). *Crop diversity*, using statistics to identify the number and relative amount of different crops in an area (more diverse agriculture will indicate a greater likelihood of herbicide sensitive crops growing in proximity to herbicide target crops)

d). *Wind direction and intensity data*, to indicate likelihood of drift problems due to aerial application.

Figure 3.2 illustrates the results of preliminary analysis of agroecoregions of interest in the US based on potential for herbicide drift impacts to non-target crops and native plants. The agroecoregions are comprised of counties with the highest percentage of acres in agricultural production, the highest percentage of crop acreage receiving herbicides, the greatest diversity of crops grown, and wind speeds >10 mph.

2. Develop list of most important terrestrial plant test species for each agroecoregion. Begin with the current EPA, ASTM, OECD, Canadian lists of test species. Ten species have been used for testing based on the current EPA Tier I and II vegetative growth tests, but the number of species selected could vary depending on the diversity of plants growing in an agroecoregion. The addition or subtraction of species to be selected will be based on the following considerations:

   a) Dominant crop species

      Based on yield, area or economic value

   b) Dominant native vegetation based on

      i) Potential native vegetation (e.g., historical based)

      ii) Current vegetation surveys

      iii) Satellite imagery or other form of remote sensing

      iv) Wildlife habitat (food and shelter)

      v) Ecological importance of species (keystone species for productivity, nutrient cycling)

c) Threatened and endangered plant species (or surrogate species in same genera)

d) Consider phylogenetic relationships to insure that a diversity of plant genotypes which may vary considerably in responses, are considered as test species.

3. For single-species test studies, conduct experiments in pots or small field plots for the most important endpoints and with experimental conditions representing the climate of interest, cultural practices and herbicide exposure characteristics typical for the crop (B, below).

4. For ecological studies and field tests, key assemblages of species for major regions will be identified.

5. For mechanistic and molecular biology studies, the species selected initially will reflect model systems currently in use for plant genomics / proteomics research. Later, the list will be broadened to include those identified for different agroecosystems.

The GIS analysis will identify specific regions of the U.S. for intensive species analysis. Based on preliminary research, the first area of interest will be the Midwestern corn belt (due to intensity of agricultural production and herbicide use), followed by California (in part because of the intensity of pesticide use data), Pacific Northwest (in part because of ability to conduct initial field work), northern plains states (location of crops of interest for both herbicide and gene flow studies) or other areas.

B. Plant test guidelines

Response Endpoints. Current short-term, vegetative growth endpoints (biomass, height), while useful to indicate general lethality of herbicides, may not provide data regarding longer-term effects on plants such as sublethal effects, the ability of plants to recover from stress, or changes in the competitiveness of plants. It also is clear that early plant growth effects may not predict latent adverse reproductive effects. The current plant tests last a maximum of 28 days. This short time period is insufficient to capture the critical reproductive phase of most plant life cycles. Not only is this aspect important for the individual plant's ability to pass along its traits, but reproductive yield is one of the most important economic aspects of agriculture. The very limited data available suggests

exposure during vegetative versus reproductive phases of growth may not have equal influences on reproduction and crop yield. Thus, we will conduct research to determine the effects of herbicides on endpoints most important for the risk assessment process.

Experiments will be conducted to determine the following:

1. What is the relationship between the time of exposure during a plant's life cycle and resultant developmental effects, both in terms of altered seed production and damage to other storage organs such as tubers or roots?

2. What are reproductive and development endpoints of particular usefulness when evaluating responses of native plants, and how best can these be quantified?

3. How do different classes of chemicals affect various specific reproductive or developmental responses?

4. Do different families of plants respond differently in terms of mechanisms of uptake, transport and degradation of different chemicals? Can we predict the specificity of this mode of action for different species and different stages of plant development?

5. What physiological processes (biochemical and molecular endpoints) are most sensitive to different chemicals?

Annual Plants. The relationship between vegetative and developmental endpoints will be determined for important annual crops from key agroecoregions by:

1. A limited number of crops will be used to determine reproductive (seed production) endpoints, building on the results from previous studies. For example, soybeans will be used a) to gain additional information on the risks to this major crop from the central U.S. corn-belt agroecoregion, b) to verify and build upon results with soybean from previous studies at WED and elsewhere, and c) to develop experimental protocol methodologies for a crop growing areas with hot summers. Peas will be used to obtain similar information, with respect to a crop growing under cooler environmental conditions.

2. Several crops will be used to determine developmental endpoints such as tuber or storage root formation and growth. Potatoes will be used due to their known susceptibility to ALSase herbicides along with other candidate crops such as sugar beets and carrots.

3. Crop plants will be grown in pots under controlled greenhouse conditions and exposed to a variety of different herbicides at different growth stages. Vegetative endpoints will be measured at 14 days after treatment to provide data that will correspond to the results from current vegetative vigor tests. Reproductive and developmental endpoints will be measured at the time of target organ maturity, i.e., mature seed or seed for reproductive endpoints and tuber maturity for a developmental endpoint. Results will be given in terms of various parameters.

4. Regression equations will be calculated to relate response to over a range of herbicide concentrations to obtain a range of EC values (Effective Concentration for a certain response), and to obtain coefficient of variability around the regression line. Such measures of variability are necessary for probabilistic risk assessments. They also indicate the possibility of sublethal plant responses at lower concentrations of the herbicides. For compatibility with the current vegetative vigor tests, EC_{25} values (EC for a 25% reduction in a response) will be calculated from the regression equations for different response parameters.

5. After basic vegetative and developmental relationships are established for annual crops, experiments will be conducted with annual native plants from key agroecoregions to determine if similar relationships occur for those species.

6. Annual plant studies will be conducted first with potted plants under greenhouse conditions to develop test protocols that can be used in multiple locations throughout the year. Experiments will then be conducted with plants in pots placed outside, and plants in soil in small field plots to develop test protocols that can effectively be used for specific crops under particular exposure scenarios.

7. As feasible, studies will also consider the use of annual native plants as test species considering reproductive and developmental endpoints in addition to general growth responses.

Perennial Plants. Reproductive and developmental responses are of particular interest for perennial crops due to the long-time frame elapsing from field planting to crop production. For native plants, responses of perennial species to stressors are key aspects of ecosystem responses. Perennial plants of interest include both herbaceous plants such as grasses where the belowground portion persists each year, as well as woody shrubs and trees. Basic aspects of the studies will be as follows:

1. Key perennial crops from agroecoregions of interest will be evaluated both to verify reproductive responses and to identify relevant experimental conditions for testing. Examples are strawberries from California and grapes from California and other regions.

2. Perennial crop plants will be initially grown in pots under controlled greenhouse conditions and exposed to a variety of different herbicides at different developmental stages. Vegetative endpoints will be measured to correspond to the results from current vegetative vigor tests and developmental endpoints will be measured at the time of target organ maturity, as feasible, i.e. mature seed or seed for reproductive endpoints from species such as strawberries and belowground sinks for other species. Results will be given in terms of EC_{25} for different response parameters at different harvest times.

3. The feasibility of using perennial crop plants in the field will be evaluated.

4. Experiments will be conducted with perennial annual native plants from key agroecoregions to evaluate the feasibility of their use as herbicide test species and to determine if native plant responses are similar to those of perennial crop plants. For example, perennial grasses, forbs (herbaceous plants other than grasses) and seedlings from woody plants characteristic of the central U.S. corn-belt agroecoregion will be obtained, propagated and evaluated as possible test species in pots. Our initial research will focus on developing the experimental conditions necessary for the successful use of these species as possible test plants for herbicide studies. Of special importance will be vernalization requirements, soil, and water

regimes. The phenology of species will be determined to optimize the time of herbicide exposure. Initially perennial grasses and forbs may be studied because their short life-cycle means that it takes a relatively short time for them to produce seed. In addition, their extensive root systems provide a belowground sink which may be affected by herbicide treatment.

5. The most advanced studies will focus on the responses of native perennial herbaceous and woody plants species to herbicides *in situ*. These would be long-term studies (optimally five years or more), primarily to measure reproductive and developmental effects. The studies likely would be conducted in primarily Oregon, but they may be conducted in other areas of interest. This work will build on the advances in research that result from the studies with annual and perennial crops and annual and perennial native plants grown in pots.

Specific Experimental Objectives and Conditions. We will determine the cultural conditions required to grow healthy test plants and herbicide treatments which produce the most field-relevant results. Examples of key experimental conditions that will be considered are:

1. plant growth containers (size of pots for plant life-cycle tests)
2. media for potted plants (mineral soil vs. artificial soil mix, mineral soil type especially critical for native plants)
3. watering protocols (amount, top vs. bottom)
4. environmental conditions (greenhouse vs. field)
5. herbicide concentrations (based on modeled exposures based on GIS analysis)
6. herbicide mode of exposure (foliar spray, soil application, timing during life-cycle of plant)
7. number of herbicides in formulation (single vs. multiple chemicals).

Our experimental objectives and conditions initially will be based on the methodologies used in current test guidelines. This will ensure that we build on past efforts while providing a path to

future test designs. We will refine the methodology based on outputs (e.g., soil type) from GIS analyses for specific agroecoregions of the U.S. Each individual experiment will contribute to address several of the research objectives. For specific experiments, details will depend on plant species chosen for the study and the response endpoints of interest. Examples of specific potential experiments which may be conducted in 2002-2003 are given in Table 4.5. An example of the research protocol used for a preliminary study to determine effects of the herbicide "Oust®" on soybeans is shown in Appendix B.

The particular experimental design (including treatments, replicates, and observations) and statistical analysis protocol for a study will depend on the objectives and hypotheses to be tested. We will consider other types of statistics to describe responses besides the commonly used EC_{25}. For example, we will consider the nonlinear curve fitting techniques such as described by Stephenson et al. (2000) which can provide a measure of the uncertainty associated with the response.

C. Future studies

Ecological Studies. Ecological studies will be designed to address the greatest areas of greatest uncertainty in the risk assessment process for chemical herbicides and other chemicals. Even though ecological studies likely will be highly herbicide- and ecosystem-specific, we hope to establish ecological study protocols that will provide data that are broadly applicable to a variety of risk assessments. Ecological studies could be conducted at different scales depending on the scientific question being asked and available resources, and could range in scale from the simple to complex. These studies will require detailed protocols including questions asked, experimental design and endpoints needed to address those questions, and types of statistical analyses and power of those statistical analyses to determine to evaluate results. Examples of the types of possible studies and a sense of how they might be staged are given below:

1. Initially, ecological information could be obtained under controlled conditions. The simplest experiments could evaluate plant competition (measured as numbers of species, biomass) with herbicide exposure under controlled conditions, possibly in large pots. These could be replacement series experiments with different densities of two to several species. Such

studies with herbicides could be similar to those with elevated CO_2 and described by Olszyk and Ranasinghe (1994).

2. Next, small plots could be used to simulate multi-species responses to chemical herbicides under controlled soil conditions (Pfleeger and Zobel, 1995). Test scenarios would include sampling of existing vegetation surrounding test plots such as field margins and more structured designs where mixtures of plants are seeded or planted at various densities and proportions prior to intentional herbicide application. End points will include production, reproduction and species interactions. The effects of herbicides on susceptibility of plants to other stresses (e.g., disease, insects, and climate) could also be studied.

3. Large field-plot studies then would be conducted to refine and further develop the methodology for determining herbicide effects at the ecological level (Taylor, 2001). The studies would be conducted, initially at sites in the Pacific Northwest, and later in various geographical locations throughout the country, targeted to answer specific herbicide risk questions of interest. The sites will be selected by the previously detailed GIS approach. The studies would be conducted adjacent to agriculture fields by a multi-disciplinary team of scientists to determine ecological effects. The studies would be based on models of the potential responses of a variety of plants to herbicides in ecosystems. Herbicide exposures would be as modeled for those systems.

4. Finally, field surveys for ecosystem effects of pesticides will be conducted by comparing different farming systems. For example, organic farming systems are now reaching sufficient maturity so that their ecological characteristics can be compared to those of conventional (pesticide using) farming systems. Studies of organic farming systems to date have focused on the effects on crops including yield and profitability, and of the general environmental effects such as changes in soil quality and nutrient runoff (Reganold et al. 2001). However, these systems could be used to test the hypothesis that low levels of herbicides can, indeed, affect native plant populations, and, hence, their suitability as habitat for wildlife populations. Other wildlife population studies could also be extended to study herbicide effects. For example, OPP's Biopesticides and Pollution Prevention Division (BPPD) currently is looking at the relationship between insecticide use and breeding bird

data, and will be exploring further linkages to understand relationships with avian populations (Brandt, E., Personal Communication, OPP).

Mechanism of Action for ALS Herbicides. Most past research on low-dose, high-toxicity herbicides has identified a single mode of action, impairment of the ability to produce branched chain amino acids. However, there is reason to believe that other process such as cross membrane transport may be impaired, especially in the species where reproductive effects have been identified (Taylor et al., 2001). Studies will be developed to evaluate alternative modes of action for ALS inhibitors, and the effects of ALS modes of action on plant structure and function. For example, in terms of transport processes within the plant, radioactive tracer studies could be performed to determine the tissues containing the herbicide or its breakdown products periodically after application. Identification of the labeled compounds will be done to verify their composition and to determine if the herbicide is directly causing the impairment or if a physiological system has been disrupted. While ALS herbicides will serve as model systems for the proposed mode of action research, we will address herbicides with other novel modes of action which may be developed during the course of this project. Studies on modes of action will be based on literature review and, if feasible, on information available from herbicide manufactures (Beyer et al., 1988).

Rapid screening genomic tests to detecting potential effects of pesticides. As currently conducted, the pre-registration evaluation of chemical herbicides or other chemicals considers a very narrow range in the genetic, economic and ecological breadth of organisms present in the highly-diverse ecosystems found in the United States. We need to develop methods that can serve as biomarkers of ecological effects of environmental stresses (McCarty et al., 2002; McCarthy, 1990). They need to be specific for herbicide effects in plants, as such as cholinesterase inhibition as a biomarker of insecticide exposure in animals (Chambers et al., 2002). Such biomarkers would enhance the ability of EPA to better understand the effects of herbicides and other chemicals on target and non-target plant species at the molecular level. Information on genes affected by herbicides and other chemicals could be assembled into a single database for structure-activity modeling of pesticide behavior and anticipated effects. Since the first detectable response of an organism to a toxin is typically a change in the expression of its genes (discernible as altered abundance of messenger RNA) it is logical to evaluate impacts of toxins initially by examining mRNA through differential display methodologies or standard microarray assays to characterize the

effects of classes of pesticides. However, in the longer-term, it would also be useful to study pesticide effects through genomic analysis of notable instances of resistance or susceptibility to pesticides. The range of methodologies involved in this latter work would allow for the collection of data from a diverse set of organisms whose full genomic sequences have not yet been completely determined, are not yet available in the public domain, or are not anticipated to be determined. Once the genetic context for responses to pesticides by a broader range of organisms is characterized, rapid screening methodologies can readily be developed and applied. From this overall approach, EPA would gain the necessary flexibility to analyze new classes of effects of pesticides. The Agency could then move from database construction to the development of microarrays specific for assessing the effects of new pesticides with similar modes of action.

Molecular Biology Tools. Molecular biology tools may allow us to identify sites of action and to elucidate modes of action for different chemical classes of herbicides. For example, while it is generally recognized that sulfonylurea herbicides are inhibitors of the enzyme acetolactate synthase and that phenoxy herbicides inhibit acetyl coA carboxylase, less is known about how other herbicides may affect the expression of genes. Weeds and crops which have spontaneously developed resistance to herbicides, and crop plants engineered to be resistant to specific types of herbicides, can each serve as means to identify genes and proteins that may be useful in mode of action studies. For example, by comparing the time course of gene expression at different developmental stages in herbicide resistant and susceptible plants prior to and after exposure to herbicides or other test chemicals or environmental stressors, one may be able to identify gene or protein sequences of interest for subsequent biochemical characterization and for use in physiological and ecological studies.

Genomics information such as DNA sequences for entire plant genomes are becoming increasingly available in the public domain (e.g., the *Arabidopsis* and *Oryza* genomes that have been sequenced and others still being worked out) and may become useful to evaluate effects of chemical herbicides. Currently, the only commercially available gene chip for plant genes is for *Arabidopsis*. However, within the next few years, it is anticipated that commercial gene chips for microarray analyses will be available for corn, wheat and soybeans. In addition to commercial gene chips, we have tools available locally at WED and through the OSU Center for Gene Research and Biotechnology to isolate, characterize and sequence nucleic acids and proteins of interest as markers

of exposure to specific kinds of agricultural chemicals. Use and critical analysis of microarray approaches (see Fig. 5-5) are expected to play a key role in these future studies (Dahlquist et al., 2002; Pan, 2002; Ramoni et al., 2002; Simon et al., 2002; Yang and Speed, 2002).

WED's ability to conduct this molecular biology research will require additional resources. The first step in the establishment of the program will be the recruitment and hiring of an EPA Postdoc familiar in current physiological/biochemical/molecular methodologies and research questions regarding herbicides and terrestrial plants.

4.4 Time line

The timing of research on plant effects from herbicides is in three phases (Table 4.6). The first in the sequence of research events was the problem formulation phase in FY2002. Then a five-year (FY 2003-FY 2007) effects research program will be launched to address the highest priority research objectives. Finally, there will be the synthesis and integration phase (from FY 2007 onward), during which time information will be analyzed and summarized to develop new tests. Further plans will be made for intensive research on new areas needed for complete risk assessments which may be beyond the scope of the current project, e.g. ecosystem response tests and genomics/proteomics based mechanistic tests.

During FY2002 the focus was on preparation of this research plan and the development of basic resources (i.e., plant growing facilities, herbicide treatment equipment, and experienced staff) necessary for plant effects research at WED. Preliminary scoping research has been conducted to develop the initial GIS procedures for species selection; to update the greenhouse, growth chamber, and nursery area infrastructure for growing plants; to gain familiarity with phenology, morphology, and productivity of a variety of possible test species; to evaluate performance of the pesticide track sprayer; to establish health and safety and quality assurance protocols to insure scientifically credible studies in the future; and to initiate preliminary experimental studies..

