



133
657
THS



3 1293 01787 6701

LIBRARY
Michigan State
University

This is to certify that the

thesis entitled

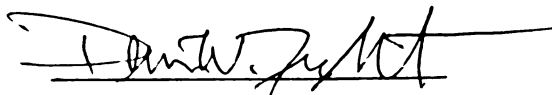
Cyanide-resistant respiration and altered
virulence in Ceratocystis fagacearum,
the oak wilt fungus.

presented by

R. Scott Shaw

has been accepted towards fulfillment
of the requirements for

M.S. Botany and Plant Pathology
_____ degree in _____


Major professor

Date May 12, 1999

PLACE IN RETURN BOX to remove this checkout from your record.
TO AVOID FINES return on or before date due.
MAY BE RECALLED with earlier due date if requested.

DATE DUE	DATE DUE	DATE DUE

CYANIDE-RESISTANT RESPIRATION AND ALTERED VIRULENCE IN
CERATOCYSTIS FAGACEARUM, THE OAK WILT FUNGUS.

By

R. Scott Shaw

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Botany and Plant Pathology

1999

ABSTRACT

CYANIDE-RESISTANT RESPIRATION AND ALTERED VIRULENCE IN *CERATOCYSTIS FAGACEARUM*, THE OAK WILT FUNGUS

By

R. Scott Shaw

Hypovirulent fungal pathogens have been implicated in biological control. Hypovirulence has been attributed to non-nuclear factors such as hypoviruses and mitochondrial (mt)-DNA mutations. Conidia from *Ceratocystis fagacearum*, the oak wilt pathogen, were exposed to ethidium bromide and UV-light to produce a slow-growing isolate (PM447) with increased cyanide (CN)-resistant respiration, indicative of respiratory dysfunction. When PM447 was used as the male in sexual crosses with wild type (WT), only 4% of the progeny were CN-resistant. The reciprocal cross produced no perithecia. Vegetative pairing of PM447 with a benomyl-resistant WT strain resulted in benomyl-resistant conidia with the PM447 morphology. These results suggested a cytoplasmic origin of the PM447 phenotype. In virulence assays using red oak seedlings, PM447 produced fewer symptoms than WT. Seedlings inoculated with PM447 and challenged two weeks later with WT also displayed fewer symptoms when compared to seedlings inoculated with WT alone.

*This is dedicated to all the people who believed in me.
Without their support this would not have been possible.*

ACKNOWLEDGEMENTS

I would like to thank Dr. Dennis Fulbright for his support and guidance while I was working on this project, along with the other members of my guidance committee, Dr. Helmut Bertrand and Dr. Frances Trail. Their input, advice and support were immeasurable. I would like to further thank the members of Dr. Fulbright's laboratory, Dr. Julia Bell, Carmen Medina-Mora, Mario Mandujano and Dr. Bertrand's laboratory, Dipnath Baidyaroy, Cathy Nummy for their friendly help and companionship over the last few years. I would like to additionally thank Dr. William L. MacDonald for kindly providing the fungal samples and for his encouragement. I greatly appreciate the support received from the Rackham Foundation, Agriculture Experiment Station and Department of Botany and Plant Pathology.

TABLE OF CONTENTS

LIST OF TABLES.....	vi
LIST OF FIGURES.....	vii
CHAPTER 1– INTRODUCTION AND LITERATURE REVIEW	
Chestnut blight and hypovirulence.....	3
Hypovirulence and double-stranded RNA.....	4
Description of dsRNA.....	6
Mitochondrial association of dsRNA.....	7
Hypovirulence associated with mitochondria.....	9
Alternative pathway of respiration.....	9
Dysfunctional mitochondria and senescence.....	11
Induction of mitochondrial hypovirulence.....	12
History and description of <i>Ceratocystis fagacearum</i>	14
Disease cycle of <i>Ceratocystis fagacearum</i>	16
Control of oak wilt.....	18
CHAPTER 2– IDENTIFICATION AND CHARACTERIZATION OF PM447	
Introduction.....	22
Material and Methods.....	25
Fungal strains and growth conditions.....	25
Mutagenesis.....	25
Respiration assays.....	27
Sexual transmission.....	28
Transmission via hyphal anastomosis.....	29
Isolation of mitochondrial DNA.....	29
Pathogenicity assays.....	32
Results.....	35
<i>nit</i> -mutant genotype determination.....	35
Induction of benomyl-resistance.....	35
Induction of cyanide-resistant respiration and alternative oxidase.....	37
Isolation of respiratory mutants.....	39
Conidial germination analysis.....	39
Sexual transmission.....	41
Transmission via hyphal anastomosis.....	44
Endonuclease digestion of mitochondrial DNA.....	46
Virulence and biological control assays.....	46
Discussion.....	56
CONCLUSION.....	60
REFERENCES.....	62

LIST OF TABLES

Table 1.	Relative growth rates of strains of <i>Ceratocystis fagacearum</i> after one week on different media. – is no growth, + is very little growth, ++ is little growth, +++ is medium growth, ++++ is lots of growth, +++++ is very much growth. N.D. = not determined.....	36
Table 2.	Percentages of alternative oxidase activity obtained from early and late germinating conidia derived from cultures of <i>Ceratocystis fagacearum</i> strain PM447 grown on potato dextrose agar. Conidia produced by PM447 germinated early and late as determined by growth diameter after eight days. These early and late germinating conidia were cultured again and each type produced both early and late germinating conidia. The early and late germinating conidia derived from early germinating conidia were described as early, early or early, late, respectively. The early and late germinating conidia derived from late germinating conidia were described as late, early or late, late, respectively. These second generation early and late germinating conidia subsequently produced a third generation of early and late germinating conidia. Percentages of alternative oxidase activity obtained from isolates are listed. Multiple numbers represents the percentages of alternative oxidase activity obtained from multiple isolates with the same germination phenotype. N.A. = not available.....	43
Table 3.	Levels of cyanide-resistant respiration expressed by ascospores obtained by crossing <i>Ceratocystis fagacearum</i> strain 63061 with PM447. Ascospores numbered 1-79, not listed, expressed 0% cyanide-resistance.....	44
Table 4.	Number of conidia with different growth phenotypes obtained from plates where fungal strains were paired together. Ben ^r = benomyl-resistant.....	45

LIST OF FIGURES

Figure 1.	Growth of <i>Ceratocystis fagacearum</i> on potato dextrose agar amended with 1 µg benomyl per ml. A fast-growing, benomyl-resistant, virulent strain Fenn (lower right) with three benomyl-sensitive strains.....	37
Figure 2.	Respiration rates of <i>Ceratocystis fagacearum</i> A) wild-type Fenn, B) wild-type Fenn + 3 µg antimycin A per ml, C) Fenn <i>nit 1/3</i> , D) PM447. Additions of cyanide (CN) and salicylhydroxamic acid (SHAM) are indicated by arrows. Numbers above each tracing represent relative respiratory activity measured as oxygen consumption over time.	38
Figure 3.	The number of surviving <i>Ceratocystis fagacearum</i> conidia after various times of exposure to ultra-violet light.....	40
Figure 4.	Conidia from <i>Ceratocystis fagacearum</i> 8 days after germination on potato dextrose agar. A) Synchronous growth pattern of wild-type Fenn conidia, B) Synchronous growth pattern of Fenn <i>nit1/3</i> conidia, C) Asynchronous growth pattern of PM447 conidia.....	42
Figure 5.	A pairing plate seven days after PM447 (left) was paired with wild-type, virulent benomyl-resistant Fenn (right).....	45
Figure 6.	Endonuclease restriction digestions of mitochondrial DNA. Lane 1, wild-type Fenn digested with <i>HindIII</i> ; Lane 2, PM447 digested with <i>HindIII</i> ; Lane 3, 1kb marker; Lane 4, wild-type Fenn digested with <i>XbaI</i> ; Lane 5, PM447 digested with <i>XbaI</i>	47
Figure 7.	Red oak (<i>Quercus borealis</i>) seedlings four weeks after inoculation. A) inoculated with sterile, distilled water, B) inoculated with conidia from <i>Ceratocystis fagacearum</i> wild-type, virulent Fenn, C) inoculated with conidia from PM447.....	48

Figure 8.	<i>Ceratocystis fagacearum</i> isolates recovered from stem sections of red oak (<i>Quercus borealis</i>) seedling. A) A fast growing isolate with wild-type Fenn morphology recovered from a seedling inoculated with wild-type Fenn. B) A slow growing isolate with PM447 morphology recovered from a seedling inoculated with PM447. Isolate with PM447 morphology is older than the isolate with wild-type Fenn morphology.....	49
Figure 9.	Disease ratings of red oak (<i>Quercus borealis</i>) seedlings four weeks after infection with either wild-type, virulent Fenn, or Fenn <i>nit1/3</i> , or PM447.....	50
Figure 10.	Disease ratings for red oak (<i>Quercus borealis</i>) seedlings inoculated with PM447 and wild-type, virulent Fenn	52
Figure 11.	Red oak (<i>Quercus borealis</i>) seedlings 4 weeks after inoculation with <i>Ceratocystis fagacearum</i> . A) inoculation with wild-type, virulent Fenn conidia alone, B) simultaneous co-inoculation with conidia from both wild-type, virulent Fenn and PM447.....	53
Figure 12.	Red oak (<i>Quercus borealis</i>) seedlings four weeks after inoculation with <i>Ceratocystis fagacearum</i> wild-type, virulent Fenn. Seedlings were inoculated with Fenn at the same time. A) Seedlings inoculated with Fenn conidia only, B) seedlings inoculated with PM447 one week prior to inoculation with Fenn.....	54
Figure 13.	Red oak (<i>Quercus borealis</i>) seedlings four weeks after inoculation with <i>Ceratocystis fagacearum</i> wild-type, virulent Fenn. Seedlings were inoculated with Fenn at the same time. A) Seedlings inoculated with Fenn conidia only, B) seedlings inoculated with PM447 two weeks prior to inoculation with Fenn.....	55

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION AND LITERATURE REVIEW

The term hypovirulence was first used by the French mycologist Grent (1965) when describing strains of the fungal chestnut pathogen *Cryphonectria parasitica* (Murr.) Barr recovered from non-lethal infections of chestnut trees. These strains were reduced in virulence or aggressiveness, were white instead of orange, produced fewer conidia and often exhibited abnormal culture morphology. These strains were capable of transferring these traits to normal appearing, virulent strains, both on the tree and in the laboratory (Grent, 1965; Van Alfen et al., 1975). Later, the transmissible or infectious nature of the hypovirulent phenotype was associated with virus-like, double-stranded (ds)RNA genomes in the cytoplasm (Day et al., 1977). It was soon apparent that the hypovirulent strains were responsible for the biological control of this chestnut pathogen known as chestnut blight.

In North America, a similar story was taking place, but on a much smaller scale in Michigan and a few other areas. However, in addition to dsRNA genomes causing hypovirulence, strains of *C. parasitica* were also recovered that lacked dsRNA, yet the virulence and phenotypic characteristics remained the same as the hypovirulent, dsRNA containing isolates (Mahanti et al., 1993). In this introduction I will describe our current understanding of chestnut blight and hypovirulence including viral hypovirulence and the more recently described form, mitochondrial hypovirulence. The second chapter describes experiments performed to extend the use of mitochondrial hypovirulence in

other fungal species in hopes of controlling other plant diseases. Our understanding of chestnut blight and continued study of the hypovirulent phenotype in this species has allowed me to pursue the hypovirulent phenotype in the oak wilt pathogen, *Ceratocystis fagacearum*.

Chestnut blight and hypovirulence

Chestnut blight was first discovered in North America in 1904 (Anagnostakis, 1982). *Cryphonectria parasitica*, the causal fungus, spread at a rate of about 30 miles a year and virtually eliminated the American chestnut (*Castanea dentate* [Mars] Birch) from its natural range. Infection started through wounds and resulted in cankers that girdled the stems, killing that portion of them stem above the canker. Some root systems have survived and still produce sprouts from the roots. These sprouts are infected by the pathogen and the disease cycle continues.

In Europe, chestnut blight was first observed in 1938 on European chestnut trees (*Castanea sativa* Mill.) in Italy (MacDonald and Fulbright, 1991). An epidemic started similar to the one observed in the United States, but in the 1950's it became obvious that the European epidemic would be much less devastating. Biraghi (1953) discovered healing or non-lethal cankers on chestnut trees in Italy. It was originally thought that the trees may be resistant to infection, but fungal isolates that were recovered from these healing cankers were debilitated and phenotypically distinguishable from isolates recovered from lethal cankers. The term "hypovirulent" was first used to describe the strains of *C. parasitica* that were recovered from these healing cankers (Grente, 1965). The debilitated strains were reduced in their ability to elicit disease symptoms in their

susceptible host, had white pigmentation instead of orange pigmentation, and the debilitated strains did not conidiate as profusely as the virulent strains.

Virulent strains elicit severe disease symptoms, leading to the death of the susceptible host after infection. After infection of a susceptible host with an hypovirulent strain of a normally virulent pathogen, there is a general decrease in the overall severity of disease symptoms (Anagnostakis, 1982). It was the less virulent fungus that was responsible for inducing the wound healing response in the chestnut trees, so the fungal isolates were described as hypovirulent.

Hypovirulence and double-stranded RNA

Van Alfen et al. (1975) used a European hypovirulent strain to biologically control virulent North American strains of *C. parasitica*. They co-inoculated American chestnut trees with American virulent and European hypovirulent strains. The cankers produced by the co-inoculations were smaller than the cankers produced by the virulent strain alone. They also co-inoculated American chestnut trees with auxotrophic mutants of *C. parasitica*, and demonstrated that the factor responsible for the hypovirulent phenotype was cytoplasmic and could be transferred via hyphal anastomosis. A virulent methionine requiring strain was co-inoculated into chestnut trees along with a hypovirulent lysine requiring strain. Only methionine auxotrophs were re-isolated from the cankers. These isolates produced significantly smaller cankers in virulence assays, indicative of hypovirulence. These workers were also able to accomplish cytoplasmic transfer in laboratory heterokaryon tests.

Two years later, hypovirulent isolates were shown to contain dsRNA, while the virulent isolates of chestnut blight did not contain dsRNA (Day et al. 1977). Fulbright

(1984) provided more evidence that dsRNA was causing the hypovirulent phenotype by curing hypovirulent (dsRNA containing) strains of their dsRNA through the application of cycloheximide. The cured strains had increased levels of virulence. These strains were able to become hypovirulent again after re-infection with dsRNA.

