USDA/APHIS Environmental Assessment in response to permit application (05-097-01r) received from University of Wisconsin for field testing of genetically engineered strains of bacterium, *Erwinia carotovora* subsp. *carotovora*

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I. Summary

USDA/APHIS has prepared an environmental assessment in response to a permit application (APHIS Number 05-097-1r) received from University of Wisconsin for controlled field tests in Waushara, Wisconsin. The field test involves four genetically engineered strains of a bacterium, *Erwinia carotovora* ssp. *carotovora*, the causal agent of tuber soft rot disease in potato. The permit application was originally submitted to conduct the field test with six genetically engineered strains of the bacterium. However, the applicant withdrew two strains, WPP191 and WPP194, from this permit on May 27, 2005 after discovering unexplained growth defects in broth culture.

The *E. carotovora* ssp. *carotovora* WPP14 strain was initially isolated from a diseased potato plant obtained from a commercial farm in Waushara County, Wisconsin (Yap *et al*., 2004). The WPP14 strain has been genetically engineered by inserting an antibiotic resistance marker gene from *Escherichia coli* into genes encoding the bacterial secretion proteins of types II and III secretion systems of *E. carotovora* using molecular biology techniques as detailed in the permit application. The inserted antibiotic resistant marker genes include the neomycin phosphotransferase (*nptII*) gene, the chloramphenicol acetyl transferase (*cat*) gene, or the aminoglycoside adenyltransferase (*aadA*) gene that encode resistance to kanamycin, chloramphenicol or spectinomycin/streptomycin, respectively. Insertion of any of these marker genes into a specific *hrp/hrc* (**h**ypersensitive **r**eaction on non-host plants and **p**athogenesis on host plants or **c**onserved among plant and animal pathogens) gene or *hrp*-mutant gene results in the disruption of the disease-causing mechanism within the bacterium by preventing the formation of the secretion proteins. This disruption is expected to make the bacterial strains avirulent/ non-pathogenic.

Bacterial tuber soft rot causes serious losses in potato production and often occurs in stored potatoes that have been frozen, injured or harvested under excessively wet conditions, in conjunction with other diseases such as late blight, leak, and blackleg. The purpose of the field trial is to use mutant constructs with defects in well-characterized genes as tools to: (1) understand the effects of specific genes on the fitness of *E. carotovora*; (2) use the results from these experiments to better understand the function of these genes in plant-bacterial interactions; and (3) compare the results obtained with *E. carotovora* mutants with those previously found for *Pseudomonas syringae* to determine if homologous genes play similar roles in fitness in different environments.

Erwinia carotovora is widely spread in the environment and commonly present on plant roots of numerous species as well as in lakes, streams, rain, and ground water (Toth *et al*., 2003; Perombelon, 2002). The applicant submitted referenced data showing that the engineered bacteria are genetically stable (Yap *et al*., 2004; Datsenko and Wanner, 2000; Matthysse *et al*., 1996). Even if the antibiotic resistant marker gene that is inserted into the *hrp* gene sequence gets deleted by a classical genetic mechanism, the resulting bacteria would be a virulent *Erwinia* strain virtually identical to the strain that is already widely prevalent in Wisconsin. The virulence assay with the mutated strains confirmed that one strain is reduced in virulence compared to the wild-type strain. This strain was mutated in the *hrp*N gene that is required for a

functional type II bacterial secretion system thereby making it unable to secrete cell-wall degrading enzymes. It also contains the green fluorescent gene (*gfp*) from *Aequorea victoria* as a visual marker in addition to *cat* gene. The other three mutated bacterial strains are not significantly reduced in virulence nor have they increased in virulence. The *nptII*, *cat*, and *aadA* genes have been safely used in many genetically engineered organisms. Additionally, the gene that encodes the green fluorescent protein has been used as a visual marker in genetically engineered organisms with no reported toxic effects.

In addition, the applicant is implementing the following containment measures to prevent persistence of the mutated bacteria in the environment: (1) harvest and remove all inoculated potato from the field throughout the course of the field trial; (2) treat with bleach or autoclave any inoculated plant material, contaminated soil, and equipment used prior to disposal; (3) plow back into the field the un-inoculated border rows to minimize spread in case of bacterial spread to these rows.