Over the following five years (FY2003-2007) the research plan will be implemented and the research will follow a general sequence of experiments moving from more toxicologically-oriented to ecologically-oriented work, to provide a range of information and tools available for ecological risk assessments. In general research will progress over time from:

Toxicological Responses---------->Ecological Responses

Annual Plants---------->Perennial Plants

Crop Plants---------->Native Plants

Individual Species ---------->Multiple Species

Vegetative Responses ---------->Reproductive Responses

Plants in Pots ----------> Plants in Field Soil

Greenhouse Studies ---------->Field Studies

Each individual experiment will address several research objectives based on questions being asked and availability of staff. In addition to whole-plant test oriented research, preliminary studies will be conducted to develop methodology needed for to address ecological questions regarding chemical herbicides and to develop mode of action and molecular tools. During the third year of the project (2005) a peer review workshop will be held to assess progress in research to that date and to finalize the most critical research to be conducted during the last two years of this plan.

At the end of the five years of the present research plan, single test species protocols will be developed and a course of action will be prepared for further work on ecological tests for chemical herbicide effects and the development of molecular tools based on preliminary research and evaluation. Should additional funds become available, the ecological and mode of action and molecular studies can be initiated sooner.

Table 4.1 Examples of current criteria for performing vegetative vigor studies for terrestrial plants and chemical herbicides (Stavely, 2002).

	Subdivision J	OPPTS Series 850	ASTM 1963-98	OECD 208 (existing)	OECD 208 (proposed)	Environment Canada
Temperature °C	NS	25 ± 3 (Day) 20 ± 3 (Night)	20-30	NA	NS	NS
re: Humidity %	NS	70 ± 5 (Day) 90 (Night)	above 30, 50 recommended	NA	NS	NS
Light Intensity	NS	350 ± 50 μmol m^{-2} s^{-1}	300-400 μmol m^{-2} s^{-1} (2000 fc)	NA	NS	NS
Photoperiod	NS	16 Light : 8 Dark	≥ 14 h light	NA	NS	NS
Carbon Dioxide ppm	NS	350 ± 50	NS	NA	NS	NS
Watering	NS	sub-irrigation preferred	sub-irrigation or overhead spray	NA	sub-irrigation preferred	NS
Soil Organic Matter	NS	≤ 3%	< 5%	NA	< 1.5%	NS
Addition of Test Substance	foliar spray or soil application	consistent with use pattern	mixed into soil, sprayed on soil, or sub-irrigation	NA	sprayed on plants	NS
Test Design (minimum)	3 replicates, 5 plants each	3 replicates, 10 plants each	3 replicates, 5 plants each	NA	4 replicates, 10 plants each	4 replicates

NS = Not specified NR = Not reported NA = Not available

Table 4.2 Summary of recent efforts to address research needs for tests of plant effects from chemical pesticides, emphasizing WED contributions and concerns regarding new low-dose high-toxicity herbicides.

1. 1990 Workshop in Corvallis, OR to evaluate non-target plant testing in subdivision J of the pesticide guidelines and define research needs. This workshop included participants from government (OPP, ORD, Region 10, state agencies, Canada, academia (state universities from Colorado, Idaho, Washington and Oregon (state universities, and industry (Du Pont, ICI,, ABC Laboratories, Kodak, Springborne Laboratories). EPA Report EPA/600/9-91/041. A summary of issues shown in Appendix 4.D.

2. 1993, 1994, 1996. WED staff published papers in support of OPP, confirming the inadequacy of present test guidelines to determine reproductive effects of low-dose herbicides. This research was supported and partially funded by OPP and Region 10.

3. 1999 WED Staff (T. Pfleeger and J. Fletcher) on the steering committee developing the non-target plants workshop for ILSA Risk Science Institute sponsored by OPP EFED. The workshop was held in 1999 in Washington, DC and was attended by government, industry and academics. One of the major goals of the workshop was to identify OPP research needs. Following the workshop staff from OPP, WED and Region 10 met for an additional day to discuss the findings of the workshop as they pertained to the research needs of OPP EFED. Background information published as SETAC Publication on Impacts of Low-Dose High Potency Herbicides on Non-target and Unintended Plant Species (Ferenc, 2001), with specific research recommendations from that publication given in Appendix 4.E.

4. Spring, 2001 WED staff became involved in the development of the NHEERL implementation plan for Goal 4 (safe communities, i.e., pesticides). The goal of this committee is to take the research needs of OPP and develop a comprehensive research strategy to fulfill those needs. OPP is a participant at these meetings.

5. June, 2001 OPP EFED convened a meeting of the a Scientific Advisory Panel in Washington, DC in conjunction with the NAFTA US-Canada Workshop on Impacts of Low-Dose High Potency Herbicides on Unintended Plant Species to discuss the Non-target test guidelines and the need for change. Staff from OPP and WED made presentations at this meeting.

6. July, 2001. Reports of non-target effects from low-dose, high-toxicity herbicide Oust in Idaho. Staff from OPP, WED and Region 10 with Idaho State officials and local growers visited sites.

7. December, 2001. United Kingdom issues draft efficacy guidelines for Effects of Non-Target Crops of Highly Active Herbicides - Including Mixtures and Sequences. This document recognizes the need for highlighting the effects of newer herbicides, but no new specifics are given regarding those tests.

8. January, 2002. Final Report of the FIFRA Scientific Advisory Panel (SAP) Meeting of June 27-29, 2001.

9. March, 2002. Memo by I. Suzenaurer (OPP, EFED) stating critical chemical pesticide effects research needs.

10. May 2002. Report from the International Workshop on Non-Target Plant Risk Assessment. January 15-17, 2002, Alexandria VA.

11. June 2002. WED staff present invited seminar demonstrating an example of a GIS -based analysis to identify crop and native plant species useful for a agro-ecoregion based plant testing protocol.

Table 4.3 Examples of reports of non-seedling stage pesticide effects on non-target or simulated non-target plants (modified from Maxwell and Weed, 2001; Ratsch and Fletcher, 1991). This list only includes direct crop effects and not carryover effects due to residual herbicide from the previous year.. The non-seedling effects may, or may not have been associated with leaf injury.

Chemical	Non-target Plant	Response	Reference
2,4-D	Fieldbeans	Flower and pod	Lyon and Wilson, 1986
chlorsulfuron	Barley	Reduced seed/fruit yield	Lemerle et al., 1993
chlorsulfuron	Cherry	Reduced fruit yield, quality	Bhatti et al., 1995
chlorsulfuron	Cherry	Reduced fruit yield	Fletcher et al., 1993
chlorsulfuron	Canola	Reduced seed yield	Fletcher et al., 1996
chlorsulfuron	Pea	Reduced seed yield	Fletcher et al., 1995
chlorsulfuron	Smartweed	Reduced seed yield	Fletcher et al., 1996
chlorsulfuron	Soybean	Reduced seed yield	Fletcher et al., 1996
chlorsulfuron	Sunflower	Reduced seed yield	Fletcher et al., 1996
dicamba	Soybean	Pre- and post-bloom	Weidhamer et al., 1989
dicamba	Soybean	Pre- and post-bloom	Al-Khatib, K. and D. Peterson, 1999
Maleic hydrazide	Soybean	Reproductive effects	Helsel et al., 1987
metsulfuron methyl	Soybean	Reduced seed/fruit yield	Boutin et al., 1999
metsulfuron methyl	Soybean	Reduced seed/fruit yield	Boutin et al., 1999
metsulfuron methyl	Soybean	Reduced seed/fruit yield	Boutin et al., 1999
metsulfuron methyl	Soybean	Reduced seed/fruit yield	Boutin et al., 1999
primisulfuron	Soybean	Pre- and post-bloom	Al-Khatib, K. and D. Peterson, 1999
sulfometuron	Potato	Reduced tuber size and quality	Westra et al., 1991
imazamethabenz	Potato	Reduced tuber quality	Westra et al., 1991
thifensulfuron / tribenuron	Potato	Reduced tuber quality	Westra et al., 1991

Table 4.4 Examples of limitations for current assessment indicators for non-target effects of herbicides on plants (adapted from Maxwell and Reed, 2001; as based on Obrigawitch et al., 1998).

Assessment Indicator	Limitations
Visual Chlorosis Anthocyanin formation	Conflicting data on correlation with subsequent crop yield
Height	Variable correlation with yield, not well suited for mature dicotyledonous plants with multi-stem or prostrate forms
Plant Biomass	Suitable when vegetative portions are harvested, but not necessarily when reproductive parts are harvested. Can be affected by increased branching. Varies in sensitivity with time during plant life-cycle

Table 4.5 Examples of experiments on effects of chemicals on terrestrial plants 2002-2003.

Time Frame	Objectives	Species	Herbicide	Conditions
Summer-Fall 2002	Evaluate possible test plant species- annual crops Corn belt, Pacific Northwest Compare vegetative and reproductive responses Determine cultural conditions Evaluate herbicide treatment conditions	Soybean Potato	Oust	Pots Outside Nursery, Greenhouse
Summer-Fall 2002	Evaluate possible test plant species- annual crops, corn belt Compare reproductive response with different herbicides Determine cultural conditions Evaluate herbicide treatment conditions	Soybean	Multiple	Pots Greenhouse
Summer-Fall 2002	Evaluate possible test plant species- annual crops Pacific Northwest Compare vegetative and reproductive responses Determine cultural conditions - cool season crop Evaluate herbicide treatment conditions	Pea	Oust	Pots Greenhouse
Winter 2002-Spring 2003	Evaluate possible test plant species- native plants, corn belt Compare vegetative and reproductive responses Determine cultural conditions Evaluate herbicide treatment conditions	Multiple	TBD	Pots Greenhouse
Spring-Summer 2003	Evaluate possible test plant species- crops corn belt, Pacific Northwest Evaluate belowground developmental responses Determine cultural conditions Evaluate herbicide treatment conditions	Potato Soybean	TBD	Pots Greenhouse Small Field Plots
Fall 2003	Evaluate possible test plant species-native plants, corn belt Compare vegetative and reproductive responses Determine cultural conditions, soil type Evaluate herbicide treatment conditions	Multiple	TBD	Pots Outside Nursery

* TBD = To be determined

Table 4.6 Time Line for Chemical Herbicides and Terrestrial Plants Research

FY2002	FY2003	FY2004	FY2005	FY2006	FY2007
Produce Research Plan OPP Workshop Regulatory Needs Literature Search Produce QA Plan Track Sprayer Protocols Health and Safety Protocols Preliminary Plant Experiments Soil Type Environment Vegetative vs. Reproductive/ Development Endpoints	Contribute to APM on Strategy for Updated Test Guidelines: Finalized Research Plan Use GIS to Identify Crops and Native Plants at Risk from Herbicides: Case Studies Corn belt California Pacific Northwest Initiate Greenhouse/Nursery experiments on Vegetative and Developmental (Seed/tuber) Responses of Annual Crops and Native Plants Initiate Field Experiments on Developmental (seed/root/tuber) Responses of Annual Crops Test Experimental Procedures for Plant Growth and Herbicide Exposure Pots in Greenhouse/Field Mineral vs. artificial soil	Use GIS to Identify Crops and Native Plants at Risk from Herbicides: Other Case Studies (example) Northern Plains Continue Greenhouse Experiments on Developmental Responses of Annual Crops and Native Plants Other Case Studies Field Experiments on Developmental Responses in Annual crops and Native Plants Plan Greenhouse and Field Experiments for Reproductive Responses of Perennial Crops Test Cultural Procedures for Optimum Plant Growth Watering Hire PostDoc for Mode of Action / Molecular Biology Studies Contribute to APM on evaluation of risk assessment methods for herbicides	Use GIS to Identify Crops and Native Plants at Risk from Herbicides Other Case Studies Continue Greenhouse and Field Experiments on Developmental Responses in Annual crops and Native Plants (other case studies) Initiate Greenhouse and Field Experiments for Reproductive an Developmental Responses of Perennial Crops and/or Native Plants Initiate Studies of Mode of Action for Developmental Effects of ALS herbicides Peer Review Workshop on Plant Effects Research Contribute to APM on regional approach to risk assessment	Produce Improved Vegetative and Reproductive / Developmental Test Methodology for Crop and Native Plant Species in Major US Agroecoregions Cultural procedures (propagation, soil, watering, field vs. greenhouse) Continue Greenhouse and Field Experiments for Reproductive and Developmental Responses of Perennial Crops and/or Native Plants Continue Studies of Mode of Action for Developmental Effects of ALS herbicides Initiate Evaluation of Molecular Basis for Developmental Signals and possible Molecular Detection of Effects Contribute to APM on draft new protocol / guidelines for vegetative vigor test	Produce Developmental Test Methodology Seed and belowground sink herbaceous species Cultural procedures Measurement Endpoints Continue Field experiments for Reproductive Responses of Perennial crops Develop Protocols for Field Ecological Research Develop Protocols for Genomics Research for Factors Controlling/Detecting Herbicide Effects Contribute to APM on draft new protocol / guidelines for reproductive /developmental test

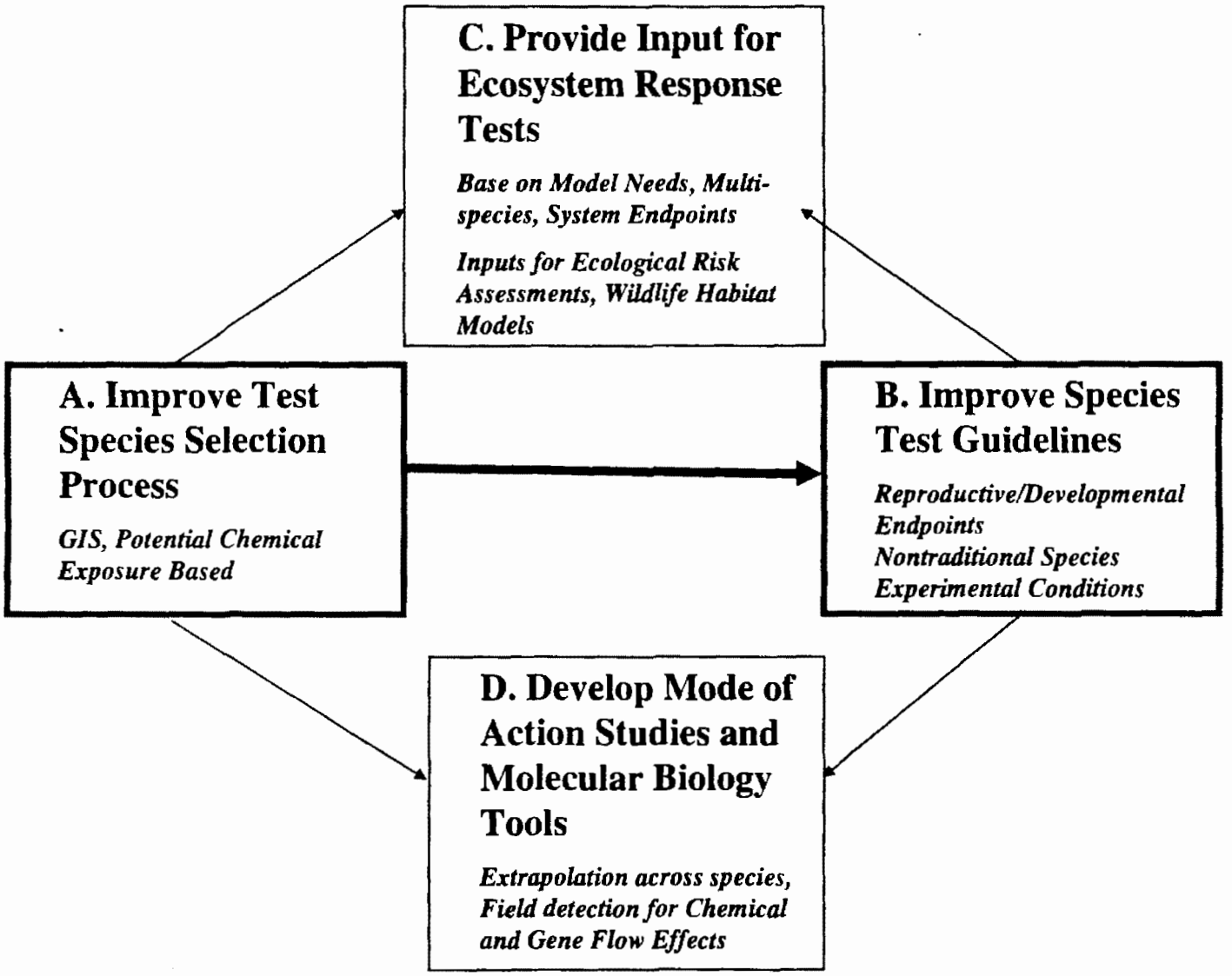

Figure 4-1 Research Objectives for Effects of Chemical Herbicides on Terrestrial Plants

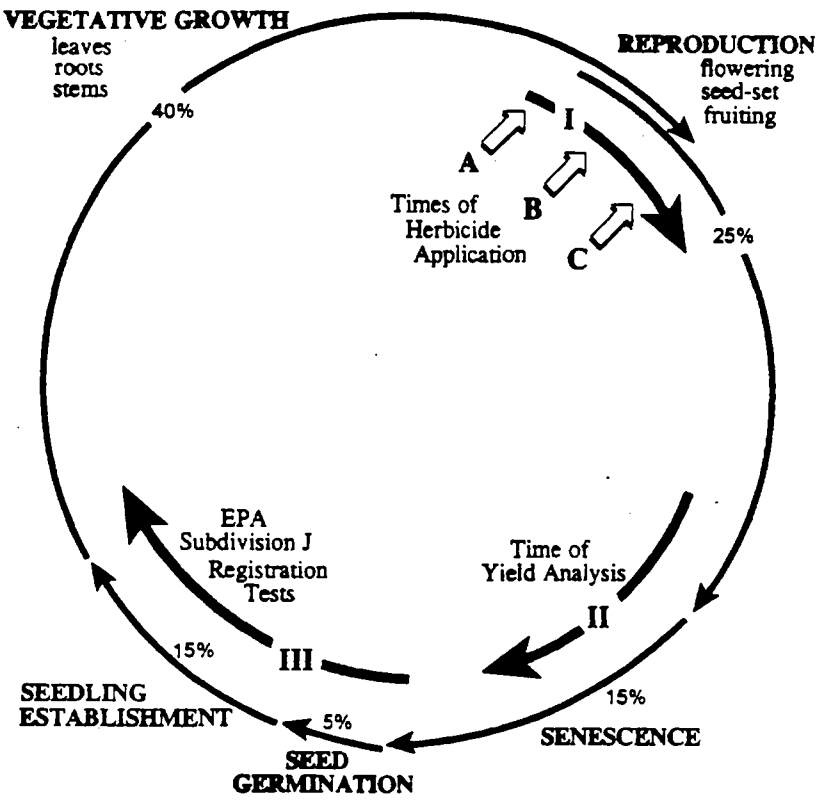

Figure 4-2. Stages in the life cycle of a plant in relation to potential herbicide applications. Arrow III indicates time of herbicide application corresponding to current EPA Subdivision J registration tests, and arrow I indicates possible times of application for reproductive / developmental tests. Critical periods of plant life-cycle are indicated outside the circle. Adapted from Fletcher et al. (1996).

