Finding of No Significant Impact and Decision Notice

Animal and Plant Health Inspection Service

Issuance of a permit to grow perennial ryegrass inoculated with
two strains of genetically engineered fungal endophytes.

The Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) has received a permit application (APHIS number 05-152-01r) from the University of Kentucky to conduct field tests with two genetically engineered strains of the endophyte *Neotyphodium* sp. isolate Lp1. A description of the field test may be found in the attached Environmental Assessment (EA) which was prepared pursuant to APHIS regulations at 7 CFR 372, promulgated under the National Environmental Policy Act. The field tests are scheduled to begin in October 2005 in Fayette County, Kentucky.

APHIS proposed three different actions to take in response to the permit application: the denial of the permit (Alternative I), the granting of the permit with no Supplemental Permit Conditions and no provision for field test reports (Alternative II), and the granting of the permit with Supplemental Permit Conditions containing additional environmental safety requirements and a requirement for the filing of field test reports with APHIS (Alternative III).

A draft EA was prepared and submitted for public comment for 30 days. 8 comments were received and addressed, where appropriate, in the preparation of the final EA, which is attached to this document.

Based on the analysis documented in its EA, APHIS has selected the action proposed in Alternative III. APHIS has determined that the proposed action will not have a significant impact, either individually or cumulatively, on the quality of the human environment and that no Environmental Impact Statement will be prepared regarding this decision.

Pursuant to its regulations (7 CFR 340) promulgated under the Plant Protection Act of 2000, APHIS has determined that this field trial will not pose a risk of the introduction or dissemination of a plant pest for the following reasons.

1. The test fungi *Neotyphodium* sp. Lp1 strains Lp1- 981 and Lp1-4175 are identical to the untransformed endophyte except for their inability to produce toxic ergot alkaloids.
2. *Neotyphodium* species are not known as animal or human pathogens, and both it and its sexually transmitted form of the species (*Epichloë* sp.) are only found in grasses.
3. Dissemination of *Neotyphodium* sp. Lp1 strains Lp1- 981 and Lp1-4175 will be prevented through physical methods, normal site security, small size of the trials, and cleaning of equipment.
4. The host range of *Neotyphodium* sp. Lp1 strains Lp1- 981 and Lp1-4175 and mode of transmission has not changed.

5. The *Neotyphodium* sp. Lp1 strains Lp1- 981 and Lp1-4175 are expected to be less toxic to herbivores than the untransformed endophyte and therefore should not pose any new dietary threat.
6. The *Neotyphodium* species has never been associated with animal or human disease and therefore will not pose a risk to human health.
7. Hygromycin B phosphotransferase (from the marker gene) does not confer any plant pest characteristics to *Neotyphodium* species.
8. Threatened and endangered species in the area are not hosts of *Neotyphodium* sp. nor do they feed on hosts of these fungi, and therefore will not be affected by the trials.

For the reasons enumerated above, which are consistent with regulations implementing the Plant Protection Act, the field trial of two strains of genetically engineered fungal entophytes introduced in perennial ryegrass is hereby authorized.

Cindy Smith
Deputy Administrator
Biotechnology Regulatory Services
Animal and Plant Health Inspection Service
U.S. Department of Agriculture
Date:

SEP 27 2005

Attachment
Finding of No Significant Impact
Response to Comments
AHPIS No. 05-062-1

On August 12, 2005, a notice was published in the *Federal Register* (70 FR 47169-47170, Docket No. 05-062-1) announcing APHIS' intent to allow a confined field release of two genetically engineered strains of the endophyte *Neotyphodium* sp. isolate Lp1 by the University of Kentucky (APHIS Permit No. 05-152-01r) and the availability of the APHIS-prepared Environmental Assessment (EA). During the designated 30-day comment period, which ended September 12, 2005, APHIS received eight comments on these documents.

One of the comments opposed the field release based on a general opposition to all genetically engineered plants. Another asked that antibiotic resistant creations only be studied in "permanently sealed facilities". The individuals did not provide justifications or supporting documentation for these statements.

One individual opposed to the field release provided two responses, one on his own behalf and the other representing a public interest group. Other members of the group were urged to support his opposition by providing comments and referring to his comments. The commenter raised several issues:

1. The commenter stated that the EA did not address the impact of the protein produced by the antibiotic resistance gene, hygromycin B phosphotransferase, other than to note that the United States Environmental Protection Agency (EPA) has granted it an exemption from tolerance. He went on to state that the toxicity and allergenicity of the protein does not appear to have been considered in the proposal. APHIS disagrees with this statement. Toxicity and allergenicity of the protein was considered in EPA's review published in the Federal Register on April 7, 2004 (69 FR 18275-18278). In the review, the EPA concluded that the lack of amino acid sequence similarity of the APH4 protein to proteins known to be mammalian toxins or human allergens further supports lack of toxicity.

2. The commenter expressed concern that the endophyte could break down in the soil and that the … "antibiotic resistance gene will be released to the soil environment where it may transform soil bacteria." APHIS does not consider this occurrence likely. The gene would not transform the soil bacteria, but it is possible that the soil bacteria could incorporate the resistance gene. Studies of horizontal gene transfer from eukaryotes (such as plants and fungi) to bacteria indicate that the frequency is very low and would be likely to become apparent within millions of years rather than within the time scale that genetically engineered plants are grown (Andersson, J.O. 2005; Nielsen et al., 1998). Horizontal gene transfer between prokaryotes (such as bacteria) greatly exceeds gene transfer from eukaryotes to prokaryotes (Jain et al., 1999). As the resistance

gene already exists in the bacterial world, gene transfer of hygromycin from plants to bacteria would not increase the current risk of hygromycin gene transfer between bacteria. Furthermore, transfer frequencies should not be confounded with the likelihood of environmental implications, since the frequency of horizontal gene transfer is probably only marginally important compared to the selective force acting on the outcome. (Nielsen et al., 1998). As pastures and grasslands are not treated with hygromycin B, there is not expected to be a selective advantage for bacteria that have incorporated the resistance gene. (Goldstein et al., 2005). One study estimated that even under optimized conditions, a transformation frequency of less than 10^{-13} (transformants per recipient) would be expected, and this would drop even lower to 10^{-16} in the natural environment due to soil conditions and a lowered concentration of DNA available to cells (Nielsen et al., 1997).

