ABSTRACT

PETTERSSON, JAN MARTIN. Diseases on Christmas Trees in Southern Sweden and Western North Carolina with Emphasis on Phytophthora Root Rot and Neonectria Canker. (Under the direction of Dr. John Frampton).

The aim of this dissertation was to investigate diseases of Christmas trees in Sweden and North Carolina, focusing on Phytophthora root rot and Neonectria canker. Both are serious Christmas tree diseases in a number of European countries and North America and may pose a threat also to Swedish Christmas tree production and forests.

Disease surveys and inoculation experiments were conducted in both countries. In North Carolina, Phytophthora root rot caused by *Phytophthora cinnamomi* has been known for decades and continues to be a major problem for the Christmas tree industry. According to a questionnaire survey, 88% of Christmas tree growers had Phytophthora root rot in their fields. In the disease survey reported here, six *Phytophthora* species were discovered on symptomatic Fraser fir (*Abies fraseri*), three of which were new to the region (*P. europaea*, *P. citrophthora*, and *P. sansomeana*). *Phytophthora cinnamomi* was still the dominating species causing disease, however, *P. cryptogea* also contributed significantly to Fraser fir losses. One management tactic used to combat Phytophthora root rot in North Carolina is to plant Eastern white pine on heavily infested sites as an alternative to Fraser fir as it is less susceptible to Phytophthora root rot. After screening Eastern white pine families for *P. cinnamomi* resistance, it is evident that this species is relatively resistant yet resistance is still under a moderately high level of genetic control. Deployment of families selected for resistance will reduce Phytophthora root rot losses although host x pathogen genetic interactions must be addressed.

In Sweden, a pioneering disease and pest survey of Christmas tree plantations was conducted in 2015. In total, 16 disease-causing pathogens and six pests were discovered. Further
studies focused on Phytophthora root rot and Neonectria canker because, based on experience from other countries, these were considered the largest disease threats to Swedish Christmas trees. In total, five identified and one unidentified *Phytophthora* species were isolated from waterways and soil samples. In addition, *P. megasperma* was isolated from a young diseased Norway spruce (*Picea abies*). Inoculation tests with the most potentially threatening species (*P. cryptogea, P. megasperma, P. plurivora*) showed minor disease development, but all species could be reisolated from inoculated Norway spruce and Nordmann fir (*A. nordmanniana*) seedlings. None of the *Phytophthora* species were widespread and therefore Phytophthora root rot is currently not considered a major problem in Swedish Christmas tree fields. However, vigilance is recommended to avoid introducing or spreading these intransigent pathogens.

From Norway spruce trees with top-dieback (up to three or four dead branch whorls), *Neonectria fuckeliana* was commonly isolated. On Nordmann fir, *Neonectria neomacrospora* was detected. Inoculation studies using *N. fuckeliana* and *N. neomacrospora* on Norway spruce and Nordmann fir, respectively, demonstrated that both pathogens caused disease. A second and much more comprehensive *N. fuckeliana* inoculation study found symptom development to be minor, contradicting the previous results.

For rapid and reliable identification of *N. fuckeliana*, a species-specific TaqMan real-time PCR-based test was developed.

Christmas tree growers in both Sweden and North Carolina rely heavily on imported seedlings. This is especially risky with respect to soil-borne *Phytophthora* species, not least when the seedlings are bare-root plants. Also, other alien plant pathogens can be introduced to Christmas tree fields by import. Lack of awareness of this disease potential may prevent early detection. Continued research on the epidemiology and impact of these “new-to-the-region”
Phytophthora and Neonectria species is needed, especially as none of these pathogens are limited to Christmas trees, and thus pose a risk to all forests.
Diseases on Christmas Trees in Southern Sweden and Western North Carolina
with Emphasis on Phytophthora Root Rot and Neonectria Canker

by
Jan Martin Pettersson

A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Forestry and Environmental Resources

Raleigh, North Carolina

2018

APPROVED BY:

_______________________________  ______________________________
John Frampton                   Marc Cubeta
Committee Chair

_______________________________  ______________________________
Howard Shew                     Gary Blank

_______________________________  ______________________________
Jonas Rönnberg                  Venche Talgø
External Member                 External Member
DEDICATION

To Tess
BIOGRAPHY

Martin Pettersson, born and raised outside of Vänersborg, a small city in western Sweden. His love for trees and nature started from climbing any tree he could find as a child. Growing up on a dairy farm surrounded by forests, he found a lot of trees. In school, he was excited to learn about nature and found field excursions to be most satisfying. His interests took him to study forestry at the Swedish University of Agricultural Sciences (SLU). During his time in the five-year forestry program, he spent three years in Umeå (northern Sweden) learning about forestry, one year in Wageningen (the Netherlands) learning about nature conservation, and one year in Alnarp (southern Sweden) focusing on researching conifer root rot caused by *Heterobasidion* species. During the summers, since he was a kid, he helped with the work on the farm, but also worked practically with forest management for different forest companies. He graduated with a Master’s degree in Forestry from SLU in 2013 and worked as a research assistant before starting his doctoral studies at North Carolina State University (NCSU) in spring 2014.
ACKNOWLEDGMENTS

I was always curious about science and what being a researcher would be like. During my PhD program I have had the opportunity to work at four different research institutions in three different countries and have spent time with a lot of amazing people. I would like to start by thanking all of the Christmas tree growers that I had the pleasure to meet over the years, growers in both Sweden and North Carolina. Without you, none of my work would have been possible or meaningful. I hope that you will find some of my results helpful and not too alarming. I further hope that my dissertation will draw attention to your special field of tree cultivation, deeply embedded in our cultural traditions.

At North Carolina State University (NCSU) I felt welcome everywhere I went. I would like to start by thanking my NCSU committee members: John Frampton, Marc Cubeta, David Shew and Gary Blank. I would also like to thank Mike Benson, Lilian Matallana, Anne-Margaret Braham, Will Kohlway, Kala Parker, Avery Barr, Maria Escanferla and Sarah Slover.

I would like to extend a special thank-you to John Frampton. I could not have asked for a better supervisor to help me along this long and bumpy road. From when I stepped off the plane in Raleigh four and a half years ago until now, you have shown me nothing but kindness and support. Not many people would lend their truck to someone they barely know! I appreciated your kindness and confidence in me (not only as a driver, but as a PhD student, too). I also appreciated your flexibility and adaptiveness, which I first experienced when you guided and counseled me through the change in my studies from pathogens on poplar and hybrid aspen to Christmas tree diseases. This was an area I didn’t even know existed. You have expanded my horizons and are one of the best researchers I have ever met. Your dedication and hard work as
well as your ability to work with numbers is impressive. Not only are you a fantastic researcher, but you also connect it to actual problems that Christmas tree growers face. I hope that someday I will be able to connect research to practice the way you have! I will also always remember how you introduced me to the incredibly beautiful southern Appalachians, welcomed me into your home and let us decorate your Fraser fir Christmas tree (with more decorations than five Swedish Christmas trees could hold!). Thanks for all the support, advice and knowledge you have shared with me.

I would also like to give extra thanks to Marc Cubeta for taking time out of your many commitments to be on my committee, for including me in your lab group, and for taking me to my first conference. Your quick thinking, ideas and ability to think outside the box never failed to fascinate me. You also win the prize for quickest to reply to emails, which was always a comfort to a stressed-out PhD student… Tack så mycket!

Thank-you, David Shew, for agreeing to be on my committee and providing valuable tips on how to handle Phytophthora cultures.

Thank-you, Gary Blank, for agreeing to become my fourth committee member and for forcing me to read “Tomorrow's Table: Organic Farming, Genetics, and the Future of Food” which gave me new insights.

Thank-you, Mike Benson, for allowing me to use your lab and for answering all of my Phytophthora questions. I hope you are enjoying your retirement and that you are catching a lot of big fish off the Outer Banks.

Thank-you, Lilian Matallana, for all your help in the lab. Your patience and answering of my endless questions was unmatched. You are a great teacher and draw amazing pedagogical
pictures. You’re also the best roommate/landlord, and I really enjoyed sharing living quarters and all the laughs we had together.

Thank you, Anne Margaret Braham, for your endless kindness. You have been my savior many times and have been so thoughtful over these years. It has really warmed my heart.

Thank you, Will Kohlway and Kala Parker, for your valuable assistance in the lab, and Avery Barr and Maria Escanferla for helping me out with all the cultures in my first study!

Thank you, Sarah Slover, for keeping track of all of my deadlines, course credits, and all the other administrative details that a confused PhD student such as myself forgets.

And thank you, Gloria Abad, for hosting a great Phytophthora workshop in Washington DC. I wish I had taken that workshop a year earlier than I did!

— Thanks, y’all!

At the Swedish University of Agricultural Sciences (SLU) I would like to thank, Jonas Rönnberg, Carl Salk, Jan-Eric Englund, Larisa Gustavsson, Michelle Cleary, Johanna Witzell, Marjan Ghasemkhani, Emma Sandell-Festin and Linda Petersson.

Special thanks go to my SLU supervisor Jonas Rönnberg. Between small children, travelling around the world for your work, lecturing, researching, a crazy virus and all your other obligations, you somehow still found time to be my supervisor! You were the instigator of this entire process and supported me even before my PhD had begun. There were many logistical and bureaucratic obstacles throughout the whole process that you were not worried about. Thank you for getting rid of these obstacles, I know it took a lot of your time and effort. Without your help and assistance with various funding applications I would not be getting a PhD at all. Thank you
for all your evening and weekend time on the telephone, particularly at the end! Throughout the whole process, you were always positive and encouraging, even when things seemed bleak. I especially enjoyed our discussions while running (and possibly trespassing) in the southern Appalachians and on the NCSU campus. I wish we could have run together more often and in Sweden too. Thank you for everything.

Thank you, Emma and Emil Sandell-Festin, for your hospitality and letting me stay at your home countless times. Especially at the end when I was extremely stressed and probably not good company at all... Your company, office and food was very much appreciated.

At the Norwegian Institute of Bioeconomy Research (NIBIO) I would like to thank, Venche Talgø, Arne Stensvand, Vinh Hong Le, Håvard Eikemo, Gunn Mari Strømeng, Erling Fløistad, Jafar Razzaghian, Andrew Dobson, Trude Slørstad, Monica Skogen, Arnaud Lefrançois and May Bente Brurberg.

A special thank you to Venche Talgø for being an amazingly supportive, dedicated, positive and hardworking supervisor. Nothing is impossible for you! I contacted you to ask about Phytophthora in the Nordic countries and suddenly you were one of my supervisors and I was using the NIBIO facilities, researching diseases that I’d never heard of and conducting studies in Norway. I don’t know how I would have gotten through this PhD without you. Even though all the projects didn’t go as planned, you remained positive, supportive and involved throughout. You made me feel like a part of the team, and your drive and ambition are contagious. You and research are like two peas in a pod. I haven’t met anyone who detects and classifies diseases on trees and shrubs like you (including people’s gardens in your free time…). Neither have I met anyone who can work instead of sleep, and is banned from work because they don’t take enough
vacation… Thank you for all the time you took to read my manuscripts and your valuable feedback. You made me feel that you prioritized my projects even though you were always busy. You are one of a kind, and I am so happy that I contacted you in 2015!

My warm thanks also to Johannes Deelstra and to Peter and Elisabeth for all your hospitality and kindness.

At Skogforsk, I would like to thank Bo Karlsson, Johan Malm, Mihály Czimbalmos, Ingrid Vos, Eva Petersson and Joakim Nilsson. Thank you for helping to fund CHAPTER 7 and 8, and for allowing me to work in your laboratory and inoculate plants in one of your greenhouses.

At the University of Copenhagen, I would like to thank Iben Thomsen and Knud Nor Nielsen, for our *Neonectria* discussions.

At the Forestry Commission (UK), I would like to thank Ana Pérez-Sierra for giving me the opportunity to practice my dissertation in front of a good crowd of researches and a few Christmas tree growers.

Thank you Kelly Olson for all your work in proof-reading my dissertation on such short notice.

Finally, thank you to my family and close friends for supporting me over these years. I know I have been away a lot of the time, but your support has been appreciated.

Hanna, you are my favorite sister. I love that our fields of study have become more and more related over the years and I am inspired by your studies and research. Now that I’m done, I
look forward to spending more time with you. And mom and dad, you are also my favorites – thank you for your continuous and unconditional support over the years. I love you and am very grateful for everything.

I would like to express my biggest and most sincere thanks to Tess. These years have been an adventure and you have been with me from the start. Not only have you consistently supported me, believed in me and inspired me – you have also agreed to be my wife. Without you this would not have been possible.

This research has received funding from the Gunnar and Lillian Nicholson Graduate Fellowship and Faculty Exchange Fund in Forestry, the Forestry Research Institute of Sweden (Skogforsk) together with Partnership Alnarp, the Rattsjö Foundation, the USDA NIFA Specialty Crops Research Initiative (2012-51181-19940) and the North Carolina Agricultural Research Service via the Christmas Tree Genetics Program. Without this funding, this PhD would not have been possible. I am grateful for your trust in me, and the opportunity to further my studies.
# TABLE OF CONTENTS

LIST OF TABLES ............................................................................................................................ xiii  
LIST OF FIGURES .......................................................................................................................... xvi  
INTRODUCTION .......................................................................................................................... 1  
   Christmas trees .......................................................................................................................... 1  
      History of the Christmas tree .................................................................................................. 1  
      Tree species cultivated as Christmas trees .......................................................................... 2  
      Production of Christmas trees and greenery ........................................................................ 6  
      Diseases that limit the Christmas tree production ............................................................... 8  
Phytophthora ............................................................................................................................. 9  
   General information about Phytophthora ................................................................................. 9  
   Many Phytophthora species are highly invasive ...................................................................... 11  
   Global nursery trade – the root of the problem ...................................................................... 11  
   Phytophthora root rot in Christmas tree fields in North Carolina ......................................... 12  
   Phytophthora root rot in Christmas tree fields in Sweden ...................................................... 13  
   Management of Phytophthora root rot .................................................................................. 14  
Neonectria .................................................................................................................................. 16  
   General information about Neonectria .................................................................................. 17  
   Neonectria canker caused by Neonectria fuckeliana on spruce trees ..................................... 17  
   Neonectria canker caused by Neonectria neomacrospora on fir trees .................................... 18  
   Neonectria canker caused by Neonectria ditissima on broadleaf trees .................................. 19  
   Scope, aim and research questions ....................................................................................... 20  
CHAPTER 1  
INFLUENCE OF PHYTOPHTHORA ROOT ROT ON PLANTING TRENDS OF  
FRASER FIR CHRISTMAS TREES IN THE SOUTHERN APPALACHIAN  
MOUNTAINS ............................................................................................................................ 26  
   Abstract .................................................................................................................................. 27  
   Introduction ............................................................................................................................ 27  
   Fraser Fir Planting Stock— The Early Years ......................................................................... 27  
   Emergence and Spread of Phytophthora Root Rot .................................................................. 28  
   Fraser Fir Planting Stock— Shift to Out-of-State Sources ....................................................... 29  
   2015 Survey of Planting Trends and Phytophthora Root Rot Discussion ............................. 30  
   Perspective ............................................................................................................................ 32  
   References ............................................................................................................................. 33  
CHAPTER 2  
INCREASED DIVERSITY OF PHYTOPHTHORA SPECIES IN FRASER FIR  
CHRISTMAS TREE PLANTATIONS IN THE SOUTHERN APPALACHIANS .................. 35  
   Abstract .................................................................................................................................. 36  
   Introduction ............................................................................................................................ 36  
   Material and Methods ........................................................................................................... 37  
   Results .................................................................................................................................... 40  
   Discussion .............................................................................................................................. 41  
   Acknowledgements ............................................................................................................... 43  
   References ............................................................................................................................. 44
# CHAPTER 8
DEVELOPMENT AND APPLICATION OF A REAL-TIME PCR ASSAY FOR DETECTION AND IDENTIFICATION OF NEONECTRIA FUCKELIANA FROM NORWAY SPRUCE

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>105</td>
</tr>
<tr>
<td>Introduction</td>
<td>106</td>
</tr>
<tr>
<td>Material and Methods</td>
<td>108</td>
</tr>
<tr>
<td>Results</td>
<td>114</td>
</tr>
<tr>
<td>Discussion</td>
<td>117</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>119</td>
</tr>
<tr>
<td>References</td>
<td>120</td>
</tr>
</tbody>
</table>

# GENERAL DISCUSSION, PRACTICAL IMPLICATIONS AND FUTURE RESEARCH

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A comparison of the Christmas tree production in Sweden and North Carolina</td>
<td>124</td>
</tr>
<tr>
<td>Pathogens that could seriously harm future Christmas tree production</td>
<td>127</td>
</tr>
<tr>
<td>Management tactics to protect Christmas tree production</td>
<td>130</td>
</tr>
<tr>
<td>Strengthening Swedish Christmas tree production and reducing the risk for disease epidemics – lessons learned from North Carolina</td>
<td>134</td>
</tr>
<tr>
<td>Future research</td>
<td>139</td>
</tr>
<tr>
<td>Phytophthora in Sweden and North Carolina</td>
<td>139</td>
</tr>
<tr>
<td>Neonectria in Sweden</td>
<td>141</td>
</tr>
</tbody>
</table>

# REFERENCES

References
LIST OF TABLES

CHAPTER 1
Influence of Phytophthora Root Rot on Planting Trends of Fraser fir Christmas Trees in the Southern Appalachian Mountains

Table 1. Summary of responses concerning planting stock from four pest management surveys conducted by the North Carolina Cooperative Extension Service between 1995 and 2014. Respondents could select multiple choices so responses do not total 100 percent (Sidebottom, unpublished data) ........................... 29

CHAPTER 2
Increased diversity of Phytophthora species in Fraser fir Christmas tree plantations in the Southern Appalachians

Table 1. Phytophthora species recovered from Fraser fir (Abies fraseri) Christmas trees displaying disease symptoms of Phytophthora root rot from sites in the southern Appalachian Mountains .......................................................... 37

Table 2. Symptoms of sampled Fraser fir (Abies fraseri) trees in Christmas tree plantations in the southern Appalachian Mountains ........................................... 40

Table 3. Shape, pore type, length, width and length/width ratio of sporangia produced on clarified V8 medium pieces flooded with soil extract of Phytophthora cryptogea, P. pini, P. citrophthora, P. cinnamomi, P. europaea and P. sansomeana ............... 41

Table 4. Observations of sexual characteristics on clarified V8 medium pieces flooded with non-sterile soil extract, for Phytophthora cinnamomi, P. cryptogea, P. citrophthora, P. europaea, P. pini and P. sansomeana ........................................... 41

Table 5. A selection of 10 out of 91 Phytophthora isolates obtained from symptomatic Fraser fir (Abies fraseri) Christmas trees in a survey of 103 sites in the southern Appalachians, 2014 .......................................................... 42

CHAPTER 3
Genetic Variation for Resistance to Phytophthora Root Rot in Eastern White Pine Seedlings

Table 1. Study design with mortality (%) and its heritability estimates for Eastern white pine (EWP) seedlings one and two years after inoculation with a Phytophthora cinnamomi culture isolated from Fraser fir (Main Study) or EWP (Supplemental Study) and non-inoculated control seedlings. The Main Study (subsample) column includes values calculated using only the 20 open-pollinated families in common with the Supplemental Study ........................................... 49
CHAPTER 4
Diseases, pests and nutrient deficiencies of Swedish Christmas trees (2015 disease survey)

Table 1. Biotic and abiotic damaging agents found in Swedish Christmas tree fields during a survey in 2015 ................................................................. 63

CHAPTER 5
Presence of Phytophthora species in Swedish Christmas tree plantations

Table 1. *Phytophthora* isolates obtained from Christmas tree fields in southern Sweden in 2015 ................................................................. 70

Table 2. Characteristics of *Phytophthora cryptogea*, *P. megasperma*, and *P. plurivora* in culture .................................................................................. 73

Table 3. Pathogenicity of *Phytophthora cryptogea*, *P. megasperma*, and *P. plurivora* on Norway spruce (*Picea abies*) and Nordmann fir (*Abies nordmanniana*). The seedlings were inoculated in the root with colonized rice grains or in the stem by mycelial plugs, and incubated in a growth chamber with 65% RH, 16°C and 18 h daylight for 7 months. Visual rating was conducted using a scale from 0 to 5, where 0 = 0-10%, 1 = 11-25%, 2 = 26-50%, 3 = 51-75%, 4 = 76-90%, and 5 = 91-100% necrotic root tips for the root rot symptoms and yellow/brown needles and wilting shoots for the foliage symptoms. For the mycelial plug inoculation, lesion length under bark (LLUB) is given in millimeters .................... 75

CHAPTER 6
Neonectria canker found on spruce and fir in Swedish Christmas tree plantations

Table 1 *Neonectria* spp. isolates obtained from six different Christmas tree fields in a disease and pest survey of 21 farms in southern Sweden in 2015 ............................... 83

CHAPTER 7
Pathogenicity of Neonectria fuckeliana on Norway spruce clones in Sweden and potential management strategies

Table 1. *Neonectria fuckeliana* isolates from Norway spruce (*Picea abies*) used for inoculation studies of young Norway spruce plants ............................................. 91

Table 2. *Neonectria fuckeliana* detection from 128 terminal leader and 64 branch cuttings of Norway spruce inoculated with a microconidial suspension, and 64 control cuttings (inoculated with water). The treatments included were shoot-topped, shoot-wounded, needles-removed, and non-wounded ........................................ 96

Table A1. Monthly average, standard deviation, minimum and maximum temperature fluctuation patterns for day and night from April 2016–March 2017 in the greenhouse where the second and third inoculation trials took place ............. 101
CHAPTER 8
Development and application of a real-time PCR assay for detection and identification of *Neonectria fuckeliana* from Norway spruce

Table 1. Norwegian and Swedish fungal isolates used to develop and validate a TaqMan real-time PCR assay for identification of *N. fuckeliana* .......................... 113

Table 2. Fungal species, host plants, GenBank accession numbers of the internal transcribed spacer 1 (ITS-1) region of the ribosomal DNA (rDNA) sequences used to verify species specificity of the Nfuc-F1/Nfuc-R1 primer pair, and countries of isolate origin ................................................................. 113

Table 3. The primer pair and probe designed for *Neonectria fuckeliana* by using Primer Express software 2.0................................................................. 116

Table A2. Daily average, standard deviation, minimum and maximum temperature fluctuation patterns for day and night for the 8-day-period following the second inoculation trial and the third inoculation trial............................... 102
LIST OF FIGURES

Introduction
Figure 1. Nordmann fir (Abies nordmanniana) (A-B) and Norway spruce (Picea abies) (C) Christmas trees in southern Sweden. Subalpine fir (A. lasiocarpa) Christmas trees in Norway (D). Photos: Martin Pettersson .................................................. 4

Figure 2. Christmas Trees in the southern Appalachian Mountains. Fraser fir (Abies fraseri) Christmas trees in Grayson County, Virginia (A). Fraser fir progeny test in Ashe County, North Carolina (B). Fraser fir adjacent to the Premium Fraser Fir Seed Orchard in Ashe county (C). Fraser fir containerized seedlings on raised benches in a greenhouse in North Carolina (D). Photos: Martin Pettersson (A,D) and Anne Margaret Braham (B-C) .................................................................. 5

Figure 3. Phytophthora root rot causing losses of Fraser fir in the southern Appalachian Mountains, North Carolina. Characteristic symptoms are tree mortality in the field (A), flagging of basal branches (B), cambial stem lesion with distinct borders between healthy and diseased tissue (C), and heavily infected root systems with sloughing necrotic roots and absence of fine roots (D). Photos: Martin Pettersson ............................................................................. 13

Figure 4. Plant symptoms and pathogen signs of Neonectria canker caused by the fungus Neonectria fuckeliana. Norway spruce Christmas tree with top-dieback where the fungus N. fuckeliana was isolated from the margin between dead and live tissue (A). Canker wound (B-C), resin flow (D), perithecia (sexual fruiting bodies) (D-E) with white spore tendrils coming out (F), sporodochia (asexual fruiting bodies) (G), cultures with mycelial growth containing conidia on potato-dextrose agar. Photos: Martin Pettersson ............................................................................. 19

CHAPTER 1
Influence of Phytophthora Root Rot on Planting Trends of Fraser fir Christmas Trees in the Southern Appalachian Mountains

Figure 1. Starting in the mid-1970s, extension programs focused on teaching Christmas tree growers how to sow their own Fraser fir seed in bareroot beds and to line-out transplants. (Photo courtesy of James McGraw, North Carolina State University, retired, Jackson County, NC, 1970s) ................................................................. 28

Figure 2. Phytophthora root rot inflicts a significant economic impact on the Fraser fir Christmas tree industry of the southern Appalachian region. Characteristic symptoms of Phytophthora root rot on Fraser fir include: (a) tree mortality, (b) flagging of basal branches, (c) cambial stem lesion with distinct borders, and (d) heavily infected root systems with sloughing necrotic roots and absence of fine roots. (Photos by Martin Pettersson, 2015) ............................................................................. 28
Figure 3. A total of 89 Christmas tree growers from 13 counties participated in a 2015 survey conducted in western North Carolina. Twenty-two growers included in the survey had Christmas tree farms in more than 1 county resulting in 123 farms from North Carolina, Tennessee (Carter and Johnson Counties), and Virginia (Grayson County).  

Figure 4. Christmas tree growers surveyed in western North Carolina (n=89) steadily shifted from locally produced Fraser fir planting stock to out-of-State stock starting in 1970, with the shift accelerating around 2000.  

Figure 5. Over a 43-year period (1970 to 2013), 64 percent of Christmas tree growers surveyed in western North Carolina shifted from using locally produced to out-of-State planting stock. Because growers with more land were more likely to shift, about 80 percent of the land base managed by the surveyed growers was planted with out-of-State planting stock by 2013.  

CHAPTER 2  
Increased diversity of Phytophthora species in Fraser fir Christmas tree plantations in the Southern Appalachians  

Figure 1. Characteristic chlorotic foliage symptom severity estimated based on a 0–4 scale (0 = 0%), (a) 1 = 1–25%, (b) 2 = 26–50%, (c) 3 = 51–75% and (d) 4 = 76–100%.  

Figure 2. Canker symptom severity width relative to the circumference of the stem. Scale 0–4: (0 = 0%), (a) 1 = 1–25%, (b) 2 = 26–50%, (c) 3 = 51–75% and (d) 4 = 76–100% of the stem width occupied by canker.  

Figure 3. Amount of root rot caused by Phytophthora species roughly determined based on a 1–3 scale where 1 = 0–30%, 2 = 31–70% and 3 = 71–100% rotted roots: (a,b) = 0–30% rotted roots. (c,d) = 31–70% rotted roots. (e,f) = 71–100% rotted roots.  

Figure 4. Colony morphology of (a) Phytophthora pini, (b) P. europaea, (c) P. sansomeana, (d) P. citrophthora, (e) P. cryptogea and (f) P. cinnamomi, after 7 days growth at 22°C on clarified V8 medium. Phytophthora cinnamomi, P. europaea and P. sansomeana had aerial fluffy culture morphology, while P. cryptogea, P. pini and P. citrophthora had a rosette-like morphology.  

CHAPTER 3  
Genetic Variation for Resistance to Phytophthora Root Rot in Eastern White Pine Seedlings  

Figure 1. Eastern white pine study seedlings in the shade house prior to inoculation (upper), inoculation of seedlings using rice grains colonized with
Phytophthora cinnamomi (lower left), and symptomatic seedlings 16 weeks after inoculation (lower right). Photos by A.M. Braham

Figure 2. Mortality (%) of Eastern white pine seedlings inoculated with Phytophthora cinnamomi at weeks 0, 8, 53 and 61. Seedlings in the Main Study were inoculated with a culture isolated from Fraser fir while seedlings in the Supplemental Study were inoculated with a culture isolated from Eastern white pine. The Main Study (subsample) represents the 20 open-pollinated families in common with the Supplemental Study. The Control Seedlings were not inoculated

Figure 3. Individual-tree (left) and family mean (right) heritability estimates (with std. error) for mortality of Eastern white pine seedlings inoculated with Phytophthora cinnamomi at weeks 0, 8, 53 and 61. Seedlings in the Main Study were inoculated with a culture isolated from Fraser fir while seedlings in the Supplemental Study were inoculated with a culture isolated from Eastern white pine

Figure 4. Height growth of Eastern white pine seedlings that remained non-symptomatic throughout the study period after inoculation with Phytophthora cinnamomi at weeks 0, 8, 53 and 61. Seedlings in the Main Study were inoculated with a culture isolated from Fraser fir while seedlings in the Supplemental Study were inoculated with a culture isolated from Eastern white pine. The Control Seedlings were not inoculated

CHAPTER 4
Diseases, pests and nutrient deficiencies of Swedish Christmas trees (2015 disease survey)

Figure 1. Map of southern Sweden with the 21 Christmas tree farms (indicated as black dots) included in a disease and pest survey in spring 2015. The star on the smaller-scale map of the Nordic countries (upper right hand corner) indicates the area where the majority of Swedish Christmas tree production is based and where the studies were conducted

Figure 2. Plant symptoms and pathogen signs of several diseases found in a survey of Swedish Christmas trees in 2015. Armillaria root rot (A-B), Cherry spruce rust (C), Chrysomyxa needle rust (D-E), Delphinella shoot blight (F), Gemmamyces bud blight (G-H), Lirula needle cast (I-J), CSNN (K), and Sclerophoma shoot dieback (L-M). Photos: Martin Pettersson
CHAPTER 5
Presence of *Phytophthora* species in Swedish Christmas tree plantations

Figure 1. Map of southern Sweden with fourteen Christmas tree farms (indicated as black dots) that were investigated for the presence of *Phytophthora* during spring 2015. In the smaller map of Scandinavia (upper right hand corner), a star indicates the area were the study was made ................................................. 68

Figure 2. Baiting for *Phytophthora* with Rhododendron ‘Cunningham’s White’ leaves; (a) anchored bait bag containing three leaves (two newly emerged and one from the previous year) and a piece of styrofoam to keep the bait near the surface; (b) Rhododendron leaves with dark, water soaked spots after baiting (old leaf on top and current year leaves underneath); (c) nine cm Petri dish with *Phytophthora*-selective medium (P10ARPH) and a PVC well (3 cm) with five sections from the leading edges of Rhododendron leaves. Photo: Martin Pettersson ................. 69

Figure 3. Morphological structures of *Phytophthora cryptogea* 250494 - (a) ovoid, non-papillate sporangium, (b) basal swelling of ovoid non-papillate sporangium; *P. megasperma* 250494 - (c) ovoid, non-papillate sporangium, (d) globose hyphal swelling; *P. plurivora* 250496 – (e) oogonia with oospore; (f) obpyriform sporangium, (g) extended internal proliferation. Scale bar = 20 μm. Photos: Martin Pettersson ................................ 73

Figure 4. Mean radial growth per day (mm/d) for *Phytophthora cryptogea* 250476, *P. megasperma* 250494, and *P. plurivora* (250496 grown on PDA at 5, 10, 15, 20, 25, 30, and 35°C. The whiskers are standard error of the mean from three replicates ................................................................. 76

CHAPTER 6
Neonectria canker found on spruce and fir in Swedish Christmas tree plantations

Figure 1. Map of southern Sweden indicating the positions of the Christmas tree fields included in a disease and pest survey in 2015 ................................................................. 83

Figure 2. Symptoms and signs of Neonectria canker on Christmas trees in Sweden: (A) dead top, (B) perithecia with emerging ascospore tendrils, and (C) sporodochia with asexual spore mass from *Neonectria fuckeliana* on Norway spruce (*Picea abies*); and (D) a dead branch tip, (E) perithecia in a needle scar, and (F) sporodochia of *N. neomacrospora* on Nordmann fir (*Abies nordmanniana*). Photos: (A, D) Martin Pettersson and (B, C, E, F) Venche .............................................. 74

Figure 3. Morphological features of *Neonectria* spp. found on spruce and fir in Sweden: (A) yellow/orange, floccose culture on PDA, and (B) macro and microconidia of *Neonectria fuckeliana*; (C) whitish, floccose culture on PDA; and (D) macro- and microconidia of *N. neomacrospora*. Both cultures were 20 days old. Scale bar = 20 μm. Photos: Martin Pettersson ............................................................... 74
Figure 4. Results from inoculation tests using map pins with mycelium of *Neonectria* spp.: (A) dead shoot and (B) canker wound on Norway spruce (*Picea abies*) 144 days after inoculation with *Neonectria fuckeliana*; (C) re-isolation of *N. fuckeliana* on PDA; (D) dead shoot on Nordmann fir (*Abies nordmanniana*) 222 days after inoculation with *N. neomacrospora*; (E) incubated Nordmann fir stems displaying white mycelium of *N. neomacrospora* in the infected area; (F) reisolation of *N. neomacrospora* on PDA. Photos: (A) Erling Fløistad and (B, C, D, E, F) Venche Talgø.................................................................75

Figure 5. Radial growth rates in millimeters per day of *Neonectria fuckeliana* (GenBank Accession No. KT350495) and *N. neomacrospora* (KT350497) grown on PDA in darkness at 5-degree intervals from 5 to 35°C ..........................................................76

CHAPTER 7
Pathogenicity of *Neonectria fuckeliana* on Norway spruce clones in Sweden and potential management strategies

Figure 1. Damage caused by *Neonectria fuckeliana* on Norway spruce (*Picea abies*) in the Nordic countries: dark necrotic canker wounds on trees in a Norwegian forest stand (left and middle, red arrows point towards clusters of perithecia of *N. fuckeliana* in or around the canker wound), and top-dieback including several branch whorls in a Swedish Christmas tree field (right). Photos: Martin Pettersson.................................................................89

Figure 2. Symptoms observed in the inoculation trials with *N. fuckeliana* on Norway spruce (*Picea abies*): Top row = pilot study (the first inoculation trial) using microconidia from isolate no. 250603 (left), isolate no. 250605 (middle), and isolate no. 250603 (right), resulting in dark necrotic canker wounds with resin production. Photographed during assessment in 2016, 2 months after inoculation. Second row = greenhouse study part I (the second inoculation trial) where four different treatments (shoot-topped [left], shoot-wounded [middle], needles-removed [right], and non-wounded) were applied to the terminal leader of actively growing and dormant cuttings. Photographed during assessment in 2017, 10 months after inoculation. Third row = greenhouse study part II (the third inoculation trial) where the biggest branch shoot was inoculated using the same four treatments as in the second inoculation trial. Photographed during assessment in 2017, 8 months after inoculation. Fourth row = field study (the fourth inoculation trial) where 7-year-old Norway spruce plants were inoculated with mycelial plugs of *N. fuckeliana* in the stem. Photographed during assessment in 2017, 11 months after inoculation. Photos: Martin Pettersson...........95
CHAPTER 8
Development and application of a real-time PCR assay for detection and identification of Neonectria fuckeliana from Norway spruce

Figure 1. Typical symptoms (cankers, branch and top-dieback) and red fruiting bodies (perithecia) caused by Neonectria ditissima on apple (Malus domestica) in Norway (A, B), N. neomacrospora on white fir (Abies concolor) in Norway (C, D), and N. fuckeliana on Norway spruce (Picea abies) in Sweden (E) and Norway (F). Photos: Venche Talgø (A, B, C, D, F) and Martin Pettersson (E)..... 110

Figure 2. The primer pair and probe were chosen based on multiple-sequence alignments of Neonectria fuckeliana, N. ditissima, N. neomacrospora and related fungi (a few from other genera) obtained from GenBank (Table 2). This figure display an alignment of parts of the internal transcribed spacer 1 (ITS-1) region of N. fuckeliana, N. neomacrospora and N. ditissima. The primer pair and probe for the N. fuckeliana (yellow) assay was designed for the ITS-1 region. Primer sequences are indicated by the arrow boxes, and the probe sequence is shown in the rectangular box in the middle. The ITS sequences were HQ840386 (N. fuckeliana), HQ840388 (N. neomacrospora) and DQ178169 (N. ditissima). Identical nucleotides are marked with an asterisk (*) and inserts with a dash (-). 115

Figure 3. Standard curve plot for Neonectria fuckeliana generated using 10-fold serially diluted DNA and the threshold cycle (Ct) value. The equation of the regression curve (y) and the coefficient of determination (R²) are shown in the graph. There were four replicates for each dilution and error bars represent the standard error from four replicate reactions. For the 10⁻⁷ dilution, error bars represent standard deviation from two replicate reactions (two reactions did not get a value within the 40 cycles) .......................................................... 117
Introduction

Christmas trees

History of the Christmas tree

Throughout history, evergreen trees have been regarded as mysterious and sacred plants, especially in the wintertime when all other plants have withered and the landscape looks deserted, dead and bare. This remains in our songs:

\[
O \text{ Christmas tree, O Christmas tree} \\
\text{how lovely are thy branches.} \\
\text{Not only green when summer’s here} \\
\text{but in the coldest time of year . . .}
\]

The evergreen tree reminded ancient peoples of the next growing season to come. In pagan nature-worship, it was heathen practice to decorate one’s home during the darkest part of the winter with branches or whole cut fir (\textit{Abies}) and spruce (\textit{Picea}) trees (Rätsch & Müller-Ebeling, 2006). This tradition dates back long before the Christian era. Pagans believed that evergreens gave shelter to friendly forest spirits and scared away bad spirits.
During the rise of Christianity, the church forbade the pagan tradition of using branches and trees to protect against evil spirits. It later changed its view, however, retaining the traditions but giving them a new Christian meaning. The oldest reference to today’s decorated Christmas tree dates back to 16th-century Germany. From there it spread throughout Europe and on to North America with German emigrants (Lauritsen, 2004). In Sweden, the first reports of decorated indoor Christmas trees are from 1741 (SkogsSverige, 2015). Noble households were first to put up trees, with commoners not adopting the tradition until the middle of the 19th century. The earliest Christmas trees were often small and placed on tables or hung from the ceiling (SkogsSverige, 2015). For centuries, people cut their Christmas trees from local forests, which is still done today but to a lesser extent. The first Christmas tree market in North America, was established in New York City in 1851. There you could buy spruce and fir trees harvested from the local mountains. In the early 1900s in North America, some pioneering tree growers started to grow Norway spruce \( Picea abies \) (L.) H. Karst and Scots pine \( Pinus sylvestris \) L. in Christmas tree plantations. By the 1950s, growers started to shear trees to increase their crown density and uniformity, as still preferred by North American consumers. In Europe, consumers prefer more open trees with layered branches, though some shearing of trees and reduction of top-shoot length is common also in Europe. The majority of Christmas trees in the world are now produced on Christmas tree farms.

**Tree species cultivated as Christmas trees**

Today, there is a wide range of Christmas trees and greenery products on the market. Real Christmas trees are mainly used in Europe, North America, Central America and South America, and to some extent in Australia and other continents. The Christmas trees are primarily fir, spruce and pine \( Pinus \) species, though other evergreens such as cypress \( Cupressus \) and cedar
(Juniperus) species are also used. In Europe and North America, a shift has taken place, where fir species with superior postharvest needle and moisture retention characteristics have increased dramatically. In Europe, Nordmann fir [Abies nordmanniana (Steven) Spach] is the dominant Christmas tree species, while noble fir (A. procera Rehd.) is the main species used for Christmas greenery. The market for both species has increased while Norway spruce has decreased. In North America, Fraser fir [A. fraseri (Pursh) Poir.] and noble fir have dramatically increased, while Scots pine (Pinus sylvestris L.) has radically decreased. Depending on the size of seedlings planted, species, management regime, site and harvesting size, it takes 4-15 years to produce a Christmas tree (National Christmas Tree Association 2017a; Chastagner & Benson, 2000).

In Sweden, Norway spruce and Nordmann fir are the main Christmas tree species (Fig. 1). Colorado blue spruce (P. pungens Engelm.), Serbian spruce [P. omorica (Pancie) Purk.], balsam fir [A. balsamea (L.) Mill.], Fraser fir, and subalpine fir [A. lasiocarpa var. lasiocarpa (Hook.) Nutt.] are also grown, but on a much smaller scale than the main species. Fir tree production has increased due to good postharvest qualities, such as better needle retention, and their production (as well as Christmas tree production in general) is centered in southern Sweden, where the winters are milder compared to the rest of the country. Subalpine fir can serve as an alternative to Nordmann fir in places where Nordmann fir does not grow well (central and northern Sweden). However, subalpine fir is uncommon in Sweden in contrast to Norway, where subalpine fir constitutes about 50% of the total fir production (Fig. 1). The other half of the fir production in Norway is mainly Nordmann fir. About 60% of all Christmas trees produced in Norway are fir and the rest mainly Norway spruce (Strande, 2017).
In the US, different regions grow different tree species. The mountains of North Carolina are home to Fraser fir Christmas tree production. Fraser fir grows naturally in the southern Appalachian Mountains and the Christmas tree production of this species in the US occurs mainly in the mountainous areas of North Carolina, Tennessee and Virginia (Fig. 2). Due to its pleasing color and superior postharvest needle retention, cultivation has increased dramatically. Other species grown as Christmas trees in the mountainous areas of North Carolina are: Canaan fir [A. balsamea var. phanerolepis (L.) Mill.], white fir [A. concolor (Gord. & Glend.) Lindl.], Nordmann fir, Turkish fir (A. bornmuelleriana Mattf.), blue spruce (P. pungens Engelm.),
Norway spruce, white spruce \([P. \text{ glauca} \,(\text{Moench}) \,Voss]\), Eastern white pine \((P. \text{ strobus} \,\text{L.})\), and Scots pine. The production of these are limited in comparison with Fraser fir.