5 EFFECTS OF GENE FLOW FROM TRANSGENIC CROPS

5.1 Introduction

A. Rationale

Statutory authority for Agency research on the ecological effects of chemical and biological pesticides is provided by the Federal Fungicide, Insecticide and Insecticide Act (FIFRA) and the Federal Food Drug and Cosmetic Act (FFDCA). Evaluations for compliance to FIFRA and FFDCA are determined in coordinated reviews of registrant applications with USDA and FDA respectively. In July, 2001, Final Rules and Proposed Rules for Plant-Incorporated Protectants (PIPs, i. e., engineered traits for crop protection expressed in transgenic plants), were published in the Federal Register (40CFR Parts 152 and 174, Appendix C). Regulated PIPs include nucleic acids and the proteins they encode to confer to weed, disease or insect pests of plants. Because of the pesticidal nature of these products, research to develop improved methods for assessing potential ecological risk is covered under the Implementation Plan for Government Performance Results Act (GPRA) Goal 4, Safe Communities.

Specific objectives of the Gene Flow Effects Project will be to address Agency needs for registration standards for transgenic plants containing PIPs that will help ensure their safety to the environment, and for human and animal food consumption. The Gene Flow Effects Project will develop methods to assess and predict the ecological effects of gene flow on plant community structure and succession. Crop protection PIPs to be evaluated may include traits for herbicide, disease or insect resistance. Methods will be developed to measure, assess and predict the effects of gene flow on selected individual species of herbaceous and woody plants and on plant community structure and succession in crop and non-crop ecosystems. Recognizing that specific plant species, plant communities and ecosystems may differ in different geographic regions of the United States, specific crops, traits and geographies will be selected and prioritized. Anticipated Project outputs for the Agency will include scientifically defensible methods that measure, assess and predict changes in plant community structure of crop and non-crop plants in terrestrial ecosystems. While gene flow may have broad potential national and international impacts, our initial emphasis at WED will be on identifying suitable test plants and engineered crop protection traits for specific geographic regions

of the United States, e. g., for herbicide, disease or insect resistant canola or grasses in the Pacific Northwest. It is possible that similar work may be carried out at other NHEERL Ecology Divisions, which would focus on gene flow, transgenic pesticidal pollen or feral transgenic plant effects in aquatic, rather than in terrestrial ecosystems.

Research carried out under this plan is expected to contribute to recommendations for short-term data requirements to be submitted by registrants for specific types of transgenic traits in specific types of crops in specific geographies. It may also result in criteria for post-registration, longer-term adverse effects plant monitoring requirements.

Gene flow effects research is anticipated to result in recommendations for measuring, monitoring and mitigating the extent and effects of gene flow from crop plants to other crop and non-crop plants. Effects of gene flow on plant community structure and succession, and on rare and endangered plant species, will be examined in both crop and non-crop ecosystems. Evaluation of the effects of gene flow from transgenic plants will focus on traits that have been developed for crop protection purposes such as herbicide, disease or insect resistance. Plant parts of special interest are pollen and seeds, since they typically are the primary means of dispersal via wind, insects, water or animals. The germination of pollen and seed respectively can bring about hybridization between compatible plant species, and establishment of a new generation of hybrid plants, which may in turn shed and disseminate hybrid pollen and result in the establishment and spread of subsequent hybrid seed progeny.

Initially gene flow effects will be evaluated empirically on a local or regional bases; longer term, they should be considered domestically and internationally on landscape levels, given the potential for movement of both pollen and seeds by biological and abiotic natural and anthropogenic means. Adverse effects of gene flow on plant associated symbionts, saprophytes, and pathogens, should also be considered, since those integral plant community components impact the sustain ability of plant ecosystems. In the longer term, probabilistic risk assessment modeling approaches will be developed, based on parameters identified in our initial empirical studies. While the potential for horizontal gene transfer between plants and microbes or plants and invertebrate or vertebrate herbivores and pollinators of plants is generally assumed to be low, such events could have adverse ecological and health effects. Accordingly, it is recommended that in the long-term, as

resources may permit, the ecological and health impacts of horizontal gene flow should be considered between plants and other types of organisms as well.

Currently, engineered crops are planted on tens of millions of acres in the US alone. Pollen from transgenic crops may hybridize with other related crops or weeds, potentially conferring resistance to crop-crop or crop-weed hybrids. If fertile, the resultant hybrids and their subsequent progeny can produce seed which may result in continued persistence and spread beyond the confines of the original intended agronomic fields. Multiple resistances to PIPs can occur simultaneously. For example, resistance (engineered and/or spontaneous in origin), to two or three herbicides belonging to different chemical classes is already showing up in canola in the UK and Canada (Orson, 2002). At a recent OPP Biological Pesticides Division and OPTS workshop in Washington, DC, high concerns about these and similar issues were expressed by attendees from EPA Regional Offices regarding potential impacts of transgenic gene flow. Due in part, to the relative scarcity of ecological (Appendix D), or human health data on effects of genetically engineered plants in the peer reviewed literature, concerns about the potential contamination of crops, feed and food with genetically modified materials have been raised by the media, activist environmental groups, commercial food processing companies and producers and consumers of organic crops. Demands have been made to label foods or ingredients derived from transgenic crops, and "GMO-free" has become a marketing tool to address the concerns of food processors, food retailers and domestic and international consumers. Legal actions against the Agency by activist groups and the high media attention paid to potential adverse ecological effects of transgenic pollen on monarch butterflies (*Danaus plexippus*) (Sears et al., 2001), allergenic effects of Starlink™ corn (Segarra and Rawson, 2001), and contamination of Mexican land races of maize with transgenic genes from cultivated corn (Quist and Chapela, 2001), further suggest the need to support Agency policies through research in assessing potential ecological effects of gene flow from transgenic crops.

Research to be done at NHEERL-WED initially will have a regional focus; e.g., in the Pacific Northwest, assessing effects of gene flow from canola to mustard and other compatible weedy or crop species or from grass crops to compatible weedy and native species. Two widely grown engineered crops, corn and cotton containing herbicide or insect resistance genes, were excluded from our proposed studies on gene flow. Our reasoning for that is based on the absence of known compatible weedy and native corn species in the continental US, and the presence of compatible

cotton relatives only in the Florida Keys, the U.S. Virgin Islands, Puerto Rico and the Caribbean, and Hawaii (Wozniak, 2002). Potential impacts of competitive ability, spread and persistence of crop-weed hybrids on plant community composition, structure, and wildlife habitat quality will first be examined by empirical approaches; later, these effects may also be examined by modeling approaches. The project is expected to have synergies with research at NHEERL-WED in the areas of effects of chemical pesticides on plants (Plant Effects) and modeling to determine the effects of multiple stressors on wildlife habitat. If areas beyond the Pacific Northwest are to be considered, collaborative research efforts with other NHEERL ecological and health effects Divisions also may ensue, e.g., to study potential impacts of the engineered genes, pollen, feral transgenic plants, and plant-derived products in aquatic habitats and on human health. Currently, literature reviews, discussions with plant breeders, molecular biologists and plant ecologists and participation in transgenic plant workshops are helping us identify species and genes of most interest and thereby refine our research strategies. Inputs for our proposed research are also being actively sought from Agency colleagues in the Office of Pesticide Programs, the National Center for Environmental Assessment and in EPA Regional offices. The research proposed herein would constitute part of an ORD-wide Five Year Initiative in Biotechnology.

B. Background

Genetic engineering permits the introduction of genes from diverse plant, microbial and animal sources into agronomic plant species. Once inserted into plants, those genes potentially may spread or flow to other compatible crops, weeds and native species. The resultant F1 hybrids, created by hybridization with transgenic pollen transported via wind or insects, may in turn self, out-cross to other compatible species, or backcross to the transgenic or non-transgenic parent. In addition to hybridization assisted scenarios, the transgenic genes may move via feral transgenic plants or seeds, i. e., over-wintering transgenic plants or seeds which escape cultivation, or via seeds that have fallen from planters, combines, trucks, or railroad cars during routine planting, harvesting, and shipping activities. Incidental transport of seeds via birds, other animals, and humans can further contribute to the unintended spread of transgenic genes beyond their intended areas of cultivation. While it is commonly argued that cultivated crops would not persist well outside of agronomic situations due to their need for high levels of fertilizer, limited information is available on

the survival, fertility and out-crossing potential of hybrids formed between crops and compatible weedy or native species. Many species in each of the two latter categories (weedy and native species), commonly thrive in low fertility soils. It may thus be anticipated that soil fertility requirements of hybrids between crops and weeds or native species may be lower than that of their transgenic crop parents. Available hybridization information, based largely on information gained with non-transgenic crops, tends to document that it can occur between specific combinations of crops and weeds. Less information is available on hybridizations between transgenic crops and weeds, or between crops or crop/weed hybrids and native species. Downstream impacts of such hybridizations on plant community composition, function and habitat quality remain hypothetical and largely unknown. The studies proposed below in the Objectives and Approach portions of this Research Plan (Sections 5.2 and 5.3 respectively) address these data gaps, developing molecular methods to track gene flow, and beginning to identify potential non-target ecological effects of gene flow.

In recent years, since the advent of commercial transgenic crops, such as cotton and corn expressing insecticidal (*Bacillus thuringiensis* subsp. *kurstaki*) genes, data are beginning to be available on potential impacts of toxin production on populations of beneficial insects, insect pests and birds. Much effort is being expended to develop strategies to minimize development of resistance in target pest populations to pesticidal toxins and to promote the useful commercial life of both plant and microbial delivery systems for pesticidal genes. These strategies include use of buffer rows of conventional crops, planting refugia, using multiple or alternative insecticidal genes, targeting tissues and times of gene expression. Some data also are available on potential non-target effects of transgenic plants on invertebrate and vertebrate herbivore and pollinator populations, or on plant-associated microbial pathogens, saprophytes or symbionts. However, the impact to the invertebrate community of transfer of pesticidal genes to non-target plant populations has not been well studied, particularly with regard to determining potential impacts on plant community structure and function. Expression of pesticidal genes in transgenic crops and unintended transfer of those genes to other crop and non-crop plant species, might each potentially result in changes in the population sizes of beneficial insects such as pollinators, as well as reducing targeted populations of insect pests or plant pathogens.

Researchers at WED are experienced in the development and use of molecular methods (Fischhoff and Watrud, 1988; Porteous, et al., 1994, 1997; Watrud et al., 1985; Watrud et al., 1987a, 1987b; Watrud et al., 1996; Watrud et al., 1998; Watrud, 2000; Widmer et al., 1996 a, 1996b; Widmer et al., 1998; Widmer et al., 1999; Winton et al., 2001). WED researchers also have prior experience with risk assessment of genetically engineered microbes and plants (Pfender et al., 1995; Seidler et al., 1998; Donegan et al., 1999; Di Giovanni et al., 1999a, 1999b). Additional key insights for the research we propose below have been provided by National Academy of Sciences reports on pesticides use (2000a) and on environmental effects of transgenic plants (2002); the Scientific Methods Workshop: Ecological and Agronomic Consequences of Gene Flow (2002) (Appendix E), meetings on biosafety and risk assessment of engineered plants (Appendix F), biotechnology references recommended by the US EPA Biotechnology Steering Committee (Appendix G), and numerous journal articles including (Bergelson et al., 1998; Dale et al.,1996; Duggan et al., 2000; Purrington and Bergelson, 1995; Quist and Chapela, 2001; Snow, 1997; Vierhelig et al., 1995; Siciliano et al., 1998). Numerous reports and publications [including those cited above as well as the National Academy of Sciences reports (2000a, 2000b and 2002) and the Scientific Methods Workshop: Ecological and Agronomic Consequences of Gene Flow from Transgenic Crops to Wild Relatives (2002)], demonstrate a clear need for more risk assessment research on products of agricultural biotechnology. The specific research questions and scientific approaches that we propose below are focused on (a) development of molecular methods to detect gene flow and (b) assessment of potential ecological effects of gene flow on the structure and functions of plant communities (Tilman, 1988) in agronomic and non-agronomic ecosystems.

5.2 Objectives

To address the overall goal to determine the effects of gene flow from transgenic crops (Figure 1.1), the proposed research will develop methods to assess the ecological effects of gene flow from transgenic plants and also the effects of feral transgenic plants, i.e., escapes from cultivation. The two major objectives of the research are (Figure 1.1):

1. to develop molecular methods to assess gene flow potential and exposure to transgenic genes in compatible weed, native and crop plant species, and

2. to assess ecological effects of exposure to transgenic genes in non-target plant species and communities, i.e., plants and associated biota (pests, pathogens, pollinators, herbivores and symbionts).

The research will focus on transgenic plants developed for resistance to herbicides, plant disease or insect pests. Pending availability, transgenic plants designed for specialty purposes such as chemical or pharmaceutical production, also would be of interest to examine in our studies. The specific scientific objectives include the ones indicated below. The studies would be designed to provide methods, protocols and data to track gene flow and its ecological consequences. Longer term, the research is envisioned to provide inputs, i.e., define parameters for probabilistic models to be used in the risk assessment of gene flow from transgenic plants.

5.4 Experimental Approach

An overview of the proposed ecological research and its fit with health effects research within NHEERL is shown in Figure 5.1. The exposure and ecological effects components of the risk assessment research for GM crops are shown in Figures 5.2 and 5.3 respectively. Figure 5.3 additionally illustrates the types of non-target potential ecological effects on plants that would be examined in plants at individual, population and community levels. The research proposed herein is focused on terrestrial habitats, primarily in the Pacific Northwest. The evaluation criteria considered in the selection and prioritization of plant species of interest as potential donors and recipients of transgenic genes in the gene flow exposure and ecological effects studies proposed below is shown in Table 5.1. Depending on potential collaborations with public and private sector researchers, the geographic scope of the research could expand both domestically and internationally. Regardless of geographic location, the research will provide methods and proof-of-concept for approaches to assess potential ecological effects of gene flow. Figure 5.4 conceptually illustrates the formation of patches of transgenic plants, which may arise as a result of gene flow resulting from wind or insect pollination of compatible species, or from the incidental transport of feral seeds. Progeny from the transgenic patches in turn, may serve as additional sources of transgenic gene flow. Figure 5.5 illustrates the types of molecular methods based largely on PCR and genomic technologies that would be used to estimate exposure to non-target plants such as weedy or native species, resulting

from gene flow from transgenic plants. Some key aspects of the gene flow research are highlighted in Figure 1.1

A. Develop molecular methods to assess gene flow

In FY2002, initial emphasis is being given to reviewing the literature and consulting with experts in the academic, government and private sectors to identify and prioritize crops and transgenic traits of greatest interest to the Agency. For example, is gene flow from herbicide resistant wheat, corn or potatoes in the northwest of greater interest than herbicide resistant gene flow from cotton in the southeast, or sugar beets in the southwest, or from corn, soybeans and sunflowers in the Midwest? In addition to regulatory needs, the technical feasibility and availability of tools will also be considered. For example, are plant materials and nucleic acid sequences of genes of interest publicly available? If not, can we access them from the private sector? How and where can we gain access to fields where the crops are/have been grown, so that we sample plant and soil materials for the presence of genes of interest? Once a selection has been made, e.g., to study the ecological effects of gene flow of a given trait such as herbicide resistance in a particular crop, research will be conducted to develop and use a quantitative polymerase chain reaction (PCR) methodology (Sambrook and Russel, 2001), to detect, monitor, and quantify the presence, persistence and spread of a targeted gene. When information on the mode or site of action of a herbicide or other pesticide is known, sequences of nucleic acids known as primers, can be selected or designed to essentially "bait" for a specific type of DNA in an environmental sample. Due to the presence of unique markers, promoters and coding sequences used in the engineering process, primers can be designed to detect the targeted transgenic DNA sequence. DNA extracted from environmental samples can then be restricted or cut with restriction enzymes that recognize specific sequences of nucleotide bases. The fragments are then "amplified" or replicated using a PCR reaction mix containing the appropriate primer, nucleotide bases, and *Taq* polymerase enzyme. Using ultra-violet light, the presence of the targeted DNA fragments in environmental samples are visualized and photographed following electrophoresis of the fragments on agarose gels. The gels also can be scanned to graphically illustrate the presence of bands of interest. Using fluorescently labeled primers, and appropriate instrumentation (such as the PE 7700 Gene Detection System

available at WED), we can determine the presence/absence of DNAs of interest, and also determine the number of copies of a given gene in a sample.

Specifically, the following approaches will be taken to develop required methods:

1. Identify and access or design primers and/or probes for hybridization for qualitative PCR or quantitative real-time PCR method to detect engineered gene trait (e. g,. herbicide resistance to glyphosate or disease resistance to a plant pathogen). Primers to be selective for gene in above and below ground plant parts of target crop (e. g., transgenic canola) and non-target weedy plant (e. g., bird's rape mustard), in crop and non-crop plant ecosystems.

2. Develop and use qualitative and quantitative molecular methods such as RT-PCR to detect genes of interest and to assess gene flow and its ecological effects on above ground and below ground plant community composition and functions.

3. Create F1 hybrids between selected donor crop and compatible recipient weedy, native or crop species and determine inheritance and expression of the transgenic gene in F1 and F2 populations.

4. Utilize Southern hybridizations, microarray and other genomic and PCR methods such as RFLPs (restriction fragment length polymorphism), SNPs (single nucleotide polymorphisms), ESTs (enhanced sequence tags), AFLPs (amplified fragment length polymorphisms), RAPDs (random amplified polymorphic DNA), SSLP (single sequence length polymorphism), microsatellite, SSR (simple sequence repeat) markers, STS (sequence tagged site) etc., to identify the presence and transmission of transgenic genes in hybrids of transgenic crops with non-target plant species. Specific examples of these and other molecular methods can be seen in Sambrook and Russel (2001), Cevera et al (2000), Denise et al. (2002), Dionsis et al. (2002), Hardegger et al. (1999), Templin et al. (2002) and Webster et al. (2002).