Since dsRNA is an infectious cytoplasmic factor, natural transmission via hyphal anastomosis can occur in vegetatively compatible strains. It is estimated that there are at least five genes governing vegetative compatibility (Anagnostakis and Day, 1979), and in order for hyphal anastomosis to occur, all five alleles must be homologous. Differences among or between loci can have different effects, but not necessarily precluding transfer of cytoplasmic factors via hyphal anastomosis. Huber and Fulbright (1992) demonstrated that different vegetative compatibility genes have specific effects on the transmission of cytoplasmic elements between different strains.

Isolates from the same vegetative compatibility group form hyphal fusions, thereby allowing the mixing of cytoplasm. The dsRNA can be transferred to dsRNA-free isolates causing them to become hypovirulent (Nuss and Koltin 1990). The dsRNA are not known to be passed on through sexual ascospore progeny, but dsRNA can be found in the asexual conidia. The ability for a hypovirulent strain to act as a biological control agent may be limited by vegetative compatibility within a population of fungal pathogens.

North American hypovirulent isolates of *C. parasitica* were discovered in Michigan by Elliston et al. (1977). Hypovirulent strains were subsequently found in several locations throughout Michigan (Fulbright et al. 1983). These hypovirulent strains were similar to European hypovirulent strains in that they were debilitated and contained

dsRNA; however, they were also different, as North American hypovirulent strains were not white, but had the same orange pigmentation as non-infected, virulent strains.

Hypovirulent strains have been isolated throughout North America including Ontario (Dunn and Boland, 1993), New Jersey, Maryland, Pennsylvania, Virginia, West Virginia, and Tennessee (Peever et al., 1997). Almost all of these strains are hypovirulent due to the presence of dsRNA.

Description of dsRNA

The dsRNA isolated from strains of *C. parasitica*, can be separated by nucleotide sequences, indicating there was genetic variation among the dsRNA (Paul and Fulbright, 1988; Enebak et al., 1994; Peever et al. 1997). Despite their variability, dsRNA hypoviruses from *C. parasitica* have many similarities and have therefore been classified into the family Hypoviridae (Hillman et al. 1995). The dsRNA from *C. parasitica* strain EP713, the type species for the genus *Hypovirus* within the family Hypoviridae, includes large, medium and small-dsRNA segments. The large (L)-dsRNA (12.7kb) is considered the genome and is divided into two open reading frames (ORF), ORF A and ORF B. When a virulent strain of *C. parasitica* was transformed with a cDNA clone of ORF B, it became debilitated and hypovirulent. This demonstrated the importance of the L-dsRNA in producing the hypovirulence phenotype (Choi and Nuss 1992). The transformation of a virulent strain to hypovirulent with cDNA from the dsRNA hypovirus was not only a step forward in understanding the cause of hypovirulence, it may also be a step forward in biological control. The transformed isolate, with cDNA of the hypovirus integrated into its nuclear genome, produced cytoplasmic-borne dsRNA hypoviruses. These were not only transmissible via hyphal anastomosis but the nuclear copy was transferred in sexual

crosses allowing hypovirulence to cross vegetatively incompatible barriers (Chen et al. 1993).

Transformants, containing full-length cDNA of the prototypic hypovirus, were inoculated on chestnut trees in Connecticut (Anagnostakis et al., 1998). Four months later, ascospores were obtained that contained the cDNA integrated into their genome. Additionally, a new canker was identified on an untreated chestnut tree and the fungus isolated from the canker was a strain with the cDNA in its genome but of a different mating type than the original transformant. This infection appeared to be caused by an ascospore derived by a field cross of the transformant with an endemic strain. Unfortunately, the experiment involved a limited, one-year introduction which is not sufficient for establishment of biological control. The experiments did demonstrate that transformants are capable of transferring hypovirulence in the field, and thus may act as biological control agents.

Mitochondrial association of dsRNA

Hypovirulence due to dsRNA has been reported in other fungal plant pathogens. These reports include *Diaporthe ambigua* (Smit et al., 1996), *Gaeumannomyces graminis* (Stanway, 1985), *Ophiostoma ulmi* (Rogers et al., 1988), *Sclerotinia sclerotiorum* (Boland, 1992) and *Ustilago maydis* (Wood and Bozarth, 1973). The dsRNA in these other fungal species may be different from that of *C. parasitica* as exemplified by the cytoplasmic dsRNAs in the Dutch elm disease fungus, *Ophiostoma* (= *Ceratocystis*) *ulmi* (Bruisman) Nannf. A disease factor of *O. ulmi* was discovered by Brasier in 1983. Isolates that contained this disease factor were debilitated and shown to transmit the disease factor to disease-free strains via hyphal anastomosis. Diseased isolates contained

10 segments of dsRNA of which seven have been eliminated as the cause of the debilitated phenotype (Rogers et al., 1986). The structures of the disease causing segments of dsRNA from *O. ulmi* were quite different from that of *C. parasitica*, suggesting a different ancestry (Hong et al., 1998). The dsRNA co-purified with mitochondria, and diseased isolates had greatly reduced levels of cytochrome oxidase (Rogers et al., 1987).

A dsRNA associated with the mitochondria has also been observed in *C. parasitica*. Polashock and Hillman (1994) isolated a strain of *C. parasitica* that was reduced in virulence when compared to the dsRNA-free virulent control. The colony morphology of the mutant was the same as the virulent-type, orange pigmentation and copious conidia production. They extracted and sequenced a small (2.7 kb) dsRNA element associated with purified mitochondria from the hypovirulent strain. Sequence analysis revealed that long open reading frames were not present unless mitochondrial translation (UGA codes for tryptophan instead of stop) was invoked. The dsRNA was transferable via hyphal anastomosis and capable of being passed on to ascospore progeny only when the mutant was used as the female in sexual crosses (Polashock et al., 1997).

Association of dsRNA with mitochondria in *O. ulmi* was the first report of its kind in a fungal system. It created hope for discovering novel approaches for biological control of fungal pathogens. Around the same time, certain hypovirulent strains of *C. parasitica* were also being studied, and mitochondrial dysfunction associated with debilitation and hypovirulence was being demonstrated.

Hypovirulence associated with mitochondria

Some hypovirulent strains of *C. parasitica* were found to be free of dsRNA hypoviruses (Fulbright, 1985). These isolates had increased levels of alternative oxidase activity, indicative of mitochondrial dysfunction (Mahanti et al., 1993). Hypovirulence was transferable via hyphal anastomosis, maternally inherited in sexual crosses, and the dysfunctional mitochondria appeared to be just as infectious as the dsRNA hypoviruses of other strains (Fulbright 1985). These factors, along with the fact that there were no detectable levels of dsRNA, suggested that mitochondrial mutations, were involved (Mahanti et al., 1993).

Alternative pathway of respiration

Most fungi, along with plants, contain two pathways for electron transport in the mitochondria (McIntosh 1994). The normal electron transport pathway that mitochondria utilize during respiration is the cytochrome pathway (Lambers 1990). This pathway, in the inner mitochondrial membrane, involves passing electrons from NADH dehydrogenase to ubiquinone. This is the first step that contributes to a trans-membrane potential which is utilized for the phosphorylation of ADP to ATP. Ubiquinone passes electrons to cytochrome *bc₁*, which then passes them to cytochrome *c*. Cytochrome *c* is the terminal oxidase of the cytochrome pathway. Passage of electrons between cytochromes generates the remaining trans-membrane potential. The alternative pathway of respiration branches from the cytochrome pathway at the ubiquinone pool and passes electrons to a single terminal oxidase, alternative oxidase. This shunt does not contribute to the trans-membrane potential and therefore fewer molecules of ATP are produced.

The alternative pathway of respiration was first hypothesized by Tissieres et al. (1953) in order to explain the abnormal respiratory phenotype of the *poky* mutant of *Neurospora crassa*. This mutant was lacking certain cytochromes, that the wild type contained, necessary for electron transfer along the cytochrome pathway. The wild type *N. crassa* respiration was cyanide-sensitive and salicylhydroxamic acid (SHAM)-resistant while the *poky* mutant's respiration was shown to be cyanide-resistant and SHAM-sensitive (Lambowitz and Slayman, 1971).

Disruptions in the cytochrome pathway leading to the induction of the alternative pathway of respiration is not only due to genetic mutations, but can also be induced in wild-type strains by respiratory inhibitors such as cyanide (Lambowitz and Slayman, 1971) or antimycin A (Roberts et al., 1980) which block electron transport along the cytochrome pathway. Other chemicals such as chloramphenicol, which block mitochondrial protein synthesis (Küntzel, 1969), can also lead to respiration via the alternative pathway. Chemicals such as SHAM block the alternative pathway of respiration (Schonbaum et al., 1971). Therefore, wild type respiration via the cytochrome pathway is characterized as cyanide-sensitive and SHAM-resistant, while alternative oxidase activity is characterized as respiration that is both cyanide-resistant and SHAM-sensitive (Lambers 1990; McIntoch 1994).

Alternative oxidase, the nuclearly encoded protein that acts as the terminal oxidase in the alternative pathway of respiration, was recognized in the *poky* mutant of *N. crassa* by probing mitochondrial protein preparations with monoclonal antibodies raised to the alternative oxidase of the plant, *Sauromatum guttatum* (Lambowitz et al., 1989).

This experiment also indicated that the alternative oxidase protein is highly conserved between plant and fungal species.

The role that alternative oxidase plays in plants and fungi is not completely clear. The alternative pathway of respiration is known to be used for heat production and to volatilize chemicals in order to attract pollinators in the Araceae family of plants (Meeuse 1975). The alternative pathway has also been hypothesized to function as an electron overflow that can be utilized to maintain respiratory activity when there is an excess of carbohydrates (Lambers 1982). Vanlerberghe et al. (1997) were able to grow transgenic tobacco cells that only respired via the alternative pathway, demonstrating that growth can be supported by alternative oxidase activity alone. They also obtained genetic evidence that alternative oxidase may regulate imbalances between carbon metabolism and electron transport (Vanlerberghe et al., 1997). The alternative pathway of respiration may additionally play a role in allowing pathogens to bypass plant defense mechanisms or to act as a defensive mechanism against competitive organisms which produce respiratory inhibitors (Lambowitz et al., 1989).

Dysfunctional mitochondria and senescence

Another phenotype associated with dysfunctional mitochondria in fungi is termed senescence. Wild-type strains of *Neurospora* are capable of growing indefinitely. But some naturally occurring strains of *Neurospora intermedia* from Hawaii, after a period of normal growth, initiate a period of slow growth and eventually stop growing all together. This growth pattern is known as senescence (Griffiths, 1992). Senescence in *Neurospora* is a cytoplasmic trait caused by mitochondrial-DNA mutations. Senescence was inherited in a strictly maternal fashion, even though in some cases the senescent cultures were

female sterile (Griffiths and Bertrand, 1984). Molecular studies found that these strains had abnormal cytochrome spectra and there was an integration of novel DNA into the mitochondrial chromosome (Bertrand et al., 1985; Bertrand and Griffiths, 1989).

Mitochondria with DNA mutations are able to accumulate and replace wild type mitochondria through a process called suppressiveness. Mitochondria that have genetic defects can proliferate at a higher rate than the non-defective, wild-type mitochondria (Bertrand, 1995). The suppressiveness phenomenon was also observed in the non-senescent *poky* mutant. Suppressiveness is also linked to senescence in that, as mutant mitochondria increase, senescence is the observed result. Suppressiveness also allows mutant mitochondria to be infectious when cytoplasmic mixing occurs during hyphal anastomosis between wild type and mutant strains. The mitochondrial mutation leading to the *poky* phenotype is non-lethal but the mutation leading to senescence in the Hawaiian strain is lethal; in other words, it is the accumulation of lethal mutations that leads to senescence while the accumulation of non-lethal respiratory mutations does not.

Induction of mitochondrial hypovirulence

Both the mitochondrial and the nuclear genomes code for proteins in the electron transport chain. Alterations, due to mutations, in either genome may block electron transport along the cytochrome pathway requiring electrons to be shunted via the alternative pathway. Using ethidium bromide and UV-light, Monteiro-Vitorello et al. (1995) induced mutations in a virulent strain of *C. parasitica*. Slow growing mutant colonies were screened for mitochondrial dysfunction by measuring for elevated levels of alternative oxidase activity. Several putative slow-growing mutants were recovered and the cytoplasmic origin was established in two isolates by cytoplasmically transferring the

respiratory defect via hyphal anastomosis. The respiratory phenotype was also maternally inherited when sexual crosses were performed. These experiments demonstrated that mutating mitochondrial-DNA could induce hypovirulence in *C. parasitica*, thereby mimicking naturally occurring cases of hypovirulence.

The selection of mitochondrial mutants of *C. parasitica* in the laboratory raised several questions. Can hypovirulence due to mitochondrial dysfunction be selected in other fungal systems where hypovirulence does not exist? If so, can we mimic this naturally occurring system in the laboratory? Can such mitochondrial mutants be utilized as agents of biological control? In an effort to answer these questions, attempts were made to select for hypovirulence due to mitochondrial dysfunction in other fungal pathogens.

Fusarium oxysporum f. sp. *basilicum*, and *Colletotrichum coccodes* were subjected to ethidium bromide and UV-light, and respiratory mutants with increased levels of alternative oxidase activity were identified (Catal, 1996). Mutants that were recovered in both fungal species were reduced in their virulence states. Unfortunately, the cytoplasmic or nuclear origin of the mutations responsible for the mutant phenotypes (increased alternative oxidase activity, reduction in virulence) in both fungal species could not be determined, conclusively.

F. oxysporum f. sp. *basilicum* lacks a sexual system and transfer of the mutant phenotype was not detected after hyphal fusion with a genetically marked virulent strain. Therefore, heterokaryon tests were performed to determine if the mutant phenotype would segregate from the nuclear marker. A hypovirulent mutant of *F. oxysporum* f. sp. *basilicum* (Pm33) with increased levels of constitutive alternative oxidase activity, and a

nitrate non-utilizing mutation (*nit3*) in its nuclear background was paired with a wild-type strain that had a complementary nit mutation (*nitM*) in its nuclear background.

Heterokaryons were formed and then screened for the *nitM* nuclear marker. Isolates with the *nitM* nuclear marker were screened for their respiratory phenotype and several were recovered with increased levels of alternative oxidase activity. Interestingly, the levels of alternative oxidase activity were lower than in the original mutant, but higher than wild type levels (Catal, 1996).

C. coccodes also lacks a sexual system, so maternal inheritance of the mutant phenotypes could not be tested in this species. Cytoplasmic transfer of the mutant phenotypes in forced heterokaryon tests was unsuccessful. This indicated that the mutation was either nuclear or mitochondrial transfer does not occur during hyphal contact in *C. coccodes* (Catal, 1996).

