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Minutes

Agricultural Biotechnology
Research Advisory Committee (ABRAC)

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Research Advisory Committee
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Guidelines for Research Involving Planned
Introductions into the Environment of
Genetically Modified Organisms

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BY THE
AGRICULTURAL, BIOTECHNOLOGY RESEARCH ADVISORY COMMITTEE
(ABRAC)

Guidelines For Research Involving Planned Introduction Into The Environment Of Genetically Modified Organisms

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I. Purpose

These Guidelines recommend practices and procedures for the safe conduct of research involving the planned introduction into the environment of certain genetically modified organisms. The Guidelines establish principles for assessing the safety of research with specific organisms and designing confinement to promote safety. They are intended to aid researchers and institutions in the design of safe experiments conducted outside contained facilities.

II. Definitions and Acronyms

II-A. Technical Terms

II-A-1. "Accessible environment" refers to the environment that can be reached by the organism and its progeny if introduced at the research site(s).


II-A-3. "Cellular Microorganisms" refers to microorganisms other than viruses and subviral structures such as viroids. (See also II-A-8.)

II-A-4. "Confinement" refers to that which restrains or limits the spread or survival of organisms and their products in research involving planned introduction of organisms into the environment. (See Section IX.)

II-A-5. "Contained facility" refers to a structure (e.g., a laboratory or greenhouse) which surrounds and encloses the organism to effectively restrict its movement outside the structure, as described in the National Institutes of Health "Guidelines for Research Involving Recombinant DNA Molecules," (Federal Register, May 7, 1986, 51 FR 16958).

II-A-6. "Genetically Modified Organism" is operationally defined as an organism whose hereditary traits have been modified by human intervention using any method that results in the introduction, rearrangement, or removal of genetic material from the genome of an organism.

II-A-7. "Genome" means the sum total of chromosomal and extra-chromosomal genetic material of a specific organism. In the case of a microorganism, it means the sum total of chromosomal and extra-chromosomal genetic material of an isolate and any descendants derived under pure culture conditions from that isolate.
11-A-8. "Managed or natural ecosystems" refers to all plants, animals, and microorganisms, and their interactions, in domesticated and wild environments.

11-A-9. "Microorganism" refers to any organism too small to be seen by the unaided eye. In practice, these organisms are classified in the kingdoms Monera, Protista, and Fungi, and the phyla Chlorophyta and Phodophyta of the kingdom Plantae (as defined by R. H. Whittacker, 1969, "New concepts of kingdoms or organisms", Science, 163:150-160), prions, viruses and subviral structures. These organisms include, but are not limited to, bacteria, protozoa, fungi, mycoplasmas, mycoplasma-like organisms, spiroplasmas, microphytoplanktons, and certain algae. Prions, as well as viruses and subviral structures such as viroids are also considered microorganisms but are classified in a separate taxonomic system.

11-A-10. "Organism" refers to any biological entity, cellular or noncellular, with the capacity for self-perpetuation and response to evolutionary forces.

11-A-11. "Parental organism" refers to the initial organism which is to be the recipient of introduced genetic material or whose genome is to be altered by removal or rearrangement of genetic material.


11-A-13. "Research involving planned introduction into the environment" refers to research outside a contained facility at a designated site(s) with appropriate confinement. (See Section IX.) It does not refer to the deliberate release of organisms beyond designated research sites or to commercial release.

11-A-14. "Safety" or "safe" refers to conditions determined with reasonable certainty to have negligible risk to human health and no unreasonable risk to managed or natural ecosystems.

II-B. Administrative Terms

II-B-1. "ABRAC" or "Agricultural Biotechnology Research Advisory Committee" is a Federal advisory committee that advises the Secretary of Agriculture through the Assistant Secretary for Science and Education on scientific and technical matters concerning biotechnological research, including research involving the planned introduction into the environment of genetically modified organisms.
II-B-2. "Department" refers to the United States Department of Agriculture.

II-B-3. "IBC" or "Institutional Biosafety Committee" is a committee at an institution that provides local expertise in aiding researchers in the use of the Guidelines. (See Section X-B.)

II-B-4. "Institution" refers to any individual, corporation, partnership, association, public or private entity, Federal agency, or other unit which conducts or sponsors research. (See Section X-A.)

II-B-5. "OAB" or "Office of Agricultural Biotechnology" is the office within the United States Department of Agriculture which serves as the point of contact for users of the Guidelines.

II-B-6. "USDA" refers to the United States Department of Agriculture.

III. Scope

III-A. General

These guidelines are intended for research involving the planned introduction into the environment of organisms that have been deliberately modified by alteration of their genome. Use of the Guidelines is not necessary for the following organisms:

III-A-1. Plants that result solely from: (a) selection, natural regeneration or traditional breeding techniques, including hand pollination or other managed, controlled pollination; (b) chemical or physical mutagenesis, and (c) plants that are regenerated from organ, tissue, or cell culture, including those produced through selection and propagation of somaclonal variants, embryo rescue, protoplast fusion, or ploidy manipulation.

III-A-2. Animals that result solely from selection, artificial insemination, superovulation, embryo transfer, embryo splitting, embryo fusion, or ploidy manipulation.

III-A-3. Cellular microorganisms modified in hereditary traits solely by one of the following means:

(a) Chemical or physical mutagenesis.
(b) The movement of nucleic acids using the physiological processes including, but not limited to, transduction, transformation, or conjugation, provided that there has been no directed addition to or
rearrangement of nucleic acids from the nucleotide sequences that are moved.

III.A.4. Microorganisms resulting from deletions, rearrangements, and amplifications, within a single genome, including its extra-chromosomal elements. Rearrangements are translocations and inversions of nucleotide sequences in the genome. This exclusion does not apply if the microorganism is deliberately modified to have (i) increased virulence or toxin production, (ii) significant changes in competitive ability or environmental requirements, or (iii) phenotypic properties that are harmful to humans or would adversely alter the environment.

III.A.5. Organisms modified by the introduction of non-coding, non-expressed nucleotide sequences that cause no phenotypic or physiological changes in the recipient microorganisms. Non-coding, non-expressed nucleotide sequences that cause no phenotypic or physiological changes in the recipient organism means the nucleotide sequences are not transcribed and are not involved in gene expression or replication and include linkers, homopolymers, adaptors, and flanking sequences.

III.B. Research Subject to Regulations

Research involving introduction into the environment of many of the organisms included within the scope of these guidelines is subject to the jurisdiction of a Federal regulatory agency and requires prior approval or clearance. This includes, for example, regulation by the Animal and Plant Health Inspection Service, USDA of plant pests under the Federal Plant Pest Act and Plant Quarantine Act and the regulation of veterinary biologics and organisms and vectors that may cause infectious diseases in animals under the Virus-Serum-Toxin Act and the Animal Quarantine Statutes. The U.S. Environmental Protection Agency regulates microbial pesticides under the Federal Insecticide, Fungicide and Rodenticide Act and other microorganisms under the Toxic Substances Control Act. Questions concerning jurisdiction of Federal agencies may be addressed to OAB. Although the guidelines may be useful to investigators preparing submissions to regulatory agencies, adherence to the Guidelines should not be viewed as a substitute for full compliance with all regulatory requirements. Experiments receiving regulatory approval by USDA or EPA are considered to automatically comply with the Guidelines.

IV. General Information

The Guidelines are based upon current knowledge and practices for safe planned introduction of genetically modified organisms into the environment. USDA will periodically revise the Guidelines in accordance with the amendment procedures in section XI to reflect new scientific information.
V. Overview: Guidelines for Safe Conduct of Research

The purpose of this section is to provide an overview of the scheme, or step-wise process, that is recommended for use by principal investigators. The conditions under which research with a genetically modified organism can be conducted safely should be assessed relative to the conditions that are normally accepted for conducting research with the parental organism. Therefore, the safety evaluation begins in Section VI with a determination of the level of safety concern for the parental organism in a specific, described environment. Section VI sets out a framework for determining which of three levels of safety concern is appropriate for the parental organism in a specific environment.

After the level of safety concern for the parental organism has been determined, the principal investigator should consider the effect of the genetic modification on safety. Section VII sets out a framework for assessing whether the modification has no effect on safety, or whether it increases or decreases safety. Knowledge of the precise modification may allow better predictability of the safety of the organism and its products so that appropriate confinement and other safety practices for the research can be selected.

At this point, principal investigators should choose appropriate confinement measures, based on the biological and ecological attributes of the modified organism. Section IX describes confinement principles that can be applied to the design of safety protocols so that the research can be conducted safely.

In summary, the conditions for safely performing research should be chosen according to the following four step process:

Step 1. Determination of the level of safety concern for the parental organism (Section VI).

Step 2. Determination of the effect of the genetic modification on safety, i.e., whether it increases, decreases, or has no effect on safety (Section VII).

Step 3. Determination of the level of safety concern for the modified organism (Section VIII).

Step 4. Determination of the confinement measures appropriate to the particular biological and ecological attributes of the genetically modified organism.
and development of a safety protocol (Section IX) so that the research is conducted in a safe manner.

Examples of research with specific organisms evaluated by this step-wise process are provided in Appendix 1.

VI. Step 1: Determination of the Level of Safety Concern for Parental Organisms

The level of safety concern for the parental organism should be determined by evaluating the attributes of the organism within the context of the environment in which the research is to be performed. (See Section VI-A.) The particular attributes of the organism should be considered along with its ecological relationships with other organisms in that environment. The attributes which should be considered are:

- the potential of the parental organism to establish itself in the accessible environment,
- the pest/pathogen status and potential of the parental organism in the accessible environment,
- other ecological relationships of the parental organism with organisms in the accessible environment,
- the potential of the parental organism for inducing genetic change in natural or managed populations in the accessible environment, and
- the potential for monitoring and control of the parental organism in the accessible environment.

A series of actions is recommended in this section to determine the level of safety concern for the parental organism. By following these actions, principal investigators will be in a reasonable position to evaluate the relative importance of specific attributes, to choose a level of safety concern for the parental organism, and to document the rationale for placing the parental organism at a particular level of safety concern.

The evaluations made under this section will not be the same for all organisms. Nor will all evaluations described in this section be relevant to every organism. At the same time, there may be additional information relevant to the level of safety concern for a particular organism that is not specifically mentioned in this section. Scientific judgement should be used in considering the available relevant information and the potential significance of any gaps in information relevant to safety.
VI-A ACTION I. Accessible Environment

Describe the environment that can be reached by the parental organism and its progeny in the absence of confinement beyond that inherent in the biology of the organism. Describe the environmental characteristics of the area in and immediately surrounding the research site and include the expected area of dispersal of the parental organism and its progeny from that location.

VI-B ACTION II. Attributes of the Organism

Describe the relevant attributes of the parental organism in the accessible environment. This should be done by addressing the questions and issues presented in this section. As noted above, the evaluation will differ among different organisms. Not all questions are relevant to all organisms.

The significance of gaps in information should be assessed along with available information. After considering the available relevant information on each of the five attributes described in this section, the degree of concern posed by the attribute should be indicated as low, medium, or high.

VI-B-1. Potential to Establish Itself in the Accessible Environment

VI-B-1-a. What are the known mechanisms of survival or persistence of the organism in the environment? Are there natural predators or other organismal relationships that affect its survival? Are there climatic and soil conditions or other abiotic factors influencing survival of the organism?

VI-B-1-b. What are the known mechanisms of dissemination of the parental organism?

VI-B-1-c. Is population size known to affect the ability of the organism to become established?

VI-B-1-d. What information is known about the competitiveness and aggressiveness of the organism in the accessible environment in relation to the ability of the organism to become established in that environment?

VI-B-2. Pest/Pathogen Status and Potential in the Accessible Environment

VI-B-2-a. What are the plausible adverse effects of the organism on the accessible environment due to its being a pest or pathogen? These include adverse effects, such as lowered productivity of economically
important organisms, damage or destruction of natural habitats, and adverse effects on human health. Will the potential extent of adverse effects, as a result of this research, be greater than already exists in the accessible environment from the organisms already present? 

**VI-B-2-b.** What is the potential for exchange of genetic information between the organism and pests or pathogens in the accessible environment? In other words, what is the likelihood of the organism becoming a pest or pathogen through an exchange of genetic information under the conditions of the research?

**VI-B-2-c.** Does the organism have any ecological characteristics that might increase or decrease its pest/pathogen potential? For example, if the organism and its relatives were restricted to a narrow set of ecological conditions (niche), does this imply that the potential to broaden that niche and become a pest is expected to be low?

**VI-B-3. Other Ecological Relationships with Organisms in the Accessible Environment**

**VI-B-3-a.** What is the importance of the organism to the structure of the community? Is the parental organism involved in any critical ecosystem functions, e.g., nitrogen fixation, inorganic nutrient uptake, key food chain component, critical habitat for key species? Is involvement in critical ecosystem functions indirect or direct? Can other organisms in the ecosystem fulfill its function?

**VI-B-3-h.** What is the ecological specificity and range of interactions of the organism with other organisms?

**VI-B-3-c.** What is the geographic range of the organism? Is the geographic range small or large? What changes might occur in the organism to broaden or narrow its geographic range?

**VI-B-3-d.** What is the habit of the organism? Is the organism free-living, mutualistic, pathogenic, parasitic, or symbiotic? Does its habit relate to potential adverse effects on the environment should it escape from confinement? Will the habit of the organism facilitate monitoring and control?
VI-B-4. Potential for Inducing Genetic Change in Natural or Managed Populations in the Accessible Environment

VI-B-4-a. Is there intrinsic genetic stability of the genome? Can the organism incorporate exogenous DNA? Are active transposable elements present? Are active viral elements present that interact with the normal genome? Have mutations been observed that have resulted in an unusual genotype or phenotype?

VI-B-4-b. Is there a natural or managed interbreeding population known? What is its size? What is the degree of genetic diversity in the population? Is there potential for genetic exchange between a “released” organism and the organisms in the natural population?

VI-B-5. Potential for Monitoring and Control in the Accessible Environment

VI-B-5-a. Is information from prior research (both within and outside contained facilities) available that has demonstrated control or management of the organism by various means, such as, biological, environmental, physical, chemical?

VI-B-5-b. What monitoring methods are available? What is their sensitivity and degree of accuracy? What is their cost?

VI-B-5-c. Are there procedures to minimize escape of the organism from the test site and to mitigate potential adverse effects?

VI-C. ACTION III. Relative Importance of Attributes

Determine the relative importance of the specific attributes in the context of the planned research. Analyze the attributes to identify those that are most critical or influential in the determination of safe research conditions.

VI-D. ACTION IV. Level of Safety Concern

Determine the level of safety concern for the parental organism. The three levels of safety concern are dependent on two criteria: (1) whether the organism poses negligible risk to human health and no unreasonable risk to managed or natural ecosystems, and (2) the ability to manage or control the organism during its planned introduction into the environment so that the research is conducted in a safe manner.
The particular attributes listed, which indicate levels of safety concern, are not exclusive. Other attributes may also indicate a particular level. Furthermore, the presence of any one attribute does not necessarily indicate a particular level, and all attributes listed need not be shown to conclude a particular level. For example, an organism that may readily become established in the accessible environment would only be of concern if other attributes indicate that such establishment would result in a risk to human health that is not negligible or an unreasonable risk to the environment. Principal investigators must exercise sound scientific judgement in evaluating the relative importance of the attributes in Action II (Section VI-C) in order to assign the level of safety concern.