B. Gene flow studies

The general approach that will be used in greenhouse and growth chamber studies and to a lesser extent in field studies will be to create constructed communities of potential donors and

compatible recipients of transgenic genes. Donor plants may consist of transgenic crops and F1 or subsequent hybrid or backcross generations between the initial transgenic crop and compatible non-crop species. The non-crop species may consist of weedy or native plants; they may also be represented by other cultivars of the primary donor transgenic crop, or of other cultivated crops with which the donor crop may be compatible e. g., canola (*Brassica napus*), is compatible with other *Brassica* species and also with wild radish (*Raphanus raphanistrum*) and with cultivated radish (*Raphanus sativa*); creeping bentgrass (*Agrostis stolonifera)* is compatible with other *Agrostis* species (Wipff, 2002), and with rabbitfoot polypogon (*Polypogon monspeliensis*). It thus may be necessary to do analyses in a number of potential recipient plant species to determine the presence and stability of transgenic genes that have originated from the original transgenic crop source or from subsequent transfer of that gene to other crop and non-crop plants.

Specific questions to address gene flow are:

1. What is the potential for gene flow from crop to other crop and non-crop plants?

2. What is the geographic proximity of the crop to compatible wild and cultivated relatives and their respective times of flowering?

3. Gene flow occurs between transgenic crops and non-transgenic crop or non-crop plants, weeds, native, rare and endangered species?

4. Can F1 hybrids and back-cross progeny of F1 progeny x each parent, formed with crop, or non-crop species and transgenic plant escapes persist, spread, outcross and produce viable, fertile seed?

5. How long will DNA from the transgenic crops or their hybrids with weedy or native species persist in the environment and remain biologically active?

C. Greenhouse/growth chamber/field studies to measure potential ecological effects of gene flow.

The methods we will use at WED to assess the ecological effects of gene flow will be focused primarily on effects on plant fitness and on plant community structure. Depending on the

specific nature of the transgenic genes, which are being evaluated, additional specific types of measurements and assays will need to be carried out. For example, via extra-mural collaborative studies with academic and other federal or state agency collaborators, data could be obtained to determine effects of gene flow from crop to weedy or native species, on insect community structure, on beneficial pollinators and on predators of targeted insect pests. Similarly, if the transgenic gene being studied has been developed for disease resistance to a specific pathogen, it would be of interest to examine potential effects of presence of the transgenic gene to responses to other plant pathogens or to symbionts.

Specific approaches/questions to be addressed in the greenhouse, growth chamber and field studies are:

1. Identify, obtain and/or create hybrids between crops and weedy or native species.

2. Assess changes in herbaceous and woody plant fitness characteristics in response to gene flow, specifically effects on developmental endpoints, vegetative biomass and seed yield, viability, fertility and dormancy.

3. Sample transgenic donor and recipient compatible plants from plant growth facilities on-site and from field plots at locations of public and private sector collaborators, for the presence of the transgenic genes and for effects on fitness of the recipient plant species. Plants and soil within and beyond donor source plots may also be of interest to sample to determine the presence and persistence and transport of transgenic DNA.

4. Detect and quantify the presence of transgenic genes in transgenic plant sources and potential compatible crop, weed and native plant species recipients. Analyze greenhouse, environmental chamber or field samples using Sybr Green or other quantitative PCR method (Heid et al., 1996). Compare results obtained with Sybr green to those obtained using commercially available engineered trait testing materials or information available in the literature, for detection of e. g., herbicide or disease resistant traits of interest.

5. Using model constructed plant communities in open top chambers and in controlled environment chambers and in field tests, test the effects of population density and spatial arrangements of donors of transgenic genes on gene transfer to compatible hosts (see Fig. 5.4). Donors may be the primary transgenic crops or F1-F4 hybrids or back cross (BC1-BC4) progeny containing the transgene of interest. Additional test conditions may include optimal and sub-optimal moisture, soil fertility, and temperature regimes, as well as application of selective pressures provided by chemical herbicide, disease inoculum or insect populations.

D. Field studies of potential ecological effects of gene flow

1. Using constructed plant communities in contained environments (growth chambers and greenhouses or fine-screened field plots), and containing F1, F2 and/or back-cross progeny between the designated transgenic crop/trait(s) of interest and non-target weedy, native or compatible crops, assess transgenic gene flow between and among potential transgenic crop donors and crop and non-crop recipients. Study ecological parameters of interest, such as those indicated below, over a multi-year period.

2. Determine effects of gene flow on plant biomass, numbers, biomass and germination of seeds, fertility of hybrids and changes to plant community composition, re: frequency and abundance of given species, hybrids, etc.

3. Depending on the engineered trait(s) chosen for specific studies (i.e., whether insecticidal for above or below ground insect or disease pests, or resistance to an herbicide), apply specific biological pest or pesticide (e.g., herbicide) selective pressure(s). Next examine potential ecological responses for above ground and below ground biota such as beneficial invertebrates and microbes. Ecological parameters to consider in examining those non-target populations include abundance, diversity and metabolic functioning.

4. Given the expected interactions between genotype and environment, long-term efforts should be made to measure gene flow and its potential impacts under a variety of environmental conditions in the field. Factors to be evaluated may include different

levels of soil fertility, moisture, climates and soil pollutants such as persistent agricultural chemicals, e.g., herbicides, fungicides, insecticides which may confer selective pressures over an extended period of time.

5. Evaluate the effects of cultural practices, including crop rotation (particularly to a crop(s) containing the same transgenic gene), tillage methods, and pesticide use, on the short-term and long-term ecological effects of gene flow from transgenic crops

6. Evaluate the effects of soil fertility on crop-weed hybrid establishment, survival seed production, out-crossing potential and non-target ecological effects by (a) creating a series of compatible transgenic crop-weed or crop-crop hybrids; plant under optimal agronomic and low fertility conditions; assay for establishment, biomass, seed production, seed germination, fertility, over-wintering survivorship, and seed dispersal distances; assay for effects on non-target beneficial organisms, pests and wildlife.

E. Inputs for prototype model

Development of a probabilistic, regionally based, ecological risk assessment model is of interest to provide predictive information on the likelihood of adverse effects related to gene flow from transgenic crops. Based on empirical data obtained from greenhouse and field studies on potential impacts of hybridization with transgenic crops, key parameters to include in model will be identified. The model will be run for one key crop/trait combination. For example, effects of gene flow from a herbicide, insect or disease resistant canola or grass crop on the diversity, functioning and biomass of plant, insect or microbial communities. Parameters to consider in developing the model include the probabilities of finding compatible crop and weeds in proximity or within hybridization range (via pollinating insects or wind); overlaps of flowering times and other similarities in phenology; seed number, biomass, and germination; fertility and out crossing potential of resultant hybrids and the abundance (population density) of compatible donor and recipient populations. Impacts of cultural practices (types of tillage, pesticide use and crop rotations), soil fertility and climate on survival of crop-weed hybrids and feral transgenic plants also will need to be considered.

1. Generate empirical data from contained (growth chamber or greenhouse) and field tests with model system(s), e.g., one crop, one or more compatible weeds or native species, one or more transgenic pesticidal traits.

2. Identify parameters to include in model, e. g., rates of out crossing from crop and from compatible weedy or native species; plant population density, seed number, plant and seed biomass, viability; fertility, over-wintering survival, seed dormancy and lateral spread of crop-weed or crop-native hybrid plants (F1-F4) and of backcross generations.

3. Obtain effects data with and without relevant selective pressures of disease, insects, agricultural chemicals (herbicide, insecticide, fungicide).

4. Obtain field data on gene flow (exposure) and ecological effects from one or more typical crop rotations with selected crop, e. g., alfalfa, wheat, oats or barley, after GMO canola.

5. Run model and compare predictions to empirical data.

F. Additional research to consider

Examine the influence of the following parameters on rates of gene transfer and ecological effects:

- nuclear vs. organelle (chloroplast or mitochondrial) inserts
- different sites of insertion, e.g., within different genomes of allopolyploids, minimizing outcrossing to compatible wild relatives
- use of different promoters with different times, places and levels of expression
- inducible vs. constitutive expression of genes
- single *vs.* multiple engineered traits; linkages; interactions

Expand Crops/Traits/Weeds of interest beyond Northwest/ex-U.S. (e. g., Canada, Mexico, western Europe):

- crop protection (herbicide, insect, disease resistance)
- crop quality (nutrient) traits
- newer generation traits: specialty chemicals, pharmaceuticals

Use genomic and proteomic analytical methods to identify molecular markers of plant fitness and development.

Evaluate effects of transgenic genes, crop pests, environmental factors and agricultural chemicals on plant gene expression.

Aspects of plant development that may be impacted by biotic and abiotic factors include pollen and seed formation and viability, shoot and root biomass, ability to overwinter and responses to symbiotic and pathogenic organisms. By using microarray approaches (Figure 5.5), identify changes in gene expression between conventional crops or weedy or native species with transgenic crops and with crop-weed hybrids that contain the gene of interest. Examination of responses of the conventional, transgenic and crop-weed hybrids at various developmental stages and under a variety of environmental conditions, including exposure to conventional agricultural chemicals, is of interest to identify potential plant molecular markers which may be diagnostic indicators of the presence of the transgenic gene or of exposure to certain environmental conditions, including exposure to chemical pesticides. Additional molecular markers that may be of interest include those which signify potential changes in the diversity and functions of microbial communities in soil that are involved in N cycling, i.e., using primer sets diagnostic for structural genes involved in nitrification (*amoA)*, denitrification *(nir)*, or nitrogen fixation (*nifH*). Information on potential changes in soil microbial communities involved in nutrient cycling are of interest, given the key roles of soil organisms in decomposition, soil fertility and plant nutrient availability

G. Specific research proposed

Sections A through F above describe the breath of research that should be addressed in a comprehensive gene flow project. Based on available resources the gene flow research will be limited at least initially, to research on two crops. Bent grass (*Agrostis stolonifera*) which is wind pollinated, and canola (*Brassica napus*), which can be both wind and insect pollinated, have been

selected as potential sources of transgenic genes to use in our gene flow studies. Other factors (see Table 5.1), in our choice of these crops include:

- Availability of essentially completely sequenced genomes for closely related plants, e. g., rice and *Arabidopsis,* which are in the same families *Gramineae* and *Brassicaceae*, as *Agrostis* and *Brassica* respectively;

- Presence of compatible crop, weedy and/or native relatives with which each can hybridize, both locally here in the Pacific northwest as well as nationally and internationally;

- Each of the selected transgenic crops will soon be or have been approved for commercial release.

The transgenic trait which currently is/will be commercially available is herbicide tolerance, a trait which is of agronomic interest and is an easily selectable marker to detect progeny and feral plants containing the transgenic gene. Other transgenic traits which may be available for research, but which are not yet commercially available for either of these crops, are ones that confer tolerance to bacterial or fungal diseases or to insect pests. Our proposed research strategy with each of these crops can be summarized as follows:

1. Determination of Gene Transfer Rates / Obtain / Produce Hybrids

a. Select specific compatible crop/weed or crop/native species of interest in particular geographies of interest using GIS based crop, weed, chemical use and plant systematics information (Fig 5.1).

b. Determine gene detection methods needs (Fig. 5.2). Are marker genes available? Is that information proprietary/can it be accessed? Will we need to develop our own markers, based on genomics research (Fig. 5.3)?

c. Obtain, find or make hybrids between the crop and weedy or native species of interest that are most likely to be found within the same geographic area and which would flower at approximately the same time (Fig 5.2).

d. Use the transgenic parents, their hybrid (F1-F4) progeny with selected species and backcross progeny (BC1-BC4), as sources of transgenic genes in controlled environment studies in growth chambers or greenhouses or screen houses in the field. Determine and compare rates of gene flow in reciprocal crosses, in the presence and absence of selective pressures appropriate to the selected transgenic herbicide, disease or insect tolerant trait (Figs. 5.2, 5.3 and 5.4).

e. Compare rates of transfer from transgenic sources in which the trait of interest is located in different locations on nuclear or organelle genes. If multiple nuclear genomes exist, assess rates of transfer from different genomes and chromosomes within those genomes (Figs. 5.2 and Fig. 5.3).

2. Evaluation of Hybrid Fitness and Ecological Effects

a. Compare ecological effects of gene flow on fitness of progeny at the plant community, population and individual species levels. Examples of effects are, vegetative and seed biomass production, germination, fertility of progeny, over-wintering survival, survival in the soil seed bank; effects on plant community composition.

b. Determine ecological effects of gene flow and related fitness changes on beneficial insects and crop pests, invertebrate herbivores; soil food web biota; and vertebrate herbivores and invertebrate and vertebrate seed predators (Fig. 5.3).

3. Develop a Probabilistic Risk Assessment Model of the Ecological Effects of Gene Flow

a. Use exposure and ecological effects information to provide inputs for a probabilistic risk assessment model (Fig 5.1), based on above-ground effects on plants, beneficial insects, insect pests and vertebrate herbivores at community, population and species levels.

b. Incorporate inputs (as available) on effects of changes below ground, on beneficial and pathogenic soil foodweb organisms.

5.5 Time Line

Table 5.2 describes identification of technical resources (plants, traits, and contacts), formulation of the research plan, hiring of molecular biology post-doc and definition of pilot studies in year one (FY 2002). In FY 2003, the research and QA plans are approved and laboratory, greenhouse and growth chamber studies are to be initiated. Pending availability of funding, cooperative agreements and inter-agency agreements will also be implemented in FY 2003. In addition to continuing intramural and extramural research with domestic collaborators, international collaborations may be implemented in FY 2004. An all investigator meeting will be convened in FY 2005 to review research progress, e.g. on gene tracking methods and on ecological effects on plant community composition in non-agronomic ecosystems. An international workshop may also be convened in the latter part of FY 2005 or early FY 2006, to call for the development of an international data collection network and potential risk assessment guidelines for evaluation of non-target effects of gene flow from transgenic plants. Based on results obtained through FY 2005, in FY 2006 it is anticipated that inputs for a probabilistic risk assessment model of ecological effects of gene flow on non-target plant populations will have been identified, so that a prototype model can be run.

Table 5-1 Evaluation Criteria and Ranking of Northwest Crops/Traits of Interest

Crop Candidates	Compatible Weedy, Native, Crop Species in Pacific Northwest	Engineered Traits Commercially Available[1] or in Development[2]	Technology Access and Genomics Information
Canola	Numerous Weedy and Crop Mustard and Radish species, Brassica Vegetable Crops	Herbicide Tolerance[1] Fungal Disease Resistance[2] Insect Resistance[2]	Herbicide tolerant canola commercially available in Canada, parts of northwest, upper midwest; *Arabidopsis* genome published; canola (*Brassica*) is also a cruciferous plant species; commercial microarrays available for *Arabidopsis*
Grass Seed/Wheat	numerous; jointed goat grass	Herbicide Tolerance[2]	Grower concerns with GMO traits limiting sales to Japan and western Europe are causing delay in commercial introduction; recent publication of genomes of two rice species could enhance genomics studies with wheat and other grass species (bent grass, rye grass) being considered for use in Pacific Northwest
Raspberries	Himalayan Blackberry	Virus resistance[2]	research at early stage; gene expression not yet at commercial level

Table 5-2 Timeline/Outputs Gene Flow Research.

FY 2002	FY 2003	FY 2004	FY 2005	FY 2006-2007
Review Literature Identify Resources: -Plants, Traits, People, Organizations Attend Workshop: - Identify Data Gaps -Select Crops/Traits, & Geographies Draft Research Plan Pilot On-Site Studies: -DNA Characteristics, Persistence, Expression, Transforming Ability Hire NHEERL Post-Doc Identify Potential IPA (academic and federal agency) and GSF collaborators	Contribute to APM on Strategy for Updated Test Guidelines: Finalized Research Plan Approved QA Plan in Place Initiate Lab and Chamber Studies With Transgenic and Parental Plants Initiate DNA Analyses of Plant and Soil Materials from Field Sites in US region(s) in which selected crop(s) are grown Hire NRC Post-doc Formulate RFA Issue RFA Identify US and International Collaborators	Continue R &D -Intramural Studies in: Laboratory Chambers Field Model Inputs Initiate extra-Mural R & D via: -Coops -IAGs: USDA-ARS DOI-NPS DOI-BLM -Contracts -CRADAs with Private Sector Identify Collaborators for International Ecological Effects and Molecular Tracking Collaborations in Multi-Year Field Studies With a Wind and With An Insect Pollinated Crop	Continue R & D -Intramural -Extramural Convene Meeting of Investigators to Review: -Findings -Methods -Problems -Define Model Parameters Convene Workshop to Develop National and International Data Collection Network	Complete: Short-Term R & D Continue: Long-Term R & D Produce: Protocols Publications Test Model Agency Reports: -Findings -Methods -White Paper on Strategies to Minimize Gene Flow Effects