Since the mitochondrial origin of the mutations in these species could not be adequately established, we wanted to see if it was possible to induce mitochondrial mutations in a fungal system that was similar to the systems in which mitochondrial hypovirulence occurs naturally. The fungus chosen for this was *Ceratocystis fagacearum* (Bretz) Hunt.

History and description of *Ceratocystis fagacearum*

A report published by the Wisconsin Agriculture Experiment Station in 1942 first described symptoms of oak wilt in dying oak trees (Anonymous 1942). The wilt symptoms in the dying oak trees were caused by a fungal pathogen (Henry et al. 1944). Trees infected with the oak wilt fungus exhibited leaf drying in the crown, including leaf curling, chlorosis, browning and necrosis of foliage, followed by premature defoliation.

The asexual stage of the causal agent of oak wilt was identified as *Chalara quercina* (Henry 1944). Based on petri dish cultures, it was noted that the mycelium becomes gray to olive green with occasional patches of tan as it ages. The hyphae were described as subhyaline to brown in color. Morphologically, the hyphae were septate, branched and 2- 6 μm in diameter. The conidiophores were similar to the sterile hyphae in morphology, except that they were slightly tapered at the tip. The conidia were described as variable in size, 2- 4.5 x 4-22 μm with a mean size of 3 x 6.5 μm . The single-celled, hyaline conidia are enteroblastic-phalidic, cylindrical, and truncated at both ends (Hunt, 1956; Upadhyay, 1981).

The discovery of the sexual stage in *C. fagacearum* was first reported in 1951 (Bretz, 1951). Isolates fell into two sexual groups (Hepting et al., 1952) now known as compatibility types A and B. The fungus is heterothallic (Bretz, 1952) and conidia act as spermatia when they come in contact with a thallus of the opposite mating type, resulting in perithecium formation (Wilson 1956). Perithecia are flask shaped and have a black, globose base (235-380 μm diameter) with a neck that can be up to 500 μm long (Hunt, 1956; Upadhyay 1981). The slightly curved, hyaline ascospores, which make up the white exudate that oozes from the tip of the neck, have average dimensions of 6 - 10 x 1.5 – 3 μm (Wilson 1956).

The pathogen could infect other types of trees including the genera: *Acer*, *Betula*, *Carya*, *Celtis*, *Fagus*, *Fraxinus*, *Gleditsia*, *Rhus* and *Ulmus* (Fenn et al.1975). It is mainly pathogenic to the genera *Castanea*, and *Quercus* within the family Fagaceae (Bretz, 1952). Disease most quickly develops in species within the genus *Quercus* and most specifically on *Quercus rubra* and *Q. borealis*.

Because this fungus is a member of the endoconidial group, its perfect stage had been identified (Bretz, 1951), and since the fungus was pathogenic on other members of the family Fagaceae besides oak, Bretz (1952) proposed the name *Endoconidiophora fagacearum*. Based on the *Chalara* anamorph, Hunt (1956) reclassified the fungus to the genus *Ceratocystis* where it has remained to this date.

The genus *Ceratocystis* was considered synonymous with *Ophiostoma* from the 1950s until the 1990s (Hunt, 1956; Upadhyay, 1981; Samuels 1993). Upadhyay is still of the opinion that the distinctions are not clear enough to separate the two genera and that the best level of classification that can be made is that all fungi that fall within this controversial group should be classified within the family Ophiostomataceae (Upadhyay, 1993). However, not every one agrees. Based on the anamorph *Chalara* and its morphological similarities with other species within the family Lasiosphaeriaceae, and the conidiogenic properties of *Sporothrix* and *Leptographium* anamorphs, Samuels (1993) suggests a separation of *Ceratocystis* and *Ophiostoma* and believes the two should be classified into two different families. For the time being it appears that the scientific community recognizes the similarities and differences between these fungal types and agrees to disagree on their formal classification until more information can be obtained.

Disease cycle of *Ceratocystis fagacearum*

Red oak trees can become infected with *C. fagacearum* by transmission of the fungus from an infected tree to an uninfected tree via root grafts. Root grafts form between compatible oak trees when their roots come into contact. This is the means of short-term dispersal of this pathogen. Long-term dispersal can be accomplished by insect vectors such as nitidulid beetles (Jewell, 1956; MacDonald and Hindal 1981). These

beetles are attracted to the fruity aroma produced by mycelial mats that grow between the cambium and the bark. The mats contain asexual conidia and sexual ascospores generated in perithecia. The relationship between *C. fagacearum* and nitidulid beetles has been described as a symbiotic relationship (Jewell, 1956). As the beetles crawl across the mats consuming the mycelia as a food source, spores adhere to their bodies. Viable conidia have also been obtained from beetle fecal material. These beetles can enter fresh wounds on healthy oak trees, thereby vectoring the fungus to a new host. They can also transfer conidia to mats of mycelia of the opposite mating type thereby spermatizing them, leading to perithecia formation. There are some areas of the country where mat production is rare, but long-term dispersal still occurs. Other insects, such as oak bark beetles that live and breed within oak wood, are thought to vector the pathogen in these areas (MacDonald and Hindal 1981). Infection is thought to occur from mid-May to early July because that is when the fungus produces the greatest amount of inoculum and beetle activity is highest (Stambaugh et al., 1955). Trees are susceptible year round and can develop symptoms shortly after infection (Stambaugh and Nelson, 1956; Skelly and Merrill, 1968). Even though insects are capable of spreading inoculum, dissemination is inefficient and very little is known about how the pathogen actually spreads to new infection centers. The role that insects play in spreading oak wilt in nature is unknown because experiments on beetle transmission have involved artificial inoculation of the insects (Stambaugh et al., 1955; Jewell, 1956). In fact, the vector-pathogen relationship may be quite inefficient, which may be beneficial to the oak tree population in North America (Merrill and French, 1995). But given the close association that the beetles have with the fungus it seems reasonable that they can play some role in dispersal.

Once *C. fagacearum* has been introduced into a susceptible oak, it spreads throughout the current year's xylem by producing hyphae that grow both inter- and intracellularly (Sachs et al., 1970). It also spreads by producing conidia that are dispersed by the xylem. By the time symptom expression is observed, hyphal fragments and conidia have been distributed throughout the tree from the roots to the crown. Fungal particles, along with tyloses and gum produced by the host, block the xylem vessels resulting in xylem dysfunction (Struckmeyer et al. 1954), leading to wilt symptoms. *In vitro*, *C. fagacearum* is thought to produce toxins responsible for wilting and browning of leaves (McWain and Gregory, 1972; White, 1955). Initial wilt symptoms on mature trees develop within six weeks after infection and death can follow shortly, usually within the same season as infection. Fungal mats can develop under the bark in such large quantities that the bark splits open and cankers are formed. *C. fagacearum* can colonize inner growth rings after the tree has died but it does not compete well with other saprophytic fungi that colonize the tree after death. The wood underneath the bark can be stained dark blue by *C. fagacearum* thereby making the timber unmarketable.

Control of oak wilt

Unfortunately, very little is known about how to control the incidence of oak wilt. The techniques utilized to stop short-term dispersal via root grafts, in a stand of oak trees, involves breaking the root grafts by trenching around infected trees. Physical and chemical means have been utilized to destroy roots that are grafted together. Since mature oak trees can have extensive root systems, trenching to break root grafts can be a monumental task that is not always successful. Chemical treatment of roots can also be hazardous to other trees present that are not susceptible to oak wilt. Injecting fungicides

directly into the vascular system has controlled symptom expression, but this approach is better as a prophylactic measure than as a cure (Appel, 1995). Other protocols involving the destruction of oaks within a fifty-foot radius of infected trees had unacceptable levels of tree loss (MacDonald and Hindal 1981). Stopping long-term dispersal by controlling populations of vectoring beetles is impractical and not likely to be effective since little is known about the role insects play in the formation of new infection centers.

Attempts have been made at biologically controlling oak wilt. Fungal mats of *C. fagacearum* were augmented with *Ophiostoma quercus*, a saprophytic colonizer of oak (Juzwik et al., 1998). It was hoped that the frequency of *C. fagacearum* propagule acquisition by nitidulid beetles would be reduced if there was competition with other inoculum. Unfortunately, there was no reduction in the amount of *C. fagacearum* conidia obtained from nitidulid beetles exposed to these mats.

Since biological control has been studied in other forest pathogens and inhibition of *O. ulmi* has been indicated by *in vitro* studies using *Pseudomonas* spp., the evaluation of endophytic bacteria as a biological control of oak wilt has been investigated (Gonzalez et al., 1995). A direct quote from the conclusion of this paper (Gonzalez et al., 1995) best describes the current conditions of biological control in this field; “Currently there exists no biocontrol agent that consistently controls a vascular pathogen of trees.”

Therefore, in order to investigate the potential of mitochondrial DNA mutations in controlling the virulence of fungal pathogens, experiments were performed to extend the use of mitochondrial hypovirulence to another fungal species, *C. fagacearum*. The goals of the research were to recover a hypovirulent mutant, establish the mitochondrial origin

of the mutation responsible for the hypovirulent phenotype, and to determine if the mutant is capable of acting as a biological control agent.

CHAPTER 2
IDENTIFICATION AND CHARACTERIZATION OF PM447

INTRODUCTION

Hypovirulent strains of *Cryphonectria parasitica* (Grente, 1965) and *Ophiostoma ulmi* (Brasier, 1983) have been found to occur in nature. In both fungal types, hypovirulence has been attributed to cytoplasmic factors. In *C. parasitica* the cytoplasmic factors are dsRNA hypoviruses (Day et al. 1977; Hillman et al., 1995) or dysfunctional mitochondria (Mahanti et al., 1993). In *O. ulmi*, cytoplasmic factors such as dsRNA that copurify with the mitochondria (Rogers et al., 1987) and DNA plasmids derived from mitochondrial DNA (Abu-Amero et al., 1995) have been identified .

Hypovirulence due to mitochondrial dysfunction is detected by assaying strains for increased levels of alternative oxidase activity. In this way, hypovirulence in *C. parasitica* and *O. ulmi* is similar to senescence in *Neurospora* (Griffiths, 1992). Hypovirulence and senescence are both associated with increased levels of constitutively expressed alternative oxidase. Alternative oxidase activity is identified through cyanide-resistant and SHAM-sensitive respiration.

The isolation of *C. parasitica* strains with dysfunctional mitochondria, as evidenced by increased levels of alternative oxidase activity, from blighted but recovering chestnut trees has led to the idea that hypovirulence may be able to be selected for in the laboratory by targeting the mitochondria for mutations. Monteiro- Vitorello et al. (1995) were able to induce mutations in laboratory strains of *C. parasitica* by using ethidium bromide and UV-light. Some of the mutant isolates had increased levels of alternative oxidase activity and were hypovirulent. The cytoplasmic origin of some of

these mutations was determined by establishing maternal inheritance and showing that the phenotype could be transferred through hyphal anastomosis. The ability to generate hypovirulence in laboratory strains of *C. parasitica*, similar to the type observed in naturally occurring isolates, provides the impetus for the idea that hypovirulence could be induced in other fungal pathogens.

Catal (1996) attempted to induce hypovirulence in *Fusarium oxysporum* f. sp. *basilicum* and *Colletotrichum coccodes*. After mutagenizing these fungal species with ethidium bromide and UV-light, mutant isolates with increased levels of alternative oxidase activity, corresponding with hypovirulence, were recovered. The mitochondrial nature of these mutations was not conclusively shown. Both fungal systems lack a sexual system so Mendalian versus non-Mendalian inheritance could not be determined. Transfer of the mutant phenotype via hyphal anastomosis was not evident in *C. coccodes*. In *F. oxysporum* f. sp. *basilicum*, heterokaryons were formed that had increased levels of alternative oxidase activity, but the alternative oxidase activity was intermediate between wild type and mutant levels.

Since the mitochondrial origin of the mutations could not be established conclusively in *F. oxysporum* f. sp. *basilicum* and *C. coccodes*, we wanted to see if it was possible to induce mitochondrial mutations in a fungal system that was more amenable to laboratory manipulations. It was important to select a fungus that had an easily inducible sexual system and a relatively simple virulence assay. It was also thought that, in order to mimic naturally occurring systems in the laboratory, we may have to use fungi related to isolates in which hypovirulence has been previously described. A fungal species that meets these requirements is *Ceratocystis fagacearum* (Bretz) Hunt.

C. fagacearum, the oak wilt fungus, is related to *O. ulmi*, the pathogen responsible for Dutch elm disease (Upadhyay, 1993). *C. fagacearum* has an easily inducible sexual system. Perithecia are formed within one week of spermatizing thalli of the opposite mating type. The virulence assay, utilizing one-month-old red oak seedlings, is relatively simple. Oak wilt is an important disease in both ornamental and forest oak trees. *C. fagacearum* is an extremely virulent pathogen, resulting in the death of an infected host generally within the same year as infection, with no known forms of hypovirulence or biological control.

To determine if mitochondrial DNA (mtDNA) mutations could be used to control the virulence of *C. fagacearum*, mutagenesis and selection for mitochondrial dysfunction was performed. Here I present evidence that a mtDNA mutation leads to a hypovirulent phenotype in *C. fagacearum*.

MATERIAL AND METHODS

Fungal strains and growth conditions

Wild type strains of *C. fagacearum* were kindly provided by Dr. William L. MacDonald (West Virginia University, Morgantown). These included strains with compatibility type A: Fenn, Baxter 9, Travis TX #378; and compatibility type B: 63061, Washington, Wisconsin 2, Wisconsin 5. Fungal strains were maintained on potato dextrose agar (PDA, Difco) at room temperature. For short term storage, isolates were kept at 5°C. For long term storage, conidial suspensions of the different strains were put into soil storage as described by Smith and Onions (1994). Vials of double autoclaved soil were inoculated with a 1 ml spore suspension and incubated at room temperature for 10 days. They were then refrigerated at 5° C until needed. To retrieve isolates out of soil storage, bits of soil were tapped out of the bottle on to PDA and incubated at room temperature.

Mutagenesis

To induce benomyl-resistance, a nuclear marker, 4 mm² plugs of mycelia from the wild-type strain Fenn were put on PDA plates amended with 1 µg benomyl per milliliter. Mycelia capable of growth were transferred to fresh PDA plates containing benomyl to confirm that they were truly benomyl-resistant. Isolates that continued to grow on benomyl medium were considered benomyl-resistant.