3. The commenter stated that transformation of gut bacteria may occur during digestion of the grass by pasture animals. The United States Food and Drug Administration has concluded that the likelihood of transfer of antibiotic genes from plant genomes to microorganisms in the gastrointestinal tract is remote (http://vm.cfsan.fda.gov/~dms/opa-armg.html). Thus the risk associated with this occurrence is negligible. Furthermore grazing animals will be excluded from the field test by two fences, further decreasing the possibility that this event will occur. In addition, hygromycin B has no clinical utility at this time or in the foreseeable future (Goldstein et al., 2005). In light of the above, APHIS does not consider the transformation of gut bacteria with hygromycin gene during the digestion of grass by pasture animals to be a significant concern.

4. The commenter provided a correlation between this transgenic endophyte and a bacterial endophyte modified with genes for degrading an organic pollutant along with genes for antibiotic and nickel resistance. It was noted that the endophyte *Burkholderia cepacia* is a human pathogen. There is no connection between that example and the transgenic fungal entophyte in this proposal. *Neotyphodium sp.* are not free living and the engineered trait was a loss of function (which could occur naturally) unlike the example given where a new activity was given to the organism. More importantly, *Neotyphodium sp.* are not known to be animal or human pathogens and are only found in grasses (Schardl C.L. and Leuchtmann A. 2005).

5. The commenter stated that the field test should have been preceded by a feeding experiment. It is important to note that the purpose of this confined field release is to generate enough seed to perform such a study in the future. Special precautions have been taken to minimize grazing by animals while seed is generated for the feeding study.

6. The commenter stated that gene disruption can lead to an intra-chromosomal homologous recombination that splices out the inserted gene, mobilizing the insert as a circular DNA unit and restoring the disrupted gene to full activity.

APHIS does not consider this to be a concern. If this DNA insertion were to precisely excise, leaving behind a functional fungal gene, the end result would be an endophyte which produces the toxic ergot alkaloids similar to wild type endophytes already widely prevalent in the environment.

7. The commenter stated that the proposal assumed the insertion of the antibiotic resistance gene would have no impact on the growth of the endophyte in the absence of antibiotic treatment. APHIS disagrees that the proposal or EA makes this assumption. The EA actually reads that "[t]he hygromycin B phosphotransferase enzyme would not confer a selective advantage on the ryegrass with transgenic endophyte as a symbiont, since antibiotics are not used on pastures, hayfields or turf. Further, the *hph* gene does not appear to confer resistance to other clinically relevant antibiotics (Wright and Thompson 1999)." The insertion results in the disruption of metabolic pathways that produce toxic alkaloids which protect the plant from herbivory. As the transgenic endophytes are dependent on the ryegrass for survival, the proposal and EA conclude that the insertion reduces the fitness of the ryegrass and the growth of the endophyte that depends on the fitness of the ryegrass for survival. It is important to note that these mutations are loss of function mutations that could occur naturally.

The remaining 4 comments referenced the above and were against permit approval.

References:

Andersson, J.O. (2005) Lateral gene transfer in eukaryotes. Cell Molecular Life Sciences 62:1182-1197.

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USDA/APHIS Environmental Assessment in response to permit application (05-152-01r) received from the University of Kentucky for field testing of two genetically engineered fungal endophyte *Neotyphodium sp.* isolate Lp1 strains introduced in perennial ryegrass (*Lolium perenne*).

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I. Summary

USDA/APHIS has prepared an environmental assessment in response to a permit application (APHIS Number 05-152-01r) received from the University of Kentucky for confined field tests in Fayette County, Kentucky of two strains of a genetically engineered fungal endophyte of perennial ryegrass. Each new mutant strain of the fungal entophyte, *Neotyphodium* sp. isolate Lp1, has a hygromycin phosphotransferase gene (=*hph*= hygromycin resistance gene) integrated into a unique nuclear chromosome locus resulting in disruption of a key gene for ergot alkaloid biosynthesis. Insertion of the *hph* gene also serves as a marker needed for screening of the gene disruptions.

Seed transmissible symbiotic fungi such as *Neotyphodium* species are common in cool-season grasses such as perennial ryegrass, which are used as pasture, forage, turf, conservation and amenity grasses. The endophytes can increase productivity and longevity of the plants, but can also make ergot alkaloids that negatively affect health of grazing livestock and vertebrate wildlife. Formal tests of the physiological and toxicological roles of ergot alkaloids may be conducted by comparing the effects of wild type (ergot alkaloid-producing) endophytes with derivatives of those endophytes in which key genes for ergot alkaloid biosynthesis have been disrupted. These transgenic endophytes were introduced into perennial ryegrass to produce plants that will not make the alkaloids that have negative health effects on grazing animals. This permit is to plant field plots of perennial ryegrass containing the symbiotic transgenic fungal endophytes, in order to generate sufficient seed for future tests of ergot alkaloid roles and effects on animals.

Data provided in the permit indicates that the endophytes are only transmitted by colonization of seeds during development on endophyte-symbiotic mother plants. These endophytes have no means of transmission/spread from one plant to another, and persist in nature only by vertical transmission in their hosts' maternal lineages. The applicant tested the plant to plant and pollen transmission of the endophyte in greenhouse experiments. Plants inoculated with the endophyte and uninoculated plants were randomly placed in the greenhouse to allow for cross pollination. The endophyte was present in all plants derived from seed produced on endophyte containing mother plants, but was not found in any of the seed progeny from mother plants lacking the endophyte.

Safeguards will be used to prevent dispersal of seed from the plants. The field plot will be fenced to discourage entry by livestock. The area surrounding the field plot will be monitored for ryegrass seedlings out to seventeen meters. After anthesis, each plant will have its panicles enclosed in a mesh bag to minimize any seed loss from shattering. Upon harvest by hand, the mesh bags will themselves be bagged and carefully transported in an enclosed vehicle. Threshing of the seed will be done indoors using a belt thresher that will be disassembled and thoroughly cleaned before use with other plant material and at the end of the operation. All waste material will be autoclaved and the recovered seed will be bagged and labeled.

On the basis of our review of this application, we conclude that field testing described in this application will not present any risk of plant pest introduction, will not have significant impact on non-target organisms or threatened and endangered species, and therefore constitutes a confined field trial. Furthermore, the risk to human health and the environment will be exceedingly low.