On the basis of our review of this application, we conclude that controlled field testing described in this application would not present any risk of plant pest introduction, would have no significant impact on non-target organisms and threatened and endangered species, and therefore constitutes a confined field trial. Furthermore, if the field test is performed under conditions outlined here and in the permit, the risk to human health and the environment would be exceedingly low.

II. Purpose and Need

II.1 Proposal:

USDA/APHIS is proposing to issue a permit for confined field release/testing in Waushara County, Wisconsin of four *Erwinia carotovora* strains that are genetically engineered through insertion of *nptII* or *aadA* or *cat*, or *cat* and *gfp* genes into genes encoding hrp secretion proteins. One strain is reduced in virulence compared to the wild-type strain whereas the other three strains are similar in virulence to the naturally-occurring wild type *E. carotovora*.

Erwinia carotovora has many of the same virulence genes as *Pseudomonas syringae*, is a root, tuber, and stem pathogen, and not a foliar pathogen (Perombelon, 2002). The soft rot *Erwinia* bacterium is found on plant surfaces and in soil where they may enter the plant via wound sites or through natural openings on the plant surface (e.g. lenticels). Once inside the plant they reside in the vascular tissue and intercellular spaces of parenchymatous tissues where they remain dormant until environmental conditions, including free water, oxygen availability and temperature, become suitable for disease development (Toth et al., 2003; Perombelon, 2002). If tubers become badly bruised during handling, the whole tuber may become infected. Infected tubers are initially cream colored, later becoming brown, slimy, and foul smelling. A distinct line in the tuber will delineate diseased from healthy tissue. The purpose of the field trial is to use mutant constructs with defects in well-characterized *hrp* genes that are similar to that of *P. syringae* as tools to (1) understand the effects of specific genes on the fitness of *E. carotovora*, (2) use the results from these experiments to better understand the function of these genes in

plant-bacterial interactions, and (3) compare the results obtained with *E. carotovora* mutants with those found for *P. syringae* to determine if homologous genes play similar roles in fitness in different environments.

The permit application was submitted to USDA/APHIS pursuant to regulations in 7 CFR Part 340 which are entitled "Introduction of Organisms and Products Altered or Produced through Genetic Engineering which are Plant Pests or which there is Reason to Believe are Plant Pests." The regulations govern the introduction (importation, interstate movement, or release into the environment) of certain genetically engineered organisms and products. A permit must be obtained before a regulated article that is a genetically engineered microorganism may be introduced into the U.S. Under the regulations, a genetically engineered organism is a regulated article if the recipient organism, the donor organism, or the vector, or the vector agent is listed in §340.2 of the regulations and meets the definition of a plant pest (as defined in §340.1) or if the Administrator determines is a plant pest or has reason to believe is a plant pest. In this submission, the recipient organism is in the genus *Erwinia* which is listed under §340.2 and has been genetically engineered using recombinant DNA techniques. Thus, the genetically engineered microorganism in this University of Wisconsin's submission is deemed a regulated article.

Generally, permitting for field trials of regulated articles is categorically excluded from requirements for an EA under APHIS NEPA implementing procedures (7 C.F.R. Section 372.5(c)(3)(i)). However, when APHIS determines that a confined field release of genetically engineered organisms involves new species or organisms or novel modifications that raise new issues, APHIS prepares an EA under an exception to this categorical exclusion (7 C.F.R. Section 372.5(d)(4)). APHIS had previously prepared an EA for a field test of *Erwinia amylovora* (permit application number 03-279-01r) and concluded that field test would not present a significant impact, either individually or cumulatively, on the quality of the human environment. APHIS is preparing this EA because this is the first request for a field test of the genetically engineered plant pathogen, *Erwinia carotovora*, which APHIS considers a new organism that potentially raises new issues. This documents that the analysis is in compliance with the National Environmental Policy Act (NEPA) of 1969 and the pursuant implementing regulations published by the Council on Environmental Quality and APHIS (42 U.S.C. 4331 et seq.; 40 C.F.R. 1500-1508; 7 C.F.R. part 1b; and 60 FR 6000-6005, February 1, 1995).