**VI-D-1. Level of Safety Concern 1 (LSC-1) Organisms.** Organisms whose ecological attributes in the specified accessible environment are understood to the extent that it can be determined with reasonable certainty that the parental organism poses negligible risk to human health and no unreasonable risk to managed or natural ecosystems. No confinement measures are required beyond those inherent in the biology of the organism and the environmental characteristics of the particular site. Some attributes that alone or in combination might indicate LSC-1 organisms are:

- **VI-D-1-a.** No history of adverse effects in the accessible environment or similar environments,
- **VI-D-1-b.** Low evolutionary potential to become a harmful organism in the accessible environment,
- **VI-D-1-c.** Low probability of survival in the accessible environment beyond the time necessary for the particular research,

**VI-D-2. Level of Safety Concern 2 (LSC-2) Organisms.** Organisms whose ecological attributes in the specified accessible environment may pose a risk to human health that is not negligible or may pose an unreasonable risk to managed or natural ecosystems, which can and must be managed or controlled by appropriate confinement or other measures so that the research is conducted with negligible risk to human health and no unreasonable risk to managed or natural ecosystems.

**VI-D-3. Level of Safety Concern 3 (LSC-3) Organisms.** Organisms whose ecological attributes in the specified accessible environment may pose a risk to human health that is not negligible or may pose an unreasonable risk to managed or natural ecosystems and no feasible confinement will ensure safe conduct of the research outside contained facilities with reasonable certainty at this time. Some of the attributes that alone or in combination might indicate LSC-3 organisms are:
VI-D-3-a. History of adverse effects in the specified accessible environment or in similar environments,

VI-D-3-b. Ability to survive and proliferate in the specified accessible environment with adverse effects,

VI-d-3-c. Non-indigenous status in the accessible environment,

VT-D-3-d. High frequency of exchange of genetic information with native populations of organisms with adverse effects,

VI-D-3-e. Lack of effective techniques to minimize escape of viable organisms or active products of the organism from the research site, or

VI-D-3-f. Lack of adequate techniques to recapture or kill escaped organisms before adverse effects occur.

VII. Step 2: Determination of the Effect of the Genetic Modification on Level of Safety Concern

The genetic modification should be evaluated in terms of its effects on the attributes of the parental organism evaluated in Step 1. Genetic modification may have no effect on safety, or it may increase or decrease safety. The genetic modification might alter the safety of the organism without changing the level of safety concern. For example, a specific modification of a LSC-2 parental organisms may reduce the safety concern, but certain confinement measures may still be necessary to achieve research with negligible risk to human health and no unreasonable risk to managed or natural ecosystems and, therefore, the modified organism would remain at the same level of safety concern (i.e., LSC-2). The effects of the genetic modification on safety must be evaluated with reference to (i) direct effects of the organism on human health or the environment, (ii) indirect effects of the organism through the substances it produces, and (iii) effects resulting from exchange of genetic material with other organisms in the accessible environment.

In Step 2, principal investigators should examine the method of genetic modification; the molecular characterization and stability of the modified genes; and the expression, functions, and effects of the modified genes. Although the process of modification alone is not a determinant of safety, such information can facilitate a determination of whether the genetic modification decreases safety concern for the modified organism (Type 1), has no effect on safety concern (Type 2), or increases safety concern (Type 3).
VII-A. Type 1: Genetic Modifications that Decrease Safety Concern for the Modified Organism

Type 1 modifications include those which delete or disrupt expression of a gene or genes known to be responsible for traits, such as, pathogenicity, fertility, survival, or fitness, in ways that increase safety of the organism. Substantial understanding of the molecular biology or other information, including relevant experience, which show that the modification is well characterized and that the gene functions and effects are adequately understood to predict safety, should be demonstrated before a Type 1 determination is made.

VII-B. Type 2: Genetic Modifications that Have No Effect on Safety Concern for the Modified Organism

Substantial understanding of the molecular biology or other information, including relevant experience, which show that the modification is well characterized and that the gene functions and effects are adequately understood to predict safety, should be demonstrated before a Type 2 determination is made.

Type 2 modifications include:

VII-B-1. Insertions of nucleic acid from any source, deletions, or rearrangements that have no phenotypic or genotypic consequence in the accessible environment, e.g., certain marker genes bearing no hazardous traits, and

VII-B-2. Insertions of nucleic acid from any source, deletions, or rearrangements that have known or predictable phenotypic or genotypic consequence in the accessible environment that are unlikely to result in additional adverse effect on human health or on managed or natural ecosystems, e.g., a storage protein gene with a more desirable amino acid balance.

VII-C. Type 3: Genetic Modifications that Increase the Safety Concern for the Modified Organism

Type 3 modifications include:

VII-C-1. Insertions of nucleic acid from any source, deletions, or rearrangements that affect the expression of genes, but the functions or effects are not sufficiently understood to determine with reasonable certainty if the modified organism poses greater risk than the parental organism, and

VII-C-2. Insertions of nucleic acid from any source, deletions, or rearrangements that have known or predictable phenotypic or genotypic consequence in the accessible environment that are likely to result in additional
adverse effects on human health or on managed or natural ecosystems, e.g., those which result in the production of certain toxins.

**VIII. Step 3: Determination of the Level of Safety Concern for Genetically Modified Organisms**

In Step 3, principal investigators should assign the genetically modified organism to one of the three levels of safety concern by considering the effect of the genetic modification on safety (Section VII) and if any affected attributes alter the level of safety concern for the modified organism compared to the parental organism (Section VI). The level of safety concern for the genetically modified organism is dependent on the same criteria applied to the determination of the level of safety concern for the parental organism, namely: (1) whether the organism poses negligible risk to human health and no unreasonable risk to managed or natural ecosystems, and (2) the ability to manage and control the organism during its planned introduction into the environment so that the research is conducted in a safe manner.

**VIII-A. LSC-1 Parental Organisms**

**VIII-A-1.** LSC-1 parental organisms with Type 1 modifications remain LSC-1 genetically modified organisms. No confinement measures are required beyond those inherent in the biology of the organism and the environmental characteristics of the particular site to conduct the research with negligible risk to human health and no unreasonable risk to managed or natural ecosystems.

**VIII-A-2.** LSC-1 parental organisms with Type 2 modifications remain LSC-1 genetically modified organisms. No confinement measures are required beyond those inherent in the biology of the organism and the environmental characteristics of the particular site to conduct the research with negligible risk to human health and no unreasonable risk to managed or natural ecosystems.

**VIII-A-3.** LSC-1 parental organisms with Type 3 modifications result in LSC-1, LSC-2, or LSC-3 genetically modified organisms, depending on the degree of increased safety concern.

**VIII-A-3-a.** If the Type 3 modification results in minimal increase in safety concern so that the risk to human health remains negligible and the risk to managed and natural ecosystems remains reasonable without the need for confinement measures beyond those inherent in the biology of the organism and the environmental characteristics of the particular site, the genetically modified organism remains LSC-1.

**VIII-A-3-b.** If the Type 3 modification increases the safety concern to the extent that risk to human health is no longer negligible or risk to the
environment is no longer reasonable, but feasible confinement and other measures are available so that the research can be conducted with negligible risk to human health and no unreasonable risk to the environment, the genetically modified organism is LSC-2.

**VIII-A-3-c.** If the Type 3 modification increases safety concern to the extent that introduction into the environment cannot be adequately managed or controlled to achieve negligible risk to human health and no unreasonable risk to the environment, the genetically modified organism is LSC-3. Research with the organism must remain in containment until there is reasonable certainty that planned introduction into the environment can be managed and controlled in a safe manner.

**VIII-B. LSC-2 Parental Organisms**

**VIII-B-1.** LSC-2 parental organisms with Type 1 modifications result in LSC-1 or LSC-2 genetically modified organisms, depending on the degree of decrease in safety concern.

**VIII-B-1-a.** If the Type 1 modification decreases the safety concern to the extent that the organism poses negligible risk to human health and no unreasonable risk to managed or natural ecosystems without the need for confinement measures beyond those inherent in the biology of the organism and the environmental characteristics of the particular site, the genetically modified organism is LSC-1.

**VIII-B-1-b.** A modification decreases the safety concern and the risk to human health is negligible and the risk to managed and natural ecosystems is reasonable only when managed by use of confinement measures beyond those inherent in the biology of the organism and the environmental characteristics of the particular site, the genetically modified organism remains LSC-2.

**VIII-B-2.** LSC-2 parental organisms with Type 2 modifications remain LSC-2 genetically modified organisms. Appropriate confinement measures are necessary for planned introduction into the environment with negligible risk to human health and no unreasonable risk to managed or natural ecosystems.

**VIII-B-3.** LSC-2 parental organisms with Type 3 modifications result in LSC-2 or LSC-3 genetically modified organisms, depending on the degree of increase in safety concern.

**VIII-B-3-a.** If the Type 3 modification increases the safety concern, but the planned introduction into the environment still can be managed or
controlled by appropriate confinement measures to achieve negligible risk to human health and no unreasonable risk to managed or natural ecosystems, the genetically modified organism remains LSC-2.

**VIII-B-3-b.** If the Type 3 modification increases safety concern to the extent that there is not reasonable certainty that planned introduction of the organism into the environment can be managed or controlled to achieve negligible risk to human health and no unreasonable risk to the environment, the genetically modified organism is LSC-3. Research with the organism must remain in containment until there is reasonable certainty that planned introduction into the environment can be managed and controlled in a safe manner.

**VIII-C. LSC-3 Parental Organisms**

**VIII-C-1.** LSC-3 parental organisms with Type 1 modifications result in LSC-1, LSC-2, or LSC-3 genetically modified organisms, depending on the degree of decrease in safety concern.

**VIII-C-1-a.** If the Type 1 modification decreases safety concern to the extent that planned introduction into the environment poses negligible risk to human health and no unreasonable risk to managed or natural ecosystems without confinement measures beyond the inherent biology of the organism or the environmental characteristics of the research site, the genetically modified organism is LSC-1.

**VIII-C-1-b.** If the Type 1 modification decreases safety concern but confinement measures beyond the inherent biology of the organism or the environmental characteristics of the research site are necessary for planned introduction into the environment with negligible risk to human health and no unreasonable risk to managed or natural ecosystems, the genetically modified organism is LSC-2.

**VIII-C-1-c.** If the Type 1 modification decreases safety concern but not to the extent that planned introduction of the organism can be managed and controlled to achieve negligible risk to human health and no unreasonable risk to managed or natural ecosystems, the genetically modified organism remains LSC-3. Research must be conducted in a contained facility until planned introduction into the environment can be adequately managed and controlled to achieve negligible risk to human health and no unreasonable risk to managed and natural ecosystems.

**VIII-C-2.** LSC-3 parental organisms with Type 2 or Type 3 modifications remain LSC-3 genetically modified organisms.
### Table 1. Level of Safety Concern for the Genetically Modified Organism

<table>
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<tr>
<th>Level of Safety Concern for the Parental Organism</th>
<th>Level of Safety Concern (LSC) for the Genetically Modified Organism</th>
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<tr>
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<td>Type of Modification</td>
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<td>LSC-1</td>
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<td>LSC-1 or 2</td>
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<td>Level 3</td>
<td>LSC-1, 2, or 3</td>
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### IX. Step 4: Confinement Principles and Design of Safety Protocols

Principal investigators should choose appropriate measures of confinement for the genetically modified organism, as indicated by the biological and ecological attributes of the organism and the level of safety concern.

Confinement measures that restrain or limit the spread or survival of organisms and their products or otherwise reduce the risk of introducing an organism into the environment, can be used to achieve safety. An experiment involving planned introduction into the environment is considered safe only when conducted under conditions determined with reasonable certainty to have negligible risk to human health and no unreasonable risk to managed or natural ecosystems.

For guidance, general principles and practices of confinement for safely conducting research are discussed. However, the appropriate design for a specific experiment will depend on the biological and ecological properties of the organism and the environmental factors unique to the research site. Examples that illustrate the application of the confinement principles are provided in Appendix 1.

#### IX-A. Application of Confinement Principles

The confinement measures used should correspond, in general, to the level of safety concern. Therefore, the need to apply confinement measures to achieve safety is related to the potential for maintaining or increasing pest/pathogen status, the nature of the ecological relationships in the environment, the potential for establishment in the environment, the potential for inducing genetic change in natural or managed populations, the potential for monitoring and control, the characteristics of the accessible environment, and the objectives of the research.
Some organisms cannot be safely managed outside contained facilities and these organisms are designated \textbf{LSC-3}. Other organisms are designated \textbf{LSC-1}, because they pose negligible risk to human health and no unreasonable risk to managed or natural ecosystems without the need for confinement measures beyond those inherent in the biology of the organism and the environmental characteristics of the particular research site(s). The planned introduction into the environment of other organisms can be safely managed only by the use of additional appropriate confinement measures.

In addition to confinement principles used to mitigate risk, all research should conform with scientific principles and practices that are generally accepted in the specific discipline. Generally accepted practices have in common some of the following features:

1. An acceptable experimental design that states the objectives, methods and procedures; describes the site; defines the source, type, and identity of the organisms used; and defines the treatments.

2. Training and supervision of personnel in safety and emergency procedures, good laboratory practices, and animal care.

3. Maintenance of verifiable records including, in addition to experimental data, an appropriate inventory of experimental units, including losses; a record of changes in the protocol and the reasons for the change; and records pertaining to maintenance of site integrity.

4. Appropriate use of statistical methods in designing the study and evaluating the data.

5. Safe disposal of excess materials at termination of the study.

Before any materials (e.g., crops or animals raised during the study) are considered for use as food for humans or feed for animals (including materials rendered for use as components of animal feed), the principal investigator must determine if such materials comply with regulations of the U.S. Food and Drug Administration issued under the Federal Food, Drug and Cosmetic Act. and regulations of USDA's Food Safety and Inspection Service issued under the Federal Meat Inspection Act and the Poultry Products Inspection Act.

\textbf{IX-B. Confinement Measures}

Confinement measures can be placed into five groups --physical, biological, environmental, chemical, and scale. The examples given for each group are not inclusive of all options available. The principal investigator is encouraged to
consult data bases in USDA's National Biological Impact Assessment Program (NBIAP). The NBIAP data bases, which can be accessed free of charge from a personal computer with a telecommunications system, provide detailed information to assist investigators in designing an appropriate safety protocol for specific organisms. For more information about NBIAP telephone (202) 401-4892; facsimile (202) 401-4888, or write to The National Biological Impact Assessment Program, Room 330-G Aerospace Building, 901 D. Street, S.W., Washington, D.C. 20250-2200.

IX-B-1. Biological. The inherent biological properties of an organism greatly affect its behavior in a specific environment. These properties include, for example with plants, whether the growth habit is annual or perennial, and whether the flowering characteristics, natural means of pollination and pollen dissemination permit cross-pollination with other plants.

Biological approaches can be used to limit survival and dissemination of organisms outside the research site and to limit the transfer of genetic information from the research organism to other organisms. Such biological approaches include genetic modifications that disable the organism, that produce sterility, and that reduce the ability of the organism to survive or to escape predators. Removal of reproductive organs and removal of organisms that are hosts for the research organism can be used to aid confinement. Permitting natural biological decay, e.g., normal death, can be an effective approach.

IX-B-2. Environmental. The choice of the research site relative to the geographical location and surrounding ecosystem, taking into account the biological and ecological attributes of the research organism, is important to creating a safe experimental design. Environmental variables, which might be utilized to be reproduction-limiting or to limit survival time and dissemination, include climate, geography or location of the research site (e.g., isolation from potential pollination species), water and nutrient supply, humidity, photoperiod, and availability of predators or host organisms in the area. Seasonal or temporal factors (i.e., time of year), may be extremely useful as well. Environmental factors can inherently contribute to the safety of a particular experimental design.

IX-B-3. Physical. Physical barriers or measures can be used to limit the survival and dissemination of organisms outside the research site. These barriers include border rows, dams, soil terraces, tillage, fences, screens, meshes, and impervious or plastic barriers.