II. Purpose and Need

II.1 Proposal:

USDA/APHIS is proposing to issue a permit for confined field release/testing in Fayette County, Kentucky of two strains of a genetically engineered fungal endophyte of perennial ryegrass.

Many pasture and forage grasses possess asexual symbiotic fungi, termed endophytes, which are only transmitted by colonization of seeds during development on endophyte-symbiotic mother plants. These endophytes have no means of transmissible spread from one plant to another, and persist in nature only by vertical transmission in their hosts' maternal lineages. These endophytes are mutualistic symbionts, because they increase fitness of host plants by protecting them from nematodes, insects and other herbivores, and sometimes against drought and heat stress, nutrient limitations, high aluminum concentrations, and other stressors (Műller and Krause 2005). Therefore, the symbionts can be important for maintenance of pasture and turf stands that prevent soil erosion and render marginal habitats agriculturally productive. Certain alkaloids help deter insect feeding on the endophyte-symbiotic grass plants, and these alkaloids fall into four classes: lolines, peramine, ergot alkaloids and indolediterpenes. The latter two classes (ergot alkaloids and indolediterpenes) can cause neuropathologies to livestock or vertebrate wildlife if ingested. Not all endophytes produce all of the protective alkaloids. The wild-type endophyte (isolate Lp1), from which the transgenics in this application were derived, makes peramine and ergot alkaloids, but does not make alkaloids of the other two classes (Panaccione et al. 2003).

Endophyte isolate Lp1 was incorporated into two perennial ryegrass cultivars in New Zealand, which were commercialized for use in pastures. One of the cultivars was withdrawn in 1993 due to the finding that the endophyte produced the toxic ergot alkaloid, ergovaline. (Bouton et al. 2002). It is hypothesized that an endophyte that produces no ergot alkaloids or indolediterpenes would have no ill effects on grazing livestock and vertebrate wildlife. Such a hypothesis is relevant to agricultural and other manmade ecosystems, and also natural ecosystems in North America and worldwide where other grasses also have ergot-alkaloid producing endophytes (Siegel et al. 1990; TePaske et al 1993 , Miles et al. 1998). However, a definitive formal test of this hypothesis has not previously been undertaken because well-controlled biological materials have been unavailable and purified or synthetic ergovaline has been too expensive for most feeding studies. To address this limitation, transgenic endophytes were produced in which the *hph* gene for hygromycin resistance was inserted within key genes for ergovaline biosynthesis, disrupting and eliminating the function of the targeted biosynthesis genes, thereby eliminating production of some or all ergot alkaloids.

One of the transgenic endophytes (Lp1-4175) has its gene for dimethylallyltryptophan synthase (*dmaW*) disrupted, and the other transgenic endophyte (Lp1-981) has its gene for lysergyl peptide synthetase subunit 1 (*lpsA*) disrupted. These endophytes were introduced into perennial ryegrass, cultivar Rosalin, and the ergot alkaloid profiles of the resulting endophyte-symbiotic plants were determined. The disruption of *dmaW* eliminated production of ergovaline and all precursors, namely clavine alkaloids and lysergic acid (Wang et al. 2004, personal communication with applicant). The disruption of *lpsA* eliminated ergovaline and other amides of lysergic acid, but not clavines or unmodified lysergic acid (Panaccione et al. 2001; Panaccione et al. 2003). In order to determine if ergovaline, its precursors, or both affect grazing mammals,

sufficient amounts of the perennial ryegrass with transgenic endophytes must be generated to allow for feeding experiments. Perennial ryegrass requires winter conditions to trigger flowering and seed set. Therefore, this permit application would allow the developer to plant field plots with perennial ryegrass cv. Rosalin plants containing the symbiotic transgenic fungi Lp1-981 and Lp1-4175, and to harvest and store the resulting seeds in preparation for future experiments.

A 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 microorganism may be introduced into the U.S.

A genetically engineered organism is considered a regulated article if it is being introduced and if the donor organism, recipient organism, vector or vector agent used in engineering the organism belongs to one of the taxa listed in the 7 CFR 340 and is also a plant pest, or if there is reason to believe that it is a plant pest. In this submission, the recipient organism is in the Class *Pyrenomycetes,* Order *Hypocreales*, which is one of the listed taxa, and it has been genetically engineered using recombinant DNA techniques. Thus, the genetically engineered microorganism in this University of Kentucky submission is deemed a regulated article. However, it is important to note that there are two life forms of the endophyte species that have resulted in dual nomenclature for this group of fungi, a sexual form *Epichloë* species, and an asexual form *Neotyphodium* species. The sexual form *Epichloë* sp. causes choke disease which suppresses flower and seed production in grasses, and is transmissible to new host plants. The asexual form *Neotyphodium* sp., (previously *Acremonium*), is seen as a mutualistic symbiont of grasses, that causes no visible symptoms and is transmitted vertically through the seeds of the host plant (Műller and Krause 2005, Schardl et al. 2004).

Generally, permitting for confined 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 the categorical exclusion (7 C.F.R.Section 372.5(d)(4)). APHIS is preparing this EA because this is the first permit request for a field test of a genetically engineered plant endophyte, *Neotyphodium* sp. with a novel modification which APHIS considers may raise new issues. This EA 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

Neotyphodium species, such as isolate Lp1, are natural symbionts of grasses, and unlike the sexual form (*Epichloë* sp.) that may cause plant disease, they can often increase the fitness of their host plants. They are not normally found in roots, but might colonize the vascular bundles in stems and leaves and will be found in the seeds. The endophytes lack a fruiting state and the only documented means of dissemination in nature is vertical transmission by host maternal lineages through seeds (Műller and Krause 2005). Transfer between plants in the laboratory is possible, but requires that the endophyte is first isolated and grown in sterile culture, then

physically introduced into grass meristems by inoculation. The methods are laborious and involve manipulations of the plant and fungal materials under conditions in which other microbes are carefully excluded and plants are carried through strict light and temperature regimes (Latch and Christensen 1985; Johnson-Cicalese et al. 2000). Such conditions do not occur in nature, which likely is in part responsible for the lack of plant-to-plant (horizontal) transmission of these *Neotyphodium* species.