II.2. Description of regulated article

The wild type (native) WPP14 strain of *Erwinia carotovora* subsp. *carotovora* is indigenous to Wisconsin. It was originally isolated from a potato field in Waushara County and has been used in experimental studies (Yap *et al*., 2004). Its virulence is typical of other indigenous *E. carotovora* strains in the U.S (Yap *et al*., 2004) and many of its virulence genes have been identified including genes encoding degradative enzymes, diverse regulatory systems and types II and III secretion systems (Chatterjee *et al*., 2002; Rantakari *et al*., 2001; Galan and Collmer, 1999; Alfano and Collmer, 1997; Mukherjee *et al*., 1997). *Erwinia carotovora* subsp. *carotovora* grows well on potato tubers and stems and injects virulence Hrp proteins, known as effectors or type III secretion proteins, into the host plant, which then interfere with host

defenses and promote bacterial growth. *E. carotovora* also secretes plant cell wall degrading proteins through the type II secretion system (Toth *et al*., 2003; Perombelon, 2002).

The field test involves four genetically engineered mutant strains (WPP60, WPP198, WPP195, and WPP40) described below, which were all derived from the wild type bacterium, *Erwinia carotovora* ssp. *carotovora strain WPP14. E. carotovora* strains WPP60 and WPP198 have been developed by inserting the streptomycin/spectinomycin or the chloramphenicol antibiotic resistant marker genes from *E. coli* into the *hrcC* or *hrpL* genes, respectively, of *E. carotovora* strain WPP14 by a marker-exchange mutagenesis process. Similarly, *E. carotovora* strain WPP195 was developed by deleting the *hrp*N gene and inserting the chloramphenicol antibiotic resistant gene and the green fluorescent gene into this locus. This mutant is unable to produce or secrete the harpin protein. The bacterial strain WPP40 has been similarly developed through insertion of the kanamycin resistance gene from *E. coli* into the *OutD* gene, which is required for a functional type II secretion system. This mutant is unable to secrete plant cell wall degrading enzymes and is avirulent. Insertion of antibiotic resistance markers within the coding region of the *hrp* genes in *E. carotovora* effectively disrupts production of the corresponding hrp protein thereby disrupting the pathogenesis (disease) process. This method of gene disruption has been effectively used in laboratory experiments (Datsenko and Wannet, 2000; Hirano *et al*., 1999). The virulence assay with the four mutated strains confirmed that one strain, WPP40, is reduced in virulence compared to the wild-type strain. The other three mutated bacterial strains are not significantly reduced in virulence nor have they increased in virulence. The applicant provided data demonstrating that insertions in these mutant strains have been stable for many generations in laboratory cultures and no reversions to wild type/antibiotic sensitivity have been detected.

Marker Genes Used as Experimental Controls: The antibiotic resistance marker genes, neomycin phosphotransferase (*nptII*), aminoglycoside adenyltransferase (*aadA*), and chloramphenicol acetyl transferase (*cat*) from *E. coli* were used in the proposed experiment to: 1) disrupt the corresponding *hrp* gene into which the marker gene is inserted, 2) allow for the initial selection of the recombinant bacterium, 3) follow the fate of the recombinant bacterium after release into the environment, and 4) differentiate the recombinant from the non-recombinant bacterium, both indigenous and introduced, after release into the environment.

The green fluorescence (*gfp*) gene, derived from the jellyfish *Aequorea victoria*, is used as a visual marker and allows the plant to produce a low level of green fluorescence under ultraviolet (UV) light.

III. Alternatives Including the Proposed Action

APHIS has considered the following three alternatives in response to the applicant's request for a permit. APHIS' preferred alternative is Alternative 3.

Alternative 1: Deny the permit: release of the regulated organism would not be authorized.

Alternative 2: Issue the permit: the test conditions proposed by the applicant would be authorized, or

Alternative 3: Issue the permit with additional conditions required by APHIS for conducting the field test.

III.1 Discussion of the alternatives:

Alternative 1: No Action/ denial of permit application. Under this alternative, release would not be authorized and research that could improve the understanding of the interaction between this pathogen and potato would not be conducted. This would affect the ability to develop better ways to control the disease. Current management and control practices for *E. carotovora* would continue at the proposed testing locations. Costs and treatment associated with disease eradication would continue.

Alternative 2: Issue the permit for the field testing under the conditions proposed by the applicant. Under this alternative, field release of the microorganisms would be authorized at the specified locations with no additional conditions outside of what the applicant provided in his request. Standard permit conditions under 7 CFR 340.4 would be required (see appendix 2). Standard management practices, including use of some pesticidal sprays, would be included as part of the field trial.