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IX-B-4. Chemical. Chemical treatments can be used to limit survival and reproduction of organisms outside the research site and to limit transfer of genetic information from the research organism to other organisms. Chemical treatments include application of herbicides, fungicides, insecticides, disinfectants, fumigants, and other materials toxic to the research organism, pH alterations, use of gametocides and other chemicals which act as reproductive control agents, and elimination of essential nutrients.

IX-B-5. Scale. By decreasing the number of organisms or the size of the research site, the possibility of rapid and widespread dissemination may be reduced. Remedial actions are easier to implement for smaller numbers of organisms and smaller research sites.

IX-C. Confinement Levels

Confinement should be designed for each particular organism and specified accessible environment, based on the ability of the organism to escape from the research site and cause non-negligible adverse effects to human health or unreasonable effects to managed or natural ecosystems. Confinement is divided into two levels.

IX-C-1. Confinement level 1. Organisms designated LSC-1, pose negligible risk to human health and no unreasonable risk to managed or natural ecosystems. Their characteristics of concern typically are of a self-limiting nature at the chosen research site, and they require no additional confinement measures beyond those inherent in the biology of the organism and the environmental characteristics of the research site. Principal investigators should adhere to practices generally accepted by the scientific discipline for the type of research study, including the general practices defined in Section IX-A.

IX-C-2. Confinement level 2. Organisms designated LSC-2 require additional confinement measures to achieve planned introduction into the environment with negligible risk to human health and no unreasonable risk to managed or natural ecosystems. The confinement measure(s) should be designed to be effective in managing the identified risk. There is no set number or type of confinement measures that should be used as the performance of the confinement measure(s) selected in mitigating risk is the important determinant. For example, if dissemination of pollen is the only identified risk factor and dissemination can be controlled by preventing flower formation, a single measure that adequately controls flower formation will be sufficient. In some cases it may be necessary to utilize a combination of confinement measures (e.g., the use of more than
one type of biological barrier, or the use of a combination of biological and physical barriers) to achieve reasonable certainty that the planned introduction into the environment is conducted with negligible risk to human health and no unreasonable risk to managed or natural ecosystems.

IX-D. Monitoring

Monitoring the movement or persistence of genetically modified organisms, their progeny or products, can provide useful information for designing future experiments and verifying the effectiveness of confinement measures. In some cases elaborate monitoring designs may be important in conducting a safe experiment, while in other cases minimal monitoring (e.g., visual observations during the course of the study) may be sufficient. The decision to monitor and the development of an appropriate monitoring protocol should be a flexible process that draws upon all pertinent available information. Investigators are urged to supply monitoring data to USDA's National Biological Impact Assessment Program so that it can be made available to other investigators through an NBIAP data base on monitoring currently under development.

X. Roles and Responsibilities

X-A. Institution

Each institution conducting or sponsoring research involving the planned introduction into the environment of genetically modified organisms is responsible for safety of the research and compliance with applicable regulations. Fulfilling this responsibility requires at least the following activities:

X-A-1. Establishment and implementation of policies that include confirmation that organisms used and conditions of research are assessed in accordance with the principles of the Guidelines;

X-A-2. Ensuring that principal investigators responsible for research involving planned introduction into the environment of genetically modified organisms comply with the Guidelines and all applicable regulations and assisting them in doing so; and

X-A-3. Ensuring that concerns of the community about planned introductions into the environment of genetically modified organisms are solicited and addressed by the institution.
X-B. Institutional Biosafety Committee and other Experts

Principal investigators may wish to seek advice from institutional biosafety committees and others expert in assessing the safety of a proposed experiment and designing adequate safety protocols.

X-C. Principal Investigator

On behalf of the institution, principal investigators are generally responsible for conducting research in a safe manner. As part of this responsibility, principal investigators should:

X-C-1. Determine whether local, state, or federal regulations and guidelines apply and adhere to the requirements;

X-C-2. Consider the principles for safety assessment and design of safety protocols described in the Guidelines; and

X-C-3. Instruct and train their staffs in practices and techniques to achieve safety and in procedures for dealing with accidents.

XI. Amendment Procedure

Proposals to change the Guidelines may be made by anyone through a written request for amendment to OAB. OAB will notify the submitter in writing that the request has been received and indicate the procedure for reviewing the request. Normally, OAB will publish the request in the Federal Register announcement for the next ABRAC meeting, if received in time for publication and if space is available on the ABRAC agenda. The Assistant Secretary for Science and Education will make a final determination on the request, usually after receiving a recommendation from ABRAC.

APPENDIX I

To assist users of the guidelines, eight examples have been evaluated using the step-wise process. These examples are presented in the following order:

(1) *Bos taurus* (domestic cattle),
(2) *Cyprinus carpio* (partially-scaled common carp),
(3) *Pinus taeda* (loblolly pine),
(4) *Brassica napus* (oil rapeseed),
(5) *Cardiochiles nigraerae* (parasitic wasp),
(6) *Drosophila melanogaster* (fruit fly),
(7) *Pseudomottas fluorescens* 2-79, and
(8) *Clavibacter xyli*, subsp. *cynodontis*. 
Appendix I

Examples of Research Evaluated
Under the Guidelines

(1) *Bos taurus* (domestic cattle),
(2) *Cyprinus carpio* (partially-scaled common carp),
(3) *Pinus taeda* (loblolly pine),
(4) *Brassica napus* (oil rapeseed),
(5) *Cardiochiles nigriceps* (parasitic wasp),
(6) *Drosophila melanogaster* (fruit fly),
(7) *Pseudomonas fluorescens 2-79*, and
(8) *Clavibacter xyli*, subsp. *cynodontis*. 
Example 1

Bos taurus

Prepared by: Harold Hafs

V-I. Step 1. Determination of the level of safety concern for the parental organism. Bos taurus in Overton, Texas. The purpose of this proposed research is to determine whether extra copies of the bovine growth hormone gene can be introduced into the germ line of Bos taurus, and whether its expression can be controlled with a prolactin promoter.

VI-A. Action I. Accessible Environment. This research will be conducted at Texas A & M Agricultural Research Center at Overton, Texas. It is a well-managed station, with cattle production facilities typical of this temperate region. The accessible environment consists of the Center and the ranches and farms surrounding the Center. Cattle released without constraints could move several miles daily, although natural and man-made barriers would limit their movements. These cattle could mate with other bovidae, in this example probably only the herds of domesticated Bos taurus and Bos indicus which are abundant around Overton and throughout most of the temperate and subtropical regions of North America. There are no wild bovidae in the Overton region. The mated animals could be distributed widely in the normal course of the cattle business, and the offspring normally would not be available for identification until birth 9 months after mating - perhaps hundreds of miles from Overton and with new owners who are unaware of the genetic history. Another possibility is inadvertent distribution of gametes from cattle, world-wide through commercial channels for artificial insemination or embryo transfer (Salisbury et al, 1978).

VI-B. Action II. Attributes of the Organism

VI-B-I. Potential to Establish Itself in the Accessible Environment: Low concern.

a) Known mechanisms of survival. Contemporary breeds of cattle can survive without husbandry (Cole and Brander, 1986; Ensminger, 1983) although coyotes, feral dogs, and wolves are predators. Progressively from south to north, cattle survival is increasingly limited by the lower temperatures (Cole and Ronning, 1974), especially above 49° latitude.

b) Known mechanisms of dissemination. Cattle can travel miles for feed, water, and cover.
c) Effects of population size. Cattle are gregarious, preferring herds. This tends to optimize proximity for mating interactions regardless of population size.

d) Ability to become established. Feral cattle are not competitive, particularly in inhabited regions which Overton typifies, and not even in regions not densely inhabited by man. Their relatively low fecundity without husbandry delays the establishment of a feral population.

VI-B-2. Pest/Pathogen Status and Potential in the Accessible Environment: Low concern.

a) Effects on accessible environment. Cattle will damage cultivated crops, and long-term grazing on semi-arid lands can modify ecosystems (Schlesinger et al, 1990). Otherwise, their effect on the environment is low.

b) Potential for exchange of genetic information. Although *B. taurus* would breed with other *bovidae*, this exchange of genetic information would be greatly curtailed because most cattle in the United States normally are confined, to control their feeding and breeding and to exclude interlopers.

c) Ecological characteristics which affect pest status. Cattle have been raised for dairy and beef from the time the Europeans settled in the United States. Given the extensive database, there is little chance feral cattle could become pests.

VI-B-3. Other Ecological Relationships with Other Organisms in the Accessible Environment: Moderate concern.

a) Importance to community. Cattle influence the ecosystem directly in proportion to their consumption of forage. They can also modify the ecosystem on the boundaries of water, such as stream banks, particularly if their population is dense. While they occupy no pivotal ecological niche, they provide essential living conditions to several insects (e.g., ticks, flies, lice, and mites) and some birds (e.g., egrets), as well as hundreds of species of dung flora and fauna which normally degrade cattle dung (Stevenson and Dindal, 1987).

b) Ecological specificity. Cattle are quite versatile, adapting to woodland, rangeland, and even swamps, provided forage, water, and cover are available.

c) Extent of geographic range. Feral cattle can survive throughout most of North America, but their survival is reduced in adverse winter climates typical of the northern states.

d) Habit. Cattle are easily identified if they escape confinement in most environments where genetic modification research should be conducted.
VI-B-4. Potential for Inducing Genetic Change in Natural or MANAMA Populations in the Accessible Environment: Moderate concern.

a) Genetic stability/mutagenicity. Given the size of the U.S. cattle population (about 50 million), unless a modified trait was intensively selected or had some huge competitive advantage, it would not have much practical genetic impact on the parental population.

b) Interbreeding nonulation size. Decades of intensive cattle breeding reveal their genome is stable, but pliable with traditional breeding methods. Mutations are known, but they occur with low frequency. A major effort has been launched to map the bovine genome (Womack, 1990).

c) Other:

i. Potential for genetic exchange. *Bos taurus* can exchange genetic information with other bovidae. Many heritable traits are known.

ii. Degree of genetic diversity. Fossil remains of cattle date back 3 or 4 million years (Blakely and Bade, 1982), and there is a broad genetic diversity. For example, 80 allogenic determinants have been described and assigned to 11 genetically independent blood groups (Stormont, 1988), and some evidence suggests the more heterozygous cattle survive longer (Schleger et al, 1977).

VI-B-5. Potential for Monitoring and Control in the Accessible Environment: Low concern.

a) History of use and control. After over 2 centuries of domestication, there is much information on methods to confine, monitor, and control cattle. They are easily recognized. If sperm or embryos are harvested from modified animals, special identification may be warranted to prevent unauthorized distribution in commercial channels.

b) Accented monitoring methods. There are numerous reliable methods to identify and monitor cattle, with perfect accuracy and low cost.

c) Control of inadvertent release. Several confinement procedures are highly effective. Control of cattle released inadvertently can be accomplished by permanent or reversible fertility control. For example, implantation with progestogen would reversibly prevent estrus and castration would permanently prevent propagation.

VI-C. Action III. Relative Importance of Attributes.

The most important consideration is that *Bos taurus* can interbreed with other *bovidae*. Therefore, in keeping with good scientific principles for cattle breeding
research, for this genetic modification research these cattle should be permanently identified and confined so as to prevent uncontrolled mating.

**VI.D. Action IV. Level of Safety Concern.**

The lowest level (1) of safety concern should be assigned to *Bos taurus* in Overton, Texas.

**VII. Step 2: Determination of the Effect of Genetic Modifications on the Level of Safety Concern.**

This hypothetical modification of the parental cattle is an insertion of a DNA construct consisting of the bovine growth hormone (GH) gene ligated to the bovine prolactin promoter using pro-nuclear microinjection into the zygote (Polge et al, 1989). Increased levels of GH in the serum serves as a reporter of expression of the exogenous gene. The product of the transgene (GH) is a normally secreted protein in cattle. The transgene construct may be activated to cause extra GH secretion by treatment of the resultant transgenic animal with a dopamine antagonist (e.g., sulpiride), or with thyrotropin releasing hormone. The extra GH secretion which may be measured in the cow’s blood by radioimmunoassay, results in improved milk and meat production. The prolactin promoter is intended to restrict expression of the GH gene to the pituitary gland, where GH and prolactin normally are secreted. Therefore by design, expression of the transgene GH should not occur in edible tissues although this must be proven. However, the extra GH secreted (as a result of the extra gene copies would find its way into edible tissues, as is normal for GH.

Little is known of the mechanism of DNA transfer, the stability of the transgene, or its genetics and the number of copies which become integrated into the genome cannot be controlled by these methods.

**As** these genetic modifications are unlikely to affect human health or ecosystems, this is a Type 2 modification (Section VII-B).

A second hypothetical example modification of cattle is an insertion of a DNA construct consisting of the bovine GH gene ligated to the a-skeletal actin promoter and to a luciferase reporter gene (Chen, et al, 1990). It would be introduced into the zygote by microinjection, and the product of the transgene is GH, as in the first example. The a-skeletal actin promoter should provide expression of the construct in skeletal muscle, and the luciferase provides a rapid sensitive method to monitor integration of the transgene. However, in addition to extra GH, these genes and their products will be present in edible tissues from the modified cattle. Since this modification raises human food safety questions, this second example may be a Type 3 modification (Section VII-C-1) because the effects are not well understood. It is incumbent on the investigator to show that these gene products pose no human health threat before the animals enter human food.
supply. For example, if the gene products are all destroyed in the digestive tract, there is no human safety issue and this example would be a Type 2 modification.

VIII. Step 3: Determination of the Level of Safety Concern for the Genetically Modified Organism.

The parental *Bos taurus* has low level ability to establish feral populations around Overton, Texas, and the proposed insertion of extra copies of the bovine growth hormone gene pose no added threat to the environment or to humans. Therefore, the modified cattle in Overton, Texas have the lowest safety concern (level 1, see table 1).

IX. Step 4: Confinement Principles and Design of Safety Protocols.

Good scientific practices and standard cattle breeding principles will provide adequate confinement for the genetically modified cattle. However, it would be prudent also to introduce another level of confinement, recognizing the potential value of the animals. An extra physical barrier or continuous monitoring would accomplish this end.

References


Example 2

*Cyprinus carpio* (partially-scaled common carp)

Prepared by: William Witt

V-I. Step 1. Determination of the level of safety concern for the parental organism: *Cyprinus carpio*, partially-scaled common carp termed "mirror" carp, in Auburn, Alabama.

The purpose of this research is to (1) evaluate the effects of the trout growth hormone gene (rtGH) on the reproductive capacity of brood carp, (2) determine whether offspring of these carp inherit the trout growth hormone gene, and (3) determine the effects of the inherited gene on the survival, growth rate, and behavior of the offspring. The research will develop basic information that may in the future be useful in developing improved fish species for commercial aquaculture through the use of recombinant deoxyribonucleic acid (DNA) technology.

VI-A. Action I. Accessible Environment

This research is to be conducted at the Alabama Agricultural Experiment Station.

VI-B. Action II. Attributes of the Organism

VI-B-1. Potential to Establish: Low concern

There are several features of the mirror carp which place them at a competitive disadvantage in natural ecosystems. For example, mirror carp have reduced survival, growth, hemoglobin percentages, and ability to regenerate fins compared to normally scaled carp (Kirpichnikov 1981). Mirror carp are also more susceptible to disease than are other carp (Suzuki, et al., 1976) and show lesser weight gains when food is readily available (Raat 1987), and when temperature conditions are unsuitable (Mishvelov 1983). Natural carp predators in the accessible environment include largemouth bass, smallmouth bass, sunfish and catfish which prey heavily on carp eggs and fry.
Other predators include introduced saltwater striped bass and hybrid striped bass.