Neotyphodium sp. isolate Lp1, the wild type endophyte, is noteworthy among perennial ryegrass endophytes for its lack of lolitrem production, whereas it produces significant amounts of ergovaline and other ergot alkaloids, as well as peramine. Additional simpler ergot alkaloids also have been detected in the endophyte including ergine, a simple amide of lysergic acid, and several precursors to lysergic acid and derivatives of these precursors collectively known as clavines. Ergovaline is an ergopeptide composed of D-lysergic acid linked via an amide bond to a three-membered peptide derived from L-alanine, L-valine, and L-proline. The assembly of the ergopeptines is catalyzed by a multifunctional peptide synthase complex named lysergyl peptide synthase. Lysergic peptide synthase is made up of two separate polypeptides, lysergic peptide synthase 2 (LPS2) and lysergic peptide synthase 1 (LPS1) (Panaccione et al. 2003).

Transgenic Lp1-4175 is derived from *Neotyphodium* sp. isolate Lp1, an endophytic fungus in *Lolium perenne* (perennial ryegrass) (Wang et al. 2004). Like its wild type, the transgenic endophyte is strictly seed-borne, thus vertically transmitted in maternal lineages of the plant. It is incapable of transmissible spread by spores, root grafts, or pollen. This endophyte was transformed with a vector bearing the bacterial gene, *hph,* encoding hygromycin phosphotransferase. A single copy of the *hph* gene was integrated into the dimethylallyltryptophan synthase (*dmaW*) locus thereby replacing and deleting a portion of the *dmaW* in the nuclear genome of *Neotyphodium* sp. isolate Lp1. Wild-type *dmaW* encodes a gene that is essential for the production of clavines, indolediterpenes, and ergot alkaloids (e.g., ergovaline). Ergot alkaloids and indolediterpenes are mycotoxins that are believed to cause toxicoses to grazing livestock and wildlife. The toxicological effects of clavines, if any, are not known. Because of the insertion of *hph*, the *dmaW* gene was inactivated and the transformed strain no longer produces clavines, indolediterpenes, or ergot alkaloids (Wang et al. 2004). The *dmaW* step takes two common and ubiquitous precursors from primary metabolism (tryptophan and dimethylallylpyrophosphate) to the first determinate step for clavine and ergot alkaloids. Without an active *dmaW*, it is expected that the precursors will be used mainly in primary metabolism and indeed, no new metabolites were observed in the knockout strain.
Transgenic Lp1-981 is similar to Lp1-4175, except that a single copy of the *hph* gene is located within, and replaces part of the fungal lysergyl peptide synthetase subunit 1 (*lpsA*) gene (Panaccione et al. 2001). The fungus lacks the ability to produce ergot alkaloids such as ergovaline but because the insertion is further down the metabolic pathway than the *dmaW* disruption, it retains the ability to produce clavines and lysergic acid. Knocking out the *lpsA* gene had no effect on the concentration of most clavines with the exception of 6,7-secolysergine which is roughly doubled. The level of lysergic acid increased by about twenty-five times the level found in the parent *Neotyphodium* sp. isolate Lp1. However, the amount of lysergic acid and derivatives in Lp1-981 corresponded to only 13% of the concentration of lysergic acid and derivatives accumulated in the Lp1 associations, indicating some type of feedback regulation in the pathway (Panaccione et al. 2003, and personal communication with applicant). This feedback regulation may explain why no new clavines were observed in Lp1-981 and the levels of most clavines were relatively unchanged.

In all other ways, both transgenic strains are similar to the untransformed wild-type endophyte. It is important to note that these mutations are loss of function mutations and are expected to occur naturally at some low frequency. The fact that they are not widespread in nature suggests that these mutations are selected against perhaps because they would not discourage herbivory by grazing mammals.

Marker Genes Used as Experimental Controls: The *hph* gene (Gritz and Davies 1983; Kaster et al., 1983) was originally isolated from a bacterium and encodes the enzyme hygromycin B phosphotransferase (Hpt). Hpt is an aminoglycoside-4-phosphotransferase that inactivates the aminoglycoside antibiotic hygromycin B, permitting cells that possess and express this gene to grow on hygromycin-containing medium. Both transgenic isolates Lp1-981 and Lp1-4175 can be grown in culture in the presence of hygromycin B at a concentration of at least 200 mg/L.

The active gene introduced into both transgenics (Lp1-981 and Lp1-4175) is the *hph* gene, modified by addition of upstream and downstream noncoding sequences derived from filamentous fungi, and cloned into the vector pMOcosX (Orbach 1994). The identical *hph* gene has been identified in three bacterial species, *Klebsiella* sp., *Streptomyces hygroscopicus* and *Escherichia coli*. The literature is obscure regarding the specific donor of the *hph* gene in plasmid pMOcosX (Orbach 1994). This *hph* gene is expressed in filamentous fungi due to a promoter from *Neurospora crassa,* which was derived from 1.2 kb of noncoding sequence upstream of the *N. crassa* gene *cpc-1*; and also has 1 kb of sequence including the transcription terminator from the downstream noncoding portion of the *trpC* gene of *Aspergillus nidulans*. The *hph* gene with fungal promoter and terminator comprise the *hph* cassette. Both the promoter and terminator of the *hph* cassette are regulatory sequences from filamentous fungi, and the applicant has found no detectable resistance to hygromycin B conferred to *E. coli* containing this *hph* cassette. The Hpt enzyme would not confer a selective advantage on the ryegrass with transgenic endophyte as a symbiont, since antibiotics are not typically used on pastures, hayfields or turf.

III. Alternatives Including the Proposed Action

APHIS has considered the following three alternatives in response to the applicant's request for a permit:

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. This is the preferred alternative.

III.1 Discussion of the alternatives:

Alternative 1: No Action/ denial of permit application--- Under this alternative, release would not be authorized and research of the endophyte *Neotyphodium* sp. could only be carried out under contained conditions such as a fully contained greenhouse. This would make it difficult to grow enough seed to enable future research on the effect these transgenic endophytes would have on grazing livestock.

Alternative 2: Issue the permit for the field testing under the conditions proposed by the applicant--- Under this alternative, field release of the perennial ryegrass plants inoculated with the genetically engineered endophyte 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).