Alternative 3: Issue the permit with additional conditions for conducting the field test. Supplemental permit conditions, based on APHIS analysis, comments from U.S. Fish and Wildlife Service, the State of Wisconsin and public comment on this environmental assessment, would be required. If warranted based on environmental risk of escape of the engineered bacteria, APHIS would require mitigating measures to prevent spread of the organism outside the test area. These measures could include spraying of the test site with antimicrobial compounds to kill the engineered bacteria and/or any other method deemed effective by APHIS.

IV. Description of the Field Test/ Affected Environment

The field experiments are based upon previous experiments completed by another research group at the University of Wisconsin (Hirano *et al*., 1999). The field trial will be conducted in Waushara County, Wisconsin on 0.2 acres of the University of Wisconsin Experiment Station near Hancock, Wisconsin.

Potato tubers and potato stems will be hand inoculated with either the mutant strain alone or in a combination with the parent strain or another mutant strain as detailed in the permit application pages 5 and 6. Planting will be conducted in a randomized complete block design with four to six blocks, each four rows wide and 25 feet long. Experimental details are described in the permit application.

Erwinia carotovora is widely spread in the environment and commonly present on plant surfaces and in lakes and soil where they may enter the plant via wound sites or through natural openings on the plant surface (Toth *et al*., 2003; Perombelon, 2002). The experimental field plot described in this permit is surrounded on all sides by other potato field experiments. To

minimize spread of the bacteria through out the research field, a perimeter fallow zone of two-row alleys, a total of 16 foot wide, will be maintained around each plot of the experiment to ensure that inoculated plants are not inadvertently commingled with plants to be used for food or feed. This perimeter area will not contain any plants that are hosts for *E. carotovora* and will be monitored throughout the field trial (as discussed below) to prevent the bacterial spread beyond the inoculated plants. The field plot is a four-year rotation plot of potato and ryegrass-corn-soybean. Ryegrass, corn and soybean are not hosts for *E. carotovora* subsp. *carotovora* and the mutant strains should not be able to survive on the rotation plants.

Additionally, standard agricultural practices of plowing, hilling, and pesticide application will be used in this field test (see impact on existing agricultural practices described below in section V.2.) The pesticide application includes Admire to control insects and Rimon, Matrix, and Bravo Zn to control different fungi. This will further prevent the bacterial spread to adjacent areas through insects.

The soil at the test site is very sandy and water does not readily run off. Additionally, there are no lakes or streams in the area. This will prevent spread of the bacteria through the water system.

The laboratory at which the testing will be conducted is located at the research station 0.5 miles from the field plots. Samples of inoculated plants collected from field plots will be transported to the field laboratory in sealed plastic bags that are kept in locked ice coolers to prevent spillage.

Field Observation, Monitoring, and Final Disposition of the Test Plants: Data on bacterial populations and incidence of disease will be collected throughout the testing periods. Site monitoring and confinement protocols have been designed to limit dispersal of the recombinant bacterium and are expected to provide the necessary degree of both biological and physical confinement. There will be no inoculated potato left on the field since they will be collected throughout the course of the experiment. Inoculated material will be treated with bleach or autoclaved prior to disposal to kill the *E. carotovora*. Any remaining plant material including the uninoculated border rows will be plowed into the soil. These methods of disposition have been successfully used to minimize the bacterial spread in previous field experiments with bacterially infected plants (Hirano *et al*., 1999).

V. Potential Environmental Impacts

Alternative I: No Action/ denial of permit request:

Field release research would not be allowed. Denying the permit would affect the collection of data on the effect of *hrp* genes on the fitness of *E. carotovora* to help identify other means to control the pathogen, since this is the purpose of the field test. Environmental impacts associated with current management practices would continue.

Alternative II: Issue the permit with no additional conditions:

The proposed field test is a controlled release of the regulated article into the environment. The bacteria, *E. carotovora*, have been mutagenized by marker exchange mutagenesis and the mutant strains were selected for their inability to produce a specific Hrp protein or secrete virulence proteins, such as plant cell wall degrading enzymes. Insertion of antibiotic resistance marker genes into the chromosome of each derivative also conferred resistance to the antibiotic. Reversion of these strains would not pose any additional environmental risk because reverted mutants will be similar to the other *E. carotovora* strains that are commonly present on these plants. The risks associated with the introduction of genetically engineered organisms are generally the same kind as those associated with the introduction into the environment of unmodified organisms and organisms modified by other genetic techniques.