**VI-B-2. Pest/Pathogen Status and Potential in the Accessible Environment: Moderate concern**

A member of the minnow family (Cyprinidae), common carp (Cyprinus carpio Linnaeus) are native to Asia and were introduced into North America in the 1880's. Since their introduction, carp have spread throughout North America and are found in a variety of habitats. The largest carp populations in the United States are found in the midwest (Welcomme 1984), with generally fewer carp in the southern United States (Courtnay and Stauffer 1984, Welcomme 1984). Because of their gregarious spawning activities, disruptive feeding habits, and propensity to displace existing fish species, carp are the most often cited nuisance fish in North America (Kohler and Stanley 1984).

Mirror carp are a naturally occurring, partially scaled, genetically selected, mutant form of scaled common carp. Mirror carp occur in waters of North America (Panek 1987), although seldom in large numbers.

**VI-B-3. Other Ecological Relationships with Organisms in the Accessible Environment: Moderate concern**

Carp generally do not displace existing fish populations by directly competing for food, but rather by physically disrupting their habitats. The feeding and spawning habits of carp can uproot aquatic vegetation and increase water turbidity which, when sufficiently disturbed, deprives plants of needed sunlight and adversely affects fish populations that depend on sight for feeding.

Those fish that rely on aquatic plants to provide shelter from predation, or who feed upon the animals that colonize the plants, are displaced when the carp uproot the plants. Many species of fish deposit their eggs directly on the sediments during spawning. The eggs are then vulnerable to incidental ingestion by carp, or to burial as the sediments which the carp suck up during feeding settle through the water column. These types of disruptions occur most commonly in those systems that support mature carp (Crivelli 1983).

Specifically, the aquatic vegetation in Sougahatchee Creek and Yates Reservoir already has been exposed to any adverse effects of shading and substrate disruption caused by the feeding and spawning habits of the existing scaled common carp populations in those waters. Yet, aquatic plants occur in Yates Reservoir, indicating that the aquatic vegetation has withstood any adverse effects caused by the small, scaled common carp population in these waters.

\[ 1 - 2 \]
The existing fish population in Yates Reservoir is a stable community with many predators. Scaled common carp reside there, but are at carrying capacity and are a minor component of the population. They have not been documented as displacing more desirable fish species. Therefore, it is apparent that Yates Reservoir will only support a limited biomass of carp for reasons such as a limited amount of suitable habitat or predatory pressure.

The presence of carp is not always detrimental to aquatic ecosystems. Carp tolerate, and indeed may thrive under conditions that have become disturbed and can no longer be tolerated by native fish (Scott and Crossman 1973). In addition, carp can uproot nuisance aquatic vegetation, and, as juveniles, provide forage to certain game fish (McCrimmon 1968; Scott and Crossman 1973).

The indigenous organisms in Sougahatchee Creek and Yates Reservoir have been exposed to possible adverse effects of the spawning habits of the existing scaled common carp populations in those waters. Yet, the ecosystem in Yates Reservoir is stable, indicating that the indigenous organisms have withstood any adverse effects caused by the existing, scaled common carp population.

**VI-B-4. Potential for Inducing Genetic Change in Natural or Managed Populations in the Accessible Environment: Low concern**

Mirror carp are a naturally occurring, partially scaled, genetically selected, mutant form of scaled common carp. Mirror carp occur in waters of North America (Panek 1988), although seldom in large numbers. Mirror carp are reported to be present in Alabama but rarely have been observed (William Reeves, personal communication). To date, mirror carp have not been reported from either Yates Reservoir (Hornsby et al. 1990) or Sougahatchee Creek (USFWS 1983).

Parental mirror carp to be used in this study are of a genetic line of captively bred mirror carp brought to AAES over 30 years ago. These fish have been exposed to a hatchery environment for many generations, including rearing at high densities, usual dependence on artificial diets, frequent exposure to low levels of oxygen and poor water quality, and lack of competition with predators for survival. Therefore, their fitness in nonculture conditions may have been affected.

In order to pass on the "mirror" gene to their offspring, sexually mature adults must successfully spawn. Because mirror carp are not intentionally stocked in natural water bodies in the United States, little information is available regarding the number of fish that would be necessary to establish a reproducing population in the accessible environment. At any rate, many
factors mitigate against male and female mirror carp being able to spawn successfully either among themselves or with scaled common carp. First, so few, if any, mirror carp would be expected to survive to sexual maturity given their competitive inferiority and the intense predation in the unmanaged ecosystem that the surviving individuals could become geographically isolated in the natural environment. Second, temporal differences in gonad development during the spawning season may vary between mirror carp and scaled common carp and effectively isolate the spawning individuals. Third, preferential mating may also serve as an isolating mechanism (Smitherman et al. 1984, Smitherman et al. 1988). Scaled common carp may prefer to spawn with other scaled common carp rather than with mirror carp. The potential does exist for the parental mirror carp to spawn with other carp (both mirror and common scaled carp) in natural or managed systems. However, available data on such crosses show that the continuous pattern of scaling in common carp is dominant over the "mirror" type of scale distribution (Kirpichnikov 1981).

VI-B-5. Potential for Monitoring and Control in the Accessible Environment: Low concern

Mirror carp are designated as such because their skin is but partially scaled as compared to the continuously scaled skin of common carp. Such a difference in appearance may be adequate to distinguish between the mirror carp and the common carp for the purpose of monitoring these conspecific fish once they are caught or are confined in an environment conducive to visualization. Monitoring of the fish in this manner would become extremely difficult if the fish reached the vast area contained in the Sougahatchee Creek and Yates Reservoir (the potential accessible environment should any of the parental fish escape the outdoor ponds). Because the mirror carp are at a competitive disadvantage in natural ecosystems the need for monitoring would be insignificant. Survivability of the mirror carp in unmanaged ecosystems would depend heavily upon predation and the availability of food. Because Yates Reservoir is crowded with bass and sunfish, very intense predator pressure would be expected on mirror carp eggs, fry, and fingerlings. In addition, most of the habitat in Yates Reservoir is not typically suited for carp and food availability would be expected to be very low.

Within the proposed outdoor ponds, control of the parental fish could be accomplished with the use of rotenone followed by detoxification of rotenone-treated water with permanganate. Such a means of control would not be advisable should the fish be introduced into the Sougahatchee Creek and Yates Reservoir environments.
VI-C. **Action III. Relative Importance of Attributes**

The most important consideration is that the parental mirror carp to be used in the proposed research were selected for this study because of specific attributes that, in general, are competitively inferior to scaled common carp. Should any of these fish become introduced into the receiving bodies of water (Sougahatchee Creek and Yates Reservoir) associated with AAES, they would then need to survive in this natural environment to inadvertently impact it. Many factors, as discussed above, should act against the establishment of a reproducing population of the parental mirror carp in these natural waters.

VI-D. **Action IV. Level of Safety Concern: Low concern**

The lowest level of safety concern should be assigned to parental mirror carp introduced into the 10 designated ponds at AAES. Even though mirror carp occur in waters of North America (Panek 1988), they are seldom found in large numbers. Also, the continuous pattern of scaling in common carp is dominant over the "mirror" type of scale distribution (Kirpichnikov 1981) which should result in the disappearance of the "mirror" trait from the gene pool altogether should spawning occur between scaled common carp and mirror carp in unmanaged systems.

VII. **Step 2: Determination of the Effect of Genetic Modifications on Safety**

VII-C. Because the effects of the proposed genetic modification of *Cyprinus carpio* are not sufficiently understood to determine with reasonable certainty if the modified organism poses greater risk then the parental organism, the modification is classified as Type 3 (increased safety concern for the modified organism).

The genetic transformation of the mirror carp that is the subject of the proposed experiment was accomplished by the chromosomal insertion of DNA from a cloning vector, pRSV-2. The recombinant plasmid, pRSV-2 contained the gene (cDNA) for rainbow trout growth hormone (rtGH) under the promotional control of the long terminal repeat (LTR) from Rous sarcoma virus (RSV) and additional apparently non-functional flanking sequences used in construction of the pBR322-derived plasmid. The LTR, a non-infectious regulatory sequence of DNA derived from RSV-RNA, functions as an efficient molecular recognition site for initiation of synthesis of rtGH protein in transgenic carp.

The growth hormone gene and its growth hormone product affect growth rate, feed conversion efficiency, and fat metabolism. Secondary or pleiotropic effects are known to occur in mammals, such as rodents and pigs that have been genetically modified.
by the introduction of a foreign growth hormone gene. Some of these secondary effects may be debilitating.

No direct scientific evidence is available on the performance and behavior of transgenic carp in non-laboratory settings. However, fish injected with fish, mammalian, or avian growth hormones grow faster and convert food more efficiently. Transgenic mirror carp have been reported to be 22% larger, on the average, than their sibling controls at the same age in cultured (laboratory) conditions (Zhang et al. 1990).

There is concern about environmental consequences relative to the worst case scenario should transgenic mirror carp be inadvertently introduced into local receiving bodies of water. The concern rests on the uncertainty as to whether the carp would exhibit superior fitness, increased growth rate, etc., as compared to scaled common carp which many consider to be a nuisance fish and which are already present in these waters. If the transgenic carp were more fit and the rtGH gene were increased in the gene pool of carp of the same species, the range and distribution of common carp in the receiving waters might be extended beyond that which now exists and result in a destabilization of the existing fish community, food web, and aquatic ecosystem. However, even if some transgenic mirror carp were to escape the AAES, few, if any, of the fish would be expected to survive, grow, and reach sexual maturity in the receiving bodies of water. Factors that would act against the establishment of transgenic carp in those waters include: the relatively small number of fish that might escape, the naturally high mortality rate during the early life history stages of the fish, the lack of suitable habitat, the large number of predatory fish in those waters, and because the experiment uses a highly domesticated fish genotype that is not likely to be well suited for survival in a natural environment where those fish must forage for food. Also, the experimental fish have been derived from mirror carp which have traits that, in general, are competitively inferior to scaled common carp.

In past AAES experiments, both transgenic and non-transgenic mirror carp reared indoors exhibited high mortality rates. Less than half of the transgenic carp reared indoors survived to two years of age. The reason for this high mortality rate is not known.

No adverse effects are expected from the use of the viral DNA sequence in the genetic modification of the carp. The viral sequence represents less than 10 percent of the viral genome, it does not code for or express any protein, and it cannot replicate and initiate an infection independently.
VIII. Step 3: Determination of the Level of Safety Concern for Genetically Modified Organisms

In Step 1 it was determined that the release of the parental organism *Cyprinus carpio* into the designated ponds at AAES is LSC-1. The modification and its possible consequences described in Step 2 is a Type 3 modification based primarily on its uncertainty. Type 3 modifications of LSC-1 parental organisms places the modified organism at LSC-1, LSC-2, or LSC-3. It is concluded that modified *Cyprinus carpio* as described in Step 2 and released into the 10 confined outdoor ponds located at AAES, Auburn, Lee County, Alabama, constitutes a LSC-2 release because feasible confinement and other measures are available such that the research can be conducted with negligible risk to human health and no unreasonable risk to the environment.

IX. Step 4: Confinement Principles and Design of Safety Protocols

The appropriate level of confinement for the LSC-2 modified *Cyprinus carpio* described above and released into the confined outdoor ponds located at AAES, Auburn, Lee County, Alabama, is Confinement Level 2.

The objective of confinement is to minimize the escape of the fish from the AAES site into receiving bodies of water (Sougahatchee Creek and Yates Reservoir). A combination of confinement measures that may include biological, physical, environmental, chemical and scale measures, should be designed into the proposed research to achieve a level of safety concern equivalent to LSC-1, i.e., negligible risk to human health and no unreasonable risk to managed or natural ecosystems.

Site selection is very important. The outdoor ponds should be located in an area that is not vulnerable to flooding and should be geographically isolated to the extent practical from drainage into natural bodies of water. The AAES ponds are located 36 feet above the estimated 100 year flood height of Sougahatchee Creek. The ponds are located over a mile from Sougahatchee Creek, and between the ponds and Sougahatchee Creek there is an impounded farm pond containing predators of carp into which water from the area drains.

The ponds should be constructed and maintained in a manner that prevents any breach in the pond barrier, such as may occur by erosion of the levees or damage by burrowing animals. The AAES pond construction with wide levees of packed clay reinforced by concrete sides and AAES maintenance procedures meet these criteria.

The most likely route of escape of fish from outdoor ponds would occur either from overflow of the ponds if the drainage system fails, or failure of the filtration system to prevent escape as water discharges from the ponds. Various systems can be used,
such as a closed water re-circulating system or a system where water is filtered before discharge into the environment. In the AAES system there is a filtered drainpipe 18 inches below the top of the pond levees and the ponds are filled to a level 5 inches below the drain pipe. Based on rainfall records, these conditions should allow the experiment to proceed under essentially static conditions with no intentional flow of water through the ponds. Any water draining from the ponds will ass through two separate filters of appropriate mesh size, depending on the age of the fish, before entering a large catch basin pond with a French drain constructed with layers of gravel and Agri-Fabric U. Should maintenance be required, flow can be directed to a second catch basin that services water draining from the hatchery.

Because outdoor ponds are accessible to predators, the AAES ponds are enclosed with an 8 foot high chain link fence to which a 1/16 inch wire mesh fence, 18 inches high is attached. Polyethylene bird netting over the fence and across the top completely encloses the ponds.

At termination of the experiment the fish will be seined from the ponds, humanely sacrificed, and buried. The ponds will be poisoned with rotenone and drained only after the rotenone is detoxified. Rotenone also will be used for any emergency termination due to extreme weather conditions and to poison the barrier farm pond in the event of a recognized escape of fish.

Important biological measures of confinement in this experiment include: 1) carp sperm and unfertilized eggs remain viable in water only for approximately one minute so their escape into the environment is not of concern; 2) fertilized eggs of carp in water sink and are adhesive, making the escape of unhatched embryos improbable; 3) brood fish maintained outdoors will be sex segregated and brought indoors for artificial spawning; and 4) the experiment will be terminated before offspring, which are not sex-segregated in the outdoor ponds, are sexually mature. Additionally, the barrier farm pond as well as the closest natural bodies of water are laden with predators of immature carp.

Another factor that decreases the probability of any detrimental effect on the ecosystem involves the scale of the research. The maximum number of transgenic fry is 25,000 and mortality in the natural environment at this life stage is only 1500. The physical barriers alone make any escape remote, let alone escape of numbers of fish large enough to be noticed. In the latter case, poisoning of the barrier pond with rotenone would minimize potential entry of transgenic fish into the natural bodies of water. If only a small number escaped undetected, they are unlikely to survive to maturity or successfully reproduce. In the absence of positive selection pressure favoring individuals with the πGH gene, no significant effect on the environment would occur.
REFERENCES:


Mishvelov, E.G. 1983. Relationship between growth rate and body water content of carp, *Cyprinus carpio* (Cyprinidae), influenced by scale cover and temperature. J. Ichthyol. 23(3):144-147.


Example 3

**Pinus taeda**, loblolly pine

Prepared by: Frank Whitmore


**VI-A Action I. Accessible Environment**

The experiment will be conducted in Ashley County, Arkansas. *Pinus taeda* is accessible to all sites surrounding the test plot. Loblolly pine has a very large natural range, extending from east Texas across the southeastern Coastal Plain and Piedmont as far north as New Jersey. The southernmost range is central Florida. The only significant break in the east-west distribution of loblolly pine, which might stop or impede gene flow, is the Mississippi River flood plain. Within the wide natural range, the species cannot survive in bottomland hardwood sites that remain flooded for several months each year.