Alternative 3: Issue the permit with additional conditions for conducting the field test. Supplemental permit conditions, based on APHIS analysis, comments from the State of Kentucky and public comment from this environmental assessment, would be required. If warranted based on environmental risk of escape of the engineered fungus, APHIS will require mitigating measures to prevent spread of the organism outside the test area. These measures could include removal and destruction of host plants, and/or any other method deemed effective by APHIS.

IV. Description of the Field Test/ Affected Environment

As mentioned, *Neotyphodium* species are asexual endophytes. This fungus is not infectious and cannot move from plant to plant. It is only transmissible in seeds from plants already symbiotic with the endophyte, or by artificial means. (Freeman 1904; Sampson 1935; 1937; Latch and Christensen 1985). Therefore, confinement of the plant and its seeds is sufficient to confine the endophytes.

Containment of research material prior to field release: The endophyte, *Neotyphodium* sp. Lp1 was originally obtained in perennial ryegrass seeds provided by Garrick C.M. Latch of the Department of Scientific and Industrial Research (now Crown Research Institute) of New Zealand. The seeds were germinated and plants (as laboratory accession number 144) were grown and maintained in a locked restricted access greenhouse. Cultures of *Neotyphodium* sp. Lp1 were derived from surface-disinfested tissues of those infected plants. Cultures of *Neotyphodium* sp. Lp1 are kept in a locked cold room near the laboratory accessible only to authorized personnel. In addition, this endophyte was introduced by inoculation into perennial ryegrass cv. Rosalin. Inoculated plants are kept in a locked greenhouse. While in the vegetative state, plants were transferred to locked growth chambers for vernalization. After vernalization, the plants were returned to the original greenhouse, where they set seed. All inflorescences were enclosed in mesh bags to minimize any seed loss due to shattering, and to facilitate seed harvest. Seeds were stored in a locked freezer. Seedlings of all plants will be started in pots in the greenhouse in July 2005. In late October 2005, they will be transferred to the field plots.

Field studies: There will be four “treatments” of perennial ryegrass: 1) cv. Rosalin containing no endophyte, 2) cv. Rosalin containing the non-engineered isolate Lp1, 3) cv. Rosalin containing Lp1-4175, and 4) cv. Rosalin containing Lp1-981. Up to 4,000 plants for each treatment will be planted in the field. Thus, 8,000 of these plants will contain a transgenic symbiont. For each treatment, the plot will have 4,000 plants spaced 18" apart in rows 36" apart. Plants will be arranged in four plots, one for each treatment. The plots will be isolated from each other by 2 meter borders kept free of vegetation and a 5 meter border of triticale. The entire perimeter of the plot will also be surrounded by a 2 meter border kept free of vegetation and a 15 meter border of triticale. The triticale borders are to prevent cross-pollination in order to help protect the genetic integrity of the ryegrass cultivar in which the endophyte resides. The borders also serve as an area to be monitored for perennial ryegrass plants as a result of dispersed seed. After anthesis, each plant will have its panicles enclosed in a mesh bag to minimize any seed loss due

to shattering. The bags will be inspected weekly for the first two weeks, and then daily until harvest. Livestock will be prevented from entering the site by two fences. There is a fence enclosing an area of approximately fifteen acres that includes the test plot, and a second fence around the two acre test plot.

Harvest of seed: Once seeds are mature, the triticale border (no longer needed to prevent cross-pollination) will be mown. The perennial ryegrass seed will be retained in the mesh bags, which in turn will be placed inside cloth bags (double-bagged). The bags will be placed in an enclosed vehicle parked on the triticale border and then transported to a dedicated limited access on-site facility for drying, threshing and seed collection. The thresher will be disassembled and thoroughly cleaned before use with other plant material, and all waste plant debris will be appropriately devitalized. Bags of seed will be double bagged, labeled, and transported in an enclosed vehicle to the storage facility at the campus of the University of Kentucky, Lexington, KY. To test for the presence of the endophyte, 90 seeds from each treatment will be germinated in the laboratory, and the seedlings tested by PCR for the mutant or wild-type *dmaW* and *lpsA* genes. Since inheritance of *Neotyphodium* spp. endophytes is strictly maternal (Austen and Scott 1998) and not via pollen, presence of the endophyte in the seed should be the same as that of the plant from which the seed is obtained.

A renewal of this permit will be sought to allow another year of seed production. If that permit is granted, the plants will remain in the field to vernalize over the winter of 2006-2007. Then, again in the Spring of 2007, panicles will be bagged after anthesis, and seeds harvested, processed and stored as described above.

Final disposition of the field trial: After completion of seed harvest in July 2006 or (if permit is renewed) July 2007, plots will be treated as follows to kill all plants and volunteers: The plots will be sprayed with glyphosate or other appropriate herbicide to kill all plants. Additional herbicide applications will be used to manage any volunteers that may arise for a period of three years. Unused seeds and their associated transgenic endophytes will be killed by autoclaving.

V. Potential Environmental Impacts

Alternative I: No Action/ denial of permit request:

Field release research would not be allowed. Further research by the applicant would be restricted to working under containment. It would be difficult under these conditions to generate the quantity of forage required to conduct experiments necessary to determine the effect of the ergot alkaloid on animals.

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 fungal entophyte, *Neotyphodium* sp. Lp1 has been mutagenized by marker exchange mutagenesis and the mutant strains Lp1- 981 and Lp1-4175 were selected for their inability to produce ergot alkaloids that are toxic to vertebrate animals. Insertion of the *hph* gene that encodes the enzyme, hygromycin B phosphotransferase inactivates the aminoglycoside antibiotic hygromycin B, permitting cells that possess and express this gene to grow on hygromycin containing medium. 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 Native Floral and Faunal Communities: *Neotyphodium* sp. Lp1 strains Lp1- 981 and Lp1-4175 - are identical to the untransformed endophyte except for their inability to produce toxic ergot alkaloids. Although *Neotyphodium* sp. Lp1 was developed in the laboratory and has not been identified in any naturalized population, it is closely related to *Neotyphodium lolii* that is found in perennial ryegrass in temperate North America. Both endophytes lack a fruiting stage and are transmitted through seed. Transfer between plants in the laboratory is possible, but requires that the endophyte first be grown in sterile culture, and then physically introduced into grass meristems under precise conditions that do not exist in nature. Therefore, plant-to-plant (horizontal) transmission of these *Neotyphodium* species is extremely unlikely.