Nitrate non-utilizing (*nit*)-mutations were induced as described by Correll et al. (1987). A basal medium was prepared as follows: 30 g sucrose; 1 g KH₂PO₄; 0.5 g MgSO₄·7H₂O; 0.5 g KCl; 20 g agar (Sigma); 0.2 ml trace element solution; 1 liter dH₂O. The trace element solution contained: 50 g citric acid; 50 g ZnSO₄·6H₂O; 10 g

$\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$; 2.5 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$; 0.5 g MnSO_4 ; 0.5 g boric acid; 0.5 g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$; 1 liter dH_2O . Minimal medium was prepared by adding 2 g NaNO_3 to the basal medium. Hyphal plugs, 4 mm² in size, were placed on chlorate medium, which was minimal medium amended with 4 mg potassium chlorate per ml and 2.55 mg aspartic acid per ml (Klittich and Leslie 1988). Fast growing sectors formed and these were transferred to fresh chlorate medium in order to insure that they were true *nit*-mutants. Sectors that continued to grow quickly were considered true *nit*-mutants and were then placed on basal medium supplemented with either 0.2 g hypoxanthine (HX) per liter, or 1 g ammonium tartrate (NH_4) per liter, or 2 g sodium nitrate per liter, or 0.5 g sodium nitrite (NaNO_2) per liter to correctly identify the nitrate-metabolism phenotypes of the strains.

Mutagenesis of conidia was performed according to a procedure modified from Monteiro-Vitorello et al. (1995) and Catal (1996). An isolate of Fenn was grown on maltose-case amino acids medium (Barnett 1953) for 10-15 days. Conidia were collected and suspended in 2 ml sterile dH_2O . The presence of conidia was verified by using a microscope. One milliliter of a spore suspension was added to 24 ml Endothia complete broth (Puhalla and Anagnostakis, 1971). Ethidium bromide was added to a final concentration of 1 μg per ml and the flask was placed on a laboratory shaker for 10 hr at 190 rpm. Five milliliter aliquots of the broth were then allocated into five different sterile Petri dishes and each plate was subsequently placed within 3-5 cm of a short wave UV-light source and exposed to the light for 0, 5, 10, 15, or 20 min. Each mutagenized spore suspension was then transferred to a sterile centrifuge tube and centrifuged in a clinical, fixed angle centrifuge for 15 min. The supernatant was discarded and the pellet was

suspended in 1 ml sterile dH₂O. The suspension was centrifuged for an additional 15 min and the supernatant discarded. The pellet was suspended in 1 ml dH₂O before being serially diluted and plated on PDA. After four days, the number of germinating conidia were counted to determine the proportion that were killed during each exposure time. Germinating conidia that were exposed to the UV-light for 20 min were individually transferred to fresh PDA, four isolates per plate. These isolates were screened for slow growth. Isolates were determined to be slow growing if their colony diameter was less than 90% of that of the non-mutagenized control after eight days. Each slow growing isolate was transferred to its own fresh PDA plate before being tested for aberrant, cyanide-resistant, respiration.

Respiration assays

Respiration assays were performed according to a procedure modified from Monteiro-Vitorello et al. (1995) and Catal (1996). Cultures were initiated by transferring 10 to 20 3-mm squares of mycelium into 150 ml flasks containing 30 ml Endothia complete broth. Flasks were placed on a laboratory shaker for one week at 190 rpm and then centrifuged in a Sorvall type spx rotor at 4962 rcf for 15 min. The supernatant was poured off and the pellet was resuspended in 10 ml Vogel's medium (Vogel, 1956). Pellet resuspended in Vogel's medium was homogenized using a Dremel high speed rotary tool. Rate of respiration was determined as rate of oxygen consumption over time. Oxygen consumption was measured by pipetting 1-2 ml of mycelium homogenized in Vogel's medium into a Yellow Springs Instruments assay chamber attached to a YSI 5300 biological oxygen monitor. The final volume of the medium in the assay chamber was adjusted to 3 ml with sterilized water. A 20% glucose solution (200 µl) was added to

insure that respiration did not halt due to lack of resources (Lambowitz and Slayman, 1971). To be sure that the solution was saturated with air before the oxygen probe was inserted into the chamber, the mixture was aerated for 10 to 20 seconds. A magnetic stirrer insured that the recorded oxygen levels represented the entire suspension and not just oxygen levels close to the probe. To inhibit respiration through the cytochrome pathway, 25 μ l of 8 mg potassium cyanide (KCN, Aldrich) per ml dH₂O was added. Twenty five microliters of Salicylhydroxamic acid (SHAM, Aldrich) solution (SHAM 80 mg per ml 95% ethanol) was added to inhibit respiration that may occur via the alternative pathway. The amount of alternative respiration activity was calculated as follows: % alternative oxidase = (% KCN/slope 100%) - (% SHAM/slope 100%). Slope 100% represents the initial rate of oxygen consumption. The percentage of respiratory activity remaining after the addition of KCN equals % KCN. The percentage of respiratory activity remaining when SHAM was added after KCN equals % SHAM.

Sexual transmission

Strains of the opposite mating types of Fenn were grown on maltose/casamino acid medium for 10 days (Barnett 1953). Since the conidia also act as spermatia, conidia from the strain that was used as the male were collected and suspended in 1 ml dH₂O. The female was spermatized by pipetting 5 μ l droplets of the conidial suspension on tufts of aerial-hyphae. Perithecia typically formed around the base of the droplets and were visible after 5-7 days. Ascospores were collected from perithecia by removing one perithecium at a time from the agar plate and gently washing it in sterile water. The perithecium was then transferred to a new 50 μ l water droplet and squashed. The ascospores could then be seen as a thin, cloudy mass in the water. The ascospores were

then suspended in 1 ml dH₂O, serially diluted, and plated on PDA. After four days, single germinating ascospores were individually transferred to fresh medium. These ascospores were tested for possible segregation of nuclear markers by transferring hyphal plugs to the appropriate phenotyping media and watching the growth characteristics. They were then assayed for their respiratory phenotype.

Transmission via hyphal anastomosis

A slow-growing isolate of Fenn was plated on PDA that had a piece of cellophane on the surface. The slow-growing isolate was grown on the surface of the cellophane for 5 days before a fast-growing isolate of Fenn was put on the plate next to it. The two strains then grew for seven days and the area where the different isolates came in contact with each other was considered the zone of interaction. Mycelium was taken from both sides of the zone of interaction. These samples were then individually suspended in 1 ml sterile H₂O and then serially diluted and plated on PDA amended with benomyl (1 µg per ml). Conidia that germinated were then transferred to fresh medium and observed for their growth phenotype. Conidia from fast and slow growing isolates were serially diluted and plated out on PDA to see if they germinated asynchronously. The resulting slow-growing conidia were then tested for their respiratory phenotype.

Isolation of mitochondrial DNA

Mitochondria were purified using a flotation-gradient procedure (Lambowitz, 1979). Fungal isolates were inoculated into 10 different 125 ml flasks containing 30 ml Endothia complete broth. Fast-growing isolates were allowed to shake for 2.5 days while flasks containing slow-growing isolates were allowed to shake for 6.5 days. The time differential allowed the cultures to produce similar amounts of mycelium in all flasks.

Six, 2-liter flasks containing 1 liter of Endothia complete medium were prepared. Each 2-liter flask was inoculated with the contents of three of the 125 ml flasks and placed on a laboratory shaker for three days. The following protocol was repeated individually for both the fast and slow growing isolates. The contents of the 2-liter flasks were filtered through Whatman filter paper using a Buchner funnel hooked up to a vacuum apparatus. The samples were washed twice with cold isolation buffer (0.44 M sucrose, 10 mM TRIS-HCl pH 7.5, 5 mM EDTA pH 8.0) and the cells were peeled off the filter paper and placed in a 1-liter beaker on ice. In the cold room, the mycelium was placed in a blender. The mycelium was covered with isolation buffer, and homogenized for 1 min. The homogenate was ground in a grinding mill and centrifuged at 4° C at 4000 rpm for 10 min using a SS-34 Sorvall rotor. The supernatant was then centrifuged again at the same speed and the resulting pellet was discarded. The supernatant was centrifuged at 16,000 rpm for 40 min using a SS-34 Sorvall rotor. The pellet was saved and any remaining isolation buffer was removed. One milliliter cold 65% sucrose buffer (65% sucrose in 10 mM TRIS-HCl pH 8.0, 0.1 mM EDTA, final pH 7.6) was added to each centrifuge tube and gently homogenized with a glass rod. All wild type or mutant mitochondria were then pooled into one tube and the total volume was brought up to 27 ml with 65% sucrose buffer. Using six Beckman centrifuge tubes, a sucrose gradient of 44%, 55%, and 65% was set up. Four and a half milliliters of mitochondrial solution was over layered first with 4 ml 55% sucrose buffer (55% sucrose in 10 mM TRIS-HCl pH 8.0, 0.1 mM EDTA, final pH 7.6) and then with 4 ml 44% sucrose buffer (44% sucrose in 10 mM TRIS-HCl pH 8.0, 0.1 mM EDTA, final pH 7.6). These gradients were then centrifuged at 41,000 rpm for 105 min using a SS-34 Sorvall rotor. The band that appeared at the 55%- 44%

sucrose interface was pipetted into a fresh 50 ml polyallomer tube. Isolation buffer was added to the tube and centrifuged at 16,000 rpm at 4° C for 40 min using a SS-34 Sorvall rotor. Then the supernatant was discarded.

Mitochondrial-DNA was prepared as described by Vierula and Bertrand (1992). The pellet was dissolved in proteinase K buffer (comprised of 10 mM TRIS-HCl, 5 mM EDTA, 0.2 mM NaCl, 0.5% SDS), incubated in a 50° C water bath for 15 minutes and incubated overnight with 40 µl Proteinase K (20 mg per ml) at 37° C. Twelve hours later, 2.5 volumes of 95% ethanol were added to the tubes containing the mitochondria and the tubes were gently agitated at room temperature for 15 min. The tubes were centrifuged at room temperature for 20 min at 10,000 rpm using a SS-34 Sorvall rotor. The pellet was washed with 70% ethanol and centrifuged for 5 min. The pellet was dried in a vacuum chamber for 30 min and then dissolved in 0.5 ml TE (10 mM TRIS-HCl pH 8.0, 1 mM EDTA). An equal volume phenol was then added before gently agitating for 10 min. After agitating, the sample was centrifuged for 10 min at 10,000 rpm using a SS-34 Sorvall rotor. The upper phase was transferred to a fresh tube. Two and a half milliliters phenol and 2.5 ml chloroform: isoamyl alcohol (24:1) was added before gently agitating again for 10 min. The sample was then centrifuged for 10 min as described above and the aqueous phase was transferred to a clean tube. Five milliliters chloroform: isopropyl alcohol was added and the sample was centrifuged for 10 min. The resulting aqueous phase was divided into two new tubes and 2.5 volumes cold ethanol and 1/10 volume 3 M sodium acetate were added before centrifuging in a HB-6 Sorvall swinging bucket rotor at 12,000 rpm for 30 min at 4° C. The supernatant was discarded and the pellet was washed with cold 70% ethanol and centrifuged at 12,000 rpm for 5 min. The pellet was

then dried under a vacuum and dissolved in 400 µl of 10 mM Tris/ 1 mM EDTA/ 1 µl RNase (20 mg per ml). The DNA was further cleaned using a cetyltrimethylammonium bromide (CTAB) protocol (Ausubal et al., 1987). One hundred microliters of 5 M NaCl was added to the DNA mix, followed by 80 µl 10% CTAB in 1 M NaCl. The DNA solution was incubated at 65° C for 10 min before adding 600 µl chloroform. The mixture was then centrifuged at 12,000 rpm for 10 min in a HB-6 Sorvall swinging bucket rotor. The aqueous phase was collected and 600 µl of isopropanol was added before centrifuging again at 12,000 rpm for 10 min. The pellet was washed twice with 70% ethanol, dried under a vacuum, and resuspended in TE.

Overnight restriction digestions of the mtDNA were performed following the recommendations of the manufacture (Gibco), with the following restriction enzymes: *Bam*HI, *Eco*RI, *Eco*RV, *Hind*III, *Pst*I, *Pvu*II, *Sal*I and *Xba*I and stopped using 0.25% bromophenol blue in 40% (w/v) sucrose in H₂O (Maniatis et al., 1982). DNA was electrophoresed in a 0.8% agarose gel with 1x TBE buffer for 13 hr at 50 v.

Pathogenicity assays

To test the virulence levels of *C. fagacearum* isolates, seedlings were inoculated with conidia. Control seedlings were inoculated with sterile H₂O. Biological control assays were performed by co-inoculating seedlings with a mixture of wild-type, virulent Fenn conidia and PM447 conidia. The control consisted of seedlings inoculated with wild-type, virulent Fenn conidia alone. Challenge inoculations were done by inoculating seedlings with PM447 and then inoculating them again, either one or two weeks later with the wild-type, virulent Fenn. Control seedlings were not inoculated with PM447 but

were inoculated at the same time on either day 7 or day 14 with the wild-type, virulent Fenn.

Seeds of *Quercus borealis* were obtained from central Michigan and from the F. W. Schumacher seed company. Seeds were stratified for 90 days at 5° C and then planted individually in 16 ounce cups containing Baccto high porosity professional planting mix. Seeds were kept moist by watering every two to three days. Red oak (*Q. borealis*) seedlings were chosen for inoculation when they were 28 to 35 days post-germination (Fenn et al. 1975). Seedlings were germinated in the greenhouse (16 hr light per day, 27°C) and then transferred to a growth chamber three days prior to inoculation in order to allow the plants to adjust to the new conditions. Growth chamber conditions were maintained at a constant 27°C with the measurable light level at 70 m^E for 16 hr a day.

Conidia from fungi growing on agar plates were used to create conidial suspensions in sterile dH₂O. Conidial suspensions were checked, using a hemacytometer, for conidia density and samples were adjusted to ~2 x 10⁵ conidia/ml. A 10 µl droplet of this suspension was put on the stem of the seedling and a 27-gauge hypodermic needle was inserted through the droplet into the xylem of the seedling. Evidence of successful inoculation was obtained by observing the droplet being taken up into the xylem of the plant. Inoculated seedlings were then watered every three days, alternating between water and Hoaglands mix (Hoagland and Arnon 1950). Inoculated seedlings were checked every other day for symptom development. After initial symptoms were observed, they were checked every day until 50 days post-inoculation or the plants were dead, whichever came first.

A numerical disease scale was created in order to relate the level of symptom expression to the amount of time it took for symptoms to develop. Different levels of symptom expression were assigned a numerical rating: 0- healthy/ no disease; 1- leaf epinasty/ slight chlorosis; 2- slight leaf curling/ wrinkled leaves/ increased chlorosis; 3- half of the plant expressing disease symptoms including brown lesions/ curled, dried leaves; 4- extensive browning of leaves, along with curling and drying of the leaves; 5- dead. The amount of time for disease development was broken into ten-day periods: 1-10, 11-20, 21-30, 31-40, 41-50. Each ten-day period was assigned a multiplication factor. Symptoms expressed within 1-10 days received a x5 score for that time period. Subsequent time periods had decreasing multiplication factors so that 11-20 days multiplied the disease score by 4, 21-30 days multiplied the disease score by 3, 31-40 days multiplied the disease score by 2, and 41-50 days multiplied the disease score by 1. The disease scores for each time period were added together to create a final disease rating. If a plant died within the first ten days after inoculation, then it received a disease rating of 75. If a plant did not develop symptoms within 50 days, then it received a disease rating of 0.

To check inoculated seedlings for colonization by *C. fagacearum*, seedlings were cut into 5 cm segments. These segments were surfaced sterilized by submerging them into a solution of 10% bleach (Clorox) for five minutes. The segments were then rinsed in sterile dH₂O for one minute and placed on either acidified PDA, or PDA containing 1 mg streptomycin per ml.

RESULTS

***nit*-mutant genotype determination**

Ceratocystis fagacearum strains Fenn, Baxter 9, Travis, TX#378, 63061, Wisconsin2, and Wisconsin5 produced many fast growing sectors after four and a half weeks of growth on chlorate medium (Table 1). Mycelia from these fast growing sectors were then transferred to fresh chlorate medium to confirm their chlorate resistance phenotype. Twelve sectors were confirmed chlorate resistant and therefore *nit*-mutants. Agar plugs, 4 mm³ in size, with hyphae on one side were transferred from culture plates to fresh media containing different nitrogen sources, in order to determine where along the nitrogen metabolizing pathway the mutations had occurred. The *nit*-mutants could be classified into two *nit*-groups, *nit1/3* and *nitM* (Table 1). Since *C. fagacearum* does not use NaNO₂ as a nitrogen source, it was not possible to separate *nit1* and *nit3* mutations. In addition, all strains of *C. fagacearum* were capable of only growing thinly on NaNO₃. Chlorate resistant isolates capable of growing thick and dark on NH₄ and hypoxanthine (HX) were classified as *nit1/3*. Isolates that grew thin and light on HX and thick and dark on NH₄ were classified as *nitM*.

Induction of benomyl-resistance

Over 320 hyphal plugs from wild-type Fenn were transferred to PDA amended with 1 µg benomyl per ml. Of those, only one was able to grow after two weeks (Figure 1). This putative benomyl-resistant isolate was then transferred to fresh benomyl containing medium in order to confirm that it was truly benomyl-resistant. It was capable of continuing to grow on benomyl media so it was considered to be benomyl-resistant.

Table 1. Relative growth rates of strains of *Ceratomyxys fagacearum* after one week on different media. – is no growth, + is very little growth, ++ is little growth, +++ is medium growth, ++++ is lots of growth, +++++ is very much growth. N.D. = not determined.

<u>STRAIN</u>	<u>PDA</u>	<u>MEDIA TYPE</u>				<u>HX</u>	<u>genotype</u>
		<u>Chlorate</u>	<u>NaNO₃</u>	<u>NaNO₂</u>	<u>NH₄</u>		
63061 (wt)	+++	-	+++ (thin)	-	+++	++	wild type
Baxter9 (wt)	++++	-	+++ (thin)	-	+++	++	wild type
Travis, TX #378 (wt)	++++	-	+++ (thin)	-	+++	++	wild type
Washington (wt)	++++	-	+++ (thin)	-	+++	++	wild type
Wisconsin 2 (wt)	++++	-	+++ (thin)	-	+++	++	wild type
Wisconsin 5 (wt)	++++	-	+++ (thin)	-	+++	++	wild type
Fenn (wt)	+++++	++	+++ (thin)	-	++++	+++	wild type
Fenn.1	+++++	++++	+++ (thin)	-	++++	+++	nit 1/3
Fenn.5	+++++	++++	+++ (thin)	N.D.	++++	+++	nit 1/3
Travis, TX #378 .1	++++	+++	+++ (thin)	-	+++	+++	nit 1/3
Travis, TX #378 .8	++++	+++	+++ (thin)	-	+++	+++	nit 1/3
Travis, TX #378 .9	++++	+++	+++ (thin)	-	+++	+++	nit 1/3
Travis, TX #378 .10	++++	+++	+++ (thin)	-	+++	+++	nit 1/3
Wisconsin 2.1	++++	+++	+++ (thin)	-	+++	+++	nit 1/3
Wisconsin 5.1	++++	+++	+++ (thin)	-	+++	+++	nit 1/3
63061.1	+++	+++	+++ (thin)	-	+++	+++	nit 1/3
Baxter9 .1	++++	+++	+++ (thin)	-	+++	++ (thin)	nit M
Baxter9 .2	++++	+++	+++ (thin)	-	++	++ (thin)	nit M
Baxter9 .3	++++	+++	+++ (thin)	-	+++	++ (thin)	nit M
PM447	+++	++	+	N.D.	+++	+	nit 1/3

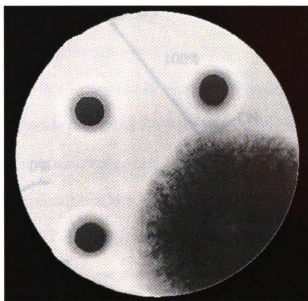


Figure 1. Growth of *Ceratocystis fagacearum* on potato dextrose agar amended with 1 μ g benomyl per ml. A fast-growing, benomyl-resistant, virulent strain Fenn (lower right) with three benomyl-sensitive strains.

Induction of cyanide-resistant respiration and alternative oxidase

Before an attempt was made to genetically induce increased, constitutively expressed, levels of the alternative pathway of respiration, it was necessary to determine if *C. fagacearum* was capable of expressing the pathway at all. The ability for *C. fagacearum* to express the alternative pathway of respiration was determined by growing *C. fagacearum* in the presence of antimycin A, a known chemical inhibitor of the cytochrome pathway. When *C. fagacearum* wild-type strain Fenn was grown in the absence of antimycin A, its respiration was completely CN sensitive and SHAM resistant (Figure 2a), indicating that all of the respiration occurred via the cytochrome pathway. After wild-type strain Fenn was grown in the presence of 3 μ g antimycin A per ml for four days its respiration was mostly CN resistant and somewhat SHAM sensitive

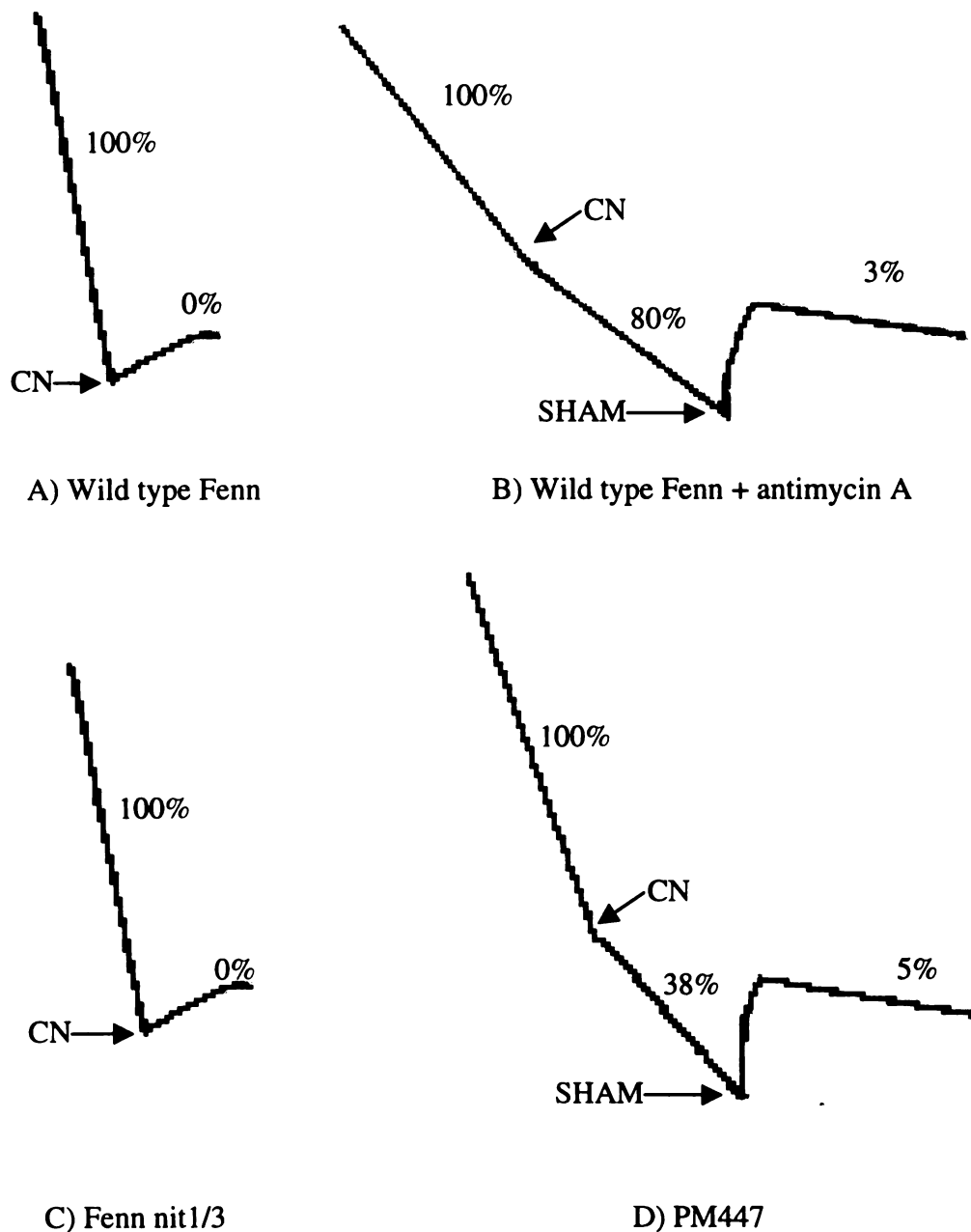


Figure 2. Respiration rates of *Ceratocystis fagacearum* A) wild-type Fenn, B) wild-type Fenn + 3 μ g antimycin A per ml, C) Fenn *nit 1/3*, D) PM447. Additions of cyanide (CN) and salicylhydroxamic acid (SHAM) are indicated by arrows. Numbers above each tracing represent relative respiratory activity measured as oxygen consumption over time.

(Figure 2b), indicating that it was capable of respiring via the alternative pathway of respiration. The *nit* nuclear marker had very little effect on the isolate's ability to respire via the cytochrome or alternative pathway (Figure 2c).

Mitochondrial DNA was targeted for mutations by treating conidia from Fenn *nit1/3* with ethidium bromide (EtBr) and UV-light. The conidia were incubated in 2 µg EtBr /ml for 10 hours and then subsequently exposed to UV-light for 0, 5, 10, 15, or 20 min respectively. The highest proportion of conidia that were killed, 99.9%, was achieved with 20 min exposure to UV-light (Figure 3). Conidia that had been exposed to UV-light for 20 min were diluted to 5×10^{-3} and plated on PDA so that they could germinate.

Isolation of respiratory mutants

Over 5000 individual mutated spores were transferred to fresh PDA. On average, control conidia obtained from Fenn *nit1/3*, not exposed to UV-light, grew 27 mm in diameter within 6 days. Mutagenized conidia that grew less than 24mm after 6 days were considered putative slow-growing isolates. Of the mutagenized conidia transferred, 521 of them grew less than 24 mm after 6 days and were transferred to fresh medium for confirmation of the slow-growing phenotype. Two hundred and forty eight conidial isolates continued to grow slowly and were therefore tested for cyanide-resistant respiration. One of them, PM447, had increased levels of constitutively expressed, cyanide-resistant and SHAM-sensitive respiration, indicating alternative oxidase activity (Figure 2d).

Conidial germination analysis

Conidia were serially diluted and plated on PDA to examine their germination phenotype. Conidia from wild-type Fenn and Fenn *nit1/3* germinated synchronously

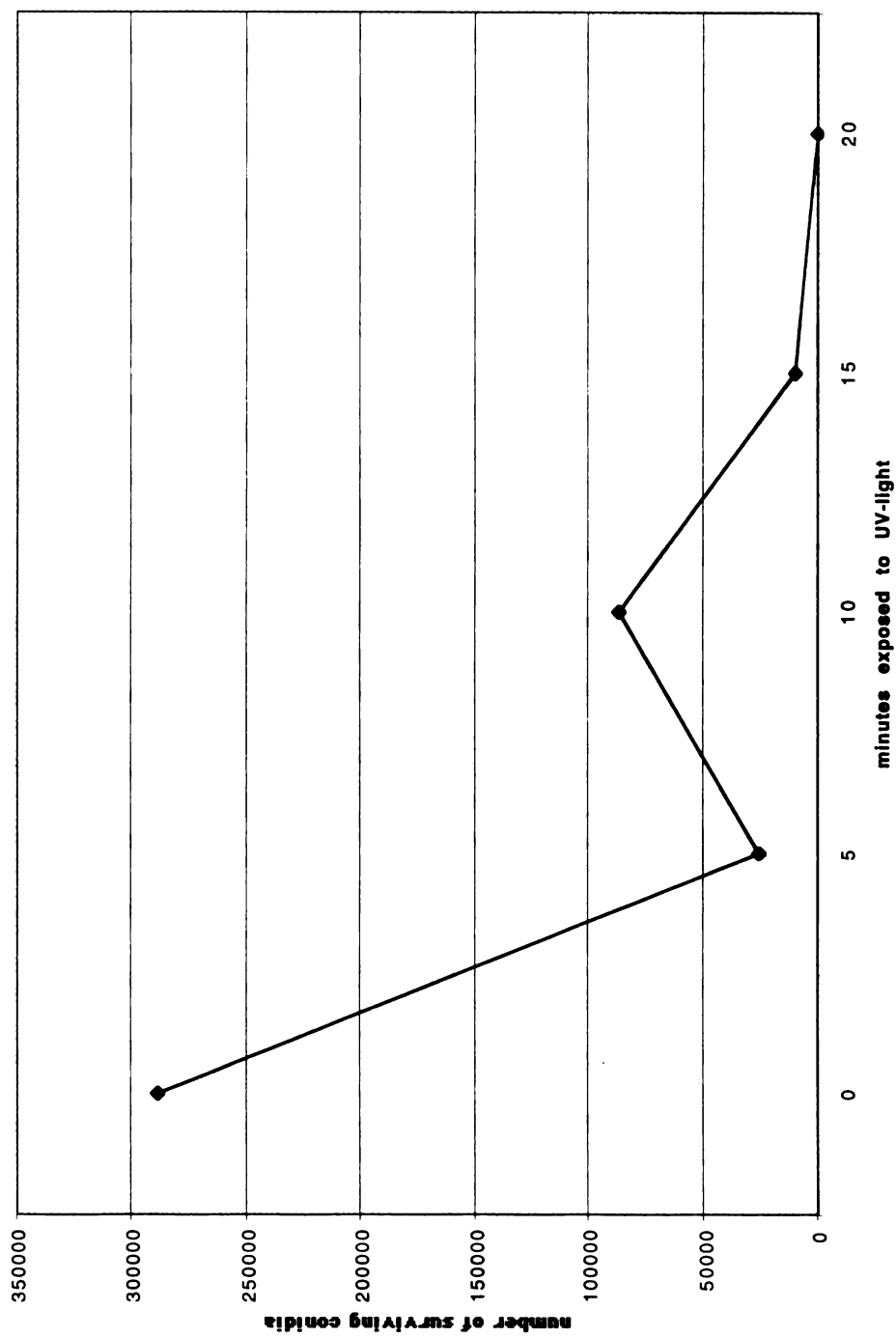


Figure 3. The number of surviving *Ceratocystis fagacearum* conidia after various times of exposure to ultra-violet light.

(Figure 4a, 4b) while conidia from PM447 germinated asynchronously (Figure 4c). Conidial isolates that germinated earlier appeared larger eight days after plating than conidia that germinated later. These larger isolates produced conidia that germinated asynchronously. Late germinating, therefore smaller after eight days, conidial isolates obtained from PM447 also produced conidia that germinated asynchronously. The asynchronously germinating conidia produced by the large and small isolates obtained from PM447 produced conidia that germinated asynchronously. All of the asynchronously growing conidia obtained from PM447 had increased levels of alternative oxidase as compared to the wild type (Table 2).

Sexual transmission

PM447, which contained the *nit1/3* nuclear marker and putative cytoplasmic mutation, was crossed with a wild type strain of the opposite mating type, 63061. Spermatizing 63061 thalli with conidia from PM447 produced dozens of perithecia. Ascospores were collected from different perithecia and 79 of the ascospores were tested for their respiratory phenotype. Three out of seventy-nine ascospores tested expressed CN-resistant respiration that was higher than the wild-type controls but lower than PM447 levels recorded at that time (Table 3). Sixty of the ascospores assayed for CN-resistance, regardless of CN-resistance, were grown on 4% chlorate medium to determine the segregation of the *nit* nuclear marker. The ascospores segregated 27:33, growth: no growth, respectively, on 4% chlorate medium ($\chi^2 = 0.6$). Five separate attempts at the reciprocal cross resulted in no perithecia being formed.

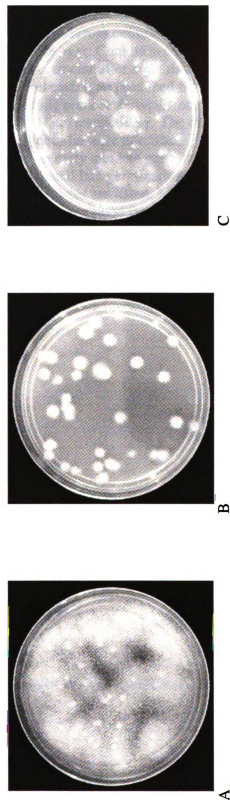


Figure 4. Conidia from *Ceratocystis fagacearum* 8 days after germination on potato dextrose agar. A) Synchronous growth pattern of wild-type Fenn conidia, B) Synchronous growth pattern of Fenn *nit 1/3* conidia, C) Asynchronous growth pattern of PM447 conidia.

Table 2. Percentages of alternative oxidase activity obtained from early and late germinating conidia derived from cultures of *Ceratocystis fagacearum* strain PM447 grown on potato dextrose agar. Conidia produced by PM447 germinated early and late as determined by growth diameter after eight days. These early and late germinating conidia were cultured again and each type produced both early and late germinating conidia. The early and late germinating conidia derived from early germinating conidia were described as early, early or early, late, respectively. The early and late germinating conidia derived from late germinating conidia were described as late, early or late, late, respectively. These second generation early and late germinating conidia subsequently produced a third generation of early and late germinating conidia. Percentages of alternative oxidase activity obtained from isolates are listed. Multiple numbers represents the percentages of alternative oxidase activity obtained from multiple isolates with the same germination phenotype. N.A. = not available.

Time for germination	Alternative oxidase (%)
Early	19, 24
Late	21, 28
Early, early	39
Early, late	27
Late, early	47
Late, late	24
Early, early, early	29, 26, 22, 20
Early, early, late	27, 23, 22, 19
Early, late, early	27, 27, 24, 20
Early, late, late	25, 17, 15, 4
Late, early, early	30, 22, 21, 17
Late, early, late	23, 18, 16, 16
Late, late, early	N.A.
Late, late, late	13, 13, 8, 8

Table 3. Levels of cyanide-resistant respiration expressed by ascospores obtained by crossing *Ceratocystis fagacearum* strain 63061 with PM447. Ascospores numbered 1-79, not listed, expressed 0% cyanide-resistance.

Isolate	CN-resistance (%)
63061	0
PM447	17-26
Ascospore 11	8
Ascospore 40	5
Ascospore 41	7

Transmission via hyphal anastomosis

The slow-growing, benomyl-sensitive PM447 was paired with the fast growing, benomyl-resistant Fenn on PDA with cellophane (Figure 5). The two isolates grew side-by-side forming a zone of interaction. Conidia were collected from both the mutant appearing and wild-type appearing sides of the zone of interaction. The conidia were diluted and plated out on PDA amended with 1 µg benomyl per ml. Only benomyl-resistant conidia were capable of growing on this medium. Conidia from the control, benomyl-resistant Fenn, obtained without pairing, were benomyl-resistant and fast-growing. All conidia obtained from PM447, without pairing, were benomyl-sensitive. Of the 1,977 benomyl-resistant conidia obtained from the pairing plate, 42 were also slow-growing (Table 4). Eight of these slow-growing, benomyl-resistant isolates produced conidia that germinated asynchronously.

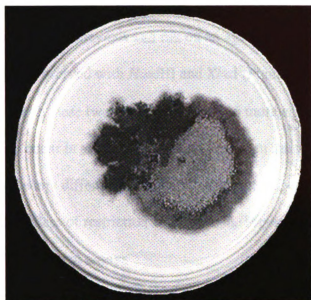


Figure 5. A pairing plate seven days after PM447 (left) was paired with wild-type, virulent, benomyl-resistant Fenn (right).

Table 4. Number of conidia with different growth phenotypes obtained from plates where fungal strains were paired together. Ben^r = benomyl-resistant.

Conidial Origin	Ben ^r Fast-growth	Ben ^r Slow-growth
Fenn Ben ^r	886	0
PM447	0	0
Paired culture	1935	42

Endonuclease digestion of mitochondrial DNA

Mitochondrial DNA was isolated from both wild-type Fenn and PM447. They were cut with various restriction enzymes and run on an agarose gel. Numerous restriction fragments were observed with *HindIII* and *XbaI* (Figure 6). Restriction reactions were repeated with these two enzymes in order to more easily identify any polymorphisms that may occur in the banding patterns between the wild type and mutant. There did not appear to be any differences in the banding pattern between the wild type and mutant mtDNA, regardless of restriction enzyme used (Figure 6).

Virulence and biological control assays

Virulence assays were done by inoculating 28 to 35-day old seedlings with conidia from wild-type Fenn, Fenn *nit1/3*, and PM447. Control plants were inoculated with sterile water. A disease rating was calculated for each seedling based on symptom development over time. Four weeks after water inoculation of the control plants, seedlings were growing vigorously and were also quite green (Figure 7a). Within two weeks of inoculation with conidia from the wild-type Fenn, seedlings began to develop symptoms. Initial symptom expression included wilting of leaves, seen as a drooping of the leaves followed by curling and drying of the leaf tissue. Four weeks after inoculation, the leaves on these seedlings were completely brown, dried and curled; many of the seedlings died (Figure 7b). A fast-growing Fenn-appearing strain was re-isolated from wilted seedlings (Figure 8a). Seedlings inoculated with wild-type Fenn had a high average disease rating of 47 (Figure 9). The disease rating of seedlings inoculated with Fenn *nit1/3* was not significantly different from the disease rating of seedlings inoculated with wild-type Fenn.

1 2 3 4 5

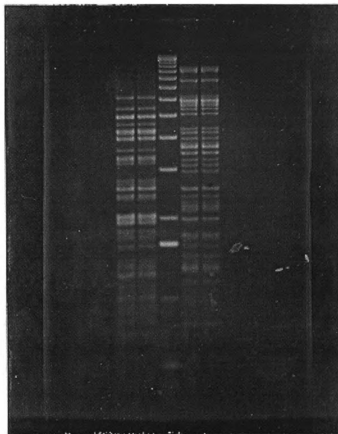


Figure 6. Endonuclease restriction digestions of mitochondrial DNA. Lane 1, wild-type Fenn digested with *Hind*III; Lane 2, PM447 digested with *Hind*III; Lane 3, 1kb marker; Lane 4, wild-type Fenn digested with *Xba*I; Lane 5, PM447 digested with *Xba*I.



Figure 7. Red oak (*Quercus borealis*) seedlings four weeks after inoculation. A) inoculated with sterile, distilled water, B) inoculated with conidia from *Ceratocystis fagacearum* wild-type, virulent Fenn, C) inoculated with conidia from PM447.



A



B

Figure 8. *Ceratocystis fagacearum* isolates recovered from stem sections of red oak (*Quercus borealis*) seedlings. A) A fast growing isolate with wild-type Fenn morphology recovered from a seedling inoculated with wild-type Fenn. B) A slow growing isolate with PM447 morphology recovered from a seedling inoculated with PM447. Isolate with PM447 morphology is older than the isolate with wild-type Fenn morphology.

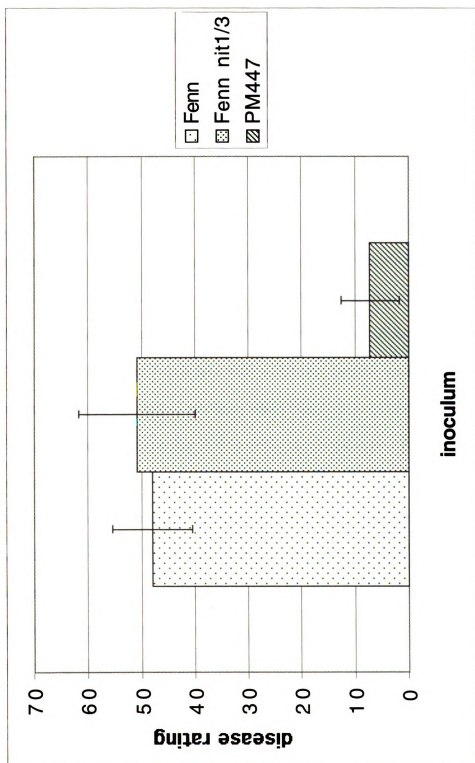


Figure 9. Disease ratings of red oak (*Quercus borealis*) seedlings four weeks after infection with either wild-type Fenn, or Fenn nit1/3, or PM447.

Two weeks after seedlings were inoculated with conidia from PM447 they started to express symptoms. These seedlings had some leaf curling, drooping of some leaves, and slight chlorosis. Four weeks after inoculation with PM447 conidia, the disease symptoms had not progressed (Figure 7c). A slow-growing, PM447-appearing, *C. fagacearum* strain was re-isolated from stem sections (Figure 8b), indicating that the fungus was still colonizing the seedlings. Seedlings inoculated with PM447 had an average disease rating of 6, which was dramatically lower than that of the wild type (Figure 9).

When conidia from wild-type, virulent Fenn and PM447 were co-inoculated into seedlings at the same time, the disease rating was not significantly different from seedlings inoculated with wild-type conidia alone (Figure 10). In both cases, seedlings were dead within four weeks (Figures 11a, 11b). Seedlings inoculated initially with PM447 and then challenged 7-days later with wild-type, virulent Fenn also exhibited the same disease rating as the control seedlings which were not inoculated with PM447, but inoculated at the same time (on day 7) with wild type (Figures 10, 12a, 12b). Seedlings that were inoculated initially with PM447 and then challenge inoculated 14-days later with wild-type, virulent Fenn, had a significant reduction in disease rating when compared to control seedlings (Figure 10). The seedlings, inoculated with PM447 and then challenged 14-days later, expressed symptoms such as brown lesions and slight curling, but symptom expression was delayed and not as severe as in the case of the control seedlings (Figures 13a, 13b).

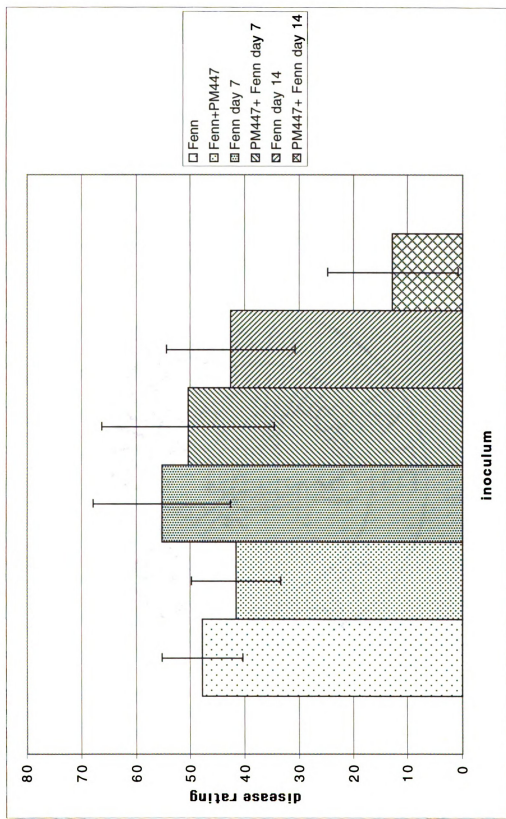


Figure 10. Disease ratings for red oak (*Quercus borealis*) seedlings inoculated with PM447 and wild-type, virulent Fenn.



A

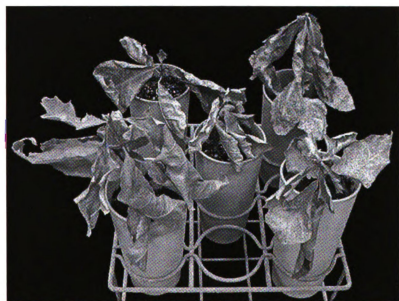


B

Figure 11. Red oak (*Quercus borealis*) seedlings 4 weeks after inoculation with *Ceratocystis fagacearum*. A) inoculation with wild-type, virulent Fenn conidia alone, B) simultaneous co-inoculation with conidia from both wild-type, virulent Fenn and PM447.



A



B

Figure 12. Red oak (*Quercus borealis*) seedlings four weeks after inoculation with *Ceratocystis fagacearum* wild-type, virulent Fenn. Seedlings were inoculated with Fenn at the same time. A) Seedlings inoculated with Fenn conidia only, B) seedlings inoculated with PM447 one week prior to inoculation with Fenn.



A



B

Figure 13. Red oak (*Quercus borealis*) seedlings four weeks after inoculation with *Ceratocystis fagacearum* wild-type, virulent Fenn. Seedlings were inoculated with Fenn at the same time. A) Seedlings inoculated with Fenn conidia only, B) seedlings inoculated with PM447 two weeks prior to inoculation with Fenn.

DISCUSSION

Hypovirulence in *Ophiostoma ulmi*, and in some cases *Cryphonectria parasitica*, has been attributed to mitochondrial dysfunction (Rogers et al., 1987; Mahanti et al., 1993; Monteiro-Vitorello et al., 1995), and may be capable of acting as a biological control. To investigate the ability to generate hypovirulence in laboratory strains of other fungal pathogens, similar to the type observed in *C. parasitica* and *O. ulmi*, a mutagenesis of *Ceratocystis fagacearum* was performed. A slow-growing mutant (PM447) with increased levels of alternative oxidase activity was recovered.

To determine if the PM447 phenotype was due to a nuclear or mitochondrial mutation, certain criteria for cytoplasmic origin were tested. This included an analysis of the conidial germination, mode of inheritance, and the ability of the mutant phenotype to be transferred via hyphal anastomosis. Since all of the conidia produced by PM447 should have the same nuclear genotype, the asynchronous germination pattern that was observed was probably due to a heterogenic cytoplasmic factor. If the timing of germination and growth rates of each conidium depended on the number of mutant mitochondria present, then conidia with a high titer of mutant mitochondria should take longer to germinate and grow the slowest. Additionally, conidia that contain mostly wild-type mitochondria should germinate and grow the fastest. Therefore, early-germinating isolates should have low levels of alternative oxidase activity and late-germinating isolates should have high levels of alternative oxidase activity. This should be true if there is a correlation between cellular populations of mutant mitochondria, germination and growth rates. However, the respiratory data does not support this idea. All conidial isolates obtained from PM447 had increased levels of alternative oxidase activity when

compared to the wild type and there was no correlation between early germination and low alternative oxidase, or late germination and high alternative oxidase (Table 2). In fact, some of the highest levels of alternative oxidase activity (39%, 47%) were observed in conidial isolates that germinated early.

Mahanti and Fulbright (1995) crossed isolates of *C. parasitica* and observed a 1:1 segregation of a nuclear marker and maternal inheritance of a mitochondrial DNA marker. When sexual crosses were performed between PM447 and a wild-type strain of the opposite mating type (63061), a non-Mendelian mode of inheritance was observed for cyanide-resistance, the putative cytoplasmic marker, while a Mendelian mode of inheritance was observed for the *nit*-mutation, a nuclear marker. When PM447 was used as the male gamete, only 4% of the ascospores tested had increased levels of cyanide-resistant respiration. In the same cross, 45% of the ascospores tested were chlorate-resistant. If the cyanide-resistant phenotype was nuclear, then a 1:1 ratio of resistant:sensitive would be expected, as was observed for the *nit*-mutation.

Unfortunately, when PM447 was used as the female gamete, no perithecia were formed. I made several attempts, utilizing a variety of growth media, and was unable to observe perithecial development suggesting female sterility. Female sterility has been previously observed in organisms with dysfunctional mitochondria. In some cases senescent isolates of *Neurospora* with mitochondrial-DNA mutations, have been shown to be female sterile (Griffiths and Bertrand, 1984).

The slow growing PM447 phenotype was capable of being transferred via hyphal anastomosis to a virulent, benomyl-resistant strain of Fenn. Benomyl-resistant isolates that germinated late and grew slowly were obtained in small numbers, suggesting that the PM447 phenotype converted the virulent strain Fenn. Mutant mitochondria must have

transferred to the benomyl-resistant strain or else there would not have been any converts recovered from the pairing plate. It's possible that the mutant mitochondria in PM447 were capable of replicating at higher rates than the wild type, as is the case in suppressive mutants of *Neurospora* (Bertrand, 1995). If the mitochondria did not replicate more quickly than the wild type it is highly unlikely that the mutation event that produced PM447 or any pairing converts would have been identified.

Based on the results of the conidial analysis, non-Mendelian mode of inheritance, and the ability for the late-germination or slow-growth phenotype to be transferred via hyphal anastomosis, I believe that the mutation responsible for the PM447 phenotype is cytoplasmic in its origin.

Restriction fragment analysis of healthy and diseased isolates of *O. novo-ulmi* revealed that a mitochondrial DNA plasmid occurred only in diseased isolates (Charter et al., 1993). Analysis of mitochondrial DNA restriction fragments from a hypovirulent mutant obtained by Monteiro-Vitorello et al. (1995) showed that certain fragments were over-expressed in the mutant mitochondrial genome when compared to the wild type. When restriction digestions of mitochondrial DNA from *C. fagacearum* wild-type and PM447 isolates were done, no polymorphisms, or over-expressed fragments were identifiable. This suggested that the mutation responsible for the PM447 phenotype is not a major deletion or re-arrangement in the mitochondrial genome and there are no accumulation-defective derivatives of mitochondrial DNA. The mutation responsible for the PM447 phenotype is probably a point mutation or a small deletion or re-arrangement not detectable by restriction analysis.

The virulence of PM447 was greatly reduced from that of the wild type. Symptoms appeared in seedlings inoculated with PM447 after two weeks, but did not

progress as quickly as in seedlings inoculated with wild type. Sometimes no symptoms were observed at all in the PM447 inoculated seedlings. Therefore, recovering the isolate with the PM447 phenotype from the inoculated, symptomless seedlings demonstrated that the fungus had colonized the plant.

In biological control assays, a reduction in symptom expression was only observed when seedlings were inoculated with PM447 and then challenged 14-days later with the wild type. When seedlings were inoculated with PM447 and then challenged 7-days later with wild type, there was no reduction in symptoms expressed. It was not known why the difference exists between the 7 and 14-day challenge inoculation. Whether or not the reduction in symptoms after inoculation 14-days later is due to a plant or fungal factor remains to be seen.

Inoculations of oak trees with mixed isolates have resulted in the survival of only one type (Barnett and Staley, 1953). Since PM447 is slower growing than wild-type Fenn, there could be a latent period necessary for PM447 to successfully establish an infection and thereby exclude the wild type.

Plants are also capable of expressing defense responses after being infected by a pathogen (Hammerschmidt and Dann, 1997). PM447 could elicit an induced resistance response that is not effective until after one week. Comparing the infection progress of PM447 to the wild type in relation to the developing time line of host responses would help identify any changes in host response to infection with PM447. Transmission of the cytoplasmic factor responsible for the PM447 phenotype, from PM447 to the wild type, could be occurring *in planta*. Given the low number of conversions obtained from plates where cultures were paired, transmission of a cytoplasmic factor is probably not responsible for the reduction in symptom expression in the 14-day challenged seedlings.

CONCLUSION

This study showed that *Ceratocystis fagacearum* could be utilized in laboratory experiments designed to investigate the role that the mitochondria play in hypovirulence. A hypovirulent mutant, PM447, was slow growing and had increased levels of alternative oxidase activity. PM447 may be capable of acting as a biological control agent. To date there are no known forms of biological control for this pathogen. Further study of PM447 may be able to help us better understand the different genetic contributions necessary for virulence and its ability to be utilized as a biological control agent. Since hypovirulent mutants, due to mitochondrial dysfunction are cytochrome deficient (Rogers et al., 1987; Monteiro-Vitorello et al., 1995), a cytochrome analysis of PM447 should be performed. A physiological and microscopic analysis of infection by PM447 may help us better understand why seedlings inoculated with this isolate do not express normal symptoms of the disease when compared to seedlings inoculated with the wild type.

It is not known why there was a reduction in symptom expression in the two-week-challenged plants and not in the one-week-challenged plants. There could be a latent period necessary for PM447 to colonize the host and thereby exclude the wild type from successfully establishing an infection. PM447 could induce host responses that are not effective until one week after inoculation. These defenses would not be able to be effective when infection occurs with a virulent strain. Developing a time line of host responses would help identify any changes in host response to infection with PM447. Pathogenicity and biological control assays need to be performed on mature trees. The virulence and biological control assays in seedling have provided evidence that PM447 is a good biological control of oak wilt in seedlings. Unfortunately, oak wilt is a problem in

mature trees, and the seedling assay may not necessarily mimic what will occur in the larger trees. Observing the long-term affects of PM447 in seedlings, and mature trees, is important if PM447 is to be used as a form of biological control. We have over-wintered seedlings inoculated with PM447 to see if they are capable of budding out after a period of dormancy, and will attempt to re-isolate *C. fagacearum* from infected seedlings that leaf out.

This study has provided evidence that laboratory induced mutations in fungal species, where no hypovirulent strains have been recovered from nature, can control the aggressiveness of a pathogen. Further studies need to be done to understand the mechanism of control by PM447. *Ceratocystis fagacearum* is a good candidate for laboratory manipulations, and may be useful in other areas of research.

REFERENCES

REFERENCES

- Abu-Amero, S. N., Charter, N. W., Buck, K. W., and Brasier, C. M. 1995. Nucleotide-sequence analysis indicates that a DNA plasmid in a diseased isolate of *Ophiostoma novo-ulmi* is derived by recombination between two long repeat sequences in the mitochondrial large subunit ribosomal RNA gene. *Current Genetics* 28:54-59.
- Anagnostakis, S. L. 1982. Biological control of chestnut blight. *Science* 215:466-471.
- Anagnostakis, S. L., Chen, B., Geletka, L. M., and Nuss, D. L. 1998. Hypovirus transmission to ascospore progeny by field-released transgenic hypovirulent strains of *Cryphonectria parasitica*. *Phytopathology* 88:598-604.
- Anagnostakis, S. L., and Day, P. R. 1979. Hypovirulence conversion in *Endothia parasitica*. *Phytopathology* 69:1226-1229.
- Anonymous. 1942. Oak wilt: a fungus disease. What's New in Farm Science. Wisconsin. Agriculture Experiment Station Bulletin 455:75-76.
- Appel, D. N. 1995. Chemical control of oak wilt. Pages 81-88 in: *Oak Wilt Perspectives: The Proceedings of the National Oak Wilt Symposium*, D. N. Appel, and R. F. Billings, eds. Information Development Inc., Houston.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Strohl, K. 1987. In: *Current Protocols in Molecular Biology*. John Wiley and Sons, New York.
- Barnett, H. L. 1953. A unisexual male culture of *Chalara quercina*. *Mycologia* 45:450-457.
- Barnett, H. L., and Staley, J. M. 1953. Isolation of compatibility types of *Endoconidiophora fagacearum* from oak wilt trees naturally and artificially inoculated. *Phytopathology* 43: 341-343.
- Bertrand, H., Argan, C. A., and Szakacs, N. A. 1983. Genetic control of the biogenesis of cyanide insensitive respiration in *Neurospora crassa*. Pages 495-507 in: *Mitochondria*. R. J. Schweyen, K. Wolf, and F. Kaudewitz eds. Walter de Gruyter and Co., New York.
- Bertrand, H., Chan, B. S-S., and Griffiths, A. J. F. 1985. Insertion of a foreign nucleotide sequence into mitochondrial DNA causes senescence in *Neurospora intermedia*. *Cell* 41:877-884.

- Bertrand, H., Collins, R. A., Stohl, L. L., Goewert, R. R., and Lambowitz, A. M. 1980. Deletion mutants of *Neurospora crassa* mitochondrial DNA and their relationship to the "start-stop" growth phenotype. *Proceedings. National Academy of Science* 77:6032-6036.
- Bertrand, H., and Griffiths, A. J. F. 1989. Linear plasmids that integrate into mitochondrial DNA in *Neurospora*. *Genome* 31:155-159.
- Bertrand, H. 1995. Senescence is coupled to induction of an oxidative phosphorylation stress response by mitochondrial DNA mutations in *Neurospora*. *Canadian Journal of Botany* 73:S198-S204.
- Biraghi, A. 1953. Possible active resistance to *Endothia parasitica* in *Castanea sativa*. Pages 643-645 in: Report of the 11th Congress of the International Union of Forest Research Organizations, Rome, Italy.
- Boland, G. J. 1992. Hypovirulence and double-stranded-RNA in *Sclerotinia sclerotiorum*. *Canadian Journal of Plant Pathology* 14:10-17.
- Brasier, C. M. 1983. A cytoplasmically transmitted disease of *Ceratocystis ulmi*. *Nature* 305:220-223.
- Bretz, T. W. 1951. A preliminary report on the perithecial stage of *Chalara quercina* Henry. *Plant Disease Reporter* 35:298-299.
- Bretz, T. W. 1952. The ascigerous stage of the oak wilt fungus. *Phytopathology* 42:435-437.
- Catal, M. 1996. Induced alternative oxidase activity and attenuation of virulence in respiratory mutants of *Fusarium oxysporum* f.sp. *basilicum* and *Colletotrichum coccodes*. Masters thesis, Michigan State University.
- Charter, N. W., Buck, K. W., and Brasier, C. M. 1993. De-novo generation of mitochondrial DNA plasmids following cytoplasmic transmission of a degenerative disease in *Ophiostoma nono-ulmi*. *Current Genetics* 24:505-514.
- Chen, B., Choi, G. H., and Nuss, D. L. 1993. Mitotic stability and nuclear inheritance of integrated viral cDNA in engineered hypovirulent strains of the chestnut blight fungus. *EMBO Journal* 12:2991-2998.
- Choi, G. H., and Nuss, D. L. 1992. Hypovirulence of chestnut blight fungus conferred by an infectious viral cDNA. *Science* 257: 800-803.
- Correll, J. C., Klittich, C. J. R., and Leslie, J. F. 1987. Nitrate nonutilizing mutants of *Fusarium oxysporum* and their use in vegetative compatibility tests. *Phytopathology* 77:1640-1646.