In the Piedmont and Coastal Plain regions of North Carolina, the Christmas tree species grown are: Eastern white pine, Virginia pine \((P. \text{ virginiana} \,\text{Mill.})\), Eastern red cedar \((\text{Juniperus virginiana} \,\text{L.})\), Leyland cypress \((\times \text{Cupressocyparis leylandii} \,'\text{Leighton Green}')\), ‘Carolina Sapphire’ Arizona cypress \((\text{Cupressus arizonica} \,\text{var. glabra})\), ‘Blue Ice’ Arizona cypress \((\text{Cupressus glabra})\), ‘Green Giant’ arborvitae \((\text{Thuja L.} \times \text{‘Green Giant’})\), Atlantic white cedar \([\text{Chamaecyparis thyoides} \,(\text{L.}) \,\text{Mills.}])\) and various spruces \((\text{Picea} \,\text{spp.})\).

\textbf{Figure 2}. Christmas Trees in the southern Appalachian Mountains. Fraser fir \((\text{Abies fraseri})\)

Christmas trees in Grayson County, Virginia (A). Fraser fir progeny test in Ashe County, North
Production of Christmas trees and greenery

Christmas trees are an important and valuable specialty crop. In 2016, the Christmas Tree Growers Council of Europe (CTGCE) surveyed Christmas tree production in member countries and estimated that 120,000 hectares are planted with Christmas trees in Europe, a production of 75 million trees sold every year. Of those, 50 million were fir (Nordmann fir, noble fir and subalpine fir), 20 million spruce (Norway spruce and blue spruce) and 5 million pine [Scots pine and black pine (P. nigra Arn.)].

The annual revenue in Europe is approximately 1.76 billion USD (1.5 billion EUR) (Danske Juletræer, 2017b). The top six Christmas tree producing countries are:

- Germany: 24 million trees/year
- Denmark: 12 million trees/year
- Poland: 6.5 million trees/year
- England: 5 million trees/year
- France: 4.5 million trees/year
- Belgium: 4.0 million trees/year

In Denmark, there are around 3500 growers, and the majority (90%) of the trees produced are exported (Danske Juletræer, 2017a). In Norway, Christmas tree farming is increasing in popularity and 1.2 million trees were sold in 2016. Due to the small size of the Swedish
Christmas tree industry, no reliable domestic estimates are available for the number of Christmas trees sold, land area cultivated, number of growers, or how many trees are imported annually. However, because Sweden is an important market for Danish Christmas tree export, the Danes have estimated Swedish consumption for 2016 at roughly 3.3 million Christmas trees (Claus Jerram Christensen, Danske Juletræer, pers. comm.). Of the trees consumed in Sweden, approximately 60% were Norway spruce, 30% Nordmann fir, 6% other Christmas tree species, and 4% plastic trees (though the accuracy of these number is uncertain). About 0.5 million trees (the majority Nordmann fir) were imported from Denmark. The Swedish production of fir is roughly 0.5 million trees. All the fir seedlings planted in Sweden are imported.

In North America, approximately 40 million Christmas trees are sold each year and the majority, 25-36 million trees, are produced in the US. The revenue from US Christmas tree production totals approximately 506 million USD (430 million EUR) (National Christmas Tree Association, 2017a; Chastagner & Benson, 2000). In the US, all of the 50 states produce Christmas trees and about 100,000 people are employed in the industry. According to the United States Department of Agriculture (USDA, 2012), the states with the largest production are:

- Oregon: 6.4 million trees/year
- North Carolina: 4.3 million trees/year
- Michigan: 1.7 million trees/year
- Pennsylvania: 1.0 million trees/year
- Wisconsin: 0.6 million trees/year
- Washington: 0.6 million trees/year
Based on a different estimate (Napier & Sidebottom, 2011), North Carolina alone harvests 5-6 million Christmas trees annually, with a wholesale value of over 100 million USD.

**Diseases that limit the Christmas tree production**

Christmas tree growers face a number of disease (primarily fungi) and pest (primarily insect and mite) problems. In Denmark and Norway, the most prominent diseases limiting fir production are current season needle necrosis (CSNN) (Talgø et al., 2010), Delphinella shoot blight [Delphinella abietis (E. Rostrup) E. Müller] (Talgø et al., 2016) and Neonectria canker [Neonectria neomacrospora (C. Booth & Samuels) Mantiri & Samuels] (Nielsen et al., 2017; Skulason et al., 2017; Talgø et al., 2010). These diseases result in needle discoloration and needle cast, shoot blight and cankers, respectively. In northern Europe, the most prominent pest problems are caused by the silver fir woolly adelgid (Adelges nordmannianae Eckst.), the balsam woolly adelgid (D. piceae Ratz.), Adelges pectinatae (Cholodkovsky), and gall mites (Nalepella shevtchenkoi Boczek and N. danica Boczek, Harding & Shi). All cause needle discoloration, needle and shoot deformation and needle cast, and can kill trees when population pressure is high (Sundbye et al., 2015). Both disease and pest problems limit the production and marketability of Christmas trees.

In Sweden, very little information was available on diseases and pests in Christmas tree fields prior to this study. No surveys had ever been conducted.

In North America, Phytophthora root rot and stem canker, CSNN, and interior needle blight are the most prominent diseases limiting production of Fraser fir and noble fir. The worst pest problems are balsam woolly adelgid, balsam twig aphid (Mindarus abietinus Koch) and spruce spider mites (Oligonychus ununguis Jacobi) (Chastagner & Benson, 2000). In North
Carolina, Phytophthora root rot and balsam woolly adelgid are the main limiting factors Fraser fir production.

For the insect and mite problems, pesticides are available. For *Phytophthora* species, of which many are serious plant pathogens, there are chemical controls for seed and transplant beds, but no chemical controls that are economically feasible for field use.

**Phytophthora**

The genus *Phytophthora* was first described by the German mycologist Anton de Bary, who studied the potato blight pathogen [*Phytophthora infestans* (Mont.) de Bary]. The name *Phytophthora* originates from Greek and means “the plant-destroyer” (*phytón* = plant and *phthorá* = destruction), which is suitable since *Phytophthora* is the cause of some of the most devastating diseases of woody plants worldwide (www.ForestPhytophthoras.org).

**General information about Phytophthora**

The *Phytophthora* genus contains many major plant pathogens. They have fungus-like structures, such as spores and mycelia, but are not classified in the kingdom of Fungi. Instead, *Phytophthora* belongs to the phylum Oomycota, in the kingdom Stramenopila. They are so-called “water molds” and more closely related to brown algae than fungi, which is reflected in their preference for wet environments such as saturated soils and moisture on foliage (Ribeiro, 2013; Erwin & Ribeiro, 1996).

*Phytophthora* species can be soil-borne and/or airborne. During favorable conditions, such as rainfall and flooding events, *Phytophthora* release spores (“zoospores”) that are moved by water (e.g., rain splash and runoff). The zoospores are motile in saturated soil and can swim short distances toward plant roots, being attracted by the root exudates (Erwin & Ribeiro, 1996).
During unfavorable conditions, such as droughts and lack of water, *Phytophthora* forms thick-walled spores, chlamydospores and oospores, in infected roots, organic debris and soil. These spores are resting structures that can survive for decades waiting for better conditions. It is therefore very difficult to eradicate *Phytophthora* once it has been introduced to a new habitat (Erwin & Ribeiro, 1996).

There are approximately 150 formally and informally described species of *Phytophthora* (Jung et al., 2016). The majority are plant pathogens, breaking down and consuming live and/or dead plant tissue, and causing the death of roots, stems and leaves on a wide variety of annual crops as well as perennial shrubs and trees (Ribeiro, 2013; Kroon et al., 2012). While some species have a narrow host range, others are so-called “biological bulldozers” (Scott et al., 2013) and can attack hundreds of different host plants. *Phytophthora* has been estimated to cause more than 60% of the fine root damage and approximately 90% of the collar rots of woody plant species globally (Jung et al., 2016), resulting in large-scale economic losses in agriculture and forestry, and is a threat to many natural ecosystems (Lamour, 2013; Erwin & Ribeiro, 1996).

Several of the most serious forest disease epidemics are caused by *Phytophthora* species, e.g. the jarrah forest dieback in western Australia caused by *P. cinnamomi* Rands (Hee et al., 2013; Shearer & Tippett, 1989), sudden oak death in California caused by *P. ramorum* (Balci & Bienapfl, 2013), dieback of alder in Europe caused by *P. alni* Brasier & S.A. Kirk (Érsek & Man in’t Veld, 2013), extensive mortality of *Larix* species in the UK and Ireland caused by *P. ramorum* (Brasier & Webber, 2010), mortality of *Austrocedrus chilensis* (D. Don) Florin & Bout in Patagonia caused by *P. austrocedrae* Gresl. & E.M. Hansen (Greslebin et al., 2007), the pine needle and shoot blight of *Pinus radiata* D. Don in Chile caused by *P. pinifolia* Alv. Durán,
Gryzenh. & M.J. Wingf. (Duran et al., 2008), and littleleaf disease of Pinus species in southeastern USA caused by P. cinnamomi (Oak & Tainter, 1988).

**Many Phytophthora species are highly invasive**

In their natural habitats, Phytophthora species are not aggressive pathogens since native plants are evolutionarily adapted to cope with them. When Phytophthora species are introduced to new habitats where plants have not evolved any defense mechanisms, however, they can cause great damage. If the environmental conditions are right, i.e. allow for survival and reproduction, Phytophthora species have the potential to destabilize entire ecosystems. Furthermore, Phytophthora species are very adaptive, and can form hybrids, which can create new combinations of characteristics potentially enabling the development of new and devastating forest diseases (Burgess, 2015; Brasier et al., 2004). One notable example is P. alni causing dieback of alder trees (Alnus spp.) in Europe (Redondo et al., 2015). Hybridization puts more trees at risk, especially those that lack immunity (resistance) to Phytophthora species.

**Global nursery trade – the root of the problem**

The global trade of plant material is responsible for spreading Phytophthora all over the world, despite regulations like phytosanitary certification. Phytophthora hitchhikes with plants or the growth media they are rooted in (Jung et al., 2013; Brasier, 2008; Erwin & Ribeiro, 1996; Shearer & Tippett, 1989), and infested nursery stock carrying Phytophthora is well-documented (Bienapfl & Balci, 2014; Parke et al., 2014; Perez-Sierra & Jung, 2013; Jung, 2009; Moralejo et al., 2009; Yakabe et al., 2009; Schwingle et al., 2007; Davison et al., 2006; Orlikowski et al., 2004; Themann et al., 2002; Lilja et al., 1996; MacDonald et al., 1994; Hardy & Sivasithamparam, 1988). The movement of soil and plants is generally considered the major
pathway for spread of *Phytophthora* species. Limiting the introduction of *Phytophthora* through inspection of imported plants is therefore extremely important.

**Phytophthora root rot in Christmas tree fields in North Carolina**

In the US, Phytophthora root rot is one of the most devastating diseases affecting Christmas tree production (Fig. 3). *Abies* species in particular, in both Christmas tree plantations and nurseries, are affected. In the southern Appalachian Mountains of North Carolina, *Phytophthora cinnamomi* has been the major cause of Phytophthora root rot for decades (Benson & Grand, 2000; Grand & Lapp, 1974). It is most prevalent in poorly drained soils, and losses of 75% have been recorded for individual fields (Benson & Grand, 2000). The average incidence of Phytophthora root rot was estimated at 9% (Benson & Grand, 2000). Based on a 100 million USD industry, annual losses due to Phytophthora root rot total approximately 9 million USD.

In North Carolina, root rot caused by *P. cinnamomi* was first reported in 1963 on Fraser fir seedlings in a nursery bed in Penrose (Kuhlman & Hendrix, 1963). The authors warned of the possibility of transferring *P. cinnamomi* by infested soil on the roots of the Fraser fir seedlings to Christmas tree production sites. This is exactly what happened. Growers bought the locally produced seedlings and Phytophthora root rot dramatically increased in the region. Eventually, this led growers to import out-of-state grown transplants and containerized seedlings instead of locally produced material. It is also well-known that other regions where seedlings are imported from have other *Phytophthora* species that cause losses in their Christmas tree production (McKeever & Chastagner, 2016). There was therefore concern about introducing new *Phytophthora* species into North Carolina on the imported plant material, which formed the background for the studies presented in **CHAPTERS 1, 2 and 3.**
Phytophthora cinnamomi is also a problem in the Piedmont and Coastal Plain regions of North Carolina, where Eastern white pine is the most cultivated Christmas tree. Eastern white pine along with other conifers is planted in the Piedmont and Coastal Plain regions because Fraser fir cannot be cultivated there due to the warm climate. In the southern Appalachian Mountains, Eastern white pine is known to have some tolerance to Phytophthora infection and is planted on sites where Fraser fir cannot be grown due to Phytophthora root rot, i.e. mainly wet poorly drained sites. However, in the Piedmont and Coastal Plain regions, Eastern white pine seems to be more susceptible to Phytophthora root rot than in the mountains.

Figure 3. Phytophthora root rot causing losses of Fraser fir in the southern Appalachian Mountains, North Carolina. Characteristic symptoms are tree mortality in the field (A), flagging of basal branches (B), cambial stem lesion with distinct borders between healthy and diseased tissue (C), and heavily infected root systems with sloughing necrotic roots and absence of fine roots (D). Photos: Martin Pettersson

Phytophthora root rot in Christmas tree fields in Sweden

No Phytophthora infection was reported in Swedish Christmas tree fields prior to the work presented in CHAPTER 5. Elsewhere in Europe, Phytophthora root rot in Christmas tree fields
has not been extensively studied. *Phytophthora* species have, however, been reported on Christmas trees in Norway (Talgø *et al.*, 2007; Talgø *et al.*, 2006) and Ireland (Shafizadeh & Kavanagh, 2005). In Norway, *P. cambivora* (Petri) Buisman was found on noble fir, *P. megasperma* Drechsler on subalpine fir, and a *P. inundata*-like species on Nordmann fir. In Ireland, *P. cryptogea* Pethybr. & Laff., *P. cinnamomi*, *P. cambivora* and *P. megasperma* were found on noble fir.

Over 20 *Phytophthora* species have also been found on a variety of different conifers in nurseries and forest plantings around Europe (Jung *et al.*, 2016). Therefore, *Phytophthora* poses a great threat to Christmas tree and bough production in European countries. Since most fir seedlings are imported into Sweden as bare-root plants, and many fir species are highly susceptible to *Phytophthora*, the Christmas tree industry may be at risk for introducing and spreading *Phytophthora* in Sweden.

**Management of Phytophthora root rot**

Since Phytophthora root rot is a soil-borne disease with hardy spore stages, it is almost impossible to get rid of it once it has been introduced to a field. Therefore, the most important preventative measures Christmas tree growers can use are site selection and use of only healthy seedlings. Seedlings that appear unhealthy should not be planted. There are easy-to-use kits for rapid field-diagnostics of *Phytophthora*, and the NCSU Cooperative Extension Service has trained Christmas tree growers in North Carolina how to use such test kits (see **CHAPTER 2**). Even though these kits are not 100% reliable, as they can cross-react with a few specific *Pythium* species, they provide an excellent tool for growers and nursery personal to test seedlings prior to planting (Lane *et al.*, 2007). Any plant tissues suspected of infection with *Phytophthora* species, can be tested on-site and results are obtained within a few minutes. This means symptomatic
seedlings with *Phytophthora* infection can be detected before being planted in the fields, helping to prevent the spread of disease.

Poorly drained soils, wet areas in the fields or fields that can be flooded by nearby streams and rivers, should not be planted with Christmas trees because they are likely to become diseased with Phytophthora root rot (Chastagner & Benson, 2000). Different fir species vary in their sensitivity to Phytophthora root rot. Fraser, noble, balsam, grand [A. *grandis* (Dougl.) Lindl.], red (*A. magnifica* A. Murr.) and Shasta (*A. magnifica var. shastensis* Lemmon) firs are among the most susceptible species (Frampton & Benson, 2012; Chastagner & Benson, 2000). Less susceptible species are Eastern white pine, Nordmann, Turkish and momi (*A. *firma* Sieb. et Zucc.) firs (Frampton & Benson, 2012; Chastagner & Benson, 2000). The less susceptible species can be planted as substitute species on sites that are prone to Phytophthora root rot. A costlier alternative is to graft a susceptible fir onto rootstock of a more resistant fir.

In nurseries, the recycling of irrigation water or use of water from nearby streams or rivers should be avoided since they are commonly contaminated with *Phytophthora* inoculum (Hong & Moorman, 2005). However, these water sources can be used if the water can be decontaminated, e.g. by UV light treatment (Zheng *et al.*, 2014). High soil moisture in nursery and transplant beds can be avoided by installing drain tiles. Phytophthora root rot in nursery beds can be controlled with chemical pesticides through soil fumigation or by other soil treatments, e.g. using Subdue (metalaxyl) or Aliette (fosetyl aluminum) as a soil drench (Chase, 1993). Other methods for sterilizing contaminated soil are steam or hot water treatment (McGovern & McSorley, 1997). However, no available chemicals can cure *Phytophthora*-infected seedlings; they can only suppress symptom development. There is therefore, a risk that seemingly healthy
nursery stock may introduce *Phytophthora* species to Christmas tree plantations (latent infection).

Another solution for managing *Phytophthora* species in nurseries, is to replace bare-root production with containerized seedlings lifted off the ground, e.g. on raised benches. In such production, containerized plants can be grown in potting mixtures of organic and inorganic materials, such as peat, perlite and vermiculite. This method has been used for some of the Christmas tree seedlings produced in North Carolina (Jill Sidebottom, NCSU, pers. comm.).

A more long-term goal to combat Phytophthora root rot is resistance breeding of Christmas tree species, perhaps in combination with genetic engineering (CHAPTER 1). The end goal is to incorporate the most popular Christmas tree species with a broad resistance to as many *Phytophthora* species as possible. It is therefore important to know exactly which *Phytophthora* species are contributing to Christmas tree mortality.

**Neonectria**

The genus *Neonectria* was first described in the 1800s and consists of a group of fungal species defined by a *Neonectria* perfect (ascosporic, sexual) and a *Cylindrocarpon* imperfect (conidial, asexual) state (Chaverri et al., 2011; Castlebury et al., 2006). Many species in the genus *Neonectria* were previously listed under the genus *Nectria*, but have been reassigned based on improved molecular phylogenetic analyses (Chaverri et al., 2011; Castlebury et al., 2006). Some species in the *Neonectria* genus are plant pathogens that cause diseases on conifer and hardwood trees (Uimari et al., 2018; Nielsen et al., 2017; Castlebury et al., 2006; Halleen et al., 2006; Hirooka et al., 2005; Kobayashi et al., 2005).
General information about *Neonectria*

Worldwide, around 50 *Neonectria* species have been identified (Kirk & Cooper, 2010; Robert et al., 2005). The genus *Neonectria* belongs to the Ascomycota phylum in the kingdom Fungi. In northern Europe, three species of the genus *Neonectria* are particularly known to cause economic losses to broadleaf and conifer trees: *N. ditissima* (Tul. & C. Tul.) Samuels & Rossman, *N. neomacrospora* and *N. fuckeliana* (C. Booth) Castl. & Rossman (Børve et al., 2018; Nielsen et al., 2017; Skulason et al., 2017; Talgø et al., 2017; Kirk & Cooper, 2010; Robert et al., 2005; Swinburne, 1975; Roll-Hansen, 1962). All are genetically closely related (Lombard et al., 2014; Chaverri et al., 2011; Castlebury et al., 2006), and *Neonectria fuckeliana* naturally occurs in the northern hemisphere (Booth, 1979; Booth, 1966; Roll-Hansen, 1962; Booth, 1959). Regarding *N. ditissima* and *N. neomacrospora*, even though they are found in Europe, the geographical origin is unclear. The common name for the disease caused by *N. ditissima*, *N. neomacrospora* and *N. fuckeliana* is Neonectria canker. Symptoms such as canker wounds, top and branch dieback and death of trees are observed throughout northern Europe (Børve et al., 2018; Uimari et al., 2018; Nielsen et al., 2017; Pérez-Sierra et al., 2016; Weber, 2014; Lilja et al., 2012) (Fig. 4A-C). In addition, resin-flow commonly occurs on infected conifers (Fig. 4D). All are wound-invading fungi that colonize new host trees by airborne, sexual spores (ascospores) from fruiting bodies (perithecia) or splash-dispersed asexual spores (conidia) produced on sporodochia (Skulason et al., 2017; Vasiliauskas & Stenlid, 1997; Roll-Hansen & Roll-Hansen, 1979; Swinburne, 1975) (Fig. 4D-H).

**Neonectria canker caused by *Neonectria fuckeliana* on spruce trees**

*Neonectria fuckeliana* has been recognized as a weak pathogen that enters wounds on Norway spruce, and has frequently been detected in stems of older trees (Vasiliauskas et al., 1996; Huse,
1981; Roll-Hansen & Roll-Hansen, 1979; Roll-Hansen, 1962). However, it has recently been
associated with canker wound, resin flow, top-dieback and mortality of young trees where no
pre-wounding was obvious. In eastern Finland, several hundred hectares of young Norway
spruce forest plantations (5-30 years old) have been infected with *N. fuckeliana* (Uimari et al.,
2018; Lilja et al., 2012). Reports of 13% and 37% of Finnish and Polish provenances,
respectively, had dying tops and blackened canker wounds associated with *N. fuckeliana* (Lilja et
al., 2012). In Norway and Denmark, the fungus has been associated with top-dieback in young
spruce stands (Talgø et al., 2017; Thomsen et al., 2016). In Northern Ireland, *N. fuckeliana* has,
since 2012, been thought to play a part in the mortality of Sitka spruce [*P. sitchensis* (Bong.)
Carr.] at several sites spread across the entire region (Richard O’Hanlon, Agri-Food and
Biosciences Institute, pers. comm.). Sitka spruce is an important exotic forest tree species in the
region.

*Neonectria fuckeliana* has not been extensively studied, and there are gaps in knowledge
concerning its basic biology, pathogenicity and infection mechanisms. Even the taxonomic status
of this species has changed several times (Castlebury et al., 2006; Booth, 1959), and it has
recently been suggested that it belongs to a completely new genus (González & Chaverri, 2017).
However, the incidence of *N. fuckeliana* seems to have increased in northern Europe over the last
ten years (Uimari et al., 2018; Talgø et al., 2017; Lilja et al., 2012). In Sweden, it is unclear
whether *N. fuckeliana* has caused any epidemic disease outbreak, but the fungus was frequently
detected in Norway spruce Christmas tree plantations in 2015 (*CHAPTER 6*).

**Neonectria canker caused by Neonectria neomacrospora on fir trees**

*Neonectria neomacrospora* is an aggressive pathogen on fir species that causes shoot-tip
necrosis, branch dieback, heavy resin flow, and often mortality (Talgø et al., 2018; Thomsen &
Nielsen, 2018; Chastagner et al., 2014). In Denmark and Norway, the fungus has caused large-scale dieback of forest stands, provenance trials, seed orchards, Christmas trees, bough plantations, and landscape plantings such as arboreta (Talgø et al., 2018; Thomsen & Nielsen, 2018; Nielsen et al., 2017; Skulason et al., 2017). It is also an emerging disease in the US (Chastagner et al., 2014) and UK (Pérez-Sierra et al., 2016). It was also recently reported in Belgium (Schmitz et al., 2017). In Sweden, no _N. neomacrospora_ epidemic has yet been reported, but the fungus was recently detected there (CHAPTER 6). Currently, there is limited information available about _N. neomacrospora_.

**Neonectria canker caused by _Neonectria ditissima_ on broadleaf trees**

_Neonectria ditissima_ causes girdling cankers and dieback of many deciduous tree species (Farr et al., 1989; Flack & Swinburne, 1977). In Norway, it has also been found on the evergreen broadleaf holly (_Ilex aquifolium_ L.) (Talgø et al., 2012). The largest economic damage occurs in commercial apple (_Malus x domestica_ Borkh.) and pear (_Pyrus communis_ L.) orchards (Weber, 2014; Farr et al., 1989; Flack & Swinburne, 1977; Swinburne, 1975). In northern Europe, _N. ditissima_, together with apple scab [Venturia inaequalis (Cooke) G. Winter], are the most serious diseases on apples (Weber, 2014). In Swedish apple production, _N. ditissima_ is the most serious disease-causing pathogen (Garkava-Gustavsson et al., 2018). There is therefore an extensive amount of information available about _N. ditissima_.

Figure 4. Plant symptoms and pathogen signs of Neonectria canker caused by the fungus *Neonectria fuckeliana*. Norway spruce Christmas tree with top-dieback where the fungus *N. fuckeliana* was isolated from the margin between dead and live tissue (A). Canker wound (B-C), resin flow (D), perithecia (sexual fruiting bodies) (D-E) with white spore tendrils coming out (F), sporodochia (asexual fruiting bodies) (G), cultures with mycelial growth containing conidia on potato-dextrose agar. Photos: Martin Pettersson

Scope, aim and research questions

A large amount of knowledge about Christmas tree diseases and pests is now available from many parts of the world, the majority from North America and Europe, including handbooks, factsheets, manuals, articles and websites dealing with Christmas tree disease and pest management. However, much of this knowledge was not available in Sweden when the research presented here began. Some projects were therefore undertaken first in North Carolina to gain knowledge and experience from a well-functioning Christmas tree industry.
CHAPTER 1 presents the results from a questionnaire survey of local Christmas tree growers about the history of Phytophthora root rot on Fraser fir in western North Carolina. The survey focuses on a shift where growers switched from locally produced bare-root seedlings to out-of-state-grown planting stock, and the extent to which Phytophthora root rot impacts today’s Fraser fir production in the southern Appalachians.

As mentioned in the Phytophthora section above, in western North Carolina, there was concern that new Phytophthora species may have been introduced into the main Christmas tree producing regions of the southern Appalachian Mountains through imported plant materials. This could potentially lead to increased economic losses for the growers. Thus, the aim of CHAPTER 2 was to investigate which Phytophthora species were associated with and contribute to Fraser fir Christmas tree losses in western North Carolina and adjacent states. Such information is important when providing management recommendations to growers in order to restrict and minimize the spread and effect of alien Phytophthora species.

In the Eastern white pine Christmas tree production in the Piedmont and Coastal Plain regions of North Carolina, where Phytophthora root rot caused by \textit{P. cinnamomi} has increased due to contaminated nursery stock, less susceptible Eastern white pine seedlings are needed to minimize losses. The aim of CHAPTER 3 was to determine whether there was variation in susceptibility to \textit{P. cinnamomi} between different Eastern white pine families. The long-term goal was to reduce Phytophthora root rot damage by selecting and cultivating only Eastern white pine families with the highest tolerance to \textit{P. cinnamomi}.

In North Carolina, both the NCSU Cooperative Extension Service and NSCU Christmas Tree Genetics Program are devoted to supporting the state’s Christmas tree growers. There are numerous extension agents helping North Carolina’s 1300 Christmas tree growers to produce the
best possible trees. This has helped to shape a successful multimillion dollar Christmas tree market, where North Carolina exports Fraser firs all around the US from October to December (NCCTA, 2015).

In Sweden, the opposite is true. Christmas tree farming is still largely a small-scale venture for the curious. Most of the seedlings available for growers who cultivate fir are imports from other countries, since few Swedish nurseries are able to offer locally produced fir seedlings for Christmas tree production. Sweden’s only Christmas tree growers association (Sydsveriges Julgran och Pyntegröntodlarförening) has approximately 100 members. Sweden is not a member of the Christmas Tree Growers Council of Europe and there are no extension agents or research programs to help the growers. The growers in Sweden are therefore mostly self-taught, though a few belong to the Danish Christmas Tree Association, whose members have access to assistance from Danish extension agents. Most Swedish Christmas tree growers lack support and much-needed information about disease and pest problems. Prior to this study, no disease and pest survey had ever been conducted in Swedish Christmas tree fields. Therefore, the initial aim of the Swedish activities of this dissertation was to find out which, if any, diseases or pests were affecting the Swedish Christmas tree industry. A number of biotic and abiotic problems were discovered (Pettersson et al., 2015), but the main focus had to be narrowed down to the most problematic of these.

Since Phytophthora species are commonly spread via nursery planting stock, and many fir species are highly susceptible, the increased import of bare-root fir plants for Christmas tree production in Sweden poses a risk for both the Christmas tree industry and the forest sector as a whole. This is especially true since such problems are known from our neighbor, Norway (Talgø
et al., 2007; Talgø et al., 2006). Therefore, a study was implemented to investigate whether *Phytophthora* species were present in or near Swedish Christmas trees fields (CHAPTER 5).

Since *Neonectria* canker is currently causing dieback in Denmark and Norway, another aim of the study was to investigate whether *Neonectria* canker was a problem on fir and spruce Christmas trees in Sweden (CHAPTER 6). In CHAPTER 7, the focus is on Neonectria canker caused by *N. fuckeliana* on Norway spruce, and CHAPTER 8 describes the development and application of a species-specific real-time PCR-based test for rapid identification of *N. fuckeliana* from Norway spruce in northern Europe.

Thus, the main scope of the dissertation work is on *Phytophthora* root rot and *Neonectria* canker, two of the most important and emerging Christmas tree diseases in Europe, that also pose a threat to the forestry industry. The knowledge gained about *Phytophthora* and *Neonectria* will be useful in future research projects and for preventing and/or reducing diseases in Swedish Christmas trees fields through management recommendations.

The specific objectives and hypotheses were (to):

- Investigate the history and influence of Phytophthora root rot on Fraser fir planting trends in western North Carolina (CHAPTER 1). The hypothesis was that loss of Christmas trees due to Phytophthora root rot has increased since the last North Carolina Christmas Tree Pest Management Survey, and that a majority of the Christmas tree growers are today using out-of-state-grown planting stock.

- Determine and characterize *Phytophthora* species associated with symptomatic Fraser fir Christmas trees sampled in the southern Appalachian Mountains. This was done to be able to
restrict the spread and effect from new *Phytophthora* species in the future through advanced recommendations to growers (**CHAPTER 2**). The **hypothesis** was that several new-to-the-region *Phytophthora* species contribute to the losses in Fraser fir.

➢ Investigate variation of resistance among 83 Eastern white pine families to *P. cinnamomi* and determine if the resistance is under genetic control. A parallel objective within this study was to test differences in aggressiveness between one *P. cinnamomi* isolate derived from Fraser fir and another derived from Eastern white pine. The long-term goal is to reduce Phytophthora root rot damage by selecting and producing families with higher tolerance to *P. cinnamomi* available for use by the Christmas tree industry (**CHAPTER 3**). The **hypothesis** was that there is large variation in susceptibility to Phytophthora root rot among different Eastern white pine families.

➢ Investigate the occurrence of diseases, pests and nutrient deficiencies in Swedish Christmas tree fields (**2015 disease survey, CHAPTER 4**) (Pettersson *et al.*, 2015). This was done to get an overview of the situation and thereby enable the selection of focus areas for the dissertation. The **hypothesis** was that several diseases were present in the Swedish Christmas tree plantations.

➢ Investigate the presence of *Phytophthora* species in Swedish Christmas tree production of both fir and spruce species (**CHAPTER 5**). This was done to build a base for future research on *Phytophthora* in Swedish Christmas tree plantations. The **hypothesis** was that several pathogenic *Phytophthora* species were present in Swedish Christmas tree plantations.
➢ Investigate the role of Neonectria in Swedish Christmas tree production of both fir and spruce species and conduct Koch’s postulates for N. neomacrospora and N. fuckeliana on fir and spruce, respectively (CHAPTER 6). The hypothesis was that N. neomacrospora and N. fuckeliana were present on, and pathogenic to, fir and spruce, respectively, in Swedish Christmas tree plantations.

➢ Determine the ability of N. fuckeliana to cause disease on Norway spruce cuttings (CHAPTER 7). More specifically to:
  o Determine how different wound types affected the occurrence and severity of N. fuckeliana infections (carried out on 3-year old and 7-year old Norway spruce trees produced from cuttings).
  o Describe symptom development of N. fuckeliana infections on cuttings and determine whether symptom development correlated with field observations.

The hypothesis was that inoculation of Norway spruce with N. fuckeliana would result in similar top-dieback symptoms as seen in Christmas tree fields, and that seedling with larger wounds would develop symptoms faster.

➢ Develop a species-specific TaqMan real-time PCR assay to identify N. fuckeliana directly from infected plant tissue on trees (CHAPTER 8). The hypothesis was that a species-specific primer pair could be found and become a useful tool for detection of N. fuckeliana.
CHAPTER 1. Influence of Phytophthora Root Rot on Planting Trends of Fraser fir Christmas Trees in the Southern Appalachian Mountains

Citation:
Influence of Phytophthora Root Rot on Planting Trends of Fraser Fir Christmas Trees in the Southern Appalachian Mountains

Martin Pettersson, John Frampton, and Jill Sidebottom

Graduate Research Assistant, Department of Forestry and Environmental Resources, North Carolina State University (NCSU), Raleigh, NC; Professor, Department of Forestry and Environmental Resources, NCSU, Raleigh, NC; Mountain Conifer Integrated Pest Management Extension Specialist, Mountain Horticultural Crops Research and Extension Center, Mills River, NC

Abstract

The Southern Appalachian Mountains are home to the attractive Fraser fir [Abies fraseri (Pusch) Poir.] that began to be cultivated for Christmas trees in the 1950s. Today, 5 to 6 million trees are harvested annually in this region, yielding a wholesale value of more than $100 million. Since the 1960s, however, Phytophthora root rot has been a problem for Christmas tree production in this region. This article gives a brief history of Fraser fir cultivation and how Phytophthora root rot has influenced planting practices. It also presents the results from surveys of Christmas tree growers about planting trends and their perspectives of the Phytophthora disease problem. Even though most growers have shifted from using locally produced bareroot seedlings to out-of-State-grown planting stock, Phytophthora root rot continues to have a major impact on Fraser fir plantations, and new Phytophthora species have recently been found on Fraser fir.

Introduction

The Southern Appalachian Mountains are home to one of the largest Christmas tree production regions in the United States. Collectively, North Carolina, Tennessee, and Virginia account for 17 percent of the 309,363 acres (125,195 ha) of the Nation’s land used for Christmas tree cultivation (USDA NASS 2014). North Carolina is the second-largest producing State, annually harvesting 5 to 6 million trees that produce a wholesale revenue of more than $100 million (Napier and Sidebottom 2011). Fraser fir (Abies fraseri [Pusch] Poir.) is the main species cultivated in the region, where it naturally grows in a small number of island-like populations on high ridges, mostly above 4,250 feet (1,300 m) (Busing et al. 1993). Christmas tree production sites are generally below the natural elevational range of Fraser fir, down to about 3,000 ft (910 m). Fraser fir has beautiful dark green foliage, a pleasant aroma, and excellent post-harvest needle retention that have made it highly desirable as a Christmas tree (Chastagner and Benson 2000).

Fraser Fir Planting Stock—The Early Years

The Fraser fir Christmas tree industry in western North Carolina and surrounding States began in the 1950s when the Toecane Ranger District of the Pisgah National Forest opened up portions of the Roan Highlands for the harvest of fir boughs and cut trees. Due to the superior quality of Fraser fir as a Christmas tree, growers became interested in planting them, which required a supply of seed and seedlings. Seed was collected primarily from Roan Mountain along the North Carolina-Tennessee border. Growers also lifted (“pulled”) wild seedlings (“wildlings”) or collected seed from other wilderness areas with, and sometimes without, permission. For instance, in the 1956–1957 planting season, the U.S. Department of Agriculture (USDA) Forest Service permitted the Haywood County 4-H Council to lift Fraser fir seedlings from thick natural reproduction under a virgin fir stand near Burnsville to use as planting stock. Landowners in the Mount Rogers area in Virginia also pulled Fraser fir seedlings and sold them (Sidebottom 2011).

In 1955, the North Carolina Forest Service agreed to produce Fraser fir seedlings. To hasten production, these were originally gathered as wildlings from natural stands, primarily near Mount Rogers, VA. The climate at the two nurseries used initially—Holmes State Nursery near Hendersonville and Ralph Edwards Nursery in Morganton—was too warm to produce good-quality Fraser fir. Therefore, the North Carolina
Forest Service established a seed orchard and a seedling production facility at the Linville River Nursery near Crossnore (Avery County) and began seedling production there in 1968 (Sidebottom 2011).

Fraser fir seedlings were also available from commercial nurseries in other States as early as the 1960s. Fraser fir seedlings were advertised for sale in what became the American Christmas Tree Journal (produced by the National Christmas Tree Association) from at least two nurseries in Pennsylvania and one in Maine. Even with these sources, limited seedling supply would plague the developing Fraser fir industry through the 1970s. The main limiting factor for Fraser fir Christmas tree production in western North Carolina during the 1970s was the lack of Fraser fir stock ready for field planting. Because of this insufficient supply, extension programs and North Carolina Christmas Tree Association meetings focused on teaching growers how to grow their own seed in beds and to line-out transplants. As a result, this practice became widespread in the region (figure 1). Simultaneously, in 1977 and 1978, five contractors lifted 1.5 million wildlings from Roan Mountain. The number of wildlings taken from Roan Mountain remained high through 2000, with about 500,000 seedlings pulled each year (Sidebottom 2011).

**Emergence and Spread of Phytophthora Root Rot**

As Fraser fir planting in the region expanded during the 1960s and 1970s, growers began to recognize a number of disease and insect problems. Particularly challenging was Phytophthora root rot, a disease first reported on Fraser fir in 1963 on nursery seedlings in Penrose (Transylvania County, NC). *Phytophthora cinnamomi* Rands was identified as the causal agent (Kuhlman and Hendrix 1963). *Phytophthora* are fungus-like organisms belonging to the class Oomycetes (water molds). *P. cinnamomi* is exotic to the region, originating from Southeast Asia, where it was first described from cinnamon plants in Sumatra (Zentmyer 1988). It is believed to have been brought into the United States through southern ports during the 1800s or earlier on exotic plants destined for gardens of antebellum estates (Crandall et al. 1945). A map published in 1945 (Crandall et al.) showed the observed range of root rot caused by *P. cinnamomi* on American chestnut (*Castanea dentata* [Marsh.] Borkh.) and chinquapin species (*Castanea* spp.) and clearly demonstrates that this pathogen had been introduced into the Southern Appalachian region well before the start of the Fraser fir Christmas tree industry. *Phytophthora* root rot affects all sizes and ages of Fraser fir. Symptoms include flagging of lower branches, stem cankers or cambial lesions with distinct borders, foliar chlorosis, reddening or browning of needles, diminished growth, and wilting of new growth, as well as darkened, sloughing, and necrotic roots (figure 2). Dying roots

Figure 1. Starting in the mid-1970s, extension programs focused on teaching Christmas tree growers how to sow their own Fraser fir seed in bareroot beds and to line-out transplants. (Photo courtesy of James McGraw, North Carolina State University, retired, Jackson County, NC, 1976b)

Figure 2. *Phytophthora* root rot inflicts a significant economic impact on the Fraser fir Christmas tree industry of the Southern Appalachian region. Characteristic symptoms of *Phytophthora* root rot on Fraser fir include: (a) tree mortality, (b) flagging of basal branches, (c) cambial stem lesion with distinct borders, and (d) heavily infected root systems with sloughing necrotic roots and absence of fine roots. (Photos by Martin Peterson, 2015)
and girdling stem infections result in decreased water and nutrient translocation and often lead to a weakened tree and eventual death (Chastagner et al. 1995, Chastagner and Benson 2000).

*Phytophthora* species can rapidly spread in saturated and waterlogged soils or by splashing rain, subsurface water flow, and run-off water. Heavy rains and flooding conditions accelerate the spread. In addition to water movement, *Phytophthora* species can be introduced into new fields by infected planting stock, contaminated agricultural tools, vehicle tires, field workers' shoes, and animals. In nurseries, *Phytophthora* species can infect all plants if the irrigation water is taken from contaminated streams or surface water and not sterilized or filtered prior to use. Irrigation and rain can splash contaminated soil from one infected seedling onto surrounding seedlings, which may also become infected. In the nursery, seedlings experience optimal conditions (i.e., they grow in well-drained, nutritive soils, under optimal temperature), and therefore may be less prone to display disease symptoms, especially when dormant. Furthermore, fungicides do not always kill *Phytophthora* species, so that diseased plants are often not recognized until they have been lifted and planted in the field.

Two investigations of the incidence of *Phytophthora* root rot in Fraser fir Christmas tree plantations in North Carolina suggest that this disease is common in the region. The first study was conducted in 1972 and average disease incidence due to *P. cinnamomi* in 14 Fraser fir plantations in 5 counties in western North Carolina was reported to be 9.6 percent (range = <1 to 90 percent)(Grand and Lapp 1974). In a more recent study, conducted in 1997 and 1998 (Benson and Grand 2000), the average disease incidence was similar (9 percent, range = 0 to 75 percent) in 58 Fraser fir plantations sampled in the same 5 western North Carolina counties. As in the earlier survey, all isolates from the field sites were identified as *P. cinnamomi*, except for one isolate of an unidentified *Phytophthora* species. In the more recent study, nursery transplant beds were also sampled and had a mean disease incidence of 2 percent (range = 0 to 12 percent). In addition to *P. cinnamomi*, *P. cactorum* (Leb. and Cohn) Schröeter, *P. destruens* Tucker, and an unidentified *Phytophthora* species were found on Fraser fir seedlings sampled in the nursery transplant beds.