V.1. Impact on the Plant: The genetically engineered strains of *E. carotovora* are identical to the indigenous strain except for their inability to produce hrp proteins to induce a disease reaction in potato and the expression of certain antibiotic resistance marker genes and green fluorescent protein. The applicant performed the virulence assay on plants with the mutant strains and found that virulence of the mutant strain WPP40 was significantly reduced, whereas the virulence of the other mutant strains was not significantly altered and remained similar to the wild type strain.

V.2. Impact on Existing Agricultural Practices: This small field test of 0.2 acres will not have any significant impact on existing agricultural practices because this test is solely for research purposes. *E. carotovora* subsp. *carotovora* causes rotting in many crops in subtropical and temperate regions and has a host range including Brussels sprout, carrot, celery, cucumber, capsicum, turnip, chicory and potato. Although naturally occurring *E. carotovora* strains that lack *hrp/hrc* genes have been found on potato (Yap *et al*., 2004), screening weeds around the potato fields did not reveal any such mutants. Therefore, the mutant hrp strains that are the subject of this release permit are also not expected to have an expanded host range.

The antibiotic resistance genes themselves should not cause these mutant strains to have any competitive advantage in the environment and would not interfere with current agricultural practices to control this disease in potato. Although spraying with streptomycin is used to control *Erwinia amylovora* on fruit trees, it is not normally used to control the soft rot disease in potatoes on this field station.

V.3. Impacts on Human Health: These experiments use resistance to the antibiotics kanamycin, chloramphenicol, and streptomycin/spectinomycin as experimental markers. The introduction of these antibiotic resistance genes, even in the event that they were transferred to new organisms, would not be expected to present a significant risk because these genes are naturally occurring in enteric bacteria such as *E. coli* (http://jb.asm.org/cgi/content/full/180/23/6408?view=full&pmid=9829956) and can transfer resistance determinants to related microorganisms (Wachsmuth *et al*., 1983). The Food and Drug Administration has previously addressed the use of antibiotic resistant marker genes and presence of their proteins in genetically engineered organisms in the 1992 policy statement on foods derived from new plant varieties (http://www.cfsan.fda.gov/~acrobat/fr920529.pdf) and in the draft guidance that was issued on September 1998 (http://www.cfsan.fda.gov/~dms/opa-

armg.html). The Environmental Protection Agency has granted the antibiotic kanamycin resistant gene (*nptII*) and the associated protein exemptions from tolerance in or on raw agricultural products when used as plant-pesticide inert ingredients (http://a257.g.akamaitech.net/7/257/2422/08aug20031600/edocket.access.gpo.gov/cfr_2003/julqtr/pdf/40cfr180.1134.pdf).

The green fluorescence (*gfp*) gene is used as a visual marker and allows the plant to produce a low level of green fluorescence under ultraviolet (UV) light. No impacts of this gene have been reported, nor are any likely to be observed.

Erwinia species are not known as animal or human pathogens. *Erwinia* species are associated with plants as pathogens, saprophytes, or as constituents of the epiphytic flora (Toth *et al*., 2003.) There are no references to association with human or animal disease even though farm workers have been exposed to *Erwinia* for decades. There should be no risk to university personnel handling the inoculated potato since they hand-inoculate potato while wearing gloves and all diseased plants are removed from the field. No potential impact of this experiment on people living in the area of the field trial test plot or any other human population can be identified.

V.4. Horizontal transfer of antibiotic resistance gene from *Erwinia* to other species: This issue has been evaluated by the Food and Drug Administration (http://vm.cfsan.fda.gov/~dms/opa-armg.html). Resistance to antibiotics is already widely prevalent in enteric bacteria and soil-borne bacteria (Wang and Liu 2004; Sengelov *et al*., 2003; Jensen *et al*., 2001; Cole and Elkan, 1979; Bronstad *et al*., 1996). Gene transfer from *Erwinia* to animals and plants is highly unlikely under the conditions of this field test (Syvanen, 1999; Syvanen and Kado, 1998).