**VI-B Action II. Attributes of the Organism**

VI-B-1. Potential to establish: **moderate concern**

Loblolly pine is classed as intolerant (Baker 1949). It will survive in some shade when in the seedling stage, but cannot compete successfully with the more tolerant hardwoods when a few years older (Baker and Langdon 1990). The species can quickly invade old fields or openings left by logging or fires if a nearby seed source exists. The limits of the natural range is determined by low temperatures to the north and high potential evapotranspiration to the west. Its southern range limit in central Florida is determined by the preponderance of deep sand soils on which loblolly pine cannot compete with other southern pines. Male and female strobili (flowers) are borne on the same tree, as in all species of *Pinus*. Flowering usually begins no earlier than 10 years, although precocious appearance of male and female strobili have been reported at 5 and 6 years, respectively (Righter 1939). Once initiated, seed production increases until about 50 years of age, and may continue for decades more. Pollen dissemination occurs from February to April, depending on latitude. Pollen is distributed by wind and can be carried for kilometers, although effective quantities are usually limited to less than 100 meters. Seeds mature and fall from cones.
in October. Seed dispersal is usually no more than 100 meters from the producing tree. Population size is not an important determinant in establishment, except that in a well-stocked stand with complete crown closure, reproduction cannot survive. Loblolly pine does not reproduce vegetatively. The most important factor in establishment on appropriate sites is the presence of openings near seed trees that are free of well-established herbaceous or woody vegetation. With adequate moisture and little or no shading, seedlings grow rapidly. If seedlings are not overtopped by hardwoods by age 3, they have a good chance to outgrow any competition (Baker and Langdon 1990). When all these conditions are met, loblolly pine aggressively occupies new sites and forms pure stands. Diseases and insects are generally not important factors in preventing the establishment of natural stands. Fusiform rust (Cronartium auercuum f. sp. fusiforme) is one of the most serious diseases of loblolly pine, and is commonly a problem in nurseries (Hepting 1971). This stem disease also causes losses in plantations.

VI-B-2. Pest/Pathogen Status and Potential: low concern

Pinus taeda has no pathogenic characteristics and is only occasionally considered a pest, mainly when trees encroach upon lawns. In the western part of its range, the species frequently hybridizes with Pinus palustris (longleaf pine), resulting in Pinus x sondereggeri (Sonderegger pine). Less common interbreeding occurs with P. echinata (shortleaf pine), P. rigida (pitch pine), and P. serotina (pond pine). All of the species with which loblolly pine hybridizes are non-pathogenic and are no more pestilient than loblolly pine.

VI-B-3. Ecological Relationships With Other Organisms: moderate concern

Loblolly pine is the dominant conifer species in the southern pine region, in economic importance, in area occupied, and in volume. On the most favorable sites, it is the fastest-growing of the southern pines and reaches the largest size. It forms extensive pure stands, especially after fires or abandonment of crop fields. It is also grown extensively in plantations throughout its range, but also as an exotic in Australia, New Zealand, and southern Africa. The most important conifer species associated with loblolly pine is shortleaf pine. Many species of southern hardwoods also occur in association with loblolly pine. Because of the intolerant nature of loblolly pine, it is not the climax type in its natural range. Its long term dominance in the southern forests requires disturbance with the resulting establishment of new stands. In undisturbed forests, the hardwoods will eventually succeed pines. The species does not have a narrow ecological specificity; however, it develops best on soils that
are slightly acid, have imperfect or poor surface drainage, a fairly thick, medium-textured surface layer, and a fine-texturea subsoil (Coile and Schumaker 1953, Zahner, 1954). This type of site is common throughout the range of *P. taeda*. The species has a broad but benign range of interactions with many other organisms, from soil microorganisms to mammals. Southern pine forests, in which loblolly pine is the most important species, are the principal habit of birds such as the pine warbler and Bachman’s warbler. The red-cockaded woodpecker, an endangered species, may be dependent upon loblolly pine. The natural range of loblolly pine is large; there appears to be little chance of changes occurring in the organism to broaden or narrow its geographic range. It is a free-living woody plant that can attain a height of 50 meters and age of 300 years. Loblolly pine trees can occasionally form root grafts that connect the vascular system of two or more trees. The ecological importance of this phenomenon is unknown. The habit of loblolly pine indicates no potential adverse effects on the environment. Loblolly pine frequently has a symbiotic relationship with mycorrhizal-forming organisms. One species, *Pisolithus tinctorius*, causes significant increases in growth rate of infected trees compared with non-infected trees (Marx et al. 1978).

**V-B-4. Potential for Induce Genetic Change in Natural or Managed Populations:** low concern

Genetic analysis of *Pinus taeda* is limited, even though the species has been studied probably as much as any other forest tree species. Provenance tests of loblolly pine have shown seed source differences in such attributes as disease resistance, growth rates, drought hardiness, cold hardiness, and wood properties. Some of these differences seem to be racial, but no distinct races have been named. Genetic variability within populations is fairly high, yielding good gains in selection and breeding programs. Active transposable elements are not known to be present. Viral elements interacting with the normal genome are not known. No unusual genotypes arising from mutations have been observed. The natural interbreeding population of loblolly is extensive and is continuous, limited by pollen and seed dispersal. Within these limits, there is a definite potential for genetic exchange between an individual “released transgenic” organism and the natural population, but only after the released organism has attained flowering age.

**V-B-5. Potential for Monitoring and Control:** low concern

Because of fast growth rate, high economic value, and wide site adaptability, loblolly pine has been established in many plantations throughout its natural range as well as in the southern hemisphere.
Practice has shown that no problems have arisen from natural expansion of these plantings. Old field invasion by loblolly pine has occurred frequently throughout the South, but this usually has been considered desirable. Monitoring to prevent escape from confinement can easily be done by periodic observation of research plots. If an experiment is maintained for several years, careful observations can be made for the appearance of strobili. At the first appearance of precocious flowering, the affected plants can be destroyed or strobili can be removed.

VT-C. Action III. Relative Importance of Attributes

Pinus taeda is an aggressive invader of open sites; in normal silvicultural practice, this attribute is considered desirable for reestablishment of stands. The ecological relationships with other organisms is complex; therefore, its introduction into new sites outside its natural range may be of concern. However, the species has never been considered a serious pest. At least 75 years of ecological observations, management, and plantation establishment throughout its native range and around the world have revealed no problems arising from invasions by the species or from its ecological relationships with other organisms. If transformed individuals were allowed to reach sexual maturity and large size, control of the dissemination of pollen and seeds would be difficult. However, for the parental organism, the relative importance of the attributes combine to yield a safety concern of no consequence.

VI-D. Action IV. Level of Safety Concern: 1

VII. Step 2: Determination of the Effect of the Genetic Modification on Level of Safety Concern

In this experiment, apical meristem cells will be transformed by biolistic particles carrying a (hypothetical) bacterial hydroxylase sequence and the chalcone synthase (CHS) promoter. This promoter is from parsley. Transformation of micropropagated loblolly pine seedlings by these constructs has been shown in greenhouse studies to cause a specific hydroxylation of a native terpenoid compound in vascular tissues, conferring upon the terpenoid a high toxicity to Cronartium quercuum f. sp. fusiforme (fusiform rust), a destructive fungal pathogen of loblolly pine stems. Micropropagules will be cultured, grown to seedlings in a greenhouse, then seedlings will be planted in an outdoor test plot where they will be inoculated with fungal spores.)

VII-C. Type 3: Genetic Modification that Increases the Safety Concern for the Modified Organism: Type of Modification: 3

The type 3 modification increases the safety concern for the modified organism. The bacterial hydroxylase to be used in this project is well
characterized and its phenotypic and genotypic consequences in bacteria are well known. Likewise, the effects of the expression of this gene in loblolly pine seedlings on infection by fusiform rust fungus are well known and predictable, at least in laboratory and greenhouse studies. The effects of this modification in the southern pine ecosystem, however, are not predictable. In the following, attributes of the modified organism are compared with those of its parental type:

1. The modified organism will have the same accessible environment as the unmodified organism.

2. The potential of the modified organism to become established in the accessible environment may be reduced if the modification adversely affects beneficial symbiotic relationships (see 4 below).

3. The pest/pathogen status or potential for such status should not be changed by the modification.

4. The effects of the modification on ecological relationships of loblolly pine with other organisms in the field are uncertain, and possibly could be detrimental to the species itself. For example, loblolly pine growth is enhanced by its association with ectomycorrhizal fungus. If the modified terpenoid that is toxic to fusiform rust also inhibits infection by or effectiveness of mycorrhizal organisms, the widespread incorporation of this genetic system in the native population by natural means might have undesirable effects upon managed or unmanaged southern pine ecosystems. It will be necessary to test the system for several years in the field to determine whether or not the modified terpenoid disturbs certain ecological relationships.

5. The modification should neither lower nor raise the potential for inducing genetic change in natural or managed populations from that of the parental organism.

6. The potential for monitoring and control of the organism should not be changed by the modification.

7. Relative importance of the attributes of the modified organism: The only attributes of the parental organism that might be changed by the modification are the ecological relationships with associated organisms. Uncertainties, such as an effect on mycorrhizae, require the assignment of the modification to Type 3.
VIII. Step 3. Determination of the Level of Safety Concern for Genetically Modified Organisms

Level of Safety Concern: 2

An LSC 1 parental organism with a Type 3 modification results in an LSC 2 modified organism if the risk of introduction of the modified organism into the environment is not acceptable, but if, with confinement measures, the risk can be lowered to negligible risk to human health and no unreasonable risk to the environment (see VII-A-3-b). Loblolly pine can be confined easily by biological means and means of scale for the duration of the experiment as proposed.

IX. Step 4. Confinement Principles and Design of Safety Protocols

Confinement Level: 2

Organisms designated LSC-2 require Confinement Level 2 (see XI-C-2). The confinement principles that will be used in this experiment are: generally accepted research practices, biological measures, and measures of scale.

Generally accepted research practices: The field containing the test plot is 1 hectare, square, equidistant on sides. Four hundred plants will be established, 200 transgenic and 200 control trees. Spacing will be 2.5 x 2.5 meters; the plot layout will be a square with 20 trees on a side, in the center of the 1-hectare field. This arrangement will provide for a 50-meter-wide isolation strip around the test plot. The experimental design will be complete randomization, with single tree plots. The soil is Lexington series, silt loam 0.5 meters deep overlaying yellow loam subsoil. Internal drainage is imperfect, surface is well-drained. Site index is 90 feet at 50 years.

Biological: The experiment will be terminated by cutting the trees after 5 growing seasons, well before any individuals should begin to flower. This should prevent escape of any transformed pollen or seed, although the plot will be monitored continually for precocious flowering. Loblolly pine does not normally regenerate from root sprouts; however the plot will be monitored for unusual sprouting for two additional years.

Scale: The small number of genetically modified plants and the wide spacing will allow for careful monitoring to detect any precocious flowering.
References


VI. Step 1. Determination of the Level of Safety Concern for the Parental Organism: Brassica napus in northwestern United States

VI-A. Action I. Accessible Environment

Accessible environment. The release of Brassica napus into the seed production area of Washington State. Given that this organism is stationary, inadvertent release requires either the spread of pollen by insects or spread of seed by animals or man. Cross fertilization of other Brassica varieties and species will reduce the value of the production seed. Inadvertent cross pollination of weeds could spread the engineered traits into the wild populations of the Washington production area.

VI-B. Action II. Attributes of the Organism

Parental Organism: Brassica napus

B. napus is extensively grown as an oil seed crop in southern Canada and is becoming an important oil seed crop in the U.S. A large portion of the elite vegetable B. napus seed is produced for the U.S. seed market in the Northwest. Cultivated Brassica species represent one of the largest and most diverse families of interrelated species and subspecies. Their diverse uses range from oils (B. napus), condiments (B. juncea), vegetables (B. oleracea), to animal fadders (B. napus). Included within the broad family are many weedy, wild Brassicas, e.g., B. nigra (black mustard), B. juncea (Indian mustard). B. napus will outcross with all of these species.

VI-B-I. Potential to Establish Itself in the Accessible Environment

a) B. napus tends to adapt well and could persist as do its closely related weedy relatives.

b) Dissemination is through seed, not by vegetative propagation.

c) The effects of population size are not well characterized. The larger the field of B. napus the more likely insects will find it and spread its pollen.
d) The aggressiveness of *B. napus* is not clearly defined. However, based on the spread of related species, *B. napus* is likely to propagate in an unmanaged ecosystem and could establish itself as the predominant species.

**VI-B-2. Pest/Pathogen Status and Potential in the Accessible Environment**

a) Wild strains of *B. napus* are often considered weeds but not a pathogen. Other weedy *Brassica* species found in the Northwest are *B. nigra*, *B. juncea*, *B. kaber*, and *B. hirta*. There are two different issues regarding the impact of *Brassica* on the environment. The first is its potential as a pest in the natural environment, the second its potential as a pest on crops.

b) The potential for exchange between the various *Brassica* species in the Northwest is relatively high for plants growing close together. Because *B. napus* is extensively grown for vegetable seed production and because there are many indigenous weeds (see 2.c for more details), exchange could effect foundation seed purity and spread new traits to weeds. This potential is high when the plants are growing in close proximity to each other.

c) Generally, *Brassica* species are pollinated by insects with little or no occurrence of wind pollination. Therefore, insect control could mitigate the spread of the modified traits. Plant separations as small as 1/2 meters result in approximately 20% outcrossing but distances as great as 1/4 mile reduce outcrossing to less than 0.05%.

**VI-B-3. Other Ecological Relationships with other Organisms in the Accessible Environment**

a) Important to community. Northwest species have low independence. They do not appear to serve any critical function in the ecosystem. However, its importance lies in its agronomic characteristics.

b) The niche for *Brassica napus* is cultivated farm fields but its weedy relatives can be found in wastelands and roadides. Because *B. napus* can cross with weedy relatives in close proximity there is a potential for spread of genes into the ecosystem.

c) The geographic range of the genus *Brassica* is worldwide.

d) *B. napus* is a free-living macroscopic organism. It is easily recognized and monitored. It has been grown in a managed ecosystems for many years and is familiar to the farming and scientific community.

**VI-B-4. Potential for Inducing Genetic Change in Natural or Managed Populations in the Accessible Environment**

a) Unless a characteristic of a genetically modified *B. napus* was intensely selected for (e.g. herbicide resistance), it would be unlikely to significantly alter the genetic composition of the natural population.
b) The natural or interbreeding population is diverse. *B. napus* has the potential for genetic exchange with other *Brassica* species. *Brassica* is considered a very genetically diverse family. (See VI-B-2-b).

**VI-B-5. Potential for Monitoring and Control in the Accessible Environment**

a) *B. napus* has been cultivated for over 100 years, and good control measures are available. *B. napus* is a higher plant, thus it is easily recognized and, except as seed or pollen, individuals do not move.

b) Accepted monitoring methods include surveying suspect areas and visual identification of plants using taxonomic keys. Herbicides that control *B. napus* are available and the spread of *B. napus* can be completely eliminated by preventing flowering or by controlling the insects that *B. napus* depends on for pollination. It is a non-vegetatively propagated annual plant. However, *B. napus* can survive in an unmanaged ecosystem and can cross with many other species. The control of inadvertent release can be accomplished through the use of cultural, and chemical methods of weed control. Cultural methods would include cultivation, mowing and hand pulling of plants. Chemical methods would include herbicides and fumigants to control plants and seed, respectively. Examples of herbicides would include 2, 4-D, dicamba, glyphosate, and bromoxynil. Weed scientists would be able to recommend the most effective control measures depending on the crop or ecological situation.

