PROCEEDINGS

WORKSHOP ON CONFINEMENT OF GENETICALLY ENGINEERED CROPS DURING FIELD TESTING

September 13-14, 2004

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EDITORS
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INTRODUCTION

A Workshop on Confinement of Genetically Engineered Crops During Field Testing (Workshop) was held on September 13-14, 2004, sponsored by Biotechnology Regulatory Services (BRS) of the Animal and Plant Health Inspection Service (APHIS), United States Department of Agriculture (USDA). Under the Plant Protection Act of 2000, APHIS regulates the safe development and release into the environment of genetically engineered plants that have the potential to pose a plant pest risk. Thus, APHIS grants permission for field testing of most genetically engineered crops. The Workshop was held to review past results and obtain an update on the most recent scientific results relevant to biological dispersal and confinement of genetically engineered crops during field testing. The Workshop concentrated on, but was not limited to, crop plants currently planted under APHIS permit for use as plant made pharmaceuticals (PMPs) and plant made industrials (PMIs).

Experts were convened to present and consider past and current information relevant to biological and physical factors that influence the design, implementation, efficacy and feasibility of measures used to confine genetically engineered plants and their progeny to the authorized field sites, including measures that can be taken to limit gene flow beyond the authorized site, commingling with other crops, and persistence of genetically engineered plants in the environment following termination of the field trial. The use of modeling to predict gene flow or to enhance the design or assessment of confinement measures was also discussed. Finally, there was consideration of where research might facilitate the design or assessment of confinement measures. Three types of plants were considered: 1) wind pollinated crops using corn as a model, 2) self pollinated crops using rice as a model, and 3) insect pollinated crops using safflower as a model.

The Workshop format was developed with a multidisciplinary steering committee. The Workshop began with a half day of speakers presenting information to the initial plenary session on cross-cutting issues to aid in subsequent break-out sessions. Break-out sessions formed around the three types of plants and each discussed three major topics consecutively: pollen confinement, seed confinement, and general confinement strategies. Each major topic within a break-out group was developed through discussions that were initiated with short presentations by members of each group. Presentations can be viewed online at: http://www.aphis.usda.gov/brs/confine_present.html.

This document of proceedings summarizes the presentations and discussions of the participants. Each break-out group had a rapporteur take notes and summarize the group discussion. This summary was presented to the plenary of participants at the conclusion of the Workshop and the summaries were used as the basis for the proceedings. Members of the break-out groups were given the opportunity to review the summaries for their group and provide comments and additional information when relevant. Editing was provided by BRS. Generally, scientific notation is used in the document. Some information found in this proceeding was provided by members of the break-out groups after the conclusion of the Workshop. The rapporteur for each group reviewed the edited document. In addition, an extensive bibliography with references dating up to July 2005 was developed to facilitate discussion at the meeting and was elaborated after the Workshop by BRS and Workshop participants.

The proceedings and bibliography should serve as a resource for those involved in the design, evaluation, and research of confinement measures for all stages of field trials of genetically engineered plants (pre-plant through post-harvest monitoring), particularly for field trials of plants engineered to express pharmaceutical or industrial products.

Steering Committee Members

- Peter Bretting, National Program Leader Plant Germplasm & Genomes, USDA-Agriculture Research Service
- Dr. Mark Condon, Vice-President, American Seed Trade Association
- Dr. Norman Ellstrand, Professor of Genetics and Director, Biotechnology Impacts Center Department of Botany & Plant Sciences University of California, Riverside
- Dr. Anne Fairbrothers, Chief, Risk Characterization Branch, U.S. Environmental Protection Agency, National Health and Environmental Effects Research Laboratory, Western Ecology Division
- Dr. Alan Galbreth, Associate Director, Indiana Crop Improvement Association and Association of Official Seed Certifying Agencies
- Dr. Jim Knuteson, Senior Scientist, Dow AgroSciences, LLC
- Dr. Margaret Mellon, Director Food and Environment, Union of Concerned Scientists
- Dr. Chris Wozniak, National Program Leader for Food Biotechnology and Microbiology, USDA-Cooperative State Research, Education and Extension Service, Plant and Animal Systems Unit

APHIS Committee Members:

- Dr. Sally McCammon, Science Advisor, Office of Science, USDA APHIS BRS
- Dr. Susan Koehler, Chief of the Environmental and Ecological Analysis Branch, USDA APHIS BRS
- Dr. Robyn Rose, Biotechnologist, USDA APHIS BRS
- Dr. Laura Bartley, American Association for the Advancement of Science Fellow with USDA APHIS BRS

ACKNOWLEDGEMENTS

The Animal and Plant Health Inspection Service, Biotechnology Regulatory Services (BRS) gratefully acknowledges the members of the Workshop Steering Committee, who aided in the development of the agenda and format of the workshop, the development of questions for the break-out groups, and provided recommendations of invited speakers and participants.

BRS also wishes to acknowledge the invited speakers and participants for their informed and thoughtful contributions to this workshop and the proceedings, in particularly the following individuals who served as rapporteurs, drafting the summary reports of the break-out sessions: Michelle Marvier for the wind-pollinated plants (corn), Karen Hokanson for the self-pollinated plants (rice), and Hanu Pappu for the insect-pollinated (safflower).

BRS acknowledges the major contribution of the workshop coordinator, Robyn Rose and the direction provided by the BRS Office of Science. Clint Nesbitt and Laura Bartley are recognized by BRS for organizing the bibliography. BRS also wishes to thank the following individuals who provided support in arranging travel, meeting facilities and meeting logistics: Kathy Balderson and Terry Hampton.

WORKSHOP AGENDA

Day 1 Monday September 13

Introductory Speakers

8:30 - 8:35 *Welcome*; Sally McCammon, Science Advisor, USDA APHIS BRS

8:35 - 8:45 *Introduction to the workshop*; Robyn Rose, Biotechnologist/Ecologist, USDA APHIS BRS

8:45 - 9:15 *Introduction to and principles of confinement*; Susan Koehler, Chief of the Environmental and Ecological Analysis Branch, USDA APHIS BRS

9:15 - 9:45 *Setting of AOSCA standards*; Allan Simons, President, AOSCA

9:45 -10:15 *Modeling tools for gene flow and confinement*; Franco DiGiovanni, Air Quality Modeller, AriZOne Inc.

10:15 - 10:30 Break

10:30 - 11:00 *Confinement analysis critical control points (CACCP) and quality control/monitoring*; Stacy Charlton, Manager, Regulatory Affairs, Syngenta Seeds, Inc

11:00 - 11:30 *Monitoring to verify confinement*; Jeff Wolt, BIGMAP, Iowa State University

11:30 - 12:00 *Bioconfinement -- molecular strategies for gene containment*; Henry Daniell, University of Central Florida

12:00 - 1:00 Lunch

1:00 - 3:00 Pollen Confinement

Breakout Group 1 - **Wind Pollinated Crops (e.g., corn**)

Integrating the biological and physical components of maize pollen dispersal; Mark Westgate, Iowa State University (speaker).

- Rapporteur - Michelle Marvier, Santa Clara University
- Facilitator - Chris Wozniak, National Program Leader for Food Biotechnology and Microbiology, USDA/CSREES-PAS

Breakout Group 2 - **Self Pollinated Crops (e.g., rice)**

Dynamics of pollen dispersal and confinement in U.S. rice; David Gealy, USDA/ARS (speaker).

- Rapporteur - Karen Hokanson, Program for Biosafety Systems
- Facilitator - Laura Bartley, AAAS Fellow with USDA APHIS BRS

Breakout Group 3 - **Insect Pollinated Crops (e.g., safflower)**

Confining safflower pollen during regeneration of germplasm seed stocks; Richard Johnson, USDA/ARS (speaker).

- Rapporteur- Hanu Pappu, Washington State University
- Facilitator - Phil MacDonald, Canadian Food Inspection Agency

3:00 - 3:15 Break

3:15 - 5:00 Continue Pollen Confinement

Day 2 Tuesday September 14

8:30 - 10:30 Seed and Volunteer Confinement

Breakout Group 1 - **Wind Pollinated Crops (e.g., corn**)

What we have learned in four years of production; Bill Horan, Horan Brothers (speaker).

- Rapporteur- Michelle Marvier, Santa Clara University
- Facilitator - Lidia Watrud, EPA/ORD

Breakout Group 2 - **Self Pollinated Crops (e.g., rice)**

Gene containment via process management; John Nelson, Rice Tech (speaker).

- Rapporteur - Karen Hokanson, Program for Biosafety Systems
- Facilitator - Michael Wach, USDA APHIS BRS

Breakout Group 3 - **Insect Pollinated Crops (e.g., safflower)**

Confinement of transgenes - seed and volunteer crops; Linda Hall, University of Alberta (speaker).

- Rapporteur - Hanu Pappu, Washington State University
- Facilitator - Bob Rose, USDA APHIS BRS

10:30 - 10:45 Break

10:45 - 12:00 Continue Seed and Volunteer Confinement

12:00 - 1:30 Lunch

1:30 - 3:30 Strategies for Confinement

Breakout Group 1 - **Wind Pollinated Crops (e.g., corn**)

Practical application of redundant systems for biological confinement; Mark Halsey, Consultant, Donald Danforth Plant Sciences Center, Program for Biosafety Systems (speaker).

- Rapporteur - Michelle Marvier, Santa Clara University
- Facilitator - Eldon Ortman (Purdue University and CSREES)

Breakout Group 2 - **Self Pollinated Crops (e.g., rice)**

Opportunities for confinement of rice; Donna Mitten, Bayer Crop Science (speaker).

- Rapporteur - Karen Hokanson, Program for Biosafety Systems
- Facilitator - Debora Hamernik, National Program Lead for USDA/CSREES/PAS

Breakout Group 3 - **Insect Pollinated Crops (e.g., safflower)**

PMP safflower confinement at Symbioses; Rick Keon, Symbioses (speaker).

- Rapporteur - Hanu Pappu, Washington State University
- Facilitator - Virgil Meier, USDA APHIS BRS

3:30 - 5:00 Discussion of Day 3 Presentations

Confinement During Field Testing of Wind-Pollinated Plant Made Pharmaceutical and Plant Made Industrial Crops Using Corn as a Model

Rapporteur: Michelle Marvier, Santa Clara University

ABSTRACT

This section summarizes the discussions of a panel of experts that were charged with discussing confinement measures for pollen and seed dispersal of wind-pollinated crops genetically engineered (GE) to contain pharmaceutical or industrial proteins. Corn was chosen as the focus of discussion by USDA scientists as an example of an outcrossing, wind-pollinated species that is well-understood and currently being used for the production of plant made pharmaceutical (PMP) proteins. This report includes a review the basic biology of the plant, pollen, and seeds, as it relates to issues and measures of confinement for field trials of PMP and plant made industrial (PMI) corn. Corn (also commonly referred to as maize) is self fertile and typically cross pollinated by the wind because of differences in floral synchrony between male (tassel) and female (silk) flowers on single plant. The mechanism by which genes are moved from one flowering plant to another in nature is through cross-pollination of sexually compatible plants so the plants with which corn can cross-pollinate are taken into account when considering gene confinement. Strengths and weaknesses, effectiveness, and feasibility of confinement measure were discussed by the panel, as well as research needs related to issues of confinement in corn. Finally, this report summarizes panel discussions and a few themes that threaded themselves throughout the workshop, including the importance of estimating human error, the need to determine threshold levels of contamination, and the tradeoffs that complicate many of the decisions surrounding confinement measures.

BACKGROUND

Basic Biology of Corn

Corn or maize (*Zea mays* ssp. *mays*) is a member of the grass family (Poaceae). Unlike most grasses, corn is monoecious with the male and female reproductive structures present on a plant, but physically separated from one another (i.e. the flowers are imperfect). Corn plants are annuals that are typically cross-pollinated by wind. Due to its large size and mass, corn pollen may also be disseminated by gravity resulting in self-pollination. Pollen is produced at the top of the plant in staminate inflorescences (tassels), whereas ovules are produced on the pistillate inflorescences (ears) sprouting from the leaf axils, located lower on the plant.

Due to approximately 8000 years of selective breeding, modern cultivated corn bears little resemblance to its native ancestors, the teosintes. Unlike teosinte, cultivated corn has a single main vegetative stem referred to as the stalk. The grain or "kernels" are larger and do not fall off the ears easily (i.e. the grain does not 'shatter'). Cultivated corn ears are four or more rows, whereas the ears of teosinte have only two rows.

Outcrossing
In Mexico, corn can potentially outcross with other subspecies and species of *Zea* including teosinte. Since teosinte does not occur naturally within the United States, outcrossing of corn with wild relatives is not an issue. The main concern within the United States, is cross pollination from one field of corn to another, rather than from corn to wild relatives.

Outcrossing with other species is, however, an important issue for many wind-pollinated species other than corn. If these species were to be used for the production of PMP or PMI proteins, then the issue of outcrossing to other species would warrant more attention.

Corn Pollen
Corn pollen grains are relatively large (measuring 50-100 microns) and the pollen coat is not sticky. Pollen shed from tassels is often captured on leaves, but grains may become re-suspended by wind because of the non-sticky surface leading to pollination of an ovule. However, much of the pollen hits the ground within 3 seconds.

It is generally difficult to save corn pollen for more than a few hours, even under laboratory conditions. Corn pollen typically retains viability for as little as 10 min, but can last as long as 3 to 5 h. The duration of viability largely depends on the corn variety and environmental conditions. For example, viability is lost slowly on moist days and quickly on dry days. Recent data indicate that the viability of corn pollen does not decrease linearly. Instead, viability generally remains relatively constant for a couple of hours followed by a rapid exponential decline (Aylor 2004).

Humidity and vapor pressure deficit (VPD) leading to water loss in grains may occur at higher altitudes, thus reducing viability if pollen is lifted high into the air by wind currents (Aylor 2003). Pollen that rises in elevation 800 to 1000 ft., and then drops down, may have reduced viability, thereby decreasing the chance of successful pollination over very long distances. Additional research is needed to determine the effect of environmental conditions on the duration of pollen viability.

For additional information on the basic biology of corn, please see
http://www.aphis.usda.gov/brs/corn.html and
http://www.inspection.gc.ca/english/plaveg/bio/dir/dir9411e.shtml
http://www.oecd.org/document/51/0,2340,en_2649_34387_1889395_1_1_1_1,00.html.

DISCUSSION SUMMARY

Pollen Confinement Measures

Dispersal of Corn Pollen
Most corn pollen falls within 2 m of the edge of a field. Pollen grains tend to remain close to their source plant because corn pollen is large and heavy. Due to its relatively rare occurrence and difficulty with data collection, there are few publications examining movement of corn

pollen at distances beyond 660 ft (Raynor *et al.* 1972; Emberlin 1999; MAFF 2000; Stevens 2004; Pleasants *et al.* 2001; Jemison and Vayda 2001). Gene flow occurring from pollen movement has been observed 400 to 800 m (1312 to 2625 feet) from the source (Jones and Brooks 1950; Salamov 1940 cf Jones and Brooks 1950, Luna *et al.* 2001; Halsey *et al.* 2005). However, these studies differ widely in methodology (e.g., source plot size), making quantitative comparisons difficult.

Geographic Isolation

Pollen dispersal has been recorded to a distance of 990 feet (60 rods) (Jones and Newell 1946). The Association for Official Seed Certifying Agencies (AOSCA) uses an isolation distance of 660 ft to achieve a 98% purity level in foundation and certified seed. A standard of 1320 ft (twice the 660 ft) is implemented for the production of foundation seed in some AOSCA agencies such as Indiana. This distance is expected to limit outcrossing to the 0.1% level and achieve seed standards for foundation material at 99.9% purity level. However, variability in direction and strength of wind speeds may affect the overall pattern of pollen movement. To accurately predict pollen movement, it may be useful to obtain meteorological data taken at short intervals and examine localized patterns of wind speed and direction at times of anthesis. Because the effect of wind on pollen movement and fertilization is random and episodic depending on the chance occurrence of loose and viable pollen, silk viability, and updrafts and gusts of wind, meteorological data on the overall pattern of wind speed and direction may not accurately predict pollen movement.

Currently, PMP and PMI open-pollinated corn field trials cannot be grown within a 1 mi radius of other corn including commercial fields, research plots, garden plots, and volunteers of corn. Alternatively, if pollination of the PMP or PMI corn is controlled by bagging, or if the GE corn is male sterile and is detasseled, the corn-free area can be reduced to a 0.5 mile radius, provided that any corn between 0.5 mi and 1 mi away is planted at least 28 days before or after the PMP corn (these additional confinement measures are discussed below). Although geographic isolation makes it more difficult to grow these types of GE corn in major corn-producing regions, planting field trials with the 1 or 0.5 mi isolation distance is technically feasible with corn.

Temporal Confinement

Temporal confinement requires that planting dates for GE and non-GE varieties are separated over time to reduce the possibility that one field will shed pollen while the other field has receptive stigmas. Current confinement options include a 28-calendar day (temporal) separation between the PMP or PMI corn and other corn located within 0.5 to 1 mi, when the GE corn tassels are either bagged or they are male sterile and detasseled.

Temporal confinement, if implemented properly, can be highly effective, but there are several factors that can reduce the degree of separation actually realized in the field. Both rainfall and temperature can alter the timing of germination, emergence, and maturation of corn plants, possibly allowing portions of nearby fields to become reproductive simultaneously despite having been planted weeks apart. In addition to weather conditions, the particular varieties of corn that are grown may alter the amount of temporal separation actually realized. Since different varieties can mature from 75 to120 days, information regarding time to maturation for

the particular varieties should be factored in when determining the duration of the separation window.

Separating plantings solely on the basis of calendar days may not be adequate to determine appropriate temporal isolation because time requires heat to be an effective isolation mechanism. Corn maturity is typically labeled in days; for example, a 120-day hybrid would reach maturity 120 days after planting. However, this system does not take into account complicated physiological processes and other factors that control growth and development of corn such as location of planting, and weather. There is a growing acceptance among seed producers to use the temperature based Growing Degree Unit (GDU) accumulation to express maturity. The GDU system currently in use for corn was proposed by the Environmental Data Service of the National Oceanic and Atmospheric Administration (formerly U.S. Weather Bureau).

Using GDU accumulation would allow the intent of the 28-day temporal isolation to be achieved more accurately. Basing GDUs on flowering, correlated with average temperature and growth, may improve the efficiency of temporal isolation. GDUs that are required to reach silking for different corn varieties, and cumulative GDUs for different locations, can be easily obtained on the worldwide web. Ideally, temporal separation would be based on emergence of the earlier fields rather than planting dates. This would provide even greater precision in obtaining the appropriate amount of temporal separation.

In summary, temporal isolation should not be viewed as a stand alone confinement mechanism. Temporal confinement combined with geographic isolation provides a higher degree of confinement than geographic isolation alone. However, it may be more appropriate to consider temporal isolation based on heat accumulation units or GDUs instead of calendar days. Information regarding corn maturity dates is useful to appropriately determine what to consider when determining the GDUs necessary to achieve isolation of viable pollen.

Detasseling

Detasseling is the physical (manual or mechanized) removal of tassels before they begin to shed pollen. This method for pollen confinement has some drawbacks. First, while it may be feasible to carefully remove all tassels from a small plot of corn, errors (e.g., missed tassels) may occur as field sizes increase. It also may not be sufficient to go through and detassel a field of any size only once because corn plants can tiller (i.e. send up reproductive shoots from the base of the plants); however, tillers would typically not be an issue since they flower 2-4 weeks after the primary crop. The cost of manual labor to detassel a large field multiple times could be prohibitive depending on the product, and there can be missed tassels. Detasseling has historically been effective for small acreage. As acreage increases, this method is harder to manage but is still an excellent method when cost is not a factor. Detasseling should be combined with other confinement measures such as geographic isolation, temporal isolation, or male sterility for larger acreages for greater confinement. Incomplete detasseling may also be useful in conjunction with other methods, such as time and distance, since the amount of pollen would be reduced, thus improving the efficacy of the other measures.

Bioconfinement
Male sterility can be a highly effective means of confinement of PMP and PMI corn. Although no measure can assure 100% confinement, using male sterility in combination with geographic or temporal isolation can greatly reduce the chance that GE corn will cross-pollinate with non-GE corn (NAS 2004).

Male sterility is a mechanism of confinement that warrants additional research and should be considered in PMP and PMI corn as new genetic technologies for confinement become available. For example, genetic use restriction technologies (GURTs), such as the use of inducible promoters to restrict expression of transgenes only upon the application of the chemical inducer (Gatz *et al.* 1992), the production of sterile seeds (Kuvshinov *et al.* 2001) and chloroplast transformation (Daniell *et al.* 1998) should be carefully considered if and when they become available in corn (Daniell 2002).

Volunteer Confinement Measures

For corn growing in temperate regions of the United States, confinement measures typically require two types of monitoring for volunteers:

1. One year post-harvest monitoring of the regulated article within the field test site and surrounding fallow zone
2. Any corn growing in the 1 or 0.5 mi radius of the field trial during the first growing season.

These monitoring activities are feasible for several reasons. First, corn seed does not remain viable beyond a single year in the field. Although corn occasionally germinates in roadsides and previously cultivated fields, it is not known to establish feral populations within the United States. Second, there is no outcrossing of corn to other species within the United States. Third, because the plants are large, it is relatively easy to spot volunteer corn plants in a non-corn field. However, some background plants such as sorghum may mask the presence of volunteer corn to various degrees (depending largely on the height and density of the vegetation). Therefore, growers of PMP and PMI corn should consider carefully what species of a non-food crop to plant in the year prior to and following a field trial in order to maximize the chances of spotting and destroying volunteer plants before they shed pollen. For example, it will be easier to identify volunteers if corn or sorghum is not grown in the year immediately following a PMP or PMI field trial. Finally, once a corn plant is spotted, they are quite easy to destroy by hand pulling, and for large areas, treatment with herbicide is usually highly effective. A contact herbicide such as glyphosate (if plants are not glyphosate tolerant) is effective to control volunteers or a pre-emergent herbicide, such as Treflan, would be effective if no crops were planted the year following production. Either or both of these could be done and would greatly reduce or eliminate the risk of volunteers but monitoring should still be required.

Monitoring for Volunteers
Although it is relatively simple to monitor for volunteer corn (compared to crops of a shorter stature), the size of the area that must be scouted can result in logistical challenges, particularly when resources are limited. In addition, it is important to monitor until the first killing freeze of autumn; monitoring that is discontinued prematurely can allow room for some late germinating

volunteers to escape detection. Planting crops that resemble corn, such as sorghum, may make monitoring volunteer corn difficult. A previous season of corn planting in the 1 or 0.5 mi radius may result in a large number of corn volunteer plants that are difficult to manage. Monitoring of volunteers of non-transgenic corn is required to maintain isolation distances from PMP and PMI corn. If sorghum is rotated with corn and planted near a PMP or PMI corn crop, it may be difficult to manage the non-transgenic corn volunteers effectively if the sorghum crop is growing within the isolation distance. Mistakes in monitoring can be made and have been made in some instances when confinement protocols were not properly followed.

Fallow Zones

Current field trials include a 50 ft fallow zone free of crops grown for food or feed immediately surrounding all PMP and PMI corn. The 50 ft fallow zone is designed to allow farm equipment to easily move around the trial site and prevent inadvertent mixing with other crops during planting and harvesting operations. The fallow zone may also serve to reduce some seed dispersal problems because volunteers are easily identified. However, a fallow zone may interact with local weather conditions to enhance pollen dispersal and soil erosion problems when this area is kept bare. Soil erosion that occurs when soil is kept exposed for long periods of time combined with wind that moves across a bare zone and then hits the edge of a corn field causing strong updrafts may allow pollen to move farther than it otherwise would. The heat radiating off the dark soil in areas where the bare ground is black (e.g. Iowa) can also cause strong updrafts and enhance pollen movement. Therefore, appropriate cover crops should be considered rather than the use of a fallow zone.

The use of certain types of vegetation is permissible within fallow zones around the edges of field trials provided the vegetation is not used for food or feed. A border of mowed grass, for example, would reduce problems of erosion and radiant heat. Another possibility is to use a border of soybean that could later be mowed after corn pollen shed is complete. The height of any plants used in the buffer zone, relative to the height of the corn field, should be considered because abrupt changes in vegetation height can affect wind flow (and therefore pollen movement) across the border-crop boundary. Security issues related to the presence of such a large fallow zone should also be considered. Fallow zones could be a clue to saboteurs that the plot is PMP or PMI corn. Additional research on use of border rows and/or surrounding cover crops to confine pollen or seeds may be helpful.

Seed Confinement Measures

Corn Seed Harvesting

Corn seeds are large and they remain firmly attached to the ear, even after drying. However, seed may be left in the field after harvest as both kernels and ears. The amount of harvest loss depends on factors such as weather conditions, the vigor of the corn stalks, the moisture level of the corn kernels, and the harvest methods used. Combines, for example, may lead to harvest losses of 0.6 - 2.5 bushels per acre. In contrast, machinery developed to harvest sweet corn and hand-picking can result in much lower levels of harvest loss. Many companies in the seed corn industry have switched their field corn picker components, such as shucking rollers that strip the ears off the stalks, with sweet corn components to lessen harvest loss. In drying buildings,

shucking beds to remove husks from ears have also been switched to those used for sweet corn resulting in gentler handling and less seed loss. Additional research is needed to determine the amount that sweet corn harvest equipment reduces field corn and popcorn seed loss, as well as research into other forms of equipment that can reduce losses during planting and harvesting.

Equipment

Seed planters and harvesters used in PMP and PMI fields must be dedicated to the GE crop, meaning that they cannot be used in any other type of field for the duration of the test. Standard Operating Procedures (SOPs) for cleaning field equipment and moving it off the test site to other PMP and PMI fields of the same variety are also necessary. SOPs must also be developed for cleaning field equipment prior to decommissioning its use in PMP or PMI production to enable its use for other purposes, such as the production of food or feed; equipment must be inspected (by USDA's Animal and Plant Health Inspection Service [APHIS]) prior to returning it to general use. Inspection of tires before equipment is moved out of a field is not currently required and would reduce the potential of seeds leaving the PMP plot. Foot scrapers on the field edges are a low-tech, inexpensive way to further reduce the likelihood of movement of seeds and pollen out of a PMP or PMI field.

Fencing and Netting

Fences around field edges—especially electric fences—may help to exclude wildlife from field trials of PMP and PMI crops. Netting over these fields could be used to reduce the presence of birds in the field although it will not prevent birds from feeding on seed left on the ground after harvest. However, it is questionable if birds are able to disperse corn since it is unlikely that whole seeds could be excreted. Animals moving in and out of fields might potentially act as seed dispersers. The combined use of fencing and netting may help increase the overall level of confinement. While fencing is practical for small acreage it may not always be practical or effective for larger acres in keeping animals out. Fencing may also draw attention to the field as being PMP or PMI, leading to vandalism or theft, which may result in gene escape. In addition, netting corn is not a practical solution and is less relevant for corn than other exposed crops, such as sunflower, since the shucks limit the birds' access to the corn seed. Further information may be useful on the extent wildlife disperses viable seed outside of the field trial.

Processing on the Field Trial Site or Indoors

Loss of seeds would be reduced if grain processing (e.g., sorting, shucking, drying, etc.) were to be performed either on the field trial site itself or within a contained facility, as is typically the case. Corn left to dry outdoors is more subject to dispersal by birds, and it is easier to clean up a spill if it occurs indoors. While a contained facility would provide a higher level of confinement, if operations are conducted on the trial plot itself, for example on a tarp, the seed would also be easy to clean up, and if missed would presumably be detected during the volunteer monitoring period.

Genetic Technologies for Seed Confinement

Genetic markers could be used to aid in the identification of kernels that contain PMP and PMI transgenes. The genetic markers could be fluorescent proteins (e.g., GFP or lux), or it may be possible to use morphological markers such as kernel color and shape that are easily identified.

However, natural markers may be ineffective due to natural variation in the commodity

germplasm or may lead to intentional sabotage. Future research is needed to identify a marker that would be easily detected but not easily obtained by other sources.

Dyes for Identity Preservation
Dyes can be used to identify grain that contains PMP or PMI proteins. These dyes can be applied at the time of harvest. The use of dyes may be a way to expedite monitoring for contamination of grain lots.

Overall Strategies of Confinement

Although geographic isolation is an important isolation mechanism, there may be the potential of low levels of pollination occurring over long distances in corn. Geographic isolation combined with other confinement measures, such as those mentioned above would further reduce the chance of gene escape through pollen movement. In general, combining measures (e.g., using geographic isolation in combination with temporal isolation) could increase the degree of confinement achieved.

Corn does not exhibit seed dormancy and its seeds will germinate quickly when exposed to adequate moisture and heat, which may result in implications for confinement. Therefore, monitoring for volunteers during the year immediately subsequent to a field trial is needed. Monitoring for 1 yr should be sufficient since it is unlikely that corn seed will remain viable in the soil for a longer period. In tropical environments, corn seed deteriorates more rapidly than temperate climates, therefore periods of less than 1 yr may also be sufficient to monitor for volunteers especially if adequate water is provided to assure seed germination. Since it is possible to grow more than one crop per season in tropical environments, this may result in the need for intensified monitoring for volunteers.

Modeling of Gene Dispersal and Confinement
Models may be useful as a tool providing understanding as to what may occur at larger scales without conducting outcrossing studies. Models of the movement of particulate air pollution have been adapted to simulate the movement of corn pollen. These models are well-developed, but further work could address at least two areas. More attention should be considered regarding the effects of irregular wind gusts. Second, interactions between airflow, vegetation structure, and topography may need more attention due to flow patterns around isolated experimental plots (with fallow buffer zone) where incoming air may be forced upwards on the leading edge of the field. It is unclear whether the enhancement of upward pollen flow at the leading edge of a cornfield is compensated for by downward flow at the trailing edge and, if so, to what extent. The importance of factors, such as updrafts and wind gusts for long distance pollen transport, may warrant additional consideration, both theoretically and experimentally. However, useful models on wind-breaks have been developed.

Economic Incentives
In addition to the geographic, temporal, genetic, and physical confinement measures described above, economic disincentives can be considered where appropriate. These disincentives may include penalties for violation of confinement requirements. Requiring that individuals sign and

take responsibility for the completion of each task in a confinement protocol can also be an effective way to reduce human error, especially if used in conjunction with economic penalties.

RESEARCH NEEDS

What are the Key Vulnerabilities in Confinement?
There is a need for the development of quantitative research on the processes involved in the entire lifecycle of PMP and PMI corn related to confinement—from the time seeds leave a developer's custody until they are planted in a field, harvested, and processed. These experiments could include detailed information about all possible routes for loss of confinement, including biological processes such as long-distance pollen dispersal, seed movement by animals, and viability of seeds following consumption by animals. This research should include routes of transgene escape that are subject to system failure and human error, such as a failure to perform appropriate monitoring for volunteers, inadvertent mixing of GE and non-GE grain, inadequate cleaning of equipment, inadequately designed equipment, equipment failures, and violations of procedures for chain of custody. There are many possible points at which confinement could be breached and data may be useful to measure the occurrence of these incidents at each of these points. Using these data to then estimate the likelihood of containment failure at critical points of various forms of human error would be useful to identify how and when errors are most likely to occur in field operations. Some data are already received by APHIS' Biotechnology Regulatory Services (BRS) in incident and inspection reports.

What Mitigation Methods Work?
Mitigation methods, that would be implemented should escape occur, are required for all PMP and PMI field trials. For example, permit holders must describe what would be done in case of a spill during shipping. Cleaning up spilled seed from a roadway is one level of mitigation, but it could be far more difficult to mitigate pollen drift off a PMP or PMI field and pollinate non-GE corn. Comparisons of the effectiveness of various mitigation methods could be performed in an experimental framework using non-GE genes released into the environment. For example, pollen containing genes for wrinkled kernels could be released into replicate fields of non-wrinkly corn. Then, various methods to eliminate plants containing this gene from the fields could be applied, and their effectiveness compared. This would involve comparing methods to detect the transgenes as well as procedures for harvesting or destroying the crop and managing volunteers.