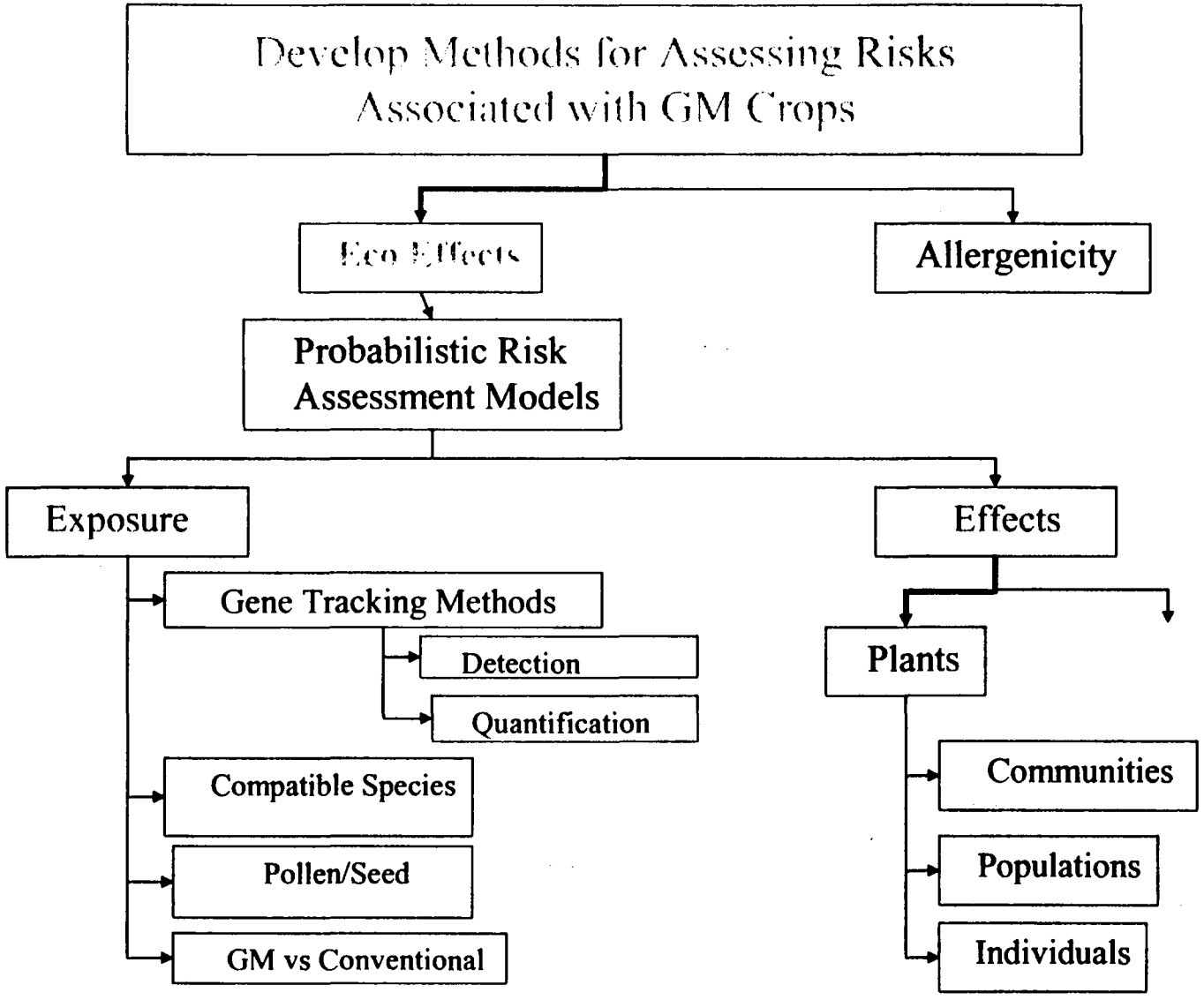

Figure 5-1 Overview of exposure and effects research needed for risk assessment of genetically modified (GM) crops

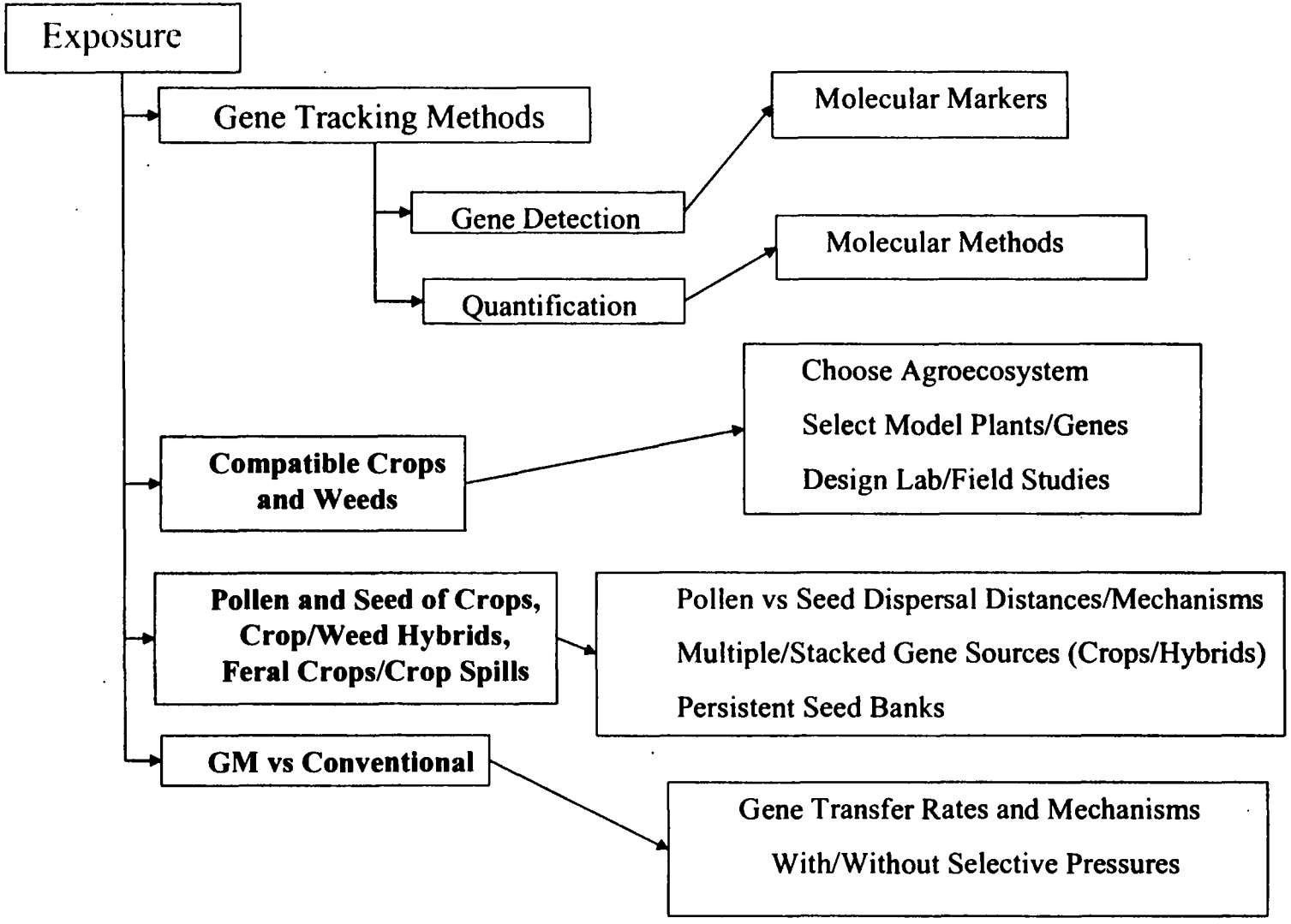

Figure 5-2 Critical path for exposure component of gene flow risk assessment.

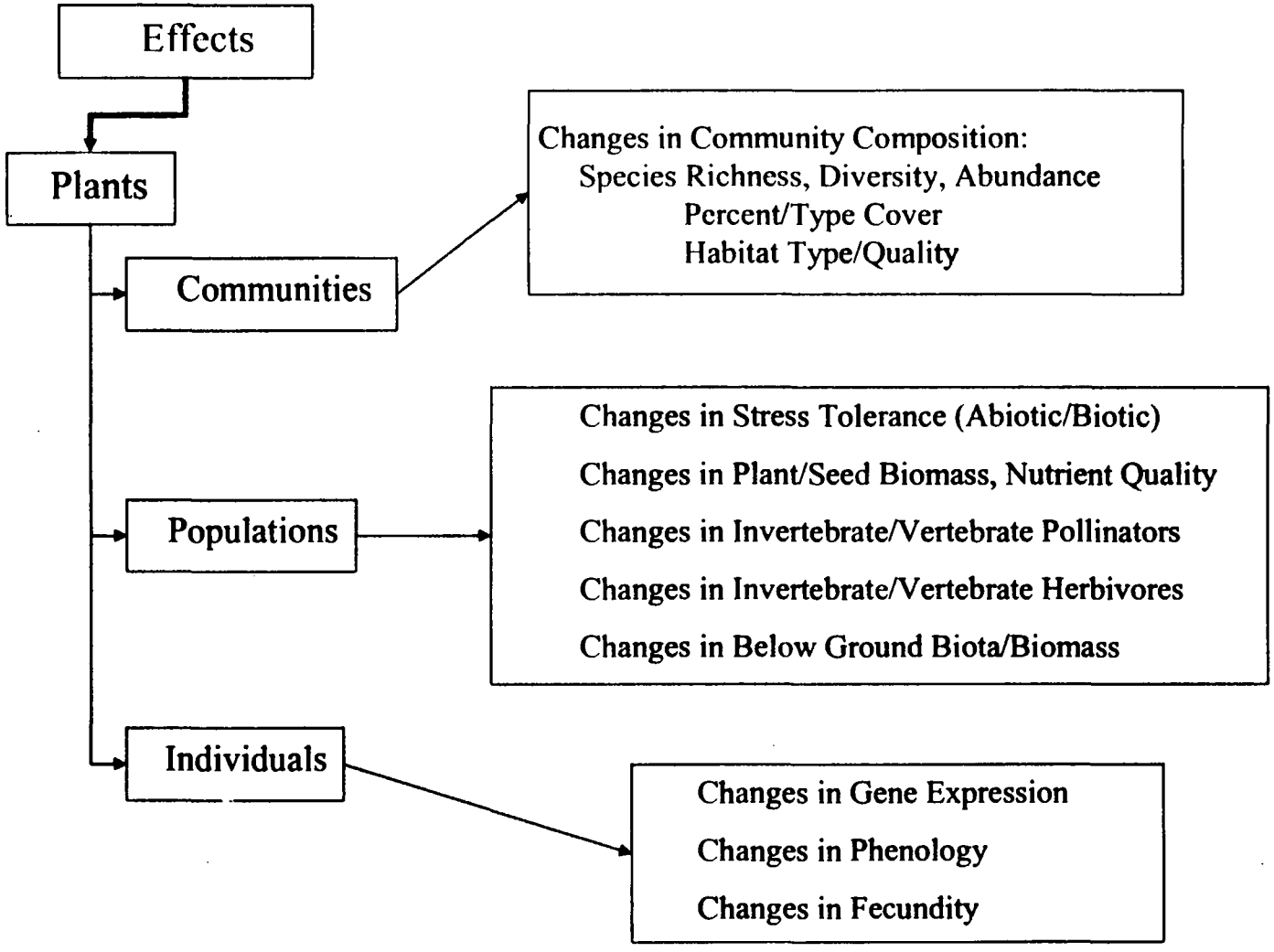

Figure 5-3 Critical path for ecological effects component of gene flow risk assessment.

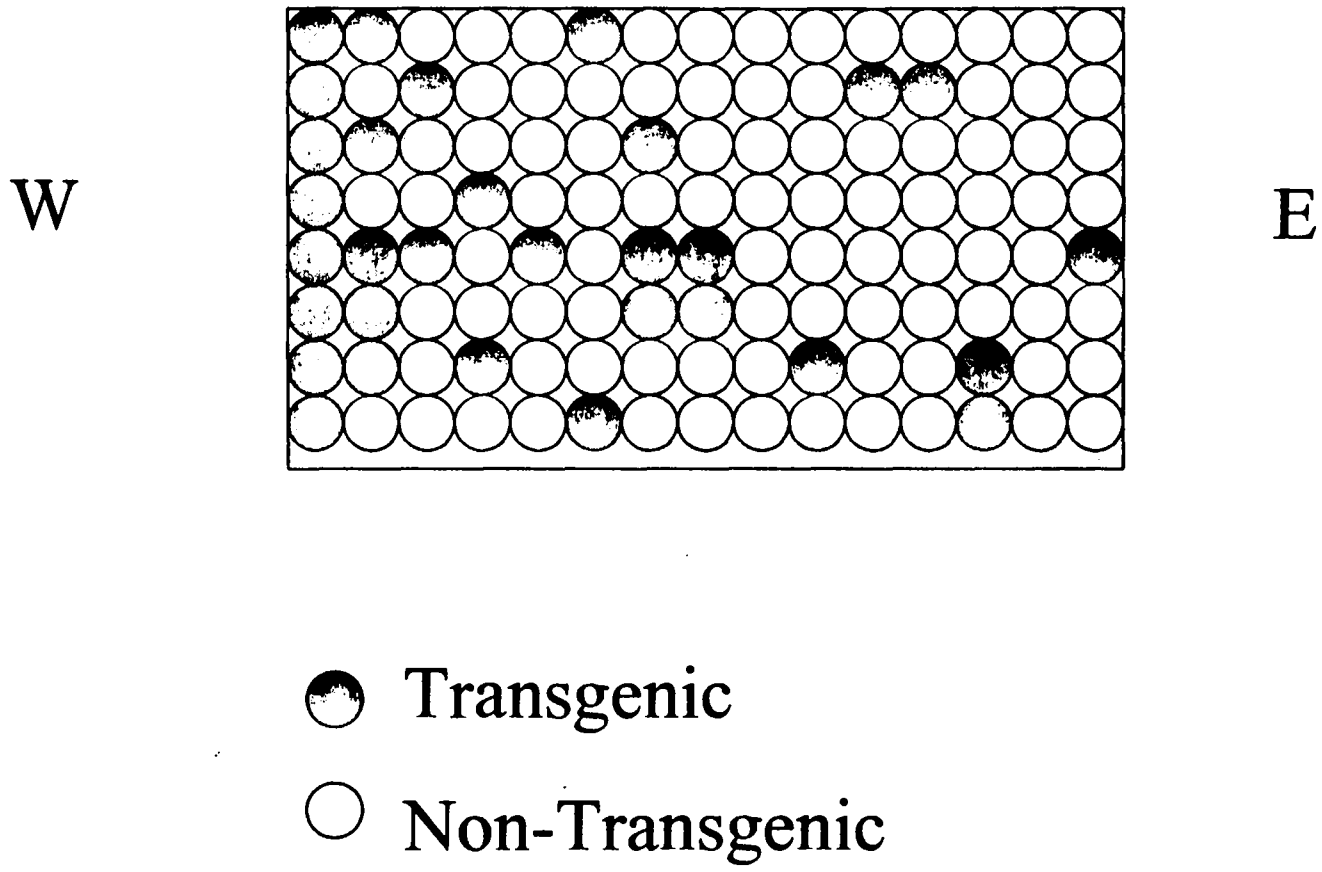

Figure 5-4 Illustration of production of transgenic patches of varying population density which may result from gene flow or from incidental transport of feral transgenic seed.

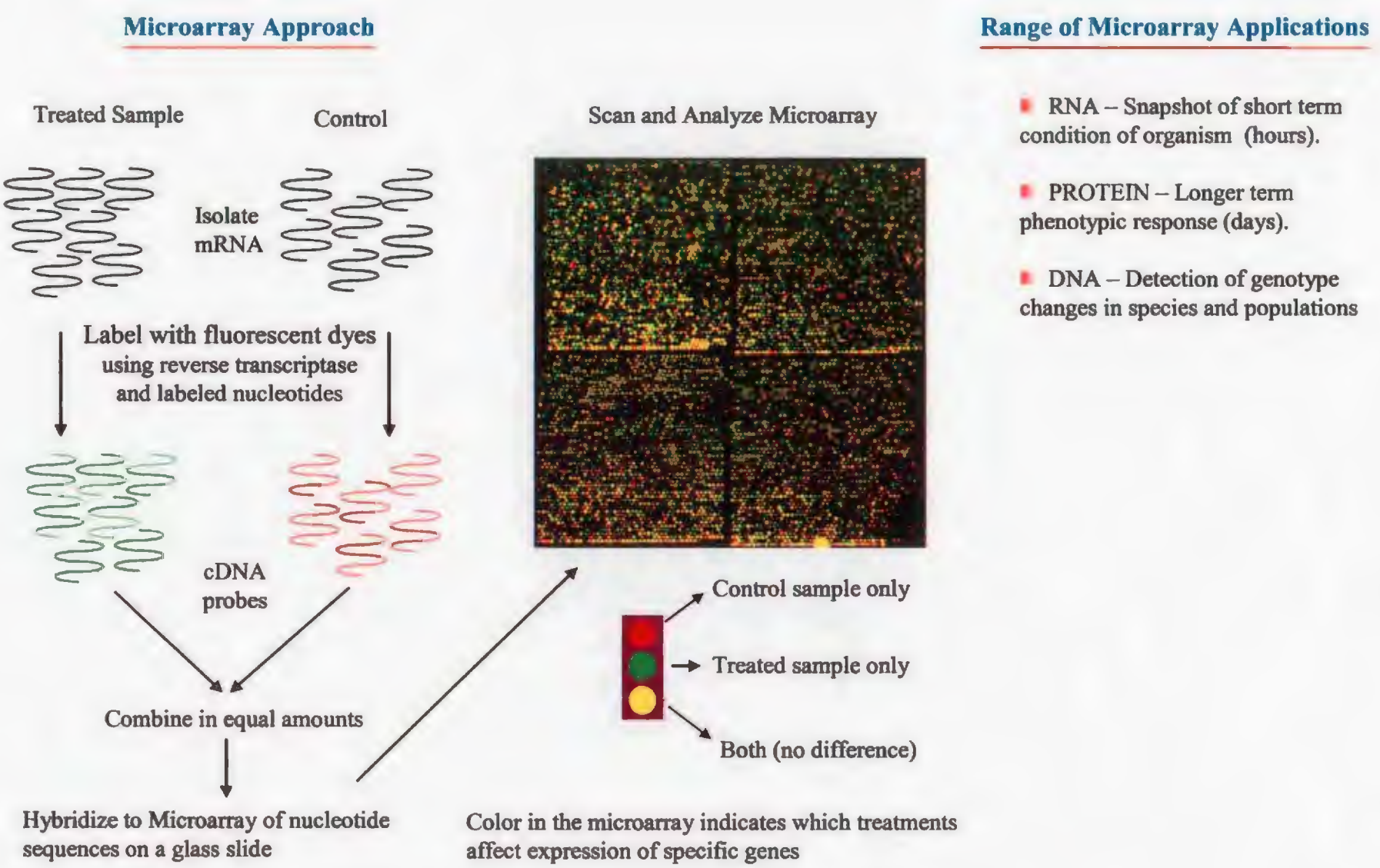

Figure 5-5. Examples of microarray methods including approaches and applications.

6 OUTPUTS AND PERFORMANCE MEASURES

The major outputs of this project will be tools to carry out ecological risk assessments (Figure 2.2).