Some endophytes attract insects that may aid in horizontal transmission. In the case of *Epichloë* species, there is a conspicuous external structure (stroma) produced, which apparently attracts the insects (*Botanophila* species, formerly classified as *Phorbia* species). These insects do not transmit *Epichloë* between grass plants, although they are indirectly involved in horizontal transmission (Bultman et al. 1995). In this case, the insects transmit spermatia between stromata, thereby initiating the sexual state of the fungus. The culmination of the *Epichloë* sexual state is maturation and ejection of ascospores, and it is the ascospores that mediate horizontal transmission (Chung, K.-R. and Schardl, C. L. 1997), (Brem, D. and Leuchtmann, A. 1999). *Neotyphodium* sp. Lp1 and other asexual *Neotyphodium* species do not interact with these flies, and fail to transmit horizontally because they do not produce stromata.

The hygromycin B phosphotransferase enzyme would not confer a selective advantage on the ryegrass with transgenic endophyte as a symbiont, since antibiotics are not used on pastures, hayfields or turf. Further, the *hph* gene does not appear to confer resistance to other clinically relevant antibiotics (Wright and Thompson 1999).

Because of the lack of horizontal transmission of the endophyte, the only means of dispersal is through seed or movement of the plants. The production design includes a 2 meter fallow zone as well as a 5 meter border of triticale between each of the four treatments. In addition, the entire plot will be surrounded by a 2 meter fallow zone, and outside of the fallow zone will be fifteen meters of triticale. The fallow zone and triticale buffer will be monitored for the presence of perennial ryegrass seedlings. To minimize seed dispersal by birds and rodents, the panicles will be bagged after pollination is nearly complete but before shattering occurs. The applicant will inspect the plot weekly for two weeks after bagging and then daily until seed harvest to ensure the bags are intact and preventing seed dispersal. After harvest the plot will be kept mown to prevent further flowering. To prevent entry by livestock, the plot is situated within a double enclosure of livestock fencing. In the event that the inspection reveals the possibility of seed dispersal by rodents, birds, or by any other means, the applicant will notify APHIS.

V.2. Impact on Existing Agricultural Practices: This small field test will not have any significant impact on existing agricultural practices because this test is solely for research purposes.

V.3. Impacts on Human Health: These experiments use resistance to the antibiotic hygromycin B as an experimental marker. The introduction of this resistance gene, even in the event that it were transferred to new organisms, would not be expected to present a significant

risk because it naturally occurs in bacteria such as *E. coli* and *Streptomyces hygroscopicus* (http://www.invivogen.com/family.php?ID=97). Furthermore, use of the antibiotic hygromycin B does not pose a risk since this antibiotic has limited therapeutic use due to its somewhat toxic nature (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&list_uids=8419155&dopt=Abstract), and it has no use in humans (http://www.forfas.ie/icsti/statements/biotech01/important.htm). The Environmental Protection Agency has granted the associated gene (*hph*) and the protein an exemption from the requirement of a tolerance in or on raw agricultural products when used as a plant-pesticide inert ingredient in cotton (http://www.epa.gov/fedrgstr/EPA-PEST/2004/April/Day-07/p7866.htm).

Both *Epichloë* and *Neotyphodium* species are not known as animal or human pathogens, and are only found in grasses (Schardl C.L. and Leuchtmann A. 2005). Conceivably, the *Neotyphodium* sp. Lp1 endophyte could survive in other species of *Festuca* or *Lolium*. However, in cross inoculation studies, this isolate was found to be incapable of maintaining a stable infection of tall fescue (*Festuca arundinacea*) (Christensen, M. J. 1995). It is important to note that the inoculations were by highly artificial means, because these endophytes are incapable of infecting plants by natural means. 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 hygromycin B resistance gene from *Neotyphodium* to other species.

The issue of horizontal gene transfer from the use of antibiotic resistance marker genes has been evaluated by the Food and Drug Administration (FDA) in 1998 (http://vm.cfsan.fda.gov/~dms/opa-armg.html). FDA concluded that the likelihood of transfer of antibiotic resistance genes from plant genomes to microorganisms in the gastrointestinal tract of humans or animals, or in the environment is remote. In addition, the rate of such transfer, if any, would be insignificant when compared to transfer between microorganisms, and would not add to existing levels of resistance in bacterial populations in any meaningful way. Nonetheless, caution should be the rule for antibiotic resistance markers that inactivate clinically important antibiotics. Some experts consulted for the report also felt that there would be little concern with use of the hygromycin resistance gene as a selectable marker. However, it was mentioned that hygromycin may have important veterinary uses and, therefore, its use should be carefully evaluated in those crops that have animal feed applications. Uses for hygromycin can be found at the website http://www.usitc.gov/tata/bground/wco/41XXX/SC41670E.pdf. This report of the World Customs Organization, Scientific Subcommittee dated November 1997 lists de-worming of poultry as the only use of hygromycin B. However, in 2002, hygromycin B was withdrawn from the market for sales and marketing reasons (Dawe and Hofacre, 2002). It appears that hygromycin has no clinical utility at this time or in the foreseeable future (Goldstein et al, 2005).

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). Hygromycin B is an infrequently used antibiotic, and antibiotic resistant marker genes for hygromycin B resistance are already widely prevalent in bacterial communities. Gene transfer from *Neotyphodium* to animals and plants is highly unlikely under the conditions of this field test (Goldstein et al, 2005).

V.5. Effects on Threatened and Endangered Species: The proposed field test is a controlled release of the regulated article into the environment in Fayette County, Kentucky. Neither the engineered *Neotyphodium* nor the *hph* gene will affect any non-target organism including any threatened and endangered species listed in Kentucky. An analysis of TES distribution in Kentucky using the U.S. Fish and Wildlife ECOS database (http://ecos.fws.gov/ecos/index.do, and Appendix I) indicated that nine threatened or endangered plant species exist or once existed in the state. These species are in the Asteraceae, Lamiaceae, Fabaceae, Brassicaceae, Rosaceae and Caryophyllaceae families. None of the listed species is in the Festuca or Lolium genera. Only species in these two genera are known to be susceptible to *Neotyphodium* species. (Christenson M. J. 1995).