In 2014, another study conducted across the Southern Appalachian region revealed that the diversity of *Phytophthora* species in Fraser fir Christmas tree plantations had increased (Pettersson et al. 2016). Six *Phytophthora* species were isolated from infected roots sampled from 82 sites in 13 counties (North Carolina, Tennessee, and Virginia). While *P. cinnamomi* remained the most prevalent species isolated (70 percent), *P. cryptogea* Pethyr. & Lafr. was relatively common (23 percent). Additionally, one or two isolates of four other species were found: *P. citrophthora* (R.E. Sm. & E.H. Sm.) Leonian, *P. europaea* Hans. & Jung, *P. pini* Leonian, and *P. sawomeeana* Leonian & Reeser.

**Fraser Fir Planting Stock—Shift to Out-of-State Sources**

As regional Christmas tree growers became more knowledgeable about the distribution and occurrence of *Phytophthora* root rot, they wanted to reduce the risk associated with contaminating clean fields with diseased planting stock. Growers gradually stopped producing their own planting stock or buying from local sources and began to import out-of-State sources of planting stock. Often seed was provided to the contracted out-of-State grower. This trend is revealed in the results of pest management surveys conducted during 1995 to 2014 by the North Carolina Cooperative Extension Service (table 1).

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Grow seedlings in outdoor beds</td>
<td>23.0</td>
<td>24.4</td>
<td>32.8</td>
<td>17.0</td>
</tr>
<tr>
<td>Didn't set Christmas trees this year in the field</td>
<td>5.3</td>
<td>30.7</td>
<td>28.2</td>
<td>30.0</td>
</tr>
<tr>
<td>Seedlings grown out of State in outdoor bed</td>
<td>Not Asked</td>
<td>14.8</td>
<td>24.8</td>
<td>59.0</td>
</tr>
<tr>
<td>Seedlings grown by NC grower in outdoor beds</td>
<td>Not Asked</td>
<td>27.4</td>
<td>15.1</td>
<td>14.0</td>
</tr>
<tr>
<td>Seedlings grown by NC Forest Service</td>
<td>34.2</td>
<td>19.0</td>
<td>14.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Seedling source was Rox River Mountain wildlings</td>
<td>14.0</td>
<td>19.2</td>
<td>6.3</td>
<td>3.0</td>
</tr>
</tbody>
</table>

NC = North Carolina
During the period of these surveys, the USDA Forest Service began limiting the number of wildlings removed from Roan Mountain, or in some years, not allowing removal of any wildlings. The survey results reflect this trend by the decreasing proportion of growers using wildlings from Roan Mountain from 1995 to 2014 (table 1). The USDA Forest Service is currently involved in a 5-year process of developing a long-term management plan for the Roan Mountain area of the Pisgah National Forest. Because of concerns with threatened and endangered species in the spruce-fir ecosystem, wild seedlings from Roan Mountain may not be available to the Christmas tree industry in the future.

Natural events also contributed to the shift toward the use of out-of-State planting stock. In September 2004, remnants of Hurricanes Frances and Ivan dropped record rainfall amounts across the region resulting in excessive soil moisture and flooding. The following spring, Phytophthora disease was widespread in Christmas tree plantations and the Linville River had spread Phytophthora inoculum through some fields of the State-run nursery. Ultimately, this nursery ceased production of bareroot Fraser fir seedlings and began producing a much smaller volume of containerized seedlings. The use of seedlings grown by the North Carolina Forest Service decreased from 34.2 percent in 1995 to 3 percent in 2014. Many growers found out-of-State contracting to be more reliable at the time when the local nurseries were in crisis.

2015 Survey of Planting Trends and Phytophthora Root Rot

In spring 2015, a survey was conducted at five different Christmas tree grower meetings in western North Carolina to determine how many growers use out-of-State Fraser fir seedlings and for how long they have done so. A total of 89 growers from 13 counties took the survey. Twenty-two of the growers had Christmas tree farms in more than one county, resulting in 123 farms from North Carolina, Tennessee, and Virginia included in the survey (figure 3). Most of the surveyed farms

![Figure 3](image-url)
were located in Avery County, NC. Of the growers surveyed, approximately 88 percent reported that they had Phytophthora root rot causing mortality in their Christmas tree fields. That is approximately 18 percent higher than what was reported in the 2006 North Carolina Christmas Tree Pest Management Survey.

On average, 64 percent of all growers surveyed were using out-of-State material (figure 4), with larger scale growers more likely to do so (figure 5). About 83 percent of the surveyed growers with more than 50 ac (>20.2 ha) of Christmas tree production were using out-of-State Fraser fir planting stock and 46 percent of these growers were purchasing seedlings from more than one State. Seventy-one percent of growers with 10 to 50 acres (4.0 to 20.2 ha) and 29 percent of growers with less than 10 ac (<4 ha) were using out-of-State planting stock. The out-of-State Fraser fir planting stock was bought from Oregon (41.2 percent), Washington (18.6 percent), Pennsylvania (17.5 percent), Michigan (17.5 percent), and Maine (5.2 percent). Clearly, a variety of locations produce the out-of-State material being planted in the Southern Appalachian Mountains.

More than half (57 percent) of the out-of-State planting stock purchased by the surveyed growers was bareroot seedlings. About 27 percent of surveyed growers purchased bareroot transplants that had been started in containers (plug+1) while only 16 percent purchased containerized seedlings grown exclusively in a greenhouse. Twenty-one percent of the growers responding to the survey reported using more than one type of planting stock.

Of the growers using out-of-State Fraser fir planting stock, 30 percent perceived an increased incidence of Phytophthora root rot in their fields since they began using out-of-State material, 47 percent said that the incidence of Phytophthora root rot had not changed, and 18 percent said that the incidence had decreased in their plantations (5 percent did not respond to this question).

![Graph showing the number of growers shifting to out-of-State planting stock over time.](image-url)

Figure 4. Christmas tree growers surveyed in western North Carolina (n=80) steadily shifted from locally produced Fraser fir planting stock to out-of-State stock starting in 1970, with the shift accelerating around 2000.
Figure 5. Over a 43-year period (1970 to 2013), 64 percent of Christmas tree growers surveyed in western North Carolina shifted from using locally produced to out-of-State planting stock. Because growers with more land were more likely to shift, about 80 percent of the land base managed by the surveyed growers was planted with out-of-State planting stock by 2013.

**Perspective**

Almost since its inception, the Fraser fir Christmas tree industry of the Southern Appalachian region has been afflicted by Phytophthora root rot. Although there is evidence that this exotic pathogen had previously been introduced into the region, undoubtedly the industry has contributed to its spread, especially through the movement of infested plant material. Once infested, land remains unsuitable for Fraser fir cultivation indefinitely. As Christmas tree growers understood the problem, they shifted toward importing out-of-State material to reduce the risk of contaminating sites with Phytophthora species, so that today only a small portion of Christmas tree plantations is regenerated with locally produced planting stock. Despite pursuing this strategy, Phytophthora root rot remains a menace to the regional Fraser fir industry. Most growers switching to out-of-State material perceive that their Phytophthora root rot problems have either stayed the same or increased since switching.

Of particular concern is the increased risk of introducing new Phytophthora species via importation of seedlings from bare-root nurseries and nurseries where containerized plants have been transplanted into outdoor beds. Most Phytophthora species are harmful plant pathogens that can cause serious and unpredictable, ecological and economic damage when they are introduced to a new environment. The Southern Appalachian climate, with its relatively warm soil temperatures and plentiful rainfall throughout the year, enhances the chance of survival and dissemination of many Phytophthora species. Further, the coexistence of multiple Phytophthora species in overlapping geographic areas increases the risk of another bleak problem—a hybridization event from which a more virulent race could evolve (Ersek and Nagy 2008).

The number of Phytophthora species isolated from Fraser fir in the Southern Appalachian region has recently increased and *P. cryptogea*, in particular, appears to have rapidly spread (Pettersson et al. 2016). Regions from which Fraser fir planting stock is imported (the Pacific Northwest, Great Lakes,
and Northeast) are known to have different Phytophthora species affecting fir, including P. cryptogea (McKeever and Chastagner 2016). For example, much of the planting stock (approximately 60 percent) used in the Southern Appalachians is produced in the Pacific Northwest where at least eight Phytophthora species have been reported to cause mortality in fir Christmas tree fields (Chastagner et al. 1990, 1995; Chastagner and Benson 2000). While most of these Phytophthora species have not yet been found in the Southern Appalachian region and there is no direct evidence that species have been introduced from other regions, vigilance is warranted because the nursery trade is known to contribute to the introduction and dispersal of plant pathogens (Jones and Baker 2007, Brasier 2008, McKeever and Chastagner 2016). Once introduced and dispersed in a new region, Phytophthora species have proven to be nearly impossible to control.

Christmas tree growers must be watchful to detect symptomatic plant material prior to and after planting. Symptomatic seedlings should be discarded; the cost of planting stock is inconsequential compared to the cost of losing Fraser fir production on a site due to the introduction of Phytophthora species. Today, suspect plant material can be evaluated with easy-to-use kits designed for rapid field-diagnosis of Phytophthora species. The North Carolina State University Cooperative Extension Service has been training regional Christmas tree growers on how to use these kits. Symptomatic seedlings may also be sent to a plant disease clinic for further verification and possible Phytophthora species identification. Growers must also employ good sanitation practices to prevent Phytophthora species spread from infested areas via equipment, vehicles, boots, water drainage, and other means.

Recently, a number of regional Christmas tree growers have begun greenhouse production of containerized Fraser fir seedlings. This movement is in its infancy and involves much experimentation with cultural aspects such as media, containers, lights, irrigation, etc. The results have been variable but some attempts are clearly on the path to achieve economically viable production systems. These efforts are encouraging and may provide a route to minimize the introduction of additional Phytophthora species to the region while also providing local income and reducing the cost of planting stock.

Although the use of genetically resistant material could offer a reasonable solution to this intractable problem, Fraser fir is generally highly susceptible to Phytophthora root rot and no useful level of resistance has been identified to the most prevalent species in the region, P. cinnamomi (Frampton and Benson 2012). Growers in the region commonly plant known infested areas with other species that have greater tolerance or resistance; eastern white pine (Pinus strobus L.), Canaan fir (A. balsamea var. phanerolepis Fern.), Nordmann fir (A. nordmanniana (Steven) Spach.), and Turkish fir (A. bornameilleriana Mattf.). Compared with Fraser fir, however, these species are generally less valuable as Christmas trees, and they sometimes succumb to Phytophthora root rot—especially on infested sites with poor drainage. Some growers in the region are piloting a more costly but effective strategy: the deployment of Fraser fir grafted onto rootstock of momi fir (A. firma Sieb. and Zucc.), the most Phytophthora-resistant fir species (Hibbert-Frey et al. 2010, Frampton et al. 2012). There is a need to evaluate the resistance of alternative Christmas tree species to the newly found Phytophthora species in the region, as well as to develop Phytophthora-resistant Fraser fir, either via genetic engineering (faster development but controversial) or a hybridization and backcross program (long-term development).

Phytophthora species will no doubt continue to plague the Fraser fir Christmas tree industry of the Southern Appalachian Mountains. Nonetheless, vigilance and the pursuit of a variety of amelioration strategies may help to reduce its future impact.

REFERENCES


CHAPTER 2. Increased diversity of *Phytophthora* species in Fraser fir Christmas tree plantations in the Southern Appalachians

Citation:
Increased diversity of Phytophthora species in Fraser fir Christmas tree plantations in the Southern Appalachians

M. Pettersson⁴, J. Frampton⁵, J. Rönnerberg⁶, H. D. Shew⁷, D. M. Benson⁷, W. H. Kohliway⁴, M. E. Escanferla⁸ and M. A. Cubeta⁵

⁴Department of Forestry and Environmental Resources, North Carolina State University, Raleigh, NC, USA; ⁵Faculty of Forestry Sciences, Southern Swedish Forest Research Centre, Swedish University of Agricultural Sciences, Alnarp, Sweden; ⁶Department of Plant Pathology, North Carolina State University, Raleigh, NC, USA

ABSTRACT
Phytophthora root rot (PRR) disease affects significant economic losses to the Fraser fir Christmas tree industry. In previous surveys conducted in 1972 and from 1997 to 1998 in North Carolina, the incidence of PRR was ~9.5% with Phytophthora cinnamomi identified as the predominant causal species isolated from infected roots of Fraser fir. Due to increased use of out-of-state planting stock since 2000, we suspected increased diversity of Phytophthora species. During 2014, we surveyed Fraser fir Christmas tree plantations in the Southern Appalachians of North Carolina, Tennessee and Virginia to determine the occurrence of pathogenic root-rotting species of Phytophthora. A weighted sampling strategy based on Christmas tree acreage was deployed to collect symptomatic Fraser fir roots from 103 commercial production fields in 14 counties. Six species of Phytophthora were isolated from infected roots sampled from 82 sites in 13 counties. Phytophthora cinnamomi, P. cryptogea and P. pinea represented 70.3%, 23.1% and 1.1% of the 91 isolates. Phytophthora citrophthora, P. europaica and P. sansserfiana accounted for the remaining 5.5% of the isolates and have not been identified in previously published Fraser fir surveys conducted in the region. The pathogenicity of P. citrophthora on Fraser fir was confirmed based on completion of Koch’s postulates.

Introduction
Fraser fir (Abies fraseri (Pursh) Poir.), the main species cultivated by Christmas tree growers in the Southern Appalachian Mountains, is native to the region growing naturally on high elevation (>1300 m) ridges (Busing et al. 1993). Fraser fir has beautiful dark green foliage, a pleasant aroma and excellent post-harvest needle retention that make it highly desirable as a Christmas tree (Chastagner & Benson 2005). In the Southern Appalachian Mountains, each year 5–6 million Christmas trees are harvested with a wholesale value of more than US$ 100 million (Napier & Sidebottom 2011). North Carolina (NC) is the second largest producing state in the US with more than 16,000 ha in production and more than 4 million trees harvested annually. Collectively, Tennessee (TN) and Virginia (VA) have approximately 4850 ha in production and harvest ~600,000 trees annually (USDA 2012). NC, VA and TN account for 17% of the 125,195 ha of Christmas tree land in the US (USDA 2012). Christmas tree production sites are generally below the natural range of Fraser fir. At lower elevation, soils are often more fertile and warm, but they sometimes have poorer drainage than soil in Fraser fir’s native range.

As Christmas tree production in the region expanded during the 1960s and 1970s, growers recognized a number of disease and insect related problems including Phytophthora root rot (PRR). In 1963, PRR was first reported on Christmas trees in Penrose, NC (Transylvania Co.) and Phytophthora cinnamomi Rands was identified as the causal agent (Kuhlman & Hendrix 1963). Since the initial identification, P. cinnamomi has spread throughout the production region and represents a serious and intractable management issue for the industry. The symptoms exhibited by Fraser fir trees infected with P. cinnamomi are: dieback (flagging) of lower basal branches, stem cankers or cambial lesions with distinct borders, foliar chlorosis and reddening of needles, diminished growth, wilting of new growth, and darkened, blighting and necrotic roots (root rot) (Chastagner et al. 1995; Chastagner & Benson 2000). Dying roots and girdling stem infections result in decreased water and nutrient transportation, leading to a weakening of the tree that is often followed by death.

Two surveys on the incidence and occurrence of PRR in Fraser fir Christmas tree plantations in NC region have been conducted and reported. The first survey conducted in 1972 (Grand & Lapp 1974) reported mean disease losses of 9.6% (range ≤ 1–90%) due to P. cinnamomi in 14 Fraser fir plantations in 5 counties in western NC. In the more recent survey, conducted in 1997 and 1998 (Benson & Grand 2000), the incidence of PRR averaged 9% (range = 0–75%) in 58 Fraser fir plantations sampled in 5 counties in western NC. All isolates from the field sites were identified as P. cinnamomi, except for one isolate of an unidentified species of Phytophthora. In this survey, nursery transplant beds were also sampled and had a mean disease incidence of PRR of 2.0% (range = 0–12%). In addition to P. cinnamomi, P. cactorum (Leb. and Cohn) Schröeter,
P. dieckeni Tucker and an unidentified species of Phytophthora were found on fir seedlings sampled in nursery transplant beds. As regional Christmas tree growers became more knowledgeable about the distribution and occurrence of PRR in NC, they began to purchase and import out-of-state sources of planting stock. A recent survey of Christmas tree growers (n = 89) in the Southern Appalachians in 2015 suggested a change in production practices since 2000 to purchase and import out-of-state grown transplants and containerized seedlings rather than locally produced bare-root transplants (Pettersson et al. 2017). Sixty-four percent of the growers surveyed used out-of-state Fraser fir planting stock on approximately 80% of the land they collectively managed (Pettersson et al. 2017). Because this imported material originates from geographic regions where other Phytophthora species cause disease on fir species (McKeever & Chastagner 2016), the possibility of introducing new Phytophthora species into NC exists.

With the increased movement and spread of Phytophthora in plant-based ecosystems throughout the world (Uchida & Baker 2007; Brasier 2008; Bienapfi & Balci 2014), we hypothesize that the diversity of root-rotting species of Phytophthora associated with Fraser fir in Christmas tree plantations in the Southern Appalachian Mountains has increased since 1998 (Benson & Grand 2008). The objectives of this study were to determine and characterize the species of Phytophthora associated with symptomatic Fraser fir Christmas trees sampled in the Southern Appalachian Mountains to be able to restrict the spread and effect from alien species of Phytophthora through advanced recommendations to growers.

Material and methods
Field site selection and sampling procedure
Fraser fir trees displaying PRR symptoms were sampled from 105 field sites using a weighted strategy based on the proportion of Christmas tree acreage from the 14 counties in NC, TN and VA (Table 1). The soil of each site was characterized as sand, sandy/loam, loam or clay and its placement recorded as ridge, slope, toe-height or valley. GPS coordinates and the elevation of each plantation were also recorded using a Garmin GPSmap 64st device.

At each field location, three 4–12-year-old trees with symptoms of PRR (branch flagging, chlorosis or the wilting of new growth) were selected and manually excavated. For each county, one to four non-symptomatic trees 5–10 m from the diseased trees were also excavated and served as a control.

The height of each sampled tree was measured, and percentage of chlorotic foliage was determined based on a 0–4 scale where 0 = 0%, 1 = 1–25%, 2 = 26–50%, 3 = 51–75% and 4 = 76–100% chlorotic foliage (Figure 1). A similar scale of 0–4 was used to determine canker width relative to stem circumference, where 0 = 0%, 1 = 1–25%, 2 = 26–50%, 3 = 51–75% and 4 = 76–100% of the stem width displaying stem canker or cambial lesion (Figure 2). The excavated roots were heavily shaken to loosen the soil from the root system and the amount of root rot was roughly determined based on a 1–5 scale where 1 = 0–30%, 2 = 31–70% and 3 = 71–100% rotted roots (Figure 3). Root systems with 0–30% rotted roots had a mass of fine roots attached to a majority of the larger roots and just a few rotted roots, whereas root systems with 71–100% rotted roots had very few fine roots and a majority of the larger roots were heavily necrotic with the bark peeling off.

The root system of each tree was placed into a resealable plastic bag (Ziploc Double Zipper Gallon Storage Bag) with a wet paper towel. Each bag was labeled, placed into a cooler, stored at ~15°C and transported to the laboratory at North Carolina State University (NCSU), Raleigh, and roots were assayed within 3–7 days. Six trips to the mountains were conducted with an average of 17 sites sampled on each trip.

Isolation
Fine roots from an individual root system of each sample tree were excised, washed under running tap water, surface sterilized for 30 s in a 10% solution of 8.25% sodium hypochlorite in deionized water, rinsed twice for 30 s in deionized water,
dried on paper towels and cut with sterilized scissors into 5–10 mm long segments. With sterilized forceps, 5 samples of 5–10 root segments were placed on 2 Petri dishes containing selective media for isolation of Phytophthora. The selective media used were PARPH-clarified V8 (CV8) medium (15 g Difco agar, 50 ml clarified V8 juice, 10 mg Delvocid (50% Pimaricin), 250 mg sodium Ampicillin, 10 mg Rifampicin sodium salt, 50 mg PCNB (pentachloronitrobenzene), 50 mg Hymexazol in 11 of deionized/distilled (dH2O) water) (Jeffers 2006). The PARPH-cV8 medium is specially designed to inhibit soilborne fungi by containing PCNB. It also inhibits most Pythium species by containing hymexazol while allowing most Phytophthora species to grow. Plates were incubated for 1–4 days at room temperature (±20°C) in a dark cabinet and examined daily for the presence of characteristic oomycete growth, such as coenocytic hyphae, using an inverted microscope. Only one isolate of each morphology type per site was retained. Hyphae were then transferred to new plates with PARPH-cV8 medium. After 1–3 days of growth, a 5-mm diameter hyphal plug was transferred to unamended cV8 medium and grown for 2–3 days at room temperature, and a small tuft of mycelium was harvested for DNA analysis.

**Molecular identification**

The mycelial tuft from each isolate was added into an individual 200 μl microcentrifuge tube with dH2O using a sterilized scalpel. DNA was extracted placing the tube containing the tuft of mycelium at 95°C for 5 min or by using the QIAGEN DNeasy Plant Mini Kit (QIAGEN, Inc., Valencia, CA, USA) following manufacturer’s instructions. The internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) was amplified by PCR, using the universal primers ITS6 and ITS4 (Grünwald et al. 2013). PCR conditions included initial denaturation at 94°C for 3 min followed by 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, with a 10 min extension cycle at 72°C. The amplified products were run on 1% agarose gel to ensure sufficient DNA amplification and assess contamination.

The amplified PCR products were purified by mixing 2 μl of ExoSAP-IT (Affymetrix, Santa Clara, CA) with 5 μl PCR product and following the manufacturer’s thermocycler instructions. The purified DNA products were submitted for Sanger sequencing at the Genomic Sciences Laboratory at NCsu. DNA sequences were trimmed and assembled using Geneious 8.1.4 (https://www.geneious.com (Kease et al. 2012)). Each sequence was subject to the BLAST algorithm in the public
and curated Phytotaphoradb.org database to characterize and identify isolates to the species level (Park et al. 2008).

Dr. Gloria Abad at CPHST Beltzville Laboratory, Center of Plant Science and Technology at USDA-APHIS-PPQ graciously helped identify DNA from one putative isolate of *P. citrophthora* (R.E. Sm. & E.H. Sm.) Leonian, *P. cryptogea* Pethybr. & Laff., *P. europaea* E.M. Hansen & T. Jung, *P. pini* Leonian and *P. sansomeana* E.M. Hansen & Reeser based on morphological characteristics and ITS rDNA sequence analysis (using ITS5 and ITS4 primers) done at North Carolina State University, Raleigh. Each isolate was extracted and amplified with three different loci: ITS (ITS4 and ITS5 primers), β-tubulin (Tub2F and Tub2R1 primers) and COI (COI-1 and COI-2 primers) and amplification conditions, modified from White et al. (1990), Kroon et al. (2004) and Robinette et al. (2011), respectively. The amplified DNA products were examined on 1.5% ultrapure agarose gel to ensure good quality DNA. The PCR products were sequenced by GENEWIZ (South Plainfield, NJ). The raw sequences were trimmed and assembled, using Geneious 8.1.4 software. Sequences were then subjected to the BLASTn algorithm against sequences of the type isolates from the National Center for Biotechnology Information (NCBI) GenBank database for *P. citrophthora*, *P. cryptogea*, *P. europaea*, *P. pini* and *P. sansomeana*.

**Morphological characteristics**

Six isolates each of *P. cinnamomii* and *P. cryptogea*, three isolates each of *P. citrophthora* and *P. europaea*, as well as one isolate each of *P. pini* and *P. sansomeana* were randomly selected for morphological identification to support DNA sequence identification.

**Colony morphology and asexual characteristics**

Agar plugs, taken from the edge of an actively growing culture of each isolate, were transferred to three replicate Petri dishes with cv8 and incubated at room temperature (±20°C). Colony morphology was assessed after 7 d. For each isolate, five agar plugs were placed in a 9-cm diameter plastic Petri dish, flooded with non-sterile soil extract.
Ueffers (2006), incubated under fluorescent light at room temperature for 12–36 h and examined daily for sporangia formation with a Nikon Inverted Microscope Eclipse TiS/L100 with a Q-Imaging QIClick, CCD Color camera and NIS-Elements: Basic Research Acquisition and analysis software package (Nikon microscope). The length/width ratio of 20 arbitrarily selected sporangia per isolate were measured and calculated.

Sexual characteristics

The homothallic species (P. europaea, P. pini and P. sansomeana) produced oogonia, antheridia and oospores in single cultures on cv8 agar incubated under ambient light and room temperature conditions. The diameter of 10 arbitrarily selected oospores was measured using a Nikon microscope as described above.

For the heterothallic species (P. cinnamomi, P. cryptogea and P. citrophthora) formation of sexual characteristics and determination of mating type were attempted by pairing P. cinnamomi and P. cryptogea and P. citrophthora isolates with known reference P. cinnamomi A1 (2322) and A2 (2333) type isolates. Plugs from heterothallic isolates were paired with the reference P. cinnamomi A1 and A2 type isolates by placing test and reference plugs 2–3 cm apart on cv8 agar. The reference mating types, A1 (P. cinnamomi A1) and A2 (P. cinnamomi A2) were also paired with each other and with themselves as positive and negative controls, respectively. The cultures were incubated in a dark cabinet at room temperature and examined every week for 4 weeks. Oospores that formed in a distinct band in the hyphal interaction zone between paired isolates were measured using a Nikon microscope.

Koch’s postulates

An inoculation test was done to complete Koch’s postulates and demonstrate pathogenicity of P. citrophthora on Fraser fir. As a positive control, P. cinnamomi was used because it has proven highly pathogenic in pathogenicity experiments (Benson et al. 1997, Frampton & Benson 2012). The experiment was conducted at the NC State Horticulture Field Lab starting 31 July and ending 23 Oct. 2015. For both Phytophthora species, a total of 15 five-year-old Fraser fir seedlings were inoculated using colonized rice grains according to Frampton and Benson (2012). The inoculum was prepared in an Erlenmeyer flask containing 25 g of rice grain and 17 ml of deionized water, that was autoclaved on two consecutive days prior to adding two mycelium plugs (5-mm diam) of P. citrophthora grown for 5 days on cv8. Seedlings were inoculated by preparing two holes with a sterilized glass rod, 1–2 cm away from the stem of the seedling and about 2.5 cm deep in the bark and peat potting mix medium. A single colonized rice grain was placed into each hole (Benson et al. 1997). Fifteen seedlings, functioning as controls, did not receive any colonized rice grains. All seedlings were watered twice a day (morning and evening for ~45 minutes by a sprinkler system) and examined for disease symptoms bi-weekly for 12 weeks.

Phytophthora citrophthora was re-isolated from necrotic roots of four arbitrarily selected seedlings, two appearing to be dead (chlorotic/red needles) and two seemingly healthy (green needles), using the above described isolation method. The colony morphology was compared with pictures of P. citrophthora cultures and 20 sporangia were measured using a Nikon microscope. Mycelium from the cultures growing on cv8 medium was harvested and genomic DNA was extracted using the QIAGEN DNeasy Plant Mini Kit. Amplification and sequencing analysis were performed as described above.

Results

Disease symptoms

Of the sampled trees, 67% exhibited branch flagging, 35% displayed wilting and 37% exhibited multiple disease symptoms. Nineteen percent of the sampled trees had no chlorotic foliage whereas 65%, 12% and 4% had 1–25%, 26–50% and 51–100% chlorotic foliage (one or several flagging branches), respectively (Table 2). Of the trees sampled, 70% had stem canker or cambial lesion at the base and 30% were healthy. Of the sampled trees root systems, 17%, 54% and 29% had 0–30%, 31–70% and 71–100% rotted roots, respectively. Tree height ranged from 20 to 205 cm; the average tree height was 93 cm.

Phytophthora recovery and identification

The symptomatic Fraser fir trees sampled, Phytophthora was recovered from 167 of 309 (54%) trees, and from 82 of 103 (79.6%) field sites from 13 counties (Table 1). Of the non-symptomatic control trees sampled, Phytophthora was recovered from three of 32 (9.4%) trees from three field sites (2.9%) from two counties.

Based on morphology (Tables 3 and 4 and Figure 4) and DNA sequence identification (Table 5), six species of Phytophthora were recovered (Table 1). Of the 91 isolates recovered, 64 (70.3%) were P. cinnamomi, 21 (23.1%) P. cryptogea, two (2.2%) P. europaea, two (2.2%) P. citrophthora, one (1.1%) P. pini and one (1.1%) P. sansomeana.

Koch’s postulates

The result of the inoculation trial fulfilled Koch’s postulates and provided evidence of pathogenicity of P. citrophthora

| Table 2. Symptoms of sampled Fraser fir (Abies fraseri) trees in Christmas tree plantations in the Southern Appalachian Mountains. |
|----------------|-----------------|-----------------|
| Trait          | Scale           | Frequency (%)   |
| Chlorotic foliage (%) | 0-30          | 17              |
| 1-25           | 12              |
| 26-50          | 14              |
| 51-75          | 12              |
| 76-100         | 23              |
| Canker width to stem diameter (%) | 0-30          | 17              |
| 1-25           | 12              |
| 26-50          | 14              |
| 31-75          | 12              |
| 76-100         | 23              |
| Amount of root rot (%) | 0-30          | 17              |
| 31-70          | 54              |
| 71-100         | 29              |
Table 3. Shape, pore type, length, width and length/width ratio of sporangia produced on clarified V8 medium plates flooded with soil extract of Phytophthora cryptogea, P. pini, P. citrophthora, P. cinnamomi, P. europaea and P. sannseana.

<table>
<thead>
<tr>
<th>Species</th>
<th>Shape of sporangia</th>
<th>Pore type of sporangia</th>
<th>Length × Width (µm)</th>
<th>Average length/width ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. cryptogea</td>
<td>Ovoid, obpyiform</td>
<td>Non-papillate</td>
<td>28.1 - (39.10) - 49.67 × 18.54 - (27.26) - 35.24</td>
<td>1.45</td>
</tr>
<tr>
<td>P. pini</td>
<td>Globate, ellipsoid, ovoid, obpyiform</td>
<td>Semi-papillate</td>
<td>28 - (39.79) - 60.01 × 10.28 - (12.71) - 35.3</td>
<td>1.47</td>
</tr>
<tr>
<td>P. citrophthora</td>
<td>Ellipsoid, oval, obpyiform</td>
<td>Papillate</td>
<td>22.2 - (26.28) - 50.3 × 17.38 - (26.0) - 32.72</td>
<td>1.39</td>
</tr>
<tr>
<td>P. cinnamomi</td>
<td>Ovoid, obpyiform</td>
<td>Non-papillate</td>
<td>26.26 - (35.98) - 52.93 × 19.32 - (26.18) - 36.05</td>
<td>1.37</td>
</tr>
<tr>
<td>P. europaea*</td>
<td>Ovoid, obpyiform</td>
<td>Non-papillate</td>
<td>38.83 - (43.33) - 45.13 × 25.4 - (28.1) - 36.98</td>
<td>1.51</td>
</tr>
<tr>
<td>P. sannseana**</td>
<td>Ellipsoid, obpyiform</td>
<td>Non-papillate</td>
<td>30.08 - (36.02) - 39.13 × 10.89 - (25.33) - 27.97</td>
<td>1.44</td>
</tr>
</tbody>
</table>

*Due to low amount of sporangia produced, sporangial characteristics were measured on three and six sporangia for P. europaea and P. sannseana, respectively.

Table 4. Observations of sexual characteristics on clarified V8 medium plates flooded with non-sterile soil extract, for Phytophthora cinnamomi, P. cryptogea, P. citrophthora, P. europaea, P. pini and P. sannseana.

<table>
<thead>
<tr>
<th>Species</th>
<th>Oospores in single culture</th>
<th>Oospores when crossed with</th>
<th>Oospores Will (2322)</th>
<th>Oospores Will (2333)</th>
<th>Oospores pleotic or aplectic</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. cinnamomi</td>
<td>No</td>
<td>Yes*</td>
<td>No</td>
<td>No</td>
<td>Pleotic</td>
</tr>
<tr>
<td>P. cryptogea</td>
<td>No</td>
<td>Yes*</td>
<td>No</td>
<td>No</td>
<td>Pleotic</td>
</tr>
<tr>
<td>P. citrophthora</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Pleotic</td>
</tr>
<tr>
<td>P. europaea</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Pleotic</td>
</tr>
<tr>
<td>P. sannseana</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Pleotic</td>
</tr>
</tbody>
</table>

*Phytophthora cinnamomi generated oospores for six isolates meaning that the isolates are of A2 mating type.

**Phytophthora cryptogea generated oospores for one isolate meaning that the isolate is of A2 mating type.

on Fraser fir. Seven of 15 (47%) Fraser fir seedlings inoculated with P. citrophthora died within the 12-week period, and the pathogen was re-isolated and confirmed with morphology and DNA sequence identification.

Discussion

In this study, we identified three species of Phytophthora from symptomatic Fraser fir Christmas trees not reported from previous studies conducted in the region, which almost exclusively found P. cinnamomi. We thereby confirm our hypothesis that the diversity of root-rotting Phytophthora species on Fraser fir Christmas trees in the Southern Appalachian Mountains has increased since 1998.

Phytophthora cinnamomi was the most abundant species recovered in the highest frequency and continues to be the main root-rotting species of Phytophthora on Fraser fir in NC. In addition to causing damage on Fraser fir, P. cinnamomi has also been isolated from seedlings of Norway spruce (Picea abies [L.] H.Karst.), balsam fir (Abies balsamea [L.] Mill.), Trojan fir (A. equi-trojani [Asch. & Sint. ex Boiss.], Mattf.) and Turkish fir (A. borreianiana Mattf.) in NC (Kenerley & Bruck 1981; McKeever & Chastagner 2016). Phytophthora cinnamomi is causing loss of Nordmann firs (A. nordmanniana Spach), white fir (A. concolor Lindl. ex Hildebr.) and Trojan fir in California (CA), and on noble fir (A. procera Rehder) in Oregon (OR) (Chastagner et al. 1995; McKeever & Chastagner 2016).

Phytophthora cryptogea is considered to be an aggressive pathogen of many fir species (Chastagner & Benson 2000; McKeever & Chastagner 2016). It has been found on Fraser fir in NC, New York (NY), Connecticut (CT) and Pennsylvania (PA), on Noble fir in Washington (WA) and OR, on Douglas fir (Pseudotsuga menziesii [Mirb.] Franco) in WA and OR, and on balsam fir in Wisconsin (WI) (Pratt et al. 1976; Chastagner et al. 1995; Hoover & Bates 2013; McKeever & Chastagner 2016). In this study, multiple matching sequences existed between P. cryptogea and the undescribed P. taxon kelmania in our BLAST analyses. Both taxa belong to the Phytophthora clade 8a complex together with P. dracaenae and P. cryptogea (Kroon et al. 2012), but since P. taxon kelmania is not yet an accepted species, the multiple matching events for P. cryptogea and P. taxon kelmania are here treated solely as P. cryptogea.

Phytophthora pini is a species belonging to the P. citricola Sawada complex (Hong et al. 2011) which consists of several closely related species that were regarded as one single species under the common name of P. citricola. Phytophthora pini and other species in this complex are known to be aggressive on fir species causing root rot of Fraser fir in NC, NY and Michigan (MI), Noble fir in WA and OR, balsam fir in CT, as well as white fir and red fir (A. magnifica A. Murr.) in CA (Shev & Benson 1981; McCain & Scharpf 1986; Chastagner et al. 1995; Chastagner & Benson 2000; McKeever & Chastagner 2016).

Phytophthora sannseana has been isolated from Fraser fir in WI, MI and NY and exhibits similar aggressiveness as P. cryptogea, and P. pini (McKeever & Chastagner 2016).

In the US, P. citrophthora has been isolated from symptomatic fir species in PA (Kil et al. 2014) and from Fraser fir in our study. Our Koch's postulates experiment performed with P. citrophthora on Fraser fir is to our knowledge the first report of this pathogen-host interaction. The mortality rate of 47% within the three-month period can be compared to 100% mortality rate for the positive control, P. cinnamomi. Although, P. citrophthora may not be as pathogenic as P. cin- namomi, it can still contribute to loss of Fraser fir. In Hungary, P. citrophthora has caused necrotic lesions and root rot of corkbark fir (A. lasiocarpa var. arizonica [Merriss] J. Lemm.), Port-Orford-cedar (Chamaecyparis lawsoniana [A. Murr.] Parfi), lavender (Lavandula angustifolia Mill.), flowering currant (Ribes sanguineum Pursh) and lilac (Syringa vulgaris L.) in six ornamental nurseries (Ubza et al. 2011).
Phytophthora europaea has since 2000 been more frequently found in eastern soil, and host associations have been established for several oak (Quercus) species (Balci et al. 2006; Balci et al. 2007). Phytophthora europaea has also been isolated from Fraser fir in WI (McKeever & Chastagner 2015) and from Christmas tree plantation soils in MI (Balci et al. 2013). To our knowledge, pathogenicity testing and Koch's postulates for P. europaea on fir has not yet been undertaken, but needs to be done in order to find out if P. europaea can contribute to loss of Fraser fir in the southern Appalachian Mountains.

To our knowledge, this is the first time P. europaea, P. sanseomeana and P. citrophthora have been isolated from symptomatic cultivated Fraser fir trees in the southern Appalachian Mountains. During a survey of US Christmas tree farms, McKeever and Chastagner (2016) found more isolates of P. cryptogea (=P. taxon keimania) than P. cinnamomi, though their sample size was limited to four sites in NC. In our study, P. cryptogea represented 23% of the samples and may be contributing to increased losses of Fraser fir in the region. Our study points to an increased diversity of root-rotting species of Phytophthora in North Carolinas Fraser fir dominated Christmas tree region. McKeever and Chastagner (2016) suggest the same scenario and their findings together with our own raise questions on how these new-to-the-region Phytophthora species are introduced and dispersed in the Fraser fir Christmas tree fields in the southern Appalachian Mountains.

The relative contribution of the nursery trade to the introduction and dispersal of plant pathogens are commonly discussed and viewed as increasingly problematic (Jones & Baker 2007; Brasier 2008; McKeever & Chastagner 2016). Many studies have found high numbers of root-rotting species of Phytophthora in nurseries and greenhouses on infested plant material and contaminated soil (Pérez-Sierra et al. 2013).
et al. 2013; Bienapf & Balci 2014). Due to fungicide use, infections can be masked or inhibited in the nurseries, and resting spores (oospores and chlamydospores) can survive unfavorable conditions for years (Juddelst & Blanco 2005; Balci et al. 2013). Once a Phytophthora species is introduced to a new geographic region, it can rapidly start to spread via rain-splash, runoff water and/or by human practices (e.g. not disinfecting tools between fields). It has proven nearly impossible to eliminate or reduce introduced Phytophthora species due to their long-term survival capability.

There are regional differences in population and species distribution of Phytophthora between the southeastern Fraser fir Christmas tree production region and the other four major US Christmas tree production regions: Northeastern, Great Lakes, Western and Pacific Northwestern regions (McKeever & Chastagner 2016). There is a greater diversity of Phytophthora species in the eastern regions that might be due to favorable climate and/or a larger number of nurseries producing Fraser fir for eastern growers (McKeever & Chastagner 2016). Most species of Phytophthora could potentially survive in the southeastern region, especially in the Southern Appalachian Mountains since the climate and mean soil temperature is warm and rainfall is plentiful throughout the whole year which benefits survival and dissemination of Phytophthora.

Fraser fir is highly sensitive to Phytophthora infections (Chastagner & Benson 2000; Kim et al. 2014) and because no useful level of resistance has been identified in Fraser fir (Frampton et al. 2013), other species should be planted on infested sites. A common regional practice has been to plant white pine (Pinus strobus L.) in Fraser fir production areas where PRR is known to be present. Although, Kirby and Grand (1975) have shown that white pine is susceptible to P. cinnamomi, it can often be successfully established on all but the most severely Phytophthora-infested sites in the Southern Appalachians, however, white pine displays a much lower tolerance to P. cinnamomi when planted on piedmont sites outside of its native range. White pine is less valuable as a Christmas tree so that growers prefer to replant sites with fir species such as (in order of Phytophthora-resistance): Canaan fir (A. balsamea var. phanerolepis Fern.), Nordmann fir, Trojan fir and Turkish fir. A more effective but costly strategy that some growers in the Southern Appalachian region have been piloting is deploying Fraser fir grafted onto rootstock of momi fir (Abies firma Siebold & Zucc.), the most Phytophthora-resistant fir species (Frampton et al. 2013; Hibbert-Frey et al. 2010). There is a need to evaluate the resistance of these alternative Christmas tree species to the Phytophthora species we newly found in the region.