V.5. Effects on Threatened and Endangered Species: The proposed field test is a controlled release of the regulated article into the environment in Waushara County in Wisconsin. Neither the engineered *Erwinia* nor the antibiotic genes or green fluorescent protein would affect any non-target organism including any threatened and endangered species listed in Wisconsin. An examination of threatened and endangered species (TES) of plants for Wisconsin listed in the U.S. Fish and Wildlife ECOS database (http://ecos.fws.gov/tess_public/servlet/gov.doi.tess_public.servlets.RegionLists?lead_region=3#WI) showed that six threatened or endangered species exist or once existed in the state. These species are in the Asteraceae, Fabaceae, Iridaceae, Orchidaceae and Ranunculaceae families. *E. carotovora* has been reported to be a pathogen of Orchids and Iris. Search of the Wisconsin Botanical Information System (http://www.botany.wisc.edu/wisflora) showed that these plants are found in Southern Wisconsin and along the shores of the Great Lakes and Lake Michigan. Only one species, Locoweed, Fassett's (*Oxytropis campestris var. chartacea*) has been reported in Waushara County. The applicant indicated that this plant is not located in the 200 acre Experimental Research station where the experimental field is located. Additionally, *E. carotovora* has not been reported to be a pathogen of this plant.

Examination of threatened or endangered animals for Wisconsin in the ECOS database listed ten species. Two species are fresh water fish which would not be impacted by this test. Examination of potentially impacted species such as birds and insects showed that the butterfly

species listed is confined to a narrowly defined habitat due to its dependence on a lupine and a violet species for their lifecycle. The listed Dragonfly species is located in aquatic habitat in Door County, Wisconsin (http://www.museum.state.il.us/research/entomology/hines/mainpage.html). All the bird species listed are either marine, estuary, or forest species whose habitats do not occur in or near these field test sites. Additionally, *Erwinia* species are not known as animal or human pathogens. Therefore these field tests should not impact any threatened or endangered species.

V.6. Cumulative Environmental Effects: The mutants do not have any selective ability to persist in the environment. Physical factors that influence the behavior of the bacteria in the environment have been identified. Screening weeds for the past year around potato fields did not reveal any naturally-occurring *hrp/hrc* mutants of *E. carotovora* even though these mutants have been found on potato. Additionally, the field is a potato-rye grass-corn-soybean rotation plot; rye grass, soybean and corn are not hosts for *E. carotovora* subsp. *carotovora*. All potato plants in the field will be inoculated by hand and all diseased plants removed from the field site through out the experiment for further laboratory testing. This further prevents the bacterial persistence in the environment.

V.7. Special Considerations: Because *Erwinia* is not a human pathogen and the small scale and research nature of the field test, this experiment will not pose disproportionately high or adverse human health or environmental effects to any specific minority or low-income group (Executive Order (EO) 12898, "Federal Actions To Address Environmental Justice in Minority Populations and Low-Income Populations," and EO 13045, "Protection of Children from Environmental Health Risks and Safety Risks.)

Alternative III: Issue the permit with additional conditions.

The potential environmental impacts under this alternative include all those noted under Alternative II. In addition, the applicant must provide BRS with a written summary of the data from the field test which will aid BRS in evaluating the potential risk of future field tests.

VI. Conclusions

On the basis of our review of this application, we conclude that controlled field testing described in this application should not present any risk of new plant pest introduction, should have no significant impact on non-target organisms and the threatened and endangered species, and therefore constitutes a confined field trial for the following reasons:

- *Erwinia carotovora* is widely spread in the environment and commonly present on plant roots of numerous species as well as in lakes, streams, rain, and ground water.
- The engineered bacteria are genetically stable. Even if the antibiotic resistant marker gene that is inserted into the *hrp* gene sequence gets deleted by a classical genetic mechanism, the resulting bacteria would be an *Erwinia* strain virtually identical to the strain that is already widely prevalent in Wisconsin.