- Day, P. R., Dodds, J. A., Elliston, J. E., Jaynes, R. A., and Anagnostakis, S. L. 1977. Double-stranded RNA in *Endothia parasitica*. *Phytopathology* 67:1393-1396.
- Dunn, M. M., and Boland, G. J. 1993. Hypovirulent isolates of *Cryphonectria parasitica* in southern Ontario. *Canadian Journal of Plant Pathology* 15:245-252.
- Elliston, J. E., Jaynes, R. A., Day, P. R., and Anagnostakis, S. L. 1977. A native American hypovirulent strain of *Endothia parasitica*. (Abstr.) *Proceedings of the American Phytopathological Society* 4:83-84.
- Enebak, S. A., Hillman, B. I., and MacDonald, W. L. 1994. A hypovirulent isolate of *Cryphonectria parasitica* with multiple, genetically unique dsRNA segments. *Molecular Plant-Microbe Interactions* 7:590-595.
- Fenn, P., Durbin, R. D., and Kuntz, J. E. 1975. Wilt development in red oak seedlings: a new system for studying oak wilt. *Phytopathology* 65:1381-1386.
- Fulbright, D. W., Weidlich, W. H., Haufler, K. Z., Thomas, C. S., and Paul, C. P. 1983. Chestnut blight and recovering American chestnut trees in Michigan. *Canadian Journal of Botany* 61:3164-3171.
- Fulbright, D.W. 1984. Effect of eliminating dsRNA in hypovirulent *Endothia parasitica*. *Phytopathology* 74:722-724.
- Fulbright, D. W. 1985. A cytoplasmic hypovirulent strain of *Endothia parasitica* without double-stranded RNA (dsRNA). *Phytopathology* 75:1328.
- Gonzalez, C. F., Appel, D. N., Brooks, D. S., Gehring, E. H., and Filer, T. H. 1995. Biological control of oak wilt. Pages. 89-96 in: *Oak Wilt Perspectives: The Proceedings of the National Oak Wilt Symposium*. D. N. Appel, and R. F. Billings, eds. Information Development Inc., Houston.
- Grente, J. 1965. Les formes hypovirulentes D'*Endothia parasitica* et les espoirs de lutte contre le chancre du chataignier. *Comptes Rendus Hebdomadaires des Seances. Academie d'Agriculture de France* 51:1033-1037.
- Griffiths, A. J. F. 1992. Fungal senescence. *Annual Review of Genetics* 26: 351-372.
- Griffiths, A. J. F., and Bertrand, H. 1984. Unstable cytoplasms in Hawaiian strains of *Neurospora intermedia*. *Current Genetics* 8:387-398.
- Hammerschmidt, R., and Dann, E. K. 1997. Induced resistance to disease. Pages 177-199 in: *Environmentally Safe Approaches to Crop Disease Control*. N. A. Rechcigl, and J. E. Rechcigl eds. Lewis Publishers, New York.

- Henry, B. W. 1944. *Chalara quercina* n. sp., the cause of oak wilt. *Phytopathology* 34:631-635.
- Henry, B. W., Moses, C. S., Richards, C. A., and Riker, A. J. 1944. Oak wilt: its significance, symptoms, and cause. *Phytopathology* 34:636-647.
- Hoagland, D. R., and Arnon, D. I. 1950. The water culture method for growing plants without soil. California. Agriculture Experiment Station Circular 337.
- Hong, Y., Cole, T. E., Brasier, C. M., and Buck, K. W. 1998. Novel structures of two virus-like RNA elements from a diseased isolate of the Dutch elm disease fungus, *Ophiostoma novo-ulmi*. *Virology* 242:80-89.
- Huber, D. H., and Fulbright, D. W. 1992. Preliminary investigations on the effect of individual *vic* genes upon the transmission of dsRNA in *Cryphonectria parasitica*. *Proceedings of the International Chestnut Conference* 15-18.
- Hunt, J. 1956. Taxonomy of the genus *Ceratocystis*. *Lloydia* 19:1-58.
- Hillman, B. I., Fulbright, D. W., Nuss, D. L., and Van Alfen, N. K. 1995. Hypoviridae. Pages 261-264 in: *Virus Taxonomy: Sixth Report of the International Committee for the Taxonomy of Viruses*, Murphy F.A. et al., eds. Springer-Verlag, New York.
- Jewell, F. F. 1956. Insect transmission of oak wilt. *Phytopathology* 46:244-257.
- Juzwik, J., Cease, K. R., and Meyer, J. M. 1998. Acquisition of *Ophiostoma quercus* and *Ceratocystis fagacearum* by Nitidulids from *O. quercus*-colonized oak wilt mats. *Plant Disease* 82:239-243.
- Klittich, C. J. R., and Leslie, J. F. 1988. Nitrate reduction mutants of *Fusarium moniliforme* (*Gibberella fujikuroi*). *Genetics* 118:417-423.
- Küntzel, H. 1969. Proteins of mitochondrial and cytoplasmic ribosomes from *Neurospora crassa*. *Nature* 222:142-146.
- Lambers, H. 1982. Cyanide-resistant respiration: a nonphosphorylating electron transport pathway acting as an energy overflow. *Physiologia Plantarum* 55:478-485.
- Lambers, H. 1990. Oxidation of mitochondrial NADH and the synthesis of ATP. Pages 124-143 in: *Plant Physiology, Biochemistry and Molecular Biology*. D. T. Dennis, and D. H. Turpin, eds. Wiley and Sons Inc., New York, New York.
- Lambowitz, A. M. 1979. Preparation and analysis of mitochondrial ribosomes. *Methods in Enzymology* 59:421-433.

- Lambowitz, A. M., Sabourin, J. R., Bertrand, H., Nickels, R., and McIntosh, L. 1989. Immunological identification of the alternative oxidase of *Neurospora crassa* mitochondria. *Molecular and Cellular Biology* 9:1362-1364.
- Lambowitz, A. M., and Slayman, C. W. 1971. Cyanide-resistant respiration in *Neurospora crassa*. *Journal of Bacteriology* 108:1087-1096.
- Li, Q., Ritzel, R. G., McLean, L. L. T., McIntosh, L., Ko, T., Bertrand, H., and Nargang, F. E. 1996. Cloning and analysis of the alternative oxidase gene of *Neurospora crassa*. *Genetics* 142:129-140.
- MacDonald, W. L., and Hindal, D. F. 1981. Life cycle and epidemiology of *Ceratocystis*. Pages 128-144 in: *Fungal Wilt Diseases of Plants*. Academic Press Inc.
- MacDonald, W. L., and Fulbright, D. W. 1991. Biological control of chestnut blight: use and limitations of transmissible hypovirulence. *Plant Disease* 74:656-661.
- MacDonald, W. L. 1995. Oak wilt: an historical perspective. Pages 7-14 in: *Oak Wilt Perspectives: The Proceedings of the National Oak Wilt Symposium*, D. N. Appel, and R. F. Billings, eds. Information Development Inc., Houston.
- Mahanti, N., Bertrand, H., Monteiro-Vitorello, C., and Fulbright, D. W. 1993. Elevated mitochondrial alternative oxidase activity in dsRNA-free, hypovirulent isolates of *Cryphonectria parasitica*. *Physiological and Molecular Plant Pathology* 42:455-463.
- Mahanti, N., and Fulbright, D. W. 1995. Detection of mitochondrial DNA transfer between strains after vegetative contact in *Cryphonectria parasitica*. *Molecular Plant-Microbe Interactions* 8:465-467.
- Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Press, New York.
- McIntosh, L. 1994. Molecular biology of the alternative oxidase. *Plant Physiology* 105:781-786.
- McWain, P., and Gregory, G. F. 1972. A neutral mannon from *Ceratocystis fagacearum* culture filtrate. *Phytochemistry* 11:2609-2612.
- Meeuse, B. J. D. 1975. Thermogenic respiration in aroids. *Annual Review of Plant Physiology* 26:117-126.

- Merrill, W., and French, D. W. 1995. Insects and the epidemiology of oak wilt. Pages 29-39 in: Oak Wilt Perspectives: The Proceedings of the National Oak Wilt Symposium, D. N. Appel, and R. F. Billings, eds. Information Development Inc., Houston.
- Monteiro-Vitorello, C., Bell, J. A., Fulbright, D. W., and Bertrand, H. 1995. A cytoplasmically transmissible hypovirulence phenotype associated with mitochondrial DNA mutations in the chestnut blight fungus *Cryphonectria parasitica*. Proceedings. National Academy of Science 92:5935-5939.
- Nuss, D. L., and Koltin, Y. 1990. Significance of dsRNA genetic elements in plant pathogenic fungi. Annual Review of Phytopathology 28:37-58.
- Paul, C. P., and Fulbright, D. W. 1988. Double-stranded RNA molecules from Michigan hypovirulent isolates of *Endothia parasitica* vary in size and sequence homology. Phytopathology 78:751-755.
- Peever, T. L., Liu, Y., and Milgroom, M. G. 1997. Diversity of hypoviruses and other double-stranded RNAs in *Cryphonectria parasitica* in North America. Phytopathology 87:1026-1033.
- Polashock, J. J., Bedker, P. J., and Hillman, B. I. 1997. Movement of a small mitochondrial double-stranded RNA element of *Cryphonectria parasitica*: ascospore inheritance and implications for mitochondrial recombination. Molecular and General Genetics 256:566-571.
- Polashock, J. J., and Hillman, B. I. 1994. A small mitochondrial double-stranded (ds)RNA element associated with a hypovirulent strain of the chestnut blight fungus and ancestrally related to yeast cytoplasmic T and W dsRNAs. Proceedings. National Academy of Science 91:8680-8684.
- Puhalla, J. E., and Anagnostakis, S. L. 1971. Genetic and nutritional requirements of *Endothia parasitica*. Phytopathology 61:169-173.
- Roberts, H., Smith, S. C., Marzuki, S., and Linnane, A. W. 1980. Evidence that cytochrome *b* is the anitmycin-binding component of the yeast mitochondrial cytochrome *bc₁* complex. Archives of Biochemistry and Biophysics 200:387-395.
- Rogers, H. J., Buck, K. W., and Brasier, C. M. 1986. Transmission of double-stranded RNA and a disease factor in *Ophiostoma ulmi*. Plant Pathology 35:277-287.
- Rogers, H. J., Buck, K. W., and Brasier, C. M. 1987. A mitochondrial target for double-stranded RNA in diseased isolates of the fungus that causes Dutch elm disease. Nature 329:558-560.

- Rogers, H. J., Buck, K. W., and Brasier, C. M. 1988. Double-stranded RNA in diseased isolates of the aggressive subgroup of the Dutch elm fungus *Ophiostoma ulmi*. Pages 327-351 in: Viruses of Fungi and Simple Eukaryotes, Y. Koltin, and M. J. Leibowitz, eds. New York.
- Sachs, I. B., Nair, V. M. G., and Kuntz, J. E. 1970. Penetration and degradation of cell walls in oaks infected with *Ceratocystis fagacearum*. Phytopathology 60:1399-1404.
- Samuels, G. J. 1993. The case for distinguishing *Ceratocystis* and *Ophiostoma*. Pages 15-20 in: *Ceratocystis* and *Ophiostoma* Taxonomy, Ecology, and Pathogenicity. M. J. Wingfield, K.A. Seifert, and J. F. Webber, eds. APS Press. St. Paul, Minnesota.
- Schonbaum, G. R., Bonner, W. E., Storey, B. T., and Bahr, J. T. 1971. Specific inhibitors of cyanide-insensitive respiratory pathway in plant mitochondria by hydroxamic acids. Plant Physiology 47:124-128.
- Skelly, J. M. and Merrill, W. 1968. Susceptibility of red oaks to infection by *Ceratocystis fagacearum* during the dormant season in Pennsylvania. Phytopathology 58:1425-1426.
- Smith, D., and Onions, A. H. S. 1994. The preservation and maintenance of living fungi. Second ed. Cab International. U.K. pgs 34-37.
- Smit, W. A., Wingfield, B. D., and Wingfield, M. J. 1996. Reduction of laccase activity and other hypovirulence-associated traits in dsRNA-containing strains of *Diaporthe ambigua*. Phytopathology 86:1311-1316.
- Stambaugh, W. J., and Nelson, J. C. 1956. Observations concerning autom infection of oak by *Endoconidiophora fagacearum* Bretz. Plant Disease Reporter: 40750-751.
- Stanway, C. A. 1985. Double-stranded RNA viruses and pathogenicity of the wheat take-all fungus *Gaeumannomyces graminis* var. *tritici*. PhD thesis. Univ. London, England.
- Struckmeyer, B. E., Beckman, C. H., Kuntz, J. E., and Riker, A. J. 1954. Plugging of vessels by tyloses and gums in wilting oaks. Phytopathology 44:148-153.
- Tissieres, A., Mitchell, H. K., and Haskins, F. A. 1953. Studies on the respiratory system of the *poky* strain of *Neurospora crassa*. Journal of Biological Chemistry 205:423-433.
- Upadhyay, H. P. 1981. A monograph of *Ceratocystis* and *Ceratocystiopsis*. University of Georgia Press, Athens pgs. 66-67.

- Upadhyay, H. P. 1993. Classification of the Ophiostomatoid fungi. Pages 7-14 in: *Ceratocystis and Ophiostoma Taxonomy, Ecology, and Pathogenicity*. M. J. Wingfield, K. A. Seifert, and J. F. Webber, eds. APS Press. St. Paul, Minnesota.
- Van Alfen, N. K., Jaynes, R. A., Anagnostkis, S. L., and Day, P. R. 1975. Chestnut blight: biological control by transmissible hypovirulence in *Endothia parasitica*. *Science* 189:890-891.
- Vanlerberghe, G. C., Vanlerberghe, A. E., and McIntosh, L. 1997. Molecular genetic evidence of the ability of alternative oxidase to support respiratory carbon metabolism. *Plant Physiology* 113:657-661.
- Vierula, P. J., and Bertrand, H. 1992. A deletion derivative of the *kalilo* senescence plasmid forms hairpin and duplex DNA structures in the mitochondria of *Neurospora*. *Molecular and General Genetics* 234:361-368.
- Vogel, H. J. 1956. A convenient growth medium for *Neurospora* (medium M). *Microbial Genetics Bulletin* 13:42-43.
- White, I. G. 1955. Toxin production by the oak wilt fungus *Endoconidiophora fagacearum*. *American Journal of Botany* 42:759-764.
- Wilson, C. L. 1956. Development of the ascogonium and perithecium of *Endoconidiophora fagacearum*. *Phytopathology* 46:625-632.
- Wood, H. A., and Bozarth, R. F. 1973. Heterokaryon transfer of virus particles associated with a cytoplasmically inherited determinant in *Ustilago maydis*. *Phytopathology* 63:1018-1021.

MICHIGAN STATE UNIV. LIBRARIES



31293017876701