**VI-C. Action III. Relative Importance of Attributes**

Evaluation of relative importance of specific factors: the most important factor is *Brassica napus* can cross with many vegetable and weedy species. Therefore, the overall characteristics of this unmodified organism are of some concern for the purpose of genetic engineering experiments. Field experiments with this organism in the State of Washington should be confined with procedures that will reduce the risk of inadvertent release. However, this crop is familiar and there are good monitoring and control devices. Furthermore, individual plants do not move and propagation is mainly through pollen dissemination. Separation distances of 1/4 mile should be adequate to prevent significant dissemination and seed.

**VI-D. Action IV. Level of Safety Concern for Parental Organisms**

*Brassica napus* would be assigned a safety condition in Washington State of 2 but would be only 1 in other areas where *Brassicas* are less prevalent both in natural and managed ecosystems.
References:


VII. Step 2. Determination of the Effect of Genetic Modifications on Level of Safety Concern

The modification of the parental organism (Brassica napus) is the insertion of a 11 Kbp piece of DNA from a disarmed micro Ti plasmid carried in Agrobacterium tumefaciens. The mechanism of transfer of DNA from the micro Ti plasmid to the plant genome is very precise and predictable, only the DNA sequences between defined border sequences are transferred.

The genetics of the transferred DNA are well understood. The DNA is integrated into the plant genome at a single locus. Occasionally the DNA is duplicated as tandem repeats either direct or inverted. In this case the 11 Kbp piece of DNA transferred to B. napus plants, has been integrated in a single copy and segregates as a single dominant Mendelian trait. Plants are available that are homozygous for the DNA.

The transferred DNA has been sequenced and contains three genes which are expressed in the plant (B. napus). The first gene, octopine synthase, (OCS), encodes an enzyme that converts the amino acid arginine to octopine. Octopine, an unusual amino acid, is presumed to be non-toxic; it is found at high levels in many mollusks (e.g. scallops). OCS gene is expressed in most plants and in most tissues of the plant. Octopine synthase has been used for many years as a marker-gene and should be considered a Type 2 modification as defined in the section VII-B-1.

The second gene contained on the 11 Kbp piece of DNA is the neomycin phosphotransferase II gene. This gene has been modified for expression as a selectable marker gene in plant cells. This gene has been completely sequenced and used extensively for the last 10 years as a marker gene. In B. napus, the enzyme produced by this gene increases the plant’s natural resistance in tissue culture to the antibiotic kanamycin. The increase in resistance is just enough to allow the researcher to select plant cells that have been transformed. The promoter used to express this gene is the well characterized nopaline synthase gene. This promoter allows NPT II to be expressed in plant tissues just
like the OCS gene. Again, this gene should be considered a Type 2 modification as defined in section VII-B-2.

The third gene contained on the piece of transferred DNA is the β-phaseolin gene whose gene product is the major storage protein found in *Phaseolus vulgaris* (the common bean). Storage proteins are well characterized and are a major source of protein for nutrition when seed crops are consumed. This gene remains seed specific when transferred as it does in the bean plant. This gene should be considered a Type 2 modification as defined in section VII-B-2.

Individually all three genes on the 11 Kbp sequence are considered Type 2 modification. Therefore, the overall, modification can also be considered Type 2.

An alternate gene for modifying the parental plant might be an herbicide resistance gene. Many are well characterized and can be expressed in all plant tissue when behind the NOS promoter. Since *Brassica* could transfer this gene to weeds it might be considered a Type 3 modification as far as safety to a managed ecosystem but only a Type 2 modification as far as safety to natural ecosystems. Further considerations will be discussed in IX.

References:


VIII. Step 3: Determination of the Level of Safety Concern for Genetically Modified Organisms

In step 1 we determined that the release of *B. napus* into the seed production area of Washington State was of safety concern level 2 but in other areas a level 1. The modification described in step 2 was a Type 2 modification with the exception of herbicide resistance. Since Type 2 modifications of a level 2 parental organism will not change the

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safety concern level of the modified organism, we conclude that *B. Napus* modified with the seed storage protein gene released into the seed production area of Washington State is a level 2 release and when released into other areas a level 1 release. See IX for a further discussion of herbicide resistance.

IX. **Step 4: Confinement Principles and Design of Safety Protocol**

The appropriate level of confinement for the modified *B. napus* described above and released into the State of Washington is confinement level 2 (IX-C-3). This confinement level requires, in addition to good agricultural research practices, additional confinement procedures most appropriate to *B. napus* that will reduce the concern to level 1.

The major concern for the release of modified *B. napus* into the production area of the State of Washington is its ability to cross with indigenous weeds and the production crops. Therefore, the most stringent confinement procedure would be to harvest the modified *B. napus* plants before they flower, and treat regrowth with a foliar active herbicide. These practices would virtually eliminate the spread of genetic information from the modified plants. No additional confinement procedures would be necessary. These procedures should meet the requirements for Confinement level 2 but are considered extreme.

Less extreme but effective confinement procedures would include: first, incorporating male sterility into the modified plants thereby eliminating the spread of pollen to other *Brassica* species; second, controlling deliberate or inadvertent spread of seed by locked gates and fencing of the field; and third, treating the field with a herbicide or soil fumigant to kill any germinating seed left behind after the crop is harvested. These procedures should also meet the requirements for Confinement level 2.

Male sterility is a very powerful tool for controlling the dissemination of pollen. In its absence, control of pollen dissemination becomes much more difficult. Geographic isolation of the research field from other crops or weeds with which *B. napus* can cross is possible but may be difficult in the State of Washington. *B. napus* pollen is disseminated mainly by insects (eg. honey bees) with negligible spread by wind or other animals. Insects, however, can carry pollen for several miles. The Association of Official Seed Certifying Agencies (AOSCA, publication #23) states that for foundation rapeseed a minimum isolation distance of 1320 ft. (1/4 mile) is necessary to keep cross pollination with undesirable species below the 0.05% level (one undesirable seed in 2000 seed). Experience with other crops has suggested that no detectable out crossing would be found if separations were increased to 1 mile. This does not mean, however, that a cross did not occur between plants one mile distance apart, rather standard sampling techniques would not detect such a rare event.

Since the gene modification is a bean storage protein gene that is seed specific, one might argue that even if this gene were transferred to either managed or natural population
it would be of no concern. Therefore, either managed or the AOSCA standards of 1/4 mile should hold. In other areas good agricultural research practices should be applied.

Finally, the type of modification and the environment will determine whether more or less stringent confinement procedures are required to reduce the level of concern to 1. In the storage protein gene example, the inserted genes are of little safety concern. Therefore, the 1/4 mile isolation distances recommended for foundation seed may be adequate even in Washington. If, however, the inserted gene were an herbicide resistance gene, then the confinement level is clearly a 2 and the one mile isolation distance may be necessary. Furthermore, if the introduction were into an environment free of *Brassica* species, then good agronomic research practices may be adequate even for a *B. napus* containing a herbicide resistance gene.
Example 5

Cardiochiles nigriceps Vierick (Braconidae)

Prepared by: Fred Gould/Ann Sorensen

V-I. Step 1. Determination of the level of safety concern for the parental organism
Cardiochiles nigriceps Vierick (Braconidae)

VI-A. Action I: Accessible environment. The intended release site for C. nigriceps is Clayton, N.C. Given the mobility of this organism, the environment accessible to a population that escapes confinement is the entire southeastern U.S. wherever its host, Heliothis virescens, occurs.

VI-B. Action II. Attributes of the Organism

VI-B-1. Potential to establish itself in the accessible environment: of high concern as long as host is available

a). Known mechanisms of survival. C. nigriceps has a few generations per season in the Southeast. It overwinters as a prepupa in the underground cocoon of H. virescens (Danks et al. 1979).

b). Known mechanisms of dissemination. C. nigriceps is a robust insect and a strong flier (F. Gould, personal observation). It can track cues from its hosts and fly directly to them. Although no studies of long distance movement (50-300 miles) have been made, it is not unreasonable to assume such movement of C. nigriceps, given proper weather conditions (Rabb & Kennedy 1979). Since C. nigriceps parasitizes the larval stage of H. virescens which stays in the vicinity of its host plant, movement of the parasite via its host is limited.

c). Effects of population size. There are no field data on this. As with most braconid parasites, an unmated female of C. nigriceps can produce fertile male offspring. Since adult females live up to 24 days, it is theoretically possible for a single female to mate with her sons and establish a population.

d). Competitiveness & Aggressiveness. C. nigriceps can parasitize over 90 percent of the H. virescens larvae in a field (Chamberlin & Tenhet 1926). When a single H. virescens larva in the field is attacked by C

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nigriceps and another parasite, Microptilis croceps (Cresson), it is C. nigriceps that is usually the sole survivor (Lewis & Brazzel 1968).

e). Other. C. nigriceps can be reared in the lab (Vinson et al. 1973), so some characteristics of a modified C. nigriceps could be assessed before release in the field.

VI-B-2. Pest pathogen status and potential in the accessible environment:
Low concern

a). Effects on accessible environment. C. nigriceps is a member of the insect family Brachoniidae of the order Hymenoptera. All species of this family are parasites of other insects (Borror & DeLong 1964). All of the species closely related to C. nigriceps are parasites of Lepidoptera (Borror & DeLong 1964, p. 554). It has been studied in detail because it is a parasite of Heliothis virescens, one of the most destructive pests of annual crops in the U.S. (Kogan et al. 1978). Quantitative studies of the effects of C. nigriceps on H. virescens populations date back to 1926 (See Chamberlin & Tenhet 1926, Bibby 1942, Snow et al. 1966, Neuzig 1963, Lewis & Brazzel 1966.) A number of studies have offered evidence that the host range of C. nigriceps is limited to two species, H. virescens and H. subflexa. The primary host is H. virescens. The secondary host, H. subflexa, feeds on Chinese lantern, a solanaceous plant that is considered a roadside weed. Laboratory and field attempts to establish nigriceps on Heliothis zea were a complete failure. Although C. nigriceps may oviposit into H. zea larvae, all of the eggs die within 48 hours (Lewis & Brazzel 1966, 1968). More recent studies have endeavored to explain C. nigriceps host specificity from a physiological perspective (Davies & Vinson 1986).

b). Potential for exchange of genetic information. There are at least 8 species in the genus Cardiochiles. These species have not been studied in detail at an ecological or physiological level. Most parasitic insects have well defined species barriers, but some "species" of insects can produce fertile hybrids (Proshold & LaChance 1974). Since C. nigriceps is the only species in the genus collected in annual crops (Danks et al. 1979), it is unlikely to have contact with other species with which it could exchange genetic material.

c). Ecological characteristics which affect pest status. Given the narrow host range of C. nigriceps and the pest status of its host, there is little if any reason for concern if C. nigriceps should become a pest. Even if C. nigriceps changed genetically to expand its range, it would be unlikely to attack beneficial insects. Almost all Lepidoptera (moths and butterflies) have herbivorous (plant feeding) larvae. If the host range changed enough
so that C. nigriceps attacked additional lepidopterans that feed on weeds, some damage could be done (some Heliothines related to H. virescens specialize on evening primrose and ragweed [C. Mitter, personal comm.]). However, given the high degree of host specialization of C. nigriceps involving both restricted physiology and restricted host-finding behavior (Lewis & Brazzel 1966), such changes are likely to involve complex and finely tuned evolutionary adjustments, not random genetic changes.

VI-B-3. Other ecological relationships with other organisms in the accessible environment: low concern

a). Importance to community. As indicated above, C. nigriceps has a very narrow host range. To the extent that its abundance dictates the abundance of H. virescens, it may alter food chains. Given that many predators and parasites that occur in natural ecosystems also feed on H. virescens in managed systems, the population density of H. virescens can influence the density of predators and parasites (and their hosts) in natural systems. Such indirect effects have not been carefully studied, but are not generally considered to be key factors in determining the ecology of any natural system.

b). Ecological specificity. As indicated above, narrow.

c). Extent of geographic range: Geographic range seems to mirror the geographic range of its host, H. virescens, which includes North and South America. Therefore, establishment is much more sensitive to the presence of its host than to abiotic factors.

d). Habit: As above.

e). Other: When pesticides are sprayed, the parasite's abundance decreases (e.g. Lewis and Brazzel 1968)

VI-B-4: Potential for genetic change in natural or managed populations in the accessible environment: = of medium concern based on number of unknowns

a). Genetic stability/mutagenicity: Details of the molecular genetics of this species are unavailable at this time.

b). Interbreeding population size: The size of natural C. nigriceps populations must be large in order to cause high levels of parasitism in an abundant, widespread pest species. Significant genetic exchange within the population would be unlikely unless the characteristic was intensely selected

\( \sqrt{3} \)
for or unless population numbers of the modified organism were
dramatically increased though inundative release of millions of wasps (e.g.
Stinner, Annu. Rev. Entomol. 1980).

c). Other:

i. Potential for genetic exchange: Given the mobility of *C.
nigriceps*, a released strain could certainly exchange information with
other populations.


VI-B-5. Potential for monitoring and control in the accessible environment:
= of high concern because of difficulty in monitoring and controlling

a). History of use and control. Pesticides have been shown to
decrease *C. nigriceps* populations (e.g. Lewis and Brazzel 1968).

b). Accepted monitoring methods. There are no routine monitoring
methods other than assessing the level of *H. virescens* parasitism. This is
labor-intensive and requires lab rearing of larvae to pupation.

c). Control of inadvertent release. Inadvertent releases would be
impossible to eliminate unless the release was in an area where *H.
virescens* or *H. subflexa* host plants did not occur. In the Southeast, this is
not a common occurrence, since *H. virescens* feeds on over 14 families of
plants (e.g. Neunzig 1963). Because of their size, *C. nigriceps* can be
enclosed in fine mesh field cages. Although these cages do not offer full
confinement, the stacking of three successively larger cages, one over the
other, will work if care is taken in the construction.

VI-C. ACTION III. Relative Importance of Attributes.

In evaluating the relative importance of specific factors, the most important
factors are: 1.) *C. nigriceps* is a beneficial parasitoid; 2) it has a narrow host
range confined to a major crop pest species and a minor weed pest species; and
3) it has a high ability to establish if its host is present. Given factors 1 and 2,
there seems to be little risk associated with *C. nigriceps’* ability to establish in the
accessible environment.

VI-D. ACTION IV. Level of Safety Concern.

The overall characteristics of the parental organism are of low concern for the
purpose of genetic engineering experiments. Therefore, *C. nigriceps* is assigned a

\[ V = 4 \]
safety condition of 1 (LSC-1).

VII. Step 2: Determination of the effect of genetic modification on level of safety concern.

The characteristic we will transfer to C. nigriceps is resistance to a pesticide (X) which is presently part of a cotton pest management program but to which C. nigriceps is currently susceptible. Conferring resistance to pesticide X would enable cotton producers to maintain their field populations of C. nigriceps to control H. virescens while controlling other pests as well.

Step 1. The attributes of the modified C. nigriceps are expected to be as follows:

1. Potential to establish itself in the accessible environment: of high concern as long as host is available
   a). Known mechanisms of survival. The survival of the modified organism is expected to be higher than that of the parental organism because of the conferred resistance to pesticide X.
   b). Known mechanisms of dissemination. Expected to be unchanged.
   c). Effects of population size. Not known.
   d). Competitiveness & Aggressiveness. These are the characteristics we will be testing for in the modified organism. We hope that competitiveness and aggressiveness will not be adversely affected by the genetic modification but the modified C. nigriceps may be less robust.

2. Pest pathogen status and potential in the accessible environment: Low concern
   a). Effects on accessible environment. The modified C. nigriceps is expected to have the same host range as the parental organism.
   c). Ecological characteristics which affect pest status. Status as a beneficial parasitoid is not expected to change.
3. **Other ecological relationships with other organisms in the accessible environment:** of low concern

a). **Importance to community.** Not expected to change.

b). **Ecological specificity.** Not expected to change.

c). **Extent of geographic range:** Not expected to change.

d). **Habit:** Not expected to change.

e). **Other:** Although the modified *C. nigriceps* will be resistant to pesticide X, it will still be susceptible to other pesticides.