- The virulence assay with the mutated strains confirmed that one strain is reduced in virulence compared to the wild-type strain. The other three mutated bacterial strains are not significantly reduced in virulence nor have they increased in virulence. However, virulent strains of this bacterium are indigenous to the area of the test.
- The antibiotic resistant marker genes do not confer any plant pest characteristics to *E. carotovora* and have been safely used in many genetically engineered organisms.
- The gene that encodes the green fluorescent protein has been used as a visual marker in genetically engineered organisms without any reported toxic effects.
- Dissemination of the bacteria will be prevented through physical methods, the small size of the trials and decontamination or appropriate disposal of application equipment.
- The bacterium has never been associated with animal or human disease and therefore would not pose a health risk.
- Native communities, including threatened and endangered species, are not in the host range of *E. carotovora* and therefore should not be affected by the trials.

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Appendix I. Standard Conditions for APHIS 2000 permits

(f) Permit conditions. A person who is issued a permit and his/her employees or agents shall comply with the following conditions, and any supplemental conditions which shall be listed on the permit, as deemed by the Administrator to be necessary to prevent the dissemination and establishment of plant pests:

(1) The regulated article shall be maintained and disposed of (when necessary) in a manner so as to prevent the dissemination and establishment of plant pests.
(2) All packing material, shipping containers, and any other material accompanying the regulated article shall be treated or disposed of in such a manner so as to prevent the dissemination and establishment of plant pests.
(3) The regulated article shall be kept separate from other organisms, except as specifically allowed in the permit;
(4) The regulated article shall be maintained only in areas and premises specified in the permit;
(5) An inspector shall be allowed access, during regular business hours, to the place where the regulated article is located and to any records relating to the introduction of a regulated article;
(6) The regulated article shall, when possible, be kept identified with a label showing the name of the regulated article, and the date of importation;
(7) The regulated article shall be subject to the application of measures determined by the Administrator to be necessary to prevent the accidental or unauthorized release of the regulated article;
(8) The regulated article shall be subject to the application of remedial measures (including disposal) determined by the
Administrator to be necessary to prevent the spread of plant pests;
(9) A person who has been issued a permit shall submit to APHIS a field test report within 6 months after the termination of the field test. A field test report shall include the APHIS reference number, methods of observation, resulting data, and analysis regarding all deleterious effects on plants, nontarget organisms, or the environment;
(10) APHIS shall be notified within the time periods and manner specified below, in the event of the following occurrences:

(i) Verbally notified immediately upon discovery and notify in writing within 24 hours in the event of any accidental or unauthorized release of the regulated article;

(ii) In writing as soon as possible but not later than within 5 working days if the regulated article or associated host organism is found to have characteristics substantially different from those listed in the application for a permit or suffers any unusual occurrence (excessive mortality or morbidity, or unanticipated effect on non-target organisms).

Appendix II. Supplemental Permit Conditions:

1. Regional Biotechnologist may conduct an inspection of the test site at the beginning of the test. Therefore, please inform our office, the State regulatory official, and the appropriate Regional Biotechnologist (see attached map) before the test begins.

2. Additional inspections may be conducted by the Regional Biotechnologist and the State regulatory official. Please notify the relevant Regional Biotechnologist and State regulatory official at least 1-week before termination of the experiment.

3. A field test data report must be submitted within 6 months after the termination of the field test. Field test reports shall include: methods of observation, resulting data, and analysis regarding all deleterious effects on plants, nontarget organisms, or the environment. The report shall also specifically include data on *Erwinia* bacterial populations and incidence of disease. This is to be collected throughout the testing period as stated in the permit. We encourage the inclusion of other types of data if the applicant anticipates submission of a petition for determination of regulatory status for their regulated article. APHIS views these data reports as critical to our assessment of plant pest risk and development of regulatory policies based on the best scientific evidence. Failure by an applicant to provide data reports in a timely manner for a field trial may result in APHIS withholding permission for future field trials.

Confidential Business Information (CBI) will be handled according to the APHIS policy statement 50 FR 38561-63.

4. BRS is to be notified of any proposed changes to the protocol given in the permit application and described in the environmental assessment associated with the issuance of the permit.

5. This approved Biotechnology Permit (APHIS form #2000) does not eliminate the permittee's legal responsibility to obtain all necessary Federal and State approvals, including: (1) for the use of any non-genetically engineered plant pest or pathogens as challenge inoculum; (2) plants, plant parts or seeds which are under existing Federal or State quarantine or restricted use; (3) experimental use of unregistered chemical; and (4) food or feed use of genetically engineered crops harvested from the field experiment.