Human Error and System Failure
The primary contributing factor to failure of confinement is human error. Incidents involving confinement failures for GE crops (but mostly *not* involving PMI or PMP crops) due to human error and system failure have occurred from factors such as, but not limited to, shipping plant material to the wrong place, equipment failure, inadequate equipment design, planting in the wrong place, and detectable contamination of parent seed lots. Despite the previous occurrence of these mistakes, the importance of human error and system failure has not been emphasized in risk assessment research efforts with GE crops to date. Quantitative analysis of these types of errors may identify the types of incidents that are most likely to occur and consequently the

process measures that should be implemented to minimize the frequency and consequence of such errors. A great deal could also be learned by looking to other industries where confinement is a major issue (e.g., oil transport and nuclear energy production). It may be possible to adapt studies of safety in manufacturing and transportation processes from these industries and apply them to the production of PMP and PMI proteins. In addition, the application of testing for the presence of transgenes is a potential method to validate product identity and evaluate seed sources, grain lots, and processed food or feed for PMP or PMI contamination as well as to determine dispersal into the environment. Costs develop due to the large number of samples needed for analysis and the need to develop multiple types of assays. Testing would probably be ineffective given the high degree of dilution of any contamination due to the vast excess of conventional and deregulated crops that are grown relative to PMP and PMI crops. For example, in 2004, total PMP/PMI crops in the United States totaled 45 acres whereas total corn was approximately 80 million acres, about a 2 million fold excess. Thus, it is difficult to justify the high costs required for such testing when methods currently are not sensitive enough, even for large scale field tests.

What is the Cumulative Effect of Redundant Systems?
Research is needed to assess the effects of implementing various confinement measures such as geographic, temporal, genetic, and physical confinement measures. In particular, the impacts of these measures as well as the combined use of different measures should be assessed. Careful consideration should be given to assure that combined confinement measures are reliable and achieve the intended result.

Does Effectiveness of Confinement Measures Change as Production Scales Up?
To date, most field trials have involved small acreages. It is unknown whether particular confinement measures and mixtures of measures (systems) that are effective on small plots will be equally effective as production scales up to larger acreages. Research addressing, for example, the effect of source size on pollen movement may help fill some of these gaps.

Modeling
To date, models to inform better confinement practices have focused primarily on pollen dispersal. These models are providing important information. Model validation with additional field data is needed. In addition, modeling efforts might be useful to examine the entire process or "life cycle," of PMP and PMI production because pollen movement is but one of many possible routes for gene escape.

Confinement Measures for Other Wind-pollinated, Outcrossing Systems.
Although corn is the only primarily wind pollinated PMP crop currently grown under APHIS permits, other wind pollinated crops may be field tested in the future. It is, therefore, worth noting that corn is quite unique among the wind-pollinated, outcrossing species. Corn possesses very large pollen grains, which tend to travel relatively short distances. Corn lacks a seed bank beyond 1 yr. Corn does not outcross to other species within the United States. Corn has large seeds, a fact that facilitates cleaning of equipment and detection of leaks during transportation and storage. Finally, corn volunteers are large, which makes them fairly easy to spot, and the volunteers are easy to control, for example, with herbicides. Other species in the wind-

pollinated, outcrossing category, including many trees and other grass species, differ from corn in at least one, if not all, of these respects. If any of these species were to be used for PMP or PMI production in the future, experience gained with PMP and PMI corn may not be directly relevant. Therefore, additional research would be needed to determine adequate confinement measures for each wind pollinated crop prior to field release.

Development of Stratified Risk Categories

Not all PMP and PMI protein types, expression levels, and expression systems warrant the same level of risk management with regards to effective confinement, therefore, developing a tiered system should be considered. For example, certain PMP and PMI proteins may be harmless to humans, wildlife (including invertebrates), aquatic, and soil organisms. If a protein has been rigorously tested and there is no finding of impact on humans and the environment, a less stringent set of confinement measures may be acceptable. On the other extreme, there may be certain PMP and PMI proteins that are harmful to the health of humans, non-human organisms, or both. In these cases, more effective confinement measures should be taken. It may be appropriate to restrict plants producing these high-risk proteins to contained facilities or disallow their production in the plant entirely. Many PMP and PMI proteins will fall in between these two extremes, and in these cases the degree and design of confinement required should be matched to both the level of risk to human and environmental health and the risk to public confidence.

ADDITIONAL CONSIDERATIONS

Tradeoffs as Opposed to "Right Answers"

Many of the decisions regarding production methods for PMP and PMI proteins are complicated by critical tradeoffs. For example, a fundamental question regarding production of these proteins is whether corn should be used as a system given that corn is such an important crop for both food and feed in the United States and throughout the world. Public concern may be alleviated if these proteins were produced exclusively in non-food crops. However, the familiarity of farmers with corn production is a major asset in terms of transgene confinement. Methods for planting, harvesting, and storing corn are well understood and equipment to perform these tasks already exists. Furthermore, food crops may provide an added safety margin for the products themselves particularly when they may be oral vaccines, topically applied pharmaceuticals, or industrial enzymes used in food processing. Another issue that warrants consideration is if PMP and PMI proteins are to be produced in corn, should their production be prohibited in major corn growing regions? The chances of co-mingling of grain supplies and of cross pollination between PMP or PMI corn and non-GE corn may be higher when production occurs within a major corn growing region. On the other hand, growing PMP or PMI corn outside of these regions may entail its own set of consequences. Growing corn outside of the best production areas could mean that more total acres would be needed in order to produce the same quantity of a protein.

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Confinement During Field Testing of Self-Pollinated Plant Made Pharmaceutical and Plant Made Industrial Crops Using Rice as a Model

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ABSTRACT

This section summarizes the discussions of a panel of experts that were charged with discussing confinement measures for pollen and seed dispersal of self-pollinated crops genetically engineered (GE) to contain pharmaceutical or industrial proteins. Rice was chosen as the focus of discussion by USDA scientists as an example of a self-pollinated species because it is well-understood and currently being used for the production of plant made pharmaceutical (PMP) proteins. This report includes a review of the basic biology of the plant, pollen, and seeds, as it relates to issues and measures of confinement for field trials of PMP and plant made industrial (PMI) rice. The summaries of the discussions surrounding the three main topics above represent the diverse experiences, opinions, and perspectives of the members of the rice panel. However, certain main ideas did seem to emerge for each of the topics, and these may be relevant to other self-pollinated crops or even to all crops. The most reliable and commonly employed confinement measure for rice is spatial isolation. Temporal isolation is also an option, although it can be difficult to implement and may not be reliable. There are other pollen confinement strategies for rice, but these are generally not reliable or not feasible. This report summarizes factors that can influence the rate of outcrossing in rice and methods of limiting gene flow from PMP and PMI field tests.

BACKGROUND

Biology of Rice

Worldwide, over 1000 cultivars of rice are grown, typically in humid tropics and subtropics. There are two cultivated species of rice, *Oryza sativa* and *O. glaberrima*, but *O. sativa* is more common and is the species cultivated for United States rice production (Hancock 1992). Within *O. sativa*, there are two major ecogeographical cultivars, indica and japonica. Indica plants are intermediate to tall in stature (except for the semidwarf) and tiller profusely. The grains are long to short, awnless, and shatter easily. Japonica plants are short to intermediate in stature. The grains are short, awnless to long-awned, and low-shattering. Japonica is grown in the cooler zones of the subtropics and in temperate zones.

In the United States, rice is grown primarily in two distinct regions: 1) southern lowland irrigated areas of Arkansas, Louisiana, Mississippi, Missouri, and Texas; and 2) the Sacramento Valley of central California. The southern states produce nearly all long grain tropical japonica rice, although Arkansas and Louisiana also grow some medium grain rice, while California produces primarily medium grain (and to a much lesser extent, short grain) japonica rice. Rice is typically drill seeded into the soil or seeded aerially, and requires irrigation or paddy (flooded) conditions to maintain the crop. Sometimes, mainly in Gulf Coast regions of Louisiana and Texas, a second

(ratoon) rice crop is produced after harvest of the first, by applying additional fertilizers and water (Livezey and Foreman 2004).

Hybrids can occur between cultivated *O. sativa* and its weedy wild relatives, *O. nivara* and *O. rufipogon*, and with its weedy conspecific *O. sativa* f. *spontanea* (red rice) (Ellstrand 2003). *O. nivara* is not found in the United States. *O. rufipogon* has been reported in the United States, mainly in an isolated population in Florida, but at least one report states that *O. rufipogon* has been eradicated in Florida (Vandriver 1992). While *O. rufipogon* is not common in the United States, there is taxonomic confusion because of its morphological similarity to *O. sativa*, which can lead to its misidentification (NAPPO 2003). Red rice, *O. sativa* f. *spontanea*, is common in the United States and is a major weed in southern rice production. It was effectively eradicated from California over fifty years ago, although it has been reported there recently and is being monitored.

Outcrossing

Rice is 98-99% self-pollinated under normal conditions. Pollen is usually shed slightly before or at the time when rice flowers open, and most pollen fertilizes a stigma in the same flower. Each rice flower opens only once, for a period of approximately 1 hr, although the female remains receptive for several days. The range in outcrossing of 1-2% is mainly due to pollen moved by wind, although insects may play a role in the movement of rice pollen as well.

There are a number of factors that can influence the rate of outcrossing. The most obvious ones are the proximity in time and space to the receptive female and pollen viability. Environmental conditions can also influence the rate of outcrossing, particularly those that affect temporal placement. For example, the opening and closing of florets with receptive females varies with the weather. The activity of insect pollinators could influence outcrossing rates and a slight increase in outcrossing has been observed when honey bees are present (Gealy *et al.* 2003); however, little is known about how much insects or other animals common to rice fields contribute to the movement of pollen. Finally, species and cultivar differences can influence outcrossing rates. For example, outcrossing is higher between *O. sativa* and *O. rufipogon*, than within *O. sativa*, and the degree of outcrossing is generally higher in indica cultivars and wild species than in japonica cultivars of *O.sativa* (Messeguer *et al.* 2001). In a recent review, maximum outcrossing rates between *O. sativa* rice and *O. sativa* weedy rice averaged approximately 0.2% (Gealy 2005).

Outcrossing could potentially be higher than 2%, but only in unusual conditions. For example, more than 3 days of temperatures less than 13ºC during the 15 days before anthesis can inhibit pollen development. During a cold spell, the rice florets are not self-pollinated, and are available to receive foreign pollen. Because the female floral structures (stigmas) remain viable at these temperatures and are receptive for several days, chilling temperatures could result in higher rates of outcrossing. This is more likely to occur in California, where rice is grown in cooler regions. Constant rains can also cause male sterility that can then leave the stigma receptive to incoming pollen resulting in higher rates of outcrossing.

Pollen Characteristics

The length of time that pollen is viable can be weather dependent. Pollen is typically viable for 10-20 min, but viability is dependent upon humidity and exposure to UV radiation (Gealy *et al.* 2003; Khush 1993). Under ideal conditions, pollen can be viable for up to 30 min, while under less ideal conditions, viability can be as short as 5 min.

Seed Dormancy and Germination

Germination of rice seeds immediately after harvest varies from 0-100%. Generally, rice seeds must be dried to a low moisture condition, less than 14% (the process of dry afterripening), to allow for germination when imbibed with water. Loss of dormancy by dry afterripening treatment is cultivar (species)-dependent and requires a few days to weeks and months if other *Oryza* species, such as *rufipogon*, are considered. The speed of dry afterripening is temperature-dependent (Cohn and Hughes 1981) up to approximately 45°C. At higher temperatures seeds can be killed by dry heat. If the seed moisture content remains at 20% or higher after harvest, seeds can remain dormant (Cohn and Hughes 1981; Cohn *et al.* 1984; Leopold *et al.* 1988). However, 100% of seeds from most commercial rice varieties will be able to germinate after one to several days of drying; 100% germination of healthy, afterripened seeds can be expected after 14 days of hydration at 30ºC or alternating 30/20ºC (Association of Official Seed Analysts 2004).

Rice seeds left in the field are rarely exposed to the optimal low moisture conditions for loss of dormancy via dry afterripening, but in typical field conditions, seeds of most rice varieties will decay before the next cropping season. Red rice, on the other hand, when buried in the field where it will not dry and where it is not subject to predation, can remain dormant and viable for an extended period of time, at least as long as 4-6 years (Goss and Brown 1939; Cohn *et al.* 1984). Even in laboratory conditions under 100% humidity, dormant red rice seed will remain viable and will not rot. In addition, some cultivated rice varieties have more residual dormancy than most, even after drying. For example, Jasmine 85 is known to volunteer under field conditions. Indica varieties also tend to have more residual dormancy than japonica varieties.

Some rice varieties, such as Jasmine 85, require more dry afterripening than other varieties; the deeper the seed dormancy observed, the more afterripening is required. The depth of dormancy can be estimated, although there is no foolproof method to do this. Seeds can be kept at a constant temperature and low humidity, and the germination of subsamples can be tested every week (Cohn and Hughes 1981; Cohn and Jodari 1997). Lack of germination is an indication of dormancy, when seed health is also confirmed by a viability test (Cohn and Hughes 1981). Generally, if dry afterripening takes more than 3-4 weeks, then seed dormancy and the potential to volunteer in the field could become an issue. Jasmine 85 has been used as an indicator variety of dormancy for afterripening/dormancy tests (Fig. 1) (Cohn and Jodari 1997). Calrose, or another line known not to remain dormant, could be used as an indicator line for no dormancy, as well. (Jasmine 85 is not grown in California, but Calrose is.) Red rice could also be used as a worst case indicator for dormancy.

Burying freshly harvested, undried rice seeds in a mesh bag in the soil, and observing seed behavior over time, can also be employed as an environmentally relevant test for seed dormancy.

However, evaluation of seed dormancy under these conditions is an expensive and time-consuming process, and will not be scaleable for use in routine germplasm evaluations.

When measuring afterripening, it is important to take fresh weight and dry weight measurements to determine the moisture content of the seed. Moisture content can vary depending upon, for example, the environmental conditions at the time of harvest, and this can affect seed dormancy (see above). Even varieties with no dormancy in lab tests have the potential to volunteer in the field, depending upon the timing and conditions at harvest, as is true in other crops without dormancy, such as maize. Dormancy is a plastic phenomenon. To date, no studies have attempted to correlate field dormancy with afterripening over time measured in the lab.

DISCUSSION SUMMARY

Pollen Confinement Measures

Geographic Isolation

Spatial isolation distance from other commercial or weedy rice is viewed as the most effective strategy for pollen confinement in rice, therefore the difficulty of providing adequate isolation, relative in size to the plot, as field trials are scaled up for commercial production, must be considered. Increasing isolation distances makes monitoring for gene escape due to pollen movement more difficult. The possibility of selecting a site for PMP rice production in a non-rice growing region, where isolation would be assured, would be difficult because of the necessary growing conditions for rice (irrigation levies, etc.) in areas where it is not typically grown. No gene flow modeling in rice has been done to date and the feasibility of modeling to determine adequate barren zones for commercial size fields is not known. A good model may be informative, but there are the usual concerns that modeling won't account for the complexity in the biological system.

Various spatial isolation distances have been considered adequate for different applications. The spatial isolation distance required by the Association of Official Seed Certifying Agencies (AOSCA) for drill-seeded, non-hybrid foundation rice seed is at least 10 ft (see http://www.aosca.org), although some states certification laws require larger distances (e.g. Louisiana requires at least 6.1 meters [19 ft and 8.25 in] between different drill-seeded varieties) (Gealy *et al.* 2003). For hybrid rice production, the spatial isolation distance is 660 ft, although that can vary by state. The International Rice Research Institute (IRRI) recommends 330 ft for hybrid rice. For GE rice grown under APHIS notification, the accepted isolation distance has been 10 ft. For PMP rice, isolation distances up to 1320 ft have been voluntarily implemented. In addition, the PMP rice plots have included a 10 ft border row and a 50 ft fallow zone around the rice plot.

A number of controlled studies with relatively small field tests indicate that hybridization due to pollen flow does not occur beyond 10 m (Beachell *et al.* 1938; Gealy *et al.* 2003; Messeguer *et al*. 2001; Zhang *et al.* 2003). Other unpublished studies have provided similar results. However, one unpublished controlled study conducted by the hybrid rice seed company, RiceTec, using a

purple rice as the pollen source and male-sterile rice as the receptor, found that a distance of 700 ft was required to avoid contamination.

There is considerably greater spatial isolation distance recommended for hybrid rice seed production, compared to non-hybrid rice. It is possible that conditions do not exist for gene flow at the longer distance in non-hybrid rice. There have been hybridization studies demonstrating no hybridization beyond the 10 m isolation distance. However, those studies were based on morphological evidence for hybridization or relied upon herbicide tolerance as the marker. Also, no controlled studies of hybridization due to pollen flow in non-hybrid rice under normal growing conditions have been done on a large scale; available results are from small scale plots or long strips of test plots that extended from the field. Small-scale plots will produce a small amount of pollen relative to large-scale plots that will produce a large amount of pollen.

Temporal Confinement

Temporal isolation requires either (or a combination of) carefully timed planting or selection of varieties with different flowering times, relative to neighboring plots. It is not always possible to know the intended planting date or varieties that are planted in neighboring plots. Planting late is usually best to ensure temporal isolation, but planting late is not desirable in rice production for other reasons, such as too short of a growing season. In addition, it may be difficult to plant at the desired time if the field is too wet. Alternatively, the same effect can be accomplished by planting a GE variety, which is later maturing than the non-transformed varieties planted in the area. The flowering period for most cultivated rice varieties in the United States is only a few days, whereas the flowering period for weedy red rice types in the southern United States is more variable in time and can range from one to several weeks (Gealy *et al.* 2003). Temporal isolation also depends on the growth environment, since flowering time, pollen viability, and female receptivity are all influenced by the weather. Unpredictable environmental conditions and herbicide applications may also make temporal isolation less effective. Although temporal isolation is not a reliable pollen confinement measure alone, it can be effective as a secondary strategy, in combination with spatial isolation, to limit pollen flow.

Molecular Markers

Molecular markers, which are currently used for other applications in rice, may be useful for documenting hybridization. However, there are drawbacks to the use of molecular markers. Molecular markers that are codominant (the method of detection produces a positively-identifiable product from both the "yes" and "no" situations) are quite robust, but a non-codominant marker is susceptible to false negatives. That is, if you must detect a product of a reaction to see that gene flow has occurred, then the lack of that product may come from no gene flow or a failed reaction. Also, it is much more expensive to assay for molecular markers than to visually screen for morphological evidence of gene flow, so morphological markers can be valuable in that many more data points can be collected at lower costs. Some markers, molecular or morphological, do not show up all of the time and data may be misinterpreted.

Volunteer Control Measures

Monitoring for Volunteers
Broad generalizations about volunteers are not appropriate since consideration is necessary as to whether volunteers can be easily controlled and scouted, or not. Typically, one to two seasons of post-harvest monitoring for volunteers is sufficient for cultivated rice. GE rice field tests are typically left fallow for 1 yr in California, and for 2 yrs in Louisiana and Texas, which is consistent with land requirements for production of foundation rice seed.

Seed Dormancy
Tilling is not recommended, or at least not deep tilling, because tilling tends to bury seeds in the soil, where seeds with some dormancy can remain dormant for longer periods of time. They are more likely to germinate if they remain close to the surface of the soil. If cultivated rice fields are left fallow with no tilling and no flooding, seeds left behind should germinate or decay.

There is a regional concern in areas where red rice is common. If there is red rice in the area, there is the potential for volunteers in the field from rice x red rice hybrids. These may have longer periods of dormancy than the cultivated rice. A regime of flooding, lightly discing, and drying can be implemented to encourage seed germination in order to remove volunteers before they are able to flower. Flooding keeps the seed on the surface of the soil. This can be done in the field and in fallow zones surrounding the field three times in the year following harvest (spring/summer). This can be used for monitoring in any rice field trial, but might be especially recommended where there is the potential for rice x red rice hybrids to occur. This protocol has been successfully implemented in test plots planted in California.

Management of Volunteers
In addition, post-harvest rice fields could be treated with herbicides to eliminate volunteers. For certified seed, it is a common practice to use crop-specific differential susceptibility to herbicides. In this case, a crop that is sufficiently different, physiologically and phenotypically, from the preceding crop is planted. For instance, volunteer corn in soybean fields is controlled by grass herbicides that kill the corn but not the soybeans and volunteer potatoes are controlled in wheat fields with broadleaf herbicides.

Another option is to plant an herbicide-tolerant crop following the field trial and treat with the herbicide to eliminate any rice volunteers. For trials of GE rice that express pharmaceutical or industrial products, crops for food or feed cannot be planted in a field where GE volunteers are being monitored. This excludes the option of planting an herbicide tolerant food crop following a GE crop unless the herbicide tolerant crop was just plowed under and not used for food or feed. For example, a PMP rice crop could be followed with glyphosate-tolerant alfalfa that was subsequently plowed under.

Volunteers are typically more manageable at a small scale. However, an effective herbicide that targets the volunteers and not the main crop, or which can be applied only to the volunteers (such as applying glyphosate only to the taller corn volunteers in a soybean field via rope-wicks that touch only the corn), in combination with an effective scouting program (if you had rice

volunteers in a field of a short crop, you can see them from yards away), then volunteers may be effectively and efficiently managed. Scale-up may require leaving large fields fallow for 1 or 2 yrs, and this becomes costly. For larger scale plantings of GE rice, particularly PMPs and PMIs, it might not be acceptable to plant in red rice-infested regions. Certified seed cannot be grown in the field if red rice is found, and the fields and the seed lots are inspected visually for the presence of red rice before certification. Growing PMP or PMI rice in an area where there is no red rice and where rice is not typically grown, but perhaps where it has been grown historically (e.g., North or South Carolina), might be an option to keep the rice confined. However, in many cases red rice can be effectively managed so planting of PMP or PMI rice may not be precluded from that region.

Seed Confinement Measures

Equipment

Any equipment (including planters, threshers, combines, augers, wagons, and carts for field transportation) that is used during the field test can contribute to seed dispersal and co-mingling. Some equipment is easier to clean than others. Combines and seed processing/seed cleaning equipment were deemed to be far more difficult to clean than threshers and augers, and planters were thought to be relatively easy to clean. In particular, it was noted that the small size and flatness of the rice seed and the clay soil in the rice-growing regions make the combines difficult to clean, even when they are taken apart. The Mississippi Crop Improvement Association has detailed guidance on cleaning and inspection of combines for certified seed. They have not ranked machines for ease of cleaning, but they do have statistics that would allow such an analysis. Typically, the removable parts are disassembled or dropped down and doors opened, and the equipment is cleaned with high pressure air and water. Methyl bromide can be used to sterilize the equipment and kill the seed, but it can corrode equipment components. High temperature treatments could also be used to kill the seed (e.g., greater than 45ºC and possibly up to 80ºC for at least a day); however, using high temperatures to kill seed may not be feasible due to potential harm to the equipment.

Dedicating equipment for the field test, or thoroughly cleaning the equipment used, is essential to prevent inadvertent seed dispersal and/or co-mingling. Dedicating equipment will generally not be feasible for small operations because of the high cost of the equipment, although the purchase of older, used equipment reduces this problem. Alternatively, planting and harvesting mechanisms that do not require equipment (i.e. done by hand) could be adopted. While this is an option for fairly small field tests, it is too labor intensive to be feasible for larger plantings. Cleaning the equipment (e.g., plot combines) can be very labor intensive. Alternative methods for cleaning equipment are possible, including heating of the equipment to render any remaining seed non-viable, but no option that was both efficient and effective was determined.

Dispersal by Birds and Mammals

The role of birds or mammals in seed dispersal is not clear, but total crop/seed loss can be attributed to birds on occasion. There may be more concern in areas where migrating birds fly over water ways. There is ongoing research on rice seed digestion in ducks, but currently the effect of digestion by birds or mammals on rice seed viability is not known. Deer and feral pigs

have also been known to invade rice fields, and although they might not ingest the rice seed, there is the potential for the rice seed to stick to these animals and be inadvertently dispersed in this way. Rodents can also infest stored seed lots if these are not covered or confined.

Mitigation Measures and Controls

Planes that seed rice fields can contribute to inadvertent seed dispersal. There are also issues with seed contamination in hulling and milling, and disposal of waste products from those processes, because such wastes may contain some viable seed. Unusually harsh localized weather events such as floods, tornados, and hurricanes could occasionally result in the displacement of large numbers of rice and weedy rice seeds from production fields, particularly in the southern states. Mitigation measures that would guard against such events are extremely limited.

Some mitigation measures to prevent seed dispersal and co-mingling should be considered. For example, a staging area may be established in a fallow zone, where any seed transfer, loading, and unloading of equipment, can take place, so that any seed left to germinate in that area can be controlled. Augers should be carefully placed over seed caddies during seed transfer operations. Tarps can also be placed on the ground under equipment during equipment cleaning and seed-transfer processes to catch any seeds that fall to the ground. However, tarps may not be useful since the field will already contain grain remaining after harvest and tarps will need to be efficiently cleaned. GE rice should not be aerially seeded, nor should non-GE rice be planted near PMP or PMI rice to avoid inadvertent seeding of fallow zones that would lead to complications in monitoring for volunteers. Another important measure to avoid co-mingling of seeds is to clearly identify the harvested seed or seed for planting with color-coded bags or another obvious system.

In addition, all stored seed lots should be protected to keep out rodents. It is difficult to keep birds and animals out of rice plots. Air cannons only work until the birds and animals get used to them. Netting can be used on small-scale plots to keep out some birds. Sometimes birds can be dissuaded by not flooding the field in the winter, because other growers will flood in the winter and attract the birds to their plots, and by promptly burning the rice straw remaining on the plots after harvest.

Disposal of rice hulls after hulling is problematic because seed could be mixed in and should be devitalized. But there is a large amount of waste generated after hulling, and incineration, which might be the simplest solution, is not allowed in California. It might be possible to use steam to devitalize seeds left on the rice hulls before disposing of them. It also may be possible to return the hulls to the field and plow them under.

Environmental conditions can contribute to a breakdown in seed confinement. For example, a heavy rain might result in seed dispersal. This is actually a regional concern; Arkansas can have 6-8 in of rainfall in a short period of time, but this rarely happens in California. Shattering and lodging are variety- and temperature-dependent, and this could contribute to inadvertent seed dispersal. Lower temperatures aid shattering. Low shattering varieties, such as Japonica types, and shorter, high stalk strength varieties, should pose less of a problem for seed dispersal.

However, the single most likely mechanism of seed confinement breakdown is human error, such as failure to adequately clean equipment, dropped seed bags, and misidentification or mislabeling of seed. For this reason, thorough and stringent standard operating procedures (SOPs) are extremely critical. Everyone who is involved in the handling of GE plant materials, including technicians, should be involved in the development of quality control processes. It is particularly important to focus on early stages of breeding to prevent inadvertent multiplication of the GE event in unintended breeding material.

Overall Strategies of Confinement

Pollen Confinement

Spatial isolation is currently the only consistently reliable option for pollen confinement. In hybrid rice production, an isolation distance of 700 ft has been shown to be effective to prevent cross contamination. The difference in isolation distances necessary to achieve acceptable levels of purity in hybrid vs. non-hybrid rice seed suggests that viable pollen, even in self-pollinated rice, can move beyond the field. The potential for pollen from PMP or PMI rice to pollinate any other rice may depend more upon the nature and size of the receptor population. For example if the receptor is male sterile or is an Indica type or other line used for hybrid rice production, it is more likely to have a higher rate of outcrossing. If the receptor population is male fertile, and is very large compared to the PMP or PMI donor population, outcrossing in the receptor population should be lower.

Volunteer and Seed Confinement

Concerning control of volunteers, seed dormancy does vary among rice varieties, and the dormancy characteristics of any new PMP or PMI rice event should and can be measured, although lab tests for seed dormancy do not necessarily correlate with dormancy in field conditions. Post-field-test monitoring and control for volunteers is necessary. Concerning seed confinement, there was clearly a consensus among the breakout group that the most likely mechanism contributing to a breakdown of confinement is human error, and the most reliable means of preventing this is to maintain and reinforce stringent SOPs for seed handling and cleaning of equipment involving seeds.

Red Rice Growing Regions

Another main idea emerged for both pollen confinement and volunteer control: PMP or PMI rice grown in regions where red rice is present may require different confinement and control measures than GE rice grown where there is no red rice. In regions with red rice, temporal isolation to prevent pollen-mediated gene flow from PMP or PMI rice to red rice will be difficult to achieve, and volunteers will need to be monitored for a longer period of time (about 5 yrs). Seed loss and dispersal due to flooding in red rice regions, particularly from larger plots, may pose a larger problem. However, it is possible to choose field test areas that have been previously certified as free of red rice by seed certifying agencies. It is also possible to flush the field of red rice prior to planting, but red rice will need to continue to be rogued from the PMP or PMI field test site to prevent cross-pollination.

Plot Scale

A final main idea that emerged in the discussions of all of the topics is that confinement measures for small-scale field tests might not be practical or effective for the larger plot sizes, which would be used for production of PMPs under confined conditions. For example, food/feed crop-free fallow zones surrounding small scale field trials, when scaled up, become cost prohibitive and more difficult to monitor. Another example is the avoidance of inadvertent seed mixing and dispersal by planting and harvesting practices without the use of equipment, which is feasible for a small field test, but is not practical at a larger scale. Human error factors might in fact be greatest for 1-10 acre size plots than for larger plots because the plots might not be big enough to warrant the use of large commercial-sized dedicated equipment, but will instead require more people working to accomplish the same tasks. For larger plots, site security may be more difficult to achieve and sites might be larger targets for sabotage.

ADDITIONAL CONSIDERATIONS

Measures to prevent insect-mediated pollination, such as bagging panicles (as is typically done for controlled pollinations), spraying insecticides at flowering time, netting and cages, border rows or trap crops, and planting wind breaks, such as sugarcane or hemp, may only be practical for small-scale plots. The effectiveness of using different wind breaks to reduce outcrossing in rice is unknown and needs further investigation. Male-sterility is not feasible in a crop such as rice that relies on self-pollination for high yield. However, male-sterile systems are routinely used in the production of hybrid rice seed. Tillage practices were only considered effective to remove red rice where rice x red rice hybridization is a concern.

The importance of site location should also be considered. Site location can be very important for both pollen and seed control, because it requires selecting sites that allow the grower to adhere to confinement protocols. For example, if it should become necessary to apply herbicides to "burn down" PMP or plant made industrial (PMI) rice plots, placement of such plots near residential areas or power lines may make it difficult for aerial application. PMP or PMI rice plots should not be planted near other rice fields that are being aerially seeded, as the seed may scatter into the PMP or PMI plot or the fallow zone surrounding it.

RESEARCH NEEDS

A number of areas for research were identified in the rice breakout group discussion. To understand pollen flow, studies that employ molecular markers might be informative, as well as those based on morphological or herbicide tolerance markers, as have been used typically for these types of studies. Studies on pollen flow from larger-sized plots might also be more informative. For these types of studies using imidazolinone resistance (Clearfield Rice) or molecular markers to study pollen movement from non-GE rice in large scale plantings, as an indication of pollen movement from PMP or PMI rice can be informative. The usefulness of wind breaks to reduce PMP or PMI rice pollen flow is also an area that could benefit from research, but such studies might require the use of male-sterile receptor plants to generate

sufficient numbers of GE progeny to detect differences. Although modeling could be informative to answer some of the questions concerning pollen movement in rice, the biological data necessary to design and/or test the models is largely not available. An effort to identify and generate the data useful for modeling was, therefore, identified as a research need. Experiments to determine the correlation between seed dormancy in lab tests and field tests would be useful. In addition, there was very little information available concerning seed dispersal by animals, in terms of the potential for different types of animals to move seed, the likelihood for this to occur, or the viability of seeds following animal digestion. Any research in this area would be useful. Finally, it was suggested that data gathered from field data reports could be analyzed and made available to continue to improve confinement protocols.

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Confinement During Field Testing of Insect-Pollinated Plant Made Pharmaceutical and Plant Made Industrial Crops Using Safflower as a Model

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ABSTRACT

This section summarizes the discussions of a panel of experts that were charged with discussing confinement measures for pollen and seed dispersal of insect-pollinated crops genetically engineered (GE) to contain pharmaceutical or industrial proteins. Safflower was chosen as the focus of discussion by USDA scientists as an example of an insect-pollinated species that is currently being used for the production of plant made pharmaceuticals (PMP). This report includes a review of the basic biology of the plant, pollen, and seeds, as it relates to issues and measures of gene confinement for field trials of PMP and plant made industrial (PMI) safflower. Confinement measures for pollen and seed dispersal of insect-pollinated crops were discussed by the experts participating in the insect pollinated crops discussion group that focused on safflower as a model. Two kinds of risk were recognized—transgene escape to conventional safflower in agriculture and to its free-living (wild) relatives. The panel generally agreed that physical isolation may be very effective as a confinement measure for crop-to-crop gene flow in safflower, since it is a minor crop grown in limited geographic areas, and achieving an effective isolation distance (>10 km or the distance of bee travel) in the United States and Canada is feasible. However, gene flow to compatible weedy relatives is expected to occur where they grow synchronously. Although effective confinement measures can be developed based on available information, additional research is needed to better understand the impact of various factors that affect confinement measures, the distribution of feral safflower and compatible relatives, and the extent of seed-mediated gene flow. This report summarizes current knowledge of factors contributing to the rate of outcrossing in safflower and methods of limiting gene flow from PMP and PMI field tests.

BACKGROUND

Basic Biology of Safflower

Safflower, *Carthamus tinctorius* L., is a member of the family Compositae (Asteraceae) tribe Cardueae, and subtribe Centaureinae (Garcia-Jacas *et al.* 2002) and of eastern Mediterranean origin. It is predominately cultivated for its seed, which is primarily used as edible and industrial oil and as birdseed. Previously, approximately 25 species of safflower were reported, but 15 annual species have been reported more recently (Li and Mündel 1996; Vilatersana *et al*. 2000a 2005). Traditionally, the crop has mainly been grown for its flowers, used for coloring and flavoring foods, making dyes, and in medicines. The crop originated in southwest Asia (where the genus is native), and moved to India and China. Currently, over 60 countries grow safflower with over half of the world's production occurring in India, mainly for the domestic vegetable oil market. Production in Argentina, Australia, Ethiopia, Kazakhstan, Mexico, and the United States constitutes most of the remainder (Ekin 2005). China also has a significant area planted to

safflower, where the florets are harvested for use in traditional medicines. The U.S. safflower acreage varies widely and the majority of production is in California; the annual acreage ranges between 100,000 and 200,000 acres (USDA 2004). It has been declining since the 1990s and the market for oilseed is in decline. The major demand in the United States of the oleic fatty acid types is for oil, and the linoleic types for paint with minor use as a birdseed.

Safflower is a highly branched herbaceous annual thistle, with a taproot and usually with sharp spines on the leaves and bracts of the flower head (capitulum). The strong central stem has a varying number of upper branches. Each branch usually has one to five flower heads, and the flower head typically has 15 to 20 (but up to 180) florets, each of which can produce a dry fruit (achene) with a single large seed. The seed oil content ranges from 30 to 45%. Flower color is usually yellow or orange, although some varieties have red or white flowers. The taproot can penetrate to 8 to 10 ft if subsoil temperature and moisture permit. As a result, safflower is more tolerant to drought than small grains (Berglund *et al*. 1998).

Outcrossing

Outcrossing between safflower crops has been reported to be anywhere from 0 to 100% (Claassen 1950; Knowles 1980), with an average between 15 and 20% (based on dominant flower-color markers). Genetic markers or characteristics that have been used to measure outcrossing include allozymes, flower color, spiny versus non-spiny, dominant white seed hull versus recessive gray strip, and high linoleic/low oleic versus low linoleic/high oleic fatty acid content. Co-dominant molecular markers such as allozyme variation have been reported for safflower and three of its weedy relatives in North America (McPherson *et al.* 2004). Bees are the main pollinator moving pollen among flowers (florets) and flower heads (Langridge and Goodman 1980). Forty species of native bees were collected on safflower blossoms in Arizona but their numbers were small compared to honey bees (Boch 1961).

Cultivated safflower originated in the Euphrates Basin and from this center of origin expanded to Egypt, Ethiopia, southern Europe and the Far East (McPherson *et al*. 2004). It can potentially hybridize with at least six species of wild *Carthamus* (McPherson *et al*. 2004). Of the four naturalized wild relatives in the New World, only *C. oxyacantha* (or *C. oxyacanthus*) and *C. creticus* (*C. lanatus* subsp. *creticus*, *C. baeticus*), have produced fertile F_1 hybrids when crossed with *C. tinctorius* (McPherson *et al*. 2004). *Carthamus creticus* and *C. oxyacantha* have been reported to occur in several U.S. states (Kartesz 2004) and are listed as noxious weeds, so they must be removed wherever they are found (thus also minimizing the potential for outcrossing).

There are some areas in the United States and Canada where no cultivated or wild *Carthamus* are currently found; hence cultivation in isolation is possible. For example, no wild relatives of safflower occur in Washington State, whereas other species have been reported in Oregon, and especially California (Kartesz 2004; Keil and Turner 1993). Overall, the sexually compatible weedy wild *Carthamus* are quite rare and their presence can be verified in each county because they are noxious weeds. One potential risk that needs to be addressed in transgenic safflower research is the possibly detrimental consequences of outcrosses with wild *Carthamus* species, or with volunteer safflower from the previous year in a subsequent year of field testing. This will

depend on the trait(s) and their potential impact on fitness of the hybrids. Properties of the hybrid should be evaluated for traits related to increased weediness in trials conducted in environments where other compatible *Carthamus* species can occur.

Dispersal/Pollination Mechanisms

Safflower is largely self-pollinated, but it is also considered an entomophilous crop because bees are a pollen vector, whereas wind is not known to be a significant dispersal agent (Knowles 1980; cf. Langridge and Goodman 1980). Morphological characteristics and the behavior of the floret in pollen presentation (Knowles 1980, Carapetian 1994) help to explain the apparent lack of significant wind dispersal, perhaps aided by the pollen grain's moderately large size, with a mean diameter of 53-56 μm (Carapetian 1994). Most pollen movement has been thought to occur within 2 m of the source (workshop discussion comment).

Of the insect pollinators, safflower is mostly pollinated by native bees since commercial honey bee colonies are not typically used. Most honey bee colonies are managed, as there are very few feral populations left in the United States due to invertebrate pests (e.g., varroa mites and wax moths) and disease (e.g., foul brood and chalk brood). However, honey bees are the most important contributors to long-distance pollen movement. The average foraging radius of honey bees from the colony is only a few hundred meters in agricultural areas and they typically do not move beyond 1.6 km (Winston 1987). However, foragers may fly up to 10 km and cover a 100 km^2 area around the hive (Seeley 1995), and there is evidence of honey bees flying several kilometers between apiaries and to safflower fields (Gary *et al.*, 1977). Although honey bees probably do not carry large amounts of safflower pollen, there will likely be some pollen movement between plants as the bee forages (e.g., Langridge and Goodman 1980). Moreover, if pollen moves bee to bee within hives and the recipient bee then carries it to the field, as may be the case in canola (Ramsay *et al.,* 1999), then honey bees may cause low-level gene escape depending on how long the pollen remains viable.

Native bees tend to fly shorter distances than honey bees. Although bumble bees typically forage close to their nests, they may travel 5 km from the nest and have been recorded to move up to 20 km (http://www.bumblebee.org/foraging.htm). The composition of bee populations (e.g., abundance and diversity) depends on the region. Further research is needed to determine which bee species visit safflowers, how many flower heads are visited in a field, and how much pollen is moved among flower heads and among plants by bees (e.g., Langridge and Goodman 1980). Additional information is also needed regarding the effect of floret morphology and color on bee abundance, and activity as well as bee preference for safflower, which depends on the nectar production. Weather conditions and time of the year also impact bee pollination levels.

Whether other organisms that visit safflower, such as lepidopterans, coleopterans and other insects also act as pollen dispersers or pollinators should be investigated. There is a need to enumerate other insects that visit/feed on safflower. Safflower seed is used as birdseed, and is subject to seed predation prior to harvest and following harvest. The impact of pre-harvest seed usage by birds and resulting dissemination of seed should be explored. Additionally, gene flow caused by birds and small mammals following harvest has not been assessed.

Pollen Competition and Viability
Issues related to pollen viability include how far pollen can travel, and how long it will stay viable on the pollinator. Bees tend to prefer foraging on viable pollen. Pollen viability is influenced by environmental factors such as relative humidity and temperature, and can vary among cultivars. Safflower is typically grown in dry conditions, where pollen is expected to desiccate rapidly. The viability of pollen is variable between safflower varieties and probably is very short, lasting less than 24 hours and perhaps into the following day (Knowles 1980). Varietal differences could be controlled through breeding programs. Production practices, such as plant density or row spacing, may also impact pollen viability and flow.

DISCUSSION SUMMARY

Pollen Confinement Measures

Geographic Isolation
Confinement may be best achieved by spatial isolation since there is not much safflower grown in the United States or Canada. Spatial confinement can be achieved by coordinating the cultivation with those who grow routinely in an area. The isolation distance possible is usually tens of kilometers. Spatial separation is used on a routine basis in growing GE crops, and the critical control point is distance between fields. Detailed information on areas where safflower (cultivated and wild relatives) grow, varieties, and production practices need to be assembled in order to identify areas where effective spatial separation can be ensured.

Temporal Confinement
Temporal isolation is also possible but may be difficult to adopt since safflower growth is indeterminate and pollen can be shed over a period of 10 to 45 days. Temporal isolation can be effective in other insect-pollinated crops such as sunflower, where pollen is only released over 7 to 10 days. The potential benefits of using a barrier crop to minimize safflower pollen flow remain to be explored. For example, non-transgenic safflower may be effective to dilute the transgenic pollen load in the environment.

Bioconfinement
Development of male-sterile lines is in progress; however, pollinators may avoid male-sterile plants and the utility of male sterility is unproven (e.g., Anjani 2005). Use of genetic approaches to achieve gene flow confinement is constrained due to the limitations on number of cultivars that can be transformed and the rate of transformation. Use of chemicals, such as repellants, may be possible on a small scale and insecticides may be an option to control pollinators.

Mitigation Measures
Mitigation measures that need to be taken depend on the crop biology, and as such, will be crop specific. Knowledge about the modes of pollination and potential pollinators is essential in developing effective mitigation measures. Adoption of fail-safe measures and continued

monitoring will minimize and mitigate risks. A "worst adverse-effect scenario" and an appropriate response plan should be prepared in advance.

Modeling Pollen Movement
There is limited information available on modeling pollen movement in insect-pollinated crops, although some research is underway (Creswell *et al.* 2005). More information is available for wind-pollinated crops, but crop generalizations are difficult because wind and insect pollination are so different. There are no models on honey bees, wasps, or other insects and their relation to weather. Evaluating the various pollinators and their behavior will help in defining certain parameters. There are ways to make some estimation. However, the input parameters have to be chosen first. It would take a complex model to account for environmental and other site-specific differences.

A recent theory, known as the portion-dilution model (PDM), has made it possible to predict pollinator-mediated gene dispersal (Cresswell *et al.* 2002; Cresswell 2003; Cresswell 2005; Cresswell and Osborne 2004). Use of the PDM requires knowledge of pollinator movements, which are in principle directly observable, and their associated pattern of flower-to-flower gene dispersal (or "paternity shadow"). Little information is available on these two factors for safflower.

Feasibility of Confinement
It is possible to mitigate human error through strong and careful oversight. PMP safflower is grown under identity preservation (IP) conditions. Detailed standard operating procedures (SOP) are in place for safflower cultivation since it grown under contract. There is a high level of process control, thus limiting the chances that the processes can go wrong. Reproductive isolation with strong oversight is feasible.

Volunteer Confinement Measures

Volunteers are not common compared to some other crops, because there is apparently no innate seed dormancy, which is a recessive trait in safflower. In addition to a lack of seed dormancy, seed shattering is rare to nonexistent. Volunteer control measures and monitoring protocols should address seed viability issues and how to mitigate volunteers the following year.

Monitoring for Volunteers
The first year of monitoring is critical to the identification of safflower volunteers to prevent their seed set. Effective monitoring and recording during the first year is required. Leaving the area fallow should be considered. However, there is lower probability of finding volunteers than for some other crops due to the lack of dormancy and seed predation. As a result, a suitably effective monitoring approach is needed. For example, if the plot size is large (e.g., 100 acres), plot monitoring by walking is not feasible because of the time requirements to identify all volunteers. Other monitoring options, such as the use of GIS (Geographic Information System) to map volunteers prior to flowering, may have applications in some instances and the information may be useful in monitoring during following years. Another option is to stipulate

that the grower should monitor the plot of interest and immediate perimeter for several years following the original safflower crop.

Volunteer Control Measures

All trials should be inspected during a post-harvest period and growers should have a redundant volunteer control plan. If a volunteer is found, growers should control immediately. A volunteer with flowers would be considered a breach of confinement unless isolation distances were maintained. Limiting the size of the plot is an option to facilitate isolation. For example, current maximum field size for confined field trials in Canada is 1 ha. Use of selective and effective herbicides to control volunteers in a prophylactic way (as a preventive) will reduce the number of volunteers that germinate. Safflower is an annual crop that can be easily pulled so physical removal is possible at young stages. Seedlings are small and often overlooked when monitoring for volunteers, but safflower should be easily seen during the subsequent early vegetative stages of the plant. Destruction of volunteer plants in research trials by autoclaving should be considered when feasible. However, a method that does not require the movement of volunteers from the site is preferred.

The relative effectiveness of each control measure under different conditions can not be estimated presently and is more applicable for crops with more of a knowledge base. Scale of plot is likely to influence the ability to identify and control volunteers. Safflower is very prone to germination immediately following harvest, so rainfall, flooding, or sprinkler irrigation will induce germination. In areas with dry weather (e.g., Alberta), more diligent monitoring is needed. Ground left fallow and sprayed with non-selective herbicides to control weeds (referred to as chemical fallow) is an option on a large field scale which would enable volunteers to be more easily identified prior to flowering. A non-food, non-feed crop that could be mowed or incorporated in the soil (e.g., a green manure crop) should be considered as an option to fallow. Food crops are not grown following a PMP-crop field trial for a defined time period during monitoring to ensure no commingling with other crops. The breakdown of volunteer confinement is a result of a process control breakdown. Effective oversight to ensure a strong and failsafe process would mitigate this risk.

Feasibility of Volunteer Control Measures

Trials are currently underway to determine the efficacy of common weed control options in crops that follow PMP safflower (Linda Hall, personal communication to editor). The seed bank dynamics of safflower have not yet been quantified and there may be a need to monitor for more than 1yr. It is not known how soon a non-transformed safflower could be grown in a field that had PMP safflower. More research is needed in this area.

Modeling of Volunteer Emergence

Safflower is a minor crop and not much is known about the dynamics of volunteer populations. Trials are underway to gather more information. However, currently there are not enough data to build a model. Therefore, current risk assessment should be based on practicality, experience, and common sense.

Seed Confinement Measures

Seed is subject to disease-mediated drop of the capitulum and short-distance dispersal by small mammals. The amount of long-distance dispersal has not been determined. Germination of dispersed seeds is affected by time of entry into the soil, soil moisture, depth of burial, and temperature. Safflower seed is more sensitive to light than some other crops, but not much is known to what degree. Seed bank dynamics of safflower may be more affected by seed predation than by dormancy. Some relatives of *C. tinctorius* have strong seed dormancy. These seed aspects require further study.

Harvesting

Seed losses from harvesting equipment is common. Absolute separation of PMP seed from the food and feed system is essential. As seed are relatively large in size and easily cleaned from equipment, a dedicated harvester may not be necessary when using a small-plot combine, depending on its design. In general, designated equipment for PMP field trials is preferable, but may not be necessary. Although safflower seed size and color offer an advantage, the use of dedicated equipment should be encouraged. Cleaning protocols for equipment should be in place and all equipment should be thoroughly inspected following cleaning especially before use outside of the PMP crop.

There is an unknown amount of loss of safflower seed during harvest (that ends up on the ground for birds and other wildlife to eat); estimates of 5% loss have been reported (Smith 1996). Rodents and birds may transport seed and provide opportunities for the seed to germinate offsite. Birds graze safflower fields. However it is not known whether birds carry the seed out of the field. Waterfowl, such as ducks or geese, also graze on seeds lying on the ground. Since it is unknown whether safflower seeds will remain viable if ingested by avian species (particularly waterfowl), research should be conducted in this area. However, it is unlikely that viable seed will be carried out of the field by birds because the large seeds are likely to be ground up in their gizzards.

Small mammals feed on safflower seed in southern Alberta and move the seed, collecting it in caches. Safflower seeds are not expected to survive through the digestive tract of mammals. Feed considerations should be taken into account since cake (i.e., compressed meal from the oil extraction process) is used as cattle feed in states such as California. Digestibility or passage of seed in cattle should be investigated. Rapid incorporation into soil and use of moisture to encourage germination should be effective to remove the potential for feeding by birds and other animals. Since spiny varieties are grown, animals are less likely to walk through the standing crop after flowering.

Mitigation Measures

Rapid soil incorporation and/or irrigation to induce germination may be effective. During small-scale trials, chickenwire cages or fine wire mesh netting were found to be effective in keeping out small rodents, such as mice and gophers. Cages should be small, durable and buried under the ground to keep rodents from burrowing under and to prevent cages from being blown away. Burying chickenwire 12 in into the ground and netting at least 6 in (but deeper to prevent

gophers) has been recommended. Window screens such as fiberglass should be avoided because of frequent tears and holes made by birds. Caging, netting, or screening should not occur until flowering to allow for proper plant growth. Seed quality may be reduced if plants are screened with bags or cloth. Since it is not feasible to cage large plots, they should be located in an area free of other *Carthamus*.

M*echanisms of Confinement Breakdown*
Rodents chewing through mesh can result in confinement breakdown. Use of baits around the outside, such as safflower seed around the safflower plots, may be effective if animals are not abundant. Another possible mechanism of breakdown is cages being knocked over under heavy winds/storms. Wooden cages with screen buried into the soil may be effective. Chickenwire buried 12 in deep is effective in keeping animals away. Employee dishonesty, carelessness, negligence, vandalism, and sabotage can contribute to the breakdown of confinement measures. Use of RFID (Radio Frequency Identification) chips with seed lots could help segregation and handling and use of motion detectors should be considered.

Use of color-coded bins for storage would minimize accidental mix up. Seed color could be introduced as a marker for PMP safflower, as markers can be bred into new varieties. However, the limited number of known markers and the additional breeding required can become a constraint.

Animals, such as wild boars and wild pigs (e.g., javelinas), may pose a threat to the integrity of confinement measures in certain geographies. Wild pigs are hard to keep out, are very destructive and will eat anything. They possess a simple digestive system and in general, a lot of seed they eat goes through their system, especially if there is a hard seed coat.

Feasibility of Mitigation Measures
Mitigation measures are scale-specific, and for larger plots where screening is not feasible, a fallow area that is treated with glyphosate should be studied to determine if this is an effective option. For small-scale plots, using a wooden frame and covering it with 12 mesh bridal veil may be effective. Care should be taken to avoid condensation, which may be conducive to diseases such as *Sclerotinia* and *Alternaria.* Human error may occur during post-harvest activities, such as labeling, handling, and storage of various materials at one place. Post-harvest confinement measures include use of appropriate containers with lids to avoid admixture during storage and transportation. Labeling, tracking, and record-keeping, along the chain of custody of material, are absolutely critical.

Main Ideas that Emerge Regarding Confinement Control of Seeds and Volunteer Plants of the Crop of Interest
Redundancy (using more than one method), as advocated in the U.S. National Research Council's report "Biological Confinement of Genetically Engineered Organisms" (NRC 2004), should be incorporated. For example, use of herbicides for volunteer control should be combined with physical monitoring and roguing (destroying individual plants). Growing a grass-type crop in subsequent years could make it easy to find and identify volunteers.

Overall Strategies of Confinement

Effectiveness of the Confinement Measures
Since safflower production is limited in the New World, spatial separation between crops can be effective in achieving pollen-flow confinement. The effectiveness of the confinement measures can be evaluated by using genetic markers but this approach is quite slow as there is a need to characterize a large number of seeds. Currently, glufosinate resistance is being used to measure the effectiveness of spatial isolation. However, the methodology available is cumbersome and increased speed and efficiency is needed. The use of microsatellite as a codominant marker, quantitative polymerase chain reaction (PCR), and enzyme-linked immunosorbent assay (ELISA) test strips that can detect 1 in 2000 seeds for glufosinate resistance, are other possible options for detecting gene movement that are currently being explored (Linda Hall, personal communication to editor). There are currently limited published data on outcrossing studies (Claassen 1950; Knowles 1980).

Critical Points for Pollen, Vegetation, and Seed Confinement
Fault analysis needs to be done. For pollen, critical points include: physical isolation from commercial safflower and sexually compatible relatives (including feral safflower); pollinator activity at the time of flowering of cultivated safflower; flowering synchrony between cultivated safflower and its sexually compatible relatives; choice of bee species as pollinator; and pollen viability. In the case of insect-pollinated crops, more information is needed for isolation distances, especially in the case of small plots versus large-scale fields. Conceivably, measures taken to confine pollen could compromise measures to confine seeds. Too much isolation may force the resident animals, such as birds, to feed on safflower seed. For example, birds may concentrate their feeding on isolated safflower fields and thereby become a source of seed dispersal. However, there is little information that such a compromise would actually occur.

An additional contribution could be made to pollen confinement by considering pollinator activity at the time of flowering, choice of pollinator bees, and pollen viability, although the feasibility of doing this is unclear. Coordination with neighbors (growers), satellite imaging to evaluate the neighboring vegetation, and male sterility using biotechnology or GURTS (Genetic Use Restriction Technologies) may improve the effectiveness of confinement measures.

Possible effects of location of the field should be taken into account. Clustering of small plots should be encouraged to limit outward gene flow. Vegetative reproduction is not a concern for safflower. The extent of seed dispersal by birds and mammals is not known. Volunteer control, reducing possible seed dormancy by encouraging germination, and chain of custody control of material during post-harvest handling (e.g., storage, transportation) would help ensure seed confinement. Mitigating seed banks from the start of the trial (e.g., scarifying the seed as in the case of alfalfa) would be effective. Use of border rows may be useful in some cases and should be considered.

Strengths and Weaknesses of Potential Combinations of Confinement Measures
Detailed record-keeping is needed to verify records and adherence to protocols. Adherence to permit conditions and having SOPs, and personnel trained in SOPs, are also needed. Knowledge

gaps about the crop biology, distribution of any weedy relatives, pollinators and pollen biology are weaknesses. Appraisal of the possibility of gene escape is needed. Protocols should be reviewed prior to approval. Weather patterns can influence pollen flow. The size of the plot can impact the effectiveness of the confinement measure. Cages may be an effective confinement measure in small plots. Management practices such as tillage, irrigation, and weed control differ from one region to another and should be taken into consideration while devising confinement measures.

Redundancy of Confinement Protocols
A critical control point analysis should be conducted to identify potential for loss of confinement. Redundancy in confinement should be ensured as critical control points are identified. Tests of redundancy need to be done on individual and stacked confinement methods.

Redundancy should be built into monitoring for volunteers, controlling volunteers, and post-harvest handling. Redundancy should be in place for activities such as packaging (e.g., double-bagging, labeling), continued training of personnel in case of turnover, and cross-training of personnel in various SOPs.

Modeling of Gene Dispersal and Confinement
Safflower is a minor crop and not much attention has been given to models. Whether models of other crops are applicable to safflower should be explored. Standardized methods are available for production of foundation seed and the methods may be applicable. "GENESYS" for modeling pollen and volunteers in canola (Colbach *et al.* 2001) may be useful for safflower. There are no models available at this time for cultivated safflower. Association of Official Seed Certifying Agencies (AOSCA) standards to produce varieties may be useful in developing a model for safflower.

RESEARCH NEEDS

Features of Experimental Design
In order to design experiments to test the confinement of PMP safflower, certain considerations should be made. Acceptable levels of confinement should first be chosen and experiments designed to achieve that goal. Volunteer control *in situ* would be useful. Sampling strategies need to be based on the desired level of confinement and the scale of trials/production fields.

Pollen Confinement Measures
Detailed information on parameters, such as pollen movement and bee behavior on cultivated safflower and its wild relatives, is lacking but research is underway to better define and describe these (Linda Hall 2004: Linda Hall personal communication with editor; Cresswell 2005). There are many gaps in our understanding of safflower biology, such as gene flow, dynamics of pollinators, floral biology/pollen viability, and pollen/seed movement/dispersal. Lack of quantitative predictions from the available insect models is due to absence of information on pollinators, how much each type of pollinator can move what quantity of pollen, and how far. Little information is available on the role of insects in enhancing seed yield, seed fate in birds

and their role in dispersal, other animals involved and seed fate in those animals, detailed location of compatible relatives (McPherson *et al.* 2004; Kartesz 2004), fitness of weedy relatives and extent of outcrossing, fitness of fertile hybrids, and longevity/persistence of seeds of relatives or hybrids in soil.

Effect of Pollinators on Confinement
The role of wasps and other invertebrates in safflower pollination needs to be examined. Wasps are considered generalists that do not need to go back to a hive and may move a small amount of pollen. Some other insect-pollinated crops (e.g., alfalfa, which has two key pollinators—alfalfa cutter bees and honey bees) may also provide information that can be used to establish isolation distances for safflower.

There is a need to investigate the floral and pollination biology of species that are relatives of cultivated safflower by monitoring them to determine the type and abundance of different floral visitors and determining which visitors are pollinators. In the case of safflower, the crop is grown under contract. As such, information on what is being grown around safflower is not difficult to obtain. However, because volunteers also contribute to pollen flow, fields from previous years must be included in the analysis.

Seed Confinement Measures
The role of birds in seed dispersal, animal behavior, animal digestibility studies, seed and pollen viability, insect biology, and behavior of pollinators also need to be explored.

Crop Biology Research
Basic data about the biology and agronomy of safflower need to be improved in order to design confined production of PMP safflower crops. This should include outcrossing frequency and distance to the crop, feral safflower, and weedy relatives, as well as volunteer control in subsequent years, seed bank dynamics of PMP safflower, and potential for commingling (contamination) of food and feed with PMP safflower. All experiments need to be conducted over multiple years in the environment(s) intended for production. These experiments should be designed with appropriate statistical designs to ensure appropriate detection consistent with thresholds set by regulators for the specific trait.

Effect of Scale on Confinement
The effects of environmental conditions on confinement do not appear to vary since production practices tend to be similar within a geographic area. Factors such as scale of the plot = impacting the confinement measures need to be investigated. Tenting or covering of the crop is used on small-scale research plots but these are not feasible for larger fields. Research is needed to evaluate the effect of plot size for different crops, such as safflower (and sunflower). It is not known if the scale of the plot would affect pollen flow in the case of insect-pollinated crops and whether there are differences in pollen flow and pollinator activity between 1 versus 100 ha, and whether isolation distances would have to be different depending on the scale. It is probably easier to monitor the confinement of pollen-mediated gene flow in one large site than many small ones. The border area around many small sites is greater than the area around one large

plot, thus increasing the probability of outcrossing beyond the field trial and potentially resulting in an increase in the amount of monitoring needed.

ADDITIONAL CONSIDERATIONS

Due to limited production of safflower in North America, distance can be effectively used for achieving isolation. Knowledge is needed in the areas of weedy relatives, pollinators, outcrossing rates, and distances to receptive plants. Little is known about the biological confinement of safflower. Rigorous oversight of the production processes must be maintained.

Redundancy in confinement measures for mitigation that should be considered include combining physical and biological confinement methods. However, more information is needed on the basic biology of safflower. Production of nectarless or male-sterile varieties may be an option for introducing biological confinement. Availability of biological methods could help make such redundancy of confinement measures a possibility.

Human error is the most likely cause of breakdown of crop confinement measures. Natural elements and lack of mitigation measures also contribute to breakdown. There may not be a need for combining methods where physical separation is feasible—sometimes extreme physical (geographic) isolation of the safflower crop is considered sufficient.

It is expected that human error or non-compliance, and the biology of the crop (e.g., insect pollination), contribute to breakdown of the confinement measures. Volunteers and the resulting flowering due to unexpected changes in weather may lead to pollen dispersal and seed set, and subsequent seed dispersal. Other possible scenarios for the breakdown include vandalism or intrusion.

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WORKSHOP PAPERS

Introduction to and Principles of Confinement

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Confinement is essential to USDA's Animal and Plant Health Inspection Services' (APHIS) regulatory approach to allowing the safe introduction into the environment of genetically engineered (GE) organisms during the research and development stage. APHIS' Biotechnology Regulatory Services (BRS) uses confinement measures for plants genetically-engineered to produce pharmaceutical and/or industrial compounds that have different pollination mechanisms and outcrossing frequencies. Examples of such measures will be presented to demonstrate how applicants and APHIS are applying confinement measures, and how the application of multiple or redundant measures has increased, along with the scale of such releases.

The origins of and history of application of this concept as it applies to field testing of GE crops can be found in: 1. its early introduction by the National Research Council in 1989; 2. its adoption into guidelines by the USDA Agricultural Biotechnology Research Advisory Committee in 1991; 3. the exploration of crop-specific confinement measures through workshops, and guidance on the use of such measures for different crops and traits as communicated in APHIS User's Guides; 4. Federal Register notices; and 5. on the Biotechnology Regulatory Service (BRS) website.

Early History of Confinement Concepts as Applied to Field Tests:
When APHIS' regulations under 7 CFR Part 340 were promulgated in 1987, the Agency required permits for the environmental release of GE plants that met the definition of a regulated article. A regulated article is an organism (e.g., plant, microorganism, arthropod or animal) that has been produced or modified using genetic engineering. Environmental release is recognized as the use outside the constraints of physical confinement/containment that are found in a laboratory, contained greenhouse, or other contained structure. Standard permit conditions to prevent the dissemination and establishment of regulated articles stipulate: 1. that the GE organism is segregated from organisms not specified in the permit; 2. it is maintained only in specified areas; 3. the introduction is subject to application of measures necessary to prevent accidental or unauthorized release (determined case-by-case); 4. mandatory reporting of accidental or unauthorized releases; and 5. authority to apply remedial measures to prevent spread in such cases.

In 1989, the National Academy of Sciences discussed the concept of confinement as practiced by plant breeders to ensure purity of breeding lines and to prevent spread of plant pathogens from experimental plots. Proven and routinely applied confinement methods were considered as applicable to field introductions of GE plants as they were to plants developed through classical breeding. Isolation requirements and other methods for production of genetically pure seed

established by the Association of Official Seed Certifying Agencies (AOSCA) were cited as appropriate, and it was acknowledged that these "allow for acceptable levels of contamination" (NAS 1989). APHIS' Users Guide, published in 1989, states that the AOSCA standards of seed purity are a basis for designing confinement conditions. Other proven and routinely applied confinement methods mentioned by the NAS and APHIS include biological, chemical, physical, geographical, environmental, and temporal controls, as well as limitation of the size of the field plot.

In 1991, the Agricultural Biotechnology Research Advisory Committee, in their Guidelines for Research Involving Planned Introductions into the Environment of Genetically Modified Organisms, described confinement as "that which restrains or limits the spread or survival or organisms and their products in research involving planned introductions of organisms into the environment." Their guidelines emphasized that confinement measures should correspond to the level of safety concern, taking into account: 1) the organisms pest/pathogen status; 2) its ecological relationships and establishment potential; 3) the effect of the genetic modification on safety; 4) the potential for inducing genetic change in natural or managed populations; 5) the potential for monitoring and control; 6) characteristics of the accessible environment; and 7) research objectives. Combinations of confinement measures, referred to as redundancy, should be used to achieve the desired level of safety in some cases, and monitoring can inform whether confinement measures are effective. The Advisory Committee's guidelines also stresses using the AOSCA standard isolation distance for the foundation class of rapeseed production to keep cross pollination from transgenic canola below 0.05% (1/2000 seed) for traits with a low safety concern, even in an area where wild relatives or other *Brassica* crops exist.

From 1990 to 1993, APHIS sponsored workshops to gather input on safeguards for field testing of corn, tomato, wheat, rice, canola, potato, and sorghum (see proceedings at http://www.aphis.usda.gov/brs/technical_resources.html). The primary focus of the workshops were to determine the potential for, and consequences of, pollen-mediated gene flow and expression in the crop and wild relatives and recommend specific safeguards to prevent or mitigate such consequences, if appropriate. There was little focus on seed dispersal or monitoring or managing volunteers. By 1993, about 400 field tests were safely performed under APHIS permits based on input, in part, from these workshops.

Confinement of Plants Under Notification:
In 1993, APHIS revised their regulations to allow a more streamlined notification process for introductions of six types of GE crops (corn, tomato, potato, tobacco, soybeans, and cotton) that meet specific criteria and performance standards to reduce risk. The rule was amended in May 1997 to cover all plants that are not noxious weeds or considered weeds in the area of release. Some traits or genes that raise risks are not eligible, such as, pharmaceuticals, genes of unknown function, products that harm non-target organisms, genes that cause disease in plants, animals, or humans, or genes from human or animal viruses, and in some cases from plant viruses. Under notification, as opposed to permits, APHIS does not require applicants to provide details on confinement measures, but they must certify that they will meet the performance standards.

APHIS' Notification Users Guide (http://www.aphis.usda.gov/brs/notification.html) provides

guidance on meeting the performance standards for the first six crops eligible for notification, and general guidance for all crops. APHIS addresses aspects of confinement at all stages of research and development, including the lab (e.g., eliminating viable vector agents): shipping and maintenance at facilities to prevent release of viable material; greenhouse operations; and field release, including, planting, harvesting, post harvest monitoring, and management. To prevent inadvertent mixing of regulated plants with plants not part of the release, developers should maintain appropriate alley ways to allow movement of farm implements and they should be cleaned before using with non-regulated plants. Developers must use a system to identify transgenic material while it is in use until it is appropriately devitalized.

The notification performance standards stipulate that regulated plants and their offspring can not persist in the environment. The guidance suggests that, in some cases, it may be appropriate or desirable to terminate the experiment prior to flowering, inhibit or remove flowers, or use male steriles. If pollinating flowers are present, developers should consider using bagging, wind breaks, border rows, temporal differences, and/or isolation distances.

APHIS clearly indicated in the notification rule preamble that flowering male-fertile regulated plants "must be separated from any foundation or breeder seed production of nonregulated plant material of the same species by at least the isolation distances for foundation seed production given in AMS regulations at 7 CFR 201.76", which were derived from the AOSCA standards. The User's Guide indicates that this standard applies to all plants tested under notification for which standards have been established, and that this can be a good starting point for designing confinement features if one takes into consideration the percentages of outcrossing assumed in those isolation distances. Methods that have been shown to give genetic isolation equal to certified seed standards are also acceptable to APHIS for field tests under notification.

However, for plants with sexually compatible wild or weedy relatives, more stringent requirements may be necessary, e.g. surveying surrounding area for the presence of these plants or selecting a site where these species don't exist. If flowering occurs, then applicants should consider the proximity to sexually compatible species, flowering cycles, the extent of outcrossing, and pollen and seed dispersal by biological or physical mechanisms.

The performance standards also stipulate that viable material is removed (e.g., through harvesting, herbicide treatment, discing, mulching, or burying) and volunteers are monitored and managed to prevent persistence. APHIS suggests that in subsequent growing seasons the site not be planted back to the same or compatible crop, and appropriate herbicides be used so that volunteer transgenic plants arising from seeds (or vegetative tissue) can be monitored and destroyed prior to flowering, for as long as seed could remain dormant in the field.

Confinement of Plants Engineered for Pharmaceutical or Industrial Use:
Plants genetically engineered to express proteins intended for pharmaceutical use do not meet notification criteria and introduction can only be done under permit. APHIS has indicated that they are unlikely to grant nonregulated status for most plant made pharmaceuticals (PMPs). FDA has indicated that the presence of pharmaceutical proteins in food could cause it to be considered adulterated. Any PMPs also intended for food or feed use must be approved by FDA

or APHIS' Center for Veterinary Biologics (CVB) and would require prior devitalization.

In 2003, APHIS strengthened its regulations for pharmaceuticals and industrials by requiring permits instead of notifications for the field release of these crops (Federal Register vol. 68 no. 151). This action helped address the increasing concern over scale-up of plant made industrial proteins (PMIs).

Information on confinement measures to be submitted in permits is stipulated in 7 CFR 340.4 and in the APHIS User's Guide. APHIS also provides additional guidance for PMP and PMI permits in Federal Register Notices, letters to the applicants, and on the BRS website (http://www.aphis.usda.gov/brs/pharmaceutical.html). APHIS requires the following information in permit applications: 1. the final and intermediate destinations of the plant and its products; 2. the location and specific design of the field test site and conditions of the release; 3. a description of the biological factors and measures that will be taken for physical and reproductive isolation of the plant and its progeny from planting through harvest; 4. how the site will be secured, monitored, and inspected; 5. plans for the termination, destruction, and disposal; and 6. post-harvesting monitoring and subsequent land use to ensure the plants, their progeny, or their active products do not persist or pose a risk in the environment. The 2002 joint FDA/USDA Draft Guidance to Industry document also provides guidance relevant to confinement for commercial production of PMPs (http://www.fda.gov/cber/gdlns/bioplant.pdf).

In March 2003, APHIS announced in a Federal Register Notice (Vol. 68 no. 46), that it would be modifying permit conditions for field tests of PMPs and PMIs for 2003 and subsequent years. The Notice also announced APHIS' intent to increase inspection, compliance, and auditing activities. The supplemental permit conditions specify the following:

- ***Planters and harvesters must be dedicated to use in the permitted test sites for the duration of the test.*** Such equipment can not be used back and forth between permitted PMP sites and non-permitted sites in a given growing season. APHIS must be notified and will inspect equipment before it is moved between permitted test sites and before it can be returned to general use to ensure it is sufficiently cleaned of seed or other plant material.
- ***Procedures must be submitted to and approved by APHIS for cleaning planters, harvesters, other types of field equipment such as plows, harrows, and discs, and equipment used to transport or off-load material.*** Cleaning procedures for production of certified or identity-preserved seeds are a good guide. Some equipment can be modified to prevent seed from being retained. Protocols must be approved by APHIS for seed cleaning and drying to minimize seed loss and spillage. APHIS has provided applicants the criteria we are using to approve cleaning protocols.
- ***Storage facilities (secured buildings, bins, or clearly delineated or fenced off areas, posted as restricted to authorized personnel) must be dedicated for the storage of equipment used to handle the seeds or other regulated articles for the duration of the field test.*** The facilities must also be cleaned according to protocols approved by APHIS, and inspected prior to return to general use.

- ***A fallow zone of at least 50 ft. is required around the perimeter of the rows of transgenic plants and any border rows.*** This allows sufficient room for equipment to access and turn around within the field without entering or commingling with other adjacent crops that would be harvested for food or feed.
- ***Production of food or feed crops during the subsequent growing season or period required for monitoring for volunteers is restricted in cases where volunteer plants could be harvested with the following crop.*** This doesn't exclude the planting of cover crops that are typically plowed under or treated with herbicide when no longer needed. APHIS has developed criteria for when variances will be granted for this condition. For example, the food/feed crop must be easy to distinguish from transgenic volunteers, be managed so that transgenic volunteers can be destroyed, and not commingled with the harvested crop.
- ***Specific options for confinement of corn field trials***. Corn is specifically addressed because, compared to other crops used as platforms for PMPs and PMIs, more companies were using corn, and there is larger acreages of corn in field trials and in commercial production, In addition, corn has a higher outcrossing rate, and therefore a greater potential for adulteration of food and feed. APHIS is also phasing out the use of border rows as a means to dilute out/compete with the transgenic pollen because of concerns about the required size of border rows, disposal of seed, and management of additional volunteer seed that could come from these rows in subsequent seasons.

APHIS decides confinement conditions for other crops on a case-by-case basis. The applicant proposes measures that APHIS and the State regulatory agency of the field trial release site review for adequacy. Data should be cited or provided to support the adequacy of the confinement measure with sufficient margin for error. Total acreage planted in APHIS approved PMPs or PMIs is not that high—134 acres in 2002, 25 acres in 2003, and <44 acres in 2004. Platform crops in 2004 include wind-pollinated corn, self-pollinating barley and rice, and insect-pollinated tobacco and safflower. Confinement conditions that have been approved for some of these permits for the different crops, and points considered are as follows:

CORN: Reproductive confinement options specified in the March 2003 FR notice include the following:

- Open pollinated PMP corn requires an isolation distance of at least one mile from any other corn. This is 8 times greater than the isolation distance of 660 ft required by AOSCA for production of foundation seed.
- Controlled pollination of PMP corn (i.e. by bagging, or by the use of male sterile plants combined with detasseling) requires at least ½ mile separation from other corn, and corn located beyond ½ mile and up to 1 mile must be planted at least 28 days before or 28 days after the PMP corn. Applicants should check every 2 days to ensure bags are secure or tassels are removed. This ensures there is no overlap in flowering. The Pew Initiative on Food and Biotechnology, the FDA, and the USDA, Cooperative State Research, Education and Extension Service held a workshop in July 2002 in Washington, D.C., on PMPs and PMIs. Data presented by Phil Eppard

of Monsanto Protein Technologies at that meeting (http://pewagbiotech.org/events/0717/eppard.php) showed that increasing temporal separation reduced the distance required to achieve genetic isolation, and that outcrossing was undetectable (less than1 kernel in <500,000) when temporal separation of 14 days was combined with an isolation distance of 750 meters (2460 ft. or 0.47 miles). (This data is now published online by Halsey *et al.*, Sept. 23, 2005 at http://crop.scijournals.org/cgi/reprint/45/6/2172.)

- Applicants can use border rows of corn as supplemental isolation, but not to reduce the isolation distance. APHIS generally requires monitoring for volunteers for 1 yr from harvest for the plot containing the transgenic corn, any additional border rows and the 50 ft fallow zone.

RICE: For reproductive confinement, in 2004, APHIS approved an isolation distance of at least 200 ft between rice expressing human lysozyme and lactoferrin and any other rice not included under the permit. This isolation distance was requested by the applicant and is 20 times the distance AOSCA suggests for foundation seed. It also includes a 50 ft fallow zone free of food or feed crops. Previously, temporal isolation (14 days) was used with a 100 ft isolation distance. The use of male-sterility is impractical for this crop because of its high degree of selfing. In addition to the isolation distance, 10 ft borders of non-transgenic rice were approved to act as a barrier to invading wildlife. Typically, transgenic rice seed is drilled or transplants are placed into flooded fields. An irrigation levee is required to prevent rice plants from being carried in irrigation water to commercial rice fields. Weed control and 1 year post-harvest monitoring period were required to detect and destroy volunteers of the transgenic Japonica type rice, which was planted in an area free of red rice.

SAFFLOWER: Safflower, though primarily self-pollinating, has < 20% outcrossing mediated by pollinators. It has no apparent seed dormancy and few wild relatives in the U.S. Permit conditions have specified 2 mile isolation from all other safflower and a 2 year monitoring period for volunteers within the plot and 50 ft fallow zone.

Future Challenges:
Questions regarding confinement remain, such as how can the value of alternative or redundant genetic or biological control methods be safely, accurately, and appropriately evaluated and applied for different crops. Many comments to the March 2003 FR notice about confinement conditions suggested that APHIS encourage or require the use of genetic or other biological methods to mitigate gene flow. Recommended methods included cytoplasmic or nuclear male sterility, chloroplast transformation to limit gene flow through pollen, cleistogamy (self fertilization without the flower opening and releasing pollen), apomixes, and other flowering characteristics that alter time or duration of flowering, or length of pollen viability. These methods could, in principle or practice, be achieved using conventionally bred varieties or genetic engineering.

Much of the gene flow data that exists are derived from small scale trials. Therefore, information is needed to extrapolate results from these models to larger scale releases. Furthermore, data generated on gene flow between commercial crops may not be accurate for

predicting gene flow to feral populations or vice-versa. In order to have models that could be used to predict gene flow, there is a need to better understand the key contextual factors, such as weather, pollinator behavior, local topography, or cropping patterns, that most influence gene flow among particular species. It is also critical to have a better understanding of the importance of scale in experimental design.

Models that can take into account the effects of all of the confinement measures—from planting to harvesting—are desired because for self-pollinating crops, seed loss and movement might be more important indicators for gene-flow outside the test site than is pollen-mediated gene flow. Hopefully, the information, resources, and discussion at this meeting will facilitate the development of such models.

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Setting of AOSCA Standards

Allan B. Simons
President, Association of Official Seed Certifying Agencies

History of AOSCA

The Association of Official Seed Certifying Agencies (AOSCA) was founded as the International Crop Improvement Association (ICIA) in 1919 as an organization dedicated to dealing with problems relating to seed multiplication. The international organization was developed when representatives of 13 U.S. states and the Dominion of Canada met in Chicago to formalize its purpose and objectives.

The overriding problem faced by early plant breeders and their farming customers was "….lack of knowledge and adequate equipment, together with carelessness on the part of seed growers [and] those who used home-grown seed, resulting in rapid varietal mixing," (Hackleman and Scott 1990). Only tiny amounts of breeder seed of new varieties were available to be distributed to many hands, often by U.S. Congressmen to favored supporters, leading to quick dilution and loss of new varieties. Furthermore, new varieties were often renamed and misrepresented without discretion, further obscuring their identity and purity.

The new ICIA's earliest efforts included developing standards for field and laboratory inspection of alfalfa, clovers and cereals, when committees were formed to standardize nomenclature and rules for the inspection of small-seeded forages and cereal grains. Certification standards for these crop kinds were adopted in 1921, followed quickly by standards for soybean (1922), open pollinated corn (1923), and open pollinated sorghum and cotton (1926.)

The fundamental concepts of certification were enumerated during these early years and included the following, (Hackleman and Scott 1990):

- Registration and certification of varieties should be based on lineage;
- The integrity of growers needed to be recognized;
- Properly qualified inspectors should conduct field inspections;
- Verifying trials should be used to establish the identification and usefulness of varieties and strains;
- It is essential to keep proper records to establish and maintain satisfactory pedigree of stocks used in registration and certification;
- [Crop and seed] purity and [seed] germination standards should be established;
- Seed should be sealed to protect both grower and purchaser;
- Weed species should be defined for inclusion within the meaning of noxious weeds as used by ICIA members; and
- A board of review should examine graded seed samples.

The ICIA continued to develop standards and refine procedures during the decades before World War II, striving always to achieve uniformity among its growing list of members. The first U.S.

Federal Seed Act was passed in 1939 to accommodate increasing interstate commerce. The act recognized the seed certification concept in Federal law. It also officially defined the registered and certified classes of seed and fostered the goal of uniformity among states. Furthermore, the regulations developed under the law permitted only officially recognized state agencies to certify seed moving in interstate commerce (Hackleman and Scott 1990).

The ICIA first conducted a comprehensive survey of the certification standards of its 34 members during 1943-1945. All requirements and procedures were reviewed and revised according to a standard outline and distributed as Publication No. 16 in June 1946. This document was widely circulated and studied in North America and Western Europe and was an important resource in the development of hybrid corn certification standards in Europe, as well as the OECD Seed Schemes for seed moving in international trade (Hackleman and Scott 1990).

The ICIA was incorporated as a non-profit charitable organization in Illinois on Nov. 29, 1951. By the late 1960s, the leaders of the ICIA sought to establish the organization's standards as statutory minimums for seed certification in the United States because a number of member agencies did not routinely comply with ICIA standards for varietal purity. This non-uniformity concerned commercial interests as they traded seed across state borders. Also, international entities that engaged in seed multiplication in the United States were unable to count on consistent production criteria being applied to their increases. And, because of their experiences under National Government certification, international entities were confused by the U.S. system of numerous state agencies. The expedient means of creating uniformity in the production of certified seed in the United States was, therefore, to incorporate certain aspects of the certification scheme into Federal law.

Accordingly, ICIA standards for land history, isolation and varietal purity in the field and seed were incorporated in the U.S. Federal Seed Regulations after enabling legislation became effective on Oct. 9, 1969. The American Seed Trade Association, the American Farm Bureau, the National Farmers Union, the National Council of Commercial Plant Breeders, and others supported the legislation. However, before this could happen, the ICIA changed its name to the Association of Official Seed Certifying Agencies in 1968 to comply with U.S. Government policy that prohibited the concept of "international" in promulgating Federal regulations. The new name also was a more accurate representation of the organization's focus after 50 years in existence.

AOSCA in 2004

Since 2004, AOSCA membership has consisted of 44 official state certifying agencies in the U.S., plus official agencies in Canada, Argentina, Australia (Hackleman and Scott 1990), Chile and New Zealand. Representatives of these agencies comprise a board of directors that is responsible for maintaining the standards and procedures of the association.

Approximately 3,750,000 acres are inspected in U.S. certification programs annually, down from as many as five million acres in the 1980s. Small grains (barley, oat, rice, wheat, and triticale) account for about 45% of the acres, while corn, grass, soybean, and cotton share approximately equally in an additional 40%. Canada inspects just over one million acres annually.

AOSCA also operates National Variety Review Boards to provide a uniform method for bringing new varieties of alfalfa and other legumes, grass, small grains, soybean, and sunflower into certification. These boards are comprised largely of plant breeders appointed by the seed trade, the Crop Science Society of America, and USDA's Agricultural Research Service, with the Plant Variety Protection Office holding an *ex officio* appointment.

AOSCA Advisory Committee

AOSCA needed support from stakeholder groups in the effort to amend the Federal Seed Act. Such groups had often been informally consulted during the course of seed certification development. The American Seed Trade Association, in particular, wanted opportunity for input in the development and revision of certification standards and procedures. For that reason, AOSCA established an Advisory Committee of interested parties in 1970 to review and comment on new certification standards and to provide a forum for AOSCA and its stakeholders. The Advisory Committee reports to the board of directors and cannot directly initiate policy or standards changes. The committee meets twice annually and now consists of the following representation, nominated by the designated organization:

- AOSCA (2002) - including Canada, with the immediate past president as chair;
- USDA (AOSCA 2001)- Seed Regulatory and Testing Branch, Plant Variety Protection Office, Animal and Plant Health Inspection Service and Agricultural Research Service;
- American Seed Trade Association (AOSCA 2001);
- Canadian Food Inspection Agency;
- Canadian Seed Institute;
- Canadian Seed Trade Association;
- Experiment Stations (Hackleman and Scott 1990) – U.S. and Canada;
- National Council of Commercial Plant Breeders;
- American Association of Seed Control Officials;
- Association of Official Seed Analysts;
- Society of Commercial Seed Technologists; and
- Foundation Seed Stocks Organizations

AOSCA Conducts Self-evaluation for Compliance with its Standards

In order to qualify as a seed certifying agency under the terms of the Federal Seed Act, an agency must enforce standards and procedures that meet or exceed the standards and procedures specified in Section 201.68 through 201.78 of the regulations promulgated by the act (Federal Seed Act Regulations 2000). The intent of Congress and the understanding with USDA at the passage of the Act, was that AOSCA would be responsible for monitoring its members' compliance with the regulations (Hackleman and Scott 1990). This self-evaluation process now involves the functioning of a Standards Evaluation Committee consisting of a principal evaluator and an alternate in each of four regions in the United States, with the AOSCA executive vice president serving as *ex officio* chair. This committee conducts an annual review of the standards and procedures of the agencies within the respective regions via a questionnaire developed by the American Association of Seed Control Officials. Results of the process are reported to the Secretary of Agriculture via the Seed Regulatory and Marketing Program of the Agricultural

Marketing Service. Deficiencies, when encountered, are addressed by the AOSCA executive committee. AOSCA also embarked on a rotation of on-site evaluations based on auditing principles in 2002 that will ensure a thorough review of documentation and procedures in each agency at intervals of about every four years.

AOSCA's Historical Basis for Certification

Early practitioners of certification had nothing other than physical appearance by which to judge the varietal purity of seed increases. Their primary objectives were to preserve new varieties from admixing and disappearance from the farming scene and to minimize the proliferation of names for popular varieties. Phenotypic evaluation of plants in the field was then, and continues to be, the basis for certification in the AOSCA system, with phenotypic evaluation of seeds as a secondary screen when feasible. Therefore, provision of a detailed description of the plant and seed is required for acceptance in certification programs. Phenotypic evaluation for traits such as disease and insect resistance, while desirable, has always been problematic because of variability in pest pressure and the pest by environment interaction, and is incorporated into just a few crop standards, primarily as a seed quality issue rather than as a varietal purity standard.

The consolidation of farming into relatively few operations and the maturation and evolution of the seed industry in Canada and the United States have contributed to widespread availability of high quality seed today. Consequently, the motives for certifying seed in certain regions of the U.S. now reside largely in meeting import requirements of foreign buyers, in assuring varietal identity of unrestricted public lines, in assisting in protection of intellectual property rights by means such as Title V of the Federal Seed Act, and in collecting licensing royalties. However, growers in other U.S regions, particularly the Western states, continue to view certification as their assurance of varietal and mechanical quality.

Establishing Certification Standards

As noted, the majority of the field and seed standards for certified seed classes that are cited in Part 201.76 of the Federal Seed Act Regulations were developed and adopted, or revised, between 1920 and 1969. Regrettably, few records exist as to the specific criteria or evidence used in their establishment. It can be assumed that because the early proponents of certification and pure seed production were expert crop breeders, agronomists, and extension workers at the land grant colleges, they used the scientific evidence at their disposal. They would have used phenotypic markers to measure extent of outcrossing as guides to establishing isolation distances for the various modes of pollination–wind, insects, selfing, and the combinations. They also would have used observation and large measures of common sense in determining requirements for land history. It is unknown, however, what criteria might have been used to establish maximum levels of varietal impurities in the field. Factors such as cosmetic appearance, economics, and the limitations of machinery in use at the time all probably influenced the final decisions. The declining level of varietal purity standards proceeding from the Foundation to Registered to Certified classes were intended to deal with the physical realities of the seed production process and the relative value of each class as a resource for the next generation.

The modern process of establishing or revising seed certification standards and procedures starts with the AOSCA commodity committee system. Committees for each major crop kind and

combined minor kinds are made up of volunteers coming from agency members and associated stakeholder organizations, and are usually chaired by experienced representatives of member agencies. The appropriate committee undertakes requests to evaluate the sufficiency of existing standards or to establish new ones. The committee then conducts an investigation of relevant information, including appropriate literature, professional and scientific testimony, production feasibility, economic impact, and grower/industry attitudes before proposing new or revised standards for field history and variances, isolation conditions, and varietal purity in the field and seed. A committee's final proposal goes to the board of directors for initial discussion. If the board of directors accepts the proposal, it is presented to the AOSCA Advisory Committee for review and comment. Measures approved by the Advisory Committee are sent back to the board of directors for a final vote. Those not approved by the Advisory Committee are sent back to the appropriate committee for reconsideration. Accepted measures become effective with the next seed production cycle. New standards or procedures are sent to the Agricultural Marketing Service's Seed Regulatory and Marketing Branch office for inclusion in the next round of revisions to Part 201.

Procedures for Verifying Compliance with Standards

Examination of records: Applicants for certification are required to supply information about the cropping history of land containing crops submitted for certification. Requirements for land history are typically based more on experience and common sense than on empirical evidence. Cultural practices exert significant influence on the likelihood of encountering the problem of volunteer plants from a previous crop in subsequent crops. Most certifying agencies will waive land history requirements when provided with evidence of cultural practices that mitigate normal risks of volunteer occurrence.

Applicants are also required to verify the eligibility of stock seed used in planting certified seed fields. In most cases, stock seed itself must have passed certification procedures for varietal purity and have been handled according to required protocol. This protocol stipulates continuous identification and sanitation of harvesting, hauling, elevating, storage, conditioning, and bagging equipment. Federal and state seed laws and regulations specify the kind and retention length of certified seed records. Generally, applicants themselves are responsible for compliance and are accredited to perform these operations without continuous oversight.

Field inspection: Field inspections are AOSCA's most significant quality control measure. AOSCA members retain a contingent of about 900 trained individuals to inspect fields. Isolations are verified according to traditional methods, such as pacing distances, observing surveyed sectional subdivisions, and vehicle odometers. Global positioning technology is also being increasingly used. Verifying adequacy of border rows or timing of flowering in offending fields accommodates allowable modifications to distance requirements. Varietal purity inspections are conducted at one or more times when the crop can be expected to exhibit traits that can be observed as detailed by phenotypic descriptions of morphology and growth characteristics. Methods of sampling for incidence of varietal impurities depend on circumstances. AOSCA maintains suggested sampling procedures for inspecting fields, which include travel patterns, determining estimated plant populations, conversion between percentage

and ratio of off-types per unit area, number of heads/plants to be counted in sub-samples, and procedures for conducting sequential sampling (AOSCA 2001).

Inspections for pollen control are a crucial aspect of certification of hybrid crops, such as corn, sorghum, sunflower, and canola. AOSCA standards stipulate maximum limits on pollen shedding by female seed parents at times when the seed parent is receptive and, in some crops, minimum incidence of shedding by the pollen parent when the female is receptive. The standards are intended to minimize the supply of adventitious pollen and/or maximize the supply of source pollen to limit outcrossing from within or outside the certified seed field.

It is worth noting that field inspection in some crops may be complicated by the presence of described "variants" that must be accounted for in determining the eligibility of the field for certification.

Seed inspection: AOSCA maintains minimum seed purity standards for presence of other varieties and off-types in the cleaned seed, as detailed in part 201.76, Table 5, of the regulations. The character of the seed analysis facility utilized and the reliable expression of phenotypic differences by particular crop kinds influence the usefulness of seed inspection. Agencies that employ seed analysts will screen certified seed lots for compliance with seed purity standards when identifiable traits permit. Agencies without access to in-house seed analysis are generally unable to consistently monitor seed for varietal purity.

Post harvest testing: Until recently, post harvest evaluation of the effectiveness of the previous certification process has focused almost solely on expression of phenotypic traits. AOSCA requires post- harvest verification of varietal purity as a condition of final certification only for hybrid seed crops of canola, cotton, and wheat. Post harvest verification of seed crop purity for final certification of hybrid corn, sorghum, and sunflower is conditional upon observations made during the growing season that might result in reduced hybridity. When conducted, post harvest evaluation of all crop kinds generally involves growouts of certified lots by individual agencies in counter season or next season plots. Agencies also have the discretion to utilize any other kind of reliable post-harvest test to verify varietal identity and purity, and some have the capability to use sophisticated protein-based testing methods. Generally, however, post harvest verification of varietal purity is currently not an AOSCA requirement for certification.

Seed conditioning: The Federal Seed Act Regulations contain requirements (Part 201.73) that must be met by processors and during processing of all classes of certified seed. These include availability of facilities to condition seed without introducing admixtures, maintaining seed lot identity at all times, keeping complete records of receipt and disposition, allowing inspection of records by the certifying agency, and identifying an onsite person to be responsible for certified seed procedures.

The role of certifying agencies in this aspect of compliance with standards is to approve seed conditioners annually. Most agencies fulfill this obligation by conducting inspections of conditioning facilities and associated records.

Miscellaneous Considerations in AOSCA Standards and Procedures

Objectives for varietal purity: The levels of varietal purity established and maintained for the certified classes of various crops must bear some relationship to what can be economically achieved as well as to what is acceptable to buyers. As previously mentioned, the traits examined in evaluating seed crops for compliance with varietal purity standards have been morphological or cultural in character during much of AOSCA's history. Inspection has focused on characters easily distinguished in the field, such as taller height, flower color, anther color, silk color, awns, pod pubescence color, early maturity, *et al.* These were among the obvious impurities that observers, official or otherwise, could see most readily and served as markers for the maintenance of varietal purity in commerce. However, less obvious traits such as shorter height, later maturity, and trivial morphological descriptors often escape notice or demand impractical time and effort to ascertain. A growing trend in describing new varieties is to name segregating impurities as genetic "variants" that certifying agencies are obligated to accept as part of the variety rather than as potentially disqualifying off-types. These add to the total of off-types that may populate a particular field. The motivation for this development is likely the cost in time and money to purify the breeder seed for a variety that may not persist in the market place.

Of course, the consequences of impurities in a seed crop have enormous impact on what kinds of impurities are acceptable and in what amounts. Certain traits can affect functional use and, therefore, economic value of a crop, and are normally subjected to very stringent standards. Examples are the presence of colored-lint plants in white-lint cotton varieties and red rice in white rice, where there is no tolerance for the impurity and acceptance is based on "None found" during field inspection. On the other hand, non-phenotypic traits derived from biotechnology that cannot be reliably distinguished in the field or seed without special tests, such as insect and herbicide resistance and PMP and PMI substances, have taken on increasing significance and offer a new challenge to AOSCA, its members and their commercial clients. Legal and social ramifications associated with such traits are significant forces that may work their way into varietal purity standards for certification.

Finally, AOSCA's objectives in assuring varietal purity and in implementing the standards and procedures it has employed for 85 years include the need to tolerate the presence of adventitious entities in certified seed. Both producers and consumers of certified seed have recognized that acceptance of realistic and achievable tolerances for impurities in seed has been an economically achievable endeavor. In contrast, the marginal cost of achieving greatly restricted tolerance for the phenotypic traits involved was usually excessive and unwarranted by their nature. Furthermore, the concept of zero tolerance for these traits has been unthinkable within the conditions of commercial seed production.

Size or scale of production units: Early seed certification workers theorized that smaller seed production units would be more affected by contaminating sources of pollen than would larger units because of the dilution effect of the larger seed volumes in the latter units during harvesting, handling, and conditioning. This idea is exemplified in current AOSCA isolation standards that differ for alfalfa fields smaller or larger than 5 acres and hybrid corn fields smaller

or larger than 20 acres in size, respectively. Any point of size distinction almost certainly was arbitrary and was, perhaps, related to a commonly recognized land unit.

The concept of dilution by scale has been implemented in more recent times with the establishment of the so-called "ten percent isolation zone" waiver of normal isolation requirements for alfalfa and cross-pollinated grasses. This concept states that the normal isolation requirement is waived if no more than 10 percent of the area of the certified seed-producing field lies within 165 feet of an offending crop. The waiver was adopted because it was difficult to achieve required isolation distances between fields of different varieties when a high percentage of growers in an area produced certified seed and the number of varieties being produced proliferated after the passage of the Plant Variety Protection Act. While the empirical evidence supporting the validity of granting this waiver most likely resides in the offices of a former AOSCA director, let us remember that competent scientists and certification personnel were responsible for conducting the research and reviews that led to the adoption of the waiver.

Establishment and Verification of Isolation Distances: We can only guess at the nature of the early efforts to establish and verify the efficacy of isolation distances adopted in the 1920s. ICIA Annual Reports of the time do not contain supporting evidence for new standards. Current methods of establishing or reviewing isolation distance requirements involve reviewing and discussing available literature and the problems or probabilities that might bear on implementation of new requirements. Factors such as pollen density, volume, and longevity may be considered, and may have been decades ago. Variability among genotypes in propensity to outcross or self-fertilize may also be a factor. Objectively measured rates of outcrossing at various distances from a pollen source are considered, when available. Advice is also sought from outside entities that have a stake in the issue.

While the practical results from long-used standards have met the needs of seed producers and buyers for decades, new challenges in maintaining and even increasing seed purity occasionally arise. The issue of adventitious presence of transgenic events in "conventional" varieties prompted AOSCA to collaborate with USDA's Foreign Agricultural Service and the American Seed Trade Association on a study of intrusion of adventitious pollen into hybrid corn seed fields (Burris 2002). In general, the study confirmed the effectiveness of AOSCA standards in producing high quality maize seed, as measured by conventional standards.

In the case of small grain isolation, we recently conducted a literature review to consider the adequacy of the existing standard of mechanical separation to deal with new transgenic traits. We learned that, while wheat pollen is light and can easily travel, its longevity is brief and its volume is low compared to corn. The extent of outcrossing in small grains, with isolations of 10 to 50 feet, might range from virtually none to 0.25% or more, depending on a variety of conditions. Occasional outcrosses might be detected at 100 and 200 feet or more. We concluded that an isolation requirement of 10 feet is sufficient between different varieties for all classes of certified small grain seed (AOSCA 2002). The efficacy of this new distance was verbally confirmed by a commercial entity that had conducted extensive outcrossing experiments in its product development program

While 10 feet is now an AOSCA standard, some agencies have adopted small grain isolation requirements of as much as 20, 30, and 50 feet as the most practical for their cultural conditions, based primarily on the prevalent width of combine headers.

To date, AOSCA has not addressed the issue of isolation distance requirements as related to the consequences of outcrossing. Obviously, contaminants that impact commercial crop market value or functional use or seed crop characteristics carry significant consequences. Until now, the consequences of outcrossing on most crop kinds have been primarily academic or cosmetic rather than functional. Awn or chaff color off-types in wheat resulting from outcrossing do not carry the same impact as red wheat in white wheat, for example. Even the latter may only result in grain grade dockage or rejection of the contaminated lot as seed stock for further multiplication. These consequences, in turn, may be minor when compared to functional traits that affect plant mortality, such as herbicide resistance, or patented traits or other traits that may involve feed and food safety. The legal and social ramifications relating to adventitious presence of new traits may be cause for AOSCA to consider more restrictive isolation requirements for certified seed production under particular circumstances.

AOSCA's Policy on Trait Testing as a Condition for Certification

AOSCA has continued to rely on inspection for phenotypic traits in varieties containing transgenic attributes and, as mentioned, has not adopted testing requirements for the presence of such traits as a condition for final certification. Discussions held in the early 1990s did not view traits achieved through transgenic technology as substantially different from similar traits achieved by conventional technology. These traits are generally proprietary and the technology owners impose minimum purity requirements on licensees. Licensees conduct extensive verification of both trait presence and incidence in seed production fields and seed crops. Nevertheless, the purity levels required to meet license agreements are normally well below AOSCA's field and seed purity standards. As a practical matter, however, licensees test conventional seed lots destined for markets that prohibit transgenic traits to assure freedom from their adventitious presence. Test results are usually supplied to certifying agencies at final certification.

Final Observations

AOSCA's primary objective during the past 85 years has been one of maintaining varietal identity and purity of a seed lot, whether in a field, a truck, a storage container, or a seed conditioning line. The guiding principles have included common sense and the honorable intentions of seed producers. The objective has been achieved using standards and procedures intended to limit entry of adventitious pollen or seed into the certified seed lot in order to meet reasonable expectations of seed purity.

AOSCA's objective differs sharply from USDA APHIS' Biotechnology Regulatory Service's objective of preventing the escape of certain traits from a field into non-regulated environments, where the consequences of such escape may present unknown environmental and legal concerns.

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Mechanistic Modeling Approaches to Pollen-mediated Gene Flow and Confinement: Summary of Presentation

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Introduction

Gene flow has historically been a concern for well-known reasons in seed production. However, it has become of wider interest recently because of the introduction of genetically engineered (GE) crops, and claims of negative environmental and human health impacts. In both Canada and the U.S., regulatory authorizations are required for the release of certain GE plants. Part of that authorization involves a risk assessment and part of the risk assessment involves considerations of pollen and gene flow. There is also interest in minimizing or eliminating gene flow and pollen flow. For both risk assessments and gene flow, minimization gene flow modeling serves as an important tool.

Pollen-mediated gene flow is only an issue for outcrossing (OC) crops, but it is important to remember that the definitions of OC and self-pollination crops are only nominal definitions and that there is a gradation between OC and self-pollinated crops, as well as between insect and wind vectored pollen dispersal. Canola is perhaps the best example of a crop type that uses different pollination modes and pollen vectors, so its usage of these modes may vary under different environmental conditions. On the other hand, corn is a good example of a crop that is highly biased toward OC and uses wind as the pollen vector. Other crops that use wind as the pollen vector are millet, oats, and wheat, although they also self-pollinate to some extent.

Of the various methods to reduce gene flow and pollen dispersal, the most common are biological and physical methods. Biological methods include genetic or bred modifications to induce male sterility. Another example is the use of temporal isolation where flowering periods of source and receptor plants are desynchronized. Physical methods potentially include distance isolation, barrier crops, and the use of windbreak-like structures. Distance isolation is dilution by distance of the dispersed pollen and leads to decreased gene flow. The use of barrier crops, around receptor plants or a receptor field, physically traps incoming pollen (for wind dispersed pollen) and/or dilutes the relative amount of foreign pollen by mixture with receptor field pollen. Windbreak-like structures, perhaps most effectively implemented by surrounding source fields, enhance the physical pollen capturing ability by their size and architecture.

Many of these techniques have been used or suggested in the past, and tests of their effectiveness have been based upon field trials. But since a key characteristic of pollen and gene flow is its variability, questions arise about the general applicability of very time- and site-specific measurements. The use of modeling has the potential to overcome that limitation.

Models – Overview

Pollen and gene flow models will become important tools to assess the risk and confinement of novel plants including GE plants. This is because they possess a number of advantages over the simple use of field-trial data:

1. Pollen and gene flow are inherently variable in time and space, so site specific measurements of gene flow cannot be applied to other sites and times. Models offer greater generalization; that is, they are relatively independent of the data used to generate them.
2. Assessments of pollen and gene flow variability are only obtainable with great effort from measurements and are seldom comprehensive. Models offer the ability to assess variability relatively easily.
3. Measurements provide no predictive ability to assess, say, a novel configuration of a barrier crop. Models can be used to predict the affects of different confinement configurations.

However, the models that have been developed for pollen and gene flow have varying abilities in delivering all the advantages stated above. Models that have been developed and used fall into three very general categories: empirical, mechanistic, and "genetic." "Genetic" models incorporate, to one extent or another, population genetic principles in their basic structure. Empirical models rely solely on statistical relationships determined from pollen or gene flow experiments and, thus, incur the disadvantage of not being generalizable. This presentation will concentrate on models that take a more mechanistic approach.

Mechanistic models are those that are based on mechanistic or chemical fundamental relationships. These models are perhaps most readily applied to the process of the atmospheric dispersal of pollen because the work applied to atmospheric pollution and climate studies have created a general understanding of the field. With regard to the insect vector, the mechanistic approach is generally not used as far as I am aware, although the work of Cresswell and co-workers at the University of Exeter in the UK is of particular note.

Although I have categorized a sub-group of models as mechanistic, the entire pollen/gene flow process cannot be dealt with on a purely mechanistic basis because some sub-processes are too complicated for us to deal with on such a fundamental level. Thus, we can subdivide the various sub-processes and model them "biologically" or mechanically. It is easy to subdivide the whole gene flow process in modeling because of the modular nature of developing computer code.

For example, the viability of pollen, as it disperses from the source plant, decreases over time, and thus distance. We do not understand all the physio-chemical systems controlling viability in detail and so we have to devise experiments where we measure pollen viability under varying environmental conditions and draw statistical relations to build an empirical sub-model for this particular process.

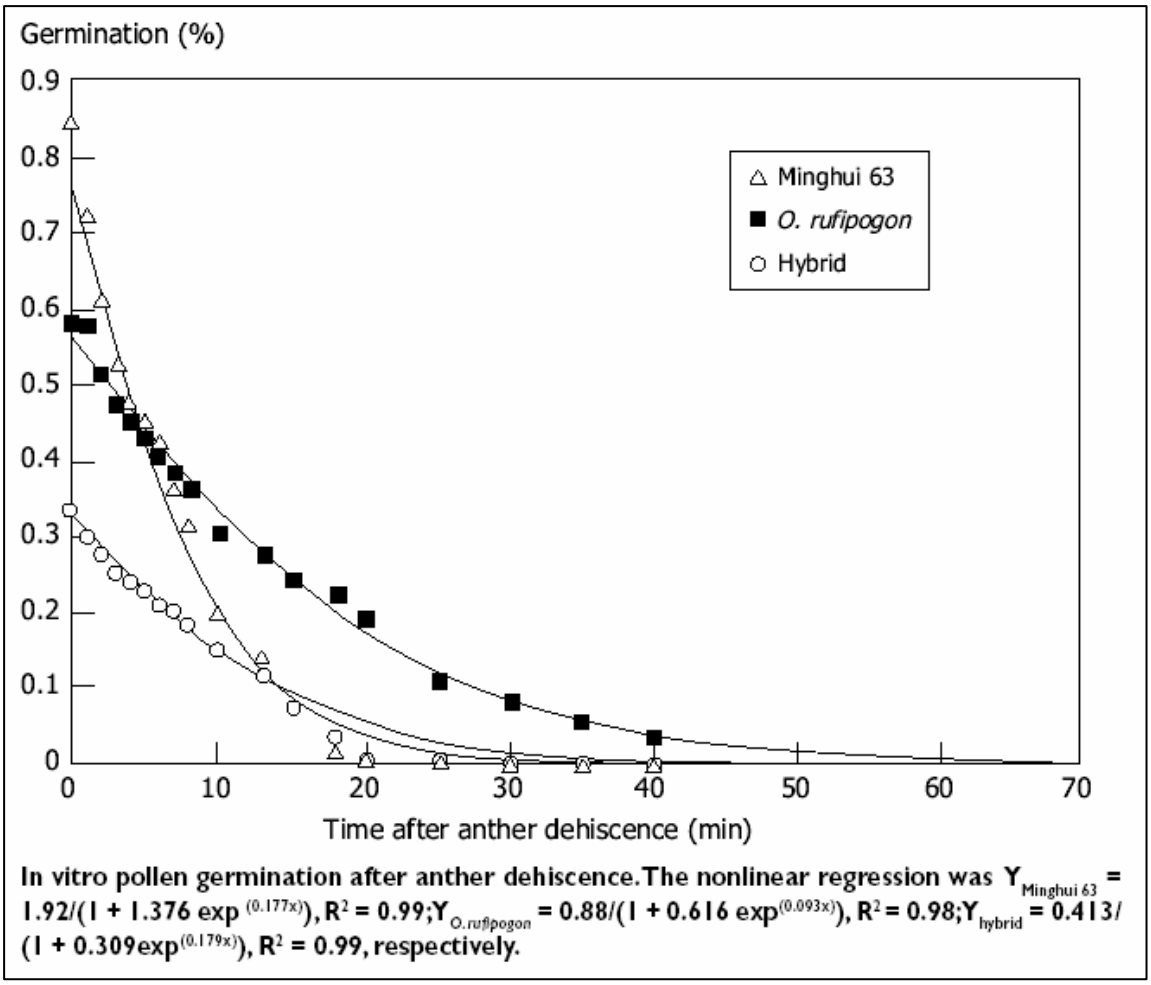

Figure 1. Variation in pollen germination versus time for different rice types (Song *et al.* 2001).

Figure 1 illustrates what can be done to produce an empirical sub-model. The non-linear regression equations can simply be used as the viability sub-model in any comprehensive model for rice gene flow, for example.

However, as a general statement, the more heavily any model relies on empirical sub-processes, the less likely it is to be easily generalizable.

Factors Affecting Pollen Dispersal by Wind

The whole process of pollen and gene flow and can subdivided into a few major steps: release of pollen from a source plant; its dispersion through the atmosphere; and its deposition. I will describe each of these three steps in more detail.

There are three main factors that characterize the emission of pollen:

1. The amount of pollen release by a plant, like many other plant processes, is controlled by

genetics and the environment. The amount of pollen released varies by plant type and the site characteristics of the plant location.

2. The height of pollen release is determined by the plant height and this, in turn, determines dispersal distance.
3. Timing of release is also determined by genetics and environment; different plants flower at broadly different times of the year. However, the exact date of maximum pollen liberation, peaking over a period of days, can be controlled by environmental factors in the season or few days before pollination. For example, heat summation techniques can be used to forecast pollination periods. Heat summation (i.e. growing degree units; GDU) is a commonly used technique for crop growth forecasting in agriculture but we have applied this technique to forecasting maximal pollen release in jack pine (Figure 2) when conducting work on modeling pollen and gene flow for tree seed production in Ontario.

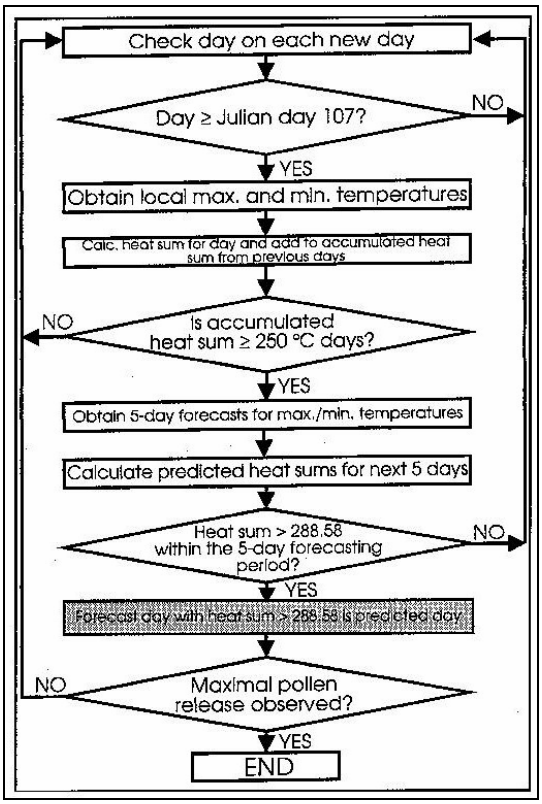

Figure 2. The algorithm presented here (DiGiovanni *et al.* 1996a) determines the start date and base temperature for heat sum predictions of maximal pollen shed of jack pine in Ontario.

Pollen release is also known to be biased toward daily cycles during the pollination period.

The dispersal distance of pollen is also affected by a number of factors:

1. Wind speed varies with height above ground according to well-established meteorological relationships, as well as within vegetation canopies. Higher wind speeds disperse pollen further.

2. Atmospheric stability can either suppress or enhance turbulence in the atmosphere and turbulence, in turn, affects dispersion as greater amounts of turbulence lead to enhanced dilution of the pollen cloud.
3. The settling velocity of pollen can also affect the dispersal distance as, not surprisingly, heavier pollen fall to the ground faster and travel shorter distances, all other factors being equal. The settling velocity is a function of pollen weight, which can also be a function of ambient relative humidity.

It is important to note that dispersal can also occur to some altitude as was found when measuring conifer pollen concentrations up to heights of 300 m upwind of a conifer tree seed production area (Figure 3).

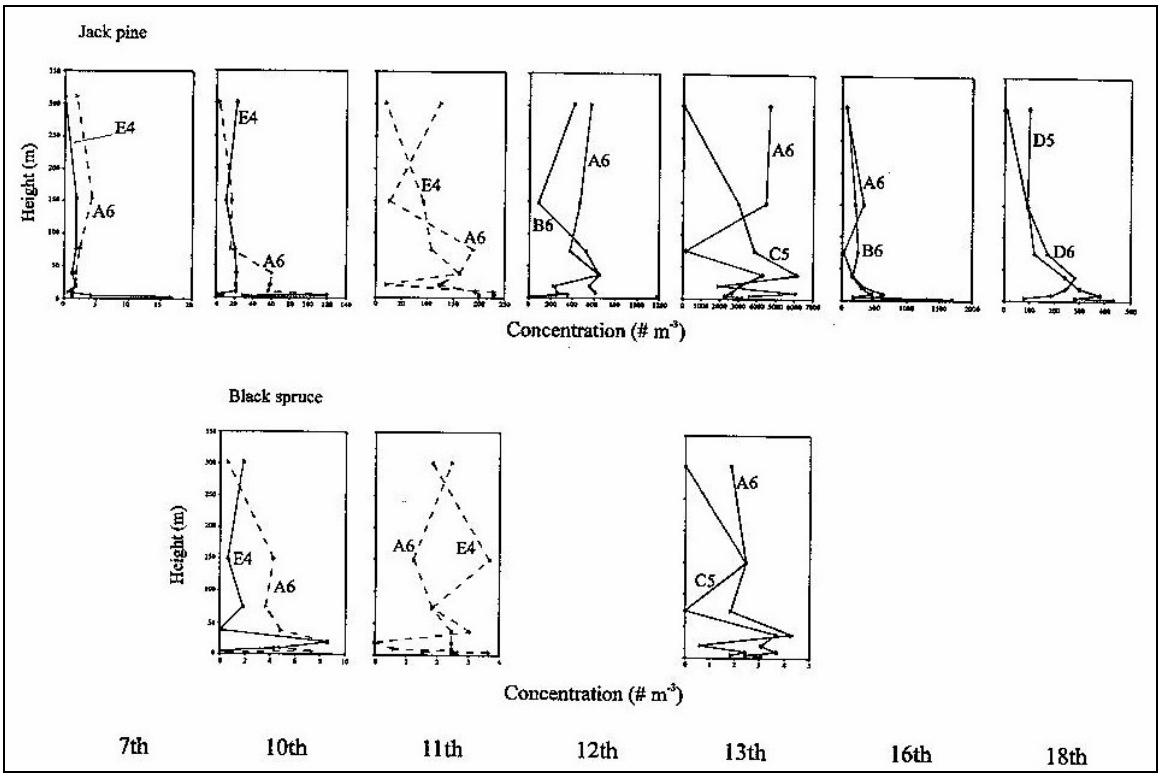

Figure 3. Measured pollen concentrations for black spruce and jack pine pollen at the edges of a conifer seed production area. Two profiles are shown within each graph with one graph for each day (in June 1993) of sampling. See original paper for details
(DiGiovanni *et al.* 1996b).

Finally, pollen can to come to rest in any one of three locations:

1. It can deposit to the ground.
2. It can be filtered by other vegetative elements as it travels through the canopy. Filtration by other plant parts is generally a function of the pollen settling velocity, the speed of the wind carrying the pollen grain and the size of the plant element. Work in aerosol science has provided some of these functional relationships that allow us to estimate this effect.

3. If the pollen is "lucky enough" to land on a stigma, and it is still viable, then pollination and gene flow can occur. Thus the addition of viability and pollen competition sub-models are required to expand a pollen flow model into a gene flow model.

Models for Wind Vector

Three of the most common types of mathematical models that can, and have been, used to simulate pollen dispersion in the atmosphere (Figure 4) are:

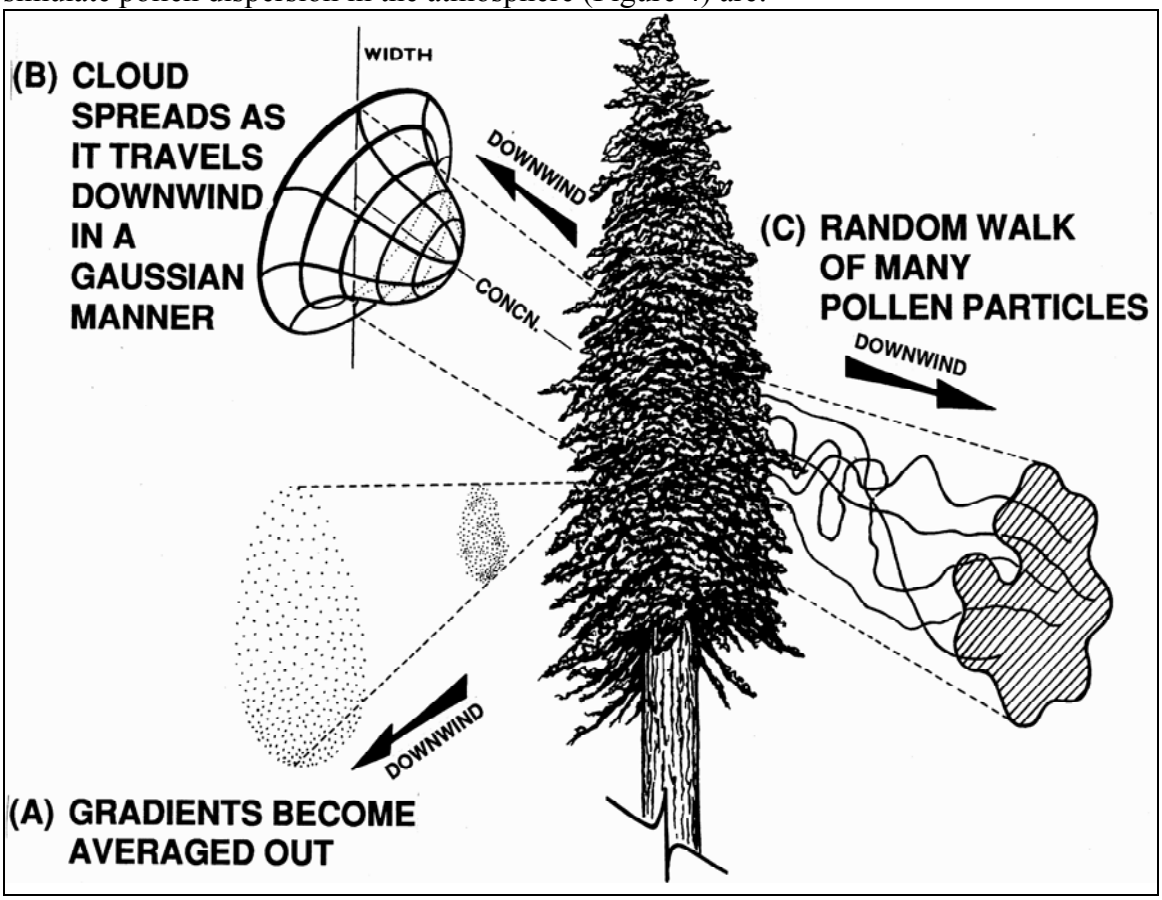

Figure 4. The most common types of mathematical models used to simulate atmospheric pollen dispersion (DiGiovanni and Kevan 1991):

A) Fickian diffusion type models, characterized by pollen diffusion, down concentration gradients;
B) Gaussian plume models, which are a particular type of Fickian diffusion model and are characterized by assuming the concentration spread follows a Normal distribution (these types of models are most popular for air pollution regulatory impact assessments); and
C) Lagrangian stochastic models, which compute a resultant pollen cloud as an ensemble of many pollen particles that have traveled in a step-wise Markovchain type pattern.

Type C is probably the most appropriate for dealing with the complexities of within-canopy dispersal and, therefore, is most appropriate for dealing with farm-scale or field-to-field gene

flow issues. However, at the landscape-scale, where within-canopy turbulent flow complexities are less significant, the other two models are probably also appropriate.

Regulatory Application for Risk Assessment

I will conclude by briefly describing our work in applying a model to aide risk assessment of wheat. This was work funded by the Canadian Food Inspection Agency (CFIA).

Methods

The work was divided into a number of logical tasks. We first developed and validated a Lagrangian stochastic dispersion model–this was modified from a version we had earlier developed for seed production isolation assessments for the forestry industry in Ontario. During the multi-stage model validation we also compared the model against an "off-the-shelf" air pollution regulatory model to provide an accuracy benchmark for the model.

We then converted it to a wheat gene flow model by incorporating the physical and biological characteristics of the wheat reproductive system. In that process, we found scant data to provide model inputs. This is, to some extent, unlike the situation with corn, where there is a much richer collection of studies available with which to formulate sub-process models. This is probably because wheat has traditionally been viewed as a self-pollinator and less attention has been paid to its OC characteristics.

The model was then run at a number of sites over the major wheat growing regions of Canada with long-term environmental data to produce large collections of "simulated" field-data. This large collection of artificial data was then used to determine the variability in gene flow both temporally and geographically. It is this quantification of variability that we believe is a key result. We're not aware of it being done for airborne pollen dispersal in this manner before and, therefore, we believe we have applied a novel concept.

Finally, the model was applied to determine the effectiveness, and variation in effectiveness, of various containment techniques.

Dealing with Ill-defined Model Inputs

Ill-defined parameters were set to a maximum value found in the literature. This action will result in model overestimates of pollen and gene flow, thus making the model "conservative." Use of a conservative environmental impacts model is allowable if applied in the correct manner. Figure 5 illustrates this concept and demonstrates the effect on model output.

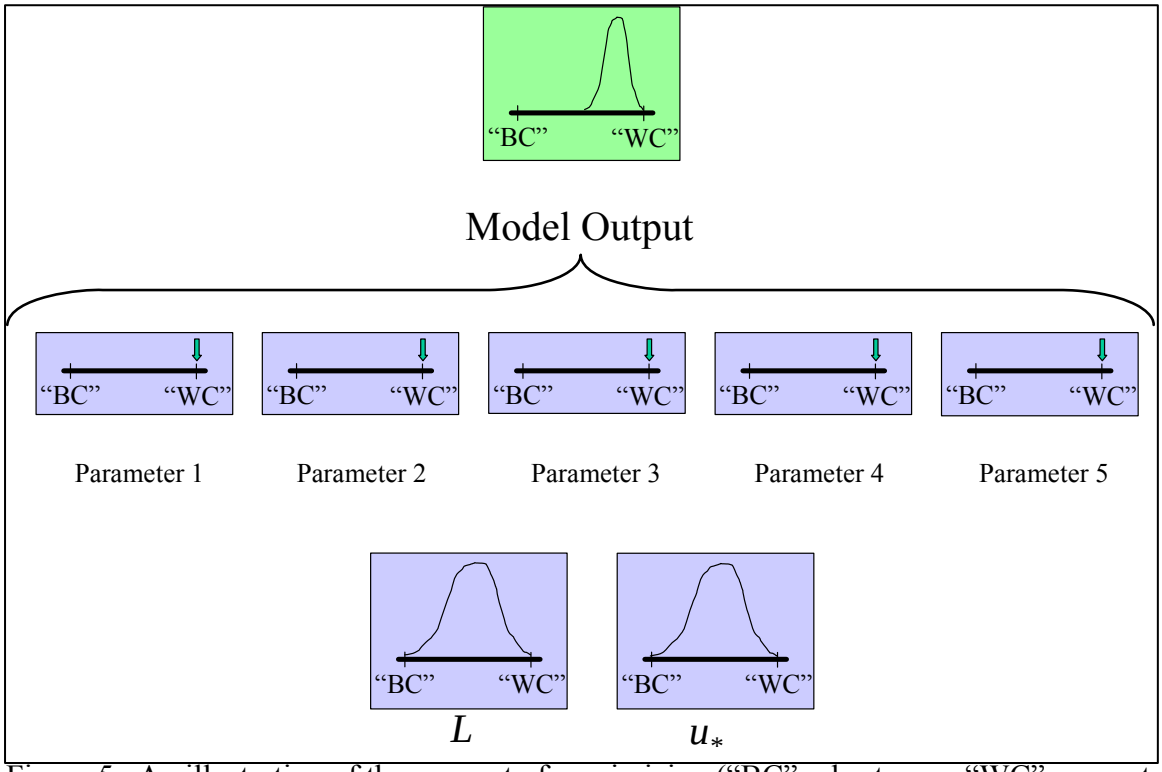

Figure 5. An illustration of the concept of maximizing ("BC" = best-case, "WC" = worst-case) ill-defined model input parameters, yet obtaining a full spectrum of data for the meteorological variables (L = Monin-obukhov length, u_* = friction velocity), which are (nominally) shown by Normal distributions. The result on model output is to retain variability (caused by the meteorological variables), yet shift the output distribution towards worst-case.

Results

Figure 6 displays the model results for predicting pollen dispersion and its variability over all sites and all years.

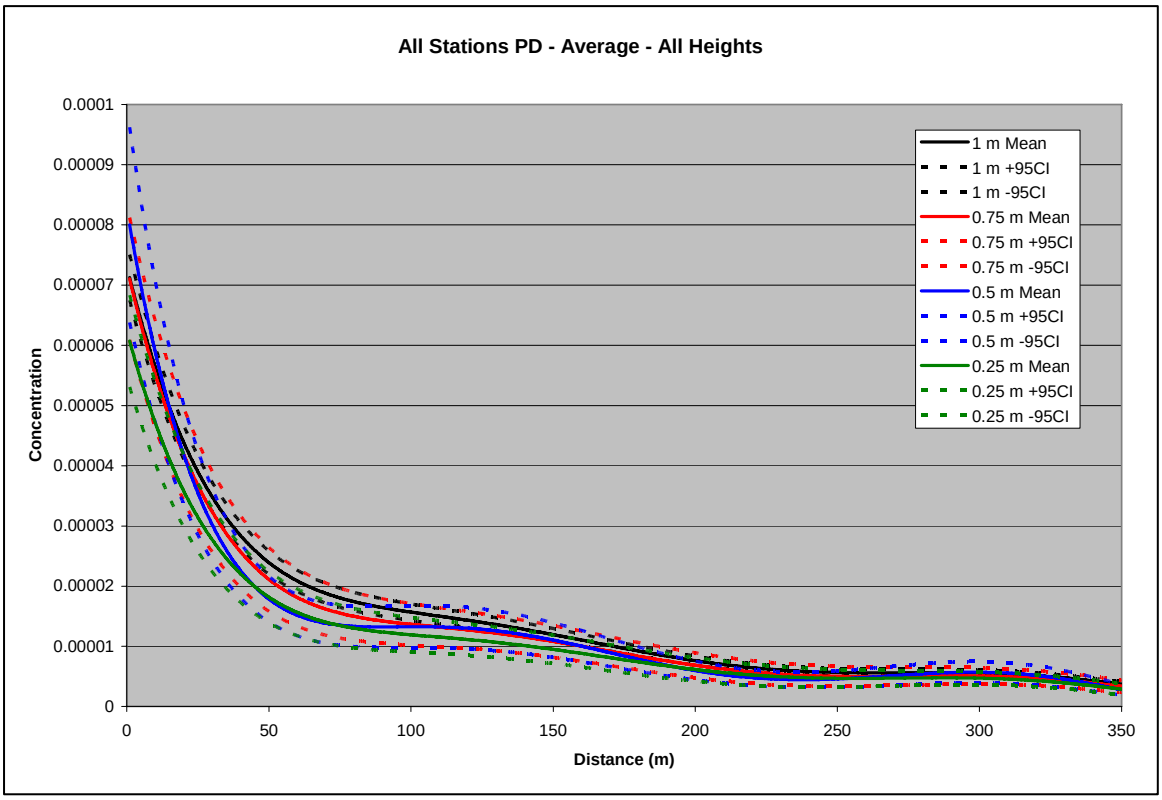

Figure 6. The averaged extent of pollen dispersion for all sites combined for each receptor height. The graph represents the change in airborne pollen concentration with increasing distance downwind of the source crop (pollen release at 1 m height) and into the receptor crop (of heights 0.25 – 1 m). The curves represent a smoothed representation of the average over all sites and years. The 95% CI's are displayed by the dotted lines on both sides of the average lines and are smoothed representations of actual values.

Figure 7 displays the change, with downwind distance, in the probability of exceedance (PE) for chosen OC thresholds for all sites combined at the 1 m receptor height, for example.

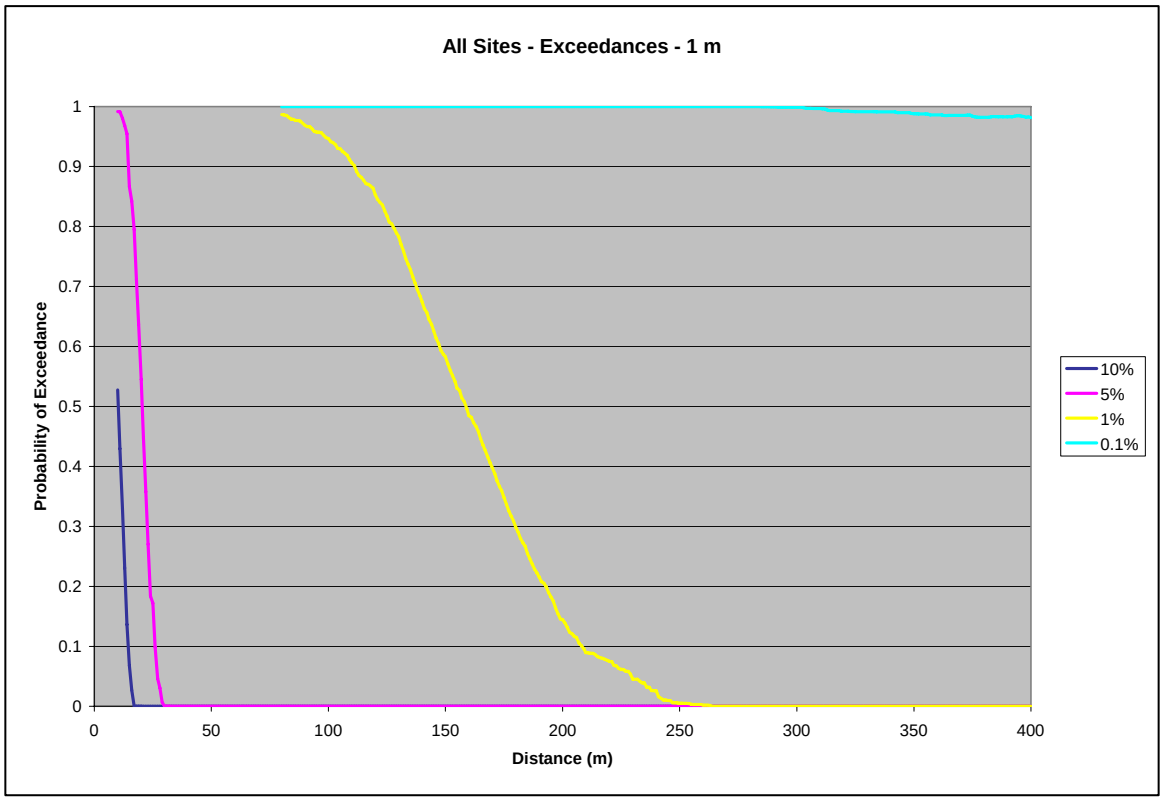

Figure 7. The graph represents the change in the probability of exceeding the said OC level with increasing distance across the receptor crop. The most direct comparison would be to define the level of success of a certain width of buffer crop.

For example, referring to Figure 7, if a 1% threshold applied, and a farmer was willing to accept failure 1 year out of 10 over the long term (≡ 10% containment failure ≡ 10% probability of exceedance), then Figure 7 indicates that a buffer strip of approximately 210 m wide is required.

The model was also applied to test the effectiveness of various isolation methods including barrier crops, barren isolation zones, windbreak-like structures, and flowering desynchronization. An illustration of typical results is given in Figure 8.

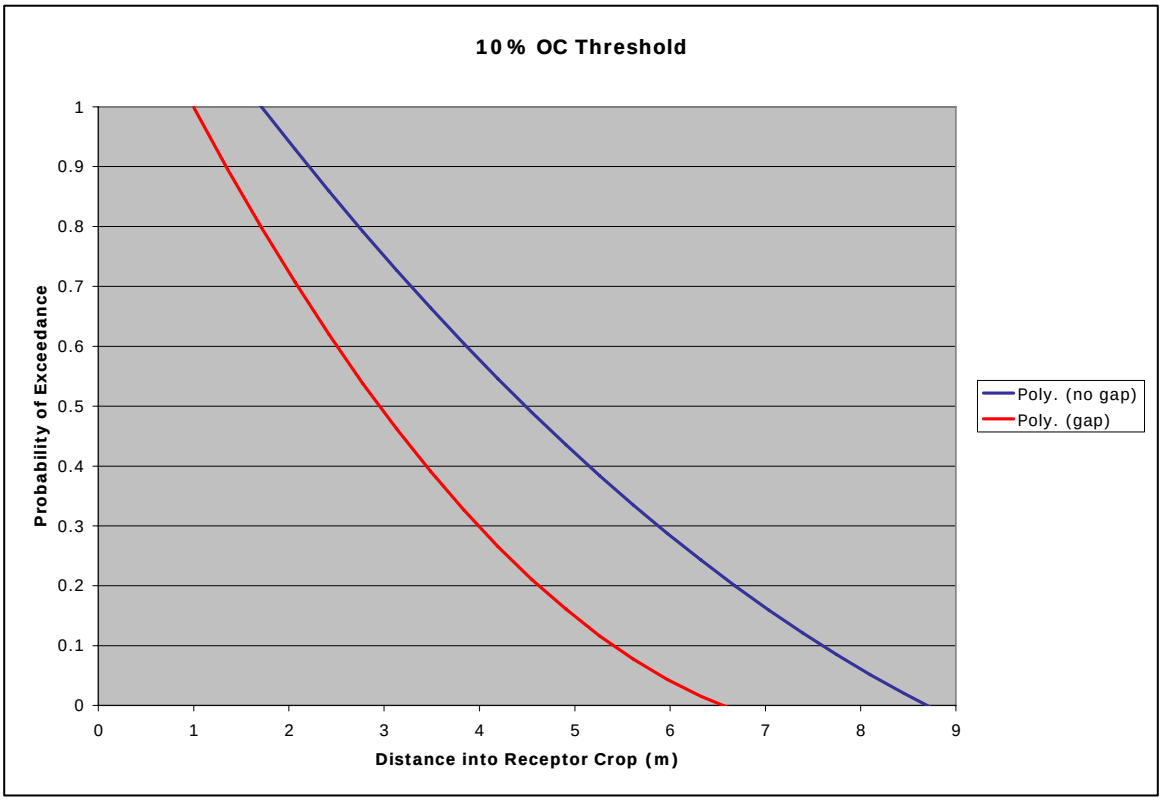

Figure 8. Smoothed model results showing the effect on the probability of exceeding a 10% OC level when comparing having source and receptor crops immediately adjacent (no gap) and 100 m apart (gap). The "gap" line indicates that lower probabilities are estimated at similar distances into the receptor crop thus quantifying the effectiveness of the gap on a probabilistic basis.

Summary

- Modeling will be a very useful tool in the regulatory risk assessment of novel plants.
- They can be used to assess present and novel containment methods.
- Maximizing the mechanistic content of models should be the ultimate goal, however, substantial use can be made of models at present.
- The appropriate model is required for the appropriate situation.
- OC and containment should be assessed on a probabilistic basis given their variability–our work for CFIA suggests such a basis.

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Monitoring To Verify Confinement

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Abstract

Public policy regarding the unintended occurrence of transgenic elements in the food supply necessitates that regulated transgenes be confined. Within this context, transgene confinement stands as a rights-based criterion for genetically engineered (GE) crops separate from the risks (human, environmental, or economic consequence) that the transgenic elements (or more specifically, their expressed products) may pose. The United States Department of Agriculture (USDA) Animal Plant Health Inspection Service (APHIS) has established permitting conditions for field testing of GE crops that produce pharmaceutical or industrial compounds in order to limit unintended occurrence of regulated transgenic elements consistent with public policy expectations.

Monitoring is used to verify that the processes for achieving confinement meet expectations. Monitoring for gene flow may be physically based (focused on detection of gene flow), process based (focused on the conditions of confinement), or model based (focused on environmental factors governing gene flow). The monitoring approach used needs to consider the nature of concern that is being addressed. For instance, the monitoring design for detecting transient (episodic) occurrence in food or feed would be different than that for accumulation from trait occurrence in the breeder's seed bank.

The monitoring threshold for concern and confinement parameters will greatly influence the feasibility of physically-based monitoring. For instance, confinement to assure that outcrossing from corn is restricted to <1% at distances within approximately 200 m of a source may be reasonably predicted and monitored, whereas, physical verification that outcrossing is near zero at 1610 m cannot be directly achieved on the basis of existing data, predictive tools, or analytical methodology. Sentinel monitoring with catch plots has been used to describe attenuation of outcrossing with distance off-source and extrapolate near source measurements to distant receptors. The veracity of this approach is not clearly established due to the aforementioned limitations regarding level of detection and modeling for distant transport. Improved monitoring and modeling approaches are emerging that allow for real time analysis of environmental conditions leading to fugitive pollen escape, and these approaches can be used to identify, isolate, and remediate unintended escapes if confinement is breached. Until validated monitoring and modeling approaches for the physical flow of genes to great distances are available, evaluating the management processes intended to achieve confinement goals stands as the most effective means to assure confinement of GE crops.

Introduction

This presentation considers monitoring strategies specifically as they relate to assuring the integrity of regulated crops being grown under confinement within field environments. The conduct of monitoring is strategic when its rationale and design are justified on the basis of risk or policy analysis, and serves to confirm the correctness of risk management decisions. At best,

monitoring is hypothesis-driven, testable, and has well-defined endpoints. The nature of the monitoring activity–indeed the overall relevance of monitoring in a given instance–is determined by the degree of residual uncertainty arising from risk and policy analysis. In describing the monitoring process for verification of confinement integrity, consideration is given to the specific context for monitoring, various approaches that can be taken for confinement monitoring, the evaluation of the adequacy of monitoring, and the suitability of monitor and respond strategies for regulated crops produced under confinement.

Monitoring Context

The advisability to undertake monitoring, as well as the nature of the monitoring for a given product of genetic engineering, will be very much context dependent. The focus of this presentation is on regulated trials involving confined field production of regulated crops. Specifically, this presentation draws on a base of experience and expertise involving plant made pharmaceuticals and industrials (PMPs/PMIs) produced using corn as the production platform. These crops are grown under conditions where crop-to-crop gene flow and ramifications to integrity of the food and feed supply are the dominant considerations for confinement.

Confinement and public policy. PMPs and PMIs represent bioactive agents for which there is no food tolerance. Thus, the intention of policies concerning the confinement of a PMP or PMI crop is to avoid adulteration of food with the regulated article (a bioactive agent). USDA APHIS has established permitting conditions for field testing of GE crops that produce pharmaceutical or industrial compounds. These conditions serve to limit unintended occurrence of regulated transgenic elements consistent with public policy expectations (USDA 2003a and 2003b). The need for monitoring within this context is, therefore, to assure or verify that the permit conditions, and actions taken to comply with these conditions, are consistent with policy goals.

The public policy position for regulation of PMPs/PMIs (as with most new technologies) represents a rights-based criterion where the primary concern is not a risk-based outcome but "the process and allowed action or activities" (e.g., Morgan and Henrion 1990). Thus, for instance, the consideration that compels monitoring is not the health consequences of unintended presence of the regulated transgenic element in food. Rather, it is the intention to eliminate the risk "independent of benefits and costs, and of how big the risks are" (Morgan and Henrion 1990). This zero risk consideration for confinement establishes the monitoring context for permitted trials with PMP/PMI crops as elimination of episodic release to the food/feed supply and assuring absences of unintended traits in the seed supply.

Confinement State-of-the-Art

Confinement for the purposes of commercial seed production is a well-established practice that is governed by seed law and developer processes intended to provide buyers with a uniform, high-quality product. For example, the Federal Seed Act mandates 99.9% purity for foundation seed and 99.5% purity for certified corn. Current industry practice meets or exceeds this standard due to the adoption of refined pre-foundation seed production processes that limit unintended presence of transgenic elements (Mumm and Walters 2001). Currently, the commercial seed supply that is labeled as non-genetically engineered is 99+% trait purity for

absence of transgenes in seed (UCS 2004). Increased emphasis on process controls for conduct of regulated field trails seeks to further improve the integrity of confinement (BIO 2004).

Breeders' seed maintenance (pre-foundation seed production) involves the tightest controls in current confinement practices. Emphasis on confinement of breeder's seed is important since undetected contamination of seed at this stage can result in far-reaching contamination of the seed supply as seed increases are made. A typical breeder's seed increase for corn consists of 30 seed each from 20 ears planted in unique rows. There are about 200 seed per ear. Typically, 5 plants per row will be selected for hand pollination and 1 plant of the 5 is advanced to the next generation. This process is repeated for a second generation. If the outcrossing frequency due to pollen in-flow for the breeder seed maintenance is 0.001 and there is no ability to detect a particular transgene, the frequency for 1 contaminant seed to be retained in the breeder's seed lot is 1 in 106. Under this scenario, when intrusion is episodic in generation 1, seed will be greater than 99.99% pure. If this same scenario takes place in generation 2, or recurs over generations, then the frequency for 1 contaminant seed to be retained in the breeder's seed lot is 1 in 250 (99.6% pure). If the breeder is able to detect and rogue off-types, the likelihood of retaining contaminant seed is further reduced from 10- to 10,000-fold. Monitoring of breeders' seed to avoid non-food trait presence minimizes the potential for magnification of unapproved traits through the seed/grain channel. For corn in a given year, about 10 acres of breeder seed is produced vs. 80 million acres of grain. Therefore, the monitoring of breeders seed is more feasible than monitoring of the general food supply. For transgenic elements, however, there remains limitations in sampling and analytical methods for monitoring these trace levels of adulteration.

Approaches to Monitoring

Confinement monitoring can involve (1) monitoring for physical presence of the trait, (2) monitoring for likelihood of escape (by accounting for pollen out-flow and the fate and channeling of seed produced within the confined trial), or (3) monitoring for integrity of the confinement process. In practice, some combination of these monitoring approaches may be used. Regardless of the monitoring approach, the monitoring will be most effective when the recognition that confinement integrity has been breached is detected early enough to allow for remediation response prior to movement of the unintended trait into food or commercial seed.

Monitor for physical presence. Monitoring for physical presence entails monitoring trait presence in a receptor field (or seed or grain lot) of concern. Direct monitoring for trait presence is restricted by analytical sensitivity, sample size constraints and a high error rate (false positives/negatives).

The feasibility of physical monitoring for trait presence when considering a zero tolerance (0% threshold) condition in seed is determined by how zero tolerance is defined. An *exact definition*of 0% lot impurity leads to the necessity to test each seed in the entire lot. A *hidden threshold*of 0% in a sample leads to a high level of uncertainty as to the meaning of the result unless there is a clear definition of the sample size. A *zero deviant plan*seeks 0% positives in a defined sample size and will be sensitive to high false positives or negatives. Hidden threshold

or zero deviant plan methodologies entail high developer risk when there is an opportunity for reanalysis of a given seed lot.

Monitoring for confinement integrity should ideally involve physical detection of the unintended trait. The monitoring threshold of concern and specific confinement conditions, however, greatly influence the feasibility of physically-based monitoring. This is because the monitoring goal for physical detection determines the scope of monitoring as well as the degree of uncertainty in the result. For instance, confinement to assure that outcrossing from corn is restricted to <1% at distances within 200 m of a source may be reasonably predicted and monitored, whereas, physical verification that outcrossing is near zero at 1600-m many not be achievable on the basis of existing data, predictive tools, or analytical methodology. This can be shown by considering examples of seed sampling methodologies. A protocol to detect and confirm 0.1% outcrossing (OC) to a receptor could entail analysis of 3000 seed and acceptance of zero positives with 5% chance of accepting a field above 0.1% OC. However, to detect and confirm 0.01% OC in a receptor field may require analysis of 100 pools of 300 seed each with acceptance of zero positives with 5% chance of accepting a field above 0.01% OC. Alternatively, one could meet the 0.01% OC threshold through analysis of 50 pools of 320 seed each and accept zero positives with 20% chance of accepting a field above 0.01%. And finally, to detect and confirm at 0% OC to a receptor would require analysis of every seed.

Indirect monitoring with sentinel plots. Use of sentinel plots represents a methodology to indirectly monitor for unintended trait presence. Sentinel monitoring with catch plots for regulated traits has been used to describe attenuation of outcrossing with distance off-source and extrapolate near source measurements to distant receptors (Eppard 2002). An array of receptor (sentinel) plots located within defined distances from a source field is used to detect and confirm a decline in a trait over distance. The results are extrapolated to the nearest field of concern. The veracity of this approach is not clearly established due to the aforementioned limitations regarding level of detection and modeling for distant transport (e.g., Aylor *et al*. 2003). In general, the methodology is effective and reasonable for traits at near distance (200 m from the source field), but is of limited practicality at the large distances (1600 m) considered with PMP/PMI confinement. This is because, in the case of corn, the limit of detection is restricted by sentinel plot sizes, and validation data are lacking to verify data extrapolation to distances much greater than 200 m.

Pollen monitoring using sentinel receptors has also been considered as an option. Pollen interception overcomes some limitations of assaying directly for the trait, but imposes other constraints that may limit its usefulness in monitoring. The major constraint is the lack of verified data to establish the relationship of pollen detected at monitoring stations to source pollen that must be viable, reach a receptive plant, compete with receptor pollen, and effectively pollinate in order for the trait to be introduced off-source.

Monitor for process integrity. Because of the limitations for direct or indirect physical monitoring for trait presence, monitoring for process integrity is more commonly practiced for confined trials of regulated materials. This will remain the case until validated monitoring and modeling approaches for the physical flow of genes to great distances are available. Permit

conditions for the confined trial identify the parameters that need to be considered in the design of compliant processes for the management of the field trial. These processes can be designed with redundant operations to address uncertainties and auditing of the processes serves as the monitoring focus. Process designs for confined trials of regulated crops have been developed and evaluated as to their integrity (BIO 2004, Christensen *et al.*2005; Wolt *et al.* 2005).

Monitor and respond. Improved monitoring and modeling approaches are emerging that allow for real time monitoring and analysis of environmental conditions leading to fugitive pollen escape, and these approaches can be used to identify, isolate, and remediate unintended escapes if confinement is breached (Hayes 2004). This monitor and respond strategy involves monitoring in conjunction with modeling to identify departures from confinement goals in real time. Should departures occur, such as high wind conditions predicted to carry source pollen beyond the confinement offset distance, there is an opportunity to identify at-risk receptor fields and to segregate potentially adulterated products from an at-risk field prior to harvest through channeling or crop destruction.

Summary

Since there is no food tolerance in place for pharmaceutical or industrial agents in food, the goal of confinement is to prevent their introduction into the food or feed supply independent of the level of risk that the particular agent may pose. Regulated field trials for PMP and PMI crops are conducted using processes to assure the adequacy of regulatory standards and developer compliance for confined field production. Monitoring activities for confined crops should focus foremost on unintended trait occurrence during variety line development and breeders' seed maintenance for food crops so as to minimize the possibility for recurrent presence of an unintended trait. As long as a plant-expressed trait has no tolerance in food, a zero risk criterion holds and monitoring should focus on process integrity. The absence of an operational definition of zero limits the ability to verify or validate monitoring strategies and models based on physical presence of the regulated trait. Effective physical monitoring must, therefore, await a change in public policy toward PMPs/PMIs that entails a risk-based criterion vs. zero tolerance.

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Transgene Confinement Via Maternal Inheritance and Cytoplasmic Male Sterility in Genetically Modified Crops

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Summary
The potential of genetically engineered (GE) crops to transfer foreign genes through pollen to related plant species has been recognized as a potential environmental concern. Until the environmental impact of novel genes on indigenous crops and weeds is thoroughly investigated, practical and regulatory considerations might require the adoption of gene confinement approaches for future generations of GE crops. To date, most molecular approaches with potential for controlling gene flow among crops and weeds have focused on maternal inheritance, male sterility, and seed sterility. This presentation focuses on the use of maternal inheritance and cytoplasmic male sterility for transgene confinement. Because no single strategy will be broadly applicable to all crop species, a combination of more than one approach might prove most effective for engineering failsafe mechanisms for the next generation of GE crops**.**

Gene Confinement
Public concerns about the environmental impact of GE crops currently limit their widespread acceptance around the world. Many of these concerns are based on the premise that such transfer could potentially result in the emergence of "superweeds" resistant to herbicides, or the introduction of undesired traits into related crop plants. Gene flow depends upon several factors, including the specific crop, its location, the potential of outcrossing with wild relatives or sexually compatible crops, the competitive nature (advantages and disadvantages) of the introduced trait, and the environmental consequences of neutral traits. Two mechanisms are responsible for the movement of genes among crops and their wild relatives or related crops: 1. dispersal in viable pollen, or 2. dissemination in seed that later germinates and produces viable pollen. This presentation focused on the dispersal via pollen. The potential for gene flow via pollen depends on several factors, including the amount of pollen produced, longevity of pollen viability, dispersal of pollen (e.g., via wind or animals), plant/weed density, dormancy/rehydration of pollen, survival of pollen from toxic substances secreted by pollinators, the distance between crops and weeds, and whether these plants are sexually receptive to the crop.

Following pollination and reproduction, dispersal of seeds from GE plants may also occur among weedy relatives during harvest, transportation and planting giving rise to mixed populations. If these GE seeds germinate, grow, and reproduce, there is a risk that interbreeding with a sexually compatible weedy species could produce a fertile hybrid. Further crossing with the weed species (introgressive hybridization) may result in new weeds that have acquired the GE trait. This again depends on the persistence of the crop among weeds and probability of forming mixed stands.

Maternal Inheritance
Three modes of plastid genome inheritance have been described: Uniparental maternal, biparental or uniparental paternal. Uniparental maternal plastid inheritance is observed in a

majority of angiosperms (Hagemann 2004). This was first described almost a hundred years ago for *Mirabilis jalapa*. Uniparental maternal inheritance is achieved through plastid exclusion from the generative cell during the first haploid pollen mitosis; all plastids are distributed into the vegetative cell and the generative cell is free of plastids. Therefore, the sperm cells formed from the generative cell are free of plastids (Hagemann 2004). If the generative cell acquires a few plastids, they degenerate during maturation and the sperm cell becomes free of plastids (Hagemann 2004). In cereals, both generative and sperm cells contain plastids but they are removed from the sperm nucleus before or during the process of fertilization.

However, rare exceptions to uniparental maternal inheritance have been reported. Occasional transmission of paternal plastids in tobacco has also been reported (Medgyesy and Marton 1986). In a few exceptions among angiosperms, such as *Oenothera* or *Medicago* (Smith *et al.* 1986), biparental plastid inheritance has been reported. This is caused by equal distribution of plastids during the first haploid pollen mitosis into generative and vegetative cells. Therefore, the sperm cells transmit plastids into egg cells. Extraordinarily, uniparental paternal inheritance of plastids has been reported in the kiwi plant (Cipriani *et al*. 1995). Aforementioned exceptions demonstrate the need to develop alternate approaches to eliminate rare paternal or biparental transmission of transgenes engineered via the chloroplast genome.

Maternal inheritance of transgenes and prevention of gene flow via pollen in chloroplast transgenic plants have been successfully demonstrated in several plant species, including tobacco (Danielle *et al.* 1998; Danielle 2002), tomato (Ruf *et al*. 2001), cotton (Kumar *et al*. 2004), and soybean (Dufourmantel *et al*. 2004). Unlike many other containment strategies, the maternal inheritance approach has already been tested in the field. Scott and Wilkinson (1999) studied plastid inheritance in natural hybrids collected from two wild populations growing next to oilseed rape along 34 km of the Thames River in the UK. These populations were assessed for the persistence of 18 feral oil seed rape populations over a period of 3 years. They analyzed several factors that would influence the movement of chloroplast genes from crops to wild relatives, including the mode of inheritance of plastids and the incidence of sympatry, (the occurrence of species together in the same area) to quantify opportunities for forming mixed populations and persistence of crops outside agriculture limits for introgression. Despite some (0.6–0.7%) sympatry between the crop and weed species, mixed stands showed a strong tendency toward rapid decline in plant number, seed return, and ultimately extinction within 3 years. Thus, they concluded that gene flow should be rare if plants are genetically engineered via the chloroplast genome.

In addition to maternal inheritance, the chloroplast genetic engineering approach offers a number of advantages, including high-level transgene expression (up to 46% of the total leaf protein) (DeCosa *et al.* 2001), multi-gene engineering in a single transformation event (Quesada-Vargas *et al.* 2005; Ruiz *et al*. 2003), lack of gene silencing, position effect and pleiotropic effects (Danielle *et al.* 2005; Grevich and Daniell 2005). Thus, maternal inheritance of chloroplast genomes is a promising option for gene confinement. Chloroplast genetic engineering has now been shown to confer resistance to herbicides (Daniell *et al*. 1998), insects (DeCosa *et al.* 2001; Kota *et al.* 1999), disease (DeGray *et al*. 2001), salt (Kumar *et al*. 2004), and drought (Lee *et al.* 2003), as well as phytoremediation of heavy metals (Ruiz *et al*. 2003). Chloroplasts have been

engineered to produce several functional vaccine antigens, including cholera (Daniell *et al*. 2001), anthrax (Watson *et al*. 2004; Koya *et al.* 2005), and tetanus (Tregoning *et al.* 2003). Chloroplasts have also been genetically engineered to produce biopharmaceuticals, including human serum albumin (Fernandez-San Millan *et al*.2003), somatotropin (Staub *et al*. 2000), interferons (Leelavathy and Reddy 2003), and other therapeutic proteins (Daniell *et al.* 2005, Danielle *et al.* 2004a, Danielle *et al*. 2004b).

Male Sterility

Male-sterility-inducing cytoplasms have been known for over a century. Cytoplasmic male sterile inbred lines have been widely used in hybrid seed production of many crops. The first application of cytoplasmic male sterility was for hybrid seed production and was a major contribution towards the "Green Revolution." The use of cytoplasmic male sterility (CMS) in hybrid seed production was recently reviewed by Havey (2004). The use of CMS for hybrid seed production received a "black eye" after the epidemic of *Bipolaris maydis* on T-cytoplasmic maize. This epidemic is often cited as a classic example of genetic vulnerability of our major crop plants. In addition to Southern corn blight (CMS-T), cold susceptibility (CMS Ogura) and *Sorghum ergot* infection in the unfertilized stigma have been reported. But these disease linkages were successfully broken by somatic cell genetics and conventional plant breeding (Havey 2004).

Hybrids of other crop plants may be produced using nuclear male sterility. A natural source of nuclear male sterility was identified in leek (Smith and Crowther 1995). Engineered sources of nuclear male sterility have been developed in model systems (Marian *et al*. 1990). GE rapeseed containing the Barstar Barnase male sterility system comprises ~10% of the commercially cultivated crop in Canada, and is one of the few GE organisms cleared for agricultural use in Europe. One problem with these nuclear transformants is that they segregate for male fertility or sterility and must be over planted and rogued by hand, or sprayed with herbicides to remove male-fertile plants.

Major investments of time and resources are required to backcross a male-sterility-inducing cytoplasm into elite lines. These generations of backcrossing could be avoided by transformation of an organellar genome of the elite male-fertile inbred to produce female inbred lines for hybrid seed production (Havey 2004). Because the male-fertile parental and male-sterile transformed lines would be developed from the same inbred line, they should be highly uniform and possess the same nuclear genotype, excluding mutations and residual heterozygosity (Havey 2004). Therefore, the male-fertile parental line becomes the maintainer line to seed-propagate the newly transformed male-sterile line (Havey 2004). A few generations of seed increases would produce a CMS-maintainer pair for hybrid seed production. An additional advantage of organellar transformation would be the diversification of CMS sources used in commercial hybrid-seed production. Transformation of the chloroplast genome would allow breeders to introduce different male-sterility-inducing factors into superior inbred lines. Introduction of a male-sterility inducing transgene into one of the organellar genomes of a higher plant would be a major breakthrough in the production of male-sterile inbred lines (Havey 2004). This technique would be of great potential importance in the production of hybrid crops by avoiding generations of backcrossing, an approach especially advantageous for crop plants with

longer generation times (Havey 2004). Moreover, transgenes that are engineered into our annual crops could be introgressed into wild crops, persist in the environment and have negative ecological consequences. Therefore, it may be necessary to engineer a male sterility system that is 100% effective (Havey 2004).

Ruiz and Daniell (2005) have recently developed a reversible male sterility system by expressing the *pha*A gene coding for β-kethiolase in transgenic chloroplasts. Prior attempts to express the *pha*A gene in transgenic plants were unsuccessful. However, in this study, the *pha*A gene was efficiently transcribed in all tissue types examined, including leaves, flowers, and anthers. Coomassie-stained gel and western blots confirmed hyperexpression of β-ketothiolase in leaves and anthers, with proportionately high levels of enzyme activity. The transgenic lines were normal except for the male sterile phenotype lacking pollen. Scanning electron microscopy revealed a collapsed morphology of the pollen grains. Floral developmental studies revealed that transgenic lines showed an accelerated pattern of anther development, affecting their maturation and resulting in aberrant tissue patterns. Abnormal thickening of the outer wall, enlarged endothecium, and vacuolation affected pollen grains and resulted in the irregular shape or collapsed phenotype. Reversibility of the male sterile phenotype was observed under continuous illumination, resulting in viable pollen and copious amount of seeds. This study results in the first engineered cytoplasmic male sterility system in plants, offers a new tool for transgene containment for both nuclear and organelle genomes, and provides an expedient mechanism for F1 hybrid seed production.

Conclusions

There is currently inadequate data on the environmental impact of specific GE traits. At present, no effective gene containment method is available for all GE crops, and considerable investment and research is needed to develop the technologies outlined above.

It is clear that the characteristics of seed and pollen production, dispersal, and potential outcrossing must be determined for each specific crop in each specific environment. Different crop species have different rates of autogamy and outcrossing, and some crops have hybridizing wild relatives only in certain geographical locations. It will also be important to allay concerns that crops engineered with altered pollination, flowering, or male sterility patterns for the purpose of gene confinement will not impact the wider biodiversity of insects, bird and wildlife in existing ecosystems.

As shown above, both biological confinement measures have been developed to control gene flow through pollen or seed. Male sterility is currently commercially utilized in Canola. It is very effective at preventing outcrossing from GE crops to weeds or related non-GE crops. However, seeds produced from nuclear male sterile GE crops by cross-pollination from weeds may become a concern because seeds of such hybrids will produce fertile pollen that would carry the GE trait. Also, pollen is not produced in a crop that makes the seed, making it less desirable for the farmer because it would require cross-pollination from a non-GE crop, or must be propagated by artificial seed. Reversible male sterile systems engineered via the chloroplast genome should address these concerns. Maternal inheritance is a promising approach for

transgene containment with added advantages of high levels of transgene expression, rapid multigene engineering, lack of position effect, gene silencing, and pleiotropic effects. Currently, chloroplast genetic engineering has been enabled in tobacco, a non-food/feed crop as a bioreactor for production of biopharmaceuticals, monoclonals, biopolymers, or to confer desired plant traits. It has also been enabled in several major GE crops, including cotton and soybean. Chloroplast transgenic carrot plants withstand salt concentrations that only halophytes could tolerate. Extension of chloroplast genetic engineering technology to other useful crops will depend on the availability of the plastid genome sequences and the ability to regenerate transgenic events.

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Integrating the Biological and Physical Components of Maize Pollen Dispersal

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Our approach to modeling pollen dispersal is to quantify and integrate three fundamental processes in maize pollination biology: pollen production, dispersal in the air, and the efficiency of pollination. We have developed mechanistic sub-models for each of these processes based on our understanding of the dynamics of the flowering process in maize, the movement of particles in a turbulent atmosphere, and the biology of pollen as a living particle. Integrating these sub-processes into a single model is the key to assessing the timing and intensity of pollen dispersal from a plant made pharmaceutical (PMP) or plant made industrial (PMI) field, as well as the risk of gene containment failure associated with the dispersal event.

The timing and intensity of pollen production is accurately simulated from simple measures of the flowering population. Basic information about percentage of plants shedding pollen, coupled with an estimate of average pollen production per plant, provides a daily estimation of pollen production that serves as the source input for the pollen dispersal model. We have observed that estimating pollen production on a daily basis leads to very accurate simulations of kernel set. Nonetheless, we are collecting hourly values of pollen shed to account for the effects of relative humidity, temperature, and wind speed on diurnal patterns. The capacity to simulate hourly variation in pollen production is critical for quantifying pollen dispersal under conditions in which atmospheric turbulence could promote long-distance transport of pollen grains.

There are a number of ways to simulate the movement of pollen grains in the atmosphere once they have been released from the anthers. Our initial approach was to use a model developed by the Environmental Protection Agency (EPA) to estimate small particle dispersal from point and surface sources (ISCST3). This model uses a gaussian equation and estimates of atmospheric conditions to predict the concentration of particles in a plume downwind of a source. This model provides the typical exponential decay of particle concentration with distance. It is fairly accurate at distances between 100-200m from the source, but tends to deposit too many grains near the source. The lagrangian (statistical) approach we are now using calculates the concentration of pollen grains as independent 'packets' downwind of the source. This model is ideal for complex terrain, can be used in concert with windbreaks, is not limited by source size, and can be used to trace the movement of particles back to the source. Initial estimates of pollen dispersal using the lagrangian model were similar to the EPA model with pollen deposition close to the source. But the lagrangian model also underestimated pollen dispersal at greater distances, relative to our field observations. Evidently, the current version does not adequately account for dispersal associated with atmospheric turbulence around the source field. We are currently incorporating a Large Eddy Simulation (LES) module into the lagrangian calculation to address this issue. We anticipate that the addition of this module will enable us to simulate dispersal of maize pollen at distances well beyond 200 m.

A critical component of the pollen dispersal sub-model is the capacity to estimate the change in pollen viability as it travels through the air. Even under favorable conditions for corn pollination, pollen must travel through air that is very dry, and it loses moisture and viability as it

does so. We have developed quantitative relationship between pollen moisture content and viability based on a number of field and controlled-environment studies. This relationship, which has been robust for a large number of genotypes, enables us to quantify the loss in pollen viability as it travels through the air of known moisture content. For relatively short trips (e.g., 200 m; the typical isolation distance between a commercial corn field and a hybrid seed field), pollen viability would remain essentially unchanged, even at low wind speeds (2 m/s). For longer trips (e.g., pollen taken up in a turbulent updraft to 1000 m, then returning to a field somewhere downwind), viability decreases predictably with the height achieved. Our predictions follow the same patterns as the measured values of Brunet *et al.* (2004), who measured viability of pollen collected at heights up to 2000 m. However, we actually calculate the viability of the pollen that has returned to 1 m and available for pollination.

Once the amount of pollen entering the atmosphere has been calculated, and its destination and viability have been established, it is essential to translate that value into a successful pollination event. The third biological sub-model makes these calculations based on our knowledge of the pollination process and the density of pollen required to affect kernel set. We have used this model to simulate kernel set in hybrid seed production fields quite accurately. The success of this approach across years, location, and genetics, confirms that we have accounted for the most important variables associated with maize pollination. We then calculate kernel set resulting from the local pollen source as well as an adventitious (foreign) pollen source. This technology enables hybrid seed producers to adjust management strategies to optimize seed production, as well as genetic purity. The same technology can be used to refine field confinement requirements to minimize the risk of an unintended pollination event at defined distance from the PMP or PMI source field.

A limitation in the utility of current models for pollen dispersal is the lack of confirming information about pollen dispersal at distances greater than 200 m from the source field. Once we have incorporated a Large Eddy Simulation module into our Pollen Dispersal model, we will have the ideal framework to confirm and simulate the extent of maize pollen dispersal at these greater distances. The next logical step would be to apply this integrated modeling approach to other wind-pollinated crops, such as grasses and cereals.

Practical Application of Time and Distance as Redundant Systems for Biological Confinement in Maize

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From **'Isolation of Maize from Pollen Mediated Gene Flow by Time and Distance',** by Mark E. Halsey, Kirk M. Remund, Christopher A. Davis, Mick Qualls, Philip J. Eppard and Sharon A. Berberich. In Press, Crop Science, Nov. – Dec. 2005. This work was supported by Monsanto Company, St Louis, MO USA.

Abstract
Studies were conducted in California to evaluate the relationship of distance and temporal separation for isolation of maize from pollen-mediated gene flow (PMGF). Kernel color was used to detect outcrossing from source plots of 0.4 to 1.2 ha to receptor plots planted at distances up to 750 m and planting intervals of up to three weeks. Outcrossing from source to receptor plots was observed up to approximately 0.0002% (1 kernel in ~500,000 kernels). Increasing temporal separation reduced the distance required to achieve genetic isolation. Outcrossing was <0.01% at 500 m when source and receptors flowered at the same time, whereas this level of confinement was achieved at 62 m when two weeks of temporal separation (335 growing degree units (GDU)) was used. No outcrossing was detected at 750 m and two weeks of temporal separation. The time main effect and the interaction of time and distance were highly significant (p <0.0001). Hence, it can be concluded that time and distance do not act independently. Isolation standards invoking both will be most realistic when derived from empirical data in which both systems are studied concurrently.

Introduction
Development of improved genetic traits in maize (*Zea mays* L.) requires both simple and robust measures to prevent pollen-mediated gene flow (PMGF) and assure isolation of new traits, whether these traits are the result of conventional breeding or of modern genetic techniques.

Distance isolation has long been used by plant breeders to assure genetic isolation, but distance alone is only one possible barrier to PMGF. Successful fertilization requires that male and female flowers be active at the same time, so that viable pollen encounters receptive silks. Such flowering synchrony – commonly called 'nicking' – is also critical to PMGF between two maize crops.

The deliberate disruption of flowering synchrony between plots or fields, with the goal of enforcing genetic isolation, is usually done by displacing planting dates and is called 'temporal separation' or 'temporal isolation'. Temporal separation is a well-established mechanism for genetic isolation in maize, but has not been extensively quantified in the same fashion as distance separation.

In practice, time and distance are deployed so that an increase in temporal separation is used to reduce isolation distance requirements. USDA guidelines for isolation of maize producing

pharmaceutical products call for 1600 m (1 mile) distance separation for fields planted at the same time, but only 800 m for fields planted four weeks or more apart. However, little empirical work has been done on gene flow restricted by distance and time together. Such information is needed to validate existing guidelines, and could also be used to develop more precise schemes for deploying time and distance together to achieve desired levels of genetic purity. A large field study was conducted in California in 2002 to evaluate the relationship of distance and temporal separation for isolation from PMGF.

Materials and Methods

A gene source plot of 1.2 ha was planted across the northern, or upwind, side of a ¼ section (800 m x 800 m) field in the San Joaquin Valley. Receptor plots were planted at six distances toward the south (downwind) of the source plot, and at five time intervals from the planting of the source plot. Each receptor plot was 4 rows wide and 6 m in length. The areas between the plots were left fallow, allowing pollen to move unhindered from the source to receptors. All distances were measured from the outside of the pollen source plot, which was construed to include a 12 m male sterile border between the source and receptor plots. The size of the source plot and the use of a male sterile border were intended to simulate an 'inbred seed production field', representing a likely source of genetically modified pollen in the production of maize with novel genetic traits. Distances from the source plot to the receptors were 30, 40, 125, 250, 500 and 750 m from the source plot. Receptor plots were planted at 3 weeks and 2 weeks before ('-3 and -2 weeks'), at the same time ('0 week'), and 2 and 3 weeks after ('+2 and +3 weeks') planting of the source plot. Small plots (2 rows by 3 m) of receptors, planted as individual hybrids (not as a blend), were placed inside the pollen source, in order to observe flowering of the individual hybrids. A schematic plot diagram is shown in Fig. 1.

The gene source used was hybrid A619 x B37, which contained the genetic markers P1-rr and R1-nj. The source hybrid was homozygous for one or both of the genetic markers. The receptor plots were a mixture of three yellow kernel maize hybrids, DK440, DK507 and DK579, ranging from 94 to 107 day Relative Maturity, and chosen to bracket the flowering time of the source plot. Where the yellow maize in the receptor plots was fertilized by pollen from the source plot, the resulting kernel had a prominent purple marker that allowed rapid visual detection of outcrossing. All primary ears were evaluated from the entire plot area in each receptor plot, which typically had 150 to 200 primary ears. Purple kernels were counted and recorded on an individual kernel basis. Where high levels of outcrossing was observed, such as in the plots inside the pollen source, estimates were visually made as percent of each ear surface occupied by outcrossed kernels. Outcrossing in this experiment was observed to 0.0002% (1 kernel in ~500,000 kernels).

No water or nutrient deficits were allowed to develop, and thus these factors did not influence the results. Planting rate was 72,000 seeds per ha, and row spacing was 93 cm. Wind velocity and direction were recorded by an on-site weather station during the period of pollen shed.

Results and Discussion

The predominant wind pattern during pollen shed was toward the southeast in the morning, shifting toward the southwest in the afternoon. Thus, the wind pattern would have blown pollen toward the receptor plots as the wind shifted from southeast to southwest.

Flowering periods of the receptor hybrids and the pollen source synchronized very well when both were planted at the same time. Outcrossing to the small plots of receptors planted within the pollen source plot averaged 43% at this planting time, but was reduced to 0.1% and 11% when receptors were planted two weeks before or after the source plot, respectively.

Total outcrossed kernels in all four replicates is shown in Fig. 2 for each distance and planting time. A total of 8 kernels was noted in plots at 750 m and planted at the same time as the source plot. No outcrossed kernels were noted at two or three weeks of temporal separation at this distance. Three weeks of temporal separation also reduced outcrossing to zero at a 500 m distance. Two and three weeks of temporal separation represented about 335 GDU and 500 GDU of crop development, respectively.

The main effect of Time, and the Time by Distance interaction were both highly significant ($p < 0.0001$) as determined by ANCOVA.

Time and distance may be employed together to limit PMGF. Increasing one factor reduces the requirement for the other in order to achieve a defined level of isolation. For example, 500 m of distance isolation reduced outcrossing at synchronous flowering to <0.01%. The same level of isolation was achieved at 60 m with two weeks separation in planting time. Measurable outcrossing occurred at 750 m and good nick, but outcrossing was eliminated at this distance by two weeks of temporal separation, representing approximately 335 GDU of crop development. This is the first practical evaluation of time and distance acting together to achieve genetic purity in maize

The observation of a significant Time by Distance interaction indicates that the relationship of time and distance for genetic confinement appears to be complex, and further research is needed to elicit the fundamental relationship between these factors when they are employed together to achieve genetic isolation of maize. Until a predictive model emerges, isolation standards invoking both time and distance will be most realistic when derived from empirical data collected on both factors acting together in specific environments.

On the basis of these results from California, the USDA isolation standards for maize producing pharmaceutical products—1600 m for crops planted at the same time, or 800 m and four weeks of temporal separation—appear to be sufficiently robust, at least for environments similar to the one studied.

Figures

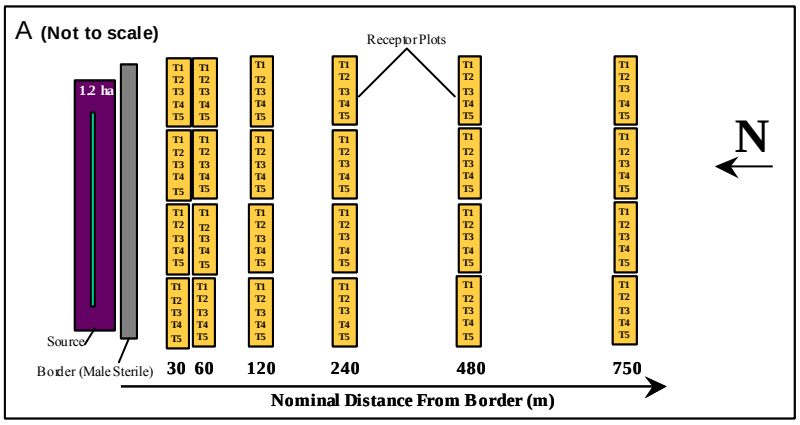

Figure 1. Experimental design. Source plot was 1.2 ha (15 m x 750 m), bordered with male sterile maize 18 m wide on the south. Four replications of receptor plots were planted at each distance and time from the source plot (T1, T2, etc. in the figure). Receptor plots were planted at 2 and 3 week intervals from the source plot. ~17.5 million kernels were evaluated.

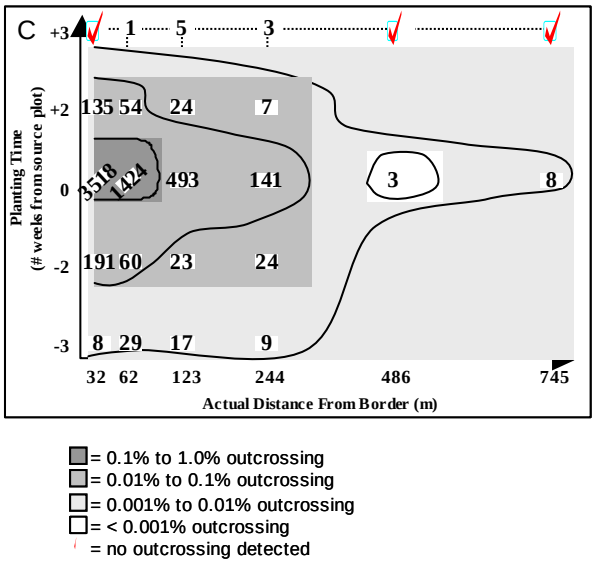

Figure 2. Total outcrossed kernels detected in four replicates of receptor plots at distance and temporal isolation intervals.

Dynamics of Pollen Dispersal and Confinement in U.S. Rice

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Rice (*Oryza sativa* L.) is arguably, the single most important food crop in the world. In the U.S. it is grown primarily in lowland irrigated areas of Arkansas, Louisiana, Mississippi, Missouri, Texas, and California. Although the U.S. produces only about 3.5 million acres of rice, it is typically the world's third or fourth largest exporter of this crop. Rice has the smallest genome, genetically is the simplest of all major cereal crops (diploid with 12 pairs of chromosomes), and has served as a useful model for crop genomics research because its genes and gene functions have a high degree of applicability to the other cereal crops.

Great improvements due to rice breeding, fertility research, mechanization, pest management research, and herbicide-based weed control have been achieved in the United States in the last half century. However, weeds remain a major limitation to optimum rice production and economic returns. Barnyardgrass and its close relatives are the most prevalent and economically significant weed species in U.S. rice. Growers apply herbicides to essentially 100% of all rice acres, largely because of these weeds. In the southern United States, weedy red rice (*O. sativa* L.) is considered to be the most troublesome weed when present, because it acts like a crop mimic of rice and has traditionally been uncontrollable in rice. California cropping systems are essentially free from red rice due to a highly effective clean seed program and exclusive use of water seeding.

Rice and weedy red rice are considered to be the same species and can readily intercross with one another. Several distinctive U.S. red rice plant types are common, especially awnless strawhull, awned blackhull, and awned strawhull. Most of these are tall-statured with medium-grain seed size and shape. Flowering dates for red rice types range from being slightly earlier than most commercial cultivars (often strawhull awnless types) to several weeks later than any modern cultivar (often blackhull awned types). Numerous other red rice types can also be found in low numbers, including short-statured long-grain types, and short-statured awned types, suggesting that these may have been derived originally from natural crosses between red rice and long-grain commercial rice (most prevalent rice in the southern United States) or between awned red rice and semi-dwarf commercial rice (introduced into the southern United States in the 1970s).

Both transgenic (glufosinate-resistant and glyphosate-resistant) and non-transgenic (imidazolinone-resistant "Clearfield" rice) herbicide-resistant rice cultivars have been developed in recent years. With the subsequent marketing of non-transgenic Clearfield rice, outcrossing between herbicide-resistant and non-resistant rice and between rice and weedy red rice has been increasingly scrutinized. Clearfield rice was first grown commercially in the United States in 2002. It has been rapidly adopted by growers due to effective red rice control, and was planted on about 15% and 25% of the rice area of the southern United States in 2004 and 2005, respectively. Clearfield rice is not grown in California. To date, no pest-resistant transgenic rice cultivars have been grown commercially in the United States.

Rice and red rice are primarily self-pollinating because most stigmas (female) are fertilized by pollen (male) produced in the same flower, and pollen shedding usually occurs slightly before or concurrent with flower opening. This is in stark contrast to corn, which produces male and female organs on different flowers, easily facilitating cross pollination. Maximum outcrossing between adjacent rice and red rice plants appears to average about 0.2 to 0.7% under field conditions, based on a large number of published, controlled experiments. However, outcrossing is highly variable. In small-scale field tests, the apparent outcrossing rates are sometimes zero, especially if the flowering periods of the two plant types do not overlap sufficiently, or if the total number of seeds sampled was too small to adequately detect very low outcrossing rates. Conversely, in a recent Louisiana report, poor herbicide performance in a large commercial field of Clearfield rice that was heavily infested with red rice, resulted in outcrossing rates of about 3%, even though outcrossing rates averaged over numerous locations were similar to the lower rates indicated above. Interestingly, a landmark report from the 1930s indicated that the average outcrossing rates between rice plants were generally similar to those reported more recently between rice and red rice, and were highly variable as well.

Outcrossing between rice and red rice can occur in both directions. In Louisiana and Arkansas studies, outcrossing has usually been much greater when pollen from red rice (tall plants) fertilized stigmas on rice (shorter plants). Thus, pollen produced by the tall red rice plants was more likely to fertilize flowers on the shorter rice plants than the reverse situation. This outcome is consistent with the notion that pollen is more likely to "rain" down (e.g. by gravity) than to be lifted upwardly (e.g. by wind currents) in order to achieve cross fertilization. This directional difference in outcrossing rate may actually reduce the introgression rates of red rice hybrid derivatives into rice fields because nearly all of the relatively large number of red rice hybrid seeds produced on rice plants, as a result of fertilization by red rice pollen, are removed from the field during harvest (modern combines can remove as much as 95% of the grain from a rice field) and will not impact the field in the future unless the grower unwisely chooses to plant this seed. Conversely, the relatively few hybrid seeds produced on red rice plants as a result of fertilization by rice pollen, are likely to shatter from the plants before harvest and remain in the field to cause future problems. Shattering rates of up to 80% are common for red rice seeds.

Outcrossing over great separation distances is mitigated by a combination of factors: 1) each rice or red rice flower opens only once, which lasts for about one hour at midday; 2) pollen released into the environment remains viable for only about 10 minutes; and 3) under calm wind conditions, most pollen grains tend to fall near to where they were produced. A complicating factor may actually facilitate both short distance and long distance outcrossing: the stigma can remain viable much longer (i.e. several days) and tolerates greater temperature extremes than pollen. Thus, flowers that do not produce viable pollen of their own (e.g. after exposure to temperatures below 16°C during pollen formation; or as in the case of male-sterile plants used in the commercial production of hybrid rice), will be more susceptible to fertilization from foreign pollen, even if it has arrived from at great distance away.

Genetic background also can affect outcrossing. Thus, cultivated rices (e.g. *O. sativa*), which rely entirely on seed production to ensure that uniform plants can be produced for future crops,

generally outcross less than wild, perennial species (e.g. *O. rufipogon*). Additionally, *japonica*-based cultivars, predominantly grown in the United States, tend to outcross less than their *indica*-based counterparts prevalent in more tropical areas of the world. In recent work with imidazolinone-resistant rice cultivars, researchers in Arkansas have shown that "CL161" rice outcrossed substantially more with awnless strawhull red rice than "CL121". The mechanisms responsible for this difference are not fully understood, but the panicle height differential between rice and red rice (very tall plant) is less for CL161 (taller cultivar) than for CL121 (shorter cultivar), which would tend to place flowers of CL161 in relatively closer proximity than CL121 to red rice flowers. Floral characteristics such as large anthers or lengthened filaments that tend to release great quantities of pollen into the environment, and large, extruding stigmas that can readily intercept foreign pollen grains can be associated with increased outcrossing rates in some cultivars.

Environmental factors can influence outcrossing. For example, outcrossing is frequently greater in the downwind or prevailing wind direction. European reports have documented a 35 times greater outcrossing rate of 1 m downwind compared to 1 m upwind from a pollen source. On the other hand, California reports concluded that prevailing wind direction did not influence outcrossing rates. Conditions, such as bright sunlight and warm temperatures that increase the degree of flower opening, and high relative humidities that increase pollen longevity, facilitate both natural outcrossing, and manual crossing performed by rice breeders. As indicated in a recent review, outcrossing between herbicide-resistant or non-resistant rice cultivars and other rice cultivars decreases rapidly with separation distance. Outcrossing is frequently undetectable at distances greater than 2 m, and seldom detectable at distances greater than 10 m. In contrast, outcrossing between a prolific pollen-producing rice and perennial wild rice (*O. rufipogon*) in Asia has been as high as 2.2% and was detectable at 43 m. This result is another reminder that a lack of detectable outcrossing at distances of 10 or 20 m in small-scale experiments does not necessarily equate to zero outcrossing.

In order to minimize seed mixing and outcrossing between U.S. certified rice fields, seed laws have traditionally required a minimum isolation distance of 5 to 6 m between different drill-seeded cultivars (precise seed placement) and 30 m between aerially-seeded cultivars (imprecise seed placement). Ultimately, pollen confinement regulations must be a compromise between restrictions that guard against extremely rare biological events (i.e. outcrossing over great distances) and practical necessities of crop management and economics. A realistic accommodation of these competing interests will combine both scientific and public policy considerations. In any event, the established limits must be measurable and verifiable using realistic and affordable methods of detection.

Opportunities for Confinement of Rice

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Abstract

The biology of cultivated rice provides many safeguards that prevent gene flow and the establishment of feral rice populations (OECD 1999). The inherent biological safeguards of cultivated rice include the strong selection for self-pollination in the centuries in which man has domesticated rice and continued to improve the crop in modern times. For example, in the United States, the purity standard for Foundation Class rice seed allows 1 in 10,000 plants as another variety and depends upon isolation distances of 10 ft (3 m) between drill-seeded varieties to achieve this level of purity (AOSCA 2004). Normal practices in rice cultivation, such as the separation of varieties by a roadway or irrigation ditch, provide sufficient isolation distance to prevent cross-pollination between different rice varieties.

No other outcrossing studies of this scale were undertaken until the advent of herbicide tolerant rice, which enables rapid screening of large numbers of seed. Two key studies completed in the United States at the rice research stations in Louisiana and California (Fischer *et al.* 2004) has yet to be published in peer-reviewed journals. However their findings are in line with work published from other regions of the world (Gealy *et al.* 2003; Messeneguer *et al.* 2001) and remain in agreement with Beachell *et al.* (1938). In Louisiana, hybrids were only detected between adjacent plants; none were detected at distances out to 21.5 m from the pollen source. In California, none were identified beyond 1.8 m; the maximum sampling distance was 16.9 m.

Today, U.S. rice production is structured to meet the needs of specific markets based upon grain type and quality, and as a consequence, is able to supply a variety of markets for special-use rice (USA Rice Federation annual report of rice distribution patterns). Market segmentation is accomplished by regional production, grain handling, and milling facilities. For the case of special rice varieties that are sources of high value proteins, the protocols call for closed production and handling systems far removed from commodity rice production. The confinement of high value protein rice varieties to dedicated farms and equipment, plus the grinding of seed before the raw product is moved to purification facilities, are more than adequate to keep these enterprises separate from the production of commodity rice. A program to monitor for volunteers and the use of distinctive rice varieties (grain and plant habit are very different for U.S. commodity rice) provide added assurance.

Taken together, these opportunities for confinement, some from nature and some from man, make rice an ideal crop for the production of high value proteins in the U.S.

Summary of Presentation: Opportunities for confinement of rice

The biology of cultivated rice provides many safeguards that can be useful for the development of confinement strategies. The self-pollinated nature of the crop is reinforced by design of the flower. The rice floret protects the stigma from outside pollen by several features. Each floret opens only once in the mid-day hours. Within the floret, the anthers are above the stigma and the timing of dehiscence is just before the glumes separate to open the floret. The result is that the

stigma surface is covered by self pollen before pollen from another source is available. If pollen successfully lands on the stigma of another rice plant, it must compete with all the self pollen in a race to reach the egg first. Only one grain of pollen will fertilize and form the embryo. The genetic differences of the parents can result in infertility by several mechanisms including embryo abortion and infertile seed. The characteristics of the pollen provide more biological barriers to gene flow. A successful cross pollination in nature must have coincident flowering, as the life of rice pollen is very short (1-9 min depending upon humidity). It is difficult for rice pollen to travel over distances as the pollen grains are heavy; they fall down and are prone to desiccation in the wind.

In 1992, the World Bank, Rockefeller Foundation, and USDA sponsored a consultation on rice biosafety. The international consultation brought together members of the rice research community and regulatory agencies representing the rice producing countries of 15 nations. The proceedings were published as a white paper to provide biosafety guidance for rice biotechnology (Clegg *et al.* 1993). Acknowledging the reproductive characteristics of domestic rice, the Rice Biosafety consultation concluded that the isolation conditions for "contained" field work would not be especially demanding. The International Rice Research Institute (IRRI) uses 10 m isolation to avoid pollen contamination in its breeding work. IRRI has conducted field studies collecting seed at 20 m and 40 m from a pollen source and found no evidence of gene flow. The symposium recommended 20 m for cases were wild relatives might be present, and 10 m to avoid cross-pollination in normal breeding cases. It is recognized that in the case of hybrid breeding, where male sterile lines are used, the isolation requirements are more demanding.

Experience in the United States for the production of certified rice seed and studies directed to identify isolation distances to maintain seed purity is instructive for setting of confinement parameters. In the early part of the 20th century, USDA established four rice breeding stations. One of the first coordinated actions at the stations was the establishment of the isolation conditions necessary between rice of different types to prevent cross pollination and thus maintain seed purity for their breeding and seed production programs (Beachell 1938). A glutinous endosperm marker gene allowed visual screening of the seed. Seed samples only needed to be dehulled and examined on a back lit table to observe the presence of a non-glutinous endosperm in a glutinous background. Four varieties were paired with germplasm of similar maturity containing a glutinous marker. The four USDA rice stations, located in Beaumont, Texas; Stuttgart, Arkansas; Crowley, Louisiana; and Biggs, California participated, each planting the same variety pairs over a period of 4 to 6 yrs. Seed was harvested from distances of 1, 2, and 3 ft (0.3 m to 1 m). The crossing range was from 0-3.4%, with the mean of all the stations being 0.45%. The California station, with its relatively higher temperature and lower humidity, recorded a mean of 0.16%.

No other outcrossing studies of this scale were undertaken until the advent of herbicide tolerant rice, which enables rapid screening of large numbers of seed. Two key studies completed in the United States at the rice research stations in Louisiana and California (Fischer *et al* 2004) have yet to published in peer-reviewed journals. However, their finding are in line with work published from other regions of the world (Gealy *et al* 2003; Messeneguer *et al* 2001) and remain in agreement with Beachell *et al* (1938). In Louisiana, hybrids were only detected

between adjacent plants. Samples were tested from distances out to 21.5 m from the pollen source. In California, no hybrids were identified beyond 1.8 m; the maximum sampling distance was 16.9 m. The researchers in these two studies provided their data for consideration by the workshop participants.

In a collaborative effort, the Louisiana State University AgCenter Rice Research Station in Crowley, LA (field study completed by Steve Linscombe and Xueyan Sha) and the Texas A&M/USDA Rice Research Station in Beaumont, TX (lab phase completed by Shannon Pinson and Faye Seaberg), planted herbicide-tolerant rice to measure pollen dispersal and outcrossing. The field design used a central plot, 6 by 6 m, seeded with 90% pollen donor, LL401, a medium grain variety, tolerant to glufosinate herbicide (genetic locus, LLRICE62), and 10% pollen receptor, the long grain variety Cypress. The central plot was surrounded by Cypress. The distance from the edge of the central to the edge of the Cypress block was 21.5 m (70 ft). Flowering was optimal for outcrossing. Fifty percent of the flowering of the donor herbicide-tolerant variety was 3 days before the recipient, conventional variety. Additional pollen donors were transplanted later to extend the pollen supply. At maturity, 50 panicles of Cypress were sampled at the border of the central plot and 2.3, 4.6, 9, 13.8, 21.5 m in 8 directions. Samples of medium grain rice were taken to serve as positive controls in the laboratory tests. In Louisiana and Texas, certified seed isolation distance is 4.6 m (15 ft).

Outcrossing was scored by screening for seedlings that could survive herbicide treatment in a lab bioassay developed by USDA researchers, Pinson and Seaberg. The bioassay required seedlings to germinate and survive in a 0.1% solution of commercial Liberty® herbicide (20% glufosinate). Two types of tests were conducted; germination of intact panicles and germination of seeds from the bulk samples which combined seed from 50 panicles, which were then sub-sampled for the survival screens.

The group tested 820 intact panicles (74,689 seed) for germination in a 0.1% solution of commercial Liberty® herbicide (20% glufosinate). Intact panicles of Cypress plants were sampled from the center and the border of a mixed planting block, and at distances of 2.3, 4.6 and 21.5 m. In addition, 80,000 bulk seed were tested in lab bioassay from the extreme (21.5m) and the border samples. In all >120,000 germinating seeds were screened. The 20 survivors of the lab bioassay (all from the mixed planting plot) were established in the greenhouse for further testing. Leaf samples of the 20 survivors in the bioassay were tested using a Polymerase Chain Reaction (PCR) protocol designed to discriminate between plants which are either homozygous, wild type, or hemizygous (hybrid) for the LLRICE62 genetic locus (PCR performed by Wuzi Xie, Bayer CropScience). Of the 20 plants, 6 were confirmed to be the result of outcrossing, 1 was confirmed to be LLRICE62, and the remaining were Cypress plants.

Surviving seedlings were confirmed with a foliar application of glufosinate herbicide and a PCR test designed to detect hybrids of LLRICE62. No evidence of pollen dispersal beyond the central plot was detected and no cluster of pollination was evident in the intact panicle assay (~70,000 seeds from the sampling distances near the pollen source). Bulk seed testing (10,000 seeds from each of the 8 sampling distances) found no survivors outside the central plot, mixed planting. Of

the approximately 117,000 germinating seeds included in the screen, only 6 hybrids were confirmed.

Seed germination lab bioassays have their limitations, including dependency on difficult-to-control conditions, space (e.g., chambers) and labor. In Bayer's experience, seed germination results can be ambiguous and require a trained eye for evaluations. However, they do allow testing without the need for environmental release, sometimes a problem for genetically engineered rice. Field screens can handle one million seed and give a clear indication of survivors. In either case, a second step to confirm the hybrid nature of the survivors is required.

In research completed at University of California-Davis (Fischer *et al.* 2004) and described in a thesis (Cheetham 2004), an extensive design was used to provide a high confidence that outcrossing, as low as 0.001%, could be detected. In California, a two year study used a field survival assay, and again survivors were confirmed by PCR. Following 2 seasons of study, no outcrossing was detected beyond approximately 2 m. Where hybrids were detected, the range of outcrossing was 0.01 to 0.4%. In excess of 1.8 million seed were screened (188 samples of 10,000 seed each). The author's reason that if no outcrossing could be demonstrated beyond 1.8 m, a reasonable isolation would be the width of a farm road (6.2 m), thus providing adequate distance to prevent pollen dispersal and a clear indication for field operations.

Contributors of data from the two U.S. studies:

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Today, U.S. rice production is structured to meet the needs of specific markets based upon grain type and quality, and as a consequence, is able supply a variety of markets for special-use rice (USA Rice Federation annual report of rice distribution patterns). Market segmentation is accomplished by regional production, grain handling, and milling facilities. The predominant rice type of the southern region's rice production is long grain, while the key markets for California rice are "Calrose" type, a medium grain. However, in addition to these two main markets, there are many regional and specialty rice markets. The production of specialty rice for demanding markets is not a new idea for the rice industry. Certification systems are already in place to support these high value markets. For example, in California and Arkansas, there is identity preserved contract production for sushi rice destined for demanding exports markets. In most rice producing states, there are growers of organic or specialty rice for small domestic mills and regional markets that are neighbors to commodity rice produced by traditional agronomic systems.

USDA market and crop reports follow the production of rice by region and type. Nathan Childes of USDA's Economic Research Service, is responsible for following rice production and projecting markets. His work is published in the Rice Outlook report (www.ers.usda.gov/publications). Because of shifts in the world market and changes in regional costs for the production and transportation of rice, there are distinct regions in the U.S. which could produce rice and are not currently doing so. These rural areas could benefit from the introduction of new crops and can be sufficiently isolated, both in distance and infrastructure, to allow co-existence of many types of rice production in the United States.

For the case of special rice varieties that are sources of high value proteins, the protocols reviewed by the USDA call for closed production and handling systems isolated from commodity rice production. A separate seed production system with delivery to the dedicated farm prevents the high value protein varieties from entering food rice production areas. The confinement of high value protein rice varieties to dedicated farms and equipment, plus the grinding of seed before the raw product is moved to purification facilities, are more than adequate to keep these enterprises separate from the production of commodity rice. A program to monitor for volunteers and the use of distinctive rice varieties (grain and plant habit are very different for U.S. commodity rice) provide added assurance.

Taken together, these opportunities for confinement, some from nature and some from humans, make rice an ideal crop for the production of high value proteins in the United States.

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Confining Safflower Pollen During Regeneration of Germplasm Seed Stocks

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Introduction

Safflower (*Carthamus tinctorius* L.) is an ancient crop with many uses (Li and Mündel 1996). Traditionally it is grown for its flowers, which are used as a dye, food coloring, flavoring, and medicinal purposes. Seeds are now mostly used to produce bird feed and a high quality edible and industrial oil (Knowles 1989). Potential expanded uses include production of transgenic pharmaceuticals (McPherson *et al*. 2004) as a biofuel, and for specialty oil types to improve the human diet (Valasco and Fernández-Martínez 2004).

The USDA-ARS Western Regional Plant Introduction Station (WRPIS) at Pullman, Washington is part of a national network of about 20 germplasm repositories that collectively make up the USDA-ARS National Germplasm System. The purpose of this system is to acquire and maintain plant genetic resources for agriculture. The WRPIS maintains the national collection of safflower germplasm, which currently includes more than 2300 accessions. These accessions, representing germplasm from more than 50 countries, are available upon request without charge to scientists worldwide.

Germplasm accessions received at genebanks usually require an initial seed increase or regeneration before the quantity and quality of seed are adequate for storage and distribution to users for research purposes. The regeneration process for safflower must consider a number of factors to minimize genetic changes potentially resulting from the process. These include the following topics, which are reviewed in this paper:

- Safflower pollination biology
- Reported outcrossing rates and genetic marker systems
- Outcrossing agents
- Outcrossing to wild relatives
- Pollen confinement in regeneration of genetic stocks

Safflower Pollination Biology

Safflower is an herbaceous annual of the family Compositae (Asteraceae). It develops from a rosette through stem elongation, branching, flowering, and seed maturity. Branching can vary widely, resulting in 15 to 150 flowering heads (capitula) per plant (Claassen 1950). Head diameter also varies widely (1 to 4+ cm), as does floret number (20 to 100) per head (capitulum). Both branching and head diameter are under environmental and genetic control.

A single plant may flower over a 10- to 40-day period owing to the different developmental stages of the heads from earlier or later branches. For a single head, the flowing process starts at the margin of the head and proceeds centripetally over 3-5 days (Claassen 1950; Knowles 1958).

The florets (flowers) of a head are tube-like, with the style enclosed by five fused anthers that are attached at the base by free-standing filaments (Fig. 1). Self-pollination occurs when the anthers dehisce before the style elongates with the stigma pushing through the pollen mass. This is the most common type of fertilization in safflower. However, dehiscence can occur after the style elongates so that the stigma has passed though the anther tube without effecting fertilization. When this has happened insect pollinators may transport pollen from one plant to another resulting in cross-pollination (Claassen 1950; McGregor 1976). Rubis et al. (1966) developed a thin-hull safflower type with delayed anther dehiscence resulting in functional male sterility.

Outcrossing Rates and Genetic Marker Systems

The majority of safflower plants are predominantly self-fertile by the process described above. Nevertheless, natural outcrossing can vary from 0 to 100% depending on the environmental conditions, genotype, and insect activity (Claassen 1950). Many species of pollinating insects are usually active in blooming safflower fields (Knowles 1958). Crossing rates measured by Claassen (1950) showed a wide range, with an average between 15 and 20% (Table 1). Studies in India referenced by Knowles (1958) gave similar results.

A weakness of outcrossing research to date has been the reliance on flower color genetics for markers to determine outcrossing rates. Flower colors in order of dominance are yellow, orange, red, and white (Claassen 1950), but possible bee preference to different flower colors has not been addressed. Other genetic marker systems could potentially be used including seed hull characteristics (white, StpStp, is dominant over recessive gray strip, stpstp), and oil fatty acids (high linoleic acid oil, OLOL, is dominant over low oleic, olol). Co-dominate molecular markers could also prove valuable; isozyme variation has been reported by Carapetian (1994).

Outcrossing Agents

Pollinating insects, especially honey bees (*Apis mellifera* L.), are frequently observed on safflower. Forty species of native bees were collected on safflower blossoms in Arizona, but populations were small compared to honeybees (Butler *et al.* 1966). In work completed in Ottawa, Canada, Boch (1961) reported that honey bees were by far the predominant visitors to safflower, collecting both nectar and pollen. This work and Eckert's (1961) suggested that in some cases honey bee colonies placed in fields may improve seed production.

Claassen (1950) showed that wind pollination was unlikely and outcrossing in safflower appears to be caused almost exclusively by insects. Some wild relatives, such as *C. oxyacantha* M. Bieb. (or *C. oxyacanthus*) (jeweled distaff thistle), have high outcrossing rates and self-incompatibility systems that ensure high levels of outcrossing.

Outcrossing to Wild Relatives

Traditionally about 25 species have been included in the genus *Carthamus* L. Ashri and Knowles (1960) divided *Carthamus* into several sections based on chromosome number (cf. Vilatersana *et al*. 2000b, 2005) (Fig. 2). Many of these species are weedy, such as the noxious *C. oxyacantha*, but the wild species also represent a rich source of genes for safflower improvement.

The 6 species with 12 chromosome pairs comprising the proposed BB genome in Fig. 2, tend to cross readily (Knowles 1980). These include safflower (*C. tinctorius*), *C. persicus*, *C. oxyacantha*, and *C. Palaestinus.* Pollen-mediated gene flow between safflower and these species through natural crossing is probable if grown in close proximity. *C. persicus* Willd. (*C. flavescens* auct. non Willd.), from Turkey, Syria, and Lebanon to Iran, is entirely self-incompatible. *C. oxyacantha*, indigenous from Iraq to Pakistan, is a mixture of self-incompatible and self-compatible types. *C. palaestinus*, found in Iraq, Jordan, and Israel, is a self-compatible species.

C. nitidus Boiss. also has 12 chromosome pairs and is found in the Middle East (Syria to Egypt), but crosses with safflower with difficulty giving sterile progeny, and is considered isolated in the genus and within section *Atractylis* (Vilatersana *et al*. 2005). Species with other than 12 pairs of chromosomes are less likely to cross with safflower, and if they do, the progeny are usually sterile (Vilatersana *et al*. 2000a). *C. divaricatus* Bég. & Vacc. (section *Atractylis*) with 11 chromosome pairs, found in Libya, may cross with safflower but gives sterile hybrids. The self-compatible *C. lanatus* L. (section *Atractylis*) with 22 chromosomes pairs, may cross with species with 12 or 10 chromosome pairs but the progeny will be mostly sterile; its F_1 crosses with safflower are sterile (Knowles 1980).

Even though safflower may cross with several other species, especially most species with 12 chromosome pairs, the distribution of wild *Carthamus* across North America is quite limited. McPherson *et al.,* (2004) concluded that New World locations could easily be found in which weedy *Carthamus* would be absent, eliminating the risk of gene flow between transgenic safflower and naturalized relatives.

Pollen Confinement in Regeneration of Genetic Stocks

As mentioned, germplasm accessions received at genebanks usually require an initial seed increase or regeneration before the quantity and quality of seed are adequate for storage and distribution to users for research purposes. After regeneration, seed longevity should be promoted by cold-storage conditions that preserve seed viability as long as possible to minimize future regenerations. But after the initial stock of regenerated seed is depleted, or if it has low germination, and if viable original seed is no longer available, the regeneration sample must be used to grow plants to replenish seed stocks.

As many as 300 or more safflower accessions may be regenerated at the WRPIS each year. The two most important considerations are plant populations and isolation. Adequate populations are needed because most safflower accessions are not highly inbred as many cultivars are. Thus, populations must be adequate to ensure a high probability of maintaining alleles that occur in relatively low frequency. A population of 100 plants will likely ensure this (Johnson *et al.,* 2004). To prevent genetic contamination by pollen, screen cages or tents are constructed that prevent insects from transferring pollen from one accession to another (Fig. 3). Since most safflower is self-compatible, insect pollinators do not normally need to be added to the cages.

Some important features of the system shown in Fig. 3 are that relatively high population numbers are utilized, plants develop normally without crowding, and the seed quality and germination from these cages have been shown to be high.

In some cases insect pollinators are prescribed. This can be for special genetic material, such as the thin-hull, male-sterile lines developed by Rubis *et al.* (1966). This material is functionally male sterile based on the late dehiscence of anthers. To properly maintain these lines insect pollinators are needed. A honey bee colony placed inside the cage ensures crossing within but not among accessions (Fig. 4). Honey bees would also be needed in wild-type materials that have self-incompatibly systems that reduce or prevent self-pollination.

Summary

Seed regeneration of insect-pollinated plant species requires an understanding of pollination biology, plant reproduction mode, outcrossing potential, and the species and behavior of pollinating insects. Safflower is normally self-compatible and self-pollinating. However, important natural outcrossing can occur at variable rates. A wide array of native and introduced insects can pollinate safflower but honey bees are the most common. Screen cages that eliminate the potential for pollination among accessions are effective for seed regeneration. Normally, pollinators are not needed within the cages as safflower will naturally self-pollinate. However, in some cases the addition of pollinators is prescribed for producing seed of special genetic material or wild, self-incompatible species. Outcrossing of safflower to wild relatives with the same number of chromosomes in section *Carthamus* and *C. creticus* can be expected if grown in close proximity without cages. Caging on a field scale would be impractical. But large areas in New World locations could easily be found in which weedy *Carthamus* would be absent, eliminating the risk of gene flow between transgenic safflower and weedy relatives (both feral safflower and the other introduced wild species).

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Confinement of Plant-Made Pharmaceuticals - Gene Flow via Seed and Volunteers of Safflower (*Carthamus tinctorius*)

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Gene flow is the movement of genetic information from one population to another. Gene flow occurs via pollen and seed dispersal, and occurs spatially and/or temporally. Pollen mediated gene flow tends to occur on a relatively small scale, while seed distribution and mechanical seed handling may result in gene flow over a much larger distance and could influence agriculture at a national and international scale. While considerable research has been directed at pollen mediated gene flow, there is less research effort directed at seed mediated gene flow.

Safflower is being adopted as a platform for the production of plant made pharmaceuticals (PMP) with a glufosinate resistance (*pat*) gene as a selectable marker (Lacey D. J. *et al.* 1998). While safflower is grown on over 80,000 ha in the United States, less than 1,000 ha are grown in Canada. The Canadian safflower crop is grown exclusively for bird food under contract production. Because of the limited acres, the ability to separate safflower crops by large distances and the end use and segregation of feed and PMP safflower may be more achievable in Canada. Additionally, wild or weedy relatives have not been reported in Canada as they have been in the United States (McPherson *et al.* 2004). Therefore, there are few concerns that PMP genes will move to related species in Canada. Seed movement remains a potential mechanism for gene flow in safflower in Canada.

Crop seeds may be dispersed pre- and post-harvest. Shed crop seed has several potential fates. Seeds may or may not persist in the soil seed bank. They may be removed through predation, moved by animals, wind, water or production equipment, or germinate to produce volunteers. Some of these volunteers may produce seed within rotational crops in subsequent growing seasons.

Safflower seed does not usually shatter prior to harvest, but if grasshopper or sclerotinia levels are high, capitula may drop intact. Both individual seeds and capitula may be lest in the field during the harvest operation. Crop seed losses from the harvest operation are generally variable. Harvest losses for safflower of 5% have been reported (Smith 1996).

Seed movement and predation by small animals has been observed in small plot experiments. In these experiments small animals have moved seed up to 30 meters McPherson (unpublished data). In addition, within post-harvest areas volunteers have observed up to 240 meters from the initial trial site. These seeds may have been dispersed by wind or animals. Seed movement by birds has not been examined in studies to date. To determine over-wintering potential of safflower, seed was spread in small plots at a density of 500 seeds m^{-2} in the fall. Less than 1% remained on or in the soil to produce volunteers in the spring. Most of this seed was consumed or moved by small animals. However, these results may have been influenced by the experimental scale. Surveys of commercial fields have identified moderate levels (1-2 plants m^2) of safflower seedlings in the year following safflower production (McPherson, unpublished data). Predation in commercial scale fields may be influenced by the quantity of seed available.

A 5% seed loss at harvest represents 117 to 168 kg ha^{-1} of seed (Mundel *et al.* 2004). In addition, spiny bracts around the capitula may protect the seed from predation when heads are lost without releasing the seeds either pre- or post-harvest.

Seed persistence studies were also conducted to examine the longevity of safflower at the soil surface and buried at two depths. Seed was placed in mesh bags to prevent predation and examined at intervals over 3 years. Seed persistence was influenced by depth, year, and site. Seeds left at the soil surface persisted and were viable longer because they did not germinate. Most buried seed germinated and therefore, did not persist after 2 years (McPherson, unpublished data). Previous studies have shown that safflower does not have prolonged innate dormancy and lacks secondary dormancy.

Admixture, the co-mingling of one crop with another, can occur through various mechanisms. Volunteer safflower may produce seed in subsequent food/feed crops, and both vegetation and/or seed containing the PMP could be harvested with the crop. Studies have been conducted over 2 years at three sites to quantify fecundity of safflower under normal cropping rotations. Preliminary results suggest that untreated volunteer safflower in canola and barley crops can produce seed leading to admixture at low levels (McPherson, unpublished data). Canola and barley are competitive, short season crops, whereas the safflower variety currently used as a PMP platform requires significantly more growing degree days to mature. Volunteer safflower seed may be immature at crop harvest and not be viable. In addition, they may not be removed from the immature capitula by the harvest operation. However, the presence of low amounts of immature seed or vegetative residue containing the PMP may be detectable by sensitive analytical methods, such as polymerase chain reaction (PCR) or immunocytochemical assays.

Herbicides applied in-crop reduced the number of safflower plants, safflower biomass, and the number of viable safflower seeds recovered from canola and barley at harvest (McPherson, unpublished data). Herbicide efficacy varied in canola, but in barley it was not significantly different regardless of the herbicide used. In canola, glyphosate applied to glyphosate-resistant canola was more effective in controlling safflower than imidazolinones used on imidazolinone-resistant canola. There was no control of safflower in conventional or glufosinate resistant canola. Therefore these crops should not be grown following glufosinate-resistant safflower. In barley in-crop applications of bromoxynil/MCPA and 2, 4-D reduced safflower significantly relative to untreated controls. While some herbicides and crop competition reduced volunteer safflower fecundity, they were not sufficient to ensure that no safflower seeds were present in seed of the harvest crop.

These studies will be used to develop best management practices to facilitate confinement of PMP safflower on a commercial scale. Control of diseases and insects during the growing season will reduce seed loss prior to harvest and increase crop yield. During harvest, loss of seed should be minimized to reduce volunteers in subsequent years. Following the harvest of safflower, tillage will reduce biotic and abiotic movement of seeds. Furthermore, burial of the seed by tillage will decrease the persistence of safflower in the seed bank. The year following the production of safflower, chemical fallow may be the best option to deplete the seed bank.

Furthermore, subsequent crops should be vigorous and in-crop herbicides should be used to reduce the risk of safflower volunteers and seed admixture.

Experimental results described are preliminary and further studies are currently in progress. Additional experiments examining seed persistence over time have been initiated with and without burial at two sites. Studies of outcrossing distance and frequency between safflower crops have been conducted at three diverse locations in Alberta, Canada and the results are being analyzed. The admixture potential of PMP safflower in subsequent crops has been conducted over 2 years at three sites. Surveys of commercial fields and quantification of safflower volunteerism and fecundity have been conducted. Finally, quantitative PCR and western blot techniques are being investigated to detect and quantify PMP safflower in conventional safflower.

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PMP Safflower Confinement at SemBioSys

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SemBioSys is a Canadian plant made pharmaceutical (PMP) company based out of Calgary, Alberta that has existed for 10 years and consists of approximately 50 employees. This company originated at the University of Calgary as a spin-off from the molecular botany lab of Maurice Moloney. Currently, SemBioSys has several products under development, which are generated from its proprietary safflower seed platform.

Safflower is appropriate for the production of PMPs based on biological characteristics that allow for a decrease in gene flow. These include the crop's bias towards self-pollination and low level of pollen transfer by wind. Gene flow from pollen movement would primarily occur from insect transportation. Since the presence of any appreciable moisture initiates seed germination, there is very low seed dormancy. Another biological factor is the fact that safflower possesses a longer than usual growth season, often allowing it to be the last crop to be harvested during a season. Agronomic practices can also be very effective in limiting the level of safflower gene flow and admixture. Such practices include:

- PMP growth outside of existing popular safflower production areas.
- Planting, cultivation, harvest, and storage of the crop with dedicated and/or use-restricted equipment to result in an identity-preserved style of production.
- Use of buffer zones in which any seed spills or movement can be easily detected and kept out of food and feed crops.
- In-season and post-season monitoring of plots, checking for any unwanted effects or plant volunteers.
- Post harvest land restrictions, such as leaving the plot fallow one season followed next by a cereal crop (cereal crops discourage safflower volunteering due to their herbicide regime).
- Seed and plant chain of custody recording to ensure that all material is accounted for.

In addition to these practices, it is important to note that there are fewer than 200,000 acres of current N. American safflower production. The majority of this production is sold to the birdseed and oil markets, residing almost exclusively in California. This low acreage enables the potential for vast isolation distances, thus further limiting unwanted gene transfer from PMP safflower to the commodity crop.

At SemBioSys, the production of PMPs and similar proteins is directed specifically to the seed because they represent natural storage organs for plants allowing proteins to remain highly stable for long periods of time when stored in a dry environment. This allows the stockpiling of the protein product within seed. In addition, the seed can be handled, shipped and stored in a variety of traditional, well-understood manners through the use of conventional combines, seed cleaning equipment, storage bags, crates, and bins. Use of these traditional handling and storage systems means that the production processes are common, well-known and don't need to be invented and

tested. SemBioSys' platform technology, the Stratosome™ Biologics System, further facilitates PMP production in seeds. This system helps offset high purification costs through the targeting of desired proteins to the seed oilbodies by genetic fusion to the oilbody protein, oleosin. The ease of removal of engineered oilbodies from the rest of the seed is the backbone strength of this system.

In order to achieve the full benefit of PMP production in safflower, the crop must be grown outdoors in an uncontained environment. This practice can result in gene flow concerns leading to the need for rigorous compliance with confinement procedures (explained below) that ensure segregation from food and feed, sharing validated methods of detection analysis, and full cooperation with inspection agencies. Development of an industry code of conduct that includes suitable compliance training programs and adherence to the BIO-developed Containment Analysis and Critical Control Point (CACCP) approach is also recommended. Like the food industry's Hazard Analysis Critical Control Point (HACCP) system, CACCP covers numerous elements of the PMP production process where containment might be an issue and assesses these so that unwanted escapes are minimized. The system covers all levels of PMP production from genetic construct development and quality control, preparation of transgenic plants and early indoor plant production, all the way through to open field growth, harvest, plant processing, and finally protein extraction. It then evaluates each of these processes based on:

- Analysis of Containment Concerns;
- Determination of Critical Control Points-Physical, Chemical, Genetic;
- Establishing Critical Limits;
- Establishing Monitoring Procedures;
- Establishing Corrective Measures;
- Establishing Verification Procedures; and
- Establishing Record Keeping and Documentation Procedures.

Throughout these processes, elements such as personnel training, contingency planning, performance verification, and site security are referenced. Using the CACCP approach in conjunction with the agronomic options listed above, SemBioSys has comprised a field guide that contains SOPs and forms that minimize the impact our PMP safflower will have on the environment and food and feed chains. These procedures cover the detailed methods that govern every aspect of outdoor field production, including site selection, training, cleaning equipment, shipping of seed, etc. Other aspects covered by CACCP that occur before and after field production are taken care of in SemBioSys' quality management system.

DISCUSSION QUESTIONS

BREAK-OUT GROUPS BY CROP TYPE AND CROP-OF-INTEREST

I. Wind Pollinated Crops (e.g., corn)

II. Self-Pollinated Crops (e.g., rice)

III. Insect-Pollinated Crops (e.g., safflower)

BREAK-OUT SESSION TOPICS AND QUESTIONS

A. Pollen-Mediated Gene Flow Confinement

During Break-Out Session A, scientific information related to confinement of pollen-mediated gene flow from crop field tests will be discussed. First, participants will establish the basic biology of pollen for the crop-of-interest and the pollen recipient (or donor) taxonomic groups of concern in the United States, that is, other crops, weeds, or/and wild relatives. Next, the strengths and weaknesses of different confinement methods that are being used or being considered for use will be covered. Methods to be discussed include the following: physical confinement methods, (e.g., spatial isolation, border rows, wind breaks, bagging, flower removal, etc.); temporal confinement methods (e.g., planting date, harvest date, growing degree days, crop variety, etc.); and biological confinement methods (e.g., male sterility, chloroplast transformation, etc.). Participants will evaluate each method in terms of effectiveness, variability under different conditions (e.g., field test scale, weather conditions, etc.), durability (e.g., ability to withstand human error, environmental variability, etc.), and feasibility (e.g., time and cost to implement, etc.), among other characteristics. Modeling of pollen mediated gene flow, and experimental methods to measure and monitor pollen-mediated gene flow will also be deliberated on. Finally, participants will be asked to summarize their discussion by identifying emergent principles of confinement of pollen-mediated gene flow from the crop-of-interest and from crops with related mechanisms of pollination, and research needs.

1) For the crop of interest, what are the pollen characteristics under typical field conditions that influence pollen mediated gene flow (e.g., pollination mechanism(s), duration of pollen viability)? Is pollen mediated gene flow influenced by variation un these characteristics under different conditions?

2) Besides confinement of pollen-mediated gene flow from the field tested crop to other crop plants of the same species, does the crop hybridize with wild or weedy relatives in the U.S.?

a) Do wild or weedy relatives of the crop occur in the U.S.?
b) Is there compatibility in the field between the crop and relatives (i.e, compatibility of time of flowering, pollination mechanisms)?

c) What is the spatial overlap of crop and relative (e.g., within field vs. external to field)? In what regions?

d) What other factors influence incidence of wild and weedy relatives and hybridization with the crop- of-interest (e.g., agricultural management conditions that influence hybridization or weedy relative incidence)?

e) Does incoming pollen flow (crop as female) pose a risk of breach of confinement for the gene being tested, or is outgoing pollen flow (crop as male) the only concern (e.g., possible detrimental consequences of hybrid seed formation on subsequent years of field testing)?

3) For the crop-of-interest, what pollen-mediated gene flow confinement measures are being employed or considered? In cases in which temporal confinement is a possibility, how useful and available is information on the growing degree days or other similar indicators?

4) For each of the pollen-mediated gene flow confinement measures in use or being considered, what is the effectiveness of the confinement measure under typical field conditions? How has effectiveness been determined (i.e., what data support a particular level of effectiveness)? How can pollen-mediated gene flow confinement be tested or monitored for in situ?

5) How does each confinement measure vary under different conditions (e.g., distance, environmental conditions, variety being grown, scale of plot, other variables)? What are the possible mechanisms of confinement break down (e.g., human error, environmental variability, biological variability, use over multiple seasons), how likely are these, and how can break down be mitigated? If applicable, are there differences between confinement achieved with each measure with gene flow to weedy or wild relatives versus other crops?

6) What is the state of the art for modeling of pollen-mediated gene flow, the effects of confinement measures, and other relevant variables (e.g., extrapolation of pollen-mediated gene flow beyond measured distances, effects of varying source and sink sizes, effects of combining different pollen confinement strategies)? Do any results from modeling impact previous responses?

7) How feasible is each confinement measure and how does feasibility vary with conditions (e.g., scale)?

8) What are the main ideas that emerge regarding confinement of pollen mediated gene flow from the crop-of-interest? Which steps are most critical for achieving pollen confinement? Which are most likely to break down? Does combining different methods of pollen confinement always lead to additive effects? (e.g., Does use of two methods that each account for 90% confinement, leads to a total of 99% confinement?)

9) Which confinement principles are generally applicable to the crop class? What factors do not apply generally and why?

10) What are outstanding research needs to better inform confinement measures for pollen-mediated gene flow for the crop of interest and similar crops?

B. Control of Volunteer Plants and Confinement of Seed-Mediated Gene Flow

During Break-Out Session B, scientific information related to control of volunteer plants and confinement of seed-mediated gene flow from crop field tests will be discussed. First, participants will establish the basic biology of seeds and, if applicable, vegetative propagules for the crop-of-interest. Next, the strengths and weakness of methods to control seeds and residual volunteer plants within *former field test plots will be discussed. Methods to be covered include physical volunteer control methods (e.g.,tillage protocols, herbicide and fire use, flooding, etc.), biological volunteer control methods (e.g., seed sterility, induced expression, etc.), and volunteer monitoring protocols. Participants will evaluate each method or protocol in terms of effectiveness, variability under different conditions (e.g., field test scale, soil conditions, etc.), durability (e.g., ability to withstand human error, environmental variability, etc.), and feasibility (e.g., time and cost to implement, etc.), among other characteristics. Subsequently, mechanisms of seed dispersal outside of the field test plots and ways to mediate it will be discussed, including unintentional co-mingling of regulated seed with other seed, equipment-mediated dispersal, and animal-mediated dispersal. As with previous topics, each confinement or control method or protocol will be evaluated in terms of effectiveness, variability, durability, and feasibility. In addition, modeling of volunteer formation and seed mediated gene flow, and experimental methods to measure and monitor seed-mediated gene flow will also be deliberated on. Finally, participants will be asked to summarize their discussion by identifying emergent principles of confinement of volunteers and seed-mediated gene flow from the crop-of-interest and from crops with related mechanisms of pollination, and research needs related to seeds and volunteers.*

1) What are the seed germination and dormancy characteristics for the crop of interest under typical field conditions? What factors effect germination and dormancy and in what ways?

2) For the crop-of-interest, what volunteer control measures and monitoring protocols are being employed or considered?

3) For each of the volunteer control measures in use or being considered, what is the effectiveness of the measure under typical field conditions? What levels of effectiveness are associated with different lengths of monitoring time for volunteers and do effectiveness levels differ geographically? What levels of effectiveness are associated with removal of volunteers at different times in the plant life cycle? How has effectiveness been determined (i.e., what data support a particular level of effectiveness)? Is planting of non-transgenic seeds in test strips in the field site an effective way to test volunteer control methods in situ? What are other possibilities for testing volunteer control methods?

4) How does each control measure vary under different conditions (e.g., depth of tilling, soil conditions, scale of plot, subsequent crops, other land use restrictions, variety being grown, other variables)? What are the possible mechanisms of volunteer confinement break down (e.g., human error, environmental variability, biological variability, use over multiple seasons), how likely are these, and how can break down be mitigated? If applicable, are there differences between volunteer control achieved with each measure if gene flow from weedy or wild relatives versus other crop individuals occurs?

5) How feasible is each volunteer control measure and how does feasibility vary with conditions (e.g., scale)?

6) What is the state of the art for modeling of volunteer emergence, the effects of confinement/control measures, and other relevant variables (e.g., modeling of weather effects on volunteer emergence)? Do any results from modeling impact on the previous responses?

7) What are points during the field testing process of seed dispersal or co-mingling (or, if relevant, vegetative propagule dispersal) related with seed handling, equipment, and transportation? How likely are these and what causes them (e.g., mechanical design, storage design, human error)?

8) What are points during the field testing process of dispersal of viable seed (or, if relevant, vegetative propagule dispersal) related with animals, weather, and other non-human factors? What animals may be involved and what are the possible volumes of seeds dispersed, dispersal distance, and likelihood of dispersal to a propagative environment?

9) What are possible mitigation measures/protocols to prevent seed dispersal or co-mingling during handling, or seed dispersal by animals or other factors and what is the effectiveness of each mitigation measure? How has effectiveness been determined (i.e., what data support a particular level of effectiveness)? How can seed confinement be tested or monitored for in situ?

10) How does each control measure for seed dispersal vary under different conditions (e.g., regional variation, other variables)? What are the possible mechanisms of confinement break down (e.g., human error, environmental variability, biological variability), how likely are these, and how can break down be mitigated?

11) How feasible is each mitigation measures/protocol for seed dispersal and how does feasibility vary with conditions (e.g., scale)?

12) What is the state of the art for modeling of seed handling methods and for modeling of seed dispersal by animals and other factors, the effects of control/confinement measures, and other relevant variables? Do any results from modeling impact previous responses?

13) What are the main ideas that emerge regarding confinement/control of seeds and volunteer plants of the crop of interest? Which steps are most critical for achieving

confinement? Which are most likely to break down? Does combining different methods of volunteer and seed confinement always lead to additive effects? (e.g., Does use of two methods that each account for 90% confinement, leads to a total of 99% confinement?)

14) Which of these ideas are generally applicable to the crop class? What factors do not apply and why?

15) What are the outstanding research needs to better inform methods of seed and volunteer confinement for the crop of interest and similar crops?

C. Overall Strategies of Confinement

During Break-Out Session C, participants will be asked to conduct an overall analysis of information and protocols related to confinement of crop field tests. Critical points of confinement during field testing and strengths and weaknesses of various confinement protocols will be identified and compared. Participants will discuss the possibility of positive or negative interactions between different confinement methods under various conditions. Redundancy of methods and other ways to mitigate weaknesses in confinement will be deliberated on. Also, experimental testing and modeling of the field test process and various confinement methods in combination will be covered. Participants will be asked to identify principles of confinement that may be generalized across different stages of a field test and among different crop types.

1) For the overall field testing process, what are the most critical points for pollen, vegetation, and seed confinement?

2) Do measures for confinement or control of different stages of field tests positively or negatively impact the effectiveness, durability, or feasibility of other methods (e.g., do any of the measures to confine pollen affect measures to control seeds?)?

3) What are the strengths and weaknesses of potential combinations of confinement measures, i.e., confinement protocols? Are there essential components that emerge for all stages (e.g., bookkeeping)?

4) How are confinement protocols impacted by various conditions (e.g, field scale, environmental conditions)?

5) What are easier points during the field testing process to build in redundancy to confinement protocols? What are critical points to build in redundancy? Which measures are most easily combined? Besides redundant measures are there other ways of mitigating weak points in confinement protocols?

6) What is the state of the art for modeling of gene dispersal and confinement throughout the field testing cycle? Do any results from modeling impact previous discussion?

7) What are features of a well designed experiment to test confinement strategies? How can confinement of active field test be efficiently and effectively monitored? What are the pros and cons of various monitoring methods and sampling strategies? Which steps of a

field trial would benefit most from in situ measurement/verification?

8) Are there principles of confinement that emerge from discussion of one class of crops (e.g., wind-pollinated) that may be general for confinement of all crops?

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PREDOMINANTLY SELF-POLLINATED CROPS

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