While there are a few strategies growers can take to reduce the impact of PRR, caution before using any planting stock should be exercised to prevent further introduction of new root-rotting Phytophthora species. Unfortunately, there has been an increasing trend of circulating nursery material among Christmas tree production regions. The 2015 survey of growers in the Southern Appalachians found that a majority (64%) of the Christmas tree growers had shifted to use of out-of-state produced planting stock (Pettersson et al. 2017). Some of these growers provide the out-of-state nursery with seeds and the nursery produces seedlings for the growers. Other growers buy out-of-state seedlings to avoid purchasing infected seedlings from local NC nurseries. Most imported Fraser fir planting stock is containerized-produced seedlings, but some seedlings are partly produced in out-of-state transplant beds. The import and movement of planting stock (especially from transplant beds) increases the probability of introducing new root-rotting species of Phytophthora to Christmas tree producing regions.

The increased number of identified Phytophthora species in this study supports the proposition that increased nursery trade has played a role in the spread of PRR. A certification system for out-of-state produced seedlings to avoid root-rotting Phytophthora being introduced to new regions would be one measure to reduce the risk of introducing new Phytophthora species. However, there are several obstacles in implementing such a certification system, one being that symptoms of PRR may be suppressed or masked for well-managed nursery seedlings, but will later emerge when seedlings are out-planted in the field. Therefore, growers must be alert to incoming symptomatic plant material and to newly out-planted seedlings displaying typical PRR symptoms. We recommend that no symptomatic seedlings should be planted unless they are first tested for Phytophthora. Testing can be done with easy-to-use test kits for rapid field-diagnostics of Phytophthora. The NCSU Cooperative Extension Service has been training regional Christmas tree growers how to use such test kits. While these kits on rare occasion cross-react with a few specific Pythium species, they can be an important tool for growers. Testing is very important, as well as contacting local extension personnel if symptomatic seedlings are found. Symptomatic seedlings may be sent to a plant disease clinic for further verification and possible Phytophthora species identification.

This study has identified six species of Phytophthora sampled from diseased roots of Fraser fir in the Southern Appalachian Christmas tree production area. Phytophthora citrophthora, P. europaea and P. sanseomeana have not been identified in previously published Fraser fir surveys conducted in the region. A validation of the host-pathogen combination, P. citrophthora and Fraser fir, was proven. This result verifies our concern for the increased diversity of Phytophthora species in the Southern Appalachian Christmas tree region. This information is important for researchers and Christmas tree growers of the region. PRR continues to be a limiting factor in the Southern Appalachian Christmas tree fields where P. cryptogae appears to have become an important pathogen; contributing to losses of Fraser fir, together with P. cinnamomi. Vigilance will be necessary to monitor the spread of the other newly found Phytophthora species and their impact on the regional Christmas tree industry in the future.

**Acknowledgements**

The authors are grateful to Avery Barr, Anne Margaret Braham and Kala Parker for their valuable technical assistance. We are thankful to Dr Gloria Abad at CPHST Bettsville Laboratory, Center of Plant Science and Technology at USDA-APHIS-PPQ, and we are also grateful to the numerous Christmas tree growers and Cooperative Extension personnel who graciously cooperated in this effort.
Disclosure statement
No potential conflict of interest was reported by the authors.

Funding
This study was supported by the Garnar and Lillian Nicholson Graduate Fellowship and Faculty Exchange Fund, the USDA NIFA Specialty Crops Research Initiative (2012-51188-19940) and the North Carolina Agricultural Research Service via the Christmas Tree Genetics Program.

References
Available from: http://www.clemson.edu/cfrts/departments/erps/research/jeffers/phytophthoraeffect.pdf
CHAPTER 3. Genetic Variation for Resistance to Phytophthora Root Rot in
Eastern White Pine Seedlings

Citation:
Frampton J., Pettersson M. and Anne Margaret Braham. Genetic Variation for Resistance to
Genetic Variation for Resistance to Phytophthora Root Rot in Eastern White Pine Seedlings

John Frampton *, Martin Pettersson and Anne Margaret Braham

Department of Forestry and Environmental Resources, North Carolina State University, Raleigh, NC 27695-8008, USA; jmpetters@ncsu.edu (M.P.); abraham@ncsu.edu (A.M.B.)

* Correspondence: frampton@ncsu.edu; Tel.: +1-919-515-7580

Received: 28 February 2018; Accepted: 22 March 2018; Published: 23 March 2018

Abstract: Deployment of genetically resistant Eastern white pine (Pinus strobus L.) planting stock could reduce economic losses to root rot caused by Phytophthora cinnamomi Rands in Christmas tree and forest plantations. This study aimed to determine the degree of genetic control of resistance to P. cinnamomi in Eastern white pine and secondarily, to compare the aggressiveness of two P. cinnamomi isolates derived from different host species. Phytophthora isolates from Fraser fir (Abies fraseri (Pursh) Poir.) and Eastern white pine were used in a main and supplemental study, respectively, including 83 and 20 open-pollinated families. In each study, two-year-old seedlings were inoculated twice each of two consecutive years and mortality was assessed biweekly for 16 weeks each year. During the first year, mortality increased over time to 18.6% and 40.4% while family variation in mortality ranged from 1.3% to 60.0% and 12.5% to 73.0% in the main and supplemental studies, respectively. At the end of the first year, individual-tree and family-mean heritability estimates were, respectively, 0.44 ± 0.0935 and 0.85 ± 0.180 for the main study, and 0.57 ± 0.216 and 0.90 ± 0.343 for the supplemental study. The P. cinnamomi isolate from Eastern white pine was more aggressive and there was a large interaction between isolates and pine families. Deploying resistant families will be complicated by this interaction but should, nevertheless, reduce economic losses.

Keywords: Pinus strobus L.; Phytophthora cinnamomi Rands; disease resistance; family variation; Christmas trees; forest genetics

1. Introduction

Eastern white pine (Pinus strobus L.) is a coniferous species with a natural range extending from southeastern Canada, through the Midwest and Northeast regions of the United States and southward through the Southern Appalachian Mountains and into adjacent upper Piedmont regions [1]. Commercially, the wood is used for lumber, cabinetry, millwork, and toys. The species is used as an ornamental and for reforestation, where it is often planted on eroded or degraded sites [1,2]. In North Carolina, 39,790 ha of Eastern white pine are in timber production [3] and 95% of the volume harvested is grown in the mountainous western region of the state [4].

Eastern white pine is also a worthy Christmas tree species with soft blue-green foliage, good post-harvest needle retention, relatively strong branches but only a slight aroma [1,5]. In North Carolina, it has traditionally been grown in the mountains for cut Christmas trees and greenery; however, growers have extended the range eastward into the Piedmont and Coastal Plain regions. There, it is grown on ‘choose and cut’ farms and often cultivated with a suite of other species including Virginia pine (Pinus virginiana Mill.), Eastern reedcedar (Juniperus virginiana L.), and various cultivars of Leyland cypress (Cupressocyparis leylandii (A.B. Jacks. & Dallim). Farjon), pure cypress (Cupressus), and arborvitae (Thuja). Eastern white pine is popular in this region, where it is the most widely planted...
species. A 2008 survey revealed that 78% of the farms in the region grow Eastern white pine and it accounts for 42% of trees sold [6].

Today, Fraser fir (Abies fraseri (Pursh) Poir.) is the most popular Christmas tree species grown in the southern Appalachians, where revenue from annual sales exceed $US 100 million in North Carolina alone [7]. However, Fraser fir is highly susceptible to root rot disease caused primarily by Phytophthora cinnamomi Rands, an introduced soil-borne pathogen with an extensive host range [8-10]. Resistance to this pathogen has not been found in Fraser fir and chemical methods of control are stop-gap at best [11,12]. Because P. cinnamomni can persist in soil for decades, infested sites are often removed from Fraser fir production. Faced with this reality, growers commonly plant Eastern white pine on infested sites where Fraser fir cannot survive. This strategy is mostly successful, the exceptions being extremely high disease hazard areas (i.e., sites with extremely poor drainage and high inoculum load) [13]. Kirby and Grand 1975 [14] demonstrated that P. cinnamomni is pathogenic to Eastern white pine. Infected seedlings showed stunting of new growth, chlorosis, necrosis, needle loss, and severe root rot. On lower Piedmont and Coastal Plain sites, especially where Eastern white pine is planted outside its natural range, it is more sensitive to Phytophthora root rot and recently an increase in disease has occurred on Christmas tree farms in this region. Annual losses of $US 15 million to the littleleaf disease complex of southern pines [15] and $US 9 million to root rot disease in Fraser fir [16], both also caused by P. cinnamomni, underscore the concern over potential losses in Eastern white pine Christmas tree and forest plantations.

Genetic resistance is widely used to ameliorate impacts of diseases caused by Phytophthora spp. in agriculture and horticulture [8], however, nothing is known about genetic resistance in Eastern white pine. As such, this study sought to ascertain the degree of genetic control, if any, of resistance to P. cinnamomni in Eastern white pine and secondarily, to compare the aggressiveness of two P. cinnamomni cultures isolated from different host species (Fraser fir and Eastern white pine). The long-term goal is to reduce Phytophthora root rot damage by identifying and deploying families with higher resistance.

2. Materials and Methods

2.1. Plant Production

The North Carolina Forest Service (NCFS) provided white pine seedlings from their Nursery and Tree Improvement Program for use in this study. The parent trees originated from the southern portion of the natural white pine range, primarily North Carolina. They were selected from natural stands (first generation) or progeny test plantings (second generation) for forest production with emphasis on total stem volume but also considering straightness, stem taper, self-pruning, crown density, and branch angle. It is unknown if any of the selection sites were infested with Phytophthora, but if so, only symptomless trees would have been selected. In 2015, one-year-old seedlings of 83 open-pollinated families grown in a greenhouse at the NCFS Linville River Nursery in Avery County, N.C., were transferred into a greenhouse at the Horticulture Field Lab, North Carolina State University, Raleigh. Seedlings of the same family had been grown in the same flats and so were rearranged into the experimental design described below while in the greenhouse and then transferred into an outdoor shade house (40% shade). All seedlings were fertilized bi-weekly with Peters 15-16-17 Peat Lite Special (150-200 ppm N) (The Scotts Co., Marysville, OH, USA) in the spring. While in the shade house, the seedlings were automatically irrigated two times daily for 20 min (about 10 mm/day) until it was discontinued for winter and the seedlings were moved back into a greenhouse. During spring 2016, seedlings were again moved outside into the shade house and top-dressed with slow release fertilizer (Multicote 6, 18-6-12 + micronutrients, Haifa Chemicals Ltd., Haifa, Israel) for another set of inoculations and disease assessments under a similar irrigation regime.
2.2. Inoculum Production and Inoculation

A large main study was conducted using a standard culture of *P. cinnamomi, 23ss04*, isolated from Fraser fir and employed in several published studies involving fir (*Abies*) and chestnut (*Castanea*) species [11,12,17,18]. For comparison, a smaller supplemental study was conducted using isolate 2334 from Eastern white pine. Inoculum of both isolates were prepared by autoclaving Erlenmeyer flasks containing 25 g of long grain rice and 18 mL deionized water two consecutive times prior to adding 3–4 corn agar plugs of the isolates [19]. The flasks were shaken daily to avoid clumping as mycelia colonized the grains. Seedlings were inoculated with colonized rice grains by making one 2 cm deep hole about 1 cm from each side of the stem, inserting a grain, and then pushing the medium back to cover the inoculum [20] (Figure 1). The first inoculation occurred in June 2015 and surviving seedlings were inoculated a second time eight weeks later in August. In June 2016, one year and one week after the first inoculation, all living seedlings were re-inoculated and again eight weeks later in the same manner with inoculum from the appropriate isolate. Depending on availability, up to three non-inoculated control seedlings of each family were grown in the shade house throughout the study in a block beside the inoculated seedlings.

![Figure 1. Eastern white pine study seedlings in the shade house prior to inoculation (upper), inoculation of seedlings using rice grains colonized with *Phytophthora cinnamomi* Randa (lower left), and symptomatic seedlings 16 weeks after inoculation (lower right). (Photos by A.M. Graham.)](image-url)
2.3. Experimental Design and Data Collection

Both studies were set up with a randomized block design including five blocks (Table 1). The main study included seedlings from all 83 families, while the supplemental study only included 20 families. Up to 15 seedlings per family were included in each block occupying one row of a tray (Forest Tray 135, Stuewe and Sons, Tangent, OR, USA). For families with insufficient numbers, seedlings were equally distributed across blocks so that the family size in the main study ranged from 40 to 75. All families selected for the supplemental study had enough seedlings to allocate 15 per block.

Biweekly disease symptom assessments began two weeks after the first inoculation and continued through week 16. In the second year, to account for winter mortality, an assessment was made immediately before the first inoculation followed by eight additional biweekly assessments. At each assessment, seedling health was assessed by estimating percent shoot (needle and stem) necrosis on a 0 to 100 scale in increments of 10 but also including 1%, 2%, and 95% levels.

In 2016, after the final disease assessment, roots from inoculated and control seedlings were tested with Agdia ImmunoStrips® (Elkhart, IN, USA) for Phytophthora. Roots from 10 randomly selected inoculated seedlings (5 dead and 5 alive) from the main study were tested, as well as 10 randomly selected control seedlings (5 dead and 5 alive).

After the study, seedlings spent the 2016–2017 winter in the outdoor shade house. Subsequent to completing their 2017 apical growth, height measurements were taken on a subset of healthy seedlings with no disease symptoms. For six families, the heights of the previous four years (2014–2017) were measured for seedlings from the main study (n = 132 total, 4–38/family), the supplemental study (n = 101 total, 9–23/family), and the control group (n = 17 total, 2–3/family).

Table 1. Study design with mortality (%) and its heritability estimates for Eastern white pine (EWP) seedlings one and two years after inoculation with a Phytophthora cinnamomi Rands culture isolated from Fraser fir (Main Study) or EWP (Supplemental Study) and non-inoculated control seedlings. The Main Study (subsample) column includes values calculated using only the 20 open-pollinated families in common with the Supplemental Study.

<table>
<thead>
<tr>
<th>Study Design (n)</th>
<th>Main Study</th>
<th>Main Study (subsample)</th>
<th>Supplemental Study</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replications</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Seedlings</td>
<td>6049</td>
<td>1420</td>
<td>1499</td>
<td>236</td>
</tr>
<tr>
<td>Open-pollinated Families</td>
<td>83</td>
<td>20</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>Seedlings/Family</td>
<td>40–75</td>
<td>53–75</td>
<td>75</td>
<td>1–3</td>
</tr>
<tr>
<td>Year 1 Mortality (%)</td>
<td>18.6 ± 1.22</td>
<td>26.4 ± 3.11</td>
<td>40.4 ± 3.94</td>
<td>0</td>
</tr>
<tr>
<td>Range of Family Means</td>
<td>1.3–60.0</td>
<td>8.0–60.4</td>
<td>12.5–73.0</td>
<td>-</td>
</tr>
<tr>
<td>Individual Tree $h^2$ ± Std. Error</td>
<td>0.44 ± 0.0935</td>
<td>0.40 ± 0.171</td>
<td>0.57 ± 0.216</td>
<td>-</td>
</tr>
<tr>
<td>Family Mean $h^2$ ± Std. Error</td>
<td>0.85 ± 0.180</td>
<td>0.83 ± 0.353</td>
<td>0.90 ± 0.343</td>
<td>-</td>
</tr>
<tr>
<td>Year 2 Mortality (%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Overall Mean ± Std. Error</td>
<td>45.7 ± 1.60</td>
<td>51.8 ± 3.79</td>
<td>58.3 ± 3.41</td>
<td>15.6 ± 2.84</td>
</tr>
<tr>
<td>Range of Family Means</td>
<td>13.3–81.1</td>
<td>22.7–3.1</td>
<td>30.6–83.6</td>
<td>0.0–100.0</td>
</tr>
<tr>
<td>Individual Tree $h^2$ ± Std. Error</td>
<td>0.29 ± 0.0871</td>
<td>0.42 ± 0.174</td>
<td>0.39 ± 0.159</td>
<td>-</td>
</tr>
<tr>
<td>Family Mean $h^2$ ± Std. Error</td>
<td>0.73 ± 0.167</td>
<td>0.83 ± 0.343</td>
<td>0.85 ± 0.3450</td>
<td>-</td>
</tr>
</tbody>
</table>

2.4. Statistical Analyses and Heritability Estimates

Prior to statistical analyses, the disease scale was simplified to a binomial mortality rating, where seedlings were considered dead (1) when they displayed 100% shoot necrosis and alive (0) otherwise. During the first four weeks of the study, some seedlings displayed obvious signs of transplant shock as a result of rearranging them into the experimental design. This confused symptom assessment so that this information was recorded and these seedlings were excluded from subsequent analyses.

Family means for mortality were calculated for each assessment period of the main and supplemental studies. Individual analyses of variance were carried out for each assessment period of the main and supplemental studies using the GLIMMIX procedure of SAS Enterprise Guide 7.11.
HFS (7.100.1.2805) (64-bit) (SAS Institute Inc., Cary, NC, USA) with block (fixed effect), family (random effect), and their interaction (random effect) as sources of variation. Because mortality was the response variable, a binomial distribution with a logistic link function was employed. This same model was used in another analysis of the main study data using only the 20 families in common with the supplemental study. Similarly, combined analyses of variance were conducted for the 20 families in common to both studies with study (fixed effect), block (study) (fixed effect), family (random effect), and their interactions as sources of variation.

Individual-tree ($h^2_i$) and family mean ($h^2_f$) heritabilities for mortality were estimated using variance components obtained from the GLIMMIX procedure in SAS as follows:

$$ h^2_i = \frac{4\sigma^2_i}{\sigma^2_i + \sigma^2_{ij} + \sigma^2_e} $$  \hspace{1cm} (1)

$$ h^2_f = \frac{\sigma^2_f}{\sigma^2_f + \frac{\sigma^2_{ij}}{b} + \frac{\sigma^2_e}{b_n}} $$  \hspace{1cm} (2)

where:

- $\sigma^2_i$ = family variance
- $\sigma^2_{ij}$ = block by family interaction variance
- $\sigma^2_e$ = error variance
- $b$ = number of blocks
- $n$ = number of families per block.

The error variance for mortality was set to $\pi^2/3 = 3.29$ in calculation of phenotypic variances, as suggested by Gilmour et al. (1985) [21] for binary traits. We assumed that the family variance component was about one-quarter of additive genetic variance [22]. Standard errors of heritability were estimated using the delta method [23].

Height data from the non-symptomatic seedlings of six families were subjected to analyses of variance for each of the four measurement years (2014–2017) using study (fixed effect), family (random effect), and their interaction (random effect) as sources of variation. Differences among the least squares means of the main study, supplemental study, and control group were compared using Tukey–Kramer tests at the $\alpha = 0.05$ level.

3. Results

3.1. Temporal Trends in Mortality

During the first year of the study, seedling mortality in the main study became evident six weeks after the initial inoculation then gradually increased, eventually reaching 18.6% at the final 16-week assessment (Table 1, Figure 2). In the supplemental study, initial mortality was also observed after six weeks, then mortality rapidly increased through the 10-week assessment after which the increase in mortality slowed and peaked at 40.4% in the final assessment. Mortality for the 20 families included in the supplemental study averaged 26.4% in the main study, slightly higher than the average of all 83 families (18.6%) (Table 1, Figure 2). No mortality was observed in the non-inoculated control seedlings during the first year (Table 1, Figure 2).

Seedling mortality occurred between the final assessment of the first year and the initial assessment and inoculation of the second year: 12.6% in the main study, 13.6% in the supplemental study, and 7.6% control seedlings. This mortality was likely due to overwintering conditions as well as disease. During the second year of the study, cumulative seedling mortality gradually increased from 31.2% to 45.7% in the main study, from 54.0% to 58.3% in the supplemental study, and from 7.6% to
15.6% in the control seedlings (Table 1, Figure 2). In the main study, final cumulative mortality for the 20 families included in the supplemental study continued to be slightly higher than the average of all 83 families (51.8% versus 45.7%) (Table 1, Figure 2). After the final year 2 assessment, none of the 10 non-inoculated controls seedlings tested positive for *Phytophthora* according to the immunostrip tests, whereas 8 out of 10 inoculated seedlings (4 dead and 4 alive) tested positive.

![Figure 2](image_url)  
*Figure 2.* Mortality (%) of Eastern white pine seedlings inoculated with *Phytophthora cinnamomii* at weeks 0, 8, 53, and 61. Seedlings in the Main Study were inoculated with a culture isolated from Fraser fir white seedlings in the Supplemental Study were inoculated with a culture isolated from Eastern white pine. The Main Study (subsample) represents the 20 open-pollinated families in common with the Supplemental Study. The Control Seedlings were not inoculated.

### 3.2. Family Variation and Heritability of Disease Mortality

Family mean mortality varied greatly at the end of the first year in both the main (1.3% to 60.0%) and supplemental (12.5% to 73.0%) studies (Table 1). Large family variation was also present after the second year for the main (13.3% to 81.1%) and supplemental (30.6% to 83.6%) studies. By week 8 of the first year, sufficient mortality had occurred so that the biweekly individual-tree and family-mean heritability estimates stabilized into a narrow range for both the main (0.43–0.49 and 0.74–0.85, respectively) and supplemental (0.52–0.57 and 0.89–0.90, respectively) studies (Figure 3). Both the individual-tree and family-mean heritability estimates dropped in value considerably at the start of the second year of the study but subsequently remained in a narrow range for both the
main (0.28–0.35 and 0.39–0.44, respectively) and supplemental (0.72–0.75 and 0.85–0.86, respectively) studies. In the main study, when only data from the sub-sample of 20 families in common with the supplemental study was used, both the individual-tree and family-mean heritability estimates were similar to the main study estimates including all 83 families after one year (Table 1). After the second year, the heritability estimates for the sub-sample of families changed little from the first year unlike the estimates for the full main and supplemental studies, which dropped somewhat between years (Table 1).

3.3. Family Interaction with Phytophthora Isolates

In the analyses of variance that included families from both studies, the family variance component estimates were zero or very low for all assessment periods resulting in no or poor heritability estimates. In these analyses, the study × family interaction variance estimates were large and averaged about 23% of the total variation. A phenotypic correlation among the family mortality means in the main and supplemental studies was not significant ($r = -0.14, p = 0.56$).

3.4. Effect of Phytophthora on Height Growth

The initial height (2014) of the sub-sample of seedlings from six families that remained non-symptomatic throughout the study was statistically different among the main study, supplemental study, and control group (Figure 4). During the subsequent three years, the height of the control seedlings and those in the main study were not statistically significant from each other. The height of the non-symptomatic seedlings in the supplemental study was initially the lowest of the three groups and this statistically significant difference widened over the measurement period (Figure 4).

![Figure 3. Individual-tree (left) and family mean (right) heritability estimates (with std. error) for mortality of Eastern white pine seedlings inoculated with Phytophthora cinnamomi at weeks 0, 8, 53, and 61. Seedlings in the Main Study were inoculated with a culture isolated from Fraser fir while seedlings in the Supplemental Study were inoculated with a culture isolated from Eastern white pine.](image-url)
4. Discussion

Eastern white pine proved to be relatively resistant to root rot caused by *P. cinnamomii*. The relatively low mortality in these studies is contrary to the 87% mortality reported by Kirby and Grand (1975). The cause of this discrepancy is unknown but may be due to their use of potentially more infectious inoculation techniques, wetter conditions, and, perhaps, more susceptible host material and/or a more aggressive *P. cinnamomii* isolate. The studies reported here were initially planned for one year but because of the relatively low mortality (18.6%) in the main study at the end of the first year, they were extended for another year with additional inoculations. In contrast to the first-year results for Eastern white pine, Fraser fir and American chestnut (*Castanea dentata* (March.) Borkh.) approached 100 and 90 percent mortality, respectively, during the same time period when challenged with the same inoculum (23s04) using the same inoculation methods, and under similar environmental conditions [12,17,18]. Furthermore, in a similar resistance screening of 32 *Abies* species, only the single most resistant species demonstrated less mortality than Eastern white pine [11]. Continuing the studies reported here into the second year did not provide additional information about genetic control of resistance. Instead, the results were confounded by the overwintering environment and the effect of the increasingly crowded conditions on both the root systems and crowns of the seedlings. These effects are manifest in the second-year mortality of the non-inoculated control seedlings (Figure 2), a sub-sample of which all tested negative for *Phytophthora*. Due to the increased environmental effects on mortality, the heritability estimates generally decreased from year one to year two, although they remained relatively stable throughout the second year (Figure 3). Future evaluations of Phytophthora root rot resistance in Eastern white pine seedlings can be relatively rapid with a final assessment between 12 and 16 weeks after the initial inoculation.

Although Eastern white pine is relatively resistant to *P. cinnamomii*, there is considerable variation among families within the species. It should be noted that the families evaluated in this study originated from selections made primarily in North Carolina, a relatively small portion of the expansive
range of Eastern white pine. The amount of variation in resistance to an introduced pathogen is rather remarkable. Possibly, a pattern of geographic variation for resistance exists, as has been shown for Trojan (Abies equi-trojani Aschers. et Sint) and Turkish (A. bornmuelleriana Mattf.) fir in Turkey [12]. Unfortunately, family deployment recommendations are complicated by the large interaction observed between the pine families and the two P. cinamomoni isolates studied. The isolate derived from Eastern white pine (supplemental study) was clearly more aggressive (40.4% mortality) than the isolate derived from Fraser fir (26.4% mortality) on the same 20 families. Interestingly, after the first year (before environmental effects considerably confounded mortality), the mortality heritability estimates for the same 20 families were higher when challenged by the isolate from white pine relative to being challenged with the isolate from Fraser fir (Table 1). However, the isolate × family interaction variance component was high and the correlation of family mortality means between studies (isolates) was not significant. This was surprising because these two isolates (among others) had been used in a previous Abies study [17]. In that study, the magnitude of mortality among four fir species inoculated with these isolates was similar with identical species mortality rankings. Further, the correlation of family mortality means between isolates 2334 and 23004 was high (r = 0.87, p ≤ 0.0001). Apparently, isolate 2334 has some attribute allowing it to be specifically aggressive to Eastern white pine. Three of the twenty families averaged 20% mortality or less across both isolates and will be recommended for deployment. Planting these families versus the most susceptible or even an average mix of families could substantially improve survival and decrease economic losses associated with Christmas tree and forestry regeneration efforts. Clearly, however, resistance evaluations of more families using isolate 2334 and other isolates derived from Eastern white pine are needed. In retrospect, it would have been preferable to have inoculated all 83 families with isolate 2334.

In addition to causing more mortality, the P. cinamomoni isolate from Eastern white pine caused a reduction in height growth in inoculated but non-symptomatic seedlings relative to the non-inoculated control seedlings, while the isolate from Fraser fir did not. It is not known whether these seedlings were infected or continually engaged in resisting infection of Phytophthora in the medium, but in either case, it appears that more resources were required to maintain defense responses to the isolate from white pine. This has important practical implications for planting stock producers, especially bare-root nurseries, that encounter Phytophthora problems. In addition to the risk of spreading the pathogen, infected non-symptomatic seedlings will likely reduce productivity when established in Christmas tree and forest plantations.

Warm soil temperatures (>50 F or >10 °C) are one of several epidemiological factors (including average soil pH (pH 4.5–6) and saturated soils) that increase damage caused by P. cinamomoni via favoring sporangium formation, zoospore release, and infection. A recent study demonstrated a dramatic increase in root rot severity and mortality of seven Abies species caused by P. cinamomoni and three other Phytophthora species under a warm (27–32 °C) versus cool (15–21 °C) environment [24]. Differences in soil temperature provide at least a partial explanation for the contrasting experiences of North Carolina Christmas tree growers, where in the mountains, Eastern white pine is deliberately planted on sites known to be infested with Phytophthora, often without obvious deleterious effects, versus in the Piedmont and Coastal Plain regions, where infected trees commonly die. Warmer soil temperatures associated with climate change will not only increase disease severity and mortality rate but are also expected to expand the range of P. cinamomoni [25], thus further increasing the threat to both Christmas tree and forest plantations.

In addition to climate change, the introduction of new Phytophthora species is another serious threat to Eastern white pine. Recently, the number of Phytophthora species contributing to losses in Fraser fir Christmas trees in the Southern Appalachians has increased [26,27]. In a 2014 survey of Fraser fir Christmas tree plantations in the region, six Phytophthora species were identified, three of which were isolated for the first time in the region [27]. P. cryptogea Pethybr. & Laff., in particular, appears to have rapidly spread. Once introduced and dispersed in a new area, Phytophthora species have proven to be nearly impossible to control and these new species are very likely to eventually
threaten Eastern white pine. As such, the challenge to finding resistance may become substantially more complex and include the need to account for variation within *P. cinnamomi*, as demonstrated by the studies reported here, as well as the need to evaluate resistance to other pathogen species.

*Phytophthora* species are certain to continue to cause losses to Eastern white pine Christmas tree and forest plantations. Nurseries, growers, and foresters all need to be knowledgeable about, and diligent in implementing, practices that reduce its spread, especially ensuring that planting stock is disease free [13,27]. Selection and use of genetically resistant material can play a key role in the overall strategy in order to ameliorate future catastrophic impacts of these intraspecific pathogens.

5. Conclusions

While Eastern white pine is relatively resistant to, or tolerant of, infection by *P. cinnamomi*, considerable variation exists among open-pollinated families within the species. There is an opportunity to reduce economic losses to the root rot disease caused by this pathogen through the deployment of genetically resistant planting stock in Christmas tree and forest plantations. In this research, the responses of white pine families interacted with the two pathogen isolates evaluated, making deployment options more challenging. Future research should be directed at better understanding this interaction and screening additional host families with a broader array of pathogen isolates.

Acknowledgments: The authors are grateful to William Kohlway, Maria Escanferla, Jonathan Pearson, Yusof Kurt, Tracynne Allison, and Rhys Porter for their valuable technical assistance. We are thankful to James West and Anna Hollifield of the North Carolina Forest Service for providing the plant material used in this study. This study was supported by the USDA NIFA Specialty Crops Research Initiative (2012-51181-19940) and the North Carolina Agricultural Research Service via the Christmas Tree Genetics Program.

Author Contributions: John Frampton designed and supervised implementation of the studies, supervised the statistical analyses, constructed the tables and figures, wrote sections of the manuscript, and crafted the final version. Martin Pettersson carried out the statistical analyses and wrote the first draft of the manuscript. Anne Margaret Ibrahim participated in the design of the studies, supervised and carried out all technical aspects, provided photographs, and participated in writing and editing the manuscript.

Conflicts of Interest: The authors declare no conflict of interest. The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

References


12. Frampton, J.; Isik, F.; Benson, D.M. Genetic variation in resistance to Phytophthora cinnamomii in seedlings of two Turkish Abies species. Trees Genet. & Genomes 2013, 9, 53–63. [CrossRef]


© 2018 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).
CHAPTER 4. Diseases, pests and nutrient deficiencies of Swedish Christmas trees (2015 disease survey)

Citation:
Introduction

Study areas in Sweden

Most Christmas tree production (and almost all fir production) is based in the southern part of the country (Fig. 1). The reason for this uneven distribution is the proximity to Danish Christmas tree production, which has had a major influence on Swedish Christmas tree production. The Danish Christmas tree provenances (particularly Nordmann fir) do not grow well further north in Sweden.

By the end of May 2015, the occurrence of diseases, pests and nutrient deficiencies was investigated in a pilot study of Swedish Christmas trees. Another goal of the study was to specifically determine which, if any, *Phytophthora* species were present in the fields or in nearby waterways. In total, 21 Swedish Christmas tree farms located in five counties (Västra Götaland, Halland, Skåne, Blekinge and Kalmar) in southern Sweden were surveyed. The farms had a mixture of fir and spruce, and one or several fields per farm were surveyed for diseases, pests and nutrient deficiencies (Pettersson *et al.*, 2015). The studies reported in CHAPTERS 5 and 6 were conducted as an outgrowth of observations made during this survey.
Figure 1. Map of southern Sweden with the 21 Christmas tree farms (indicated as black dots) included in a disease and pest survey in spring 2015. The star on the smaller-scale map of the Nordic countries (upper right-hand corner) indicates the area where the majority of Swedish Christmas tree production is based and where most of the survey was conducted.

Methods and materials

The methods employed for this survey are described in CHAPTERS 5 and 6.
Results

This pilot study conducted in 2015 was the first disease and pest survey undertaken on Swedish Christmas trees. The data from the survey were published in the Danish trade journal Nåledrys (Pettersson et al., 2015).

The occurrence of disease-causing pathogens, pests and nutrient deficiencies in Christmas trees in southern Sweden is displayed in Table 1. Among the diseases caused by pathogens, Neonectria canker and Phytophthora root rot have the largest potential to become serious problems. Neonectria canker caused by *N. fuckeliana* and *N. neomacrospora* led to top and branch dieback on spruce and fir, respectively. *Neonectria neomacrospora* had never been reported in Sweden before, and was therefore investigated for pathogenicity and reported in CHAPTER 6. *Neonectria fuckeliana* had not been reported to cause top-dieback on Norway spruce in Sweden before, and was therefore investigated for pathogenicity on Norway spruce (CHAPTER 6 and 7). Both of these studies were conducted to find out whether Neonectria could become a major problem for the Christmas tree and forestry industries. The *Phytophthora* species found were worrisome; see section 3.3 below (CHAPTER 5). However, other diseases were also observed (Table 1), and a brief description of the pathogens most problematic in addition to Neonectria and *Phytophthora* species is given below:

- **Armillaria root rot** (*Armillaria* spp.) can infect all Christmas tree species and destroy roots, result in slow growth rates and eventually lead to mortality. Early symptoms are difficult to detect, but severe root rot results in yellowing and subsequent browning of all needles. Signs of the pathogen such as white mycelial fans and dark rhizomorphs can be detected by looking
under the bark at the root collar or examining the root system (Fig. 2A-B). Sometimes clusters of yellow mushrooms (fruiting bodies) appear around the base of infected trees.

➢ **Cherry spruce rust** *[Thekopsora areolate (Fr.) Magnus]* alternates between spruce and bird cherry (*Prunus padus* L.) and infects new shoots. Infected shoots become blackened and often S-shaped (bending towards the infection site) (Fig. 2C). The fungus has a two-year life cycle where wind-borne basidiospores from bird cherry leaves infect young spruce shoots and cause them to bend. If the alternative host (bird cherry) is removed from areas surrounding the Christmas tree farm, the fungus cannot complete its life cycle.

➢ **Chrysomyxa needle rust** *[Chrysomyxa abietis (Wallr.) Unger]* attacks needles on new shoots of spruce. Infection results in small yellowish spots, which develop into bigger spots or cross-bands. Under severe disease pressure, all needles on new shoots can become chlorotic. In the following spring, infected parts of the needles swell up and a yellow-orange, waxy cushion appears (Fig. 2D-E). Basidiospores are released from these fruiting bodies. The spores can only infect soft needles of new shoots. Infected needles can remain on the tree for more than a year before they fall off. The damage by this rust fungus can be extensive in Norway spruce Christmas tree fields.

➢ **Delphinella shoot blight** *[Delphinella abietis (E. Rostrup) E. Müller]* attacks needles on new shoots of firs. The needles start to yellow and turn brown/gray in color (Fig. 2F). Numerous small black pseudothecia develop on the diseased needles. The buds normally survive, but severe infections may result in shoot dieback. Delphinella shoot blight is currently a problem in Norway and western USA (Chastagner *et al.*, 2017; Talgø *et al.*, 2016).

➢ **Gemmamyces bud blight** *[Gemmamyces piceae (Borthw.) Casagr.]* attacks and kills spruce buds, which become black, skewed and covered with black pycnidia (Fig. 2G-H).
Gemmamyces bud blight causes epidemics on Colorado blue spruce in central Europe (Černý et al., 2015).

- **Lirula needle cast** [*Lirula macrospora* (R. Hartig) Darker] causes needle discoloration on spruce. Infection of current-year needles occurs under humid or rainy weather conditions during the shoot-elongation phase. The symptoms (brown needles) appear the year after infection (two-year cycle). The fruiting bodies are elongated and black forming a distinctive black band around the base of infected needles can be seen with the naked eye (Fig. 2I-J).

- **Sydowia polyspora** (Bref. & Tavel) E. Müll. is involved in two different diseases on Christmas trees:
  - **Current Season Needle Necrosis (CSNN)** causes needle discoloration on fir. Symptoms are yellow/red discolored bands that appear on needles 2-4 weeks after shoot elongation (Fig. 2K). Small, black pycnidia develop on infected needles. Severe infections may lead to total discoloration of most of the needles and subsequent heavy needle cast.
  - **Sclerophoma shoot dieback** damages newly emerged shoots on spruce and fir. They become necrotic and may bend downwards (Fig. 2L-M). On the dead shoots, numerous black pycnidia appear.

Table 1 also includes a number of other biotic (pathogens, insects, mites, wildlife) and abiotic (nutrient deficiencies) damaging agents, though these are not further described.
Table 1. Biotic and abiotic damaging agents found in Swedish Christmas tree fields during a survey in 2015.

<table>
<thead>
<tr>
<th>Damaging agent</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathogen (disease)</td>
<td></td>
</tr>
<tr>
<td>Armillaria spp. (Armillaria root rot)</td>
<td>A. nordmanniana, P. pungens</td>
</tr>
<tr>
<td>Camarosporium sp.</td>
<td>P. pungens</td>
</tr>
<tr>
<td>Chrysomyxa abietis (Chrysomyxa needle rust)</td>
<td>P. abies, P. pungens</td>
</tr>
<tr>
<td>Delphinella abietis (Delphinella shoot blight)</td>
<td>A. nordmanniana</td>
</tr>
<tr>
<td>Gemmamyces piceae (Gemmamyces bud blight)</td>
<td>P. pungens</td>
</tr>
<tr>
<td>Herpotrichia juniperi (black snow mould)</td>
<td>P. pungens</td>
</tr>
<tr>
<td>Lirula macropora (Lirula needlecast)</td>
<td>P. abies, P. pungens</td>
</tr>
<tr>
<td>Lophodermium piceae (Lophodermium needle cast)</td>
<td>P. pungens</td>
</tr>
<tr>
<td>Neonectria fuckeliana (Neonectria canker)</td>
<td>P. abies</td>
</tr>
<tr>
<td>Neonectria neomacrospora (Neonectria canker)</td>
<td>A. nordmanniana</td>
</tr>
<tr>
<td>Phytophthora spp. (Phytophthora root rot)</td>
<td>P. abies (soil and water)</td>
</tr>
<tr>
<td>Rhizosphaera kalkhoffii (Rhizosphaera needles cast)</td>
<td>P. pungens</td>
</tr>
<tr>
<td>Siroccoccus strobilinus (Siroccoccus blight)</td>
<td>P. pungens</td>
</tr>
<tr>
<td>Sydowia polyspora (Current season needle necrosis)</td>
<td>A. nordmanniana, A. procera</td>
</tr>
<tr>
<td>Sydowia polyspora (Sclerophoma shoot dieback)</td>
<td>A. nordmanniana, P. abies</td>
</tr>
<tr>
<td>Thekopsora areolata (Cherry spruce rust)</td>
<td>P. abies</td>
</tr>
<tr>
<td>Insect and mite (pest)</td>
<td></td>
</tr>
<tr>
<td>Adelges abietis (pineapple gall adelgid)</td>
<td>P. abies</td>
</tr>
<tr>
<td>Adelges viridis (spruce pineapple gall adelgid)</td>
<td>P. abies</td>
</tr>
<tr>
<td>Adelges pectinatae</td>
<td>A. nordmanniana</td>
</tr>
<tr>
<td>Adelges nordmannianae (silver fir woolly adelgid)</td>
<td>A. nordmanniana</td>
</tr>
<tr>
<td>Adelges piceae (balsam woolly adelgid)</td>
<td>A. nordmanniana</td>
</tr>
<tr>
<td>Nalepella species (gall mites)</td>
<td>A. nordmanniana</td>
</tr>
<tr>
<td>Nutrient deficiencies + wildlife damage</td>
<td></td>
</tr>
<tr>
<td>Magnesium (Mg) deficiency</td>
<td>A. nordmanniana</td>
</tr>
<tr>
<td>Manganese (Mn) deficiency</td>
<td>A. nordmanniana</td>
</tr>
<tr>
<td>Damage by wildlife (deer, vole, etc.)</td>
<td>A. nordmanniana, P. abies, P. pungens</td>
</tr>
</tbody>
</table>
Figure 2. Plant symptoms and pathogen signs of several diseases found in a survey of Swedish Christmas trees in 2015. Armillaria root rot (A-B), Cherry spruce rust (C), Chrysomyxa needle rust (D-E), Delphinella shoot blight (F), Gemmamyces bud blight (G-H), Lirula needle cast (I-J), CSNN (K), and Sclerophoma shoot dieback (L-M). Photos: Martin Pettersson
CHAPTER 5. Presence of *Phytophthora* species in Swedish Christmas tree plantations

Citation:
Presence of Phytophthora species in Swedish Christmas tree plantations

Martin Pettersson 1,2*, John Frumpton 2, Jonas Rönberg 1, May Bente Brurberg 3,4 and Venclo Talgo 3

1 Southern Swedish Forest Research Centre, Swedish University of Agricultural Sciences, P. O. Box 49, SE-23053 Alnarp, Sweden
2 Department of Forestry and Environmental Resources, North Carolina State University, 2800 Faucette Dr, Raleigh, NC 27695, USA
3 Norwegian Institute of Bioeconomy Research, P.O. Box 115, 1431 Ås, Norway
4 Norwegian University of Life Science, P.O. Box 5003, NO-1432 Ås, Norway

*Correspondence: martin.pettersson@slu.se; Tel.: +46-070-896-6334

Abstract
Phytophthora cryptogea, P. gonapodydes, P. laciustris, P. megasperma, P. plurivora and an unknown Phytophthora species were isolated from waterways and soil samples in Christmas tree fields in Southern Sweden. In addition, P. megasperma was isolated from a diseased Norway spruce (Picea abies) plant from one of the fields in Svalöv. Root inoculation tests were sequentially carried out with one isolate from each of the three species P. cryptogea, P. megasperma, and P. plurivora, all known pathogens on conifers. The same three isolates were used to study a few morphological features to confirm the identification, and temperature-growth relationships were carried out to see how well the organisms fit into Swedish climate conditions. Seedlings of Norway spruce and Nordmann fir (Abies nordmanniana) were inoculated in either the roots or the stems. None of the isolates caused extensive root rot under the experimental conditions, but all three species could be re-isolated from both Norway spruce and Nordmann fir. Phytophthora root rot is currently of minor concern for Christmas tree growers in Sweden. However, the Phytophthora isolations from soil and water indicate the presence of this damaging agent, which may lead to future problems.

Keywords: Phytophthora cryptogea, Phytophthora megasperma, Phytophthora plurivora, Abies, Picea, root rot

Introduction
In Sweden, more than 3 million Christmas trees are sold annually. Norway spruce [Picea abies (L.) H. Karst] and Nordmann fir [Abies nordmanniana (Steven) Spach] are the most popular and economically important Christmas tree species in Sweden, accounting for most of the sales. Other species grown as Christmas trees are noble fir (A. procera Rehder), subalpine fir [A. lasiocarpa (Hook.) Nutl.], Fraser fir [A. fraseri (Porsch) Poir.], Colorado blue spruce (P. pungens Engelm.), Serbian spruce [P. omorica (Pancie) Purk.] and a few others of minor importance. Fir trees are mainly grown in southern Sweden where the winters are mild compared to northern Sweden.

Alien invasive pathogens, such as Phytophthora species, threaten conifers around the globe, including Christmas trees. These pathogens have spread long-distances through global plant trade and especially Phytophthora species have been introduced throughout European nurseries, landscape and forest plantings (Jung et al. 2016).

Phytophthora root rot causes great economic losses in the Christmas tree production in USA. There, multiple Phytophthora species are responsible for severe root and collar rot of fir species, which have become a major limitation to the Christmas tree production, especially in areas with poorly drained soils (McKeever and Chastagner 2016). In Europe, Phytophthora damage on fir Christmas trees has only been reported from Ireland (Shaflizadeh and Kavanagh 2005) and Norway (Talgo et al. 2006; Talgo et al. 2007). However, a recent study in Norway, Belgium and Denmark showed that several Phytophthora species are present in waterways associated with Christmas tree production (Talgo et al. 2017). On spruces (Picea spp.), which are commonly used as Christmas trees in the Nordic countries, Phytophthora
species have been reported on seedlings in Germany (Jung and Baschke 2004). In Sweden, several Phytophthora species have been found in waterways, natural environments and/or forests (Cleary et al. 2017; Jonsson et al. 2003; Redondo et al. 2015), but prior to this report, Christmas tree fields were never surveyed.

Diseases symptoms of Phytophthora root rot on Christmas trees are chlorosis of foliage, stunting and wilting of new growth, and tree mortality. These symptoms are caused by lack of water and nutrients due to destroyed roots. Sometimes reddish brown cambial discoloration can be seen on the lower stem (Chastagner and Benson 2000). The cambial discoloration may develop into a sunken, wounded area (canker or lesion) where resin flow sometimes occurs (Talgo and Chastagner 2013). There is a sharp border between the infected reddish brown tissue of the canker and the healthy tissue, the so-called leading edge of the infection. Dead branches (flagging) are often associated with destroyed cambial layer in the stem at the base of the branches (Chastagner and Benson 2000).

Phytophthora species reported on Christmas trees in USA are P. cambivora, P. cactorum, P. capsici, P. citricola, P. citrophthora, P. cryptogea, P. megasperma, and P. tunarii (Chastagner and Benson 2010). Among these species, only P. cambivora and P. tunarii have been recorded on Christmas trees in Europe (Sharifzadeh and Kavanagh 2005; Talgo et al. 2006; Talgo et al. 2007).

Phytophthora species can rapidly spread in the environment by sporangial formation in wet soil and the subsequent release of zoospores that may follow runoff water from e.g., nurseries, landscape plantings, planted forest sites, and agricultural fields, to streams and rivers. The infested nursery pathway is reported to be the main route for Phytophthora species to invade new areas (Jung et al. 2016). Moreover, due to resting spores (oospores and chlamydomospores), Phytophthora species can survive unfavourable conditions for many years and are nearly impossible to get rid of once introduced into an area (Hayden et al. 2013; Judelson and Blanco 2005).

The majority of the Christmas tree fir seedlings are imported into Sweden as bare-root plants, thus, posing a risk of introducing Phytophthora species (Jung et al. 2016). Knowing that Phytophthora root rot is a serious problem in many Christmas tree producing regions of the world (McKeever and Chastagner 2016; Sharifzadeh and Kavanagh 2005; Talgo and Chastagner 2013), including Sweden’s neighbouring country, Norway (Talgo et al. 2006; Talgo et al. 2007), we aimed to investigate presence of Phytophthora in the Swedish Christmas tree production and hypothesised that several species are present. We focused on true fir species since they are known to be sensitive to Phytophthora root rot (Talgo and Chastagner 2013). However, non-Swedish provenances of Norway spruce are also imported and used in Swedish plantations, hence these were included in the investigation.

Materials and Methods

Field survey

In May 2015, twenty fields on fourteen Swedish Christmas tree farms located in five counties in Southern Sweden were surveyed for Phytophthora, two in Västra Götaland, two in Halland, seven in Skåne, two in Blekinge, and one in Kalmar (Fig. 1). Trees were mainly grown on agricultural land and the age of the trees varied from 1 to 10 years. The fields ranged in size from less than half a hectare up to 30 hectares. The position of each farm was recorded with a Garmin GPS-device.
Isolation methods
Three commonly used sampling methods known to be efficient in detecting Phytophthora species, were used in the survey:

1. Trees were visually inspected for Phytophthora symptoms, such as chlorosis of foliage, flagging (dead basal branches), and basal sunken areas with resin flow. Symptomatic trees were uprooted and investigated for root rot. Only symptomatic trees were sampled. In fields less than half a hectare, all trees were included (commonly 3000-5500 trees are planted per ha). For fields larger than half a hectare, all trees grown in lower, wet or poorly drained areas were inspected. In addition, five randomly chosen transects of 300 trees (1500 trees per field) were checked for Phytophthora symptoms. The root systems of any tree with Phytophthora symptoms were dug up, and selected roots were put in resealable plastic bags, stored in a cooler and brought to the laboratory within 1 week.

2. Soil samples were taken with a small garden spade from fields with wet and poorly drained areas, not necessarily connected to symptomatic trees. Each of them was a bulk sample including five arbitrary selected spots per field (approximately 1 L/sample). The samples were stored in plastic boxes (25 x 17 x 6 cm) in a cooler.

3. Leaves from Rhododendron ‘Cunningham’s White’ were used to bait in streams and ponds in or adjacent to the Christmas trees. These streams or ponds receive drainage and runoff water from the fields during rainy periods. The Rhododendron leaves were harvested from disease-free plants kept at NIBIO. Current and last year leaves where kept apart in separate containers and stored in a cooler. In the field, two current year leaves and one last season leaf were placed in mesh bags, each with a styrofoam floater to keep the bait near the surface (Fig. 2a). The reason for using both young and old leaves was to secure an active leading edge (border between green and necrotic tissue) of a potential Phytophthora infection. Old leaves have by experience at NIBIO proven to resist infection longer than young leaves. The latter is important if inoculum level of an aggressive Phytophthora is so high that the young leaves turn completely black during the baiting period. Young leaves are important to catch smaller amounts of inoculum. The bait bags were anchored to a nearby tree or a shrub, left in the water for 6-8 days, retrieved, and then shipped to the lab. Since this study was conducted early in the season when water temperatures in Sweden are fairly low, a longer timeframe was needed for the baiting (Ghimire et al. 2009; Werres et al. 2007).
Fig. 2 Baiting for Phytophthora with Rhododendron ‘Cunningham’s White’ leaves; (a) anchored bait bag containing three leaves (two newly emerged and one from the previous year) and a piece of styrofoam to keep the bait near the surface; (b) Rhododendron leaves with dark, water soaked spots after baiting (old leaf on top and current year leaves underneath); (c) nine cm Petri dish with Phytophthora-selective medium (P10ARPH) and a PVC well (3 cm) with five sections from the leading edges of Rhododendron leaves. Photo: Martin Petersson

Roots of symptomatic plants where washed clean from soil under tap water, investigated for lesions, and segments (0.5-1 cm long) for isolation were dissected from symptomatic roots using a sterile scalpel. The soil samples, were completely submerged in deionized water and swirled for 1 minute using a glass rod. The mixed sediment suspension was left standing over night for the water to become clear, before 5 Rhododendron leaves were put on the water surface (abaxial surface down) as baits for possible Phytophthora zoospores. After 4 to 8 days, depending on symptom development, leaves with dark or water soaked lesions were removed and gently rinsed under running, deionized water. Sections from the leading edges of necrotic lesions on the leaves where removed using a sterile scalpel.

Bait leaves from waterways with dark or water soaked lesions (Fig. 2b) were washed and sectioned in the same fashion as the leaves from the soil baiting. Root and leaf sections from all three sampling methods, were placed onto the Phytophthora-selective agar P10ARPH (17 g corn meal agar, 1000 ml distilled water, 10 mg pinarnicin, 0.25 g ampicillin, 0.01 g rifampicin, 1.0 ml dimethylsulfoxide, 0.1 g pentachloronitrobenzene, and 0.05 g hyphoxol) (Telfer et al. 2015). An autoclaved PVC ring (3 or 4 cm in diameter) was placed in the middle of each Petri dish before the agar had solidified, to create a well. Five to 10 root segments and 3 to 5 leaf sections were placed inside the well (Fig. 2c), which enclosed bacterial and fungal growth, while hyphae from oomycetes grew below the ring and emerged on the agar outside the ring. Mycelium emerging on the outside of the ring was transferred to Potato Dextrose Agar (PDA; Difco Laboratories, Detroit, MI) and V8 juice agar (V8A; 354 ml Campbell Vegetable juice V8, 1416 ml distilled water, 3.5 CaCO3, 26.5 g Bacto™ agar).
Molecular identification

Mycelia from pure cultures resembling *Phytophthora* species, were harvested from the agar plates with a sterile scalp and homogenized by grinding thoroughly with a pestle and mortar with liquid nitrogen. DNA from the homogenized mycelia were extracted using DNeasy® Plant Mini Kit (Qiagen Inc., Valencia, CA, USA). Amplification of the internal transcribed spacer (ITS) region of nuclear rDNA and the COI mitochondrial DNA was performed using the primer pairs ITS5 and -ITS4 primers and OomCox1Rev- and Fm85cod primers, as described by White et al. (1990) and Robideau et al. (2011), respectively. The PCR products were sent to GATC (Germany) for sequencing. Raw sequences were trimmed, assembled and manually controlled using Geneious version 8.1.4 (http://www.geneious.com) (Kearse et al. 2012). They were then compared with sequences in GenBank using the basic local alignment search tool (BLAST) (Altschul et al. 1990), and results were used to identify isolates to species level. The obtained gene sequences were deposited in GenBank and their accession numbers are described in Table 1.

### Table 1  *Phytophthora* isolates obtained from Christmas tree fields in southern Sweden in 2015

<table>
<thead>
<tr>
<th><em>Phytophthora</em> species</th>
<th>Location 1</th>
<th>Source 2</th>
<th>Isolate no.</th>
<th>ITS GenBank Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. cryptogea</em></td>
<td>Svalöv, S</td>
<td>Soil</td>
<td>250476</td>
<td>KT350500 MGB17500</td>
</tr>
<tr>
<td><em>P. gonapodydes</em></td>
<td>Borås, VG</td>
<td>Soil</td>
<td>250477</td>
<td>KT383038 MGB17501</td>
</tr>
<tr>
<td><em>P. gonapodydes</em></td>
<td>Borås, VG</td>
<td>Bait</td>
<td>250478</td>
<td>KT383039 MGB17502</td>
</tr>
<tr>
<td><em>P. gonapodydes</em></td>
<td>Halmstad, H</td>
<td>Soil</td>
<td>250479</td>
<td>KT383040 MGB17503</td>
</tr>
<tr>
<td><em>P. gonapodydes</em></td>
<td>Lund, S</td>
<td>Soil</td>
<td>250480</td>
<td>KT383041 MGB17504</td>
</tr>
<tr>
<td><em>P. gonapodydes</em></td>
<td>Lund, S</td>
<td>Bait</td>
<td>250481</td>
<td>KT383042 MGB17505</td>
</tr>
<tr>
<td><em>P. gonapodydes</em></td>
<td>Lund, S</td>
<td>Bait</td>
<td>250482</td>
<td>KT383043 MGB17506</td>
</tr>
<tr>
<td><em>P. gonapodydes</em></td>
<td>Svedala, S</td>
<td>Bait</td>
<td>250483</td>
<td>KT383044 MGB17507</td>
</tr>
<tr>
<td><em>P. gonapodydes</em></td>
<td>Sjöbo, S</td>
<td>Bait</td>
<td>250484</td>
<td>KT383045 MGB17508</td>
</tr>
<tr>
<td><em>P. gonapodydes</em></td>
<td>Karlstörn, B</td>
<td>Bait</td>
<td>250485</td>
<td>KT383046 MGB17509</td>
</tr>
<tr>
<td><em>P. gonapodydes</em></td>
<td>Karlstörn, B</td>
<td>Bait</td>
<td>250485</td>
<td>KT383047 MGB17510</td>
</tr>
<tr>
<td><em>Phytophthora</em> sp. 3</td>
<td>Kristianst, S</td>
<td>Soil</td>
<td>250486</td>
<td>KT383048 MGB17511</td>
</tr>
<tr>
<td><em>P. lacustris</em></td>
<td>Högås, S</td>
<td>Bait</td>
<td>250487</td>
<td>KT383049 MGB17512</td>
</tr>
<tr>
<td><em>P. lacustris</em></td>
<td>Kristianst, S</td>
<td>Bait</td>
<td>250488</td>
<td>KT383050 MGB17513</td>
</tr>
<tr>
<td><em>P. lacustris</em></td>
<td>Kristianst, S</td>
<td>Bait</td>
<td>250490</td>
<td>KT383050 MGB17514</td>
</tr>
<tr>
<td><em>P. lacustris</em></td>
<td>Sjöbo, S</td>
<td>Bait</td>
<td>250491</td>
<td>KT383051 MGB17515</td>
</tr>
<tr>
<td><em>P. lacustris</em></td>
<td>Mörlönga, K</td>
<td>Bait</td>
<td>250492</td>
<td>KT383052 MGB17516</td>
</tr>
<tr>
<td><em>P. megasperma</em></td>
<td>Borås, VG</td>
<td>Soil</td>
<td>250493</td>
<td>KT383053 MGB17517</td>
</tr>
<tr>
<td><em>P. megasperma</em></td>
<td>Svalöv, S</td>
<td>Roots</td>
<td>250494</td>
<td>KT383054 MGB17518</td>
</tr>
<tr>
<td><em>P. megasperma</em></td>
<td>Svalöv, S</td>
<td>Bait</td>
<td>250495</td>
<td>KT383055 MGB17519</td>
</tr>
<tr>
<td><em>P. plurivora</em></td>
<td>Munkedal, VG</td>
<td>Bait</td>
<td>250496</td>
<td>KT383049 MGB17520</td>
</tr>
<tr>
<td><em>P. plurivora</em></td>
<td>Halmstad, H</td>
<td>Bait</td>
<td>250497</td>
<td>KT383055 MGB17521</td>
</tr>
<tr>
<td><em>P. plurivora</em></td>
<td>Svalöv, S</td>
<td>Bait</td>
<td>250498</td>
<td>KT383056 MGB17522</td>
</tr>
<tr>
<td><em>P. plurivora</em></td>
<td>Svedala, S</td>
<td>Bait</td>
<td>250499</td>
<td>KT383057 MGB17523</td>
</tr>
<tr>
<td><em>P. plurivora</em></td>
<td>Svedala, S</td>
<td>Bait</td>
<td>250500</td>
<td>KT383058 MGB17524</td>
</tr>
<tr>
<td><em>P. plurivora</em></td>
<td>Svalöv, S</td>
<td>Soil</td>
<td>250501</td>
<td>KT383059 MGB17525</td>
</tr>
</tbody>
</table>

1 Counts are given as abbreviation: VG = Västra Götaland; H = Halland; S = Skåne; B = Blekinge; K = Kalmar
2 Roots = roots of symptomatic plants; Soil = soil from wet areas in the Christmas tree fields; Bait = Rhododendron leaves used for baiting in streams and ponds
3 The unknown *Phytophthora* species had ITS and COI sequences most similar to *P. inundata* and *P. humicola*, respectively
Morphological features
Some morphological features were assessed to confirm identity for three isolates used in an inoculation test described below: *P. cryopoea* (isolate no. 250476), *P. megasperma* (isolate no. 250494), and *P. plurivora* (isolate no. 250496). The characteristics of each isolate were compared to the species descriptions in Q-bank (www.q-bank.eu) and/or Phytophthora Database (www.phytophthoradb.org), as well as the original description of the species. Colony morphology was assessed on V8A and PDA plates that were incubated in daylight at room temperature (20±1°C) for 2 weeks. The colony morphology was recorded after 7 and 14 days, respectively.

For sporangia production, non-sterile soil extract was prepared as described by Ristaino et al. (2010) with slight modifications: 15 g dried field soil was stirred in 1000 ml distilled water for 4 hours, followed by allowing it to settle overnight. The supernatant was filtered through two layers of coffee filter paper, centrifuged at 6000 rpm for 15 min and filtered again in the same manner. Part of the soil sample used for preparing the non-sterile soil extract was baited with *Rhododendron* leaves to make sure it was free from *Phytophthora*. For each isolate, five mycelial plugs, 5 mm in diameter, were cut from the edge of 4-day-old cultures grown on clarified V8 juice agar (cV8A; 100 ml of buffered and clarified V8 juice, 900 ml deionized water, and 15 g agar) (Jeffers 2006). The plugs were placed in separate empty 9 cm Petri dishes, with the mycelia side facing up, and flooded with the non-sterile soil extract. The Petri dishes were incubated in daylight at room temperature (20±1°C) for 24-36 hours. Among characteristics of emerging sporangia, width and length were measured for 20 randomly selected sporangia using a light microscope (Leica DM2000) connected to a camera (Leica DFC320) and the Olympus cellSens Entry computer software. The length/width ratio was calculated based on mean values of the sporangia. Cultures on cV8A, incubated in the dark at 20±1°C for one month, where checked regularly for emergence of oospores.

Temperature-growth relation
Temperature-growth relationships for the three selected isolates were tested by measuring radial growth rates on PDA. Three replicates of each isolate were incubated in the dark at 5, 10, 15, 20, 25, 30, and 35°C like described by Teller et al. (2015).

Pathogenicity test
Inoculations were carried out on 75 Norway spruce (10-15 cm tall) and 75 Nordmann fir (20-30 cm tall) seedlings with selected isolates of *P. cryopoea*, *P. megasperma*, and *P. plurivora*, the same ones as used for the morphological description. The seedlings were grown in trays, with drainage holes, that were placed in fitting non-perforated plastic boxes. The potting medium was a mix of peat soil and sand (4:1). A small sample of the growth medium, from around the roots of each seedling, was collected and baited with *Rhododendron* leaves to make sure they were free from *Phytophthora*. After 8 days with no dark or water soaked spots emerging on the *Rhododendron* leaves, the seedlings were determined free from *Phytophthora* infection.

For the inoculation of roots, *Phytophthora*-colonized rice grains were used. Each *Phytophthora* isolate was grown for 21 days on sterilized long-grain white rice in 250 ml Erlenmeyer flasks with caps sealed by Parafilm®. The flasks containing 25 g of rice grains and 17 ml of deionized water had been autoclaved overnight (for 20 hours) prior to adding two squares (5 x 5 x 5 mm) of actively growing mycelium on corn meal agar (CMA; Difco Laboratories, Detroit, MI). The flasks were gently shaken by hand once a day to avoid aggregations of the rice grains and to promote uniform colonization. After 21 days, 100% colonization of the rice grains was verified by plating 25 grains of each *Phytophthora* isolate on PDA. Each *Phytophthora* isolate was used for inoculation of the soil of 10 seedlings of Norway spruce and 10 of Nordmann fir, by making three holes about 2 cm deep with a sterilized glass rod in the growth medium 1-2 cm from the stem (in a triangular pattern) and placing a single colonized rice grain into each hole before closing (Benson et al. 1997). For controls, autoclaved, non-colonized rice grains in the soil were used for five Norway spruce and five Nordmann fir seedlings.

The seedlings were removed from the containers and the extent of foliar symptoms was rated by visually estimating the proportion of yellow/brown needles and wilting shoots on a scale from 0 to 5, where 0 = 0-10%, 1 = 11-25%, 2 = 26-50%, 3 = 51-75%, 4 = 76-90%, and 5 = 91-100%. Stem symptoms at the base, such as cambial discolorations or cankers were noted if present. The amount of root rot was estimated by carefully removing the growth medium from the roots in running tap water and determining the proportion of necrotic root tips using the same scale as for the foliage. Ten symptomatic root tips per
seedling were plated onto P10ARPH agar to recover the pathogen. Resulting 
Phytophthora cultures were 
sub-cultured on V8A and PDA media for comparing the morphology with the original isolates.

Stems/branches of Norway spruce and Nordmann fir seedlings were inoculated by mycelial plugs of 
14-day-old cultures grown on PDA (10 seedlings of each species per isolate). A wound was made by 
removing one needle and inoculation carried out by adding a mycelial plug (d = 5 mm) onto the needle 
scar and covering with a wet cotton pad (dipped in autoclaved, deionized water), and wrapping with 
Parafilm®. All Norway spruce seedlings were inoculated on stem needle wounds, while 50 % of the 
Nordmann fir seedlings were inoculated on needle wound on the stem and 50 % on the largest branch. 
Ten of each tree species were inoculated with plain PDA plugs, to serve as negative controls.

The inoculation test was carried out in a growth chamber with 65 % RH, 16°C and 18 h daylight (dark 
from 2100 - 0300). All the seedlings were regularly watered throughout the experiment and the water 
level at the bottom of the plastic boxes were kept at approximately 1 cm throughout the experiment. After 
seven months of incubation, all seedlings were examined for visible symptoms of Phytophthora damage. 

For the stem/branch inoculation, the lesion lengths under the bark at the inoculation point were 
measured with a ruler after carefully removing the bark with a sterile scalpel. Phytophthora species from 
inoculated plants were isolated as described above.

Results
Field survey
Of the 14 Christmas tree farms surveyed, 9 out of 20 fields had wet soil that were sampled and 3 out of 
20 fields had trees with Phytophthora symptoms. Only three plant samples from three different farms, 
two Nordmann fir plants and one Norway spruce plant, had symptoms resembling Phytophthora root rot. 
Both Nordmann fir plants were newly established, had yellowing foliage and necrotic roots, but no 
Phytophthora was recovered. The Norway spruce plant was young (estimated 2-4 years old), grown in a 
low and wet part of the field, had reddish foliage and root rot, and Phytophthora was recovered. Nine soil 
samples were taken from wet areas from nine farms and seven generated Phytophthora. A total of 30 bait 
bags were distributed over 13 farms (one site had no nearby surface water to be baited) and eighteen 
water baits generated Phytophthora. In total, Phytophthora species were recovered from roots, soil and/or 
bait samples from 12 different farms and resulted in a total of 26 isolates (Table 1).

Molecular and morphological identification of the obtained isolates generated six Phytophthora species; 
P. cryptogea, P. gonapodyides, P. lacastris, P. megasperma (sensu stricto), P. plurivora, and an 
unknown Phytophthora species (the ITS and COI sequences were most similar to P. inundata and P. 
humicola, respectively). All species except P. cryptogea and the unknown Phytophthora species were 
present at several locations (Table 1). Phytophthora megasperma was isolated from the symptomatic 
Norway spruce plant.

From the three isolates that were studied morphologically on V8A, P. cryptogea and P. megasperma 
had dense aerial fluffy colony morphology, while P. plurivora had rosette-like morphology. On PDA, all 
three isolates had more or less cotton-like colony morphology. The sporangial characteristics are 
described in Table 2 and shown in Fig. 3. The P. plurivora isolate produced gametangia in culture (Fig. 
3e), confirming that it is homothallic, while P. cryptogea and P. megasperma did not produce gametangia 
in cultures. However, P. megasperma produced cemulate globose hyphal swellings.

Optimal temperature for mycelial growth was 25°C for all three isolates. Mean daily growth rate 
(mm/d) on PDA at optimum temperature for P. cryptogea, P. megasperma and P. plurivora were 4.2, 3.6 
and 2.7 mm, respectively. No visible growth could be detected at 35°C (Fig. 4).

Phytophthora gonapodyides and P. lacastris, two closely related aquatic species (Nechwatal et al. 
2013), were not known from the literature to be harmful to conifer trees, and P. gonapodyides isolated 
from Rhododendron bait leaves, did not cause any disease symptoms in an inoculation experiment with 
several Christmas tree species (Talgo et al., unpublished data). Therefore, P. gonapodyides and P. 
lacastris were not included in further studies.
### Table 2 Characteristics of Phytophthora cryptogea, P. megasperma, and P. plurivora in culture

<table>
<thead>
<tr>
<th>Phytophthora species (isolate no.)</th>
<th>Sporangial characteristics in vitro</th>
<th>Other characteristics in single culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. cryptogea (250476)</td>
<td>Ellipsoid, ovoid, obpyriform</td>
<td>Average length: width ratio</td>
</tr>
<tr>
<td></td>
<td>Non-papillate</td>
<td>L = 34.7 – (40.0) – 44.9 W = 26.0 – (30.1) – 34.4 1.5 -</td>
</tr>
<tr>
<td>P. megasperma (250494)</td>
<td>Ellipsoid, ovoid, obpyriform</td>
<td>Abundant production of intercalary globose hyphal swellings</td>
</tr>
<tr>
<td></td>
<td>Non-papillate</td>
<td>L = 34.7 – (45.2) – 62.5 W = 25.1 – (30.5) – 37.1 1.5</td>
</tr>
<tr>
<td>P. plurivora (250496)</td>
<td>Ovoid, obpyriform</td>
<td>Abundant production of oogonia and antheridia</td>
</tr>
<tr>
<td></td>
<td>Semi-papillate</td>
<td>L = 28.8 – (49.1) – 62.2 W = 20.5 – (37.1) – 47.1 1.3</td>
</tr>
</tbody>
</table>

1 The sporangial characteristics are based on 20 randomly selected sporangia per species. The sporangia were obtained from Petri dishes with clarified V8A pieces flooded with non-sterile soil extract
2 Length (L), Width (W), Min – (average) – max [μm]
3 Phytophthora cryptogea and P. megasperma failed to produce oogonia and antheridia in single culture

---

**Fig. 3** Morphological structures of Phytophthora cryptogea 250494 - (a) ovoid, non-papillate sporangium, (b) basal swelling of ovoid non-papillate sporangium; P. megasperma 250494 - (c) ovoid, non-papillate sporangium, (d) globose hyphal swelling; P. plurivora 250496 - (e) oogonia with oospore; (f) obpyriform sporangium, (g) extended internal proliferation. Scale bar = 20 μm. Photos: Martin Pettersson
Fig. 4 Mean radial growth per day (mm/d) for Phytophthora cryptogea 250476, P. megasperma 250494, and P. plurivora (250496 grown on PDA at 5, 10, 15, 20, 25, 30, and 35°C. The whiskers are standard error of the mean from three replicates.

Pathogenicity test
Rice grain inoculation in the soil of Nordmann fir seedlings resulted in a few seedlings that displayed root rot and reduced amount of fine root mass; three, two and two seedlings for P. cryptogea, P. megasperma, and P. plurivora, respectively. Similarly, for the 10 Norway spruce seedlings, zero, two and three seedlings displayed some form of root rot, swelling of roots and reduced amount of fine root mass after inoculation with P. cryptogea, P. megasperma and P. plurivora, respectively (Fig. 5). None of the Norway spruce and Nordmann fir seedlings displayed any foliage damage after rice grain inoculation.

For the Nordmann fir seedlings inoculated with mycelial plugs on the needle scars of stems, average lesion lengths under the bark were 26.8, 8.8 and 3.8 mm for P. cryptogea, P. megasperma and P. plurivora, respectively (Table 3). In the same order, average lesion lengths under bark for the five branch inoculated seedlings were 31.4, 52.2 and 9.6 mm, respectively (Table 3). Two of the seedling branches inoculated with P. megasperma had dieback. One Nordmann fir seedling, stem inoculated with P. cryptogea, displayed major foliage damage.

For the Norway spruce seedlings, the average lesion lengths under the bark for the stem inoculated seedlings were 17.2, 23.5 and 5 mm for P. cryptogea, P. megasperma and P. plurivora, respectively (Table 3). Of these seedlings, three, four and zero displayed dead tops from the wound up, respectively. The rest of the seedlings displayed no foliage damage.

P. cryptogea, P. megasperma and P. plurivora were re-isolated from colonized rice grain inoculated roots, needle-scar-inoculated stems of Norway spruce, and stems and branches of Nordmann fir seedlings. All re-isolated cultures from each host-pathogen combination displayed the same morphology as the isolates that were originally used for inoculation, hence fulfilling Koch's postulates for P. cryptogea, P. megasperma and P. plurivora.

None of the control seedlings displayed any Phytophthora root rot or foliage symptoms and all the needles scars healed (Fig. 5). No Phytophthora outgrowth was obtained on the P10ARPH medium from the control seedlings inoculated with non-colonized rice grains in the soil, or plain PDA plugs on the needle scars. However, one Nordmann fir control seedling had a necrotic main root, but a detailed root investigation displayed a Pythium undulatum infection, most likely already present when the seedling was purchased.
<table>
<thead>
<tr>
<th>Tree species</th>
<th>Phytophthora spp. (isolate no.)</th>
<th>Root rot (foliage)</th>
<th>LLUB Stem (foliage)</th>
<th>LLUB Branch (foliage)</th>
<th>Re-isolation success</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abies nordmanniana</td>
<td>P. cryptogea (250476)</td>
<td>0.5 (0)</td>
<td>26.8 (0.4)</td>
<td>31.4 (0.4)</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>P. megasperma (250494)</td>
<td>0 (0)</td>
<td>8.8 (0)</td>
<td>52.2 (0)</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>P. plurivora (250496)</td>
<td>0.4 (0)</td>
<td>3.8 (0)</td>
<td>9.6 (0)</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0%</td>
</tr>
<tr>
<td>Picea abies</td>
<td>P. cryptogea (250476)</td>
<td>0 (0)</td>
<td>17.2 (0.6)</td>
<td>-</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>P. megasperma (250494)</td>
<td>0.1 (0)</td>
<td>23.5 (0.8)</td>
<td>-</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>P. plurivora (250496)</td>
<td>0.125 (0)</td>
<td>5 (0)</td>
<td>-</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>-</td>
<td>0%</td>
</tr>
</tbody>
</table>

1 Average root rot with average foliage symptoms in brackets
2 Lesion length under bark (LLUB) in millimetre for the stem inoculated seedlings (ten Norway spruce and five Nordmann fir seedlings with foliage symptoms in brackets)
3 Lesion length under bark in millimetre for the branch inoculated seedlings (five Nordmann fir seedlings with foliage symptoms in brackets)
4 Re-isolation success given by percent seedlings where Phytophthora was re-isolated from roots and stem/branch segments
Fig. 5 Norway spruce (Picea abies) and Nordmann fir (Abies nordmanniana) seedlings inoculated with P. cryptogeae 250476, P. megasperma 250494, and P. plurivora 250496: (a) Norway spruce negative control seedlings inoculated with non-colonized rice grains in the soil; (b) Norway spruce seedling inoculated with P. megasperma colonized rice grains in the soil, displaying swollen roots, root rot, and fine roots; (c) Nordmann fir negative control seedling inoculated with sterile PDA plugs in the needle scars; (d, e, f) Nordmann fir seedlings, inoculated with PDA plugs containing P. cryptogeae in the needle scars, displaying a dead branch over the inoculation point and stem cankers; (g) Norway spruce seedlings inoculated with plain PDA plugs in the needle scars as a negative control (left), and with PDA containing P. megasperma (right); (h, i) Nordmann fir branches inoculated with PDA plugs containing P. megasperma; (j) Norway spruce, subalpine fir (Abies lasiocarpa), and Fraser fir (Abies fraseri) seedlings three months after inoculation with Phytophthora colonized rice grains in the soil. Phytophthora cryptogeae caused no above ground symptoms of Norway spruce (left), but severe foliar symptoms of subalpine fir (middle) and Fraser fir (right). Photos: (a-i) M. Pettersson and (j) Erling Flostad.
Discussion
Phytophthora root rot is one of the most severe and problematic diseases for Christmas tree cultivation in USA (Chastagner and Benson 2000). In Europe, the only reports about Phytophthora root rot on Christmas trees are from Ireland (Shafighadeh and Kavanagh 2005) and our neighbouring country Norway (Talgo et al. 2006; Talgo et al. 2007). In addition, Phytophthora species have been found in waterways associated with Christmas tree production in Norway, Denmark and Belgium (Talgo et al. 2017). The detection of Phytophthora species associated with Christmas trees in the other two Nordic countries was the reason for conducting the Swedish survey, and subsequently testing of pathogenicity of the most likely species threatening the production.

Only three young plants across 14 Swedish Christmas tree farms displayed symptoms resembling Phytophthora root rot, but Phytophthora was recovered from only one Norway spruce and identified to P. megasperma. Thus, it seems that few Phytophthora species have reached the small-scale Swedish Christmas tree production and that Phytophthora root rot presently is not a problem for the production. This is good news for the Swedish Christmas tree business. Compared to a Phytophthora root rot survey conducted in North Carolina (Pettersson et al. 2017a), the detection of the disease in the survey reported here was very low given the extensive surveying. However, there is a need for vigilance because we found that there are several Phytophthora species present in the soil from low or poorly drained areas in the fields. Several Phytophthora species were also present in streams and ponds adjacent to the Christmas trees. These findings call for awareness of a potential future problem.

In total, we obtained one isolate of P. cryptogea (from soil), three isolates of P. megasperma (from a Norway spruce plant, soil and baits) and six isolates of P. plurivora (one from soil and five from baits). Thus, our hypothesis that several Phytophthora species are present in Swedish Christmas tree plantations, cannot be rejected.

Since the majority of the planting stock used for Christmas tree cultivation in Sweden is imported from European tree nurseries, we argue that the risk of introducing new and more aggressive Phytophthora species are high as demonstrated by Jung et al. (2016). An example of such events took place in Norway, where high mortality caused by Phytophthora was observed in a Christmas tree field with newly established bare root plants that originated from imported nursery stock (Talgo et al. 2007). Phytophthora species can hitchhike on an array of plant species without being noticed and fungicides often counteract detection by suppressing disease development during, and a period after, transport (Jung et al. 2016).

Only later, when the fungicide has stopped working, and the imported plant material have been planted in the fields, the disease starts developing. This is because treatments with fungicides only inhibit disease development temporarily but do not eradicate Phytophthora or cure infections. Resting spores in soil and plant tissue can survive unfavourable conditions like drought and extreme temperatures for many years. If such resting spores are introduced with soil on transplants, they may not germinate and produce zoospores until they are exposed to very moist conditions or planted in poorly drained areas.

Although modest, our study showed that Sweden has a quite healthy Christmas tree production nearly free from Phytophthora root rot. To maintain it this way, measures must be taken to prevent introduction of other and potentially more aggressive Phytophthora species on imported planting stock. Optimally, the supply of Christmas tree seedlings should therefore move to Swedish nurseries and strict sanitation procedures according to the best management practices for Phytophthora (CANGC 2008) should be implemented.

Phytophthora gonapodyides and P. laccatris were the most commonly isolated species in our study. Both species have previously been detected in water in Christmas tree areas in Norway and Denmark (Talgo et al. 2015). Little attention was given to these two species in this study since they are both considered weak pathogens, associated with aquatic habitats (Braiser et al. 1993; Nechwatal et al. 2013; Novak et al. 2015) and never reported to infect conifer tissue. Furthermore, in an inoculation test (Talgo et al., unpublished data), where Norway spruce, Fraser fir and subalpine fir seedlings were inoculated with P. gonapodyides, none of the tree species developed any disease symptoms. Consequently, this study focused on P. cryptogea, P. megasperma and P. plurivora, which have been reported as serious pathogens of conifers (Shafighadeh and Kavanagh 2005; Talgo et al. 2007; Rykoven et al. 2013; McKeever and Chastagner 2016).

The temperature-growth relationships for P. cryptogea, P. megasperma and P. plurivora (Fig. 4), such as an optimum growth rate of about 20-25°C, and no growth at 35°C, agrees with the literature for each species (Hansen and Maxwell 1991; Ho and Jong 1991; Jung and Burgess 2009). Although optimal growth temperatures for the three Phytophthora species do not exist in Swedish soils (Jungwist et al.
2014), they are all growing within a temperature range that enables them to invade and establish in our climate (Fig. 4). Furthermore, they are all non-host specific pathogens likely to find multiple Swedish host plants susceptible to infections, although it may still take time before they are widely distributed and established.

*Phytophthora cryptogea* and *P. megasperma* are aggressive pathogens of fir species in the cooler areas of the Pacific Northwest, USA (Chastagner and Benson 2000) and both have been isolated from dying noble fir Christmas trees in Ireland (Shaftsadze and Kavanagh 2005). *Phytophthora megasperma* was also isolated from stem cankers of subalpine fir Christmas trees in Norway (Talge et al. 2007) and roots of one dying Norway spruce plant in this study. *Phytophthora cryptogea* has previously been reported to cause disease in agricultural fields of spinach (*Spinacea oleracea* L.) and it proved pathogenic to a wide range of other vegetables in Sweden (Larsson and Gerhardsson 1990, 1992). *Phytophthora cryptogea* and *P. megasperma* were also the most aggressive species in our inoculation tests of Norway spruce and Nordmann fir. Hence, both species may become problematic for Swedish Christmas tree and bough production, especially in saturated soils which favour disease development. Therefore, it is important to maintain good soil drainage and avoid planting susceptible conifer species on sites with saturated soils.

*Phytophthora plurivora* has been isolated from diseased Fraser fir Christmas trees in USA (McKeever and Chastagner 2016) and from soil and/or roots of Norway spruce, European silver fir (*A. alba* Mill.), Scots pine (*Pinus sylvestris* L.) and Douglas fir (*Pseudotsuga menziesii* D. Don) in Europe (Jung and Burgess 2009; Jung and Blaschke 2004). In Sweden, Cleary et al. (2017) demonstrated pathogenicity of *P. plurivora* to several Swedish conifer and deciduous tree species. However, in our pathogenicity test, *P. plurivora* was the least pathogenic species and there are currently no published reports of *P. plurivora* on European Christmas trees. Therefore, *P. plurivora* may be less of a problem for the Christmas tree production, especially with the rather short rotation times for Christmas trees.

In our pathogenicity test, Norway spruce and Nordmann fir seedlings generally displayed minor symptoms seven months after inoculation with *P. cryptogea*, *P. megasperma*, and *P. plurivora*. None of the isolates caused extensive root rot or dieback under the experimental conditions, but all three species could be re-isolated from both Norway spruce and Nordmann fir. A higher disease severity than what was observed in our pathogenicity test was expected for the three *Phytophthora* species. However, Norway spruce and Nordmann fir are among the most *Phytophthora* resistant Christmas tree species (Benson et al. 1997). Higher resistance was also confirmed for Norway spruce in another inoculation test (Pettersson et al., unpublished data) were Norway spruce, Fraser fir and subalpine fir seedlings were inoculated with *P. cryptogea* in the soil. Again, Norway spruce did not develop severe symptoms of infection whereas high seedling mortality for Fraser fir and subalpine fir was evident (Fig. 5). Similarly, in a pathogenicity tests of 7 larch species (balsam fir, Canaan fir, Fraser fir, noble fir, Nordmann fir, Turkish fir, white fir) tested against 4 *Phytophthora* species (*P. cambivora*, *P. cinnamomi*, *P. sp. kalmiana*, *P. pinea*), Nordmann fir had the highest survival rate (McKeever 2017, personal communication).

The overwhelming use of Norway spruce and Nordmann fir Christmas trees in Sweden is likely one reason why no significant *Phytophthora* root rot has been reported even though several *Phytophthora* species are present in the fields. However, with increasing use of other fir Christmas trees species in Sweden, the risk of *Phytophthora* root rot increases.

The fact that we found six *Phytophthora* species in the small-scale Christmas tree production in Sweden is new knowledge demonstrating how widely distributed these pathogens have become. Furthermore, this is the first time *P. megasperma* has been isolated from roots of Norway spruce in Sweden. Since *P. cryptogea*, *P. megasperma* and *P. plurivora* could infect Norway spruce and Nordmann fir, they are not only of concern for the Swedish Christmas tree production, but potentially also to Swedish forestry, as they could effectively spread with surface runoff, streams and rivers to nearby forests. Hence, large economic values would be at risk if these species or more aggressive *Phytophthora* species are introduced to forests from nearby Christmas tree fields.

According to Ersek and Nagy (2008), closely related *Phytophthora* species can hybridize and switch hosts. With a continued spread along waterways and via nursery pathways to plantations and natural forests, the *Phytophthora* species found in this and other Swedish surveys (Cleary et al. 2016; Cleary et al. 2017; Jönsson et al. 2003; Redondo et al. 2015; Larsson and Gerhardsson 1992) increase the risk of hybridization events, which could result in more aggressive pathogens. Furthermore, due to the predicted climate changes, the Swedish conditions may become more suitable for *Phytophthora* species that are
adapted to higher temperatures (Roos et al. 2011), e.g. P. cantharum, the major pathogen in the production of Fraser fir Christmas trees in North Carolina (Pettersson et al. 2017a).

Even though our survey did not reveal Phytophthora disease outbreaks in the Swedish Christmas tree fields, there is need for vigilance since several Phytophthora species are present and have caused mortality to Christmas trees in the neighboring country Norway, where the climate is similar to Sweden. The Phytophthora disease outbreaks in Norway, prove that colder climatic regions are not guaranteed to escape problems once Phytophthora has been introduced. Thus, both Christmas tree producers and forest owners in the Nordic countries need to be aware of the risks of introducing Phytophthora species on nursery stock, and the risk of spreading Phytophthora from Christmas tree fields to forests and natural environment. Even if not causing mortality, it is possible that P. cryptogea, P. megasperma and P. plurivora may negatively affect the Swedish forestry by causing long-term growth losses.

Information leaflets about Phytophthora root rot, with symptom description, are needed. Easy-to-use test kits for rapid field-diagnostics of Phytophthora species are available, and such techniques may become very useful in early disease detection of Phytophthora root rot. In North Carolina, where Phytophthora root rot is an extensive problem in Christmas trees, extension agents have been training regional Christmas tree growers on how to use such test kits (Pettersson et al. 2017a; Pettersson et al. 2017b). Since the Swedish Christmas Tree Association encompasses just over 100 growers, no Swedish Christmas tree extension personnel or forest pathology extension personnel are currently available for advising growers. Hence, testing and surveying fails short.

Import of seedlings is a high-risk pathway for introducing plant damaging Phytophthora species, and the lack of control and knowledge may hinder early detection. Authorities need to be made aware of this situation. In further projects, more information on the epidemiology and impact of Phytophthora species in Swedish Christmas tree production sites, with the subsequent risk for natural ecosystems, should be investigated.

Compliance with Ethical Standards

Funding
This study was funded by the Gunnar and Lillian Nicholson Graduate Fellowship and Faculty Exchange Fund, and Partnership Atnarp.

Conflict of Interest
The authors declare that they have no conflict of interest.

References


Talgo, V., Schmitz, S., Chandelier, A., Brunberg, M. B., & Thomsen, I. M. (2017). Butting for Phytotaphora in waterways associated with Christmas tree production in Norway, Belgium and Denmark. NIBIO BOOK, 36(6), 80-82.


CHAPTER 6. Neonectria canker found on spruce and fir in Swedish Christmas tree plantations

Citation:
Neonectria Canker Found on Spruce and Fir in Swedish Christmas Tree Plantations

Martin Pettersson and John Frampton, Department of Forestry and Environmental Resources, North Carolina State University, Raleigh 27695 USA; Jonas Rönneberg, Southern Swedish Forest Research Centre, Swedish University of Agricultural Sciences, 22053 Mjärup, Sweden; and Vennesa Talge, Biotechnology and Plant Health Division, Norwegian Institute of Bioeconomy Research, 1431 Ås, Norway

Accepted for publication 1 September 2016.

Although Norway spruce (Picea abies) remains the dominant Christmas tree grown in Sweden, fir (Abies spp.) production has steadily increased since the 1980s, especially Nordmann fir (A. nordmanniana). In May 2015, twenty-one Christmas tree farms in southern Sweden (Fig. 1) were surveyed to identify prominent disease and pest problems. Visual inspection found dead tops of Norway spruce on more than 50% of the farms. Often one third of the upper part of the tree was dead. Additionally, dead shoots were found on eight Nordmann and noble fir (A. procera) trees.

Samples from symptomatic trees were incubated in moist chambers and/or wood samples were plated on potato dextrose agar (PDA) after submerging the samples for 10 s in 70% ethanol and 90 s in 0.5% NaOCl solution. Morphological identification of pure cultures was confirmed by sequencing the internal transcribed spacer (ITS) region of the ribosomal DNA using the primers ITS4 and ITS5. Sequences were compared with published sequences using the National Center for Biotechnology Information (NCBI) GenBank BLASTn tool; 99 to 100% identity was found for all isolates. Accession numbers were assigned using the BankIt Submission Tool (Table 1). Among the identified fungi were two Neonectria species: N. fuckeliana from Norway spruce and N. neomacrospora from Nordmann fir. This is the first report of N. neomacrospora in Sweden and of N. fuckeliana causing top dieback on young spruce.

Neonectria fuckeliana was identified from dead tops of Norway spruce at six different sites in the counties of Västra Götaland, Halland, and Skåne (Fig. 2A). Cultures were successfully isolated on PDA from wood samples taken at the margin of the necrotic zone of three diseased Norway spruce trees (Fig. 3A). Incubated wood samples from three other Norway spruce yielded characteristic, red fruticules (perithecia) and mycelial cushions (sporodochia) with aseptate spore mass (Figs. 2B and 2C). White spore tendrils emerged from perithecia after 3 to 5 days incubation. Asexual spores, both multiseptated (1 to 6 septa) macroconidia of the Cylindrocarpon stage and microconidia of the Acremonium stage, developed (Fig. 3B). Macroconidia measured 32.6 to 68.3 (mean 45.4) × 2.4 to 4.3 (mean 3.0) μm (n = 25). Neonectria neomacrospora was isolated on PDA (Fig. 3C) from Nordmann fir at three different sites, two from dead shoots of Christmas trees (Fig. 2D) in Västra Götaland County and one from an older tree in an ornamental planting in Skåne County (Table 1). The isolates were 100% identical to N. neomacrospora sequences reported to the NCBI GenBank nucleotide database. Red perithecia and sporodochia were also found on incubated Nordmann fir branches (Figs. 2E and 2F). Macroconidia (1 to 3 septa) of the Cylindrocarpon stage and microconidia of the Acremonium stage developed (Fig. 2D). Macroconidia measured 13.5 to 29.1 (mean 21.4) × 2.4 to 4.2 (mean 3.1) μm (n = 25). Inoculation tests were performed using map pins contaminated with mycelia inserted into stems of seedlings and the map pins were left standing in the inoculation site throughout the experimental period according to Talge and Steinwand (2013). Results verified that N. fuckeliana (Accession Nos. KT350496 and KT350495) and N. neomacrospora (KT350497) were patho-

![FIGURE 1](image_url)

Map of southern Sweden indicating the positions of the Christmas trees included in a disease and pest survey in 2015.

| TABLE 1 | Neonectria spp. isolates obtained from six different Christmas tree fields in a disease and pest survey of 21 farms in southern Sweden in 2015. |
| Species | Location | Isolate No. | GenBank Accession No. |
| N. fuckeliana | Skåne | 250469 | KT350493 |
| N. fuckeliana | Skåne | 250470 | KT350496 |
| N. fuckeliana | Skåne | 250471 | KT350495 |
| N. neomacrospora | Västra Götaland | 250472 | KT350497 |
| N. neomacrospora | Västra Götaland | 250473 | KT383060 |
| N. neomacrospora | Skåne | 250474 | KT383061 |

Corresponding author: Martin Pettersson. Email: jrpetter@ncsu.edu.
Symptoms and signs of Neonectria canker on Christmas trees in Sweden: (A) dead top, (B) perithecia with emerging ascospore tendrils, and (C) sporodochia with asexual spore mass from Neonectria fackeliana on Norway spruce (Picea abies); and (D) a dead branch tip, (E) perithecia in a needle scar, and (F) sporodochia of N. neomacrospora on Nordmann fir (Abies nordmanniana). Photos: (A, D) Martin Pettersson and (B, C, E, F) Venche Talga.

Neonectria fackeliana causes cankers and dieback of Monterey pine (Pinus radiata) in New Zealand (Crane et al. 2009). It also commonly occurs on Norway spruce (Picea abies) in Scandinavia, where it is regarded as a weak pathogen. However, N. fackeliana was recently reported to cause severe cankers in Norway spruce forests in Finland (Lilja et al. 2012). A similar situation has been observed in Norway (Talgo et al. 2015). Neonectria neomacrospora was found on fir species in North America and Norway more than 50 years ago (Booth 1979), but no severe damage was observed until recently. Since 2008, N. neomacrospora has been isolated from 19 fir species in Norway, Denmark, and western United States and has caused epidemic outbreaks and mortality (Talgo and Thoresen 2015). Follow-up studies on distribution and management of both Neonectria spp. will be undertaken. In particular, N. fackeliana should be followed especially closely because it affects Norway spruce, the most important forest tree in Sweden.
FIGURE 4
Results from inoculation tests using map pins with mycelium of *Neonectria* spp.: (A) dead shoot and (B) canker wound on Norway spruce (*Picea abies*) 144 days after inoculation with *Neonectria fuckeliana*; (C) re-isolation of *N. fuckeliana* on PDA; (D) dead shoot on Nordmann fir (*Abies nordmanniana*) 222 days after inoculation with *N. neomaculosa*; (E) inoculated Nordmann fir stems displaying white mycelium of *N. neomaculosa* in the infected area; (F) re-isolation of *N. neomaculosa* on PDA. Photos: (A) Erling Fleistad and (B, C, D, E, F) Venche Talgø.
Radial growth rates in millimeters per day of *Neocentria fasciata* (GenBank Accession No. KT359495) and *N. neocentria* (KT359497) grown on PDA in darkness at 5-degree intervals from 5 to 35°C.

---

**LITERATURE CITED**


CHAPTER 7. Pathogenicity of *Neonectria fuckeliana* on Norway spruce clones in Sweden and potential management strategies

Citation:
Pathogenicity of *Neonectria fuckeliana* on Norway Spruce Clones in Sweden and Potential Management Strategies

Martin Pettersson 1,*, Venche Talgo 2, John Frampton 3, Bo Karlsson 4 and Jonas Rönnberg 1

1 Southern Swedish Forest Research Centre, Swedish University of Agricultural Sciences, P.O. Box 49, SE-23053 Alnarp, Sweden; jonasa.ronnberg@slu.se
2 Division of Biotechnology and Plant Health, Norwegian Institute of Bioeconomy Research (NIBIO), P.O. Box 115, 1431 Ås, Norway; venche.talgo@nibio.no
3 Department of Forestry and Environmental Resources, North Carolina State University, 2800 Faucette Dr., Raleigh, NC 27695, USA; frampton@ncsu.edu
4 The Forestry Research Institute of Sweden (Skogforsk), Ekebo 2250, SE-26890 Svalöv, Sweden;
bo.karlsson@skogforsk.se
* Correspondence: martin.pettersson@slu.se; Tel: +46-070-895-6334

Received: 8 February 2018; Accepted: 27 February 2018; Published: 28 February 2018

Abstract: The fungus *Neonectria fuckeliana* has become an increasing problem on Norway spruce (*Picea abies*) in the Nordic countries during recent years. Canker wounds caused by the pathogen reduce timber quality and top-dieback is a problem for the Christmas tree industry. In this study, four inoculation trials were conducted to examine the ability of *N. fuckeliana* to cause disease on young Norway spruce plants and determine how different wound types would affect the occurrence and severity of the disease. Symptom development after 8–11 months was mainly mild and lesion lengths under bark were generally minor. However, *N. fuckeliana* could still be reisolated and/or molecularly detected. Slow disease development is in line with older studies describing *N. fuckeliana* as a weak pathogen. However, the results do not explain the serious increased damage by *N. fuckeliana* registered in Nordic forests and Christmas tree plantations. Potential management implications, such as shearing Christmas trees during periods of low inoculum pressure, cleaning secateurs between trees, and removal and burning of diseased branches and trees to avoid inoculum transfer and to keep disease pressure low, are based on experiments presented here and experiences with related pathogens.

Keywords: *Picea abies*; Nordic countries; canker; microconidia; inoculation; lesion length; infection

1. Introduction

*Neonectria* canker, caused by the fungus *Neonectria fuckeliana* (C. Booth) Castl. & Rossman, is a disease of Norway spruce (*Picea abies* (L.) Karst.) and other spruce species. For the Nordic forestry sector, Norway spruce is of great economic and ecological importance. Norway spruce is also the dominant Christmas tree species in Sweden, accounting for more than 50% of sales. *Neonectria* canker can diminish the value of Norway spruce by causing stem defects of timber trees or top-dieback of several branch whorls (Figure 1), the latter being especially destructive in Christmas tree fields [1]. In addition to dark canker wounds and dying tops (Figure 1), symptoms caused by *N. fuckeliana* on Norway spruce often include heavy resin-flow [2,3]. Affected trees may become more vulnerable to insect pests and decay fungi, as well as susceptible to breakage by wind, snow, and ice. Infections may also result in slower growth. Mortality of young trees may occur but, more commonly, the terminal leader shoots along with the top 3–4 whorls die [1].
Figure 1. Damage caused by *Neonectria fuckeliana* on Norway spruce (*Picea abies*) in the Nordic countries: dark necrotic canker wounds on trees in a Norwegian forest stand (left and middle, red arrows point towards clusters of perithecia of *N. fuckeliana* in or around the canker wound), and top-dieback including several branch whors in a Swedish Christmas tree field (right). Photos: Martin Pethersson.

Signs of the fungus are globose, red fruiting bodies (perithecia) on the bark of dead stems, branches, and in and around canker wounds, mainly on larger dying or dead trees. Despite the small size (300–400 μm diam.), they are visible to the naked eye. *Neonectria fuckeliana* has three spore states for colonizing new host trees [4–6], where the ascosporos (sexual state) are produced inside the perithecia are likely the major dispersal propagules [4,6]. Under humid conditions, ascospores are forcibly ejected through a small opening (ostiole) on the top of the perithecia and distributed by wind. Asexual conidal pustules (sporodochia) are rarely seen in nature and the significance of this state in the life cycle is not fully understood. Two types of conidia are produced on the sporodochia: microconidia (*Acremonium* state) and macroconidia (*Cylindrocarpon* state), the former is massively produced in culture. Observations from related fungal canker pathogens, for example, *Neonectria ditissima* (Tul. & C. Tul.) Samuels & Rossman have shown that both macro- and microconidia are important for short-distance dispersal [7].

The main infection pathways are via openings such as pruning wounds, dead branches, branch stubs, or cracks due to wind or frost [4–6]. The infection rate of *N. fuckeliana* increases when large numbers of ascospores or conidia land on fresh wounds under moist weather conditions with temperatures between 15–25 °C [4,8]. For radiata pine (*Pinus radiata* D. Don) stands in New Zealand, where *N. fuckeliana* is an aggressive pathogen, successful infection by *N. fuckeliana* depends on the time of year that the trees are pruned [9] and on host genetics [10]. *Neonectria fuckeliana* has previously not behaved as an aggressive pathogen on Norway spruce in the Northern Hemisphere. In older European studies conducted on larger Norway spruce trees, *N. fuckeliana* was regarded as a weak pathogen or a saprophyte [5,11–13]. However, the current situation with *N. fuckeliana* in Finland [2,3], the other Nordic countries [14], and in Northern Ireland (Richard O’Hanlon personal communication) [15] indicates that changes have taken place. In the Nordic countries, disease incidence of Neonectria canker has increased in some regions during the last ten years [1–3,16]. Possible explanations for this increase include mild winters and wet growing seasons, which provide better living and dispersal conditions for the fungus. With global warming and current climate change projections, the increase in disease pressure may intensify [17]. In Eastern Finland, *N. fuckeliana* has infected stands of young Norwegian spruce plantations (5–30 years old) leading to reduced timber quality due to canker wounds and significant mortality [2,5]. In a provenance trial, Lilja et al. (2012) found that 13% and 37% of Finnish and Polish provenances, respectively, had dying tops and blackened wounds caused by this fungus [2]. *Neonectria fuckeliana* has also been reported in young spruce stands and Christmas tree fields in Sweden, Denmark, and Norway [1,4,16,18]. Since 2012 in Northern Ireland, *N. fuckeliana* has been implicated in the disease and mortality of Sitka spruce (*Picea sitchensis* (Bong.) Carr.), an important tree species in the region, at several sites spread across the entire region (Richard O’Hanlon personal communication) [15].

The same changes have been observed in Norway with other ascomycetes that have been present in the country for decades, for example, *N. neomacrosora* [19] and *Delphiniella abietis* [20]. Both pathogens have caused epidemics on fir (*Abies* spp.) during the last ten years, which can be linked to increasing temperature and precipitation [18,21].
Neocorticium fuckeliana has not been extensively studied, and there are gaps in knowledge concerning its basic biology, pathogenicity, and infection mechanisms. Even the taxonomic status of this species has changed several times [22,23] and it has recently been suggested that it belongs to a completely new genus [24]. However, infection studies where Norway spruce seedlings were inoculated with N. fuckeliana using a map pin technique [25] resulted in canker wounds, resin flow, and shoot dieback, similar to observations of infected Norway spruce trees in the field [1]. The study suggested that further investigation into the pathogenicity of N. fuckeliana was needed to be able to justify preventive or sanitary actions.

The primary objective of this study was to determine the ability of N. fuckeliana to cause disease on younger Norway spruce rooted cuttings in order to better understand and manage N. fuckeliana infections in younger Norway spruce plantations. Four specific aims were outlined: (1) Determine how different wound types would impact the occurrence and severity of infections on three-year-old Norway spruce cuttings and seven-year-old Norway spruce trees; (2) Describe symptom development after inoculation with microconidia; (3) determine if symptom development of the cuttings correlated with field observations of top-dieback; and (4) estimate the potential threat to young Norway spruce plantations and discuss potential management implications.

2. Materials and Methods

2.1. Plant Material and Location

A pilot inoculation study was undertaken to examine the usefulness of microconidia as an inoculation source for young Norway spruce plants. A total of 32 two-year-old seedlings were used. The inoculation study was carried out in a growth chamber at NIBIO, Norway, with 65% relative humidity (RH), a temperature of 20 °C, and 18 h daylight (dark from 21:00–03:00). The seedlings were regularly watered.

A greenhouse inoculation study (the main experiment) was undertaken to examine the susceptibility to N. fuckeliana of actively growing and dormant Norway spruce cuttings, and the importance of wound type. Active and dormant three-year-old cuttings were inoculated when they were approximately 44 and 37 cm tall, respectively. The experiment was conducted at Skogforsk, Sweden.

In March 2016, 500 dormant cuttings were placed into a freezer (−4 °C) to maintain dormancy, while 500 were placed into a greenhouse to start a third growing season. The cuttings in the freezer were transferred to a refrigerator for acclimatization by the time the cuttings in the greenhouse started to break bud in early May. When all the actively growing cuttings had shoots that were at least 5 cm long (end of May), the dormant cuttings were moved from the cooler to the greenhouse. Both the actively growing and dormant cuttings were re-potted into potting trays (inside measurement 50 × 29 × 9 cm) with sphagnum containing eight cuttings each (in mid-May and end of May, respectively). A total of 120 potting trays (960 cuttings), were distributed onto five benches (24 potting trays per bench).

The cuttings were regularly watered and the temperature in the greenhouse followed outdoor temperature fluctuation patterns. However, the temperature in the greenhouse was kept above 0 °C. The temperature was continuously measured with three HOBO water temperature Pro V2 data loggers (Onset Company, Bourne, MA, USA) hanging above the cuttings in the greenhouse.

A field study employing seven-year-old Norway spruce trees was undertaken to test whether stem infections would result in similar top-dieback symptoms as observed on Norway spruce in Finnish and Norwegian forest stands and in Swedish Christmas tree fields [1–3,14]. The trees were planted in rows, with different families in succession. A total of 180 plants from twelve different families grown in the edge rows were used for the study.

2.2. Inoculum Preparation

Three N. fuckeliana isolates collected from infected Norway spruce trees were used for the inoculation studies (Table 1). All isolates were identified based on morphology and sequencing.
of the internal transcribed spacer (ITS) region of ribosomal DNA using ITS1 and ITS5 primers [26]. Pathogenicity of isolate no. 250605 and 250606 had not been tested before, but isolate no. 250471 had proven to be pathogenic to Norway spruce in a previous inoculation study [1].

Table 1. *Neoeucalyptus fuchelianus* isolates from Norway spruce (*Picea abies*) used for inoculation studies of young Norway spruce plants.

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>GenBank Accession No.</th>
<th>Location *</th>
<th>Collected</th>
</tr>
</thead>
<tbody>
<tr>
<td>250471</td>
<td>KT300495</td>
<td>Sóslo, Skåne</td>
<td>2015</td>
</tr>
<tr>
<td>250605</td>
<td>MF793144</td>
<td>Knutstorp, Skåne</td>
<td>2016</td>
</tr>
<tr>
<td>250606</td>
<td>MF493145</td>
<td>Inestorp, Skåne</td>
<td>2016</td>
</tr>
</tbody>
</table>

* Municipality and county in Sweden.

For the pilot inoculation study, one 14-day-old Potato Dextrose Agar (PDA) culture for each of the three isolates in Table 1 was used.

For the greenhouse study, isolates no. 250471 and no. 250605 were used for inoculation. Both isolates were grown on poor Difco™ PDA medium enhanced with needles and wood chips of Norway spruce (Poor-PDA*) to stimulate microconidial production. The medium was prepared by suspending 13 g of commercial PDA powder in 1 L of deionized water, adding 75 needles from one living Norway spruce branch, plus 15 cut branch segments (0.5–1.0 cm) of one living and one dead Norway spruce branch. The suspension was heated with frequent agitation, and boiled for one minute before autoclaving at 121 °C for 15 min.

For each isolate, a microconidial spore suspension was prepared by pouring 20 mL deionized water onto two 19-day-old cultures grown on Poor-PDA*. They were swirled gently for 30 s and the microconidial spore suspensions were poured into separate beakers. The number of microconidia was counted using a Fuchs-Rosenthal hemocytometer under a Leica DMLB compound microscope. The spore concentration was adjusted to approximately 5 × 10⁷ conidia/mL (5000 spores per 10 μL). To determine germination of the conidial inoculum before and after inoculation, a serial dilution was prepared and 10 μL aliquots (three replicates per dilution) were plated and streaked out onto PDA Petri plates using a sterile spatula. Plates were incubated at room temperature and colonies were counted after 4 days.

For the field study, 14-day-old PDA cultures of isolate no. 250471 and no. 250605 (Table 1) were used as inoculums.

2.3. Experimental Design

For the pilot inoculation study, the seedlings were organized in four rows in a plug tray. Three rows (one for each isolate) had nine seedlings and one row of five control seedlings.

For the greenhouse study, a randomized split-block design of four blocks (benches) was used. Each block was vertically split into actively growing and dormant cuttings (main plot factor) and horizontally split into isolate no. 250471 or isolate no. 250605 (main plot factor) (i.e. inoculation with spore suspension of *N. fuchelianus* from isolate no. 250471 or no. 250605). Each main plot contained four different treatments (subplot factors) with 12 replicate cuttings for each treatment and block.

For the field study, 10 Norway spruce families were inoculated with isolate no. 250471 and two families were inoculated with isolate no. 250605. For each family, 15 trees were selected; ten trees were randomly inoculated and the remaining five trees were controls.

2.4. Inoculation Trials

Four inoculation trials (2.4.1–2.4.4) were performed with the following number of plants: 32, 960, 480, and 180, respectively. In the same order, the number of seedlings inoculated with *N. fuchelianus* was 27, 769, 384, and 120. The remaining seedlings in each of the four trials were used as controls, that is, inoculated with water.
2.4.1. Pilot Study, First Inoculation Trial

In the pilot study, nine two-year-old seedlings were used per isolate. The microconidial solution for inoculations was prepared by carefully pipetting up and down 15 μL of water on the 14-days-old *N. fuckeliana* culture and placing the droplet onto a 1 cm artificial stem wound made with a scalpel to reveal the sapwood. The five control seedlings received water instead of spore suspension. Wounds were left open (not covered).

2.4.2. Greenhouse Study Part I, Second Inoculation Trial

Four different treatments were applied to the terminal leader of both actively growing and dormant cuttings: (1) shoot-toppled treatment: approximately 2 cm of the terminal leader was cut using sterilized secateurs; (2) shoot-wounded treatment: 5–10 mm of the bark and cambium in the middle of the terminal leader was removed using a sterilized knife; (3) needles-removed treatment: ten needles were removed from one area in the middle of the terminal leader; and (4) non-wounded treatment: the terminal leader was left intact. Tools were sterilized between trees by dipping them in 70% ethanol and drying with paper towels.

Each treatment was inoculated with spore suspension within 1 min after wounding. A micropipette was used for applying 10 μL of suspension to each wound of the shoot-toppled and shoot-wounded treatments. The needles-removed and the non-wounded treatments were sprayed with 1 mL of spore suspension per plant.

All cuttings were inoculated over three consecutive days from 31 May to 2 June 2016. Before inoculation, a resealable plastic bag with the bottom cut open was fitted over the terminal leader and the top whorl of the cutting, and tightened around the stem at the internode between two whorls using cable ties (2.5 × 100 mm) according to a method by Thomsen et al. (personal communication) [27]. The resealable front of the bag was opened and the treatment was executed before resealing the bag. For the needles-removed and the non-wounded treatments, the plastic bag enabled spraying suspension onto the terminal leader without contaminating other plants. The shoot-toppled and shoot-wounded treatments received a small piece of moist cotton inside the bag before resealing to give a sufficient amount of humidity to all treatments. After only a few hours, a moist film could be seen on the inside of the plastic bag. The bags were kept on for 5 days to enable germination of the microconidia.

The control cuttings received the same wound treatments, but were inoculated with sterile water, that is, mock inoculation, instead of the *N. fuckeliana* spore suspension. Half of the control cuttings were covered with plastic bags while the other half were uncovered to control for the effects of the plastic bags, that is, increased moisture and fluctuations in temperature.

2.4.3. Greenhouse Study Part II, Third Inoculation Trial

When there were no symptoms present on the cuttings in the greenhouse study part I (second inoculation test) after more than two months the third inoculation trial was conducted on all the cuttings in two of the blocks (benches 1 and 3). The shoots of the cuttings that were actively growing by the start of the greenhouse study part I had finished elongation, hardened, and set buds, while those that were dormant at that time were now actively growing and not fully elongated.

The largest lateral branch leader was chosen to receive the same treatment as its terminal leader during the previous inoculation. This branch was marked with tape. Plastic bags were not used during the third inoculation due to the risk of another heatwave. The foliage of the cuttings was kept moist by using a hose with mist spray nozzles once an hour during the day for 5 days after inoculation.

2.4.4. Field Study, Fourth Inoculation Trial

In a field study, 180 seven-year-old Norway spruce plants were inoculated with *N. fuckeliana* on the 16th of July 2016. Ten families were inoculated with the isolate no. 250471 and two families inoculated with the isolate no. 250605. Of the 15 trees for each family, ten trees were randomly selected.
and inoculated with mycelial plugs of *N. fuckeliana*, and the remaining five trees were inoculated with clean PDA plugs as controls.

The trees were wounded prior to inoculation by making shallow holes (10–15 mm deep) in the sapwood of the stem, located 1–3 cm above the third branch whorl (counted from the top). The holes were made by using a cordless power drill with an 8 mm drill bit. The plug (with or without mycelium) was placed into the hole with the surface facing the center of the tree. Mycelial plugs were used because they are a fast and secure way of initiating an infection. A moist cotton tuft was used to seal the wound. The cotton plug was kept moist during the first 5 days by spraying it with water once a day, and left in the inoculation site throughout the experimental period.

2.5. *Symptom Evaluation*

The appearance and development of plant symptoms (such as visible depression around the inoculation point, stem canker formation, color loss of needles, brown needles, wilting of shoots, necrosis, and top-dieback), and presence/absence of pathogen signs (such as perithecia and/or sporodochia) were monitored monthly.

Final symptom evaluation and harvesting of the inoculation trials occurred after two months for the pilot study (first inoculation trial), ten months for the greenhouse study part I (the second inoculation trial), eight months for the greenhouse study part II (third inoculation trial), and eleven months for the field study (fourth inoculation trial).

For each plant, notes of visible plant symptoms and pathogen signs were taken, and the lesion length under bark (mm) was measured after removal of a thin layer of bark to reveal the cambium. The length of any visible lesion was measured from the lower wound edge towards the stem/root of the plant.

For the pilot study, all seedlings were sampled by removing the needles around the inoculation point, scraping the bark with a scalpel to reveal the sapwood at the wound area, and excising that stem segment for resolation.

All the cuttings from the greenhouse study part I and II were cut using sterilized secateurs, and needles were stripped from the inoculated leader. A knife was used to remove the bark and cambium to reveal the sapwood at the inoculation point. The inoculation point and at least 1 cm below and above, or any visible depressions around the inoculation point, were removed under aseptic conditions, placed into 1.5 mL micro centrifuge tubes, and stored into a −20 °C freezer to secure material for later testing if necessary. However, for samples selected for investigating pathogen survival by isolation to culture, the stem/branch segments were split in half and placed into two different 1.5 mL micro-centrifuge tubes. One of the tubes was stored in a −20 °C freezer, while the other was used directly for resolation.

For the field study, a 20 cm stem segment with the inoculation wound in the middle was cut, wrapped in paper, put into a marked plastic bag and transported in a cooler to the laboratory. In the laboratory, the samples were longitudinally split at the inoculation point to reveal any discoloration and the lesion length under bark was measured. One half of each sample was stored in the freezer while the other half was used for resolation onto PDA and incubation in a moist chamber to observe any development of typical *N. fuckeliana* signs.

2.6. *Resolation from Inoculated Plants*

Stem segments from the inoculation points were dissected into 5–10 mm segments, surface sterilized for 10 s in 70% ethanol followed by 90 s in 0.5% NaOCl and plated on PDA in 9 cm Petri dishes. The plates were incubated at room temperature in the dark and monitored daily for growth of *N. fuckeliana* or until the plates were overgrown by other fungi or bacteria (7–21 days).

2.6.1. *Resolation from the Pilot Study, First Inoculation Trial*

After two months, all seedlings were harvested and investigated for pathogen survival. Identification of *N. fuckeliana* was based on morphological observations of the resulting cultures.
2.6.2. Reisolation and Molecular Detection from the Greenhouse Study Part I and II, Second and Third Inoculation Trial

From the greenhouse study part I, 128 out of 768 inoculated cuttings and 64 out of 192 control cuttings were investigated for the presence of *N. fuckeliana* through culture-based and molecular-based identification techniques described below. Specifically, one row from both active and dormant cuttings for each of the four benches with inoculated cuttings, and two rows from both active and dormant cuttings from the control bench were examined.

From the greenhouse study part II, a total of 64 out of 384 inoculated cuttings and 32 out of 96 control cuttings were investigated for presence of *N. fuckeliana* following the same methods as for the greenhouse study part I.

2.6.3. Reisolation and Molecular Detection from the Field Study, Fourth Inoculation Trial

For the field study, 36 trees (three randomized trees per family, two inoculated, and one control) were investigated for the presence of *N. fuckeliana* through reisolation and incubation. Twelve of these plants (six inoculated and six control) were tested molecularly. A wood chip (1-2 cm) was removed under sterile conditions from the zone between the dead and alive tissue. The wood chip was taken from the longitudinal area of the depression on the side with the longest sunken area, either above or below the inoculation point. The wood chip was surface sterilized and plated onto PDA. After the wood chip was removed, the rest of the sample was incubated for 14 days in a moist chamber to determine if sporulation would take place. Each sample was inspected under the dissection microscope and a sterilized needle was used to carefully transfer microconidia from the wood sample to PDA supplemented with 0.5 mg/mL of streptomycin sulfate (PDA).

2.7. Identification

2.7.1. Culture-Based Identification

Identification of *N. fuckeliana* was based on morphological observations of the cultures on PDA and spore characteristics described in earlier work [1].

2.7.2. Molecular-Based Identification

The samples that were stored in the freezer were analyzed for the presence of *N. fuckeliana* DNA. They were assessed by using a *N. fuckeliana* Taqman real-time PCR-based test developed by Pettersen et al. (2017) [28]. Each stem/branch segment was ground into a fine powder in liquid nitrogen using a mortar and a pestle. The DNeasy® Plant Mini Kit was used to extract DNA from 0.1 g of the homogenized samples. The Taqman assays with NF-fw1/NF-rv1 primer pair, were used to amplify any extracted *N. fuckeliana* DNA. The reactions were performed in 20 μL reaction mixtures containing 0.6 μL of each primer, 1.6 μL of probe (2.5 μM), 10 μL of SsoAdvanced Universal Probes Supermix (2x), 5.2 μL of mQ H2O, and 2 μL of genomic DNA (1/10 diluted after extraction), and run on 0.2 mL 96-well PCR plates. The samples were amplified and analyzed with Thermo Fisher Scientific CFX96 Real-Time PCR Detection System (BIO-RAD, USA) under the following conditions: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 66 °C for 30 s. All reactions had two technical replicates. For each 96-well plate, two negative controls containing sterile mQ water were included. Results were analyzed with Bio-Rad CFX Manager software v2.0.

2.8. Statistical Analysis

Data analyses for the greenhouse study part I and II were performed using the statistical software SAS Enterprise Guide 7.11 HF3 (SAS Institute, Cary, NC, USA).

Data for the sum for the sub-plots of lesion length under bark, that were normally distributed and had homogenous variances, were subjected to an ANOVA for criss-cross (or split block) design using PROC GLIMMIX. Non-normally distributed data was transformed with log-transformation. The same
analyses were run with the non-wounded and the needles-removed treatments were excluded (due to
the low number of symptoms observed). The Tukey’s honestly significant difference (HSD) test was
used to identify the significant differences ($p \leq 0.05$) among treatment means.

The molecular-based identification data was analyzed using PROC GLIMMIX with binomial
distribution and logit link for the criss-cross design.

The culture-based identification data was not statistically analyzed due to low number of
isolates retrieved.

3. Results

3.1. Results from the Inoculation Trials

3.1.1. Results from the Pilot Study. First Inoculation Trial

All 27 microconidial inoculated seedlings had swollen, non-healing wounds with resin flow
(Figure 2). Six seedlings had necrotic cambium all the way around the wound, but the needles in the
top were still green although losing color. All nine seedlings per isolate had *N. fuckeliana* outgrowth
on PDA. The wounds on the five control seedlings were healing and no *N. fuckeliana* outgrowth was
detected on PDA.

![Figure 2. Symptoms observed in the inoculation trials with *N. fuckeliana* on Norway spruce (*Picea abies*): Top row = pilot study (the first inoculation trial) using microconidia from isolate no. 250603 (left), isolate no. 250605 (middle), and isolate no. 250603 (right), resulting in dark necrotic canker wounds with resin production. Photographed during assessment in 2016, 2 months after inoculation. Second row = greenhouse study part I (the second inoculation trial) where four different treatments (shoot-topped [left], shoot-wounded [middle], needles-removed [right], and non-wounded) were applied to the terminal leader of actively growing and dormant cuttings. Photographed during assessment in 2017, 10 months after inoculation. Third row = greenhouse study part II (the third inoculation trial) where the biggest branch shoot was inoculated using the same four treatments as in the second inoculation trial. Photographed during assessment in 2017, 8 months after inoculation. Fourth row = field study (the fourth inoculation trial) where 7-year-old Norway spruce plants were inoculated with mycelial plugs of *N. fuckeliana* in the stem. Photographed during assessment in 2017, 11 months after inoculation. Photos: Martin Pettersson.](image-url)
3.1.2. Results from the Greenhouse Study Part I and II, Second and Third Inoculation Trial

In the greenhouse study part I, a small percentage of the cuttings developed disease symptoms, such as non-healing wounds with resin flow, and they all belonged to the shoot-topped and shoot-wounded treatment (Figure 2). The needles-removed and non-wounded treatment did not develop any disease symptoms. None of the control seedlings developed any disease symptoms and the wounds healed. There was a statistically significant difference among treatments for lesion length under bark (p < 0.0001) and a significant growth stage treatment effect (p = 0.0006). When the needles-removed and the non-wounded treatments were removed from the statistical analyses, lesion length under bark was significantly different (p < 0.0001) for dormant shoot-topped versus dormant shoot-wounded treatments (Table 2). Furthermore, dormant and active growing plants were significantly different for shoot-topped (p = 0.0013), but not for shoot-wounded. For lesion length under bark, there were no significant differences among growth stages or isolates, and no significant interaction was found.

Table 2. Neocctria fucetiana detection from 128 terminal leader and 64 branch cuttings of Norway spruce inoculated with a microconidial suspension, and 64 control cuttings (inoculated with water). The treatments included were shoot-topped, shoot-wounded, needles-removed, and non-wounded.

<table>
<thead>
<tr>
<th>Growth Stage</th>
<th>Treatment</th>
<th>Min-Mean-Max (mm)</th>
<th>Std (S) (mm)</th>
<th>(%)*</th>
<th>Tukey ***</th>
<th>Reisolation (%)</th>
<th>PCR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active</td>
<td>needles-removed</td>
<td>1-24-5</td>
<td>1.7 (5)</td>
<td>5</td>
<td>-</td>
<td>19</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>non-wounded</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>shoot-topped</td>
<td>1-3-12-8</td>
<td>2.0 (19)</td>
<td>20</td>
<td>B</td>
<td>38</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>shoot-wounded</td>
<td>2-7-14-19</td>
<td>6.2 (8)</td>
<td>8</td>
<td>BC</td>
<td>25</td>
<td>94</td>
</tr>
<tr>
<td>Dormant</td>
<td>needles-removed</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>13</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>non-wounded</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>shoot-topped</td>
<td>1-7-13-5</td>
<td>10.2 (5)</td>
<td>56</td>
<td>A</td>
<td>0</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>shoot-wounded</td>
<td>1-15-2</td>
<td>6.7 (2)</td>
<td>2</td>
<td>C</td>
<td>6</td>
<td>69</td>
</tr>
<tr>
<td>All</td>
<td>1-15-45</td>
<td>8.5 (88)</td>
<td>11</td>
<td>-</td>
<td>11</td>
<td>53</td>
<td></td>
</tr>
</tbody>
</table>

Biggest Branch Shoot (Greenhouse Study Part II) ****

<table>
<thead>
<tr>
<th>Growth Stage</th>
<th>Treatment</th>
<th>Min-Mean-Max (mm)</th>
<th>Std (S) (mm)</th>
<th>(%)*</th>
<th>Tukey ***</th>
<th>Reisolation (%)</th>
<th>PCR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active</td>
<td>needles-removed</td>
<td>1-4-5-8</td>
<td>5.0 (2)</td>
<td>2</td>
<td>-</td>
<td>13</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>non-wounded</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>shoot-topped</td>
<td>2-13-5-52</td>
<td>8.4 (94)</td>
<td>98</td>
<td>A</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>shoot-wounded</td>
<td>1-12-1-5</td>
<td>10.7 (80)</td>
<td>92</td>
<td>A</td>
<td>44</td>
<td>100</td>
</tr>
<tr>
<td>All</td>
<td>1-12-9-51</td>
<td>9.6 (180)</td>
<td>48</td>
<td>-</td>
<td>27</td>
<td>91</td>
<td></td>
</tr>
</tbody>
</table>

* The minimum, mean, maximum, standard deviation and sample size for lesion length under bark measured in millimeters were based on plants with a measurable lesion length under bark (that is, excluding all the plants with zero values from the analyses); ** Percent of plants that had a measurable lesion length under bark; *** In the Tukey Grouping analyses for lesion length under bark for the greenhouse study part I and II, the needles-removed and non-wounded treatments were excluded. The Tukey Grouping analyses were based on all the plants (that is both the plants with and without a measurable lesion length under bark was included in the analyses); **** At the time of the greenhouse study part II, all the cuttings were actively growing.

In the greenhouse study part II, more cuttings than in the greenhouse study part I developed disease symptoms, such as non-healing wounds with resin flow, and they all belonged to the shoot-topped and shoot-wounded treatment (Figure 2). Again, the needles-removed and non-wounded treatment did not develop any disease symptoms. None of the control seedlings developed any disease symptoms and the wounds healed. There was a statistically significant difference among treatments for lesion length under bark (p < 0.0001). When needles-removed and non-wounded treatments were removed from the statistical analyses, the lesion length under the bark was not significantly different between the shoot-topped and shoot-wounded treatments (Table 2). For lesion length under bark,
there was no statistically significant difference among growth stage, isolate, and no interaction effects were significant.

For the molecular identification, there was no statistically significant difference for any of the factors. For the reisolation data, there were not enough isolates to make meaningful data analyses.

The wounds of the control cuttings, with or without plastic bags, inoculated with sterile water, had healed smoothly after inoculation of the terminal leader and the biggest branch shoot. No *N. fuckeliana* outgrowth was detected on PDA and *N. fuckeliana* could not be detected molecularly.