4. **Potential for genetic change in natural or managed populations in the accessible environment:** = of medium concern based on number of unknowns

a). **Genetic stability/mutagenicity:** Not known.

b). **Interbreeding population size:** Not expected to change.

c). **other:**

   i. **Potential for genetic exchange:** The mobility of the modified *C. nigriceps* means it could exchange information with other populations.

   ii. **Degree of genetic diversity:** Not known.

5. **Potential for monitoring and control in the accessible environment:** = of high concern because of difficulty in monitoring and controlling

a). **History of use and control.** The modified *C. nigriceps* will be still be susceptible to some pesticides.

b). **Accepted monitoring methods.** Not expected to change.

c). **Control of inadvertent release.** Not expected to change.

Step 2. **Method of genetic modification:**

We are assuming that resistance to pesticide X can be conferred by transfer of a resistance gene from bacteria and that the *C. nigriceps* embryo can be transformed by biolistic particles coated with this bacterial DNA
sequence. Alternately, it may be transferred via a transovarial virus (there are reported transovarial viruses in braconids). Although the resistance gene we transfer may be well-characterized, its insertion into C. nigriceps may cause other effects which are difficult to predict. This type of modification can be characterized as a Type 3 modification. It represents an insertion of nucleic acid that affects the expression of genes but its functions or effects are not sufficiently understood to determine with reasonable certainty whether the modified organism poses greater risk than the parental organism. Therefore, we propose to initially raise the modified C. nigriceps in laboratory confinement and assess the modified strain before release in the field. However, once released, a pesticide resistant C. nigriceps may be intensely selected for in the field so this type of modification (which may increase fitness and chance of genetic change in surrounding populations) is of higher concern.

Level of Safety Concern: 2

In step 1, we determined that the parental organism, C. nigriceps, is of safety concern 1. The modification described in step 2 is a Type 3 modification. Therefore, we conclude that we are dealing with a level 2 release.

IX. Step 4: Confinement principles and design of safety protocols:

IX-A. Application of Confinement Principles.

The appropriate level of confinement for the modified C. nigriceps described above and released into Clayton, N.C. is confinement level 2. This confinement level requires, in addition to those practices generally accepted by entomologists, additional confinement procedures most appropriate to C. nigriceps that will reduce the safety concern to level 1. C. nigriceps has a few generations per season and overwinters as a prepupa in the underground cocoon of its host. Adult females live up to 24 days, can produce fertile males without mating, are strong fliers (up to 300 miles) and track their host quite efficiently. Their host, H. virescens, is widespread in the southeast on over 14 host plant families.

During the small scale field release experiment, we hope to determine if modified C. nigriceps adults can survive application of pesticide X, successfully parasitize H. virescens in the presence of competing parasitoids and complete their life cycle under field conditions.

IX-B. Confinement Measures.

To ensure that modified C. nigriceps adults will stay within their release site, we will confine them in fine mesh field cages. The cages will be stacked to ensure
total confinement with the base of the fine wire mesh cloth on the cage partially buried underground. Elimination of the primary host species, H. virescens, within a 300 mile radius, is not economically feasible. Release on a barrier island would be a remote possibility.

Pre-release laboratory experiments with modified C. nigriceps may give us some information about any changes in behavior which may affect their host range or ability to compete with unmodified C. nigriceps and other competing parasitoids. These observations will be confirmed under field conditions within the test cages.

Ultimately, if the modified C. nigriceps adults perform to expectations, we will release them and allow them to successfully establish in the field.

References


Wene, G. 1943. Sagaritis provancheri (D.T.), an important parasite of the tobacco budworm. J. Econ. Entomol. 36: 333.
VI. Step 1. Determination of the Level of Safety Concern for the Parental Organism: *Drosophila melanogaster*, Oregon-R wild type laboratory strain for release in vicinity of wine production facility, Davis, California.

   VI-A. Action I. Accessible Environment: The proposal concerns five releases of 500,000 male flies (genetically modified to produce sterility) in and around a wine production facility that has had several problematic infestations of *Drosophila melanogaster*. In the absence of confinement at the release point, these highly mobile, minute flies could disperse widely to exploit other sources of food common in the Davis area such as tomato processing plants, garbage bins, and fallen fruit.

   VI-B. Action II. Attributes of the Organism

   VI-B-1. Potential to Establish Itself in the Accessible Environment -- high concern

   VI-B-1-a. Known mechanisms of survival: The potential of laboratory strains to become established is significant (Barker et al. 1990). *Drosophila melanogaster* has a cosmopolitan distribution widely associated with, but not restricted to, human dwellings, agricultural and industrial habitats, including orchards, vineyards, food processing areas and refuse dumps (Patterson and Stone 1952, Oldroyd 1964, Parsons and Stanley 1981). Thus, food resources for these flies are available throughout the year. Eggs are deposited on fermenting fruits. The larvae feed through two instars on the yeast flora and pupate on nearby surfaces. Adults reinvade cool climates annually because they have no diapausal mechanism for overwintering. Natural predators, larval parasitoids, competitors, and pathogens can be important regulators of natural population levels below economic thresholds (Carton 1986, Ashburner 1989).

   VI-B-1-b. Known mechanisms of dissemination: Dispersal is wide and is accomplished by flight of the organism (Taylor and Powell 1970).
1983), movement on wind currents, and transport of fruit infested with larvae or eggs. Drosophila adults have been captured in large numbers in nets attached to ships sailing hundreds of kilometers from land (Wolf et al. 1986).

VI-B-1-c. Effects of population size: Inundative field releases of hundreds of thousands of flies are accomplished rather easily due to mass rearing techniques known for Drosophila (Knipling 1966). The greater the number released, the greater the probability of (1) establishment in the target environment, (2) dissemination of released flies away from the experimental site, and (3) introgression of the natural gene pool with traits from laboratory-reared flies.

VI-B-1-d. Aggressiveness: Although individuals of the wild type laboratory strain are likely to establish, they may be less competitive than the locally occurring populations in the natural environment due to selection through many generations of growth under laboratory conditions Ashburner (1989). In natural populations, interspecific competition, especially between the two cosmopolitan species D. melanogaster and D. simulans, has been inferred from indirect evidence including overlap in resource use and from changes in their relative proportions in native populations (Barker 1983).

VI-B-2. Pest/pathogen Status and Potential in the Accessible Environment -- Moderate concern

VI-B-2-a. Assess effects on accessible environment: Wild type D. melanogaster have been cultured in the laboratory for over 80 years representing about 2000 generations (Carlson 1966). In natural populations, D. melanogaster can be a significant pest in a wide range of crops, especially by serving as a vector for undesirable microorganisms (Fitz-Earle and Holm 1983). In addition, Drosophila contamination is a standard for rejecting processed fruits and juices (USDA 1983). Presumably, the adverse effects of released flies would depend upon the number of flies released as compared to the number already existing in the accessible environment.

VI-B-2-b. Potential for exchange of genetic information: Under laboratory conditions, wild type strains of Drosophila melanogaster can interbreed and form sterile hybrids with a small number of sibling species, including D. simulans which is also cosmopolitan (Lemunier et al. 1986). Although, D. melanogaster may form
hybrids in the wild, these offspring are likely to be inviable and will certainly be sterile.

VI-B-2-c. Ecological characteristics which affect pest status: Wild-type laboratory strains are not known to contain any traits that would lead to increased pest status in naturally occurring populations. Significant changes in the ecological characteristics of *D. melanogaster* would affect their pest status if, for example, the released insects or their offspring were able to (1) excrete a toxin or that makes contaminated food unsafe for human consumption, (2) expand their host range to fresh fruit through internal oviposition, (3) vector more virulent pathogens, or (4) disrupt the gene pool of natural *D. melanogaster* populations, or laboratory colonies of these flies, as genetic standards and informational assets to research (Ashburner 1989, Templeton 1979).

VI-B-3. Other Ecological Interrelations with Other Organisms in the Accessible Environment -- Moderate concern

VI-B-3-a. Importance to community: The role of *D. melanogaster*, a free-living, nonparasitic organism, in macro-community structure is one of nutrient cycling. It is one of the major species responsible for the early stages of fruit decomposition in domestic and, to a lesser degree, natural habitats worldwide. Its immediate interactions within the fruit involve a rich community of yeasts and bacteria as well as other insect species exploiting rotten fruit. It is possible that other species of Drosophila which exhibit ecological overlap with *D. melanogaster* could replace any essential role in critical ecosystem functions that the parental organism may play. *D. simulans* seems to have a similar niche, but lacks the genetic variability that *D. melanogaster* populations exhibit. One unique feature of *D. melanogaster*, however, is its utility as a model system in research. Used as a laboratory animal for almost a century, it represents an information bank unparalleled in the study of genetics and evolution. Therefore it is extremely important to consider both the integrity of laboratory wild-type stocks for their consistency with previous studies and the naturally-occurring gene pools as raw material for ecological/behavioral studies.

VI-B-3-b. Niche specificity: It feeds on and vectors (mechanically) a broad range of yeasts in rotting fruits (Begon 1982). These pathogens enhance the suitability of the food for their own use. Unlike notable pests such as the Mediterranean fruit fly, *D.*
melanogaster is a tiny insect that deposits eggs on the surface of the fruit. The inability to insert its eggs into fresh material is an important factor restricting the flies to the exploitation of rotting material.

VI-B-3-c. Extent of geographic range: The geographic range is worldwide, including tropical and temperate natural areas, especially moist forest habitats (Oldroyd 1974), as well as a wide range of habitats resulting from human activities.

VI-B-3-d. Aggressiveness: Interspecific interactions include the movement of yeasts and other rot-promoting organisms to fruit resources, and the exploitation of these rotting fruits with a number of other insects. The competitive ability of these flies has not been quantified, so their importance in structuring the community of microorganisms and other decomposers is not known.

VI-B-4. Potential for Inducing Genetic Change in Managed or Natural Populations in the Accessible Environment -- Moderate concern

VI-B-4-a. Intrinsic Genetic Stability/Mutagenicity: Wild-type flies from laboratory cultures have no unusual properties with respect to genetic instability, uptake of exogenous DNA or the presence of viral elements (Lindsley and Grell 1967, Ashburner and Thompson 1978). Natural populations of D. melanogaster tend to harbor and exchange transposable P-elements more than do "wild type" laboratory strains of the fly (Murphy and Sved 1990, Ashburner 1989). An extremely large variety of lab mutants exist in culture, but most of these would not be competitive with naturally-occurring populations in the field.

VI-4-B-b. Interbreeding population size: This organism is ubiquitous in nature; originating in tropical Africa, it has invaded tropical and temperate areas worldwide. The extreme rapidity with which an inherited trait may spread through a population can be derived from the recent model of an inherited incompatibility factor which has been tracked as it has moved from southern to northern California (Turelli and Hoffmann 1991). Although selective pressures in the accessible environment will ultimately determine the effect of established flies after release, the high degree of genetic diversity in natural populations (Tracey and Ayala 1974) would reduce the likelihood of significant changes in the genetic structure of the population.
VI-B-5. Potential for Monitoring and Control in the Accessible Environment --Low concern

VI-B-5-a. History of use and control: It is difficult to contain the highly mobile adult stage of *D. melanogaster* under laboratory conditions; therefore, field releases pose severe problems for physical containment. For some field experiments, biological containment methods are possible. A high degree of containment can be obtained through induced sterility or major disabilities (e.g. blindness and flightlessness).

VI-B-5-b. Accepted monitoring methods: Adult flies can be marked en masse with fluorescent powder before release. Although genetic markers are available for the identification of all life stages, they would be a modification to the pure wild-type straubs. Methods for monitoring marked fruit flies include fermenting bait traps, sticky traps, Malaise traps, and light traps, which could provide some information on movement of the released individuals. However, recapture rates are typically very low for the vagile adults.

VI-B-5-c. Minimizing escape of organisms from the field site: Mesh cages would reduce escape of the flies, especially if entrances had double doors with an anteroom equipped with attractants to capture escapees. More effective means include physical disabilities and use of only immature stages of the fly.

VI-C. Action III. Relative importance of attributes: The most important attributes of this organism in terms of safety concerns for its use in field releases are its (1) pest status, (2) wide geographic range, and, because of concerns about containment and potential mitigation, (3) mobility and small size. In the context of the planned research, each of these factors will be minimized by the genetic modification chosen by the investigators (i.e. male sterilization).

VI-D. Action IV. Risk Level of Parental organism: *Drosophila melanogaster* can be a significant pest on a wide range of crops. Thus, the release of 2,500,000 laboratory-reared flies into the target area would be likely to exacerbate the pest problem caused by naturally-occurring flies. It is an exceptionally safe and well-known organism with a long history of human contact; however, the difficulty of preventing the escape of this highly mobile, minute organism poses a major problem for the field testing of genetically modified flies unless biological containment is used.

\[ \sqrt{7} = 5 \]
VII. Step 2. Effect of Modification on Level of Safety Concern: Type 1

The proposed genetic modification is male sterility, an established technique for suppressing pest populations. Sterile males for such field releases have been produced in the past by subjecting the flies to chemosterilants or irradiation prior to release. To circumvent the sometimes deleterious effects of these traditional methods on the vigor of male flies, but retain the ability to mass produce the sterile insects, modified Drosophila will be produced by introducing a trait for temperature-sensitive male sterility. This modification will be the result of a rearrangement of the genome to mimic a mutation that has been produced on some occasions in laboratory strains (Fitz-Earle and Holm 1983). The trait allows flies to retain fertility at their optimum rearing temperature under laboratory conditions (17-19 degrees C). The sterility trait will be expressed upon release at the target site which, in summer, has high ambient temperatures. The production of sterile males constitutes a Type 1 modification because released organisms will have a transient effect if used in summer, resulting only in a reduction in the production of pestiferous maggots in the target population.

VIII. Step 3. Risk Level for the Modified Organism: Level 1

The trait has been characterized under laboratory conditions and does not constitute a change that is likely to produce unpredictable effects on the modified organism in the field. Because the modification itself constitutes a type of biological containment, it reduces the risk level of a Type 2 parental organism to a Type 1 organism when modified.

IX. Step 4. Confinement. No confinement is necessary beyond the accepted practices of entomological experimentation, which include standard measures to insure that each cohort indeed has the introduced trait before release, coupled with normal population monitoring to determine the effectiveness of the experimental control program. The likelihood that inductive releases of sterile males will select for parthenogenetic reproduction in naturally-occurring females is low, but such a development should be taken into consideration in the monitoring program.

References:


\[ \sqrt{7} - 7 \]


USDA. 1983. Procedures for determining contamination levels of processed food by foreign materials. Handbook No. xxxx (this ref needs revision)

VI. Step 1. Determination of the level of safety concern for the parental organism:  
*Pseudomonas fluorescens* 2-79

VI-A. Action I. Accessible Environment. The accessible environment would include wheat plants and soils in plots and in close proximity to the test plots. Limited distribution can occur in water and to the roots of in-row plants.

