2015 Project Abstract
For the Period Ending June 30, 2019

PROJECT TITLE: Preventing a New Disease of Pines in Minnesota
PROJECT MANAGER: Robert A. Blanchette
AFFILIATION: University of Minnesota
MAILING ADDRESS: Department of Plant Pathology, 1991 Upper Buford Circle, 495 Borlaug Hall
CITY/STATE/ZIP: St. Paul, MN 55108
PHONE: 612-625-0202
E-MAIL: robertb@umn.edu
FUNDING SOURCE: Environment and Natural Resources Trust Fund
LEGAL CITATION: M.L. 2015, Chp. 76, Sec. 2, Subd. 06d as extended M.L. 2018, Chp. 214, Art. 4, Sec. 2, Subd. 20

APPROPRIATION AMOUNT: $371,000
AMOUNT SPENT: $371,000
AMOUNT REMAINING: $0

Sound bite of Project Outcomes and Results
Heterobasidion root disease is a serious new pathogen of red and white pine that has been found in southeastern Minnesota. New diagnostic tools were developed, tested and successfully diagnosed samples with the pathogen. Selection of native fungi antagonistic to this pathogen were found and their biocontrol potential has been evaluated.

Overall Project Outcome and Results
A new invasive tree disease called *Heterobasidion* root disease is a serious threat to Minnesota's red and white pines as well as other conifers. It is considered the most economically important disease of pines throughout the Northern temperate regions. In recent years, the pathogen has moved through Wisconsin and is now found on red pine in southeastern Minnesota. New molecular methods to identify the pathogen were developed and are being used to successfully identify the pathogen from field samples. Monitoring disease progression has been initiated and spores of the pathogen appear to be moving into new areas. Finding this disease early is essential so that control procedures can be initiated to limit the spread of this disease. Control methods for Minnesota have been evaluated and management guidelines were developed in collaboration with the Minnesota Department of Natural Resources. A series of videos on the biology and control of this disease as well as information on our research activities to find biocontrol agents were developed. These educational materials are being widely used by foresters and landowners in Minnesota as well as in other states. Although some control options are available, research was carried out to identify the possibility of new biocontrol methods that could be used. Native fungi that are antagonistic to the pathogen were tested for their potential use as biological control agents. Several were found to be effective and are ready for field testing. This work has helped to limit the spread of this pathogen in Minnesota and has provided new information on potential future biological control methods. The detection protocols that were developed have been found to be very effective for monitoring this pathogen and can now be adapted and used to survey for other invasive forest pathogens that may affect Minnesota's trees.

Project Results Use and Dissemination
To disseminate important information obtained from the project we developed four videos that explain the identification, biology and management options for Heterobasidion root disease.
https://www.youtube.com/watch?v=IRO8eLmHqn0
https://www.youtube.com/watch?v=4woY5IC40RA
https://www.youtube.com/watch?v=1_B6g45OGWU
https://www.youtube.com/watch?v=Y7-jU5LzOgA

These videos provide resource managers and the public with needed information to identify the disease and the most current options for control. Collaboration with the Minnesota Department of Natural Resources in making these outreach materials has resulted in their widespread use and they have become an important resource for limiting the spread of this new invasive disease in Minnesota. Other scientific publications from project results are in the process of being published these include:

Surveys Results for *Heterobasidion irregulare* in Minnesota

Fungal community analysis of red pine stumps in managed stands across Minnesota

Antagonistic interactions between basidiomycete fungi and *Heterobasidion irregulare*

Environment and Natural Resources Trust Fund (ENRTF)
M.L. 2015 Work Plan Final Report

Date of Report: June 30, 2019

Final Report

Date of Work Plan Approval: June 11, 2015

Project Completion Date: June 30, 2019

PROJECT TITLE: Preventing a New Disease of Pines in Minnesota

Project Manager: Robert A. Blanchette

Organization: University of Minnesota

Mailing Address: Department of Plant Pathology, 1991 Upper Buford Circle, 495 Borlaug Hall,

City/State/Zip Code: St. Paul, MN 55108

Telephone Number: (612) 625-0202

Email Address: robertb@umn.edu

Web Address: http://forestpathology.cfans.umn.edu

Location: All counties in Minnesota

Total ENRTF Project Budget:		
	ENRTF Appropriation:	**$371,000**
	Amount Spent:	**$371,000**
	Balance:	**$0**

Legal Citation: M.L. 2015, Chp. 76, Sec. 2, Subd. 06d as extended M.L. 2018, Chp. 214, Art. 4, Sec. 2, Subd. 20

Appropriation Language:

$371,000 the first year is from the trust fund to the Board of Regents of the University of Minnesota to establish early detection for heterobasidion, an invasive root rot fungus, and develop efforts to prevent its spread and reduce its impact. This appropriation is available until June 30, 2018, by which time the project must be completed and final products delivered. Carryforward; Extension (a) The availability of the appropriations for the following projects are extended to June 30, 2019: (6) Laws 2015, chapter 76, section 2, subdivision 6, paragraph (d), Preventing New Disease of Pines in Minnesota

I. PROJECT TITLE: Preventing a New Disease of Pines in Minnesota

II. PROJECT STATEMENT:

Heterobasidion root rot is a serious invasive disease of red and white pine trees that has been recently found in Winona County, Minnesota. The goal of this project is to survey for *Heterobasidion* root rot in forest and urban landscapes where native red and white pines are located so infection sites can be detected as early as possible. New molecular screening methods will be used to identify the pathogen, management plans to fight the disease will be prepared and native biological control agents studied so we can reduce the impact of this disease to conifers growing in Minnesota forests and urban landscapes.

A new invasive tree disease called Heterobasidion root rot is a serious threat to Minnesota's red and white pines as well as other conifers. It is considered the most economically important disease of pines throughout the Northern temperate regions. In the United States it causes over 1 billion dollars in losses annually. It also has tremendous ecological impacts on forest health and productivity. Minnesota has been free of this very destructive tree disease until 2014. Over the past decade it has become well established in Wisconsin and has spread quickly throughout the state. The Wisconsin Department of Natural Resources reports in 2014 that it has been confirmed in 24 counties and three of these counties are adjacent to Minnesota. This pathogen attacks the roots of trees and moves from tree to adjacent tree underground causing circles of dead trees. Once in an area, the fungus grows through the roots and expands causing greater and greater mortality. The fungus produces fruiting bodies with spores at the base of dead trees and these disseminate overland to start new infections. The disease is caused by a complex of *Heterobasidion* species but the species currently in Wisconsin and recently found in southeastern Minnesota is *H. irregulare*. This project will establish effective guidelines to manage this disease which can be implemented immediately as new disease infection centers are found. It will also investigate potential biological control methods that can be used for long term prevention.

III. OVERALL PROJECT STATUS UPDATES:

Project Status as of *[January 1, 2016]***:**

Our surveys have focused on red and white pine growing areas in southeastern Minnesota where the original introduction of *Heterobasidion* root disease had been found. In addition, surveys have been initiated in the east central region of Minnesota since the disease has been found in Wisconsin counties near the Minnesota border. At the site where the disease had been first found, many additional fruiting bodies of the fungus were evident on standing dead trees and cut stumps. Samples from all of the surveyed sites are being processed in the laboratory using a DNA diagnostic method and traditional isolation methods to obtain the pathogen in culture. Pathogen specific primers for molecular detection have been developed. A real time PCR system was purchased and protocols with several chemistries for analyses are being developed. Progress has been excellent and we are on schedule with surveys, research and outreach activities.

Project Status as of *[September 1, 2016]*:

Surveys have continued to focus on red and white pine growing areas in southeastern Minnesota and in the east central region of Minnesota. In addition, surveys at sites in the North Central region of Minnesota have been carried out. The initial site where the disease was found is continuing to expand with the addition of more fruiting bodies of the pathogen being produced and additional trees dying by the underground expansion of the pathogen. All of the samples collected during surveys throughout the state have undergone assessment using a DNA diagnostic method as well as traditional isolation methods to obtain the pathogen in culture. Our original

DNA diagnostic method, which consisted of using pathogen specific primers with a traditional PCR machine has given way to a different new and much improved DNA diagnostic method using a real time PCR system. The real time PCR system is more sensitive and is able to quantify the amount of DNA of the pathogen in each sample analyzed. This helps determine if the pathogen is present in a sample as well as the amount of the pathogen DNA, which is correlated to the stage of infection. The overall progress of the project has continued on track and no problems have been encountered.

Project Status as of *[April 1, 2017]*:

Southeastern Minnesota and the east central region of Minnesota remain the focus of surveys in red and white pine growing areas. The disease has been expanding at the original site in Winona County where Heterobasidion root rot was found and the Department of Natural Resources has decided to try and completely eradicate the disease from this site. All trees in this stand have been cut and the stumps will be removed and burned along with the slash to eradicate the fungus from the site. However, this disease is most likely present at other sites in Minnesota and our work continues to focus on locating trees dying from this disease. Samples from additional surveys in the state have been processed using a DNA diagnostic method as well as the traditional isolation methods to obtain the pathogen in culture. A more robust DNA extraction method has also been tested to process the samples. The new protocol for DNA extractions does a more efficient job of obtaining fungal DNA from wood of pines. The DNA protocol involves the use of plant DNA extraction kits that are able to remove wood resins and extractives that can inhibit or contaminate DNA causing problems for the sequencing analyses. The real-time PCR system also is being utilized and is displaying higher resolution results. Diversity studies of native fungi as well as competition studies with *Heterobasidion* are also continuing. The project has maintained good progress toward accomplishing all objectives. Improvements in processing methodology and in extraction procedures are providing excellent results. Educational outreach activities are also underway. A series of short videos have been developed that will be used to inform the public about the disease. One of these can be seen at this link: https://www.youtube.com/watch?v=dDA6BZ62mNw

Heterobasidion root rot has been expanding at the original site where it was found in Winona County. A red pine stump can be seen in this photo with numerous fruiting bodies.

Project Status as of *[September 1, 2017]*:

Additional surveys have been and are still being carried out in southeastern Minnesota and in the east central region of Minnesota. These surveys have been more frequent than past surveys and an abundance of new samples were collected from locations where *Heterobasidion* could potentially be found. The DNA diagnostic method and real-time PCR system established previously is used to process these woody samples from the field. The samples have also been processed with traditional isolation techniques to obtain the fungal pathogen in pure culture. These isolations also reveal other native fungi of interest. Additionally, spore samplers have been placed in different locations to track the progress of this devastating disease as it moves into new locations in Minnesota. Diversity studies conducted have revealed interesting results as to why the disease might be present in some locations and not in others. Competition studies being conducted are also elucidating potential new antagonists to *Heterobasidion*. Outreach is also continuing with the release of new educational videos:

https://www.youtube.com/watch?v=4woY5IC40RA

https://www.youtube.com/watch?v=1_B6g45OGWU

The overall progress of the project is on track and new results obtained are giving insights into this disease and new ways to control it.

Project Status as of *[January 1, 2018]*:

Surveys are still underway in southeastern Minnesota and in the east central region of Minnesota. Additionally, surveys have been conducted in Itasca State Park. Itasca State Park is of interest because of two *Heterobasidion* sporophores found in the park in the late 1970s. These early collections may represent a different species. We surveyed the park for a second time in late 2017. No *Heterobasidion* was found. Due to the size of the park and non-specific location of the previous findings of *Heterobasidion* in Itasca it is important to continue surveys in the park. The DNA diagnostic method and real-time PCR system established previously was used to process these woody samples from the field. The samples have also been processed with traditional isolation techniques to obtain the fungal pathogen in pure culture. These isolations also reveal other native fungi of interest. New and more efficient spore samplers have been acquired, tested and will be deployed this coming spring. These collectors will be able to detect the amount of spores in the air and will be an effective way to monitor the spread of the pathogen and provide information for potential stands that could have infestation. Analysis of the Illumina high-throughput DNA sequencing data on the diversity of fungi from different sites is continuing. Antagonism assays are also continuing and nearing completion. These studies provided new information on antagonists and their interaction with *Heterobasidion.* Additionally, the studies are providing important new information on the biology, epidemiology, and management of this disease. A new educational video has also been released that explains the research underway. This is the third video produced for the public and can be viewed at:
https://www.youtube.com/watch?v=Y7-jU5LzOgA

A request is being made for authorization to move funds from equipment to supplies and from salaries to supplies. When the purchase of the qPCR was made (equipment), several components to analyze samples had to be purchased separately and these funds were charge to the supplies budget (not from the equipment budget). These items had to be purchased using supply funds and this resulted in $2,663 in excess in the equipment budget. We request transferring this amount to supplies. It appears that our original supply budget did not reflect the expenses for supplies that were needed and we have a small deficit. During our first year of the project, the PI was able to obtain some other sources of funding that were used for salaries and this has resulted

in some salary savings for the project. We request a small amount of funds be moved from the salaries to the supplies budget to provide the needed funds for expenditures that will be needed until the end of the project. Amendment Approved by LCCMR 1/12/2018.

Project Status as of *[July 1, 2018]***:**

Request for no cost extension was made December 2017 to extend the project until June 30, 2019 to complete several projects involving biological control and evaluation of new spore trapping methods to survey for *Heterobasidion*. Amendment Approved: May 30, 2018

Surveys are continuing with an expanded focus to use spores to identify the pathogen in air samples using different types of spore collectors. The spore collectors trap spores from air samples made over a period of days and can be a more efficient way to locate where *Heterobasidion* is present. The spore collecting systems being used are designed to capture spores and molecular methods of identification are used to determine if spores of the pathogen are present. A more sensitive qPCR assay is being utilized to detect spores from the air samples. This qPCR assay is also being adapted for use on woody material to detect minute amounts of *Heterobasidion* DNA. Field surveys will be concentrating on areas where the spore survey results show that the spores of the pathogen are present. Additionally, samples collected by the Minnesota DNR from sites with possible root rot mortality on pines have been processed and will continue to be processed during the coming months to detect *Heterobasidion*. Other studies include analysis of the metabarcoding data (the sequencing of all fungal DNA in wood samples) that is examining the diversity of fungi at native and non-native red pine sites. The information from this study is helping to determine sites where *Heterobasidion* may be considered high hazard versus those that are less susceptible and low hazard. It also is providing important information about the succession of fungi at these sites. Antagonism assays on culture media have been completed and assays involving more complex substrates are underway. These studies will provide a better picture as to what naturally occurring antagonists will perform the best against *Heterobasidion* as potential biocontrol agents.

Project Status as of *[January 1, 2019]***:**

Excellent progress and significant findings continue to be obtained on the project. Spore sampling was conducted at different locations in Minnesota to determine if *Heterobasidion* spores were present. The spore sampling focused on the use of rotation impaction samplers, which are active samplers, more sensitive than those previously used and they allow for quantification calculations to determine spore concentrations in the air. The results show low quantities of spores are present in the eastern areas of Minnesota. The number of spores being found indicates *Heterobasidion* is in the early stages of infection. Additional field surveys were conducted at locations of concern including Itasca State Park. The analysis of the metabarcoding data from red pine stumps in southeastern and northern Minnesota (sequencing of all fungal DNA in wood samples) is being finalized and prepared for publication. Antagonism assays are being conducted on woody substrates in the laboratory and the field. This includes wood discs and wedges in the laboratory and wood discs in the field. The results will provide us with more defined information as to what antagonists would perform the best as potential biocontrol agents against *Heterobasidion* in Minnesota.

Overall Project Outcomes and Results:

The project has been successful and has produced excellent results that are currently being prepared for several publications. New diagnostic tools were developed and tested extensively on samples processed for the presence of *Heterobasidion*. These tools can continue to be used to successfully diagnose samples for this new pathogen. In collaboration with the Minnesota DNR, samples were collected and processed from sites of high

concern. Additionally, the detection methods of spore sampling were carried out and were successful. The spore sampling protocol was able to detect very low quantities of *Heterobasidion* and determine if there was potential for new infection. Small numbers of spores were detected at different sites in eastern Minnesota. However, the number of spores captured at these sites in Minnesota were very low. Research underway in Canada to monitor Heterobasidion movement in Ontario forests suggests that the number of spores needs to meet a certain threshold otherwise new infections may not occur. Since some spores are present and being detected at new sites in Minnesota. It will be very important to continue monitoring this pathogen. The detection methods being used have been found to be very effective and can now be used to survey for other invasive forest pathogens in future studies. Antagonism assays studying native fungi and how they interact with *Heterobasidion* have been completed and valuable results were gained from these experiments. Several native fungi showed their ability to outcompete and outgrow *Heterobasidion* on woody substrates in the lab and the field. These fungi could be acting as natural biological control agents in the field and could be exploited and utilized in the future as biological control agents to be applied in the field to protect trees from attack from *Heterobasidion*.

Amendment Request 8/8/19
We request an amendment to change Activity 2 accordingly:
Travel expenses from $9500 to $6714

To Change Activity 3 accordingly:
Personnel from $106,635 to $107,204
Equipment tools and supplies from $13,863 to $16,471
And Travel from $2,500 to $2109.

This resulted from minor overages in personnel and equipment tools and supplies and slightly less travel than anticipated while still accomplishing all project outcomes.
Amendment Approved by LCCMR **9/22/2019**.

IV. PROJECT ACTIVITIES AND OUTCOMES:

ACTIVITY 1:
Description: Develop new diagnostic tools for rapid detection.

Molecular primers and probes (DNA sequences) that are specific to the pathogen, will be designed for use in a diagnostic kit that can be used to rapidly identify the pathogen directly from wood samples obtained from the field. DNA will be extracted from *Heterobasidion* cultures and PCR products obtained. These products will then be cloned into *E. coli* and sequenced. Specific primers for *Heterobasidion* will be designed for nested and real time PCR assays on the basis of the alignment of the nuclear ribosomal ITS region of *Heterobasidion* from Gen Bank accessions. Multiple pairs of primers will be screened *in silico* using Basic Local Alignment Search Tool (BLAST) to explore all available *Heterobasidion* sequences and other fungi that can potentially cross-react. Primers that only match *Heterobasidion irregulare* will be tested using genomic DNA from different *Heterobasidion* isolates. Wood tissue samples collected from forest stands (known *Heterobasidon* infected trees and healthy trees for controls) will be homogenized in lysis buffer. DNA will be extracted, tested and nested PCR methods completed to confirm the primers are effective at detecting the pathogen and no other wood inhabiting fungi. The primers will also be optimized for the real time PCR protocol and specific probes will be designed and optimized in a similar fashion as previously published. Standard fungal isolation will be attempted for each tree sample to compare detection rates with those for the PCR methodologies. This will provide a highly sensitive and accurate detection method to target this pathogen. The developed methodology will be

used to assay field samples from surveys (see Activity 2) carried out in forested and urban landscapes over the duration of this grant. A service for identifying this disease will be established in the Plant Disease Clinic, which will become a center for diagnosing the disease for the North Central region of the US.

Summary Budget Information for Activity 1:

ENRTF Budget: $ 116,567

Amount Spent: $ 116,567

Balance: $0

Outcome	Completion Date
1. Develop and test molecular primers and probes for diagnostics	December 31, 2015
2. Molecular diagnostic kit developed for evaluation of field samples	August 30, 2016
3. Molecular diagnostic methods used for pathogen detection on samples from field surveys	June 30, 2019

Activity Status as of *[January 1, 2016]*:

Heterbasidion specific primers have been developed in the ITS region of rDNA that have been optimized in a nested PCR protocol. These primers target very specific DNA sequences that are unique to *Heterobasidion* and will only amplify DNA in samples that have *Heterobasidion* DNA in them. The protocol has been tested rigorously and optimized and has been found not to amplified fungal DNA other than *Heterobasidion irregulare* in samples tested. Thus far these primers have been used in a nested PCR protocol and were pivotal in detecting the first site of *Heterobasidion* in Minnesota. However, the nested PCR protocol has disadvantages because it is prone to contamination and is time consuming. In September, we purchased a real-time PCR system (Figure 1) allowing for a one step detection method with superior sensitivity. We are currently working on optimizing this detection method with the *Heterobasidion* primers. Following extraction of total DNA from a wood sample taken from a tree suspected of having *Heterobasidion* root disease (HRD) it is mixed with reagents and primers and placed in the real-time system for analyses. In a relatively short time, an amplification curve reveals whether the DNA from the disease has been detected and amplified (Figure 2). In addition, a slightly different approach is also being developed using specific primers and probes (Taqman) that utilize different methods for protection. This will provide two rapid and efficient diagnostic methods for detecting the disease from wood samples.

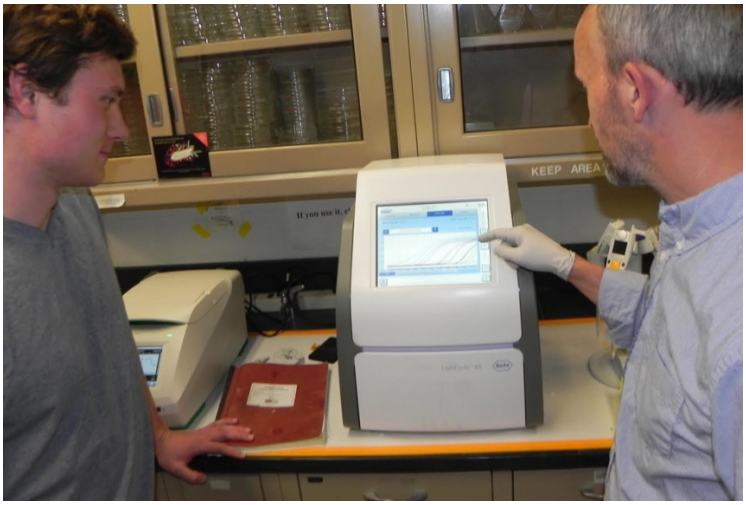

Figure 1. Real time PCR detection system optimizes sensitivity and reduces contamination for pathogen detection in field samples.

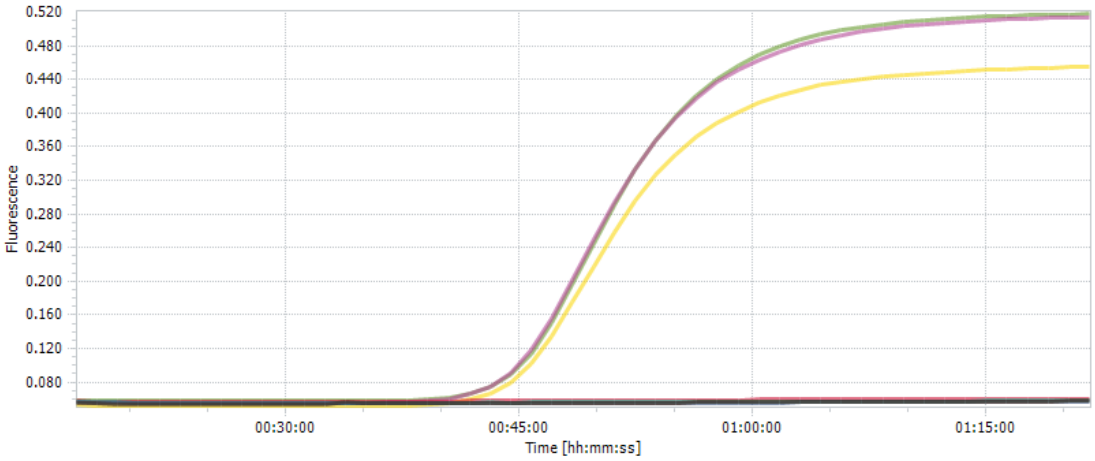

Figure 2Graph showing an amplification curve following a real-time PCR run. The curves show amplification of Heterobasidion DNA indicating the sample is positive for Heterobasidion root disease.

Activity Status as of *[September 1, 2016]*:

Diagnostic methodology for identifying *Heterobasidion* from field samples has been further developed involving the use of a real time PCR detection system. This method has replaced the previous detection method, which involved the use of a nested PCR protocol with *Heterobasidion* specific primers. The real time PCR has superior sensitivity and a streamlined protocol that reduces opportunities for contamination. This new procedure starts with the genomic DNA being extracted from wood samples collected using the cetyl-trimethylammonium bromide (CTAB) method. The CTAB method has been modified for wood samples in order to extract a higher concentration of total DNA to increase the sensitivity of the test. The extracted DNA is quantified and tested for purity using a nano-spectrophotometer. The DNA is then used in the real time PCR (qPCR) instrument and when used with a standard can identify if a sample has *Heterobasidion* present, and can also quantify the amount of *Heterobasidion* DNA. The qPCR protocol involves the use of a pair of primers that select for *Heterobasidion* and related genera. In addition to the primers, a taqman probe is used that is specific for *Heterobasidion irregulare,* the species that is attacking trees in Minnesota. This method has proven to be much faster and more sensitive than the nested PCR protocol previously used.

To identify fungal fruiting bodies, DNA is extracted from samples and amplified using a primer set specific for basidiomycete fungi and then sequenced. The sequence is then compared to other sequences in a large database to determine if the fruiting body sample is definitively *Heterobasidion*.

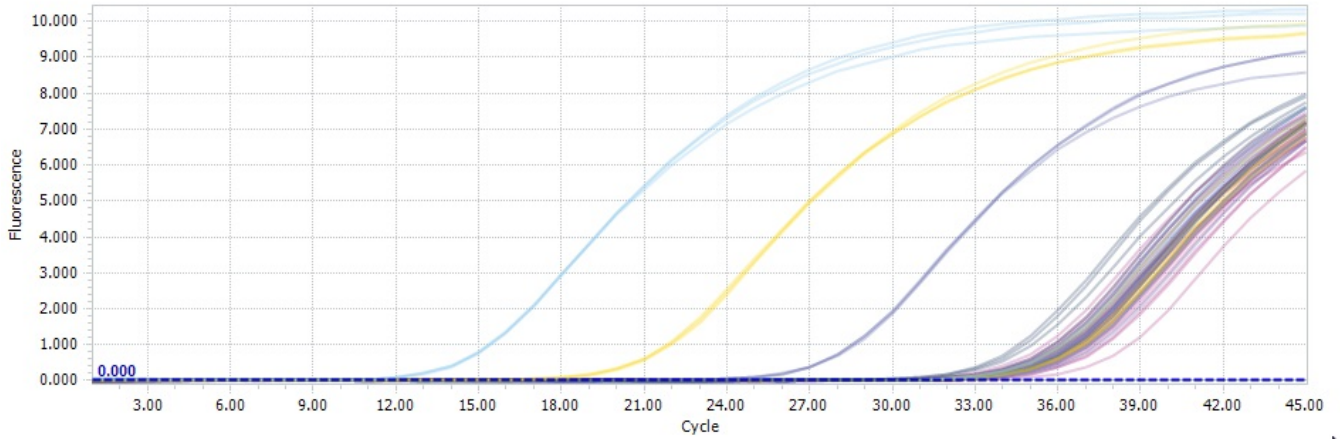

Figure 3. Amplification curve following a real-time PCR run of samples from Banning and Wild River State Park. This graph shows the amplification of the positive controls (left three curves) and little to no amplification of the wood samples demonstrating there

Activity Status as of *[April 1, 2017]*:

The real-time PCR detection system developed for identifying *Heterobasidion* is continuing to be used to process samples collected from the field. We have continued to refine and optimize methods by determining cutoff values for positive samples and reducing possible contamination. With complex substrates such as wood, effective detection can be a challenging task. During a real time PCR run there can be weak real time PCR signals that are represented by high quantification cycle (Cq) values, which could be questionable. Work is being performed in order to determine a reliable Cq threshold to determine a positive reaction for specific detection. Also, a new DNA extraction method is being used that is more effective at removing fungal DNA from wood without inhibitors or contamination. The DNA extraction methods being used are now able to recover high quality DNA from tough samples such as wood. We are also using a bead beating technology to grind samples that helps to facilitate DNA extraction. Additionally, different solutions used during extraction help with the removal of inhibitors, proteins, metabolites, and salts to ensure clean high quality DNA.

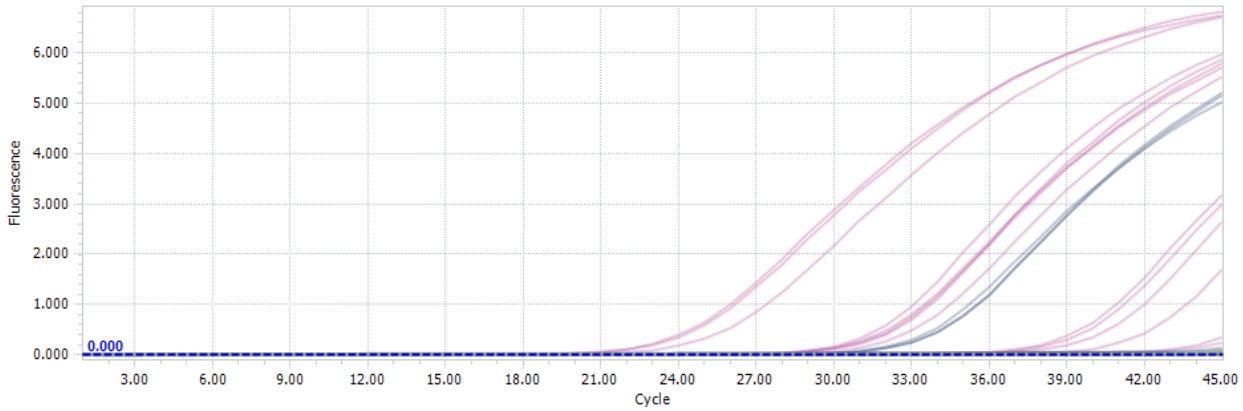

Figure 4. Successful amplification curve following a real-time PCR run with samples extracted with our new extraction methods. This graph shows the amplification of the positive controls and one new positive sample (left curves) with little to no amplification o

Activity Status as of *[September 1, 2017]*:

Samples that are collected in the field during surveys for *Heterobasidion* undergo the real-time PCR detection system previously developed. The refinement of this detection system is still continuing by trying to reduce contamination and determine effective cutoff values. Additionally, determining reliable quantification cycle (Cq)

values for determining if a reaction is positive is being narrowed down. Specific detection of possible positive samples can be challenging, especially samples with low amounts of *Heterobasidion* DNA. The real-time PCR detection system will also be used for determining the amount of *Heterobasidion* spores collected from spore samplers placed in the field. First, slides coated with petroleum jelly and containing spores collected in the field will undergo a modified DNA extraction protocol. The protocol will help separate the spores from the petroleum jelly. The spores can then undergo a standard DNA extraction. After the DNA has been obtained from the spores, it will undergo the real-time PCR protocol. This protocol will quantify the amount of *Heterobasidion* DNA from each location. These results will give us further information about the presence of this disease.

Activity *Status* as of *[January 1, 2018]*:

The real-time PCR detection system previously developed is still effective for processing samples collected in the field during surveys. However, refinement of the detection system is moving forward with determining effective cutoff values and reducing contamination. New spore samplers acquired will collect spores that will undergo a DNA extraction and then the real-time PCR detection system will be used to quantify the amount of spores on the spore sampler rods. A new assay is under development to determine basidiospore counts by using a ribosomal ITS TaqMan assay. Basidiospore equivalents per rod will be calculated by translating Ct values using standard curve equations. Spores can then be quantified and provide further information about the possible location of an infestation. The total amount of spores will be quantified weekly by determining spores sampled per m^3, $m^{-2}h^{-1}$, and cumulative spores deposited on a 30 cm stump.

Activity Status as of *[July 1, 2018]*:

A new qPCR assay recently developed by Canadian researchers for the detection of *H. irregulare* has been tested with excellent results. This method is now being used as a sensitive and more effective method to definitively detect *H. irregulare* even when exceedingly small amounts of DNA are present. The new assay currently being utilized has a higher level of sensitivity with detection of only 5 to 10 ITS region copies. The assay is also capable of detecting as low as one spore per microliter. To develop the qPCR assay for spore counts, a mono basidiosporal isolate of *H. irregulare* is being used. Serial dilutions are used to generate standard curves to determine the number of spores collected in the field. The importance and prevalence of *H. irregulare* conidia in dispersion is not fully known, but it is expected that spores that are trapped will be mono-nucleus basidiospores. This assay will help us determine how many basdiospores are present at certain locations and the extent of *Heterobasidion* infestation at each site.

The new qPCR assay has also been used to reanalyze past samples that had low concentrations of *Heterobasidion* DNA, and based on the cycle threshold value most of these samples were deemed negative. A few of the samples, however, when reanalyzed appeared to have small amounts of *H. irregulare* DNA (ITS copies). Two of the samples had all three replicates amplify, which solidifies that these samples could be positive (Fig. 5) and small numbers of spores appear to be in the area. However, no isolations made from the sites have yielded *Heterobasidion.*

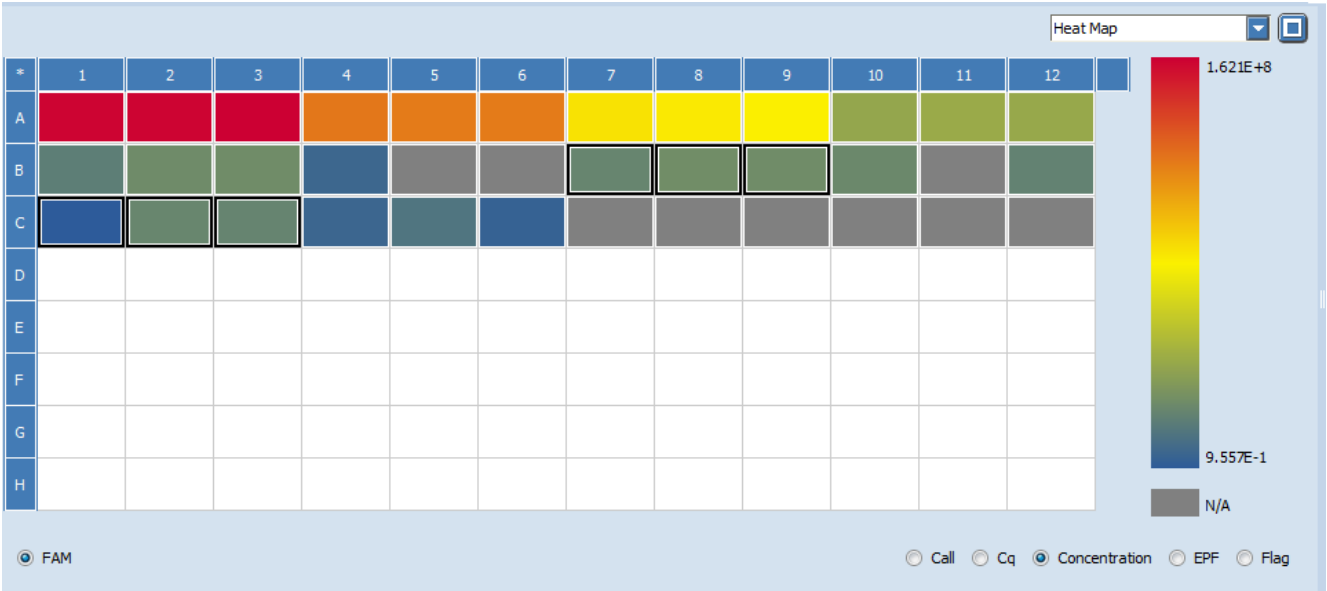

Figure 5. A heat map of samples that were previously analyzed and were questionable in regards to being positive for H. irregulare. A new analysis shows rows A (wells 1 to 12) and B (wells 1 to 3) of positive controls amplifying at known concentrations. High con

Activity Status as of *[January 1, 2019]*:

The qPCR assay developed by Canadian researchers for the detection of *Heterobasidion* has been used to process samples from the rotation impaction samplers. The results from these assays have shown low amounts of spores are present at certain locations in eastern Minnesota. The ability to detect very small numbers of spores demonstrates the sensitivity level of the assay. A synthetic standard was used to generate the standard curves. The standard used is the whole ITS region of *H. irregulare* and is synthesized using gblock by Integrated DNA Technologies (IDT). This standard provides us with an exact number of copies of DNA per microliter. After running the qPCR assay the total number of spores per rod can be determined. With further calculations based on total spores per rod and exposure time, we can determine spores per m^3, $m^{-2}h^{-1}$, and cumulative spores deposited on a 30 cm stump. These results help to determine the locations that are of high concern and places where new infections from *Heterobasidion* are likely to occur

The qPCR assay was also used to analyze additional past samples. A few of these samples had low amounts of *H. irregulare* DNA, but the majority of the samples were negative. For the samples with low quantities of *H. irregulare* DNA surveys were carried out in the area to find infection sites. Additionally, the same qPCR assay is being used for antagonism assays to detect quantities of *H. irregulare* in competition studies with native fungi.

Final Report Summary:

The qPCR assay has been tested and used extensively to process samples. It has been used to test woody substrate samples and to process samples from the rotation impaction samplers to quantify spores. The assay has high sensitivity and can detect low amounts of DNA for woody and spore samples. These assays can be used in the future to continue to monitor for *Heterobasidion* and can be adapted with different primers and probes to be used for the detection of other important invasive forest pathogens.

The qPCR assay was also used for to detect quantities of *H. irregulare* DNA in competition studies with native fungi. Large wood discs were placed in the field around stumps with fruiting bodies of *H. irregulare*. The discs were treated with a spore solution of native fungi and then left in the field for 9 weeks. The discs were then

processed with a DNA extraction procedure and the qPCR assay was used to detect *H. irregulare* DNA. Some of the treated discs showed a lower amount of *H. irregulare* DNA (Figure 28- located in section for Activity 3) and demonstrated their ability to be antagonistic towards *H. irregualre* as well as their potential to be developed as a biological control agent.

ACTIVITY 2:

Description: Survey and identify infected trees.

A statewide survey will be initiated utilizing Department of Natural Resources, county and federal data bases and information from state, county, industry, and federal foresters to identify plantations and other sites in the state that may be infected. These will include locations near the identified disease centers that are in adjacent counties to Wisconsin, sites identified using information such as stand age and thinning history and locations of unexplained pockets of mortality. Additional surveys will be done on ornamental pines located in the areas of greatest concern in Minnesota (cities in counties next to existing Wisconsin infection centers). Wood samples obtained from the surveys will be placed in sterile collection bags and brought to the laboratory for processing. The new diagnostic procedures described above will be used to detect the fungus in wood samples in its earliest stage of colonization. Additional microbiological testing will be done to confirm the pathogen and extent of its colonization.

Summary Budget Information for Activity 2:

ENRTF Budget: $ 128,649

Amount Spent: $ 128,649
Balance: $ 0

Outcome	Completion Date
1. Complete first surveys for the disease in all Minnesota counties adjacent to Wisconsin	December 31, 2016
2. Complete surveys of pine in other forest and urban areas of Minnesota	June 30, 2019

Activity Status as of *[January 1, 2016]*:

Currently, surveys are being conducted to determine the extent of the infestation of *Heterobasidion* in Minnesota. These surveys are focusing on the southeastern counties of Minnesota, which includes Wabasha, Winona, Sherburne, and Houston Counties. The surveys are also focusing on various state parks and state forests with a focus on the first surveys taking place along the eastern region of Minnesota from Pine County south towards the Twin Cities metro area. The sites surveyed and to be surveyed in southeastern Minnesota have been selected because of their close proximity to a site where *Heterobasidion* was found to be present. The sites from Pine County south to the metro area are being surveyed because of their proximity to the Wisconsin border, where the fungus has been found causing disease in pine plantations. Previously, *Heterobasidion* root rot has been confirmed in 24 counties in Wisconsin (reported by the Wisconsin Department of Natural Resources). Aerial maps of sites to survey are being provided by the Minnesota Department of Natural Resources. For surveys in Minnesota, sites with dead and dying red and white pine occurring in plantations that have been thinned in the last 10 years are a main focus but other locations where pine mortality is taking place are also being surveyed. At each site, samples consisting of small segments of woody material collected from the base or the roots of dead and dying trees are taken to the laboratory. Additionally, any basidiocarps found are collected. These samples are used for molecular methods of identification and also cultured on growth media to determine if the pathogen is present.
Sites surveyed that have produced negative results via DNA tests for *Heterobasidion* include: Kruger Campground in Wabasha County, Sand Dunes State Forest in Sherburne County, Itasca State Park, Great Bluffs

State Park, St. Croix State Park, Chengwatana State Forest, General C.C. Andrews State Forest and red pine stands in Andover in Anoka County, Clearwater in Wright County, Houston County, Wabasha County, Winona County, Hay Creek Township in Goodhue County, and sites in Lanesboro in Fillmore County.

A site in Banning State Park, also located in Pine County, had a stand that exhibited pine mortality. Six samples were collected from the site. A positive DNA test was found in one of the samples and the site was revisited for additional sampling. Seventeen samples were collected and brought back for laboratory analysis. Positive DNA tests were found again in a few of these samples but the fungus was not isolated. During the initial stages of infection, the fungus can be very difficult to isolate since it is just getting established and colonization localized to underground root systems. Banning State Park was also recently re-surveyed and an additional nine samples were collected. These are currently being analyzed.

Six different stands in Wild River State Park exhibiting varying degrees of pine mortality were surveyed and 42 samples were collected. Positive DNA tests were found in a few of these samples but the fungus was not isolated. Wild River State Park was recently re-surveyed and an additional 44 samples were collected. These samples are currently being analyzed.

Two sites in Hay Creek Township in Goodhue County had multiple pockets of pine mortality. Initially, five samples were collected and one positive DNA test was detected. Hay Creek Township was re-surveyed in August and an additional 28 samples were collected. One of these samples exhibited a positive DNA test. Hay Creek Township was also recently re-surveyed and an additional eight samples were collected. These are currently being analyzed.

A different method of detection was tested at sites suspected of having *Heterobasidion* present. In December, small wood disks used as spore traps were placed and left in the understories of Banning and Wild River State Parks for 24 hours. These disks were then retrieved and brought back to the Forest Pathology Laboratory at the University of Minnesota for examination. They are currently being tested for the pathogen using molecular diagnostic methods as well as traditional culturing methods.

We are continuing to revisit the sites that gave positive molecular results in an effort to isolate the pathogen so that we can confirm its presence with a culture of the fungus. Prior data suggests that disease severity may heavily depend on site conditions, for instance soil type. No sporophores were found at these DNA positive sites in Banning or Wild River State Parks, which indicates that this may be a very recent introduction. Although confirmation of the disease is needed by culturing, the repeated positive results from molecular tests indicate that the disease appears to be in its initial stages of infection at the sites.

Figure 5. Basidiocarp of Heterobasidion 14rregular on a red pine in SE MN (left photo) and root rot center in a red pine stand in Wild River State Park (right photo). Fruiting bodies can take several years to form after the initial infection and may not be present in many sites surveyed in Minnesota for this reason.

Activity Status as of *[September 1, 2016]*:

Surveys are still being conducted throughout Minnesota. These surveys are mainly focused on southeastern counties, state parks and forests in this region as well as the eastern region of Minnesota from Carlton County south towards the Twin Cities metro area. Aerial maps are the main tool used to locate and navigate to the stands, which are provided by the Minnesota Department of Natural Resources.

Our initial surveys were done in southeastern Minnesota and focused on stands with pockets of dead and dying trees that have been thinned. The main avenue the pathogen uses to infect living trees is to enter the surface of cut stumps and then moves into the root system of the neighboring trees. As trees are killed, circles of dead pines can be found. Additional sites surveyed in southern Minnesota include: red and white pine stands in Wabasha County, Kruger Campground in Wabasha County, Great Bluffs State Park in Winona County, Zumbro Bottoms State Forest in Wabasha County, Hay Creek Township in Goodhue County, Houston County, and Fillmore County. A total of 308 samples were collected from these sites. Twenty-one of the samples collected in Winona County tested positive for *Heterobasidion*. The remaining samples tested negative with the nested PCR protocol as well as the qPCR protocol.

Additional surveys conducted in northern Minnesota included Lake Itasca State Park. The DNR had reported several root rot centers in old growth red pine stands within the park that were suspect. St. Croix State Park located in Pine County exhibited a large amount of red pine mortality via aerial imagery and was surveyed. The cause of mortality was contributed to *Armillaria* root rot and beetle attack by *Ips pini.* A site in Wild River State Park also had a large mortality center that was caused by *Armillaria* root rot. Other sites surveyed included: Andover in Anoka County, Clearwater in Wright County, Sand Dunes State Forest in Sherburne County, Banning State Park in Pine County, Chengwatana State Forest in Pine County, General Andrews State Forest in Pine County, William O'Brien State Park in Washington County, Franconia Bluffs Scientific and Natural Area in Chisago County, a site in Kanabec County, sites in Ottertail and Pope Counties, Sherburne National Wildlife Refuge in Sherburne County, the Cloquet Forestry Center in Carlton County, and sites in Isanti and St. Louis Counties. A total of 250 samples were collected from northern Minnesota with all of them testing negative for *Heterobasidion* when analyzed with the nested PCR protocol or the qPCR protocol.

Figure 6. Sampling from stumps and woody debris for Heterobasidion. Wood samples are brought into the laboratory for DNA extraction and processing.

The initial positive DNA tests from Banning State Park, Wild River State Park, and Hay Creek Township in Goodhue County that were previously reported have been proven to be negative. The initial DNA tests involved the use of the nested PCR protocol method. This method is less precise and more prone to contamination. These samples were processed again with the real time PCR method and were shown to be negative. Since this method has superior sensitivity, we have determined that the pathogen has not become established at these sites. Additional testing by making isolations also has not provided cultures of the fungus. Continued surveys are underway at these suspect locations and new samples are being analyzed. To date, no fruiting bodies have been found at these locations. Since spores of the fungus coming into the sites (by wind or insects) may be a possible reason for the initial positive reactions some additional testing was done. A wood disk method used as a spore trap were placed in the understories of Banning and Wild River State Parks. These wood disks were evaluated in the laboratory and did not yield positive results for *Heterobasidion*. The wood disks were tested for the pathogen using the qPCR molecular diagnostic method as well as traditional culturing methods.

Future surveys will take place at suspect locations in northern and southern Minnesota to help further elucidate the distribution of *Heterobasidion* and to monitor its movement in the state. Our diagnostic methods are also now available to be used in the Plant Disease Clinic on samples from suspect trees sent in from the public.

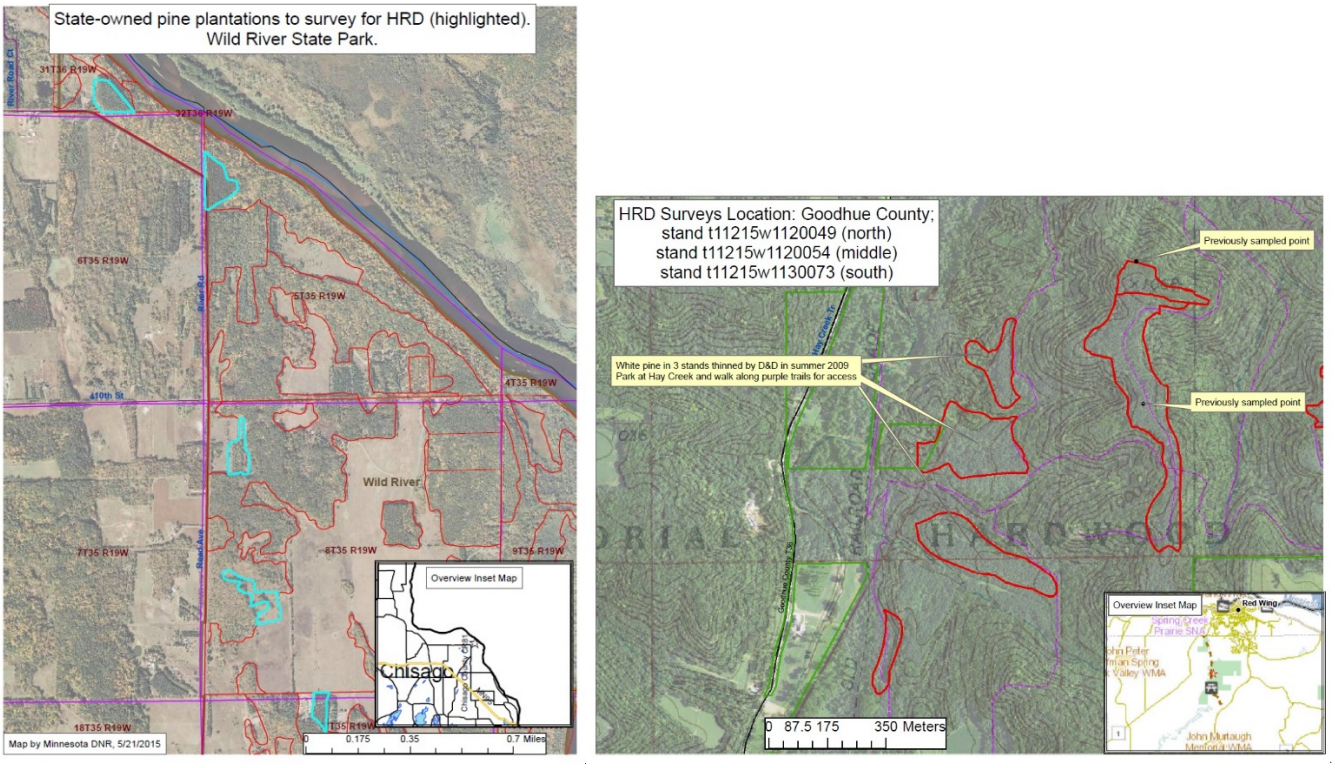

Figure 7. Aerial maps of red pine stands in Wild River State Park (left photo) and Hay Creek Township in Goodhue County (right photo). Samples were collected from these sites and then analyzed in the laboratory.

Activity Status as of *[April 1, 2017]*:

Surveys are still being conducted throughout Minnesota. These surveys are mainly focused on southeastern counties, state parks and forests in this region as well as the eastern region of Minnesota from Carlton County south towards the Twin Cities metro area. Aerial maps, which are provided by the Minnesota Department of Natural Resources, are the main tool used to locate and navigate to the stands.

The initial positive DNA tests from Banning State Park, Wild River State Park, and Hay Creek Township in Goodhue County that were previously reported were tested again and were negative. These samples were

extracted with our new DNA extraction methods. The samples were also processed with the improved real-time PCR method and were shown to be negative. Also, additional testing by making isolations also did not yield cultures of the fungus.

Surveys conducted since September 2016 were in Hay Creek Township, Krueger Campground, and Little Falls. Samples were obtained from each of these sites. After processing with DNA extractions, qPCR, and isolations onto specific media, these samples were proven to be negative for *Heterobasidion*. Additional surveys were not conducted during the winter months due to snow covering up stumps and the bases of trees. Fruiting bodies are also difficult to detect in the winter.

Surveys are now starting again with the snow not present in the majority of potential sites to survey. These future surveys will continue to focus on suspect locations in northern and southern Minnesota to help further elucidate the distribution of *Heterobasidion* and to monitor its movement in the state. It is still pertinent to conduct surveys to identify the location of the disease, so forest managers can quickly enable management plans for forests impacted by *Heterobasidion*.

Activity Status as of *[September 1, 2017]*:

The amount of surveys has increased since June 2017 to further detect the distribution of *Heterobasidion*. The surveys are still focused on southeastern counties, state parks and forests in this region as well as the eastern region of Minnesota from Carlton County south towards the Twin Cities metro area. In addition to aerial maps provided by the Minnesota Department of Natural Resources (DNR), ForestView was also used. ForestView is an interactive map on the Minnesota DNR website that contains different information on forest stands that belong to the state of Minnesota. With ForestView, different stand attributes can be viewed that could be helpful in locating stands infected by *Heterobasidion*. Furthermore, with the stand information obtained from ForestView, foresters from the Minnesota DNR were contacted to discern if these stands were harvested and had trees removed. Tree removal from stands promotes the infection biology of *Heterobasidion*, so stands that had harvesting performed could be more likely to harbor this disease.

A total of 332 samples were collected since June 2017. Surveys started in southeastern Minnesota. The locations included sites in Winona and Houston Counties, Carley State Park, Kruger and Hay Creek Management Areas. A total of 106 samples were collected from these sites. The focus then shifted to north central Minnesota. Locations surveyed here included Wild River State Park, General C.C. Andrew State Forest, Saint Croix State Forest, Nemadji State Forest, and Banning State Park. Samples from Nemadji and General C.C. Andrews State Forest and Banning State Park are still being processed.

Of the samples processed there are a set of possible positive samples for *Heterobasidion*. These samples were collected from Houston County, General C.C. Andrews, and St. Croix State Forest. The samples were processed with the DNA extraction kit and our real-time PCR protocol. The samples had small quantities of *Heterobasidion* DNA present, but also amplified late in the reaction. Samples that amplify late in PCR reactions have delayed or high Cq values, decreased efficiency, and low expression. These samples will be re-processed with the real-time PCR protocol. Additionally, isolation of these samples onto selective media is occurring. Isolating the sample onto media will definitively demonstrate that *Heterobasidion* is present in the wood.

In addition to collecting samples from different state forests and parks we have recently set up spore samplers to detect airborne *Heterobasidion* spores (Fig. 8). The spore samplers were built consisting of a long hanger that holds a motor that rotates and holds two microscope slides. These slides are coated with petroleum jelly that will collect spores as they are rotating. The spore samplers are set up in a transect starting in a positive location

in Wisconsin and then spore samples are placed moving closer to Minnesota. These slides are then collected and the petroleum jelly containing the spores can undergo a DNA extraction. Using our real-time PCR protocol we can quantify the amount of spores from each location where a spore sampler was placed. This study will further help us determine how present *Heterobasidion* is moving closer to Minnesota. The spore samplers will also be placed in different locations of concern in Minnesota to quantify how prolific *Heterobasidion* potentially is at those sites.

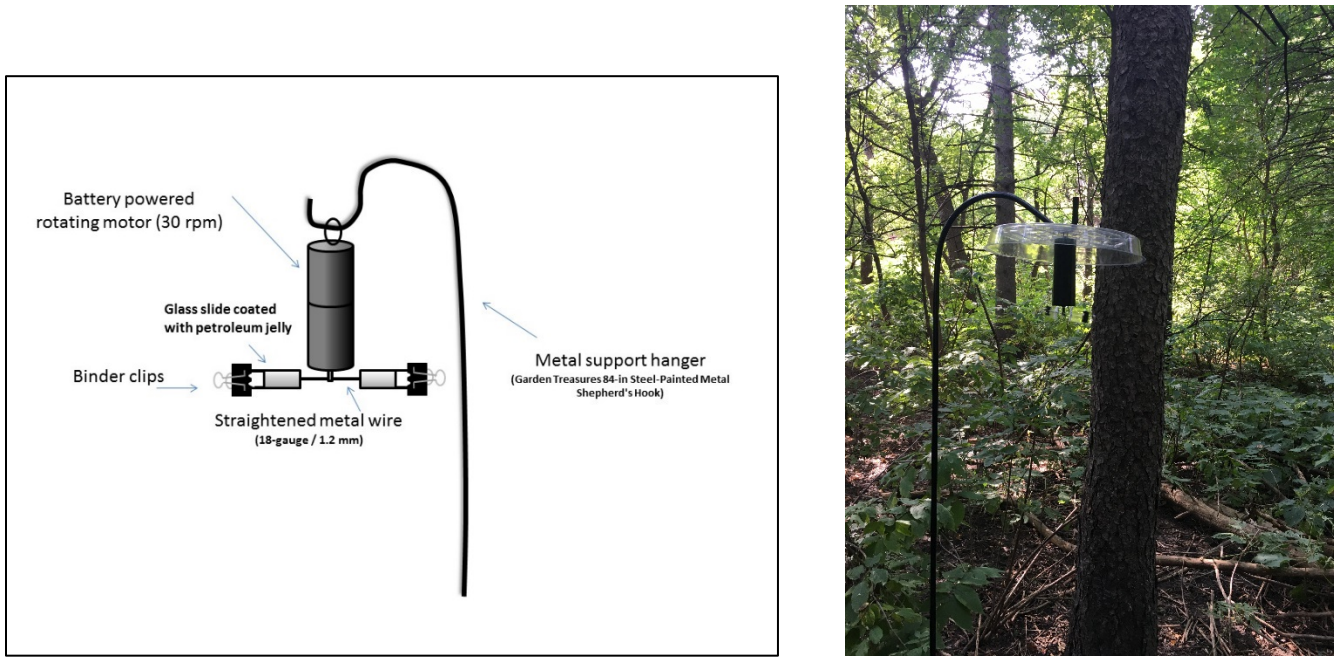

Figure 8. Diagram of a spore sampler showing the different parts that compose the sampler (left). Spore sampler that has been placed in Minnesota (right).

Activity Status as of *[January 1, 2018]*:

Additional surveys were conducted in the fall of 2017. Surveys focused on Itasca State Park and locations in southeastern Minnesota. Aerial maps provided by the Minnesota Department of Natural Resources (DNR), satellite imagery, and the Minnesota DNR tool ForestView were used for surveying the sites.

Samples collected previously from Houston County, General C.C. Andrews, and St. Croix State Forest in the fall of 2017 were reprocessed. These samples had small quantities of *Heterobasidion* DNA present, but also amplified late in the reaction. As previously stated, samples that amplify late in PCR reactions have delayed or high Cq values, decreased efficiency, and low expression. Re-processing with the real-time PCR showed high Cq values and thus low quantities of *Heterobasidion* DNA. Additionally, sequencing directly from the wood provided sequences of different wood decay fungi, but no *Heterobasidion* species.

A total of 18 samples were collected from Itasca State Park. This park is of interest because of two sporophores that were found in the late 1970s on two separate occasions. The description of the location where the sporophores were collected is not very descriptive, so it is a challenge to locate the exact area of the previous collections. The large size of the park also poses a challenge in locating the possible collection site. The samples we collected in the fall of 2017 underwent the molecular assays established and were deemed to be negative. A lot of the samples collected were sporophores that resembled *Heterobasidion* and these underwent a direct DNA extraction and sequencing. All sequences resulted in different wood decay fungi. Additionally, spore samplers were placed at different locations to detect possible *Heterobasidion* spores in the park. After analysis with real-time PCR all slides processed were negative. This may be due to the late fall time of collection. New

and more sensitive spore samplers will be placed in the park in the near future (Fig.9) and samples taken over the spring, summer and fall. The spore samplers will also be used in other areas along Minnesota's border with Wisconsin.

An additional 11 samples were collected from southeastern Minnesota from Great River Bluffs State Park and Winona County. These samples were processed with our molecular techniques and isolated onto media, but were determined to be negative. Also, spore samplers were placed in Great River Bluffs State Park for testing.

Future surveys will still consist of collecting samples from the field, but more focus will be shifted to detecting spores using spore samplers. Detecting spores will facilitate detecting the location of new infestations of *Heterobasidion*. During the spring and summer of 2018, spore samplers will be set up at different distances from a known infection site and also at sites of high interest, such as Itasca State Park. Spore samplers have recently been shown to be very effective for monitoring in south eastern Ontario, which is an area where *Heterobasidion* is moving into.

Figure 9. Spore sampler in Itasca State Park (left). A new spore sampler being tested contain two small rods that are programmed to spin at specific time intervals and collect airborne spores. This sampler (right) appears to be more sensitive and efficient at collecting the very small spores produced by Heterobasidion.

Activity Status as of *[July 1, 2018]*:

Current and future surveys are focused on spore sampling from previously sampled sites and new locations. However, samples are still being processed from those collected by the Minnesota DNR. Samples were collected by the Minnesota DNR from red pine stands in Sand Dunes State Forest, Princeton, and Prior Lake in 2018. A similar pattern of symptoms was impacting red pine trees at these sites. The trees had dieback and mortality was also present. The symptomatic trees were in concentric pockets, which is distinctive for *Heterobasidion*. No signs of *Heterobasidion* or *Armillaria* root rot were present in the field, such as mycelial mats or fruiting bodies. These samples were processed with the new qPCR assay and isolations made onto culture media. From the samples processed to date, all were negative. It's possible that red pine pocket mortality caused by the fungus

Leptographium is causing the problem at these sites. *Leptographium* was also isolated from one of the woody samples.

Spore samplers are currently being deployed in the field. Spore samplers are placed at different distances from a known infection site to determine how far basidiospores can be dispersed. Seasonal patterns of spore deposition are being examined to determine what time of the year results in the highest amount of spores. Spore samplers are deployed at the positive site in Winona County where *H. irregulare* was eradicated. Also, Itasca State Park will be surveyed based on the finding of *Heterobasidion* fruiting bodies (likely a different species) on two separate occasions in the late 1970s. Deploying spore samplers at other sites previously surveyed that have a high likelihood of having *H. irregulare* are being carried out. Additionally, collaboration with the Minnesota Pollution Control Agency is allowing us to use and process filters from their air samplers at different locations around Minnesota. These samples are being collected to monitor air quality, but will also be used to assay for the presence of *H. irregulare* using our molecular diagnostic methods.

Activity Status as of *[January 1, 2019]*:

Spore sampling was conducted at sites of high concern for *Heterobasidion*. The spore samplers used were rotation impaction samplers (Fig 10.). Each sampler consists of two small plastic rods that are coated with silicone. The samplers are programmed to spin four minutes every hour. This amount of exposure time limits the amount of dust and pollen that could accumulate on the rods and thus impact analysis. The samplers were deployed at each site for approximately one week and then collected. The rods were processed in the lab and DNA was extracted. The qPCR assay was then used to determine the amount of spores per rod. The spore samplers were deployed from September to November. Deployment time was based on previous literature, which concludes that the fall has the highest dispersal of spores from *H. irregulare*. Spore samplers were first placed at a location in Wisconsin that was infected and had fruiting bodies present. Results from this location confirmed that the spore sampling protocol was working. Samplers were then deployed in Minnesota at Minnieska, Great River Bluffs State Park, Hay Creek, Elba, Vinegar Ridge Recreation Area, William O'Brien State Park, Wild River State Park, and Itasca State Park (Fig. 11). The results show a large amount of spores at the known infected location in Wisconsin. The amount of spores is significantly lower at the locations in Minnesota. Somewhat higher concentrations were found in Minnesota at locations near the border of Wisconsin. Previous literature states that a deposition rate of 5 basidiospores m^{-2} h^{-1} may be required to initiate new infection sites. The results from our samplers indicate that none of the Minnesota sites meet that requirement as the amount of spores per m^{-2} h^{-1} is below 5. However, even with the low numbers of spores and thus a lower risk of infection, the spores are present and infection could occur. In areas where spores are being detected, management to control the disease, including treating stumps with borax or a biological control agent is warranted.

In other surveys carried out, samples from the field were collected from trees that were possibly associated with *Heterobasidion*. The Minnesota DNR collected and delivered samples from Sand Dunes State Forest, Princeton, and Prior Lake during the past few months. These samples were processed with our molecular techniques and isolated onto media, and no *Heterobasidion* was detected. Other potential causes of mortality and secondary fungi were determined. Itasca State Park was also surveyed when spore samplers were being deployed in the park. No *Heterobasidion* was found after using our molecular techniques and isolating onto media. Additionally, other possible pathogenic fungi and secondary fungi were identified.

Figure 10. A rotation impaction sampler deployed in Minnieska, Minnesota.

Location	Sampling Week	Total Spore Count	Spores per m^3	Spores per m^{-2} h^{-1}
Burr Oak , Wisconsin	9/18/2018	32465.00	932.70	76.01
Minnieska	9/18/2018	256.20	8.42	0.78
Great River Bluffs State Park	9/26/2018	70.27	2.31	0.22
Hay Creek	9/26/2018	19.82	0.64	0.06
Elba	10/3/2018	44.56	1.46	0.14
Vinegar Ridge Recreation Area	10/3/2018	9.06	0.30	0.03
William O'Brien State Park	10/10/2018	84.40	2.77	0.26
Wild River State Park	10/10/2018	44.81	1.47	0.14
Itasca State Park (Location 1)	10/23/2018	0.00	0.00	0.00
Itasca State Park (Location 2)	10/23/2018	0.00	0.00	0.00

Figure 11. Number of H. irregulare spores collected at different locations in Wisconsin and Minnesota.

Final Report Summary:

The State of Minnesota was surveyed extensively for *Heterobasidion*. Over 130 different stands were surveyed in Minnesota with the focus being in southeastern Minnesota since this is the location where the pathogen was first detected. Close to 1,000 samples were collected, processed and tested for the presence of *H. irregulare*. The majority of the samples were processed with our molecular techniques, first with traditional PCR and then qPCR, which is a more sensitive method of detection. A large number of samples were also used for culturing and to isolate onto media. These methods did not yield a definitive positive result for *H. irregulare*. The results

pointed to other fungi that could be causing the mortality to red pine trees. One common fungus found was *Armillaria*, which is another root rot pathogen. Another common fungus was *Coniophora puteana*, which causes a brown cubical rot. Additionally, different wood decay fungi were also found in samples. Some of these wood decay fungi can quickly colonize stumps or trees after they are cut are wounded.

Sites of high concern were used as locations for spore sampling to take place. Rotation impaction samplers were used at these sites. For a positive control, a site was used in an infected stand of trees in western Wisconsin near the border to Minnesota. There was a vast amount of spores detected at this location, which confirmed the methods used were successful at identifying the pathogen. Spores were also detected in Minnesota with some locations showing higher numbers of spores. This information could help in deciding where to conduct more detailed surveys and where to collect samples in the field. However, the number of spores detected at these sites of concern in Minnesota were below the threshold for the number needed to initiate an infection. These levels were established by previous research being conducted in Canada by other researchers. Nevertheless, spores of *H. irregulare* were still detected at several new sites, which is a concern. Future monitoring for *H. irregulare* is essential to determine the numbers of these spores and their potential to start new infection centers.

ACTIVITY 3:

Description: Establish a monitoring network for diagnostics and study methods for control including the evaluation of biological control agents.

There are several integrated control procedures that have been used to limit the continued spread of this disease in other parts of the country. We will evaluate the various cultural methods used to limit disease spread such as preventing movement of infected material from an area, cut and burn infected trees that could sporulate, clear cut stands that have extensive infection, thin stands during periods of the year when spores are not present, treat stumps with fungicides (boron stump treatment) to prevent new infections, clean logging equipment used in infected sites etc. In addition, investigations evaluating our native pioneer colonizing fungi for potential use for biocontrol will be carried out. Fungi will be isolated from field locations, identified by DNA sequencing and tested for biological control activity in laboratory assays. Successful candidate organisms will also be tested under field conditions. This work will follow previous protocols that have proven to be successful to identify fungi that out compete forest pathogens.

Guidelines for controlling the disease in woodlands as well as in urban landscapes in Minnesota will be developed. An integrated approach that considers each of these control measures will be developed with our state cooperators and formalized into a decision-support framework based on forest age, proximity to known infections, and long-term economic and ecological objectives. Short term and long range management plans will be developed with stakeholders from Minnesota and Wisconsin and a decision-support framework for guiding disease management plans obtained. This framework will take into consideration long-term economic and ecological objectives, forest conditions (e.g., age, density, and site quality) and proximity to known infections to help aid the development of management prescriptions for minimizing impacts and spread of this disease.

Summary Budget Information for Activity 3:	**ENRTF Budget:**	**$ 125,784**
	Amount Spent:	**$ 125,784**
	Balance:	**- $ 0**

Outcome	Completion Date
1. Control guidelines developed for woodlands and urban landscapes	June 30, 2016
2. Biological control agents found and tested	June 30, 2019
3. Informational and training programs for detection and control completed	June 30, 2019

Activity Status as of *[January 1, 2016]*:

In addition to the surveys being conducted to elucidate the distribution of *Heterobasidion*, collections are being made of native fungi that are pioneer species colonizing pine wood that may have antagonistic activity to Heterobasidion. These native pioneer fungi are possibly acting in localized areas as antagonists to *Heterobasidion*, inhibiting its spread. Wood and fruiting body samples are being collected from freshly cut stumps and from the base or the roots of dead or dying trees in areas where pine mortality is found. Samples of native fungi have been collected at each of the survey sites for *Heterobasidion*. These samples are isolated onto semi-selective media to culture Basidiomycetes. They are then isolated again to obtain a pure culture. These cultures then undergo a DNA extraction, PCR, and sequencing to determine the species of the fungal culture. A substantial amount of native fungi have been isolated and their antagonism towards *Heterobasdion* will be tested in laboratory studies in the future. Once certain fungi have demonstrated their antagonistic properties, they can possibly be used as a biocontrol method for *Heterobasidion*. Additionally, this collection of native fungi will help us better understand the biodiversity of fungi in managed Minnesota conifer forests elucidate the role they play in inhibiting *Heterobasidion* and in healthy forest ecosystem functioning.

Activity Status as of *[September 1, 2016]*:

A variety of different native pioneer species of fungi have been isolated and cultured in conjunction with *Heterobasidion* surveys. These fungi were isolated from wood and fruiting bodies that were collected from stumps and from the base and roots of dead and dying trees where pine mortality was found. The isolations took place on a semi-selective media to culture basidiomycetes and re-isolated to obtain a pure culture. The identification of the various fungal cultures obtained was determined by performing a DNA extraction followed by sequencing the ITS region of rDNA. A preliminary competition study on media was also performed to determine if they had antagonistic abilities against *Heterobasidion*. The extent of growth inhibition was examined and the fungi that performed the best against *Heterobasidion* will be used in further competition studies. Additionally, another study is under way that is examining a broader view of the diversity of fungi in northern versus southern Minnesota. Samples have been collected from stumps in red pine stands that were thinned 1 year, 3 years, 5 years, and 10 years ago. This will provide information on the dominant fungi that are present and provide insight into the successional sequence of fungi over time. Traditional isolation methods are being used as well as additional molecular methods to give a more refined picture into the fungi that exist in southern and northern red pine stands and provide large numbers of possible biological control candidates for testing against *Heterobasidion*. Naturally occurring biological barriers to *Heterobasidion* may exist in northern forests where red pine is native. These fungi could have application to help stop the spread of the disease in southeastern Minnesota where pines are not in their native range.

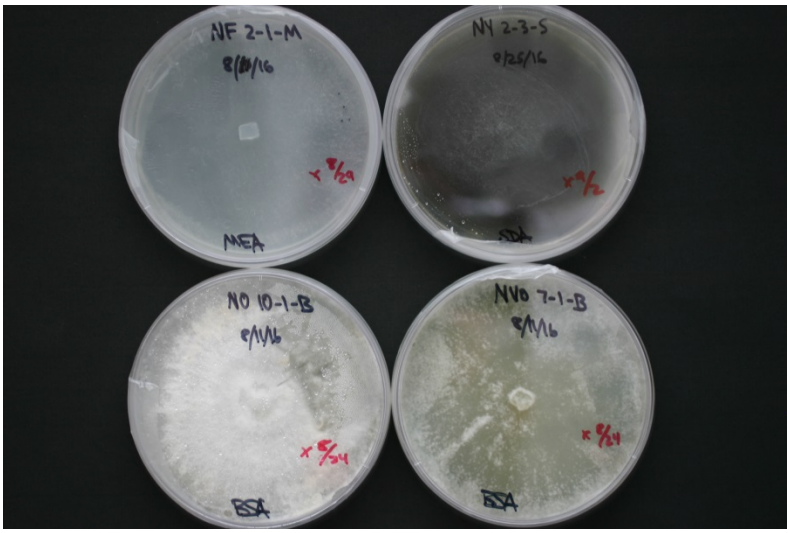

Figure 8. Pure cultures of selected pioneer species of fungi isolated from different red and white pine plantations. These isolates are being identified using DNA sequencing and used in interaction studies for biocontrol.

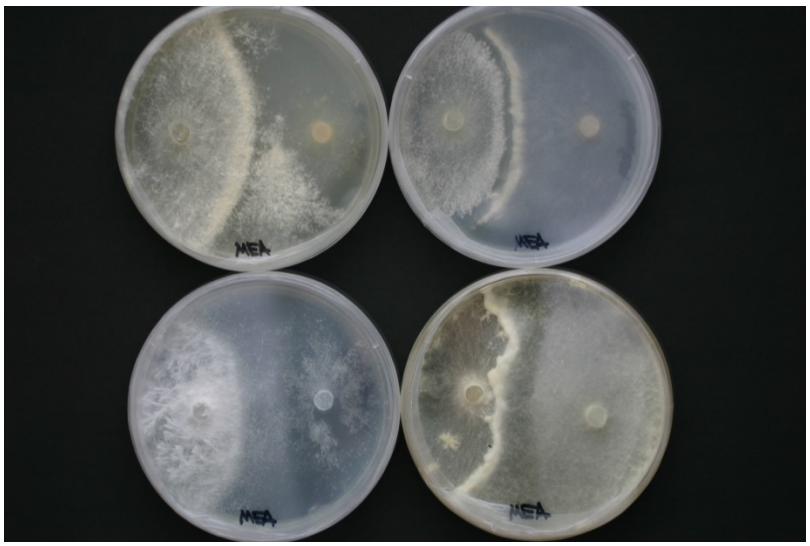

Figure 9. Laboratory antagonism studies evaluating the potential of isolates for biological control. Some fungi are antagonistic and produce an inhibition zone while others overgrow Heterobasidion and can be used for control by resource capture.

Activity Status as of *[April 1, 2017]*:

Isolations of native pioneer species of fungi from *Heterobasidion* surveys have been completed (Fig. 10 and 11). Isolations have also been completed from a study where samples were collected from red pine stumps thinned 1 year, 3 years, 5 years, and 10 years ago. This study will give insight into the successional sequence of fungi over time and why *Heterobasidion* might be present in some sites and not in others. This study also has involved the use of next generation sequencing. Samples have been collected from different age stumps from thinning harvests of trees made across two different regions of MN (northeastern and southeastern Minnesota) and total DNA has been extracted. Working with the University of Minnesota Genomics Center (UMGC), total DNA extracted from the stumps has been sequenced. The sequencing has produced a large amount of data, shown as reads per sample that will be further analyzed in the next few months (Figure 12). The reads will be used to identify and quantify fungal species for each sample. We hypothesize different patterns of fungal succession in different aged stumps and fungal communities in the northern vs the southern sites. This will help to elucidate the pioneer colonizing fungi present in these forests that have the potential to be antagonists to *Heterobasidion*.

The different fungi compiled and used in past preliminary competition studies are being used in dual culture tests (Table 1). These tests will examine inhibition and over growth of the pathogen performed by the antagonistic fungi. Future studies will look at these interactions on different substrates and different temperature regimes to identify suitable candidates for biological control studies against *Heterobasidion*.

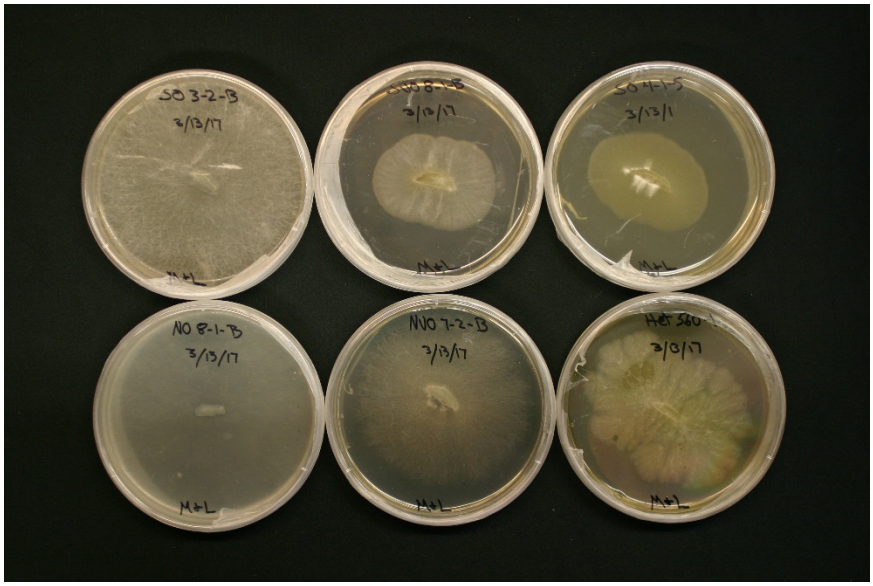

Figure 10. Representative cultures of antagonistic fungi for use in competition studies. The names of some of these fungi are listed in Table 1.

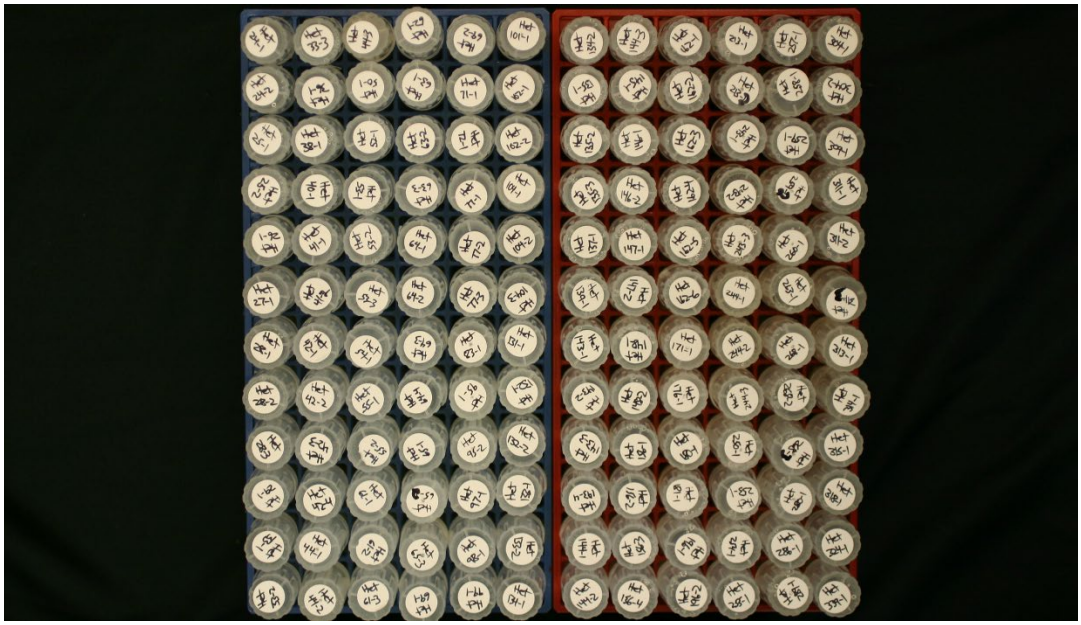

Figure 11. Collection of fungal cultures that were obtained from surveys for Heterobasidion. These fungi represent different taxa that are early colonizers of cut red and white pine. These isolates are being used in studies to identify the best antagonists that can be used for controlling Heterobasidion.

Pioneer colonizing fungi that have been isolated and sequenced

Phlebiopsis gigantea	*Irpex lacteus*
Bjerkandera adusta	*Hydnopolyporus fimbriatus*
Scytalidium album	*Phlebia tremellosa*
Pholiota cf. spumosa	*Peniophora cf. limitata*
Scytinostroma sp.	*Trametes velutina*
Phanerochaete sordida	*Dichomitus squalens*
Perenniporia subacida	*Antrodia serialis*
Sistotrema brinkmannii	*Fomitopsis pinicola*
Clitopilus hobsoni	*Phaeolus schweinitzii*
Coniophora arida	*Resinicium bicolor*

Table. 1. Selection of native fungi that were isolated and sequenced for use in competition/antagonism studies.

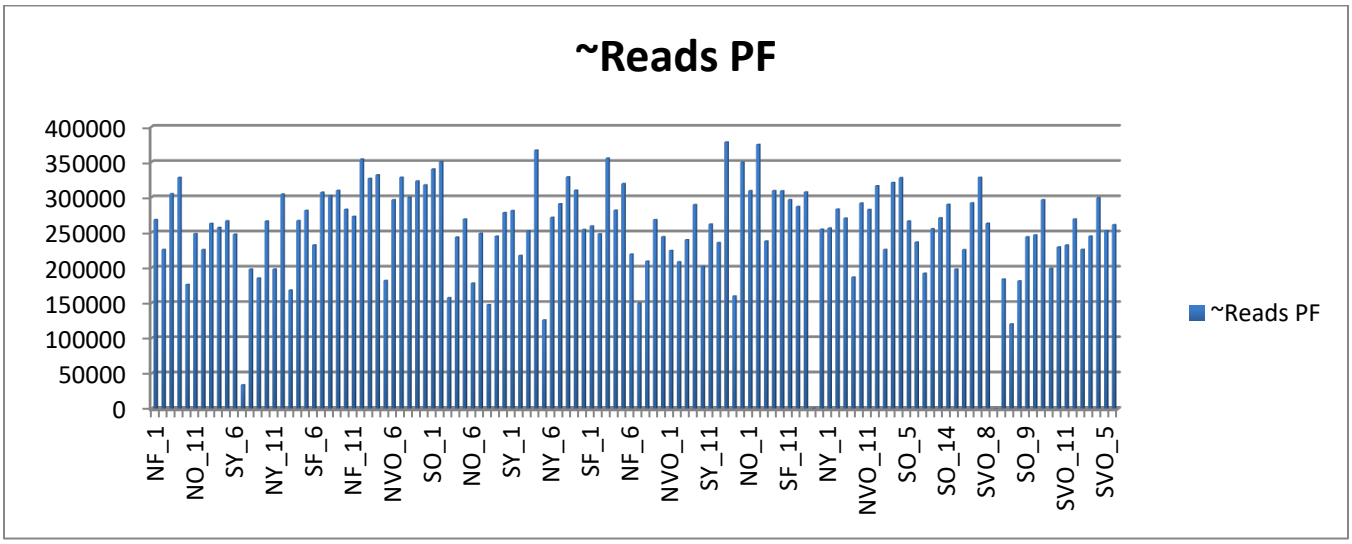

Figure 12. Next generation sequencing results showing number of reads per sample from stumps in northeastern and southeastern Minnesota. These reads will be further analyzed to determine the diversity of fungi in Minnesota and their possible impact on Heterobasidion.

Activity Status as of *[September 1, 2017]*:

Analysis from the stump study where samples were collected from red pine stumps thinned 1 year, 3 years, 5 years, and 10 years ago has revealed interesting results. These results include the successional pattern of fungi over time in red pine stands and why *Heterobasidion* might be present in some sites and not in others. *Phlebiopsis gigantea*, a biocontrol of *Heterobasidion*, was found to be more present in northern Minnesota than southern Minnesota via the culture and microbiome based study (Figs. 13 and 14). This could result in sites in southern Minnesota being more susceptible to *Heterobasidion*. However, the inference space for this study is small, with only one location in each ag`e class, but could be further investigated. Additional analysis from the microbiome dataset is underway that will present more detail into the succession of fungi and other antagonists to *Heterobasidion*. Initial dual-culture tests to examine native fungi and their antagonism towards *Heterobasidion* have taken place. Additional fungi collected were added to the list to be used in dual-culture tests. The results have revealed fungi with antagonistic properties towards *Heterobasidion* (Fig. 15). The fungal cultures that performed the best demonstrated 75-100% inhibition of *Heterobasidion*. With the dual-culture tests nearing completion, the next studies will involve different substrates including wood blocks, and logs to further examine the interaction of native fungi and *Heterobasidion*.

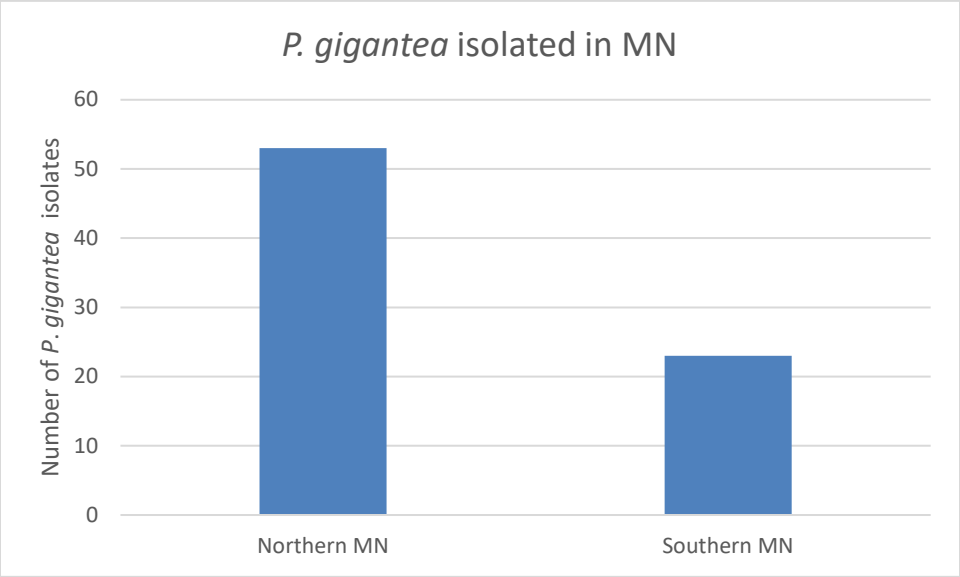

Figure 13. Culturing results show Phlebiopsis gigantea was more easily isolated from sites in northern MN compared to southern MN.

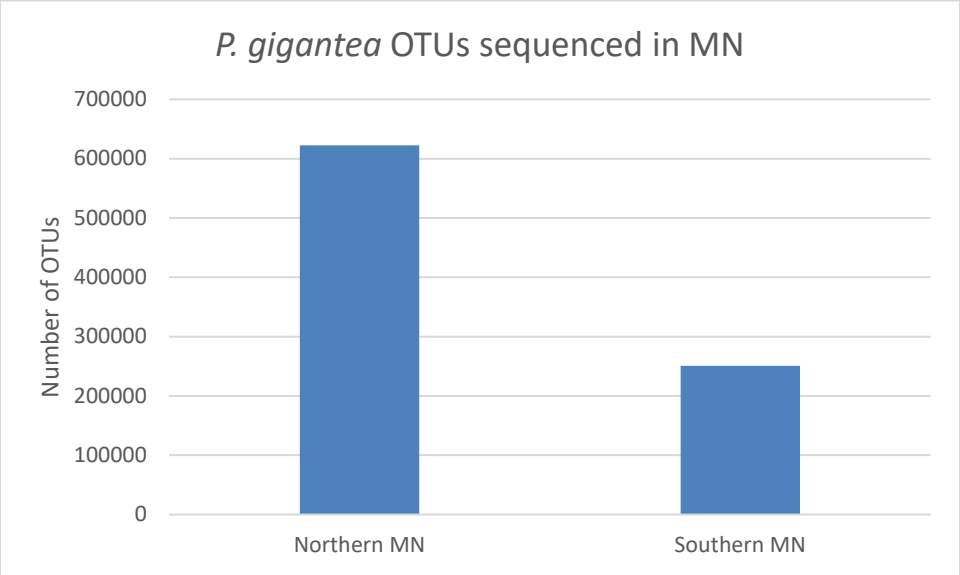

Figure 14. Next-generation sequencing results show Phlebiopsis gigantea was more abundant in stumps in northern MN compared to southern MN.

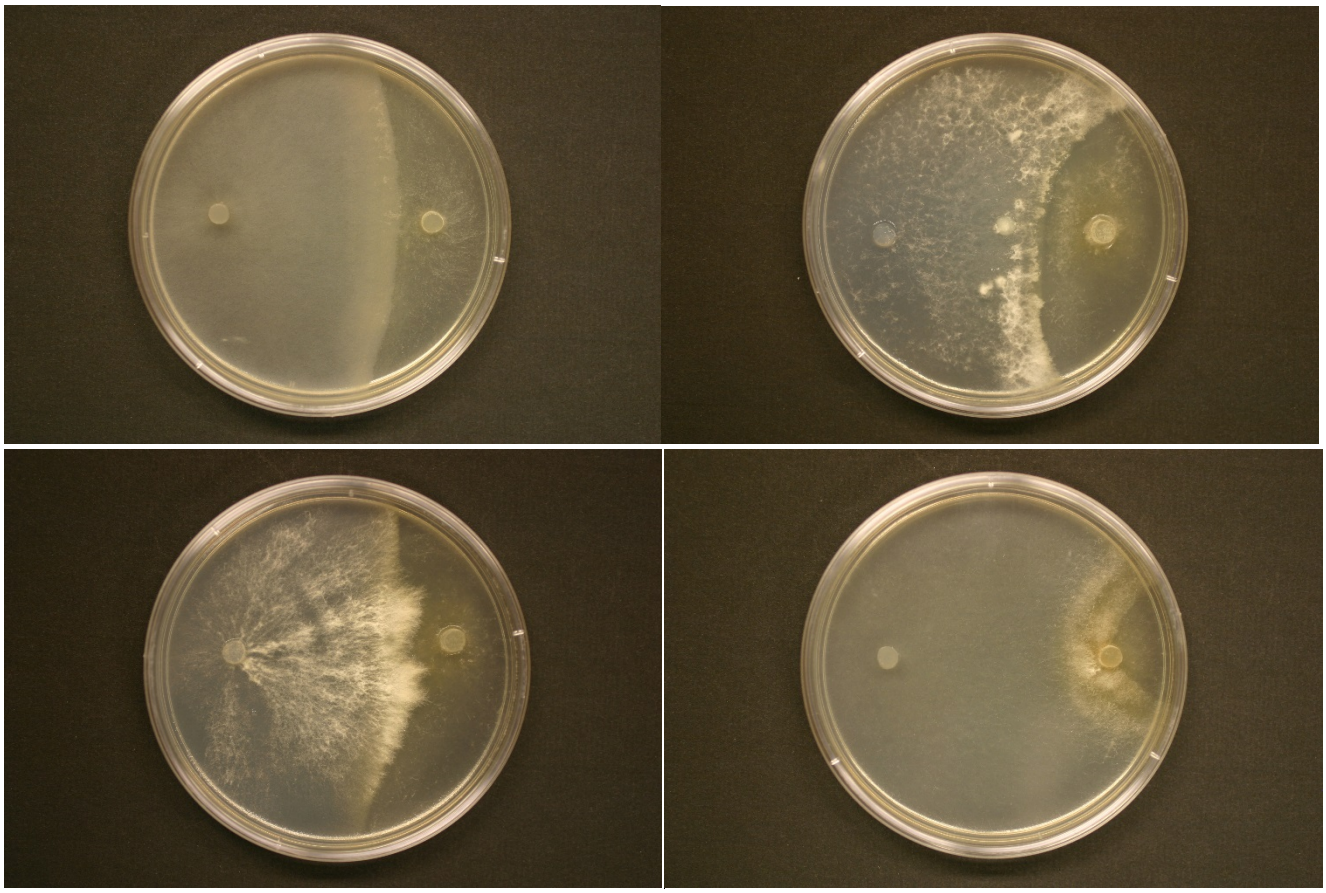

Figure 15. Antagonistic effect of different native fungi (on the left) against Heterobasidion irregulare (on the right) after two weeks of growth.

Activity Status as of *[January 1, 2018]*:

Analysis of cultures and data from the study where samples were collected from red pine stumps thinned 1 year, 3 years, 5 years, and 10 years ago is near completion. Analysis of the data from the metabarcoding part of the study was parsed to display the top ten most sequenced fungi from the native and non-native range of red pine (Figs. 16 and 17). The figures demonstrate the higher abundance of *Phlebiopsis gigantea* in the native range of red pine. The use of *P. gigantea* as a biocontrol agent would have great potential in Minnesota and it appears that a degree of natural biological control by *P. gigantea* may be taking place. Additionally, in the native range of red pine, the second most sequenced fungus is *Resinicium bicolor*. This fungus has demonstrated antagonistic properties in previous studies against *Heterobasidion*. Also, two of the other most sequenced fungi in the native range are basidiomycetes that are currently being used in antagonism studies and could show antagonistic properties. Overall, this is important because this group of fungi could act as effective biocontrol agents and manipulation of their populations in a stand could increase their occurrence making these sites much less susceptible to *Heterobasidion*.

The non-native range of red pine had much less *P. gigantea* and these other basidiomycete antagonists were not present in the top ten fungi sequenced. Dual-culture studies are near completion with selected fungi to be used in additional studies. These studies will involve different substrates including wood blocks, and logs to further examine the interaction of native fungi and *Heterobasidion*.

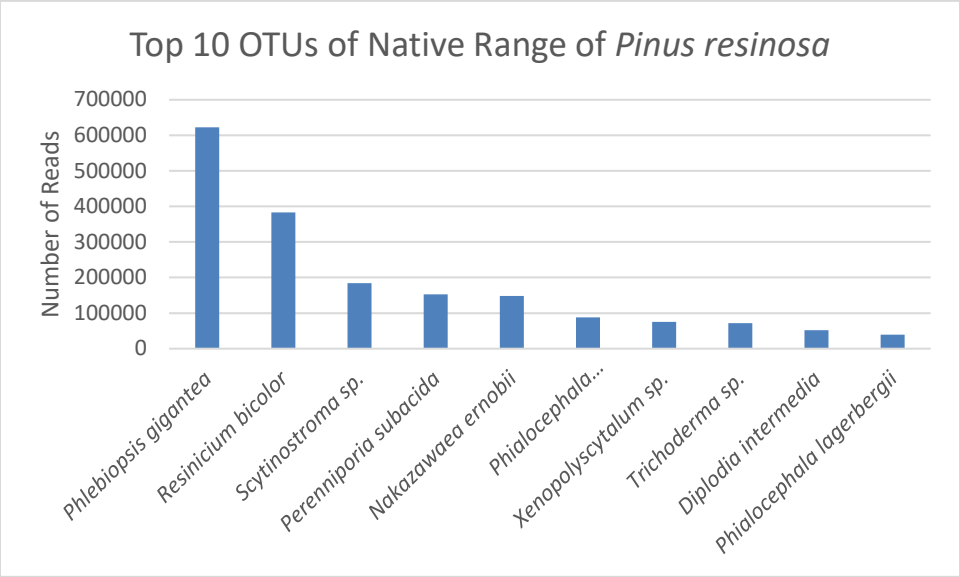

Figure 16. Metabarcoding sequencing results display proven and potential antagonists with a high amount of reads in the native range of red pine.

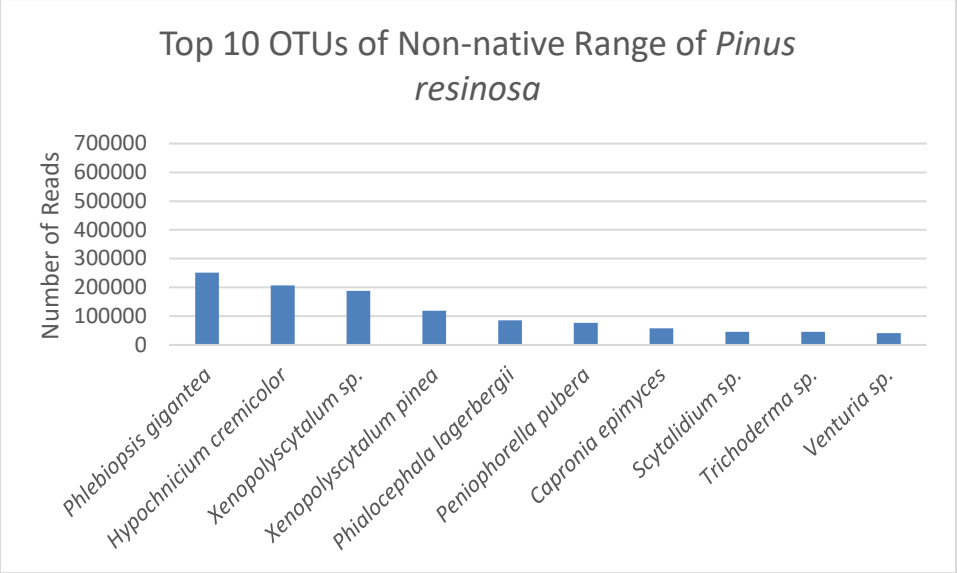

Figure 17. Metabarcoding sequencing results display less proven and potential antagonists with a low amount of reads for P. gigantea in the non-native range of red pine.

Activity Status as of *[July 1, 2018]*:

Analysis of cultures and data from the studies where samples were collected from red pine stumps thinned 1, 3, 5, and 10 years ago is being prepared for publication. The metabarcoding data, which gives information on the abundance and identification of all fungi in a sample, has shown that antagonists are more abundant in the native range of red pine as compared to the non-native range (southeastern Minnesota). Differences in the amount of *Phlebiopsis gigantea,* an antagonist of *Heterobasidion*, in the native red pine range is one of the major results. Overall, more basidiomycetes are present in the native sites as compared to the non-native range of red pine across all sites thinned at different years (Fig. 18.) Different diversity tests were also conducted including the Shannon and Simpson diversity indices. Both diversity indices show that there is more fungal diversity in the native range of red pine compared to the non-native range. The index is 0 to 1 with samples having a lower value being more diverse (Fig. 19). The diversity could be less in the non-native range of red pine due to habitat fragmentation. The majority of red pine sites in southern Minnesota are isolated and surrounded

by agricultural land compared to northern Minnesota where red pine forests are more continuous. Generally, a more diverse fungal community provides for more efficient ecosystem services.

The first phase of antagonism assays is complete with a handful of antagonists showing reduced growth of H. irregulare in media (Fig. 20). The second phase of antagonism assays is focusing on antibiotic assays and using different substrates (Fig. 21). Antibiotic assays determine if any antagonists are using antibiosis as their method of antagonism. This includes the release of secondary metabolites that inhibit growth of *Heterobasidion*. Other substrates being utilized involve red pine wood disks to provide a better idea of how the antagonist and pathogen interact. This ultimately with help lead to developing new biocontrol agents for *H. irregulare*. We currently have a total of 72 different basidiomycetes that have been isolated and are available for experimentation on other projects. These isolates are being proposed for use in new studies for natural products biosynthesis and for degrading pesticides.

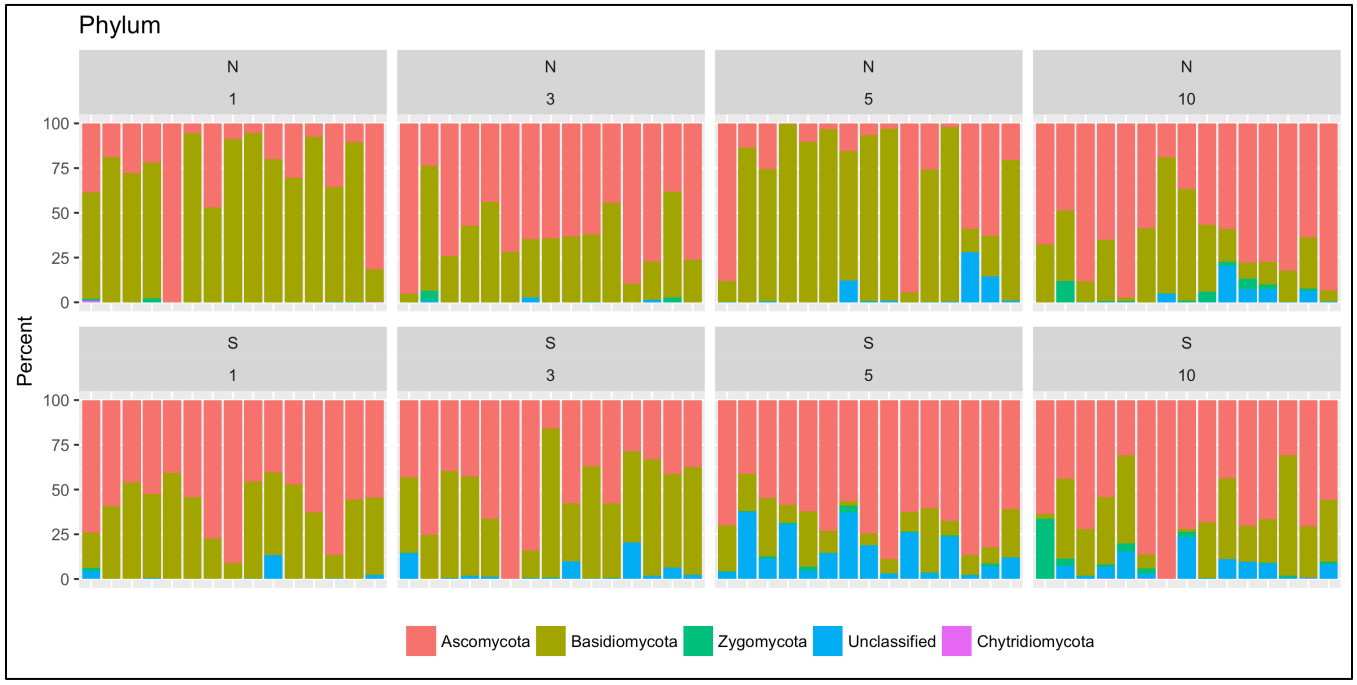

Figure 18. Metabarcoding sequencing results based on phylum show more Basidiomycota are present in the northern native red pine sites (top rows labelled N) as compared to the southern range of red pine (bottom row labelled S). The green bars show the percent of isolates that are Basidiomycota.

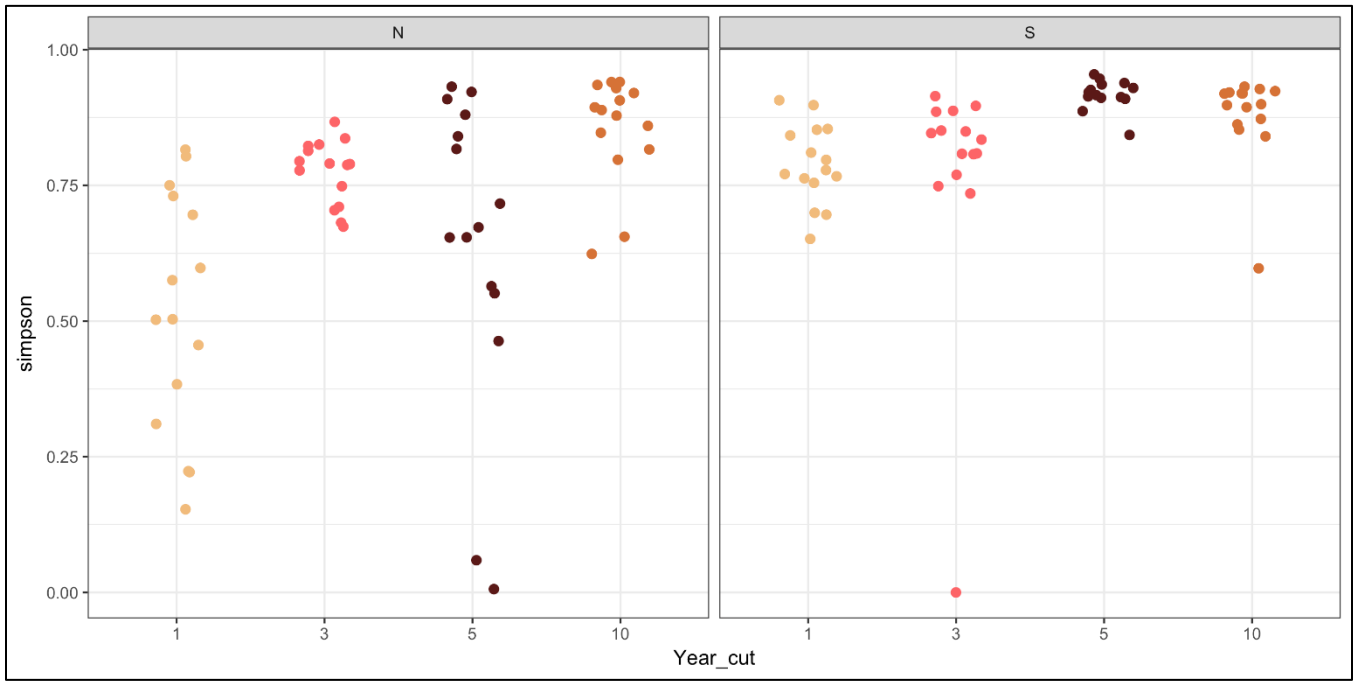

Figure 19. The Simpson Diversity Index revealing that the fungal communities are more diverse in the native or northern range of red pine (left) compared to the non-native or southern range (right).

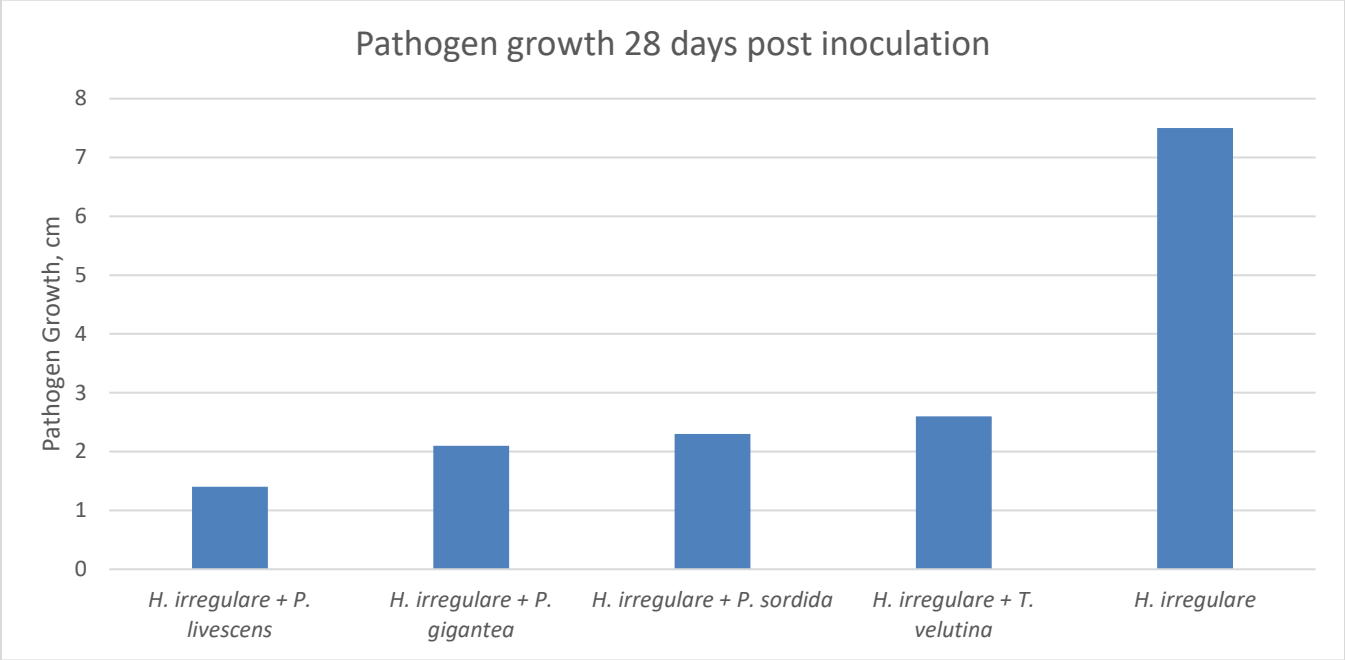

Figure 20. The growth of H. irregulare 28 days post inoculation exhibiting reduced growth with four different antagonists in media.

Figure 21. A laboratory setup for the culture based antibiotic assay (left) and red pine wedges to be used in competition studies (right). These studies will provide further information on the activity of different fungal antagonists towards H. irregulare.

Activity Status as of *[January 1, 2019]*:

Analysis of cultures and metabarcoding data from the studies where samples were collected from red pine stumps thinned 1, 3, 5, and 10 years ago is being prepared for publication. The results have shown a higher abundance of *P. gigantea* in northern Minnesota compared to southern Minnesota. The southern sites are former agriculture lands and have not been forested as long as the northern sites. Because of this, the abundance of antagonists is lower at the southern sites. Additionally, the spore sampling rods from the rotation impaction samplers will be processed with the qPCR assay using primers and probes for P. gigantea to determine if the quantity of spores is higher in northern than southern Minnesota. This will corroborate our results from the isolating and metabarcoding data.

Antagonism assays have continued on woody substrates in the lab and the field. Thin cross sections (0.5-0.8 cm) from red pine trees are being used in dual-inoculation assays (Fig. 22). These are being used to see if results are similar to those performed on media. Results thus far indicate antagonistic fungi are behaving similarly on wood discs as on different media. These fungi are still showing antagonism on sterile wood discs, which indicates they are potential biological control agents. A different study is using wood wedges split from a cross section of red pine (Fig. 23). These wood wedges are used in soil microcosms that involve colonizing a wedge with an antagonistic fungus and then transferring it to a microcosm with *H. irregulare*. The wedges are transferred at different colonization time periods of 1, 7, 14, and 28 days. This will determine if the antagonist can keep *H. irregulare* from colonizing the wedge after these various time periods. Additionally, the opposite is being conducted with *H. irregulare* first colonizing the wedge and then transferred to the microcosm with the antagonist. This will help determine if different antagonistic fungi can replace *H. irregulare* after the same colonization time periods. These studies have been done using *P. gigantea* initially, which has demonstrated it can prevent *H. irregualre* from colonizing at all time periods (Fig. 24). Another study was conducted in the field where disks of red pine were treated with different native fungi and placed around stumps that had *H. irregulare* fruiting bodies (Fig. 25). This study will provide valuable information about the effectiveness of these native fungi for use as biological control agents.

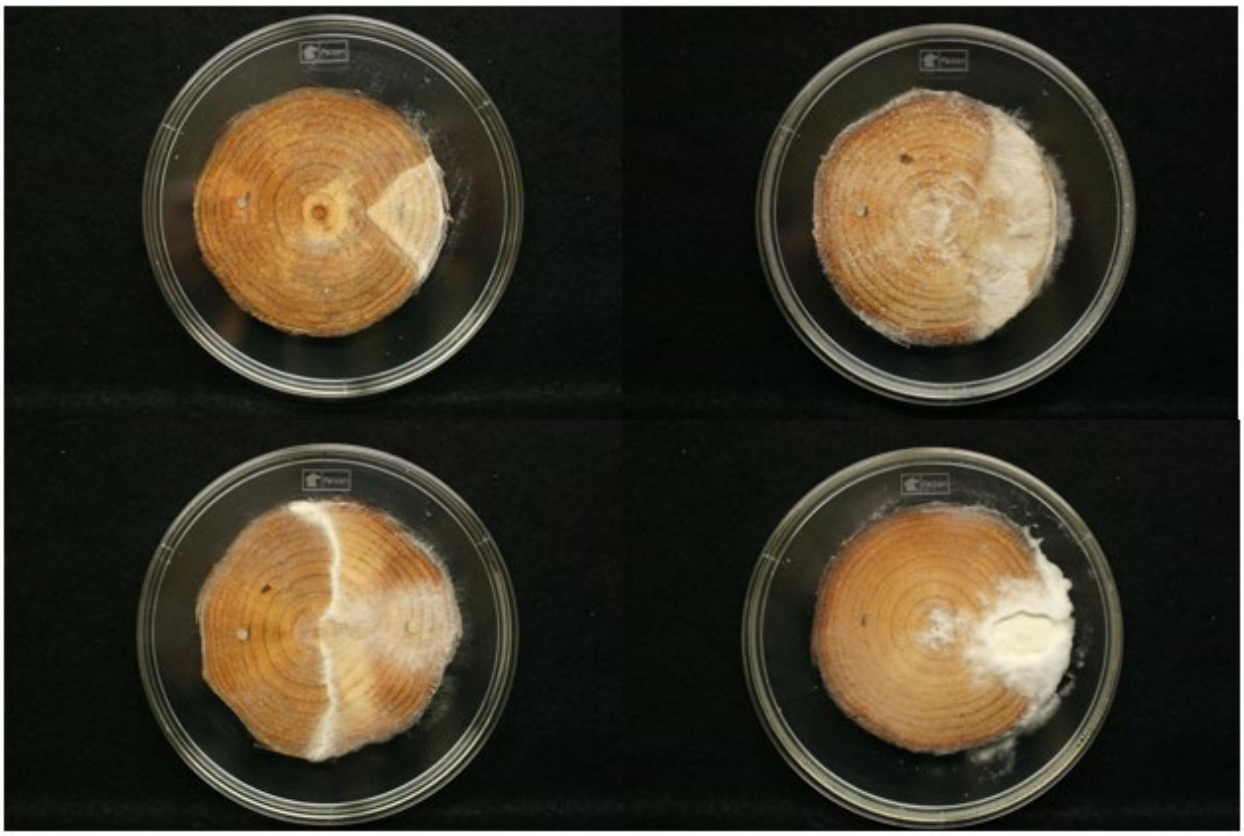

Figure 22. Antagonism assays on wood disks demonstrating native fungi with antagonistic properties inhibiting Heterobasidion (Upper right and left photo, lower left photo). A control disk with H. irregulare (lower right).

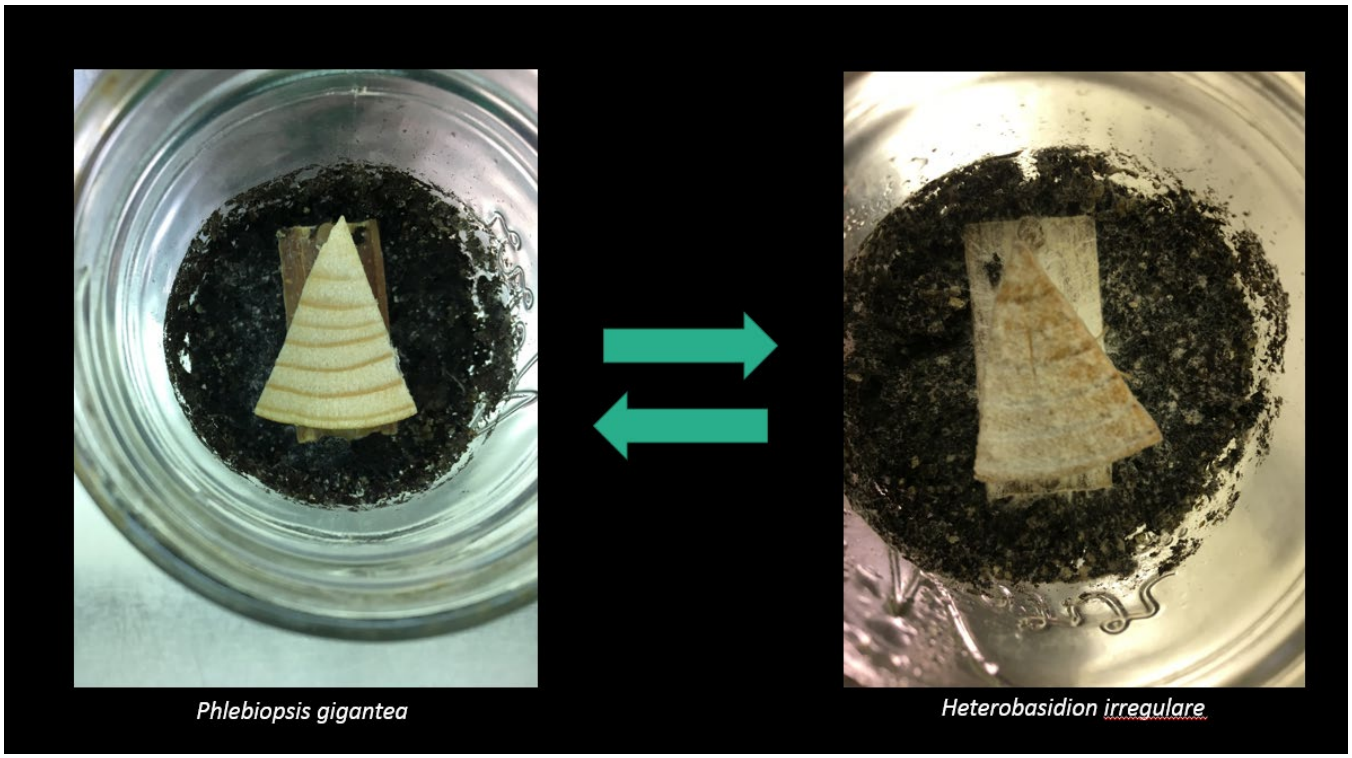

Figure 23. Antagonism assays being conducted in soil microcosms. The microcosm on the left is colonized with P. gigantea and then transferred to a jar colonized with H. irregulare to examine if H. irregulare can colonize the wood wedge. The opposite is then performed with the pathogen being colonized first and then transferred to the microcosm with the antagonist.

Colonization Time with *P. gigantea*	Total Wood Chips (4 per plate)	*H. irregulare* cultures	*% H. irregulare*	*P. gigantea* cultures	*% P. gigantea*
1 Day	48	0	0	46	95.83
7 Days	48	0	0	48	100.00
14 Days	48	0	0	48	100.00
28 Days	48	0	0	47	97.92

Figure 24. Results for soil microcosms initially colonized with P. gigantea and transferred to microcosms of H. irregulare for 56 days.

Figure 25. Red pine wood disks treated with different native fungi are placed around a stump (center) with fruiting bodies of H. irregulare.

Final Report Summary:

Information on the biology of this pathogen and guidelines for control of Heterobasidion in Minnesota have been developed and are presented in a series of short videos that have been widely distributed.
https://www.youtube.com/watch?v=IRO8eLmHqn0
https://www.youtube.com/watch?v=4woY5IC40RA
https://www.youtube.com/watch?v=1_B6g45OGWU
https://www.youtube.com/watch?v=Y7-jU5LzOgA

In addition a web site that has a summary of results and all of the above videos is at:
http://hrdmn.cfans.umn.edu

Publications are being prepared for the studies regarding the metabarcoding study of red pine stumps and antagonism assays between *H. irregulare* and native fungi. The metabarcoding data and cultures isolated from stumps thinned 1, 3, 5, and 10 years ago revealed interesting results. They have shown that a possible native biological control exists in northern Minnesota where red pine is native and has been established for many years. The red pine sites in southeast Minnesota are out of the native range of pines and have been planted on

former agricultural land. Previous literature has stated that former agricultural land is the most susceptible to H. irregulare. Results have shown that Phlebiopsis gigantea, an established biological control agent, is more abundant in northern Minnesota than southeast Minnesota. Additionally, other pioneer fungi were more abundant in northern Minnesota as well.

Antagonism assays have also been completed and results are being prepared for publication. The first phase was dual-inoculation assays in media and the native fungi that performed the best were used in later assays. The next set of assays were dual-inoculation assays on thin wood discs with 14 different antagonists. These were set up similar to the assays on media. Isolations were taken at different locations on the discs to see how abundant the antagonist and pathogen were on the discs after 56 and 63 days. Half of these fungi were isolated more abundantly than *H. irregulare* (Figure 26). The next assay involved wood wedges split from a cross section of red pine. These wood wedges were used in soil microcosms that involved colonizing a wedge with an antagonistic fungus and then transferring it to a microcosm with *H. irregulare*. The wedges were transferred at different colonization time periods of 1, 7, 14, and 28 days. This determined if the antagonist could keep *H. irregulare* from colonizing the wedge after these various time periods. Additionally, the opposite was conducted with *H. irregulare* first colonizing the wedge and then transferred to the microcosm with the antagonist. This helped determine if different antagonistic fungi can replace *H. irregulare* after the same colonization time periods. The fungi that performed the best were *Phlebiopsis gigantea* (current biocontrol agent for *H. irregulare*) and *Phanerochaete livescens* (Figure 27). Finally, a study was conducted in the field where disks of red pine were treated with different native fungi and placed around stumps that had *H. irregulare* fruiting bodies. Plates were flooded with sterile water to make a spore and hyphal solution. This spore/hyphal solution was then applied to discs in the field with a brush. These discs were brought back to the lab and underwent a DNA extraction and qPCR to quantify the amount of *H. irregulare* DNA present. The treated discs with less *H. irregualre* DNA would have the more antagonistic fungus that was applied (Figure 28). The application method might not have worked for a couple antagonistic fungi, such as *Phanerochaete livescens*, which performed well in other assays, but poorly in this one. This could be due to the culture of *P. livescens* not producing spores or aerial hyphae, so the amount of inoculum applied to the wood discs was low. However, this could rule out *P. livescens* as being a viable biological control option compared to the antagonistic fungi since it would be more challenging to apply it in the field). Overall, these antagonism assays provided useful information on how these antagonists interact with *H. irregulare* and their potential to be used a biological control agents.

H. irregulare* vs. *P. gigantea	
H. irregulare	*P. gigantea*
0	100
H. irregulare* vs. *P. sordida	
H. irregulare	*P. sordida*
25	65
H. irregulare* vs. *Phlebia sp.	
H. irregulare	*Phlebia sp.*
11.67	76.67
H. irregulare* vs. *Phlebia cf. tremellosa	
H. irregulare	*Phlebia cf. tremellosa*
13.33	86.67

H. irregulare* vs. *P. livescens	
H. irregulare	*P. livescens*
13.33	86.67
H. irregulare* vs. *S. complicatum	
H. irregulare	*S. complicatum*
20	100

Figure 26. Mean percentage of wood chips isolated from the sapwood colonized by H. irregulare or the antagonist after 56-63 days.

Colonization Time with *P. gigantea*	*H. irregulare*	*P. gigantea*
1 Day	0	95.83
7 Days	0	100
14 Days	0	100
28 Days	0	97.92

Colonization Time with *H. irregulare*	*H. irregulare*	*P. gigantea*
1 Day	0	100
7 Days	0	75
14 Days	0	100
28 Days	0	100

Colonization Time with *P. livescens*	*H. irregulare*	*P. livescens*
1 Day	0	100
7 Days	0	62.5
14 Days	0	100
28 Days	0	100

Colonization Time with *H. irregulare*	*H. irregulare*	*P. livescens*
1 Day	0.00	91.67
7 Days	10.42	75.00
14 Days	37.50	62.50
28 Days	68.75	18.75

Figure 27. Mean percentage of wood chips isolated with H. irregulare or the antagonist after being colonized for different periods of time and transferred to a jar with the opposing fungus.

Antagonist	*H. irregulare* ITS copies
Irpex lacteus	9,316
Phanerochaete livescens	11,317.40
Phlebiopsis gigantea	2,166.38
Sistotrema brinkmannii	4,234.20
Stereum complicatum	2,841.76
Trametes velutina	9,853.96
Uncultured Stereum clone	14,414.80
Heterobasidion irregulare control	12,673.20

Figure 28. The quantity of H. irregulare DNA on wood discs treated with different antagonistic fungi in the field.

V. DISSEMINATION:

Description:

A comprehensive outreach plan utilizing web based and other materials will be initiated. Online resources will include e-newsletter articles, extension factsheets, a website devoted to this project, webinars, and social media updates. Printed materials will consist of scientific journal articles and extension bulletins as well as more general outreach methods using columns in newspapers and various on line informational sites. In addition, the University of Minnesota Plant Disease Clinic will be directly involved in analyzing samples for this work and will be another informational source to the public through their bulletins, outreach activities and first responder plant disease program.

Activity Status as of *[January 1, 2016]*:

Discussions with the Minnesota Department of Natural Resources, the US Forest Service and Natural Resource Departments from Wisconsin, Michigan and Ottawa, Canada have begun to develop a comprehensive plan for control of this root rot disease. A factsheet for *Heterobasidion* has been developed. Online outreach information is being developed.

Activity Status as of *[September 1, 2016]*:

Further discussions have taken place involving the Minnesota Department of Natural Resources, the US Forest Service and Natural Resource Departments from Wisconsin, Michigan, and Ottawa, Canada. These discussions have revolved around creating an inclusive plan for control of this pathogen depending on the region where it is occurring. Online outreach information is currently being developed in conjunction with these agencies. The outreach information includes a website that is currently being constructed.

Activity Status as of *[April 1, 2017]*:

A series of short informational videos are being made to provide information about Heterobasidion Root Disease to the public. These videos focus on identification of the pathogen and disease management options. The videos were filmed by University of Minnesota technology staff and will be online in the near future.

An outreach article as well as a presentation for the Minnesota Mycological Society (MMS) was completed. The Minnesota Mycological Society is composed of amateur mycologists and they are frequently out looking for fungi and can act as citizen observers to help detect the disease. Collaboration work is also continuing with the Minnesota Department of Natural Resources, the US Forest Service, and Natural Resource Departments from Wisconsin, Michigan, and Ottawa, Canada.

Activity Status as of *[September 1, 2017]*:

Additional short informational videos have been made to provide information about Heterobasidion Root Disease to the public (Fig. 18). These new videos are focused on management and ways to prevent *Heterobasidion* from infecting new stands. These videos have been filmed by University of Minnesota technology staff and are currently available online. One of the videos is in collaboration with the Minnesota DNR. The new videos can be viewed at:
https://www.youtube.com/watch?v=4woY5IC40RA
https://www.youtube.com/watch?v=1_B6g45OGWU

Collaboration has also continued with different groups to promote awareness of this pathogen and also to collaborate on different studies. These groups include: Minnesota Mycological Society, Minnesota Department of Natural Resources, the US Forest Service, and Natural Resource Departments from Wisconsin, Michigan, and Ottawa, Canada.

Figure 18. Filming of different educational videos discussing management and research for Heterobasidion root rot disease.

Activity Status as of *[January 1, 2018]*:

A new short informational video about *Heterobasidion* has been created that provides an overview of the research on this disease that we have underway. This video and two previous videos have been promoted on social media outlets such as Facebook and Twitter with much success (Fig. 19).The new video along with the previous videos were filmed by University of Minnesota technology staff and are currently available online. One of the videos is in collaboration with the Minnesota DNR. The new videos can be viewed at:
https://www.youtube.com/watch?v=4woY5IC40RA
https://www.youtube.com/watch?v=1_B6g45OGWU
https://www.youtube.com/watch?v=Y7-jU5LzOgA

Collaboration has also continued with different groups to promote awareness of this pathogen and also to collaborate on different studies. These groups include: Minnesota Mycological Society, Minnesota Department of Natural Resources, the US Forest Service, and Natural Resource Departments from Wisconsin, Michigan, and Ottawa, Canada.

Figure 19. Promotion of online videos on Twitter and other social media outlets has increased dissemination of information regarding Heterobasidion root disease.

Activity Status as of *[July 1, 2018]*:

Mentioned previously, a collection of videos were created to disseminate important information about this disease. We now have four videos that explain the identification, biology and management options for Heterobasidion root disease.

https://www.youtube.com/watch?v=IRO8eLmHqn0
https://www.youtube.com/watch?v=4woY5IC40RA
https://www.youtube.com/watch?v=1_B6g45OGWU
https://www.youtube.com/watch?v=Y7-jU5LzOgA

A presentation was given at a UMycoNet meeting. The UMycoNet consists of a collaborative network of different laboratories at the University of Minnesota studying fungi. A presentation has been accepted for the Upper Midwest Invasive Species Conference in Rochester, MN. This will be a great way to disseminate information about this disease and current research. Collaboration is continuing with different groups to promote awareness of this pathogen and also to collaborate on different studies. These groups include: Minnesota Mycological Society, Minnesota Department of Natural Resources, the US Forest Service, and Natural Resource Departments from Wisconsin, Michigan, and Quebec, Canada. Collaboration with Natural Resources Canada is supporting the developing qPCR assays for the detection *H. irregulare* spores.

Activity Status as of *[January 1, 2019]*:

A presentation was given at the Upper Midwest Invasive Species Conference (UMISC) in Rochester, MN. This was a great venue to present research that has been carried out. Collaboration continues with the Minnesota Mycological Society, Minnesota Department of Natural Resources, the US Forest Service, and Natural Resource Departments from Wisconsin, Michigan, and Quebec, Canada. Cooperation with the Wisconsin DNR has been excellent as more extensive spore sampling and a field experiment was conducted in Wisconsin.

Final Report Summary:

A presentation was given at the Forest Health Workshop in Walker, MN. This workshop was attended by employees in the Minnesota DNR, the US Forest Service, Minnesota counties, and the University of Minnesota. It was a great opportunity to present and meet employees who would have to manage for this forest disease. Additionally, a presentation was given at a state cooperators meeting with the US Forest Service and DNR agencies from Minnesota, Wisconsin, Iowa, Ohio, and Michigan. Again it was good to present and discuss with employees who have or will deal with the management of this pathogen. A web site that has a summary of information and all of the educational videos on the biology and control of *Heterobasidion* Root Disease has been set up at: http://hrdmn.cfans.umn.edu

Publications are being prepared regarding the research on *H. irregulare*. The following are tentative titles for these publications:

- Surveys for *Heterobasidion irregulare* in Minnesota
- Fungal community analysis of red pine stumps in managed stands across Minnesota
- Antagonistic interactions between basidiomycete fungi and *Heterobasidion irregular*

VI. Project Budget SUMMARY:

A. ENRTF Budget Overview:

Budget Category	$ Amount	Overview Explanation
Personnel:	$ 312,540	Funding for a graduate student, research scientist and 3 undergraduate students to carry out the proposed activities. Undergraduate students: $18,000 (100% salary, 0% benefits). 30% FTE each year for 3 years. Graduate Student: $132,000 (56% salary, 44% benefits) 50% FTE for 3 years. Research Laboratory Scientist. $162,540 (71% salary, 29% benefits) 75% FTE each year for 3 years.
Equipment/Tools/Supplies:	$14,460	Supplies for the lab and field work
Capital Expenditures over $5,000:	$32,000	Real Time PCR for the rapid and efficient diagnostics to be conducted on field samples obtained from throughout the state.
Travel Expenses in MN:	$12,000	Travel for disease surveys and sample collection as well as to conduct the biological control investigations
TOTAL ENRTF BUDGET:	**$371,000**	

Explanation of Capital Expenditures Greater Than $5,000:

Real time PCR diagnostic system ($32,000.)- This is a molecular diagnostic system that allows precise detection of pathogens (using specific DNA primers) and is essential for developing a rapid and efficient method of disease identification from field samples. This state of the art equipment will be used for assays to detect the fungus in wood and will be used for all sample assays during the 3 year period. It will then be used in the Plant Pathology Diagnostic lab to continue a service of disease detection on samples sent by the public for analysis.

Number of Full-time Equivalents (FTE) Directly Funded with this ENRTF Appropriation:

4.65 over 3 years

B. Other Funds:

Source of Funds	$ Amount Proposed	$ Amount Spent	Use of Other Funds
Non-state			
	$0	$0	
State			
In Kind Support for Project Leader	$225,897	$37,650	Salary and fringe for 1 month per year for 3 years (41,900). University indirect costs at 52% ($183,997)
TOTAL OTHER FUNDS:	**$297,897**	**$37,650**	

VII. PROJECT STRATEGY:

A. Project Partners:

Professor Robert Blanchette will be the project leader and coordinate activities. He will take part in the development of the diagnostic kits oversee the surveys and direct outreach activities. Dr. Brett Arenz from the Department of Plant Pathology will take part in outreach and training programs and lead the laboratory diagnostic activities in the Plant Disease Clinic. Dr. Benjamin Held is a research scientist in the Department of Plant Pathology and will be involved with the development of rapid molecular procedures for identifying the pathogen, take part in the field surveys and training programs. A graduate student in the Department of Plant Pathology will investigate the use of new biological (aggressive pioneer fungal colonists) control agents. Faculty in the Department of Forest Resources at the University of Minnesota will be involved with the development of management plans for Minnesota's woodlands, will help coordinate surveys and contribute to training programs. Non funded partners that will help with surveys and sample collection include the MN Dept. of Agriculture, Minnesota DNR, Wisconsin DNR and U.S. Forest Service.

B. Project Impact and Long-term Strategy:

The main goal of this project is to identify this new invasive tree disease as early as possible as it moves into Minnesota, prepare an effective defense to fight the disease and reduce its impact to our native conifers growing in forests and urban landscapes. Once the diagnostic procedures are developed, the Plant Disease Clinic can continue to evaluate samples for the people of Minnesota after the end of this proposal . Other State agencies will use the control management guidelines developed from this project long into the future.

C. Funding History:

Funding Source and Use of Funds	Funding Timeframe	$ Amount
University of Minnesota Rapid Response Funds. To begin preliminary surveys and develop sample processing protocols for the project	2013 to 2014	$72,000

IX. VISUAL COMPONENT or MAP(S):

Proposal # O84-D
Title: Preventing a New Disease of Pines in Minnesota
Project Manager: Blanchette

Circles of dead trees in Wisconsin killed by the root rot fungus

Fruiting bodies of the fungus on the base of dying pine trees

The Problem:

Heterobasidion is an invasive root rot fungus that attacks all conifers. Once it gets into a tree it moves through the roots from tree to tree causing expanding circles of death. The Disease is in Wisconsin causing serious losses with infection centers at the border to Minnesota

What is being proposed:

Develop rapid screening methods of detection

Survey and sample trees to identify the disease

Initiate a campaign to educate the public about the disease.

Establish a monitoring network for the state at he University of Minnesota's Plant Disease Clinic.

Development management plans for Minnesota to reduce the impact of the disease

Goals:

To identify the disease as early as possible as it moves into Minnesota and prepare an effective defense to fight this disease and reduce its impact to our native conifers growing in forests and urban landscapes. The program will help keep our pine resources healthy.

X. RESEARCH ADDENDUM:

See attached

XI. REPORTING REQUIREMENTS:

Periodic work plan status update reports will be submitted no later than January 30, 2016, September 30, 2016, April 30, 2017, September 30, 2017 and January 30, 2018. A final report and associated products will be submitted between June 30 and August 15, 2018.

Environment and Natural Resources Trust Fund
Final M.L. 2015 Project Budget

Project Title: Preventing a New Disease of Pines in Minnesota
Legal Citation: M.L. 2015, Chp. 76, Sec. 2, Subd. 06d
Project Manager: Robert A. Blanchette
Organization: University of Minnesota
M.L. 2015 ENRTF Appropriation: $371,000
Project Length and Completion Date: 3 Years, June 30, 2019
Date of Report: June 30, 2019

ENVIRONMENT AND NATURAL RESOURCES TRUST FUND BUDGET	Activity 1 Budget 2/12/2018	Amount Spent	Activity 1 Balance	Acivity 2 Budget 9/22/19	Amount Spent	Activity 2 Balance	Activity 3 Budget 9/22/19	Amount Spent	Activity 3 Balance	TOTAL BUDGET	TOTAL SPENT	TOTAL BALANCE
BUDGET ITEM												
Personnel (Wages and Benefits)	$81,270	$81,270	$0	$112,135	$112,135	$0	$107,204	$107,204	$0	$300,609	$300,609	$0
3 Undergraduate Students. $18,000 (100% salary, 0% benefits). 30% FTE each year for 3 years												
1 Graduate Student. $132,00 (56% salary, 44% benefits) 50% FTE each for 3 years												
Benjamin Held, Laboratory Scientist. $162,540 (71% salary, 29% benefits)75% FTE each year for 3 years												
Equipment/Tools/Supplies	$5,960	$5,960	$0	$9,800	$9,800	$0	$16,471	$16,471	$0	$32,231	$32,231	$0
Field Supplies:collection bags, GPS unit, hatchets, saws, sampling materials, storage contaniers.												
Lab supplies: Culture media for growing microorganisms, agar, antibiotics for culturing, ethyl alcohol and other chemicals, costs for molecular sequencing, molecular primers, probes and reagents, petri dishes, culture containers, microbiology diagnostic reagents, sterile bags, pipettes and pipette tips,DNA extraction kits, cloning kits, autoclave bags, culture tubes, isolation tools, laboratory gloves, incubation chamber, biocontrol microcosm plates and jars.												
Capital Expenditures Over $5,000	$29,337	$29,337	$0	$0	$0	$0		$0	$0	$29,337	$29,337	$0
Real time PCR diagnostic system ($32000.)- a molecular diagnostic system that is able to detect specific DNA primers. Real-time PCR is being proposed for use in this project because it has become the standard for rapid and accurate pathogen detection and analysis. This equipment is different than traditional PCR that is used for DNA amplification because it involves using florescent probes attached to primers that measure amplification as it occurs, in one reaction. It offers a more sensitive and precise analysis, a more rapid turnaround time and has reduced sample handling thereby reducing the possibility for contamination. Target DNA can also be quantified which aids in determining the extent of colonization by the pathogen in field samples.This specialized new equipment is currently not available. We do not have this equipment in our research lab, the Plant Disease Diagnostic Lab or other research laboratories in the facility. For the work outlined in our proposal, a real time pcr is essential since large numbers of samples will be processed and it would be used continuously for the project.												
Travel expenses in Minnesota	$0	$0	$0	$6,714	$6,714	0	$2,109	$2,109	$0	$8,823	$8,823	$0
Travel to survey sites throught Minnesota. Mileage $9,000; lodging $2,000; meals $1000 over 3 years												
COLUMN TOTAL	**$116,567**	**$116,567**	**$0**	**$128,649**	**$128,649**	**$0**	**$125,784**	**$125,784**	**$0**	**$371,000**	**$371,000**	**$0**