Examination of threatened or endangered animals listed for Kentucky in the ECOS database listed thirty-three species. Most are fish, mollusks and bats. These would not be impacted by this test. Examination of potentially impacted species such as birds showed that the three bird species listed in Kentucky would not be found in this habitat. Similarly, the only mammal besides bats listed in Kentucky is the eastern cougar, which would be unlikely to be found at the site of the field release. Therefore these field tests should not impact any threatened or endangered species.

V.6. Cumulative Environmental Effects: Three primary factors should prevent persistence of the endophytes at the test sites: 1) the endophytes lack a fruiting state and are only transmitted through seed. Seed dispersal will be unlikely because the panicles will be bagged after anthesis; 2) dispersal by birds and other animals will be minimized by bagging and fencing; and 3) at the conclusion of the field test, the plants will be destroyed and the area monitored for three years. Perennial ryegrass seed is not expected to remain viable in the soil for longer than three years based on previous seed dormancy studies. (Rampton and Ching 1966). Thus, the engineered endophytes are not expected to persist in the environment.

V.7 Special Considerations. Because *Neotyphodium* sp. Lp1 is not a human pathogen and this is a small scale research trial, 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. The additional supplemental conditions (see Appendix III) allow BRS to add inspections of the site as needed to ensure that the applicant is following all procedures and conditions described in the application, and to monitor the disposal of regulated material. It also contains requirements added to ensure that the release is confined. 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. Because of the need to ensure that the field test is confined, and the need for further information necessary to evaluate future field tests, alternative III is the preferred alternative.

VI. Conclusions

- The test fungi *Neotyphodium* sp. Lp1 strains Lp1- 981 and Lp1-4175 - are identical to the untransformed endophyte except for their inability to produce toxic ergot alkaloids.
- *Neotyphodium* species are not known as animal or human pathogens, and both it and its sexually transmitted form of the species (*Epichloë* sp.) are only found in grasses.
- Dissemination of *Neotyphodium* sp. Lp1 strains Lp1- 981 and Lp1-4175 will be prevented through physical methods, normal site security, small size of the trials, and cleaning of equipment.
- The host range of *Neotyphodium* sp. Lp1 strains Lp1- 981 and Lp1-4175 and mode of transmission has not changed.
- The *Neotyphodium* sp. Lp1 strains Lp1- 981 and Lp1-4175 are expected to be less toxic to herbivores than the untransformed endophyte and therefore should not pose any new dietary threat.
- The *Neotyphodium* species has never been associated with animal or human disease and therefore will not pose a risk to human health.
- Hygromycin B phosphotransferase (from the marker gene) does not confer any plant pest characteristics to *Neotyphodium* species.
- Threatened and endangered species in the area are not hosts of *Neotyphodium* sp. nor do they feed on hosts of this fungi, and therefore will not be affected by the trials.

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VIII. Agency Contact

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Appendix I. Threatened and Endangered Plant and Animal Species in Kentucky
(http://ecos.fws.gov/tess_public/servlet/gov.doi.tess_public.servlets.UsaLists?state=KY)

COMMON NAME	LATIN NAME	FOUND IN FAYETTE COUNTY KENTUCKY?	FAMILY	FOOD / HABITAT OF SPECIES IN FAYETTE COUNTY
ANIMALS				
Gray Bat	*Myotis grisescens*	No	Vespertilionidae	
Indiana Bat	*Myotis sodalis*	Yes	Vespertilionidae	Flying insects are the typical prey items; diet reflects prey present in available foraging habitat.
Virginia Big-eared Bat	*Plecotus townsendii virginianus*	Unknown	Vespertilionidae	Flying insects are the typical prey items; diet reflects prey present in available foraging habitat.
Cumberland Bean (pearlymussel)	*Villosa trabalis*	No	Unionidae	
Tubercled Blossom (pearlymussel)	*Epioblasma torulosa*	Unknown	Unionidae	Medium to large rivers in gravel riffles
Catspaw (pearlymussel)	*Epioblasma obliquata obliquata*	Unknown	Unionidae	Inhabits large rivers with a sand/gravel substrate
Clubshell	*Pleurobema clava*	No	Unionidae	
Cumberlandian Combshell	*Epioblasma brevidens*	No	Unionidae	
Blackside Dace	*Phoxinus cumberlandensis*	No	*Cyprinidae*	

Duskytail Darter	*Etheostoma percnurum*	No	Percidae	
Relict Darter	*Etheostoma chienense*	No	Percidae	
Bald Eagle	*Haliaeetus leucocephalus*	No	Accipitridae	
Cumberland Elktoe	*Alasmidonta atropurpurea*	No	Unionidae	
Fanshell	*Cyprogenia stegaria*	No	Unionidae	
Winged Mapleleaf (mussel)	*Quadrula fragosa*	No	Unionidae	
Pink Mucket (pearlymussel)	*Lampsilis abrupta*	No	Unionidae	
Oyster Mussel	*Epioblasma capsaeformis*	No	Unionidae	
Cracking Pearlymussel	*Hemistena lata*	No	Unionidae	
Dromedary Pearlymussel	*Dromus dromas*	No	Unionidae	
Littlewing Pearlymussel	*Pegias fabula*	No	Unionidae	
Rough Pigtoe (mussel)	*Pleurobema plenum*	No	Unionidae	
Orangefoot Pimpleback (pearlymussel)	*Plethobasus cooperianus*	No	Unionidae	
Piping Plover	*Charadrius melodus*	No	Charadriidae	

Fat Pocketbook (mussel)	*Potamilus capax*	No	Unionidae	
Eastern Cougar	*Puma (=Felis) concolor couguar*	Unknown	Felidae	Unlikely to be found on research facility
Northern Riffleshell (clam)	*Epioblasma torulosa rangiana*	Unknown	Unionidae	Medium to large rivers in gravel riffles
Tan Riffleshell (clam)	*Epioblasma florentina walkeri (=E. walkeri)*	Unknown	Unionidae	Freshwater habitat
Ring Pink (mussel)	*Obovaria retusa*	No	Unionidae	
Palezone shiner	*Notropis albizonatus*	No	Cyprinidae	
Mammoth Cave Shrimp	*Palaemonias ganteri*	No	Atyidae	
Pallid Sturgeon	*Scaphirhynchus albus*	No	Acipenseridae	
Least Tern	*Sterna antillarum*	No	Laridae	
White Wartyback (pearlymussel)	*Plethobasus cicatricosus*	No	Unionidae	
PLANTS				
Price's Potato Bean	*Apios priceana*	No	Fabaceae	
Braun's Pocket Cress	*Arabis perstellata*	No	Brassicaceae	
Cumberland Sandwort	*Minuartia cumberlandensis*	No	Caryophyllaceae	
Cumberland Rosemary	*Conradina verticillata*	No	Lamiaceae	
Eggert's Sunflower	*Helianthus eggertii*	No	Asteraceae	
Whitehaired Goldenrod	*Solidago albopilosa*	No	Asteraceae	

Short's Goldenrod	*Solidago shortii*	No	Asteraceae	
Virginia Spiraea	*Spiraea virginiana*	No	Rosaceae	
Running Buffalo Clover	*Trifolium stoloniferum*	Yes	Fabaceae	Habitat most commonly is mesic woodlands in partial to filtered sunlight.