VI-B. Action II. Attributes of the Organism:

*Pseudomonas fluorescens* 2-79, also designated as NRRL B-15132 in the USDA-ARS culture collection at the Northern Regional Research Center, Peoria, Illinois, was originally isolated from roots of wheat grown in a Ritzville silt loam from a field at the Washington State University Dry Land Research Unit, Lind, Washington. The field had been cropped continuously to wheat for 14 years and take-all decline had developed (Weller and Cook, 1983). Take-all decline is a spontaneous diminution of take-all following two or three consecutive, severe outbreaks of the disease during wheat monoculture. Take-all is caused by the fungus *Gaumannomyces graminis* var. *tritici* (Ggt) and is the most important root disease of wheat worldwide. It is thought that 2-79 contributed to the natural suppressiveness of the soil to take-all. Strain 2-79 has been tested as a biological seed treatment (approximately $10^8$ cfu per seed) at multiple sites in the U.S., Great Britain, Europe and Australia. In tests in the Pacific Northwest of the U.S. significant disease suppression and yield increases (10-26%) occur about two-thirds of the time. Strain 2-79, like most biocontrol agents performs inconsistently (Weller, 1988). The principal mechanism of take-all suppression by 2-79 has been shown to be production of the antibiotic, phenazine-1-carboxylic acid (Thomashow and Weller, 1988; Thomashow et al., 1990; Bull et al., 1991). Residual suppressiveness is due mainly to competition and possibly under some conditions to siderophore and anthranilic acid production (Hamdan et al. 1991; Ownley et al., 1991; Weller et al., 1988).

VI-B-1. Potential to Establish Itself in the Accessible Environment = 1:  

a) Application, colonization and survival: Root colonization is the first
essential step needed for introduced fluorescent pseudomonads to suppress take all of wheat. The bacteria must become established along the root in sites most susceptible to pathogen infection. The process of colonization of wheat roots by 2-79 applied to the seed occurs in two phases: attachment and passive carriage (distribution) of the bacteria along the root during root elongation (phase I); and establishment, multiplication and survival on the root and in the rhizosphere in competition with indigenous microflora (phase II). Phase I of root colonization probably depends mainly on the bacteria becoming attached on or near the root tips. As the roots elongate, some bacteria may then be carried downward on the root tips while others are left behind. Long-distance movement along the elongating root by 2-79 occurs in the absence of percolating water, although water movement can greatly enhance the distribution of bacteria along roots (Parke et al., 1986). During phase II of root colonization, the introduced organism, adapted to the rhizosphere, becomes established in its niche and expands its population to the limits of the niche. Other organisms that are not ecologically competent are displaced by indigenous microorganisms soon after phase I transport (Howie et al. 1987; Weller, 1988). Strain 2-79 does not produce spores (Palleroni, 1984).

_Pseudomonas fluorescens_ 2-79 has been used as a model to study colonization, multiplication, and survival of take-all suppressive pseudomonads on roots in the field when introduced on seed of either winter or spring wheat. In a study (Weller, 1983) on winter wheat at Pullman, Washington (Palouse silt loam) lasting 245 days, seeds were coated with approximately $10^8$ cfu of 2-79 per seed and the root system was sampled throughout the growing season. During the first month after planting, 2-79 was present at over $10^6$ cfu/0.1g (2.5-cm length) of seminal root. During the fall and winter, the population of 2-79 on the roots, whether infected or not infected with Ggt, declined to a minimum level in March to $2.8 \times 10^3$ cfu/0.1g root. With the onset of spring, the population of 2-79 increased nearly tenfold on infected roots and remained fairly steady until harvest. In contrast, the population of 2-79 continued to decline on roots of the plants without take-all. The above is the typical population trend for 2-79 on wheat.

Colonization of individual roots by strain 2-79 also has been studied (Weller, 1984). A population gradient developed along the length of the root, with the largest population near the seed and a significant linear decline in the population at progressively greater distances toward the tip. The population profile along the root was described by a series of curves, each representing the changes in the population of 2-79 on specific sections of the root over time. On the section 0 to 3 cm below the seed, $10^5$-$10^6$ cfu/cm root were detected from the onset and no increase in the population was evident in later samples. However, on sections further along the root where initial populations were near $10^4$ cfu/cm of root, the population increased up to 100-fold, with average apparent doubling times of 15-67 hr.

b) **Fate of indigenous microorganisms:** In the Weller (1983) study, during the
first two months after seeding, the introduced bacteria displace a large portion of the indigenous rhizosphere microorganisms on sections of the root within 1 to 3 cm of the seed. For example, 2-79 made up more than 90% of the total indigenous fluorescent pseudomonads and up to 50% of the total aerobic bacteria. However, by the end of the growing season (245 days after planting) 2-79 comprised only 0.002% of the total aerobic bacteria. The effect of 2-79 on specific genera or species of nontarget, beneficial bacteria (i.e. rhizobia) has not been studied. However, based on current studies the effects would not be permanent and would be negligible by the end of the season.

There are two reports on evaluating the nontarget effects on fungi by strain 2-79. Strain 2-79 had no effect on the ability of VA mycorrhizal fungi to colonize roots of cucumber (Paulitz and Linderman, 1989). Nor was the population of introduced Trichoderma harzianum altered by the addition of 2-79 at a population as large as $10^7$ cfu/g raw soil (Bin et al., 1990).

c) Location of introduced bacteria: When strain 2-79 is introduced into the soil via seed treatment the population remains confined to the root and the rhizosphere soil (Weller and Rovira, unpublished). Almost 95% of the cells of 2-79 are located in the rhizosphere soil with the remaining on or in the root. Fluorescent pseudomonads are well adapted to growing in the rhizosphere and do not survive well in the bulk soil. The movement of 2-79 in blowing dust has not been studied. However, it was shown that the bacteria did move from wheat roots to the roots of lentils that were growing as a weed among the rows of wheat.

d) Seasonal effects on population size: All studies of the population dynamics of 2-79 show a similar pattern regardless of the type of wheat (spring or winter) or location of the study. The population is greatest immediately after planting and gradually declines throughout the growing season.

e) Microcosm studies: Studies of root colonization by 2-79 in the greenhouse and growth chamber have been conducted. The purpose of the studies was to determine the effects of biotic and abiotic factors on root colonization. Several of the factors studied included matric potential (Howie et al., 1987), rhizosphere pH (Howie, Cook and Weller, unpublished), temperature (Bull and Weller), bulk density (Ownley, Heron and Weller), wheat cultivar and indigenous microflora. Populations of strain 2-79 introduced on seed in three different soils were greatest on wheat roots at -0.3 bars to -0.7 bars. Strain 2-79 attained the greatest population size on roots with a rhizosphere pH of 6.0 to 6.5 (Howie, Cook and Weller, unpublished). The Population of strain 2-79 was stimulated by the presence of Ggt and Rhizoctonia solani and reduced by Pythium spp. (Mazzola and Cook, 1991).
a) **Mutualism:** In a broad sense the relationship between 2-79 introduced on a wheat plant and the wheat plant can be described as mutualistic. Both organisms benefit. The wheat is protected against take-all disease and the bacteria are provided with a source of nutrients in the form of root exudates.

b) **Plasmid status:** Strain 2-79 contains no detectable plasmids. *In vitro* the strain can mate with *Escherichia coli* and other pseudomonads.

c) **Pathogen/pest status:** Strain 2-79 is not a pathogen. There is no recognized potential that it could become a pest or pathogen. It has never been reported to cause disease on any plant.

**VI-B-3.** Ecological relationships = 1:

a) **Site of colonization:** As indicated above (1a) strain 2-79 remains principally with the roots of the target plant. Transient effects on other microorganisms may occur, but the principal effect is competitive colonization in the presence of the fungal pathogen.

b) **Survival in other habitats:** Strain 2-79 has been studied in several agroecosystems worldwide and in all cases the population dynamics appear to be the same.

**VI-B-4.** Potential for inducing genetic change = 1

**Incorporation of exogenous DNA:** Exogenous DNA can be incorporated into 2-79. Examples include transposons, *lacZ*Y genes from *E. coli*, and 2,4-diacetylphloroglucinol genes from *P. aureofaciens* Q2-87. These foreign genes do not appear to affect the ecological competence of 2-79. Strain 2-79RNL3 performed identically to 2-79 in the field.

**VI-B-5.** Potential for monitoring and control = 1: Strain 2-79RN10 is the rifampicin and nalidixic acid resistant version of 2-79. 2-79RN10 has been use extensively in the studies of root colonization described above. When media are amended with the antibiotics the bacteria can be selectively isolated from soil or roots.

Overall level of safety concern = 1.

**VI-C. Action III. Relative Importance of Attributes**

The parent organism, or a strain with spontaneous selectable markers for antibiotic resistance, is of very low safety concern, especially since transient
increases in opulation are directly correlated with the presence of a serious pathogen of wheat.

VI-D. Action IV. Level of Safety Concern = 1

VII. Step 2. Type of Modification = 2

Type of modification: The marker gene lacZ\textsubscript{Y}, from Escherichia coli (Drahos, et al. 1986), was transferred to P. fluorescens 2-79. This is a neutral modification with respect to performances in the field; only reisolation onto selective media demonstrates the ability to utilize lactose (Cook et al., 1991).

VIII. Step 3. Safety concern of the modified organism = 1

A study was conducted on the fate of a lacZ\textsubscript{Y}-marked (Drahos et al., 1986) 2-79 derivative designated as 2-79RNL3. Winter wheat was coated with approximately $3 \times 10^8$ CFU/seed and sown in a field at Pullman, Washington (Cook et al., 1991). The population dynamics of both wild-type and genetically engineered strains were identical and similar to those previously reported for 2-79 (Weller, 1983). In both of the above studies, in the absence of wheat, the population of 2-79 dropped to a level undetectable by plate counts, that had no impact on the agroecosystem.

In a 1988 field study with 2-79 and 2-79RNL3 the bacteria did not move from one row to the next that was 12 inches away.

Insertion of the lacZ\textsubscript{Y} tracking genes into 2-79RNL10 allows even greater precision in isolating the introduced bacteria from the environment. Populations in the range of about 10 cfu/g soil can be detected.

Since P. fluorescens 2-79, previously designated as safety concern level 1, was modified with well-characterized marker genes, the level of safety concern remains unchanged, at level 1.

IX. Step 4. Confinement

Confinement principles that are used in these experiments include good agricultural research practices, small scale, monitoring more frequently than with unmodified organisms and potential fallowing of the site to enable natural population decrease to occur (especially see Cook et al., 1991).

REFERENCES

Bin, L. Knudsen, G.R., and Eschen, D.J. 1990. Influence of an antagonistic strain of
Pseudomonas fluorescens on growth and ability of Trichoderma harzianum to colonize sclerotia of Sclerotinia sclerotium in soil. Phytopathology 81:994-1000.


Example 8

Clavibacter xyli subsp. cynodontis
Prepared by: Anne Vidaver

VI. Step 1. Determination of the level of safety concern for the parental organism:
Clavibacter xyli subsp. cynodontis MD69a in Beltsville, Maryland

VI-A. ACTION I. Accessible Environment
The accessible environment is inoculated plants as well as uninoculated plants,
soil, and water in proximity to those inoculated.

VI-B. ACTION II. Attributes of the Organism Relevant to the Level of Safety
Concern

VI-B-1. Potential to Establish = 1.

a) The only known host for C. xyli subsp. cynodontis MD69A under
natural conditions, appears to be Bermuda grass (Davis et al. 1984; Carlson
1987). Under natural conditions, no deleterious effects (e.g., stunting, overt
disease) have been observed (Carlson 1987, 1988, 1989).

b) Dissemination to other plants or insects is transient and rare. No
effectivedissemination appears to occur in soil or water (Carlson 1987, 1988,
1989). Mechanical transmission (i.e., by cutting tools, such as lawn mowers)
appears to be the main mechanism of transmission.

c) As population size increases, at least up to $10^6$ CFU/gm upon
inoculation, establishment within the plants increase (endophytic phase).
However, the populations decline in certain cultivars and in harvested plants.
The bacterium is not aggressive in the compatible environment.

VI-B-2 Pest/Pathogen Status and Potential = 2.

a) Clavibacter xyli subsp. cynodontis MD69a was originally isolated
from Bermuda grass (Cylodon dactylon L.). It has no known adverse effects
on humans or the environment, except that some cultivars of maize may suffer
a yield depression under a high inoculum load (Carlson 1987). Some methods
of artificial inoculation of seed may transiently delay germination (Carlson
1989).
b) *C. xyli* subsp. *cynodontis* MD69a showed no detectable difference in DNA restriction fragment length polymorphism analysis nor in protein profiles by SDS-PAGE, in comparison with a large number of strains (Carlson 1987). No DNA homology was detected by RFLP analysis between this strain and other representative, unrelated plant-associated coryneform bacteria. The bacterium has no detectable endogenous plasmids, nor were prophage detected. In laboratory tests, modified MD69a was not able to transmit nor exchange the integrated DNA sequences with other bacteria, even to the same genus and species. Therefore, the probability of genetic exchange with other strains of this subspecies or other microorganisms is extremely low.

c) *C. xyli* subsp. *cynodontis* MD69a is widespread in Bermuda grass. Experiments show poor survival of the strain outside of inoculated plants and gradually decreasing populations in debris from inoculated plants, as well as in soil and water.

VI-B-3. **Ecological Relationships with Other Organisms** = 1.

a) The community in which *C. xyli* subsp. *cynodontis* MD69a survives best is with plants rather than with other microorganisms. Therefore, it is not known to have any role in community structure. Some related coryneform bacteria are known pathogens of plants and may occur sporadically in some cultivated ecosystems (Vidaver 1982; Davis 1986).


VI-B-4. **Potential for Inducing Genetic Change in Natural or Managed Populations** = 1

*C. xyli* subsp. *cynodontis* MD69a could incorporate exogenous DNA, transposons, plasmids, etc., but is of limited potential to affect humans or the environment adversely, because of poor persistence and loss of introduced DNA (Carlson 1987, 1988, 1989). Strains of the organism appear very homogeneous (Davis et al. 1984; Carlson 1987). Other species could incorporate MD69a DNA, but there is no evidence of such exchange.
Numerous field tests have been conducted with C. xyli subsp. cynodontis MD69a (Carlson 1987, 1988, 1989). No indication of unacceptable spread or deleterious effect on plants have been detected. Routine differential plating techniques are successfully used to isolate and enumerate the test organism (Carlson 1987, 1988, 1989). Isolation from Bermuda grass outside the plot areas could be practiced (two meters or more).

Liquid inoculum spills or solid spills would be quickly inactivated (within hours to days), and no effective insect transmission would be expected (Carlson 1987, 1988, 1989). If the modified organism does not behave similarly, soil fumigation, plant incineration, or plowing under could be used in small-scale field tests to decrease the probability of spread and potential unwanted effects, such as leaf scorch of plants or greater than negligible killing of non-target insects.

C. xyli subsp. cynodontis MD69a is problematic in terms of its status as a quasi-pathogen under high inoculum loads for certain maize cultivars. The restricted habitat, poor survival outside of plants, poor dissemination, and unlikelihood of genetic exchange outside the subspecies warrant a very low level of safety concern (Carlson 1987, 1988, 1989).

The bacterium Clavibacter xyli subsp. cynodontis has been modified with the gene for endotoxin production from Facillus thuringiensis subsp. kurstaki, with the corn borer as the target pest. The bacterium and the toxin have both been well characterized in detail in both the scientific literature and in documents submitted to the EPA. The original toxin-producing bacterium is sold commercially. The modified bacterium has a decreased replication rate relative to the parent in the inoculated plants. The modified organism has been examined by EPA under FIFRA, and USDA under APHIS-PPA.

Except for insecticidal activity against environmentally and economically deleterious target insects, none of the attributes of Clavibacter xyli subsp. cynodontis are
changed with respect to safety concern as a result of the genetic modification. Therefore, the overall level of safety concern remains at level 2.

IX. Step 4. Confinement Measures

The principal additions to good agricultural research practices would be (1) additional monitoring to observe effects on maize cultivars and non-target insects both above and below ground; and (2) consideration of destruction and/or dispersal of those cultivars if disease symptoms should appear.

REFERENCES:


