FHP TECHNOLOGY DEVELOPMENT PROJECT PROPOSAL

PROJECT STATUS: New, not previously funded.

PROJECT NUMBER: NA-1999-05

PROJECT TITLE: Development of a Molecular Protocol for Detecting the Ash Dieback Bacterium, a New Threat to Northeastern Forests

SUBJECT: Disease Management; Survey, Evaluation and Monitoring; pathogen detection; ash dieback bacterium

PROJECT OBJECTIVE: To develop an operational protocol for sample collection, DNA extraction and specific detection of the ash dieback bacterium (ADB).

BRIEF DESCRIPTION OF PROJECT: A newly-discovered bacterium is causing extensive damage to Iowa's ash trees. The distribution and extent of the disease is unknown. Symptoms are easily confused with the less serious ash yellows disease. Existing methods (e.g. DAPI tests) for detection cannot distinguish between the ash yellows phytoplasma and the new bacterium. This project would develop a new laboratory protocol (based on PCR technology) to detect the bacterium and distinguish it from ash yellows phytoplasma. The project would focus on developing a reliable and efficient sampling method with a highly specific set of PCR primers.

FHP PERSON WHO WILL LEAD THE PROJECT: Linda Haugen

JUSTIFICATION: Biotechnology has provided powerful tools for the characterization and detection of microorganisms, but the forest health profession has not, in general, taken advantage of these technologies. It is because of the use of outdated technologies that a new bacterial pathogen on ash (Fraxinus spp.) has gone undetected until 1998. The symptoms of ABD had been noted in Iowa and elsewhere in the Northeastern Area since the late 1980s (Gleason et al., 1997; Luley et al, 1992). However, ABD was confused with ash yellows (Sinclair et al., 1996), which is caused by a wall-less phytoplasma that is less aggressive than the ADB, a walled bacterium.

Diagnosis of ash yellows has been based routinely on a protocol using the fluorescent stain DAPI (4,6-diamidino-2-phenylindole), which binds to DNA (Gleason et al., 1997; Luley et al., 1992; Sinclair et al., 1996). Sieve tubes of plant phloem are devoid of nuclei, so DAPI fluorescence in the sieve tubes indicates the presence of microbial DNA. It has been assumed that DAPI+ trees were infected with the ash yellows phytoplasma, although it is becoming increasingly recognized that other prokaryotic pathogens are also found in sieve tubes. Two surveys of declining ash in the Midwest found many DAPI+ trees, which were diagnosed as ash yellows, although the symptoms were not consistent with and were more severe than ash yellows (Gleason et al., 1997; Luley et al., 1992).

Our examination in 1998 with molecular tools of the DAPI+ trees in Iowa (Gleason et al., 1997) showed that the sieve tube inhabitant was not the ash yellows phytoplasma, but rather, a highly virulent proteobacterium. Unlike the ash yellows phytoplasma, the ADB causes severe crown dieback in the absence of witches' brooms. Also, the ADB can be readily inoculated into ash under greenhouse conditions, and it kills inoculated white ash in just a few weeks.

We have successfully used the polymerase chain reaction (PCR) to detect the proteobacterium in ABD trees. The technique requires extraction of DNA from diseased plant tissue and the use of primers (short stretches of DNA with sequences unique to ADB) to make copies of a stretch of the 16S ribosomal gene (rDNA) of the ADB. We have confirmed the presence of the ADB in diseased white ash, green ash, blue ash, and lilac in Iowa, where the disease is epidemic. The detection of ADB is highly correlated with thin crowns and branch dieback, but the geographic extent of ABD is unknown.

It is quite clear that DAPI and other non-molecular tests are insufficient for diagnosing tree diseases caused by phloem-inhabiting prokaryotes, which are unculturable and difficult to detect (Avila et al., 1998; Bruton et al., 1998; Davis et al., 1996, 1998; Jagoueix et al., 1994). These pathogens may be responsible for many of our unexplained declines of hardwood species. More precise tests are needed. The technology for such tests is available but awaits development. We currently have a PCR-based test that can distinguish the ash yellows phytoplasma from the ADB, but this test needs further development to be used on an operational level. We propose to refine this technique and develop a sampling protocol that will improve efficiency and broaden the testing and awareness of ABD.