Appendix II. **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) Consistent with standard permit conditions at 7 CFR 340.4(f) (10), APHIS shall be notified verbally immediately upon discovery and in writing within 24 hours in the event of any accidental or unauthorized release of the regulated article. For immediate verbal notification, contact the following APHIS staff in the order indicated below.

1. APHIS BRS Deputy Administrator's office [phone numbers: (301) 734-7324; (301) 734-5716; (202) 720-4383)]. Indicate that you wish to report an unauthorized or accidental release of a regulated article to the BRS Regulatory Division Director; or in that person's absence, to the Chief of either the BRS Biotechnology Permit Program Operations staff or the Biotechnology Risk Assessment staff, or the permit reviewer. In the event that one of these persons cannot be reached, contact:
2. The appropriate APHIS PPQ Regional Biotechnologist.
3. The appropriate APHIS State Plant Health Director.

Contact information is maintained at the APHIS Biotechnology Regulatory Services website at http://www.aphis.usda.gov/brs.

Unless otherwise directed, written notification should be sent to:

Animal and Plant Health Inspection Service (APHIS)
BRS Regulatory Division (2) Director, Rm. 5B54
4700 River Rd. Unit 147
Riverdale, MD 20737.

When the regulated article or associated host organism is found to have characteristics substantially different from those listed in the permit application, or suffers an unusual occurrence (excessive mortality or morbidity, or unanticipated effect on non-target organisms), APHIS shall be notified as soon as possible but no later than within 5 working days. In such cases, notice should be sent to:

Animal and Plant Health Inspection Service (APHIS)
Chief, Biotechnology Permit Program Operations, Rm. 5B53
4700 River Rd. Unit 147
Riverdale, MD 20737.

Appendix III. *Proposed* Supplemental Permit Conditions

Permit: 05-152-01r, University of Kentucky, Endophyte – *Neotyphodium* sp.

1. The Biotechnology Regulatory Services or a Regional Program Manager (Biotechnology) may conduct an inspection of the test site at the beginning of the test. Therefore, the applicant is required to notify our office, the State regulatory official, and the appropriate Regional Biotechnologist at least one week prior to the start of the test.

2. Additional inspections may be conducted by a Plant Protection and Quarantine Officer. The permittee is required to notify the Regional Program Manager (Biotechnology) and the State Official at least 1-week before termination of the experiment.

3. Within 28 calendar days after release, a report must be submitted that includes the following information for each field test site:

A. A diagram of the sites, with sufficient information to locate it.
B. The total acreage of the test plots.

Fax the report to the following APHIS personnel:
1. The Chief, Biotechnology Risk Assessment Staff at Area Code (301) 734-8669
2. The PPQ Regional Biotechnologist (fax number enclosed)
3. The State Regulatory Official (CBI-Deleted copy only)

4. A field test data report must be submitted within 6 months after the termination of this permit for each of the field tests growing or installed during this permit. Field test reports shall include: methods of observation, resulting data, and analysis regarding all deleterious effects on plants, nontarget organisms, or the environment. We encourage the inclusion of other types of data if the applicant anticipates submission of a petition for determination of non-regulated 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 the withholding of permission by APHIS for future field trials.

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

5. The procedures, processes, and safeguards which will be used to prevent escape, dissemination, and persistence of the transgenic organism and its progeny at each of the intended destinations as described in the permit application and in these supplemental permit

conditions must be strictly followed. The permittee must maintain records sufficient to verify compliance with these procedures, including information regarding who performed the activity. Biotechnology Regulatory Services should be notified of any proposed changes to the protocol referenced in the permit application and described in the environmental assessment associated with the issuance of the permit.

6. The permittee will install a fence around the estimated 2 acre field plot for the purpose of keeping livestock out of the field plot. The applicant will regularly inspect the fencing to ensure it is in working condition.

7. The permittee will encircle the field plot with a two meter fallow zone and a fifteen meter buffer zone planted with triticale. After the triticale is mown to facilitate harvest of the ryegrass seed, the border area will be thoroughly inspected for the presence of perennial ryegrass seedlings. If seedlings are found in the border area, the applicant will notify APHIS (in accordance with standard permit condition #10), make note of it in the field data report, and destroy the plant.

8. During the period that the panicles are bagged, the permittee will inspect the plot weekly for the first two weeks, then daily until harvest to ensure that all panicles have bags, and that the bags are in suitable condition to contain the seed. The daily inspections will continue until seed harvest. The permittee will notify APHIS (in accordance with standard permit condition #10) in the event there is evidence of possible seed dispersal.

9. The test site shall be monitored for any volunteer seedlings or plants for (3) three years after the completion of the test; if any volunteer seedlings or plants are found, they should be destroyed before flowering. The field plot will remain fallow and volunteers controlled as described in the permit application. The livestock fencing must be kept in place and in working condition until completion of the monitoring period.

10. 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.

Under the Plant Protection Act, individuals or corporations who fail to comply with these conditions and authorizations, or who forge, counterfeit, or deface permits or shipping labels may receive civil or criminal penalties, and may have all current permits canceled and future permit applications denied.

The permit holder is responsible for the disposition of the organisms throughout the duration of the permit. If the permit holder leaves the institution where the organisms are kept, all organisms must be destroyed, unless a new individual assumes responsibility for the continued maintenance and submits an APHIS Form 2000 application and obtains a permit prior to the permittee's departure.