In the germination tests, mycelia emerged from the microconidia on all the PDA plates.

3.1.3. Results from the Field Study, Fourth Inoculation Trial

In the field study, a small percentage of the plants developed disease symptoms, such as slightly sunken areas around the wound and resin flow (Figure 2). However, this also appeared for a few of the non-inoculated controls. There was a statistically significant difference ($p < 0.0001$) in total lesion length under bark between inoculated and control trees across both isolates. The average lesion length under bark was 53.3 mm for the inoculated trees and 41.2 mm for the control trees. There were also significant differences ($p < 0.0001$) and ($p = 0.001$) in lesion length under bark between families for the inoculated and control trees, respectively. However, all 24 inoculated trees and 11 out of 12 control trees had *N. fuckeliana* growing out on PDA. For the incubated samples, no sporodochia could be seen within the 14-day incubation period. However, microconidia could be seen under the dissection microscope for 22 out of 24 inoculated plants and 11 out of 12 control plants. These microconidia germinated to form cultures on PDAS.

*Neonectria fuckeliana* could be molecularly detected in the wood tissue in 6 out of 6 inoculated plants and 6 out of 6 control plants.

3.2. The Temperature in the Greenhouse Study Part I and II

On average over the whole year, the temperature in the greenhouse was 13.5 °C, but right after the greenhouse study part I started in June, a heat wave brought the temperature to above 30 °C for several (2–10) hours per day during the 5-day incubation time.

After the greenhouse study part II started, the greenhouse was approximately 4 °C cooler than during the inoculations in June.

The monthly average, standard deviation, maximum and minimum temperature can be seen in Appendix A Table A1. There is a significant difference ($p < 0.0001$) in mean temperature between the 8-day period of the greenhouse study part I (24.5 °C) and the greenhouse study part II (20.9 °C) (Appendix A Table A2).

4. Discussion

*Neonectria* canker on Norway spruce caused by *N. fuckeliana* has become an increasing problem in the Nordic countries during recent years [1–3,14,16,29,30]. Factors influencing the pathogenicity of *N. fuckeliana* on Norway spruce are currently unclear. However, these studies demonstrated that:

1. *N. fuckeliana* infections can take place during different growth stages (i.e., dormant and active). This was evident from the greenhouse study part I, where no clear difference could be found for lesion length under bark between dormant and active cuttings for all treatments. However, the fungus was more frequently reisolated and detected molecularly from active cuttings. The lower detection frequency from dormant cuttings was surprising, since dormant Norway spruce does not have the same immediate defense reaction to wounding as actively growing trees [31]. It is unclear what caused the difference in detection frequency, but there was a clear difference between age and character of the inoculated tissue for the dormant and active cuttings. The inoculated shoots of the dormant cuttings were one year old, woody and brown, whereas the shoots of active cuttings were a few months old and had softer greenish juvenile tissue (semi-woody).
(2) *Neoeutria fucelliana* needs an open wound as an entry point when inoculated with microconidia. This was clear from the greenhouse study parts I and II. None of the non-wounded cuttings developed symptoms and the fungus could not be reisolated; whereas the opposite was true for the wounded cuttings.

(3) Treatments with larger wounds, such as the shoot-topped and shoot-wounded treatments had larger lesion length under bark and resulted in higher reisolation frequencies. This was clear from the greenhouse study parts I and II. *Neoeutria fucelliana* was seldom or not at all reisolated from the needles-removed and non-wounded treatment, respectively. However, the shallow damage inflicted by the needles-removed treatment proved to be a large enough entry point for *N. fucelliana* to cause an infection on the active cuttings. In contrast, none of the dormant cuttings with needles-removed were positive for *N. fucelliana* reisolation. Therefore, it seems that removing needles from young actively growing shoots is more serious in terms of pathogen attack than removing needles from older and dormant shoots.

(4) It was evident that wound treatments of control cuttings inoculated with water healed smoothly, whereas wounds of microconidia inoculated cuttings healed slower or not at all. Therefore, we can conclude that some microconidia survived the high temperatures that occurred right after the inoculation of the greenhouse study part I. However, it is likely that the microconidia germination was abnormal or negatively affected by the heatwave as fewer cuttings were positive for *N. fucelliana* reisolation and molecular detection compared to the greenhouse study part II. The larger lesions in combination with the higher detection of *N. fucelliana* in the greenhouse study part II, suggests that the lower temperatures at that time had a positive influence on the survival of the microconidia. It also seems likely that the incubation technique with plastic bags was redundant or even had a negative impact as temperatures inside the plastic bags may have exceeded the greenhouse temperatures.

However, of the factors influencing the pathogenicity of *N. fucelliana* on Norway spruce, much still remains unknown and several inconsistencies are evident from this study. These include latent period, temperature, microconidia inoculation technique, and the "background" inoculum from the environment (natural infection).

It seems evident from our experiments that *N. fucelliana* can remain in a latent infection state for a long time before disease symptoms emerge. Latent infections are also reported for *N. ditissima* [32-34] and the latent period varies with wound size and spore concentration. Larger wounds and higher spore concentration shorten the latent period. This may be the reason for the slower disease development in the greenhouse study part I and II compared to the pilot study. It is unknown how much more concentrated the undiluted spore suspension for the pilot study was. Judging from the spore concentration preparation of the greenhouse inoculation study (using approximately $5 \times 10^5$ conidia/mL), it was two to three times higher. For the field trial, mycelial plugs containing *N. fucelliana* hyphae with microconidia were used to give a fast and secure way of establishing an infection without risking inoculum failure due to climatic conditions. However, as in the greenhouse study, none of the inoculated trees developed the top-dieback as observed in the pilot study, or seen in Norway spruce stands in Finland [23] and Christmas tree fields in Sweden [1].

Temperatures at the time of inoculation and over the summer periodically exceeded 25 °C. Temperatures over 25 °C have been proven to negatively affect spore germination, resulting in abnormal germination [4] and slower mycelial growth [1]. For the pilot study, the temperature was continually kept at 20 °C, which is in the range of favored growth temperature for the fungus. This may be another explanation for the slower disease development in the greenhouse study part I and II compared to the pilot study.

Microconidia may not be the optimal spore type for inoculation. In New Zealand, inoculation trials with *radiata* pine using *N. fucelliana* microconidia and ascospores showed that both were highly infectious and there was no significant difference in infection level between the two spore types [8]. Based on this, we chose microconidia for our studies since they are readily produced in
culture. However, the inoculation technique of applying microconidia spore suspension onto artificial wounds may not have been optimal because resin produced by the cuttings may have hindered spore germination (Figure 2). Larger wounds such as bark stripping by deer or tree felling damage from forest management actions do not seal as fast as the artificially created wounds on the cuttings. This may be one of the reasons for the lower amount of disease development in our greenhouse inoculation trials (resin flow sealed the wounds). From our greenhouse inoculation trials with low reisolation frequencies, the potential of *N. fuckeliana* to cause disease in natural settings may easily be underestimated. This is obvious from the results from our outdoor inoculation trial where the background level of *N. fuckeliana* infections must have been high. However, one also has to bear in mind that *N. fuckeliana* has coexisted with Norway spruce through generations in the Nordic countries; thus, the host probably harbor a certain degree of resistance towards the pathogen. In New Zealand, on the other hand, *N. fuckeliana* is an alien, invasive species with great impact on radiata pine, a host that has had no chance to build up resistance.

For the field trial, there were almost no difference between inoculated and control trees regarding reisolation and molecular detection of *N. fuckeliana*. There are two possible explanations for this. The trees could have been contaminated with *N. fuckeliana* before the trial began or they could have become contaminated by natural inoculum at the time of wounding. Since the trial was conducted outdoors, some background level of *N. fuckeliana* infections could be expected [8]. Latent natural infections are widely reported in literature where *N. fuckeliana* has been isolated from wounded and healthy looking stems of Norway spruce [5,12,13,35]. Furthermore, a light rain occurred during part of the inoculation process and the temperature was under 20 °C. Mild and wet weather conditions are favorable for pathogen sporulation, dispersal, and infection of *N. ditissima* [33,36,37] and likely also for *N. fuckeliana*. If the trees in the field trial received the same background inoculum level, then the larger lesion length under bark (approximately 12 mm or 46%) for the inoculated trees was likely due to the additional inoculum of *N. fuckeliana* mycelial plugs. This suggests that the inoculation was probably successful despite the ambient inoculum. There were also significant family differences for lesion lengths under bark. Therefore, screening Norway spruce material in breeding programs may be helpful in determining sensitivity towards *N. fuckeliana*.

The role of *N. fuckeliana* as a wound pathogen is supported by literature [4–6]. However, the relatively slow disease development seen in this study questions the role it has in the top-dieback of Norway spruce. Previous research done in Sweden, Norway, Denmark and Finland shows that *N. fuckeliana* has been found in fields and forests, and seems to be a potential threat to Norway spruce [1–3,14,16,29,30]. There may be large differences between isolates given that sexual outcrossing is common for *N. fuckeliana* [6]. Another explanation may be the effect climate changes may have. *Delphinella abietis* is an example from Scandinavia where an epidemic outbreak was related to increased temperature and precipitation [18]. Furthermore, damage by *N. neomacrospora* on fir has dramatically increased during the last years in Scandinavia [38,39]. It is suggested that the increase is due to milder winters and wetter growing seasons, which benefit dissemination and infection success of *N. neomacrospora*. *Neocryptria ditissima* also seems to have increased during the last decade in the Nordic countries [1–3,14,16,29,30]. Damage by *N. neomacrospora* and *N. fuckeliana* may therefore continue to increase due to global warming and climate change projections [17].

Our inoculation trials have revealed some complexity in doing inoculation experiments with *N. fuckeliana*. The fungus was difficult to reisolate onto PDA. Even though the infected woody tissues were surface sterilized before being plated out, other fungi such as *Sagitta polyspora*, *Fusarium sp.*, *Alternaria sp.*, and *Penicillium sp.* were commonly isolated and morphologically identified. *Neocryptria ditissima* has also been found difficult to isolate [40]. Hence, a more selective media for *N. fuckeliana* would be useful. Even though the molecular identification is much more sensitive, it needs to be complemented with isolates to prove that the fungus is alive inside the wood tissue. Furthermore, no statistically significant difference for the molecular identification for any of the factors could be found. This was likely due to the limited number of samples analyzed (one sixth
or 192 inoculated cuttings) due to limited resources. A difference might have been found between the shoot-topped and non-wounded treatments if the molecular sample size had been larger for the greenhouse inoculation trial.

The effect of temperature and wetness duration on *N. fuckeliana* infections needs to be clarified. In our experiments, the disease was more severe in the pilot study even though the plants were not kept moist after inoculation. The greenhouse inoculation study parts I and II, in which the plants were kept moist and at higher temperature than in the pilot study for several days, less severe disease symptoms developed and even at a slower pace. It is possible that a higher dose than $5 \times 10^5$ conidia/mL would be preferred as a higher spore concentration would shorten the latent period [32]. We also lack information about the epidemiology of the fungus. This includes how the inoculum pressure varies with climatic conditions throughout the year and how the infection success varies with wound age. However, it is clear from our inoculation trials that even small wounds such as needle scars are sufficient for *N. fuckeliana* infections. Hence, growers who annually shear their trees to make a denser Christmas tree area are at higher risk of getting *N. fuckeliana* contaminations through natural inoculum. Therefore, implementation of proper management strategies may become necessary to avoid future top-dieback in Christmas tree fields.

A key element in keeping any crop healthy is removal of inoculum sources to keep the disease pressure low. Even though we have not yet observed perithecia developing on the Christmas trees with top-dieback, we strongly recommend that diseased trees are removed and burnt. We argue against the practice of piling up removed trees next to the fields or discarding the diseased trees in the nearby forests. Such management may result in a buildup of inoculum that could spread and infect nearby forests and Christmas trees. Often sections of Christmas tree plantations are the same species (monocultures) planted in dense straight rows. Good sanitation practices are important as such an environment could become an incubation chamber for diseases to multiply. Diseases may enter from a forest to a Christmas tree plantation, and later re-enter to the forest or vice versa. Therefore, if a Norway spruce Christmas tree field has top-dieback caused by *N. fuckeliana* and is located close to a Norway spruce forest, it may be important to manage the disease in both in the Christmas tree field and the nearby forest. Furthermore, good hygiene practices, such as cleaning tools between pruning different trees and especially between fields, are important to avoid inoculum transfer.

Future research can include a standardized inoculation method for comparing aggressiveness of different isolates of the fungus on different tree provenances, or preferably on cuttings (clones) to avoid differences in susceptibility between individuals within a provenance. Other studies examining the growth rate of inoculated and non-inoculated Norway spruce plants under different temperature regimes are needed to determine growth loss due to *N. fuckeliana* infections and clarify the role of temperature on symptom development. To come up with better management tactics both for forestry and Christmas tree production, such as thinning operations and shearing during periods with low inoculum pressure, we need to learn more about the life-cycle of *N. fuckeliana*. This means that future research also needs to include factors such as variation in spore dissemination thought the year, in order to reduce the probability of infection from this fungus. Under natural conditions, the trees are exposed to a number of pests and diseases that may influence the damage potential of *N. fuckeliana*; thus, further research on such interactions are needed.

3. Conclusions

*Neonectria fuckeliana* needs an open wound as an entry point for causing disease on Norway spruce. Data from our inoculation trials showed that larger wound treatments resulted in larger lesion length under bark as well as higher detection frequencies of the fungus. Infections took place in both actively growing and dormant Norway spruce cuttings. The ability of *N. fuckeliana* to cause infection on inoculated Norway spruce is clearly dependent upon the technique used. Further inoculation studies are necessary to create a standardized inoculation method for reliable comparison of different *N. fuckeliana* isolates.
Symptom development of the cuttings in this study was generally minor. This does not correlate well with the recent observations in Sweden, Finland, and Norway, where *N. fuckeliana* has caused cankers, heavy resin flow, and top-dieback on young Norway spruce, suggesting it has become a more aggressive pathogen. This study is more in line with older studies describing *N. fuckeliana* as a weak pathogen on Norway spruce. Thus, further research into the epidemiology and pathogenicity of the fungus is needed to increase the understanding of why *Neectria* canker has become an increasing problem on Norway spruce in the Nordic countries during recent years.

Proper sanitation practices in Christmas tree plantations and new management strategies in young spruce forest stands are needed to prevent and control the disease.

Acknowledgments: We want to thank Johan Malm, Miklós Czimbalmos, Ingrid Vos, Eva Petersson, and Joakim Nilsson at Skogforsk for providing and watering the cuttings in the greenhouse. We like to thank Ilon Thomsen, The Department of Geosciences and Natural Resource Management, University of Copenhagen, and Larisa Gustavsson, The Department of Plant Breeding, Swedish University of Agricultural Sciences, for advice on inoculation techniques. We would also like to thank Carl Sak, The Swedish Forest Research Centre, Swedish University of Agricultural Sciences, and Jan-Eric Englund, Department of Biosystems and Technology, Swedish University of Agricultural Sciences, for valuable assistance with R and SAS statistical software, respectively. We also wish to thank Monica Skogen and Torde L. Størstad at NIBIO for laboratory assistance. This study was supported by the Partnership Alnarp, the Raisjo Foundation, and the Gunnar and Lillian Nicholson Graduate Fellowship and Faculty Exchange Fund.

Author Contributions: Martin Pettersson initiated, designed, and carried out the inoculation trials; followed by evaluation, reisolation, molecular detection, and writing of this manuscript; all in close collaboration with the coauthors.

Conflicts of Interest: We declare no conflicts of interest.

Appendix A

Table A1. Monthly average, standard deviation, minimum and maximum temperature fluctuation patterns for day and night from April 2016–March 2017 in the greenhouse where the second and third inoculation trials took place.

<table>
<thead>
<tr>
<th>Month, Year</th>
<th>Average</th>
<th>StdDev</th>
<th>Max</th>
<th>Min</th>
</tr>
</thead>
<tbody>
<tr>
<td>April, 2016 Day</td>
<td>11.6</td>
<td>3.5</td>
<td>25.8</td>
<td>4.5</td>
</tr>
<tr>
<td>Night</td>
<td>8.7</td>
<td>3.0</td>
<td>21.6</td>
<td>5.0</td>
</tr>
<tr>
<td>May, 2016 Day</td>
<td>22.7</td>
<td>7.5</td>
<td>41.1</td>
<td>6.6</td>
</tr>
<tr>
<td>Night</td>
<td>15.1</td>
<td>6.1</td>
<td>39.8</td>
<td>6.9</td>
</tr>
<tr>
<td>June, 2016 Day</td>
<td>24.9</td>
<td>8.0</td>
<td>52.5</td>
<td>8.6</td>
</tr>
<tr>
<td>Night</td>
<td>16.4</td>
<td>6.3</td>
<td>46.2</td>
<td>6.5</td>
</tr>
<tr>
<td>July, 2016 Day</td>
<td>29.5</td>
<td>7.9</td>
<td>51.1</td>
<td>10.3</td>
</tr>
<tr>
<td>Night</td>
<td>20.1</td>
<td>5.9</td>
<td>44.2</td>
<td>8.9</td>
</tr>
<tr>
<td>August, 2016 Day</td>
<td>24.1</td>
<td>7.0</td>
<td>46.6</td>
<td>10.2</td>
</tr>
<tr>
<td>Night</td>
<td>16.2</td>
<td>5.8</td>
<td>39.7</td>
<td>8.8</td>
</tr>
<tr>
<td>September, 2016</td>
<td>21.4</td>
<td>7.3</td>
<td>42.6</td>
<td>7.0</td>
</tr>
<tr>
<td>Night</td>
<td>15.0</td>
<td>5.8</td>
<td>37.3</td>
<td>7.1</td>
</tr>
<tr>
<td>October, 2016</td>
<td>10.6</td>
<td>4.4</td>
<td>34.7</td>
<td>3.7</td>
</tr>
<tr>
<td>Night</td>
<td>8.7</td>
<td>3.1</td>
<td>29.1</td>
<td>4.1</td>
</tr>
<tr>
<td>November, 2016</td>
<td>6.8</td>
<td>2.2</td>
<td>15.3</td>
<td>3.3</td>
</tr>
<tr>
<td>Night</td>
<td>6.0</td>
<td>2.1</td>
<td>13.8</td>
<td>3.5</td>
</tr>
<tr>
<td>December, 2016</td>
<td>7.0</td>
<td>1.6</td>
<td>12.6</td>
<td>3.8</td>
</tr>
<tr>
<td>Night</td>
<td>6.7</td>
<td>1.6</td>
<td>12.1</td>
<td>3.9</td>
</tr>
<tr>
<td>January, 2017</td>
<td>6.0</td>
<td>1.5</td>
<td>12.4</td>
<td>3.6</td>
</tr>
<tr>
<td>Night</td>
<td>5.6</td>
<td>1.3</td>
<td>11.0</td>
<td>3.7</td>
</tr>
<tr>
<td>February, 2017</td>
<td>8.1</td>
<td>4.0</td>
<td>25.9</td>
<td>3.8</td>
</tr>
<tr>
<td>Night</td>
<td>6.6</td>
<td>3.0</td>
<td>21.7</td>
<td>3.8</td>
</tr>
<tr>
<td>March, 2017</td>
<td>10.2</td>
<td>5.2</td>
<td>30.5</td>
<td>4.1</td>
</tr>
<tr>
<td>Night</td>
<td>7.2</td>
<td>3.9</td>
<td>27.5</td>
<td>4.5</td>
</tr>
</tbody>
</table>

* Measured by three HOBO water temperature Pro V2 data loggers (Onset Company, Bourne, MA, USA).
Table A2. Daily average, standard deviation, minimum and maximum temperature fluctuation patterns for day and night for the 8-day-period following the second inoculation trial and the third inoculation trial.

<table>
<thead>
<tr>
<th>Date</th>
<th>Mean</th>
<th>Stdev</th>
<th>Max</th>
<th>Min</th>
<th>Date</th>
<th>Mean</th>
<th>Stdev</th>
<th>Max</th>
<th>Min</th>
</tr>
</thead>
<tbody>
<tr>
<td>31 May Day</td>
<td>24.1</td>
<td>3.7</td>
<td>32.3</td>
<td>17.6</td>
<td>16 Aug Day</td>
<td>20.8</td>
<td>5.4</td>
<td>33.6</td>
<td>11.8</td>
</tr>
<tr>
<td>Night</td>
<td>18.5</td>
<td>1.8</td>
<td>22.6</td>
<td>16.7</td>
<td>Night</td>
<td>13.5</td>
<td>5.3</td>
<td>30.6</td>
<td>10.8</td>
</tr>
<tr>
<td>1 June Day</td>
<td>28.8</td>
<td>6.3</td>
<td>37.0</td>
<td>18.4</td>
<td>17 Aug Day</td>
<td>23.8</td>
<td>6.2</td>
<td>33.7</td>
<td>11.0</td>
</tr>
<tr>
<td>Night</td>
<td>23.7</td>
<td>5.8</td>
<td>35.8</td>
<td>17.6</td>
<td>Night</td>
<td>14.7</td>
<td>5.7</td>
<td>29.0</td>
<td>10.0</td>
</tr>
<tr>
<td>2 June Day</td>
<td>32.2</td>
<td>7.0</td>
<td>44.2</td>
<td>18.6</td>
<td>18 Aug Day</td>
<td>21.2</td>
<td>4.5</td>
<td>31.8</td>
<td>13.1</td>
</tr>
<tr>
<td>Night</td>
<td>20.7</td>
<td>6.0</td>
<td>37.3</td>
<td>17.5</td>
<td>Night</td>
<td>15.3</td>
<td>3.8</td>
<td>25.3</td>
<td>11.7</td>
</tr>
<tr>
<td>3 June Day</td>
<td>29.3</td>
<td>7.0</td>
<td>40.3</td>
<td>17.2</td>
<td>19 Aug Day</td>
<td>24.3</td>
<td>6.6</td>
<td>42.2</td>
<td>13.5</td>
</tr>
<tr>
<td>Night</td>
<td>19.3</td>
<td>6.7</td>
<td>37.2</td>
<td>16.0</td>
<td>Night</td>
<td>16.6</td>
<td>4.8</td>
<td>29.2</td>
<td>14.0</td>
</tr>
<tr>
<td>4 June Day</td>
<td>31.7</td>
<td>11.4</td>
<td>51.8</td>
<td>18.8</td>
<td>20 Aug Day</td>
<td>21.3</td>
<td>4.1</td>
<td>28.9</td>
<td>13.7</td>
</tr>
<tr>
<td>Night</td>
<td>18.8</td>
<td>9.9</td>
<td>45.7</td>
<td>13.7</td>
<td>Night</td>
<td>16.8</td>
<td>4.1</td>
<td>27.8</td>
<td>13.9</td>
</tr>
<tr>
<td>5 June Day</td>
<td>27.0</td>
<td>7.3</td>
<td>42.5</td>
<td>13.0</td>
<td>21 Aug Day</td>
<td>26.9</td>
<td>7.7</td>
<td>42.6</td>
<td>16.2</td>
</tr>
<tr>
<td>Night</td>
<td>15.9</td>
<td>6.4</td>
<td>34.8</td>
<td>12.0</td>
<td>Night</td>
<td>17.2</td>
<td>4.7</td>
<td>31.8</td>
<td>13.9</td>
</tr>
<tr>
<td>6 June Day</td>
<td>24.6</td>
<td>7.0</td>
<td>36.8</td>
<td>12.7</td>
<td>22 Aug Day</td>
<td>23.3</td>
<td>5.9</td>
<td>36.2</td>
<td>13.1</td>
</tr>
<tr>
<td>Night</td>
<td>15.1</td>
<td>6.2</td>
<td>31.5</td>
<td>10.4</td>
<td>Night</td>
<td>16.5</td>
<td>5.3</td>
<td>31.0</td>
<td>12.7</td>
</tr>
<tr>
<td>7 June Day</td>
<td>26.4</td>
<td>7.6</td>
<td>39.9</td>
<td>10.3</td>
<td>23 Aug Day</td>
<td>24.7</td>
<td>5.6</td>
<td>37.7</td>
<td>15.4</td>
</tr>
<tr>
<td>Night</td>
<td>14.9</td>
<td>7.0</td>
<td>32.1</td>
<td>9.5</td>
<td>Night</td>
<td>17.3</td>
<td>4.9</td>
<td>31.6</td>
<td>12.9</td>
</tr>
<tr>
<td>Average</td>
<td>24.5</td>
<td>8.3</td>
<td>51.8</td>
<td>9.5</td>
<td>Average</td>
<td>20.9</td>
<td>6.5</td>
<td>42.6</td>
<td>10.0</td>
</tr>
</tbody>
</table>

* Measured by three HOBO water temperature Pro V2 data loggers (Onset Company, Bourne, MA, USA).

References
1. Pettersson, M.; Frampton, J.; Ronnberg, J.; Talgo, V. Neoanectria canker found on spruce and fir in Swedish Christmas tree plantations. Plant Health Prog. 2016, 17, 202–205. [CrossRef]
21. Talgo, V.; Eikemo, H.; Thomsen, L.M.; Chastagner, G.A.; Pettersson, M.; Nielsen, K.N.; Perez-Sierra, A. Climatic conditions related to recent outbreaks of *Neocryptospora coniospora* on Abies spp. in Europe and USA. In Proceedings of the Conference Program from the Working Party Meeting Invasive Forest Pathogens & Implications for Biology and Policy, IUFRO 7.02.02 Foliage, Shoot and Stem Diseases of Forest Trees, Niagara Falls, Canada, 7–11 May 2017; p. 68.
24. González, C.D.; Chaverri, P. *Curvularia*, a new genus to accommodate *Neocryptospora fuckeliana* and *C. coniospora* sp. nov. from *Pinus radiata* in Chile. *Mycol. Prog.* 2017, 16, 1015–1027. [CrossRef]
25. Talgo, V.; Stensvand, A. A simple and effective inoculation method for *Phytophthora* and fungal species on woody plants. *EPPO Bull.* 2013, 43, 276–279. [CrossRef]
27. Thomsen, L.M. (University of Copenhagen, Copenhagen, Denmark). Personal communication, 2018.


© 2018 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).
CHAPTER 8. Development and application of a real-time PCR assay for detection and identification of Neonectria fuckeliana from Norway spruce

Martin Pettersson, a,b# Venche Talgø, c Jonas Rönnberg, b May Bente Brurberg c,d#

a Department of Forestry and Environmental Resources, North Carolina State University, North Carolina, Raleigh, USA

b Southern Swedish Forest Research Centre, Swedish University of Agricultural Sciences, Alnarp, Sweden

c Division of Biotechnology and Plant Health, Norwegian Institute of Bioeconomy Research, Ås, Norway

d Department of Plant Sciences, Norwegian University of Life Sciences, Ås, Norway

#Address correspondence to Martin Pettersson, jmpetter@ncsu.edu and May Bente Brurberg, may.brurberg@nibio.no

Abstract

Neonectria canker on spruce (Picea spp.), caused by the fungus Neonectria fuckeliana, has become an increasing problem in northern Europe during the last decade. Conventional detection methods such as isolation and morphological identification of N. fuckeliana are labor intensive and challenging due to competition from fast growing secondary fungi and bacteria. In this study, a species-specific TaqMan real-time PCR assay was developed for rapid identification and detection of N. fuckeliana. The internal transcribed spacer 1 (ITS-1) region was used to design a species-specific primer for the real-time PCR assay. The specificity of the primer pair was
confirmed at species level, allowing for fast and reliable detection of the pathogen directly from infected tissue. The species-specific TaqMan assay for *N. fuckeliana* can complement, and partly replace, the culture-based system for identification and it will be a useful tool for rapid and accurate identification of this pathogen in samples from forest, Christmas tree and nursery surveys. Furthermore, it has a potential for resistance screening of Norway spruce clones and families.

**Keywords**: ITS region, forest, Christmas trees, top-dieback, canker, northern Europe.

**Introduction**

*Neonectria fuckeliana* (C. Booth) Castl. & Rossman, the causal agent of Neonectria canker on spruce (*Picea* spp.), is an increasing problem in northern Europe (Pettersson et al. 2016; Lilja et al. 2012; Thomsen and Nielsen 2018; Talgø et al. 2018b). The fungus causes black resin covered canker wounds on branches and/or stem and top-dieback, which reduce timber quality and result in economic losses that negatively affect the forest and Christmas tree production. Mortality has also been observed. There are no known curative chemical control measures for Neonectria canker. For *N. fuckeliana*, we are not aware of any fungicide trials in the field, and the seasonality of infections and thus timing of any applications are unclear.

Two closely related *Neonectria* species also cause Neonectria canker on trees in northern Europe; *Neonectria ditissima* (Tul. & C. Tul.) Samuels & Rossman, which is a pathogen of many broad leaf tree species and especially problematic in commercial apple (*Malus × domestica* Borkh) production (Farr et al. 1989; Flack and Swinburne 1977; Weber 2014; Swinburne 1975);
and *N. neomacrospora* (C. Booth & Samuels) Mantiri & Samuels being an aggressive pathogen on fir species (*Abies* spp.) where it has caused epidemics in Norway and Denmark (Nielsen et al. 2017; Skulason et al. 2017; Talgø et al. 2018a; Thomsen and Nielsen 2018) during the last decade and also been detected in other countries in Europe (Schmitz et al. 2017; Perez-Sierra 2018; Pettersson et al. 2016). *Neonectria fuckeliana* is specifically associated with damage of Norway spruce (*Picea abies* L. Karst) in northern Europe (Pettersson et al. 2016; Uimari et al. 2018), but from Northern Ireland, damage has also been reported on Sitka spruce [*Picea sitchensis* (Bong.) Carr.] (Hanlon and Fleming 2018).

In the past, *N. fuckeliana* was considered a weak pathogen on Norway spruce (Huse 1981; Roll-Hansen 1962; Roll-Hansen and Roll-Hansen 1979; Vasiliauskas et al. 1996). However, the recently observed damage described above indicates that *N. fuckeliana* has become a more serious pathogen of Norway spruce, especially in young forest plantations (5-30 years old). It is unknown why this situation has occurred, however, in Finland where significant damages in young Norway spruce plantations have been evident, *N. fuckeliana* has been found in association with the spruce bark tortrix moth, (*Cydia pactolana* [Zeller]) (Uimari et al. 2018). The insect and the fungus seem to contribute to the increased extent of the damage. The increase may also partly be attributable to changes in climate, such as milder winters and wetter growing seasons (Kjellström et al. 2005; Roos et al. 2011), which positively affect the virulence of many fungal pathogens and may negatively affect tree susceptibility (Hopkins and Boberg 2012).

*Neonectria fuckeliana* is known as a wound invading fungus that colonizes new host trees through airborne sexual spores (ascospores) dispersed from fruiting bodies (perithecia) and/or with splash dispersed asexual spores (conidia) produced on sporodochia (Roll-Hansen and Roll-Hansen 1979; Vasiliauskas and Stenlid 1997). The conventional method used to detect *N.*
*fuckeliana* from infected plant material is isolation on artificial growth medium followed by
morphological and/or sequence analysis of the Internal Transcribed Spacer (ITS) region of the
ribosomal DNA (rDNA) identification (Huse 1981; Roll-Hansen 1962; Roll-Hansen and Roll-
Hansen 1979; Vasiliauskas et al. 1996). For more rapid identification, a DNA-based detection
method (an end-point PCR assay) has been developed for *N. fuckeliana* by Langrell (2005). With
the increased incidence of Neonectria canker in northern Europe (Lilja et al. 2012; Pettersson et
al. 2016; Uimari et al. 2018), the number of Neonectria canker samples received at diagnostic
laboratories has also increased, creating a demand for fast, reliable and simple detection methods
for *N. fuckeliana*. TaqMan real-time PCR has several advantages over conventional PCR. It is
faster, more sensitive and has the major advantage of being able to quantify the amount of
pathogen (Schaad and Frederick 2002). Furthermore, TaqMan real-time PCR is more sensitive
than SYBR Green real-time PCR, because it detects specific amplification products only.
The aim of this study was to develop a more rapid, simple and sensitive detection method for *N.
fuckeliana* using TaqMan real-time PCR. Identification and quantification of *N. fuckeliana*
directly from environmental samples will ease surveying efforts and provide valuable tools for
future studies of this plant pathogen.

**Material and Methods**

**Fungal isolates for primer validation.** From samples collected in Sweden and Norway, cultures
that were morphologically resembling *N. fuckeliana* and closely related fungi *N. ditissima* and *N.
neomacrospora* were isolated from stems and branches of Norway spruce, broadleaf trees (apple
trees (*Malus × domestica*) and one linden tree (*Tilia* sp.)) and fir (*Abies* spp.), respectively. All
hosts displayed typical Neonectria symptoms (cankers, top and branch dieback) and signs
perithecia) on the bark (Fig. 1). Tissue samples were cut with a knife, placed in resealable plastic bags and stored in a cold storage until examined in the laboratory within a week. Fungi were isolated on potato dextrose agar (PDA) from the leading edge of canker wounds and transferred to a new plate of PDA to obtain axenic cultures. All isolates were grown at room temperature (~21°C). The fungal isolates used in the experiment are presented in Table 1.

**Tissue samples for primer validation.** The same procedure as above was used for collecting samples for direct testing of infected plant material with the developed specific primer for *N. fuckeliana*. Neonectria cankers with perithecia were collected from Norway spruce, rowan (*Sorbus aucuparia* L.), and subalpine fir [*Abies lasiocarpa* (Hooker) Nuttall] and Siberian fir (*A. sibirica* Ledeb.). In the same order, the fungi were presumed belonging to *N. fuckeliana*, *N. ditissima*, and *N. neomacrospora* based on host species, wound type and morphology of perithecia (molecular identification was not conducted to confirm identity). From rowan, perithecia of *Nectria cinnabarina* (Tode) Fr. were collected. In addition, samples from trees presumed to be disease free (no symptoms or signs of the pathogens present) were collected to be used as controls.
Figure 1. Typical symptoms (cankers, branch and top-dieback) and red fruiting bodies (perithecia) caused by *Neonectria ditissima* on apple (*Malus domestica*) in Norway (A, B), *N. neomacrospora* on white fir (*Abies concolor*) in Norway (C, D), and *N. fuckeliana* on Norway spruce (*Picea abies*) in Sweden (E) and Norway (F). Photos: Venche Talgø (A, B, C, D, F) and Martin Pettersson (E).

**DNA extraction.** Genomic DNA was extracted using the DNeasy® Plant Mini Kit (Qiagen ®) according to the manufacturer’s recommendations. Mycelia from the pure cultures, infected plant material and dissected perithecia (removed from the bark with a scalpel) were ground in liquid nitrogen using a mortar and pestle. DNA was amplified using the ITS1 or ITS5 and ITS4 primers (White et al. 1990). Concentration and purity of the DNA were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific) and/or examined by agarose gel electrophoresis (1%). Morphological identification of the fungal cultures collected from Sweden and Norway.
was confirmed by ITS sequencing of the rDNA and using BLAST on GenBank with 99-100% identity.

**Primer design and validation of primer specificity.** Primers and probes were selected based on multiple-sequence alignments of *N. fuckeliana, N. ditissima, N. neomacrospora*, other *Neonectria* species (*N. candida, N. coccinea, N. hederae, N. lugdunensis, N. major, N. punicea, N. ramulariae, N. tsugae*) and fungi belonging to other genera (*Dactylonectria estremocensis* and *Nectria cinnabarina*) obtained from the GenBank (Table 2). The difference between *N. fuckeliana* and either *N. ditissima* or *N. neomacrospora* in the ITS region is approximately 20 base pairs (Talgø et al. 2009). The ITS region was selected to design a species-specific primer pair and probe for *N. fuckeliana* using Primer Express software v2.0 (Applied Biosystems). The primer set with the lowest penalty in Primer Express software v2.0 was selected. Initial testing of primers was done using SYBR Green real-time PCR.

**TaqMan assay optimization.** Real-time PCR was used to validate the species-specific primer pair (with probe). A 1:10 dilution of extracted DNA was used in the assay. The reactions were performed in 20 µL reaction volumes containing: 0.6 µL (10 µM) of each primer, 1.6 µL probe (2.5 µM), 10 µL SsoAdvanced Universal Probes Supermix (2×), 5.2 µL MQ H₂O and 2 µL genomic DNA (1/10 diluted after extraction). The reaction mixtures were run on 0.2 ml 96-well PCR plates. The samples were amplified using the Thermocycler CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD, USA) with the following conditions: 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 66°C for 30 s. All reactions had two technical replicates and for each 96-well plate there were negative controls (sterile MQ water). The primer
concentration and the annealing temperature were optimized to increase the efficiency of the real-time PCR. Results were analyzed using Bio-Rad CFX Manager software v2.0.

The specificity of the TaqMan assay was validated by testing the 1:10 dilution of the extracted DNA from cultures and/or perithecia and wood samples presumed to be infected by either *N. fuckeliana, N. ditissima* or *N. neomacrospora*. In addition, DNA from a culture of *N. ramulariae* and *Chondrostereum purpureum, Dactylonectria estremocensis, Ilyonectria* sp., and *Phacidioecnis washingtonensis*, and DNA from perithecia of *Nectria cinnabarina* were included in the experiments. Genomic DNA from species closely related to *N. fuckeliana* (i.e. *N. neomacrospora, N. ditissima, D. estremocensis*, and *Ilyonectria* sp.) were used as controls. DNA from disease-free host tissues and sterile MQ water were used as negative controls.

**Efficiency determination.** A standard curve analysis was conducted. The detection limit for the TaqMan assay was determined through a six 1:10 dilution series (four replicates for each dilution). Each series started from a 1:10 dilution of genomic DNA extracted from a pure *Neonectria* species isolate (no. 250605). The serial dilution ranged from 6.8 ng/µL to 6.8 fg/µL. The concentration of DNA was measured on a Qubit fluorometer (2.0) using the Qubit dsDNA HS Assay Kit.

**Accession numbers.** The GenBank accession numbers for the sequences reported in this paper are MF593143 to MF593152.
Table 1. Norwegian and Swedish fungal isolates used to develop and validate a TaqMan real-time PCR assay for identification of *N. fuckeliana*.

<table>
<thead>
<tr>
<th>Fungal spp.</th>
<th>Isolate no.</th>
<th>GenBank accession no.</th>
<th>Host</th>
<th>Origin</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chondrostereum purpureum</td>
<td>250727</td>
<td>–</td>
<td><em>Malus × domestica</em></td>
<td>Norway</td>
<td>2017</td>
</tr>
<tr>
<td>Dactylonectria estremocensis</td>
<td>250561</td>
<td>–</td>
<td><em>Fragaria ananassa</em></td>
<td>Norway</td>
<td>2015</td>
</tr>
<tr>
<td>Ilyonectria sp.</td>
<td>250685</td>
<td>–</td>
<td><em>Pinus tabuliformis</em></td>
<td>Norway</td>
<td>2016</td>
</tr>
<tr>
<td>Neonectria ditissima</td>
<td>250439</td>
<td>–</td>
<td><em>Malus × domestica</em></td>
<td>Norway</td>
<td>2015</td>
</tr>
<tr>
<td><em>N. ditissima</em></td>
<td>250440</td>
<td>–</td>
<td><em>Malus × domestica</em></td>
<td>Norway</td>
<td>2015</td>
</tr>
<tr>
<td><em>N. ditissima</em></td>
<td>250503</td>
<td>–</td>
<td><em>Malus × domestica</em></td>
<td>Norway</td>
<td>2015</td>
</tr>
<tr>
<td><em>N. ditissima</em></td>
<td>250555</td>
<td>–</td>
<td><em>Malus × domestica</em></td>
<td>Norway</td>
<td>2015</td>
</tr>
<tr>
<td><em>N. ditissima</em></td>
<td>250743</td>
<td>–</td>
<td><em>Tilia sp.</em></td>
<td>Norway</td>
<td>2017</td>
</tr>
<tr>
<td><em>N. fuckeliana</em></td>
<td>250603 MF593143</td>
<td></td>
<td><em>Picea abies</em></td>
<td>Norway</td>
<td>2016</td>
</tr>
<tr>
<td><em>N. fuckeliana</em></td>
<td>250626 MF593147</td>
<td></td>
<td><em>Picea abies</em></td>
<td>Sweden</td>
<td>2016</td>
</tr>
<tr>
<td><em>N. fuckeliana</em></td>
<td>250627 MF593148</td>
<td></td>
<td><em>Picea abies</em></td>
<td>Sweden</td>
<td>2016</td>
</tr>
<tr>
<td><em>N. fuckeliana</em></td>
<td>250628 MF593149</td>
<td></td>
<td><em>Picea abies</em></td>
<td>Sweden</td>
<td>2016</td>
</tr>
<tr>
<td><em>N. fuckeliana</em></td>
<td>250632 MF593152</td>
<td></td>
<td><em>Picea abies</em></td>
<td>Sweden</td>
<td>2016</td>
</tr>
<tr>
<td><em>N. neomacrospora</em></td>
<td>250431</td>
<td>–</td>
<td><em>Abies lasiocarpa</em></td>
<td>Norway</td>
<td>2014</td>
</tr>
<tr>
<td><em>N. neomacrospora</em></td>
<td>250463</td>
<td>–</td>
<td><em>Tsuga heterophylla</em></td>
<td>Norway</td>
<td>2008</td>
</tr>
<tr>
<td><em>N. neomacrospora</em></td>
<td>250686</td>
<td>–</td>
<td><em>Abies sibirica</em></td>
<td>Norway</td>
<td>2008</td>
</tr>
<tr>
<td><em>N. neomacrospora</em></td>
<td>250696</td>
<td>–</td>
<td><em>A. concolor</em></td>
<td>Norway</td>
<td>2008</td>
</tr>
<tr>
<td><em>N. ramulariae</em></td>
<td>2017-52</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Phacidiopycnis washingtonensis</em></td>
<td>250732</td>
<td>–</td>
<td><em>Malus × domestica</em></td>
<td>Norway</td>
<td>2017</td>
</tr>
</tbody>
</table>
Table 2. Fungal species, host plants, GenBank accession numbers of the internal transcribed spacer 1 (ITS-1) region of the ribosomal DNA (rDNA) sequences used to verify species specificity of the Nfuc-F1/Nfuc-R1 primer pair, and countries of isolate origin.