Ash trees are not only significant components of the hardwood forests of the Northeastern area, they are also among the most commonly planted trees in urban forests. The impact of the ABD epidemic in many Iowa communities is alarming, and many foresters say the disease in white ash has gotten much worse in the last four years. It is imperative that we determine the extent of this disease in order to develop sounder management practices. It is especially important that infected nursery stock not be distributed, but the currently applied technology for diagnosis is inadequate.

Detection of microbial pathogens, particularly unculturable or fastidious microorganisms, has been extremely difficult and has impeded recognition of exotic pests and their distributions. The ABD problem will be a good model system for the development of a highly specific, biotechnological approach to pathogen identification. This proposed project would refine tools that would not only help to clarify the distribution and impact of the new ADB, but also point the way for the detection of other exotic and difficult to detect pathogens that threaten our forest resources.

URGENCY: This is a newly discovered disease, and at present we do not know its geographic extent. To date, we have only confirmed the pathogen in central and eastern Iowa, although we believe it is in western Iowa and possibly North Dakota. Natural spread from Iowa may be slow, but infected nursery stock is an excellent inoculum source and will likely prove the most serious factor in the spread of the pathogen throughout the urban ash populations of the Northeast and elsewhere. Minimizing the distribution of infected nursery stock is believed crucial to disease management. We must know the geographic extent of the pathogen to determine which nursery production

areas need to be monitored for the disease. However, we need to further develop the PCR protocol for detecting the pathogen before we can efficiently and routinely test for the presence of the pathogen.

Many white ash stands in eastern Iowa have greater than 50% of the ash trees dead or dying, and most communities in eastern and central Iowa have 10 to 20% of their green ash infected with what is now known to be ABD (Gleason et al., 1997). The severity of this disease, especially on white ash, points to the exotic nature of the pathogen. This is not a normal component of eastern hardwood forests.

We should be able to slow the spread of the pathogen through a specific and unambiguous detection protocol. However, each year of delay will result in more foci of disease arising in urban forests. At present, there appears to be no reasonable control except for replacing diseased trees will non-susceptible genera, so the establishment of new foci appears to result in the irreversible loss of ash as a street tree in that urban forest. The economic and environmental impact on the surrounding natural stands of ash is not as clear but is expected to be more severe than the loss in urban forests.

LINK TO NATIONAL FHP TECHNOLOGY DEVELOPMENT PRIORITIES: The project addresses the following technical committee priority: ``Develop or improve detection, analysis, and management of exotic pests including weeds, especially technologies or methods that contribute to our ability to prevent introductions or to detect and eradicate new introductions.'

Fastidious microorganisms have been extremely difficult to identify and detect, and this has led to misdiagnosis and the failure to recognize exotic pathogens around the world. Serious epidemics by exotic, phloem-inhabiting prokaryotic pathogens have erupted with devastating consequences, especially in fruit and extractive tree species. The most notable epidemics caused by exotic phytoplasmas would include lethal yellows of palm in the Caribbean, Florida, Mexico and western Africa; lethal decline of palm in eastern Africa; sandal spike in India; and paulownia witches' broom in Asia (Rayachaudhuri and Maramoroosch, 1996). Each of these epidemics was well underway before the detection technology was available to contain the spread. Besides the applicability to ash bacterial dieback, which we hope is limited to the western Midwest, the technology we propose to develop here will lead to protocols for diagnosis of phytoplasma diseases and other diseases caused by microorganisms that are difficult or impossible to culture.

SCOPE OF APPLICATION: The protocol to be developed should be of immediate use throughout North America, wherever Fraxinus species are grown as ornamentals. Although it is hard to predict the technologies available in future decades, the PCR approach using rDNA genes is cutting edge and should be useful for many years. Further, this should help set the standard for detecting other fastidious microorganisms of importance to forestry.

RESEARCH BASIS: The disease in Iowa has been noted in two studies by others (Gleason et al., 1997; Luley et al., 1992) and provides a good basis for the development of the detection protocol. We just began the molecular detection of ADB in 1998, and we have no publications to cite as a basis. However, we did present a poster at the

September 1998 meeting of the North Central Forest Pest Work Conference. In that poster we presented data on completion of Koch's postulates (characterization of the symptomatology, association of the putative agent with the disease, inoculation of healthy plants with the agent and reproduction of the symptomatology, and association of the agent with the symptomatic inoculated plants). Association of the agent with the disease was done through PCR amplification of a portion of the rDNA gene of the pathogen. The DNA sequencing of a portion of this amplified fragment allowed comparison to the 16S rDNA sequences of other prokaryotes, and this analysis showed the ash dieback bacterium to be a member of the beta proteobacteria and unrelated to the phytoplasmas.

The use of PCR to detect fastidious prokaryotes has been available for about 10 years and has been frequently used to characterize such organisms (Avila et al., 1998; Davis et al., 1996, 1998; Jagouiex et al., 1994). We (Harrington and Wingfield, 1995; Harrington et al., 1998; Sung et al., 1997; Witthuhn et al., 1998, 1999) have used similar rDNA technology to develop detection tools and to study the evolution of fungal pathogens of trees. The Armillaria diagnostic test (Harrington and Wingfield, 1995) has found widespread use throughout the world and is now the accepted diagnostic technique for this difficult group of fungi. Thus, we are well versed in the technology to be developed.

The use of PCR technology has allowed us to recognize that a pathogen other than the ash yellows phytoplasma is causing the disease in Iowa and to complete Koch's postulates with the ash dieback bacterium. We are repeating inoculation studies, electron microscopy and full sequencing of the 16S rDNA of the ADB, and these basic studies will be incorporated into a manuscript to be submitted within the next 6 months to a refereed journal.

METHODS: We currently have effective primers and protocols for amplifying the 16S rDNA gene fragments of the ADB and the ash yellows phytoplasma, but improvements are needed to increase efficiency and sensitivity. In 1999, we will sample symptomatic white and green ash trees monthly from April through November. When available, leaf samples will be taken, along with twig, branch, stem and root flare samples. Extractions will be made from petioles and mid-ribs of leaves or from the inner bark (phloem). We will compare different methods for extruding the bacteria from sieve tubes. Different DNA extraction protocols will be attempted to optimize yields, improve efficiency, and lower costs. We will perform DNA sequencing on some of our PCR products to make sure that no other prokaryote is being detected. If rDNA of another prokaryote is detected, new primers that are more specific to the pathogens will be developed.

Once the protocol is identified, specificity of the primers can be easily tested by extracting DNA from plants with various diseases caused by fastidious prokaryotes (e.g., ash yellows, elm phloem necrosis, oak scorch, sycamore scorch). At least 100 symptomatic ash will also be tested to see how effective the protocol is in detecting the pathogen, and at least 10 of these PCR products will be partially sequenced to show that it is of the ADB. Healthy ash trees (at least 100) will also be tested to assure that no false positives are found.

MEASURE OF SUCCESS: The success of a detection protocol is based on how widely the technique is utilized and the value of the information obtained with the technique. We hope the protocol will be suitably efficient and economical so that university labs across the region will routinely test symptomatic trees in their area for ADB, the ash yellows phytoplasma, and other fastidious prokaryotes. Also, it is hoped that a public or private lab will use the protocol to provide a fee-based service for the diagnostic test. The Iowa State University Plant Disease Clinic may be able to provide such a service.

Beyond the scope of this project, we intend to apply the technology to determine the extent of the disease in Iowa, determine its host range, and to determine how much of the unexplained decline of ash in the Northeastern Area is associated with ADB. Thus, the technology should ultimately result in delineation of the geographic extent of the pathogen and some impression on its impact; and the extent to which this information is obtained will largely determine the measure of success of the project. Beyond these measures, we believe the technology will expand the awareness of such exotic prokaryotes and their impact on our forest resources.

COOPERATORS:
Linda Haugen, U.S. Forest Service Forest Health Protection.
Tom Harrington, Professor of Plant Pathology, Iowa State University (ISU). Coordination of the project, experimental design, assessment of results, writing of manuscripts. (5% of time).
Chris Feeley, Graduate Research Assistant, Forestry, ISU. Location of sample trees, sample collection, training of hourly employees. (25% of time).
Woody Hart, Professor of Entomology, ISU. Co-advisor of Chris Feeley. (less than 5% of time).

PRODUCTS: A standard protocol for type of sample, DNA extraction method, and PCR analysis will be developed for detection of the ash dieback bacterium. The sampling protocol will include the suitable times of the year and the type of plant tissue (midribs, petioles, or inner bark of twigs, branches, stems or root flares) to be collected. This procedure will be published in a refereed journal and will be freely available to the public. Publication will be in the year 2000, though we will make the procedure available in late 1999.

PUBLICATION: As stated above, the procedure will be published in a refereed journal article.

TECHNOLOGY TRANSFER: In addition to publication in a refereed journal, we will present our findings to other forest health practitioners at the North Central Forest Pest Workshop and other similar forums. Between these forums and the scientific literature, all those wishing to have samples analyzed or wishing to study the disease should be knowledgeable of the technique and should be able to have samples analyzed. ISU may be able to offer the diagnostic test on a per fee basis if another public or private agency does not do so. The US Forest Service FHP staff will also make this information known

through e-mail lists, contacts with state forest health specialists and other appropriate outlets.

PROJECT DURATION: This project will be completed in 1 year, commencing January 1, 1999 and ending December 31, 1999. It will, thus, involve federal fiscal years 1999 and 2000.

LONG TERM BUDGET:
FHP Funding Needed by FY: FY99, 1/1/99 to 9/30/99- $18,000;
FY00, 10/1/99 to 12/1/99-$4,000

ISU will contribute 5% of Tom Harrington's salary ($5727, including benefits), 25% of Chris Feeley's stipend ($3500), and the use of ISU facilities. We have applied for an additional $16,000 from Iowa State University to initiate basic studies on ash bacterial dieback. If this funding is received, it would help in the development of the protocol, although it would not diminish the needed funding from FHP. The Iowa Nursery and Landscape Association (INLA) is contributing $4000 in support of the ash dieback research at ISU.

FY 1999 BUDGET REQUEST:

Item	Requested FHP	Iowa State University	INLA
Admin:			
Salary	6000	9227	1000
Overhead	0	0	0
Travel	3000	0	1000
Procurements:			
Contracting- DNA Sequencing	2000	0	0
Equipment	0	0	0
Supplies	7000	0	2000
Other	0	0	0
TOTAL FY 99	18,000	9227	4000

STDP Production Function

PROJECT NUMBER: NA-1999-?

PROJECT COST:

Year:	1	2	Total
STDP request	$18,000	$4,000	$22,000

ISU contribution	$ 9,227	0	$ 9,227
Iowa Nursery Assoc.	$ 4,000	0	$ 4,000
Total Budget	$31,227	$4,000	
$35,227			

PROJECT OBJECTIVE: This project will optimize sample collection, DNA extraction and specific detection of the ash dieback bacterium and distinguish it from the ash yellows phytoplasma. With this methodology, we will be able to survey the geographic distribution of the ABD pathogen. We presume that if we can determine the distribution of the pathogen, we will be able to limit its spread to uninfected locations by preventing the movement of infested nursery stock and other plant material. Without this methodology, we cannot distinguish ABD from other phloem-inhabiting pathogens, we cannot determine the distribution of the pathogen, and we cannot evaluate the impact of this disease. The primary quantifiable benefit calculated in this production function is the avoidance of tree removal/replacement costs in urban landscapes, which will accrue as we apply the methodology to determine the distribution of the pathogen and limit its spread. Benefits in natural woodland stands were not quantified, but these benefits, particularly related to detection of the pathogen and limiting its spread, could be substantial. However, until we have the tools developed to assess impact of this pathogen, an economic analysis in woodlands would be very difficult.

ASSUMPTIONS:
1) This is a newly introduced pathogen, and does not yet occur outside of Iowa.
2) Without a means to accurately detect and manage the pathogen, we assume that 100 new foci of disease will be established.
3) With an accurate tool to detect the pathogen, we assume that by limiting movement of infected material we will be able to reduce that to 20 new foci.
4) We assume a population of 10,000 ash trees in each focus, considering that the population may be spread over several small to mid-size cities in a region.
5) We assume a loss rate with an r-value of 0.46 and an initial introduction of 10 trees at each focus. Using standard disease progress models (i.e., logit transformation; Manion, 1981), 1.0 % of the population would be diseased at 5 years after the start of the focus (i.e., 100 diseased trees per focus at five years post introduction).
6) Cost of tree removal = $300, cost of tree replacement = $200. If we assume that 50% of the trees removed are replaced, the average cost per tree for removal/replacement is $400.
7) Without the detection tool, 10,000 trees would need to be removed/replaced over a 5-year period.
8) With the detection tool, 2000 trees would need to be removed/replaced over a 5-year period.
9) To simplify calculations, we assume tree mortality in each focus to be equally distributed throughout the 5-year period.
10) Each year for the five-year period, 200 samples will be tested to monitor nursery stock. The cost for each sample will be $50 ($25 for sample collection and $25 for the PCR test). With 200 samples processed each year for 5 years, this would be $50,000. (Discounted over 5 years, this comes to a total of $44,520).

BACKGROUND DATA:

1) The disease has been identified in many communities in Iowa. Surveys in Iowa cities using DAPI tests indicated the following percentage infected green ash trees: 19.4% in Waterloo, 18.8% in Davenport, 13.9% in Burlington and Iowa City; and 11.1% in Des Moines and Fort Dodge (Gleason et al., 1997). A subsample of these DAPI positive trees in Iowa City all proved to harbor the ash dieback bacterium and not the ash yellows pathogen based on our PCR technique. Although we do not know how long the pathogen has been in Iowa, the 1% figure for an incidence level 5 years after introduction appears to be an underestimate.

2) There are no sources of information on spread rate for this phloem-limited prokaryote. The closest available data is for lethal yellowing of palm, caused by a phytoplasma. Data on the spread of this disease in Africa (Maramorosch, 1996) indicate a fairly slow annual linear spread rate, perhaps 2 miles/year in the absence of human mediated spread. For a disease with low linear spread rate, significant reduction in spread of the disease can be accomplished by preventing new epicenters of disease through movement of infected material (e.g., nursery stock).

3) The r-value for the increase of an elm phloem necrosis epidemic in Indiana was r=0.46 (Manion, 1981).

4) "Green ash is among the most commonly planted trees in Midwestern U.S. cities and towns, often constituting 20 to 40% of the urban forest." (Gleason et. al., 1997).

CALCULATIONS:

Expenditure & Output Values (EOV) without project:
$400/tree x 10,000 trees = $4,000,000 for 5 years,
which = $3,561,457 when discounted over 5 years.

Expenditure & Output Values (EOV) with project:
$400/tree x 2000 trees = $800,000 for 5 years.
which = $712,292 when discounted over 5 years.

Benefit (Change in EOV) Attributable to Project:
B= $(with) - $(without) = (-712,292) - (-3,561,457) = $2,849,165

Benefit/Cost Ratio:
B/C = $(with) - $(without) / $(STDP funding + contributed funds + monitoring costs)
= ($2,849,165)/($21,846 + 13,227 + 44,520)= $2,849,165/$79,593 = 35.80

Benefit Attributable to STDP:
= $(STDP cost) x (B/C) = $21,846 x 35.80 = $782,087

PNV of Project:
= $(Benefit) - $(Cost) = $2,849,165 - $79,593 = $2,769,572

PNV of STDP:

= $(Benefit attributable to STDP) - $(STDP cost) = $782,087 - $21,846 = $760,241LITERATURE CITED:

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