- The **Regional Analysis and Interpretation** component will develop databases useful for both chemical herbicide and gene flow studies including a spatial database of potential pesticide exposure to non-target plants, a database of pesticide effects on plants, and a database of crop planting dates, pesticide use dates and weed emergence; as well as several case studies to identify and prioritize potentially important uncertainties.

- The **Effects of Chemical Herbicides on Terrestrial Plants** component will contribute refined plant testing methodologies for herbicide exposures, experimental conditions, and response endpoints for risk assessments. The methodology can be used to develop new individual plant tests and multispecies ecological tests for herbicide responses. The chemical herbicides and terrestrial plants area will also include mode of action and molecular effects tools for future development.

- The **Ecological Effects of Gene Flow from Transgenic Crops** component will contribute gene tracking methodology which will be used to develop metrics of potential ecological effects of gene flow from GM-crops to compatible native, weedy and crop species in agronomic and non-agronomic ecosystems.

Project outputs will be in the form of scientific papers, protocols, data sets, and a GIS platform. These outputs will meet the objectives of the Government Performance and Results Act (GPRA) by contributing to a series of Annual Performance Goals (APGs) which are major achievements across ORD laboratories and Annual Performance Measures (APMs) which are specific milestones at the Division Level and contribute towards the APGs. Table 6.1 indicates the APGs and APMs for this project for 2003 through 2008. These are also shown in the project critical path (Figure 2.2) and individual time lines for the regional analysis (Table 3.1), chemical herbicide (Table 4.5), and gene flow components of this project (Table 5.2). The 2003 APM will be met primarily by the publication and implementation of this research plan. All three areas of research,

regional analysis, chemical herbicides, and gene flow will contribute to this APM. The APMs for 2004 and 2005 will concern risk assessment and regional approaches to species selection and risk assessment for chemical herbicides. They will include contributions from the regional analysis and chemical herbicide areas of this project and, and will be based on peer reviewed manuscripts, databases, presentations and other outputs. The APMs for 2006 and 2007 concern protocols for improved tests of plant effects from chemical herbicides. They will be based on the chemical herbicide research. A possible APM for 2008 (beyond the five year timeline for this project) would integrate outputs from all three areas of research, and take the form of a variety of regional ecological risk assessment tools including models and a GIS platform.

Table 6.1 Annual Performance Measures and Goals for the Pesticide Research Project.

APG 19
Develop improved tools and models to assess and predict human health and ecological risk from exposure to commercial chemicals and microorganisms. Attainment of this goal will include the successful completion of the suite of annual performance measures.
Reporting. ALD Contact: Tim Gleason

APM 208
Strategy for research to update herbicide testing guidelines and to evaluate gene flow
Contact: David Olszyk (WED)
2003 NHEERL/ WED/ RCB

APG 97
Provide an improved capability to assess the ecological risks associated with high potency herbicides.
Reporting. ALD Contact: Jack Fowle

APM 167
Evaluation of Risk Assessment Methods for Herbicides
Contact: E. Henry Lee (WED)
2004 NHEERL/ WED/ RCB

APM PROPOSED
Guidelines for regional approach to selection of plant species for herbicide risk assessment (WED)
2005 NHEERL/ WED/ RCB

APM PROPOSED
Draft revised protocol / guidelines for vegetative vigor test with crops and selected native plants
2006 NHEERL/ WED/ RCB

APM PROPOSED
Draft new protocol / guidelines for reproductive/developmental endpoints with annual species
2007 NHEERL/ WED/ RCB

APM PROPOSED
Refined regional assessment tools for assessing risk to plants from herbicides and gene flow based on GIS framework and probabilistic risk assessments
2008 NHEERL/ WED/ RCB

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7 PROJECT MANAGEMENT AND QUALITY ASSURANCE

7.1 Management Responsibilities

This project is coordinated with other projects within WED that address other EPA Goals (e.g,. Goal 8, Sound Science), and with projects in other NHEERL Divisions which address other aspects of Goal 4 research (e.g. health related questions). Coordination is necessary not only to make sure that key agency needs are addressed without repetition among Projects and Divisions and that resources (staff, funds) are used effectively, but to make sure that scientific information and ideas can be "cross-fertilized" among scientists working in different areas. Line management to make sure the project achieves its goals is as follows:

A. NHEERL/Associate Laboratory Director. The WED Pesticides Project is part of a coordinated NHEERL effort to characterize human health and ecological effects of pesticides. Within NHEERL the multi-year plan is being developed to elucidate the rationale and overall objectives for this research. The Associate Laboratory Director for pesticides (Goal 4) related research is Dr. Jack Fowle, located at the RTP headquarters of NHEERL.

B. Branch Chief. At WED the Project is managed within the Risk Characterization Branch and comes under the general responsibilities of the Ecosystem Characterization Branch Chief. The Branch Chief is responsible for ensuring that the Project research meets the objectives of Goal 4 and that all technical outputs meet the quality requirements of the Division, Laboratory and Agency. The Branch Chief also is the direct line manager to the Project Leader, and can apply Branch resources to resolve project issues. The Branch Chief is responsible for coordinating the WED Goal 4 research with the research of other NHEERL Divisions through the ALD and the Resource Characterization Team (RCT). Dr. Anne Fairbrother is ECB Branch Chief.

C. Project Leader. The Project Leader is management's principle contact with the Project and is responsible on a day-to-day basis for the performance of, and coordination of, research within the Project. The Project Leader works with the Branch Chief and Principal Investigators on a collaborative basis to accomplish those goals. The Project Leader also is a member of the WED Science Council. Dr. David Olszyk is Pesticide Project Leader.

D. Principal Investigators. The Principal Investigators (PIs) are responsible for the scientific questions being addressed by the three main areas within the project (Figure 1), and for managing day-to-day decisions concerning the research within their area. They also are responsible for managing resources (e.g. EPA and Senior Environmental Employee/National Asian Pacific Center on Aging (SEES), Level of Effort (LOE) contract, purchase orders) to accomplish their area's research goals. They serve as Work Assignment Mangers for the WED LOE contract, advisors for postdoctoral associates, mentors for interns and other scientific and technical staff. PIs and their areas of research within the project are as follows (note that all PI scientists may collaborate in all three areas of the project on a limited basis):

- **Effects of Chemical Pesticides on Terrestrial Plants,** Dr. Thomas Pfleeger, Dr. John Fletcher, Dr. David Olszyk
- **Ecological Effects of Gene Flow from Transgenic Crops,** Dr. Lidia Watrud
- **Regional Analysis and Integration,** Dr. Henry Lee, Dr. Thomas Pfleeger

Based on resources we will add staff and/or work collaboratively with other ORD and EPA organizations to develop the risk assessment tools.

E. Project Scientists. Under the direction of the PIs, the project Scientists work directly on the research in a specific area and may be from a variety of organizations including the EPA, the SEES staff, LOE Contract, guest workers and others. The Project Scientists' assist in the development and implementation of experimental protocols, conduct the research, process experimental data, and assist in the production of scientific documents (journal papers, presentations, briefings). The Project Scientists follow Project Quality Assurance and Health and Safety protocols.

7.2 Communications

The Pesticides Project is a sophisticated, very complex, multi-discipline research endeavor. Completing the Project successfully will require continual communication among project participants, at all levels. Communication will be fostered by regular weekly meetings of the PI's to: (1) coordinate sampling and various experimental activities, (2) exchange data and information

about the various tasks, (3) share scientific information, and (4) refine and/or modify the Research Plan, the QAPP and the SOPs. The PIs and other Project participants will meet as necessary to: (1) coordinate experimental activities (e.g., availability of equipment, harvests, etc), (2) exchange information, and (3) resolve possible problems. For work conducted through the on-site Level of Effort Contract (LOE), technical direction will be from the EPA Work Assignment Manager to the Contractor Work Plan Manager.

The Project Leader will meet regularly with the Branch Chief to keep her informed on the status of the research. The Project Leader and Branch Chief will participate in WED Science Council meetings to insure that the Pesticides Project research is coordinated with other WED research efforts. Contact will be made on a regular basis through appropriate levels (e.g. P.I., Project Leader, Branch Chief, Associate Director for Science, Division Director) to insure that the research responds to agency Goal 4 needs and is coordinated with other NHEERL and ORD research efforts. Finally, the PIs and other project scientists will be active participants in scientific meetings relating to non-target effects of chemical pesticides and GM crops, including hosting a scientific workshop in those areas, tentatively in year three of the project.

If the full complement of resources is not available, this plan will be implemented to the extent feasible with available resources to achieve the most critical of all the important goals of the project. For example, the plant effects from chemicals research may be limited to developing an improved vegetative vigor test for annual plants if funds are severely restricted.

7.3 Quality Assurance (QA)

In order to produce reliable data of known quality and to meet EPA and WED QA requirements this project has a QA Project Plan. In developing the Project's QA organizational structure to meet the QA goals, five essential QA/QC elements were addressed: (A) QA/QC responsibilities and research responsibilities, (B) communications, (C) document control, including the importance of standard protocols for the experiment especially Standard Operating Procedures (SOPs) for experimental data collection. In addition, we have separate QA issues to consider specific to the chemical herbicide (D) and gene flow research (E).

A. QA and Research Responsibilities

WED management and research staff share responsibility for implementing the Laboratory's QA policies, and they are accountable for those aspects of QA/QC associated with their work areas. The QA Responsibilities in this Quality Assurance Project Plan (QAPP) were derived from Section 1.0 of the US EPA, NHEERL, Western Ecology Division Plan (U.S. EPA 1995). The general Project QA/QC organizational structure can be described as follows:

Division Director. The Division Director has ultimate responsibility for all research conducted, funded, or managed within the division. They must approve the division Quality Management Plan . Within the Office of the Division Director, the **WED Quality Assurance Manager** is responsible for ensuring that all WED QA activities are in compliance with agency QA policy and guidance. He/she reports to the Associate Division Director for Science.

Branch Chief. The RCB Branch Chief is responsible for the quality of all research conducted, funded, or managed within the Branch and for ensuring that all technical outputs meet the quality requirements of the Laboratory and Agency. The Branch Chief also is the direct line manager to the Project Leaders, and can apply Branch resources to resolve QA issues. The Branch Chief's key QA responsibilities include: a) review and evaluation of work on QA implementation and progress, b) review the quality of outputs generated by each project, and c)review and evaluate audit and performance evaluation reports.

Project Leader. The Project Leader is responsible for production of the QA Project Plan (QAPP) and oversees all QA management aspects of the project. The Project Leader determines quality criteria based on the intended use of the results to be generated, and communicates these criteria to the Project participants. The Project leader conducts periodic reviews of QA procedures and data gathered with them within the project and writes a report and implements QA procedure changes if necessary based on those reviews.

Principal Investigators. The PIs are responsible for carrying out specific research areas within the project and for insuring the quality of the results generated by those areas. They approve specific SOPs and other QA documents relative to their areas.

Project Scientists. Project Scientists work directly on the Project's research and QA/QC procedures. The Project Scientists' key QC/QA responsibilities include: a) assist in writing SOPs as necessary, b) implementation of the SOPs, c) evaluation and documentation of QA methods used for measurements.

B. Communications:

The periodic project meetings will be used to refine and/or modify the Research Plan, the QAPP and the SOPs. The PIs and other Project participants also will meet as necessary to: (1) coordinate experimental activities (e.g, availability of equipment, harvests, etc), (2) exchange information, and (3) to resolve possible problems.

C. Document Control

The QAPP and experimental protocols define the key aspects of the Pesticides Research Project QA program , consequently, it is important that all Project participants have access to these documents. The Project Leader will be responsible for maintaining the original signed copies of the QAPP and approved SOPs and any other QA documents. These will be kept in room 203 of the Main Building. Paper copies of the Research Plan and QAPP will be available for Principle Investigators. In order to minimize paper copies, electronic versions of the Research Plan, QAPP and all other QA documents will be available to the Principal Investigators and all other Project participants on the server: NABU/Pesticides/EPA/QA. It is the responsibility of individual Project participants to print out paper copies of the documents required for their work. The Project Leader will ensure that version numbers of the approved QAPP and each approved SOP, EP or OP are correct and changed as necessary. If the QAPP or a SOP is revised, the Project Leader will ensure that relevant members of the project are notified. At that time any previous version of the document is to be discarded by the user. However, copies of older versions of SOPs and other QA documents shall be retained by the Project Leader in the files in room 203 of the Main Building in order to document collection procedures prior to the current versions of SOPs

Although the QA/QC elements described above are highly interdependent to successfully execute the Project's research and QA programs, SOPs have an especially critical role in these

programs. A SOP is the keystone element upon which experimental procedures will be based, and Management and Project personnel will interact in the other four elements of the Project QA program. Because the Project's QA organizational structure depends heavily on SOPs, their format was structured to serve as guidelines for all Project personnel to accomplish both the scientific and QA/QC procedures of the. SOPs should follow the same format as the QAPP, modified as needed. In addition to describing methodology for collection of data, SOPs include information as to processing of data and location of files and/or databases. Statistical analysis procedures will be documented in descriptions for individual experiments and/or in methods sections for manuscripts. The SOPs will be reviewed periodically, and re-authorized as necessary

D. Special Health and Safety Considerations for Chemical Herbicide Studies.

Since all active ingredients used in the chemical herbicide studies are considered to be toxic materials, their purchase, storage, use, and fate must be accounted for in the Project Health and Safety Plan. This plan also describes use of the track sprayer for plants in pots grown in greenhouses, growth chamber, or an outside nursery area. It will be modified to include new herbicides or field plot herbicide treatments as specific experiments are designed.

E. Special QA Considerations for Gene Flow Studies.

The gene flow research is developmental, involving evaluation and testing of new procedures for which there may be no existing protocols. Nevertheless, all researchers shall be required to accomplish the Quality Assurance procedures described in existing and new SOP's in order to comply with EPA's Quality Assurance Program. Researchers will comply with all relevant current SOP's and provide new SOP's as required for the execution of specific tasks. QA support will be provided for plant and microbial growth facilities (e. g., incubators, environmental chambers), coolers, freezers, ovens, autoclaves, pipettors, balances, reverse osmosis (R. O.) water supplies. A waiver for exploratory and preliminary research used in the development of skills in the WED Pesticides Project was obtained as described in the Quality Assurance Request for Exemption memo of November 7, 2002.

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Appendix A Potential research on effects of chemical herbicides on aquatic plants.

Potential research on effects of chemical herbicides on aquatic plants.

Rationale

The aquatic research needs of OPPTS in support of FIFRA and TSCA are quite broad. These include expanding the types of aquatic tests required to include representative species from a variety of aquatic plant communities ranging from freshwater wetlands to marine alga systems (see attached table). The environmental conditions of the test systems needs to be characterized and standardized. At least two reproductive tests are needed and will have to be developed. The higher tier or level tests needs to be developed including multi-species microcosm, mesocosm and field tests. Research is also needed to determine the linkage between laboratory tests and field results. The research needed to improve the aquatic plant risk assessments will require a wider level of expertise than the terrestrial component simply because the needs span from fresh water systems to marine systems.

There are currently two aquatic tests required for pesticide registration (listed below). One for algae and the other for the vascular plant *Lemna*. This data is extrapolated to protect all freshwater and marine algae, plants and communities in the United States and therefor probably the world.

a) Algal toxicity tests, Tiers I and II - The intended use is for developing data on the acute toxicity of chemical substances and mixtures in aquatic environments subject to environmental effects test regulations and was written specifically for *Selenastrum capricornutum* (fresh water green alga) and *Skeletonema costatum* (marine diatom). It is also used for *Anaabaena flos-aquae* and *Navicula pilliculosa*. The test occurs in flasks and lasts up to 96 hours. The end point is number of cells per unit volume which is turn is used to determine EC50's

b) Aquatic Plant Toxicity Test Using Lemna spp., Tiers I and II -The intended use is for developing data on the phytotoxicity of chemicals to the aquatic environment using the freshwater aquatic plants *Lemna gibba* and *L. minor*. The test takes place in a vessel and lasts up to 14 days. The end point is number of fronds which are used to calculate EC5s,50s, and 90s along with LOECs (Lowest Effect Concentration) and NOECs (No Effect Concentration).

Examples of Needed Research

The algal test requires experimental evaluation of toxicity endpoints, test organisms and test conditions. These issues have not been resolved in the scientific literature and a program to resolve

Table 1. Additional species suggested for aquatic testing for the protection of nontarget plants from phytotoxic chemicals grouped by growth form / environment and type of exposure.

Group		Additional Species
1	Freshwater algae	Green algae: *Scenedesmus subspicatus; Chorella vulgaris; Chlamydomonas reinhardi; Chlamydomonas eugametos.* Blue-green algae: *Anabaena cylindrica; Microcystis aeruginosa* Diatom: *Nitzchia* sp.; *Craticula cuspidata*
2	Marine algae	Diatom: *Thalassiosira pseudonana* Dinoflagellate: ND Red algae: ND Golden-brown algae: *Macrocystis pyrifera*
3,5	Floating vascular	*Nuphar* sp; *Nymphaea* sp.; *Spirodela* sp.
4	Submersed vascular	*Ceratophyllum* sp.; *Vallisneria americana*; *Elodea canadensis, Egeria densa, Potamogeton perfoliatus; Najas* sp.
6,7	Emergent vascular	Monocot: *Spartina pectinata; Scirpus acutus; Phalaris arundinacea* Dicot: *Nelumbo lutea; Rorippa nasturium-aquaticum*

ND - not determined

1 - 4 aquatic exposure
5 - 7 foliar exposure

Appendix B Example experimental protocol for a study on vegetative and reproductive responses of a major crop.

Appendix B. Example experimental protocol for a study on vegetative and reproductive responses of a major crop.

This experiment will address the Pesticide Project Goal of developing information for improved plant testing guidelines to be used in evaluating the potential effects of pesticides on terrestrial plants. The first proposed experiment is a comparison of herbicides effects on plants growing in different environments, treated with a herbicide at different growth stages, and representing two crops with different economic endpoints.

Objectives:

1. To compare the relative response of crop plants to a herbicide when grown in greenhouse vs. field.
2. To compare the relative response of crop plants to herbicide exposure at an early vegetative growth stage vs. a mature reproductive/full developed stage of growth.
3. To compare the relative response to a herbicide of a crop with a seed production economic endpoint vs. a crop with a storage root production economic endpoint.
4. To evaluate possible physiological indicators of noninjurious effects of herbicides on plants.
5. To evaluate the performance of the track sprayer under experimental conditions and to evaluate herbicide application QA procedures.

Plant Species:

Soybean, seed, 4 days from planting to germination, 65 days to flowering (different variety), harvest date will be based on seed development

Potato, tubers, 10 days from planting to germination, flowering (and presumably initiation of tuber development) 28 days from germination, harvested approximately 50 days after germination or when tubers are fully developed

Pots/Soil/Seeding:

Pot Size: Soybean- 6" diameter x 5 3/4" deep green plastic pot, Potato- 10" diameter x 12" high black plastic pots; saucers will be placed under each pot

Soil: Sandy loam soil, sterilized by OSU Horticulture Dept. Samples of the soil used for soybean and potatoes (different batches) will be send to the OSU soil analysis lab. to determine fertility (N, P and K concentrations), texture, pH and carbon concentration.

Fertilizer: Osmocote incorporated in soil at time of potting, 10

Appendix C Federal regulation for Plant-Incorporated Protectants; Final Rules and Proposed Rule (40 CFR 152 and 174, Part IV).

Federal Register

Thursday,
July 19, 2001

Part IV

Environmental Protection Agency

40 CFR Parts 152 and 174
Plant-Incorporated Protectants; Final Rules and Proposed Rule

ENVIRONMENTAL PROTECTION AGENCY

40 CFR Parts 152 and 174

[OPP–300369B; FRL–6057–7]

RIN 2070–AC02

Regulations Under the Federal Insecticide, Fungicide, and Rodenticide Act for Plant-Incorporated Protectants (Formerly Plant-Pesticides)

AGENCY: Environmental Protection Agency (EPA).

ACTION: Final rule.

SUMMARY: The substances plants produce for protection against pests, and the genetic material necessary to produce these substances, are pesticides under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), if humans intend to use these substances for "preventing, repelling or mitigating any pest." In this rule, EPA finalizes certain of the proposed rules published in 1994, 1996, and 1997. Specifically, EPA changes the name of this type of pesticide from "plant-pesticide" to "plant-incorporated protectant"; clarifies the relationship between plants and plant-incorporated protectants under FIFRA; exempts from FIFRA requirements plant-incorporated protectants derived through conventional breeding from sexually compatible plants; and establishes a new part in the Code of Federal Regulations (CFR) specifically for plant-incorporated protectants. Procedures are also set forth for Confidential Business Information (CBI); any claim of confidentiality must be substantiated when the claim is made. This rule will benefit the public by ensuring that public health and the environment are adequately protected while reducing burden on the regulated community, thereby potentially reducing costs for consumers.

DATES: This rule is effective September 17, 2001.

FOR FURTHER INFORMATION CONTACT: Philip Hutton, Biopesticides and Pollution Prevention Division, Office of Pesticide Programs (7511C), Environmental Protection Agency, 1200 Pennsylvania Ave., NW., Washington, DC 20460; telephone number: (703) 308–8260; e-mail address: hutton.phil@epa.gov.

SUPPLEMENTARY INFORMATION:

I. General Information

A. Does this Action Apply to Me?

You may be potentially affected by this action if you are a person or company involved with agricultural biotechnology that may develop and market plant-incorporated protectants. Potentially affected categories and entities may include, but are not limited to:

Categories	NAICS codes	Examples of potentially affected entities
Pesticide manufacturers	32532	Establishments primarily engaged in the formulation and preparation of agricultural and household pest control chemicals
Seed companies	111	Establishments primarily engaged in growing crops, plants, vines, or trees and their seeds
Colleges, universities, and professional schools	611310	Establishments of higher learning which are engaged in development and marketing of plant-incorporated protectants
Establishments involved in research and development in the life sciences	54171	Establishments primarily engaged in conducting research in the physical, engineering, or life sciences, such as agriculture and biotechnology

This table is not intended to be exhaustive, but rather provides a guide for readers regarding the types of entities potentially affected by this action. Other types of entities not listed in the table could also be affected. The North American Industrial Classification System (NAICS) codes have been provided to assist you and others in determining whether or not this action might apply to certain entities. To determine whether you or your business may be affected by this action, you should carefully examine the provisions in 40 CFR part 174. If you have any questions regarding applicability of this action to a particular entity, consult the person listed under FOR FURTHER INFORMATION CONTACT.

B. How Can I Get Additional Information, Including Copies of this Document and Other Related Documents?

1. *Electronically.* You may obtain electronic copies of this document, and certain other related documents that might be available electronically, from the EPA Internet Home Page at http://www.epa.gov/. To access this document, on the Home Page select "Laws and Regulations", "Regulations and Proposed Rules," and then look up the entry for this document under the "**Federal Register**—Environmental Documents." You can also go directly to the **Federal Register** listings at http://www.epa.gov/fedrgstr/. To access information about EPA's program for biopesticides go directly to the Home Page for the Office of Pesticide Programs at http://www.epa.gov/pesticides/biopesticides.

2. *In person.* The Agency has established an official record for this action under the docket control number OPP–300369B. The official record consists of the documents specifically referenced in this action, any public comments received during an applicable comment period, and other information related to this action, including any information claimed as Confidential Business Information (CBI). This official record includes the documents that are physically located in the docket, as well as the documents that are referenced in those documents. The public version of the official record, which includes printed, paper versions of any electronic comments submitted during an applicable comment period, is available for inspection in the Public Information and Record Integrity Branch (PIRIB), Rm. 119, Crystal Mall #2, 1921 Jefferson Davis Highway, Arlington, VA, from 8:30 a.m. to 4 p.m., Monday through Friday, excluding legal holidays. The PIRIB telephone number is (703) 305–5805.

II. Under What Authority Is EPA Issuing The Rule?

A. FIFRA

This rule is promulgated under the authority of FIFRA section 3 and section 25(a) and (b) (7 U.S.C. 136a and 136w(a) and (b)) and FFDCA section 346a and 371.

FIFRA section 3(a) provides, with some exceptions, that no person may distribute or sell in the United States any pesticide that is not registered

determining whether a pesticide chemical residue is safe. EPA must consider "available information regarding the aggregate exposure levels of consumers . . . to the pesticide chemical residue and to other related substances, including dietary exposure under the tolerance and all other tolerances in effect for the pesticide chemical residue, and exposures from other non-occupational sources." (21 U.S.C. section 346a(b)(2)(D)(vi)). Consequently, a finding that a pesticide qualifies for a tolerance exemption could also demonstrate that the pesticide chemical meets the first exemption criterion of low probability of risk with respect to human health risks arising from other non-occupational routes of exposure. Such a pesticide also meets the second FIFRA exemption criterion of no likely unreasonable adverse effects, with respect to human health risks arising from all non-occupational exposures, if the risks resulting from use of that pesticide are consistent with the FFDCA section 408 exemption standard, and the potential benefits of use outweigh any human health risk even in the absence of regulatory oversight.

However, FIFRA does not provide for exemption of a pesticide in food based solely upon consistency with the FFDCA section 408 exemption standard. At a minimum, EPA also must evaluate risks arising from occupational exposure to humans and determine that such risks meet both exemption criteria. In addition, EPA must evaluate the risks to the environment from the pesticide and determine both that the pesticide poses only a low probability of environmental risks, and that use of the pesticide is not likely to cause any unreasonable adverse effects on the remainder of the environment in the absence of regulation under FIFRA.

III. What is the Background for this Rule?

This final rule establishes certain basic parameters of EPA's regulatory program under FIFRA for plant-incorporated protectants. In this rule, EPA defines the scope of products subject to FIFRA jurisdiction, and identifies the category of products over which it will exert regulatory oversight. EPA also establishes certain fundamental definitions to clarify what will be subject to regulation as a plant-incorporated protectant. The rule also finalizes certain regulatory procedures specific to plant-incorporated protectants. This document also provides some guidance on the way in which the Agency intends to interpret the existing regulations for these products until it is able to establish additional regulations specific to plant-incorporated protectants.

Specifically, the rule clarifies that plants used as biological control agents remain exempt from FIFRA requirements, but that plant-incorporated protectants are not. Second, the rule exempts plant-incorporated protectants derived through conventional breeding from sexually compatible plants. Third, this final rule establishes a new 40 CFR part 174, specifically for plant-incorporated protectants; any additional regulations specific to plant-incorporated protectants will be codified in 40 CFR part 174. The final rule also imposes a requirement at § 174.71, that any person producing an otherwise exempt plant-incorporated protectant for sale and distribution, who obtains any information regarding adverse effects of this otherwise exempt plant-incorporated protectant on human health or the environment report that information to EPA. Finally, the rule includes a provision that any claim of confidentiality must be made at the time of submission and substantiated at the time the claim is made.

A. What Is a Plant-Incorporated Protectant?

Plants have evolved, and thus naturally possess, various mechanisms to resist pests. The mechanisms of resistance can be varied, including, for example, structural characteristics of the plant, the production of metabolites that have toxic properties, biochemical cascades resulting in localized necrosis of plant tissue, or the production of specific toxic substances in response to pest attack. Humans have for approximately 10,000 years selected and bred certain plants as sources of, for example, food, feed, and fiber, and a frequently selected characteristic was the ability to resist pests. More recently, humans have developed scientific techniques by which traits from any living organism, including an ability to resist pests, can be introduced into a plant. When humans intend to use substances involved in these mechanisms in plants for "preventing, destroying, repelling or mitigating any pest," the substances are pesticides under the FIFRA definition of pesticide, regardless of whether the pesticidal capability evolved in the plants or was introduced by breeding or through the techniques of modern biotechnology.

The genetic material necessary for the production of such a pesticidal substance also meets the FIFRA statutory definition of a pesticide. Such genetic material is introduced into a plant with the intent of ultimately producing a pesticidal effect even though the genetic material may not, itself, directly affect pests. The pesticidal substance, along with the genetic material necessary to produce it, produced and used in living plants, is designated a "plant-incorporated protectant" by EPA.

Plant-incorporated protectants are primarily distinguished from other types of pesticides because they are intended to be produced and used in the living plant. This difference in use pattern dictates in some instances differences in approach. For example, because the plant-incorporated protectant is produced by the plant itself and used in the living plant, exposure considerations in risk assessments may be different, although as noted in Unit VII.D.2., the risk assessment framework used for other types of pesticides can be used for plant-incorporated protectants.

B. Does the Rule Have Any Relevance to Other Types of Pesticides?

Nonviable plant tissues, organs, or parts that are used as pesticides, will not be subject to the provisions of this rule, which will be codified in regulations at 40 CFR part 174. Rather, such pesticides are subject to the regulations found in 40 CFR parts 150 through 173 and 40 CFR parts 177 through 180. An example of this type of pesticide would be the powder, produced by drying and grinding cayenne peppers, dusted on plants to protect them from pests.

Substances that are isolated from a plant's tissues and then applied to plants for pest control will not be subject to the regulations in 40 CFR part 174. Rather these types of pesticides in formulations such as those for foliar application are subject to regulations found in 40 CFR parts 150 through 173 and 40 CFR parts 177 through 180. An example of this type of pesticide would be pyrethrum isolated from chrysanthemum plants, formulated with other ingredients for foliar application, and sprayed on other plants for pest control.

Substances that are synthesized will not be subject to the regulations in 40 CFR part 174. Such pesticides are subject to regulations found in 40 CFR parts 150 through 173 and 40 CFR parts 177 through 180. An example of this type of pesticide is the herbicide, atrazine.

C. What is the History of this Rule?

This rule is an additional step in fully implementing the "Coordinated Framework for Regulation of

A. What Are the Key Features of the November 23, 1994, **Federal Register**?

In the November 23, 1994, **Federal Register** document (59 FR 60519), EPA proposed to: first, clarify how the exemption at 40 CFR 152.20 relates to plants used as biological control agents and to plant-incorporated protectants; second, exempt under FIFRA section 25(b)(2), plant-incorporated protectants that are derived from plants closely related to the recipient plant, except for a requirement that sellers or distributors of an otherwise exempt plant-incorporated protectant submit to EPA any information they may obtain regarding potential unreasonable adverse effects caused by an exempt plant-incorporated protectant; and third, establish new part 40 CFR part 174 specifically for plant-incorporated protectants. This document also contained a proposed rule on substantiation of any claim of confidentiality at the time the claim was made.

1. *Clarification of exemption at 40 CFR 152.20; status of plants used as biological control agents with regard to FIFRA requirements.* In the November 23, 1994, **Federal Register** document, EPA proposed to amend 40 CFR 152.20 to clarify that plants used as biological control agents are exempt from FIFRA requirements under section 25(b)(1). The proposed amendment at 40 CFR 152.20 would also indicate that this exemption does not apply to plant-incorporated protectants and would refer the reader to 40 CFR part 174 for regulations, including a listing of exemptions, on plant-incorporated protectants.

2. *Proposed exemption of plant-incorporated protectants derived from plants closely related to the recipient plant.* In 1994, EPA described three options for defining when a plant-incorporated protectant would be exempt because it is derived from plants closely related to the recipient plant. EPA proposed to exempt plant-incorporated protectants derived from plants closely related to the recipient plant based on the rationale that the probability of new exposures from this group of plant-incorporated protectants is very low. Option 1, the Agency's preferred option, used sexual compatibility, including hybridization achieved by wide and bridging crosses, as a measure of relatedness between plants. Under this option, plant-incorporated protectants would be exempt from all FIFRA requirements, except for the adverse effects reporting requirement, if the genetic material that leads to the production of the pesticidal substance is derived from plants that are sexually compatible with the recipient plant and has never been derived from a source that is not sexually compatible with the recipient plant. Recipient plant was described as the plant into which the plant-incorporated protectant is introduced and in which the plant-incorporated protectant is produced. Sexually compatible, when referring to plants, was described as capable of forming a viable zygote through the fusion of two gametes including the use of bridging or wide crosses between plants.

Option 2 would utilize the rank of genus as the taxonomic standard for describing closely related plants such that plant-incorporated protectants derived from plants classified in the same genus as the recipient plant would be exempt from all FIFRA requirements, except for the adverse effects reporting requirement. Taxonomy is a system of orderly classification of organisms according to their presumed natural relationships. Taxonomy reflects current scientific observations about phenotypic, and to a certain extent, genotypic, similarities between organisms.

Option 3, also an alternative option, would utilize both the taxonomic rank of genus and sexual compatibility to describe closely related plants. This option would exempt from all FIFRA requirements, except for the adverse effects reporting requirement, plant-incorporated protectants derived from plants classified in the same genus as the recipient plant, as well as plant-incorporated protectants derived from plants sexually compatible with the recipient plant. Under Options 1 and 3, plant-incorporated protectants derived from plants sexually compatible with the recipient plant would be exempt even if the source and recipient plants are classified in different genera.

None of the options offered by the EPA were intended to exempt a plant-incorporated protectant that has been modified so that it is significantly different functionally from the plant-incorporated protectant as it occurs in the source organism (59 FR 60524).

i. *Associated definitions.* In 1994, pertinent definitions associated with the proposed exemptions included:

"Bridging crosses between plants" would be the utilization of an intermediate plant in a cross to produce a viable zygote between the intermediate plant and a first plant, in order to cross the plant resulting from that zygote with a third plant that would not otherwise be able to produce viable zygotes from the fusion of its gametes with those of the first plant. The result of the bridging cross is the mixing of genetic material of the first and third plant through the formation of an intermediate zygote.

"Wide crosses between plants" would be to facilitate the formation of viable zygotes through the use of surgical alteration of the plant pistil, bud pollination, mentor pollen, immunosuppressants, *in vitro* fertilization, pre-pollination and post-pollination hormone treatments, manipulation of chromosome numbers, embryo culture, or ovary and ovule cultures, or any other technique that the Administrator determines meets this definition.

In 1994, EPA also presented a definition for plant-pesticide, now termed plant-incorporated protectant, and definitions of active and inert ingredient for plant-pesticides.

"Plant-pesticide" was defined as a pesticidal substance that is produced in a living plant and the genetic material necessary for the production of the substance, where the substance is intended for use in the living plant.

"Active ingredient," when referring to plant-incorporated protectants only, was defined as a pesticidal substance that is produced in a living plant and the genetic material necessary for the production of the substance, where the substance is intended for use in the living plant.

"Genetic material necessary for the production" was defined as: Genetic material that encodes for a pesticidal substance or leads to the production of a pesticidal substance and regulatory regions. It does not include noncoding, nonexpressed nucleotide sequences.

"Inert ingredient," when referring to plant-incorporated protectants only, was defined as any substance, such as a selectable marker, other than the active ingredient, and the genetic material necessary for the production of the substance, that is intentionally introduced into a living plant along with the active ingredient, where the substance is used to confirm or ensure the presence of the active ingredient.

"Living plant" was defined as a plant that is alive, including periods of dormancy, and all viable plant parts/organs involved in the plant's life cycle.

"Noncoding, nonexpressed nucleotide sequences" were defined as the nucleotide sequences that are not transcribed and are not involved in gene expression. Examples of noncoding, nonexpressed nucleotide sequences include linkers, adapters, homopolymers, and sequences of restriction enzyme recognition sites.

ii. *Potential exemption criterion based on process.* The Agency also requested

174.71, plant-incorporated protectants that are derived through conventional breeding from sexually compatible plants. The exempt plant-incorporated protectants represent a subcategory of the plant-incorporated protectants described in Option 1 in the November 23, 1994. **Federal Register** document (59 FR 60522). (EPA is seeking additional comment in a supplemental document published elsewhere in this issue of the **Federal Register** on whether all plant-incorporated protectants derived from plants sexually compatible with the recipient plant should be exempt from FIFRA requirements, regardless of how they are introduced into the recipient plant.)

The following language appears in 40 CFR 174.25 to describe this subcategory:

A plant-incorporated protectant is exempt if all of the following conditions are met:

(a) The genetic material that encodes the pesticidal substance or leads to the production of the pesticidal substance is from a plant that is sexually compatible with the recipient plant.

(b) The genetic material has never been derived from a source that is not sexually compatible with the recipient plant.

The following language addressing inert ingredients in plants derived through conventional breeding from sexually compatible plants is added to 40 CFR 174.485, subpart X:

An inert ingredient, and residues of the inert ingredient, are exempt if all of the following conditions are met:

(a) The genetic material that encodes the inert ingredient or leads to the production of the inert ingredient is derived from a plant sexually compatible with the recipient food plant.

(b) The genetic material has never been derived from a source that is not sexually compatible with the recipient food plant.

(c) The resides of the inert ingredient are not present in food from the plant at levels that are injurious or deleterious to human health.

1. *Associated definitions.* Pertinent definitions associated with the exemption include:

"Bridging crosses between plants" means the utilization of an intermediate plant in a cross to produce a viable zygote between the intermediate plant and a first plant, in order to cross the plant resulting from that zygote with a third plant that would not otherwise be able to produce viable zygotes from the fusion of its gametes with those of the first plant. The result of the bridging cross is the mixing of genetic material of the first and third plant through the formation of an intermediate zygote.

"Cell fusion" means the fusion *in vitro* of two or more cells or protoplasts.

"Conventional breeding of plants" means the creation of progeny through either: The union of gametes, i.e., syngamy, brought together through processes such as pollination, including bridging crosses between plants and wide crosses; or vegetative reproduction. It does not include use of any one of the following technologies: Recombinant DNA; other techniques wherein the genetic material is extracted from an organism and introduced into the genome of the recipient plant through, for example, micro-injection, macro-injection, micro-encapsulation; or cell fusion.

"Genome" means the sum of the heritable genetic material in the plant, including genetic material in the nucleus and organelles.

"Recombinant DNA" means the genetic material has been manipulated *in vitro* through the use of restriction endonucleases and/or other enzymes that aid in modifying genetic material, and subsequently introduced into the genome of the plant.

"Sexually compatible," when referring to plants, means a viable zygote is formed only through the union of two gametes through conventional breeding.

"Source" means the donor of the genetic material that encodes a pesticidal substance or leads to the production of a pesticidal substance.

"Vegetative reproduction" means: In seed plants, reproduction by apomixis; and in other plants, reproduction by vegetative spores, fragmentation, or division of the somatic body.

"Wide crosses" means to facilitate the formation of viable zygotes through the use of surgical alteration of the plant pistil, bud pollination, mentor pollen, immunosuppressants, *in vitro* fertilization, pre-pollination and post-pollination hormone treatments, manipulation of chromosome numbers, embryo culture, or ovary and ovule cultures.

Pertinent associated definitions in 40 CFR 174.3, several of which are discussed in Unit VII.B.8., include:

"Active ingredient" means a pesticidal substance that is intended to be produced and used in a living plant, or in the produce thereof, and the genetic material necessary for the production of such a pesticidal substance.

"Genetic material necessary for the production" means both: Genetic material that encodes a substance or leads to the production of a substance, and regulatory regions. It does not include noncoding, nonexpressed nucleotide sequences.

"Inert ingredient" means any substance, such as a selectable marker, other than the active ingredient, where the substance is used to confirm or ensure the presence of the active ingredient, and includes the genetic material necessary for the production of the substance, provided the genetic material is intentionally introduced into a living plant in addition to the active ingredient.

"Living plant" means a plant, plant organ, or plant part that is alive, viable or dormant. Examples of plant parts include, but are not limited to, seeds, fruits, leaves, roots, stems, flowers and pollen.

"Noncoding, nonexpressed nucleotide sequences" means the sequences are not transcribed and are not involved in gene expression. Examples of noncoding, nonexpressed nucleotide sequences include, but are not limited to, linkers, adaptors, homopolymers, and sequences of restriction recognition sites.

"Pesticidal substance" means a substance that is intended to be produced and used in a living plant, or in the produce thereof, for a pesticidal purpose during any part of a plant's life cycle (e.g., in the embryo, seed, seedling, mature plant).

"Plant-incorporated protectant" means a pesticidal substance that is intended to be produced and used in a living plant, or in the produce thereof, and the genetic material necessary for the production of such a pesticidal substance. It also contains any inert ingredient contained in the plant, or produce thereof.

"Produce thereof," when used with respect to plants containing plant-incorporated protectants only, means a product of a living plant containing a plant-incorporated protectant, where the pesticidal substance is intended to serve a pesticidal purpose after the product has been separated from the living plant. Examples of such products include, but are not limited to, agricultural produce, grains and lumber. Products such as raw agricultural commodities bearing pesticide chemical residues are not "produce thereof" when the residues are not intended to serve a pesticidal purpose in the produce.

"Recipient plant" means the living plant in which the plant-incorporated protectant is intended to be produced and used.

Other definitions, relevant for plant-incorporated protectants only, can be found at 40 CFR 174.3. In this final rule, "plant" means an organism classified using the 5-kingdom classification system of Whittaker (Ref. 1) in the kingdom, Plantae. Therefore, the term "plant" includes, but is not limited to, bryophytes such as mosses, pteridophytes such as ferns,

Table 2. Examples of non-target and unintended effects of engineered plants

Trait	Plant	Effects	References
Insect resistance	cotton and potato	Changes in size and diversity of soil microbial, nematode and microarthopod populations; changes in soil enzyme activity	Donegan et al., 1995; Donegan et al., 1996
	tobacco	Changes in soil respiration; changes in size and diversity of protozoa, nematode and microarthopod populations	Donegan et al., 1997
Disease resistance	tobacco	Decrease and delay in arbuscular mycorrhizal infection	Vierheilig et al., 1995
Herbicide resistance	*Arabidopsis*	Gene outcrossing	Bergelson et al., 1998
	beets	Gene outcrossing	Dietz-Pfeilstetter and Kirchner, 1998
	canola	Gene outcrossing	Chevre et al., 1997; Lefol et al., 1991; Purrington & Bergelson, 1995
	canola	Change in endophytic and rhizosphere microbial populations	Siciliano et al., 1998
Specialty uses: Lignin-Peroxidase	alfalfa	Changes in rhizosphere and soil microbial populations	Di Giovanni et al., 1999; Donegan et al., 1999
	alfalfa	Reduced shoot biomass and changes in shoot macronutrient content	Donegan et al., 1999
	alfalfa	Reduced shoot biomass; changes in macronutrient and micronutrient content; decreased mycorrhizal infection	Watrud et al., 1998
Auxin, Enzymes	aspen	Altered wood anatomy and shoot growth; change in lignin structure	Tuominen et al., 1995; Lapierre et al., 1999
Pigments	petunia	Loss of color	MacKenzie, 1990

Appendix E Agenda for a "Scientific Methods Workshop: Ecological and Agronomic Consequences of Gene Flow from Transgenic Crops to Wild Relatives," Columbus OH, 5-6 March, 2002.

Scientific Methods Workshop:

Ecological and Agronomic Consequences of Gene Flow from Transgenic Crops to Wild Relatives

Meeting Proceedings

The University Plaza Hotel and Conference Center
The Ohio State University
Columbus, OH

March 5th and 6th, 2002

Steering Committee:

Dr. Allison Snow (Chair and Co-PI), Ohio State University
Dr. Carol Mallory-Smith (Co-PI), Oregon State University
Dr. Norman Ellstrand, University of California at Riverside
Dr. Jodie Holt, University of California at Riverside
Dr. Hector Quemada, Crop Technology Consulting, Inc., Kalamazoo, MI
Logistical Coordinator: Dr. Lawrence Spencer, Ohio State University

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SUMMARY

Gene flow from transgenic plants to wild relatives is one of the major research areas targeted by USDA's Biotechnology Risk Assessment Research Grants Program (BRARGP). We received funds for a two-day workshop that will bring together researchers who study the prevalence and consequences of gene flow from transgenic crops to weeds and other wild relatives. On the first day, speakers will discuss the general context for gene flow research, the information needs of USDA-APHIS, EPA, and the biotechnology industry, and case studies of specific crop-wild complexes, including cucurbits, brassicas, sunflower, sorghum, rice, wheat, maize, strawberry, poplar, and turfgrasses. Written summaries of these talks are included below. On the second day, break-out groups will discuss the advantages and disadvantages of various approaches for studying the occurrence of gene flow and various effects of gene flow (fitness effects of transgenes in wild relatives, effects on population dynamics, indirect community effects, and effects on the genetic diversity of wild relatives). The crops, wild relatives, and regulatory issues we discuss will focus on the USA, but much of the workshop will be relevant to similar situations in other countries. Proceedings from the workshop will be posted on an internet website that will be publicized in professional journals and newsletters. Bridging the fields weed science and plant ecology, this workshop will help define the most appropriate and rigorous empirical methods available for studying questions related to gene flow from transgenic crops to weedy and wild relatives.

BACKGROUND AND GOALS

Gene flow between crops and free-living, noncultivated plants is often considered to be an undesirable consequence of adopting transgenic crops (e.g., NRC 1989, NRC 2000). This process occurs when pollen moves from a crop to its wild or feral relative – or *vice versa* – and genes from their offspring spread further *via* the dispersal of pollen and seeds. In addition, some crops, such as oats, radish, and oilseed rape, can proliferate as feral weeds. Although crops and weeds have exchanged genes for centuries, transgenes can confer novel, fitness-related traits that were not available previously, and the same transgenes can be introduced into many different crops, increasing the potential for their escape (e.g., resistance to the herbicide glyphosate). A fundamental question, then, is what impacts could single or multiple transgenes have on the abundance and distribution of wild relatives? From a regulatory perspective, it is useful to compare the effects of transgenes to effects of nontransgenic crop genes that spread to wild and/or weedy populations, keeping in mind that certain traits developed through the introduction of transgenes (e.g. herbicide tolerance, herbivore and pathogen resistance, and resistance to harsh environmental conditions) have been produced through traditional breeding as well.

As a starting point, we need to determine which crops hybridize spontaneously with wild and/or weedy relatives in a given country or region. In cases such as sunflower, squash, and radish, the crop and the weed represent different forms of the same species, and crop-to-wild plant gene flow occurs whenever these forms grow near each other. In sunflower and radish, crop genes are known to persist for many generations in wild populations, even when first-generation wild-crop hybrids produce fewer seeds per plant than wild plants (e.g., Whitton et al. 1997, Snow et al. 2001). Gene flow can also occur when crops and weeds are more

Appendix F Examples of meetings on biosafety and risk assessment of engineered plants (Watrud, 2000).

Table 1. Examples of meetings on biosafety and risk assessment of engineered plants

Meeting/Title	Location/Year Held	Editor/Publisher/Date/Organizers
1st International Symposium on the Biosafety Results of Field Tests of Genetically Modified Plants and Microorganisms	Kiawah Island, SC, 1990	MacKenzie, D.R., Henry, S.C. (eds.). Agricultural Research Institute, 1991.
Pesticidal Transgenic Plants: Product Development, Risk Assessment and Needs	Annapolis, MD, 1990	US EPA, Office of Pesticide Programs, 1991
Workshop on Safeguards for Planned Introduction of Transgenic Oilseed	Ithaca, NY, 1990	USDA, Animal and Plant Health Inspection Service, 1990
Symposium on Ecological Implications of Transgenic Plant Release	College Park, MD, 1992	Levin, M. and R.J. Seidler (eds.), Blackwell Scientific Publ., Oxford, UK. *Mol. Ecol.* 3:1-90, 1994
2nd International Symposium on the Biosafety Results of Field Tests of Genetically Modified Plants and Microorganisms	Goslar, Germany, 1992	Casper, R., Landsmann, J. (eds.). Biologische Bundesanstalt fur Land- und Forstwirtschaft, 1992
Toward Enhanced and Sustainable Agricultural Productivity in the 2000's: Breeding Research and Biotechnology	Taipei, Taiwan, 1993	Academia Sinica, Nankang, Taichung District Agricultural Improvement Station, 1994
3rd International Symposium on the Biosafety Results of Field Tests of Genetically Modified Plants and Microorganisms	Monterey, CA, 1994	Jones, D.D. (ed.), University of California, 1994
OECD Workshop on Ecological Implications of Transgenic Crop Plants Containing *Bacillus thuringiensis* Toxin Genes	Queenstown, New Zealand, 1994	Hokkanen, H.M.T. (ed.), University of Helsinki, Finland, 1994
Herbicide-resistant Crops: a Bitter or Better Harvest?	Memphis, TN, 1995	Southern Weed Science Society, Champaign, IL, 1995
Dialogue on Risk Assessment of Transgenic Plants: Scientific, Technological and Societal Perspectives	Dornach, Switzerland, 1997	Heaf, D. (coordinator), Ifene, UK, 1997
4th International Symposium on the Biosafety Results of Field Tests of Genetically Modified Plants and Microorganisms	Tsukuba-machi, Japan, 1997	Matsui, S., Miyasaki, S., Kasamo, K. (eds.). Japan International Research Center for Agricultural Sciences, 1997
Virus-resistant Transgenic Plants: Potential Ecological Impact	Godollo, Hungary, 1997	Tepfer, M. (ed.), Springer-Verlag, Berlin, Germany, 1997 Schiemann, J. and R. Casper (Organizers)
5th International Symposium on The Biosafety Results of Field Tests of Genetically Modified Plants and Microorganisms	Braunschweig, Germany, 1998	Biologische Bundesanstalt fur Land- und Forstwirtschaft Braunschweig, Germany

Appendix G Biotechnology references recommended by the USEPA Biotechnology Steering Committee.

Biotechnology References
Recommended By the Biotechnology Steering Group

1. EPA's Bt crops reassessment document. All together it exceeds 400 pages so you might want to start with the Overview. The Science Assessment Chapters are a thorough review of what we had for the initial registration and what we have learned since. See http://www.epa.gov/pesticides/biopesticides/reds/brad_bt_pip2.htm. Also, Janet Andersen can provide a wordperfect file of the document

2. USDA Biotechnology Risk Assessment Research Grants Program – http://www.reeusda.gov/1700/funding/brargp.htm
   The workshop proceedings and contributed papers from Ecological and Agronomic Consequences of Gene Flow from Transgenic Crops to Wild Relatives can be accessed at http://www.biosci.ohio-state.edu/~lspencer/gene_flow.htm. This workshop was funded by USDA biotech risk assessment research grants program and Chris Wozniak from BPPD attended. Chris has a great deal of expertise in this area.

3. EC-supported research into the safety of Genetically Modified Organisms – http://europa.eu.int/comm/research/quality-of-life/gmo/

4. USDA's Biotechnology Risk Assessment Research Grants Program Home Page (look for the summaries of sponsored research) – http://www.reeusda.gov/crgam/biotechrisk/biotech.htm
   (Also found on this site are three summaries of research in 1994,1995 and 1996 sponsored by USDA, EPA and Environment Canada.)

5. Genetically Modified Pest-Protected Plants: Science and Regulation (NAS) -- http://www.nap.edu/books/0309069300/html/

6. Environmental Effects of Transgenic Plants: The Scope and Adequacy of Regulation – http://www.nap.edu/catalog/10258.html

7. The Plant Journal - http://www.blackwell-science.com/tpj/gm
   (NB: The Kuiper et al. paper on biotech foods).

8. Information Systems for Biotechnology
   http://www.isb.vt.edu/news/2002/news02.Apr.html
   (NB: This latest issue (April 2002) of the ISB News contains a summary of the gene flow conference held in Columbus, Ohio recently and a summary of the recent series of reports in PNAS on non-target effects from Bt corn. Some might be interested in scanning earlier issues in the ISB archives.)

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13. Resistance Mangement
    a. Binns MR, Nyrop JP, van der Werf W (2000) Sampling and Monitoring in Crop Protection, CABI Publishing, New York.

    b. Bourguet D, Genissel A, Raymond M (2000) Insecticide Resistance and Dominance Levels, J. Econ. Entom. 93:1588-1595.

    c. Caprio MA (2001) Source-Sink Dynamics Between Transgenic and Non-Transgenic Habitats and Their Role in the Evolution of Resistance, J. Econ. Entom. 94: 698-705.

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y. Williamson, M. 1996. Can the Risks from Transgenic Crop Plants be Estimated? Trends Biotechnol. 14:449-450.

TECHNICAL REPORT DATA

(Please read Instructions on the reverse before completing)

1. REPORT NO.	2.	3. RECIPIENT'S ACCESSION NO.
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4. TITLE AND SUBTITLE: Research Plan: Effects of Chemical Herbicides and Gene Flow on Non-target Plants		5. REPORT DATE
		6. PERFORMING ORGANIZATION CODE
7. AUTHORS: David M. Olszyk, Ph.D.		8. PERFORMING ORGANIZATION REPORT NO.: WED-03-113
9. PERFORMING ORGANIZATION NAME AND ADDRESS: Western Ecology Division, National Health & Environ. Effects Res. Lab., 200 S.W. 35th St., Corvallis, OR 97333		10. PROGRAM ELEMENT NO.
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15. SUPPLEMENTARY NOTES

16. ABSTRACT

This project supports EPA's mission to protect human health and to safeguard the natural environment — air, water, and land — upon which life depends. Specifically, we address EPA's responsibility to prevent pollution and reduce the impacts from pollution to communities and ecosystems (Government Performance and Results Act (GPRA) Goal 4, "Safe Communities"). To achieve this goal, EPA's Office of Prevention, Pesticides, and Toxic Substances (OPPTS) requires scientifically credible information and methods for use in assessing health and ecological risks from products used in commerce, including chemical pesticides and genetically engineered plants. OPPT regulates chemical and biological pesticides primarily under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) administered through the Office of Pesticide Programs (OPP). Other acts and programs, especially the Toxic Substances Control Act (TSCA), and the Federal Food, Drug and Cosmetic Act (FFDCA) are administered by OPPTS's Office of Pollution Prevention and Toxics (OPPT) to provide for protection of the environment from chemicals and biological pesticides. In the past, protection of ecological resources has received minimal attention under these regulations compared to concerns regarding impacts on human health. Recently, however, awareness of adverse effects from drift of new low-dose high-toxicity herbicides to non-target crops and native vegetation has heightened awareness of the need to improve tests for effects of chemical herbicides to plants. Similarly, public concern regarding the release of genetically engineered plants and the adoption of the "Final Rules and Proposed Rules for Plant-Incorporated Protectants" (40CFR Parts 152 and 174) have increased the need for tools to evaluate the risks from engineered plants and gene flow from engineered crops to other plant species. Thus, OPP and OPPT need tools to assess ecological risks from transgenic crops, improved methods for spatially explicit ecological risk assessments, new methods to provide for efficient and effective gathering and interpretation of herbicide hazard identification and dose-response data, and investigations of the potential effects of high priority hazards.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
native plants / risk assessment / genetic engineering / terrestrial plants		

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