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Host plant</th>
<th>GenBank accession no.</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dactylonectria estremocensis</td>
<td>Vitis vinifera</td>
<td>JF735320</td>
<td>Portugal</td>
</tr>
<tr>
<td>Nectria cinnabarina</td>
<td>Acer pseudoplatinus</td>
<td>AF163025</td>
<td>–</td>
</tr>
<tr>
<td>Neonectria candida</td>
<td>Pyrus sp.</td>
<td>KU588183</td>
<td>Netherlands</td>
</tr>
<tr>
<td>N. coccinea</td>
<td>–</td>
<td>JF268759</td>
<td>–</td>
</tr>
<tr>
<td>N. ditissima</td>
<td>Malus sp.</td>
<td>DQ178169</td>
<td>Netherlands</td>
</tr>
<tr>
<td>N. ditissima</td>
<td>Malus sp.</td>
<td>KM515891</td>
<td>Netherlands</td>
</tr>
<tr>
<td>N. hederae</td>
<td>Hedera helix</td>
<td>KC660520</td>
<td>France</td>
</tr>
<tr>
<td>N. fuckeliana</td>
<td>Picea sitchensis</td>
<td>HQ840386</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>N. fuckeliana</td>
<td>Picea abies</td>
<td>HQ840387</td>
<td>Austria</td>
</tr>
<tr>
<td>N. fuckeliana</td>
<td>Picea abies</td>
<td>KT350495</td>
<td>Sweden</td>
</tr>
<tr>
<td>N. fuckeliana</td>
<td>Picea abies</td>
<td>KT350496</td>
<td>Sweden</td>
</tr>
<tr>
<td>N. fuckeliana</td>
<td>Picea abies</td>
<td>KT438903</td>
<td>Sweden</td>
</tr>
<tr>
<td>N. lugdunensis</td>
<td>Populus fremontii</td>
<td>KM231762</td>
<td>USA</td>
</tr>
<tr>
<td>N. lugdunensis</td>
<td>–</td>
<td>KM515896</td>
<td>China</td>
</tr>
<tr>
<td>N. major</td>
<td>Alnus incana</td>
<td>JF735308</td>
<td>Norway</td>
</tr>
<tr>
<td>N. neomacrospora</td>
<td>–</td>
<td>HQ840388</td>
<td>Canada</td>
</tr>
<tr>
<td>N. neomacrospora</td>
<td>Abies nordmanniana</td>
<td>KT350497</td>
<td>Sweden</td>
</tr>
<tr>
<td>N. neomacrospora</td>
<td>Abies nordmanniana</td>
<td>KT383060</td>
<td>Sweden</td>
</tr>
<tr>
<td>N. neomacrospora</td>
<td>Abies nordmanniana</td>
<td>KT383061</td>
<td>Sweden</td>
</tr>
<tr>
<td>N. punicea</td>
<td>–</td>
<td>JF268768</td>
<td>Austria</td>
</tr>
<tr>
<td>N. ramulariae</td>
<td>Malus sylvestris</td>
<td>JF735313</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>N. tsugae</td>
<td>Tsuga heterophylla</td>
<td>KM231763</td>
<td>Canada</td>
</tr>
</tbody>
</table>

Results

Primer design and validation of primer specificity. The positions of the species-specific primer pair (Nfuc-F1/Nfuc-R1) and the internal probe (Nfuc-P1) for N. fuckeliana are shown in Fig. 2, and the characteristics are given in Table 3. Amplicon length was 112 bp for the N. fuckeliana TaqMan PCR assay.
**TaqMan assay validation.** In this study, the Nfuc-F1/Nfuc-R1 primer pair successfully amplified the ITS-1 region of the extracted DNA of all the *N. fuckeliana* isolates (Table 1). There were no cross reactions with other closely species of *Neonectria* (e.g., *N. ditissima* and *N. neomacrospora*) and fungi from other genera (Table 1), or any of the negative controls (DNA from disease-free host tissues and sterile MilliQ water).

**Efficiency determination.** The *N. fuckeliana* TaqMan assay successfully detected and amplified DNA through six orders of magnitude in a 1:10 serial dilution (isolate no. 250605), starting at 6.8 ng/μL and ending at 6.8 fg/μL. The assay demonstrated reproducible and dependable Ct values in 40 cycles of repeated assays. The detection limit for the assays with the primers was 6.8 fg/μL per reaction. Two of four reactions at this low level were successful. Ct values were plotted against the logs of the initial amounts of *N. fuckeliana* DNA and showed a linear relationship. The standard curve had efficiency, slope, intercept and correlation coefficient ($R^2$) values of 87.4%, -3.666, 20.537 and 0.999, respectively (Fig. 3).

**TaqMan assay on plant material.** The TaqMan assay successfully amplified the specific target DNA from *N. fuckeliana* perithecia in bark and *N. fuckeliana* infected wood tissue samples. The TaqMan assay did not amplify DNA from perithecia and wood samples presumed to be infected based on disease symptoms and pathogen signs on the tree by *N. ditissima* and *N. neomacrospora*, and perithecia from *Nectria cinnabarina*. These results verified that the TaqMan assay is specific in targeting *N. fuckeliana* DNA in infected symptomatic and non-symptomatic Norway spruce plants. Plant tissue and potentially other secondary fungi present as endophytes inside the Norway spruce plants, did not inhibit the detection of *N. fuckeliana*. 
Figure 2. The primer pair and probe were chosen based on multiple-sequence alignments of \textit{Neonectria fuckeliana}, \textit{N. ditissima}, \textit{N. neomacrospora} and related fungi (a few from other genera) obtained from GenBank (Table 2). This figure displays an alignment of regions of the internal transcribed spacer 1 (ITS-1) region of \textit{N. fuckeliana}, \textit{N. neomacrospora} and \textit{N. ditissima}. The primer pair and probe for the \textit{N. fuckeliana} (yellow) assay was designed for the ITS-1 region. Primer sequences are indicated by the arrow boxes and probe sequence is shown in the rectangular box. The ITS sequences were HQ840386 (\textit{N. fuckeliana}), HQ840388 (\textit{N. neomacrospora}) and DQ178169 (\textit{N. ditissima}). Identical nucleotides are marked with an asterisk (*) and inserts with a dash (–).

\textbf{TABLE 3} The primer pair and probe designed for \textit{Neonectria fuckeliana} by using Primer Express software 2.0.

<table>
<thead>
<tr>
<th>Primer/probe</th>
<th>Target gene</th>
<th>Sequence (5’→3’)</th>
<th>Positions*</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nfuc-F1</td>
<td>ITS-1</td>
<td>(5’-CCAAACCCCTGTGAACATACCT-3’)</td>
<td>48-69</td>
<td>112</td>
</tr>
<tr>
<td>Nfuc-R1</td>
<td>ITS-1</td>
<td>(5’-CTGAATACAGTATCTTCTGAGTAA CACGATT-3’)</td>
<td>129-159</td>
<td></td>
</tr>
<tr>
<td>Nfuc-P1</td>
<td>ITS-1</td>
<td>(5’-CGCCAGAGGACCCCA-3’)</td>
<td>103-117</td>
<td></td>
</tr>
</tbody>
</table>

*Oligonucleotide positions within the sequences of the internal transcribed spacer 1 (ITS-1) region (accession no. HQ840386 for \textit{N. fuckeliana}).
Figure 3. Standard curve plot for *Neonectria fuckeliana* generated using 10-fold serially diluted DNA and the threshold cycle (Ct) value. The equation of the regression curve (y) and the coefficient of determination (R²) are shown in the graph. There were four replicates for each dilution and error bars represent the standard error from four replicate reactions. For the 10⁻⁷ dilution, error bars represent standard deviation from two replicate reactions (two reactions did not get a value within the 40 cycles).

**Discussion**

The species-specific TaqMan assay that was developed for rapid detection and identification of *N. fuckeliana* from both fungal culture and infected plant tissue, proved to be sensitive and efficient for detection and quantification of *N. fuckeliana* and had no cross-reactions with other species of *Neonectria*. Thus, field and laboratory precautions used to exclude DNA contamination from other organisms and/or obtaining pure fungal cultures will not be required,
which will be time saving. The standard curve showed that the primer pair amplified the DNA it was designed for with high efficiency and reproducibility. The observed sensitivity for the assay will allow detection of the pathogen from non-symptomatic tissue samples. This was demonstrated in Pettersson et al. (2018) where the developed species-specific TaqMan assay for *N. fuckeliana* was used to complement the culture-based identification method. In an outdoor *N. fuckeliana* field inoculation trial of Norway spruce, *N. fuckeliana* was re-isolated and detected in wood tissue from both inoculated and non-inoculated (control) plants. Another valuable feature with our *N. fuckeliana* TaqMan assay is that the amount of DNA in samples can be compared to each other through relative quantification; a trait that may be useful for screening potential disease resistance in spruce plants.

The successful amplification of *N. fuckeliana* DNA from diseased wood and bark tissue implies either that the PCR inhibitor in the wood and bark did not inhibit the assay or that the 1:10 dilution of the extracted DNA was sufficient to lower the PCR inhibitor content.

PCR-based methods do not differentiate between living and non-living cells. Therefore, identification of the pathogen must still include conventional detection methods to show that *N. fuckeliana* is alive and may cause disease. As there is no selective growth medium for *N. fuckeliana*, common media such as PDA or malt extract agar are commonly used. However, due to competition from other fast growing fungi, this method often requires a larger amount of diseased wood tissues to be plated out to increase the chances of successful isolation. This is a problem when, for example, investigating small wounds on seedlings (Pettersson et al. 2018). The described TaqMan assay, can detect DNA from very small amounts of tissue (6.8 fg/µL) when the fungus cannot be isolated.
Reasons for the increasing number of reports of damage caused by *N. fuckeliana* from northern Europe remain unclear, but there is enough damage in young Norway spruce plantations to conclude that this disease should be considered a serious threat to spruce forests and Christmas tree plantations in the region. Further research is needed to develop efficient management strategies based on increased knowledge of the pathogens life cycle and pathogenicity. For that purpose, the *N. fuckeliana* TaqMan assay may benefit ongoing and future epidemiological studies in nurseries, forest stands, Christmas tree plantations, and seed orchards. In addition, the TaqMan assay could be potentially used to screen for resistance against *N. fuckeliana* in plant material. It is well known that there are large genotypic variations in susceptibility to Neonectria canker in other conifer species, e.g. in subalpine fir towards *N. neomacrospora* (Skulason et al. 2017).

**Acknowledgements**

This study was supported by the Partnership Alnarp and the Gunnar and Lillian Nicholson Graduate Fellowship and Faculty Exchange Fund, and the Rattsjö foundation. We thank Monica Skogen, Arnaud Lefrançois, and Trude L. Slørstad at NIBIO for valuable technical assistance.
References


General discussion, practical implications and future research

A comparison of Christmas tree production in Sweden and North Carolina

There are many differences between Christmas tree production in Sweden and in North Carolina. First of all, the extent of the annual production, roughly 2.8 million trees in Sweden (of which many are taken from the forest) versus 4.3 million trees in North Carolina (produced on Christmas tree farms). Other differences include variation in tree species, growing conditions, diseases and pests and, not least, resources (extension service etc.) available for the Christmas tree growers. In both parts of the world, however, Christmas trees are an intensively managed crop where weeding, fertilizing and shearing are conducted annually. Growers in both Sweden and in North Carolina also rely heavily on imported seedlings.

In Sweden, there is often a variety of tree species within Christmas tree fields, though sections of the fields are usually monoculture areas where trees are planted densely in straight rows (Fig. 1). In the mountains of western North Carolina, most Christmas tree plantations are large monocultures of Fraser fir (Fig 2). The higher tree species variation in Swedish Christmas
tree fields obviously contributed to the fact that so many different diseases were found in the 2015 disease survey (see CHAPTER 4, Table 1).

In Sweden, as in other Christmas tree-producing countries, there is a trend towards growing firs instead of spruce. The production of Nordmann fir on agricultural land in particular has increased lately, while the number of Norway spruce Christmas trees taken from the forests has decreased. The latter is partly due to increased spruce Christmas tree production on agricultural land.

A majority of the Christmas tree growers in both Sweden and North Carolina use imported seedlings to plant their fields. In North Carolina, seedling production was originally in-state, but has gradually shifted to out-of-state plant material (CHAPTER 1). In Sweden, growers depend on imported fir seedlings because Swedish production is lacking. This situation poses a great risk of introducing new Phytophthora species to the country (CHAPTER 5).

In the 2015 disease survey of Swedish Christmas trees where we found 18 different Christmas tree diseases, Phytophthora root rot was one of them; however, in CHAPTER 5 we showed that Phytophthora root rot is currently of minor concern for Christmas tree growers in Sweden. On the other hand, the presence of several Phytophthora species found in the 2015 disease survey may lead to future spread and damage, including problems such as hybridization events (Érsek & Man in't Veld, 2013). In North Carolina, we surveyed only for Phytophthora root rot, which is dominant disease problem seen in Christmas trees fields (CHAPTER 1, 2 and 3). The incidence of Phytophthora root rot in Christmas trees in North Carolina caused by P. cinnamomi has earlier been reported to be approximately 10% (Benson & Grand, 2000; Grand & Lapp, 1974). In our Phytophthora survey in North Carolina (CHAPTER 2), we concluded that
several other *Phytophthora* species besides *P. cinnamomi* are contributing to the loss of Fraser fir in North Carolina today.

The amount of resources and information available to Christmas trees growers in North Carolina and those in Sweden differs significantly. The NCSU Cooperative Extension Service is of great importance to the growers, and NCSU Christmas Tree Genetics Program is continuously providing new knowledge and guidance. Efficient management tactics have been developed for most diseases and pests (other than Phytophthora root rot) and are incorporated into integrated pest management (IPM) programs. To detect Phytophthora root rot on symptomatic seedlings, extension workers train Christmas tree growers to use *Phytophthora* test kits for rapid field-diagnostics (CHAPTER 2). These easy-to-use test kits are important for growers, to avoid planting seedlings contaminated with *Phytophthora* in their fields. Growers can also send symptomatic seedlings to a plant disease clinic to receive accurate disease diagnostics often with the pathogen species identified. In Sweden, Christmas tree farming is a small business with few resources available to the growers. They can, however, access some information if they belong to Sweden’s one Christmas tree growers’ association (Sydsveriges Julgran och Pyntegröntodlarföreningen), and a few Swedish growers belong to the Danish Christmas tree association. The Swedish association has approximately 100 members, but it is not a member of the Christmas Tree Growers Council of Europe (CTGCE). Swedish growers therefore miss out on a lot of research, information and other opportunities. In comparison, the Norwegian Christmas tree grower association (Norsk Juletre) has 470 members, seven local associations and is a member of CTGCE (Strande, 2015b).
Pathogens that could seriously harm future Christmas tree production

Besides Neonectria canker and Phytophthora root rot, several other disease-causing pathogens, of the 16 detected in the 2015 disease survey of Swedish Christmas trees, may become problematic. Delphinella shoot blight, in particular, constitutes a potential risk for future production of fir Christmas trees (Talgø et al., 2016). Sydowia polyspora is also a troublesome pathogen on Christmas trees (Talgø et al., 2010). With respect to rust fungi, Chrysomyxa abietis may become the most problematic since it has no alternative host that can be removed to control the disease. One must also be aware of buildup of Armillaria root rot after 2-3 generations of Christmas tree plantings. Gemmamyces bud blight caused by Gemmamyces piceae was found on diseased Colorado blue spruce (CHAPTER 4, Fig. 2G-H) and may also become more serious. Currently, it is an emerging disease that has caused epidemics on Colorado blue spruce in central Europe (Černý et al., 2015). However, most of these diseases are not yet widespread in Swedish Christmas tree fields. One reason for this could be that many pathogens are limited by the low winter temperatures in northern Europe (Hopkins & Boberg, 2012). Our temperature-growth experiments in CHAPTER 5 and 6 also demonstrated this for P. cryptogea, P. megasperma, P. plurivora, N. neomacrospora and N. fuckeliana, where all grew slowly at low temperatures (5°C) and faster at higher temperatures (15-25°C). However, with global warming, it is predicted that the future climate in northern Europe will become warmer and likely wetter (Kjellström et al., 2005). Changes in the sensitive balance between plants, pathogens and the environment (the disease triangle concept) can have widespread effects on plant disease (Garrett et al., 2006).

Increased temperature and changes in precipitation patterns may negatively affect tree susceptibility and positively affect the virulence of many pathogens (Hopkins & Boberg, 2012). The negative effects on trees are due to climatic stresses that increase trees’ susceptibility to
pathogens. For many pathogens, warmer and wetter conditions are conducive to disease development, as these conditions benefit growth, spore dispersal and spore germination. With the predicted climate change scenarios, many pathogens will likely spread north.

Neonectria canker caused by *Neonectria neomacrospora* is likely the largest potential threat to Swedish Christmas tree production of fir. We detected it on Nordmann fir in several fields (CHAPTER 6), and it is causing epidemics on fir species in Denmark (Nielsen *et al.*, 2017; Skulason *et al.*, 2017) and Norway (Talgø, 2015; Talgø *et al.*, 2009). *Neonectria neomacrospora* was also added to the Alert List of the European and Mediterranean Plant Protection Organization (EPPO) in summer 2017 (EPPO, 2017). Phytophthora root rot caused by several *Phytophthora* species is likely the second largest threat to fir production in Sweden. The *Phytophthora* species already present in Swedish Christmas tree fields have the potential to spread, hybridize and emerge as more aggressive pathogens (CHAPTER 5). There is also an imminent risk of introducing other aggressive *Phytophthora* species via imported plant material (Jung *et al.*, 2016). Neonectria canker caused by *Neonectria fuckeliana* has emerged more recently as a pathogen and is strongly associated with top-dieback on Norway spruce (Fig. 4A). Neonectria canker was one of the most common problems in our Christmas tree disease survey (CHAPTER 6), and wounds from shearing Norway spruce Christmas trees are large enough for *N. fuckeliana* to cause disease (CHAPTER 5).

The most prominent risks for Christmas tree production in North Carolina are the continued spread of Phytophthora root rot in the fields and the introduction of new *Phytophthora* species on imported plant material. Neonectria canker has not been found on conifer trees in North Carolina. However, *N. neomacrospora* has been found on a total of 16 fir species in the Pacific Northwest (Chastagner *et al.*, 2014) where a lot of North Carolina’s seedlings are
produced. *Neonectria neomacrospora* inoculation tests show it to be pathogenic to several species, including Nordmann fir and noble fir (Chastagner et al., 2014). *Neonectria neomacrospora* thus constitutes a threat to fir production in North Carolina and, if introduced, must be taken seriously.

The majority of disease-causing pathogens found in our disease surveys can spread rapidly. *Neonectria neomacrospora*, *N. fuckeliana*, *D. abietis* and *G. piceae* all belong to ascomycota fungi that spread with wind-dispersed spores. The *Phytophthora* species found, on the other hand, spread through water. None of the pathogens found in our surveys are limited to Christmas trees, but all could cause disease in forests, parks, arboreta and gardens. Pathogens can spread from Christmas tree fields to neighboring forests or vice versa. This was suggested in **CHAPTER 6**, where we could not find any fruiting bodies of *N. neomacrospora* or *N. fuckeliana* in the Christmas tree fields. It is therefore likely that the inoculum came from nearby forests or gardens. Christmas trees may be more susceptible to diseases than forest trees due to the many wounds created by annual shearing. If diseases are not properly managed in Christmas tree fields, they may become a source of inoculum where pathogens can multiply and spread to nearby forests and landscape plantings.

Invasive alien pathogens, can arrive in Christmas tree fields via imported plant material. Examples of this include *Phytophthora* species on nursery stock or *N. neomacrospora* on full-grown Christmas trees. Pathogens can then spread to native forests and/or Christmas tree fields. Christmas tree fields can therefore be an entry point and a bridge for diseases to enter and re-enter forest landscape plantings. It is often easier to detect diseases and pests on the intensively managed Christmas trees than it is to detect diseases on forest trees. For Neonectria canker caused by *N. fuckeliana* (which may be difficult to isolate), detection from environmental
samples can now be aided and simplified with the use of the *N. fuckeliana* TaqMan assay (CHAPTER 8).

**Management tactics to protect Christmas tree production**

The organisms causing the diseases and pests found in the 2015 disease survey (CHAPTER 4, Table 1) are normally not problematic in their native habitats and ecological niches. However, in intensively managed Christmas tree plantations, often monocultures, these organisms can become major problems (Talgø & Fløistad, 2015). Management of diseases and pests in Christmas tree fields should consider the integrated pest management approach as outlined by the eight principals of the EU directive regarding IPM (Barzman et al., 2015). To summarize, the eight principles address: 1) prevention and suppression, 2) monitoring, 3) decision-making, 4) non-chemical methods, 5) pesticide selection, 6) reduced pesticide use, 7) anti-resistance strategies, 8) evaluation.

Therefore, to gain control of pests and diseases in Christmas trees, the following factors are important:

- Planting only healthy and disease-free seedlings
- Planting appropriate species for the site conditions, e.g. planting more resistant trees on *Phytophthora* infested soils (CHAPTER 3)
- Maintaining good field hygiene and strict sanitation practices, e.g. removal of diseased trees and burning of debris
- Using biosecurity measures such as disinfection of tools, equipment, footwear and vehicles between fields
Scouting for diseases and pests based on general disease and pest activity

Using biological treatments such as stimulating naturally occurring beneficial arthropods (ladybugs, predatory mites, etc.) as a tactic to control pests (Sundbye et al., 2015)

Applying pesticides to control pests and diseases in the fields

It is especially difficult to protect against Phytophthora root rot in Christmas tree fields as it is found mainly underground in the soil (Chastagner & Benson, 2000). No chemicals on the market can cure Phytophthora root rot in Christmas tree fields. Therefore, growers need to take precautions to prevent the introduction and further spread of *Phytophthora* in their fields.

Important biosecurity measures are:

- Cleaning and disinfecting tools and equipment, especially between fields
- Cleaning shoes, boots and gloves from soil and organic debris between fields
- Cleaning tires, wheels and wheel wells of trucks, tractors and other vehicles regularly
- Avoiding driving in fields when they are wet and sticking to the roads in the fields
- Planning to visit sites with Phytophthora root rot last

Any soil or organic debris carried from one field to another increases the risk of spreading *Phytophthora*. Growers (and their workers) should carry personal biosecurity kits containing items for cleaning and disinfection in their trucks. These kits should contain a plastic box, boot tray or bucket to clean items in, clean water, alcohol-based disinfectant, and a sprayer, hard brush, sponge and boot-tread scraper. Planting healthy nursery stock and having good drainage in the fields may also help to diminish the problems associated with Phytophthora root rot.
The nursery industry needs to understand the consequences and implications of producing and trading with infected stock, especially *Phytophthora* species. Fungicides rapidly select for resistant strains of *Phytophthora*, and will not kill *Phytophthora*. Instead, the use of fungicides often masks infections, making them harder to detect. It is important that nurseries and researchers collaborate to manage *Phytophthora* diseases.

For many airborne, fungal pathogens, such as *Neonectria* species, mild and moist weather conditions are ideal for infection (Swinburne, 1975). Several management tactics, focusing on cultural treatments and good field hygiene, may reduce the damage caused by such pathogens:

- Providing good airflow through the field, and thereby improving the microclimate by planting parallel to the most prominent wind direction, avoiding dense planting, carrying out proper weeding, and removing the lowest branches of the Christmas trees
- Keeping disease pressure low by removing diseased shoots, branches or whole trees from the field and burning the debris to avoid development of fruiting bodies
- Avoiding unnecessary wounding of trees by machinery etc. as many fungi (such as *N. fuckeliana*) require an open entry point for infection (CHAPTER 7)

The last point is very difficult to avoid, however, since annual shearing of the trees creates multiple wounds. We therefore recommend shearing the trees during dry, cold periods, preferably during frost. This minimizes the chance of spores landing on the wounds. However, some canker pathogens, such as *N. ditissima*, can infect older wounds (either by penetrating the tree’s developing defense barriers or entering through unhealed areas on the wound site) if a
sufficient period of wetness occurs. This likely applies for *N. neomacrospora* and *N. fuckeliana* too, as they are closely related to *N. ditissima* (Lombard *et al.*, 2014; Chaverri *et al.*, 2011; Castlebury *et al.*, 2006).

For most Christmas tree diseases in Europe, no fungicides trials have been conducted. However, to protect against *N. neomacrospora* on fir, for example, which is a problem in Norway and Denmark, fungicides are used; Norwegian growers use copper oxide (Nordox 75 WG) during early shoot elongation (Talgø *et al.*, 2015b), and Danish growers have a dispensation to use captan (Merpan 80 WG) (SEGES, 2017).

For common and widespread pests, such as adelgids and mites found in the 2015 disease survey, efficient management tactics exist, including chemical pesticides (Sundbye *et al.*, 2015). Before recommending specific treatments, however, Swedish growers require more knowledge of both common diseases and pests that occur on both spruce and fir Christmas trees to ensure proper identification of the damaging agent. They need knowledge about how to diagnose and treat the diseases reported in the 2015 disease survey. Currently, there are few growers who have access to such information. There is no Swedish Christmas tree extension specialist to turn to for knowledge and guidance. Therefore, the main message for the time being is to maintain good field hygiene.

For widespread diseases, such as Neonectria canker caused by *N. fuckeliana*, which was present in many Christmas trees fields in Sweden (CHAPTER 6), and likely comes from nearby forests, efficient management strategies are needed to avoid further top-dieback. We learned from CHAPTER 7, however, that the pathogenicity of *N. fuckeliana* is complicated and dependent on many factors. In that paper, we conclude that, to provide more efficient management strategies, more knowledge is needed about the life-cycle and factors that influence
successful infection by *N. fuckeliana*. To determine whether *N. fuckeliana* poses a danger to Christmas tree and forest production of Norway spruce, we also need to know if the disease pressure is high during shearing time. At present, we are unable to provide substantiated advice regarding the best time to shear Christmas trees to avoid diseases.

**Strengthening Swedish Christmas tree production and reducing the risk for disease epidemics – lessons learned from North Carolina**

The Christmas tree industry in North Carolina has grown to be the second largest in the US (NCCTA, 2015), and the majority of North Carolina-grown Christmas trees are exported to other states (John Frampton, NCSU, pers. comm.). However, there is much competition for land in the mountains suitable for Fraser fir Christmas tree production today. Consequently, some production has been moved to poorer sites with heavier soils, which are more prone to Phytophthora root rot. In Sweden, the opposite is true. Sweden has a small production and is not self-sufficient in Christmas tree production. There is no shortage of land that could be used for Christmas tree cultivation, but there is a lack of interest. This might be due to Sweden having had a profitable forestry industry for centuries and a focus that has rarely moved beyond the forestry framework.

The potential for expanding Christmas tree production in Sweden is large as land is available and cheap. Furthermore, our study (**CHAPTER 5 and 6**) showed that Sweden has a relatively healthy Christmas tree production, almost free from the most aggressive pathogens that are causing devastating losses in neighboring countries (Skulason *et al.*, 2017; Talgø *et al.*, 2016; Talgø *et al.*, 2010; Talgø *et al.*, 2007; Chastagner & Benson, 2000). Moving into the future, Sweden can learn from other countries’ problems and hopefully avoid serious diseases, such as
Phytophthora root rot in North Carolina (CHAPTER 1, 2 and 3) or Neoneectria canker and CSNN in Denmark (Nielsen et al., 2017; Skulason et al., 2017; Talgø et al., 2010), that have emerged along with those countries’ large Christmas tree industries. To maintain a healthy, expanding industry, preventive management actions should be taken now.

One such measure would be to move Christmas tree seedling production to Swedish nurseries and implement strict sanitation [best management practices (CANGC, 2008)]. This would prevent the introduction, establishment and spread of new and more aggressive Phytophthora species that could devastate the Swedish Christmas tree and forestry industries. It would also likely be the most effective management action to reduce the risk of Phytophthora root rot, and thereby avoid a situation similar to the one in North Carolina, where Phytophthora grew to be a major problem when growers bought infected seedlings and contaminated their own fields. Such preventive actions would benefit Christmas tree growers, as well as Swedish ecosystems. Preventative measures to stop harmful organisms from entering are much simpler and effective than reactive measures, such as trying to eradicate or contain introduced alien pathogens. Reactive measures can be extensive and expensive, without achieving the intended results. For example, P. cinnamomi was introduced to Christmas tree fields in North Carolina, and they have since found no economically feasible measures that can be taken to stop the spread and destruction (CHAPTER 1 and 2).

To boost Swedish Christmas tree production, public awareness of the present Christmas tree opportunities is needed. Currently, Christmas trees are mostly a topic for a few weeks before Christmas and receive very little press during the rest of the year. In order to change this, politicians and other stakeholders must be made aware of the opportunities. Sweden could also
become a member of the CTGCE to gain access to the latest information and research, including techniques for improving Christmas tree quality in the form of introducing quality standards and application of pesticide guidelines based on the most recent pesticide research. There is also benefit to be gained through the exchange of ideas and information between Christmas tree growers. The CTGCE also promotes the use of real Christmas trees, which compete with plastic Christmas trees.

Another major improvement would be to establish fir landraces and seed orchards to be used in Swedish Christmas tree production. This would increase the Swedish Christmas tree industry’s competitiveness and reduce its dependency on imported fir trees, which may not always be the right provenance for Sweden. In conversations with Swedish Christmas tree growers (while conducting the disease survey), several growers requested plant material specially adapted for Swedish climatic conditions. For example, growers north of Skåne requested hardier and more frost-tolerant Nordmann fir. In northern Sweden, the climate is too harsh for production of Nordmann fir Christmas trees, but there are other high-quality fir species popular in other countries, such as subalpine fir (A. lasiocarpa var. lasiocarpa), corkbark fir [A. lasiocarpa var. arizonica (Merriam) Lemmon II] and balsam fir (A. balsamea), that would likely do well in northern Sweden.

All of these fir trees have dense foliage, deep needle colors, good needle retention, narrow crowns, and a pleasant fragrance suitable for a Christmas tree (NCTA, 2017b; Madsen & Sigurgeirsson, 1998). Both subalpine fir and corkbark fir are grown extensively in northern and eastern Norway, and to a lesser extent in Denmark. An inter-Nordic research program for subalpine and corkbark fir Christmas trees was initiated in 1999, in Norway, Denmark, Iceland
and Finland (Madsen & Sigurgeirsson, 1998). The aim of the program was to find provenances of subalpine fir and corkbark fir with good Christmas tree qualities and high survival in Nordic climates. Subalpine and corkbark fir have already become a new niche product on the European market and are a high-value Christmas tree. Several provenances of these two firs have proven to grow well in Nordic climates (Skulason et al., 2018; Fløistad et al., 2017; Skage et al., 2012; Hansen et al., 2004). In Norway, subalpine fir has rapidly become the dominant Christmas tree species and has only been cultivated since the beginning of the 2000s (Strande, 2015b; Strande, 2015a).

Balsam fir is another popular Christmas tree species grown mostly in North America (Chastagner & Benson, 2000). The balsam has similar characteristics as its close relative, Fraser fir. The tree grows naturally over large geographical areas in eastern Canada and the US (NCTA, 2017b). Hence, some provenances are likely well-suited to the northern Swedish climate. In our survey, we saw that several growers were experimenting with growing balsam fir in southern Sweden.

Subalpine, corkbark and balsam fir have the possibility of enriching northern Sweden with alternatives to Norway spruce Christmas tree production. Hopefully, several growers would welcome the opportunity of a high-valued, fast-rotation tree crop in contrast to the long rotations of forestry, where large areas are needed to make good revenue. Eventually, cultivation of subalpine, corkbark and balsam fir could help to distribute Christmas tree production more evenly across the country, instead of its current, main concentration in southern Sweden.

The inter-Nordic research program for subalpine and corkbark fir Christmas trees has identified several provenances for the harsher Nordic climate (Skulason et al., 2018; Fløistad et al., 2017; Skage et al., 2012; Hansen et al., 2004). Provenances with a higher tolerance to
adelgids and pathogens such as *N. neomacrospora* and *D. abietis* have also been identified (Nielsen et al., 2017; Skulason et al., 2017; Talgø et al., 2016). However, several good Christmas tree provenances are highly susceptible to *N. neomacrospora* and it is recommended that they not be planted in Denmark where the disease pressure is high (Skulason et al., 2017). If Sweden remains almost free of this pathogen, these provenances could be grown here. This is another reason for starting domestic Christmas tree seedling production in Sweden. This would make Sweden more self-sufficient in seedlings and avoid further introduction of *N. neomacrospora*. However, sensitivity to pathogens is still one of the most important factors to consider when selecting Christmas tree seed sources. Genotypes that are less susceptible to pathogens should be selected.

Sweden can benefit from the research that has already been done in the inter-Nordic research program. Seeds could be imported from the provenances most likely to match the Swedish climate. However, Sweden should also conduct similar research on subalpine and corkbark fir sources in several locations in the country. This would help to find genetic material with the highest survival rate and best Christmas tree qualities for the Swedish climate. The best material could then be selected to create Swedish seed orchards for Christmas tree growers.

Establishing landraces and seed orchards with subalpine, corkbark and balsam fir in Sweden could benefit growers and potentially also foresters. As the climate warms, more tree species are likely to grow well in Sweden. To reduce the risk of climate stress or epidemic disease wiping out monocultures of native trees, a more species-diverse forestry may be beneficial.
Going forward, Sweden has options. One is to maintain the status quo and continue as we have been. This involves continuing to import seedlings and Christmas trees without any controls, guidelines or standards for minimizing the risk of diseases entering the country. No attempt to obtain reliable statistics of the number of growers, or the amount of Christmas trees produced in Sweden and imported from other countries. No responsibility taken to provide information and resources to the Christmas trees growers.

Another alternative is to start helping the industry by producing guidelines and making decisions that will help Sweden to avoid the mistakes made in other countries. This involves collaboration between government agencies, researchers and growers, similar to what exists in North Carolina, to ensure sustainable development and competitiveness of Swedish Christmas trees.

**Future research**

*Phytophthora in Sweden and North Carolina*

In both Sweden and North Carolina, our disease surveys found more *Phytophthora* species than what was previously known. Even though we cannot say how or from where the new *Phytophthora* species arrived, a large number of species is more problematic than a few. This is because different *Phytophthora* species infect different hosts, which means that more tree species are at risk. Also, it may be necessary to develop planting stock with a greater number of resistant genes. Different *Phytophthora* species can also hybridize with one another, in some cases creating a more aggressive pathogen (Ersek & Nagy, 2008). We suspect that the import of seedlings is bringing in new *Phytophthora* species. Therefore, a rigorous sampling of incoming plant material should be conducted.
Phytophthora species have been detected on many different plant species in commercial nurseries in Europe (Jung et al., 2016) and North Carolina (Warfield et al., 2008; Benson & Grand, 2000). Furthermore, Christmas tree nurseries in North Carolina have on several occasions sold growers seedlings infected with Phytophthora (John Frampton, NCSU, pers. comm.). To stop the spread of already-present Phytophthora species, nurseries in Sweden and North Carolina should also be surveyed. In Sweden, no large-scale surveys have been conducted that map the occurrence of Phytophthora species. Such a survey is needed, because it is important to know what Phytophthora species are present and likely already spread into Swedish ecosystems.

In North Carolina, screening for Phytophthora-resistant tree species that also have good Christmas tree qualities has proven to be difficult, and most fir species tested are highly susceptible to *P. cinnamomi* (Frampton et al., 2013; Frampton & Benson, 2012; Frampton & Benson, 2004; Benson et al., 1997). However, CHAPTER 3 shows that the tactic of planting Eastern white pine on heavily infested soils, may be a good one for the Southern Appalachian Mountains. Furthermore, since there are large family differences in mortality to *P. cinnamomi*, it is possible that cultivation of the most resistant families could reduce the problem of Phytophthora root rot in the Piedmont and Coastal Plain regions of North Carolina. The results of CHAPTER 3 encourage family selection for tree improvement programs, to develop more resistant planting stock of Eastern white pine. This would benefit both the Christmas tree industry and the timber industry.

A possible future solution to combat Phytophthora root rot is to genetically engineer Fraser fir for resistance to Phytophthora root rot. Genetic engineering has been used to solve complex tree problems without drastically changing the genetic makeup or phenotypic appearance of many species. A few examples are freeze-tolerant eucalyptus (*Eucalyptus* spp.),
insect-resistant poplar (*Populus* spp.) and loblolly pine (*Pinus taeda*) with increased wood
density (National Academies of Sciences, 2016). The American chestnut tree has also been
enhanced to resist the blight fungus (*Cryphonectria parasitica*). Two resistance genes have been
incorporated that significantly enhance the resulting transgenic trees’ resistance to the chestnut
blight fungus (Newhouse et al., 2014). As genetic engineering techniques become more powerful
and the cost of engineering less prohibitive, this is likely a tactic that could be applied and could
be successful for Fraser fir in the near future. However, for such work to move forward, it is
important to know the basics, such as which *Phytophthora* species contribute to mortality to
Christmas trees (CHAPTER 2).

**Neonectria in Sweden**

When Christmas trees have top-dieback, they cannot be sold and entail a loss for the Christmas
tree grower (Fig. 4A). For a reliable estimate of the top-dieback severity in Christmas tree fields,
a more thorough investigation focusing only on top-dieback is needed. Furthermore, non-
symptomatic trees must be examined to learn whether they carry latent infections. For this task,
the species-specific PCR-based test for *N. fuckeliana* developed in CHAPTER 8 will be a good
tool for rapid and reliable identification and quantification of *N. fuckeliana*. The distance to
nearby Norway spruce forests should be measured and investigated for fruiting bodies to get an
estimate of how far *N. fuckeliana* inoculum can travel. Spore-trapping in Christmas tree fields
and forests should be combined with weather data to determine what weather conditions favor *N.
fuckeliana* spores release, yielding information about the life-cycle of *N. fuckeliana*. This data in
combination with data on the distance and presence of *N. fuckeliana* in nearby forests and
Christmas tree fields would help us to model and predict the risk of local *N. fuckeliana*
epidemics.
Neonectria fuckeliana is likely to harbour a broad spectrum of virulence factors in its genome. The United States Dept. of Agriculture Fungus–Host Database (https://nt.ars-grin.gov/fungaldatabases/) lists 59 fungus-host combinations for N. fuckeliana. More studies are therefore needed to understand the nature of the pathogenicity of N. fuckeliana and to find out if it is an underestimated threat to Norway spruce production. Large numbers of N. fuckeliana isolates should be collected from different geographical sites in Sweden and neighbouring countries to conduct genome-wide association (GWA) studies. The genotypic data yielded can be used to estimate differences between geographically diverse isolates and to assess the population structure in Sweden versus other countries. Since, N. fuckeliana is a sexually recombining fungus and has been in northern Europe for generations, a high genetic variability in the population structure in Sweden is expected. The aggressiveness of the different, geographically diverse, N. fuckeliana isolates should be phenotyped through inoculation of Norway spruce clones. By associating aggressiveness with the genotype of N. fuckeliana, it should be possible to learn how genetic variation across the genome correlates with aggressiveness of the fungus. Hopefully, genomic regions can be identified for general and host-specific virulence on Norway spruce.
REFERENCES (for the Introduction and General discussion)


*Plant Disease*, 98(1), pp. 134-144.


*Mycological Papers*, 104, pp. 1-56.


https://doi.org/10.1371/journal.pone.0134225.

CANGC (2008). Nursery industry best management practices for *Phytophthora ramorum* to prevent the introduction or establishment in California nursery operations. *Version 1.0.*
California Association of Nurseries and Garden Centers. Available from:

http://www.suddenoakdeath.org/pdf/cangc_bpm_FINAL.pdf [27 Feb 2017].


important threat for Colorado blue spruce cultivation. Joint IUFRO 7.02.02 “Foliage,
shoot and stem diseases of forest trees” and 7.03.04 “Diseases and insects in forest

potted ornamentals: University of Florida, IFAS, Central Florida Research and Education
Center-Apopka.


associated with root rot and stem canker of Noble fir Christmas trees in the Pacific

blight and Grovesiella canker on Abies lasiocarpa in western USA. Scandinavian Journal
of Forest Research, 32(5), pp. 432-437.

Phytophthora spp. Phytopathology, 80, p. 887.


European canker at SLU, Sweden: knowledge gained, tools developed, lessons learned.


González, C.D. & Chaverri, P. (2017). *Corinectria*, a new genus to accommodate *Neonectria fuckeliana* and *C. constricta* sp. nov. from *Pinus radiata* in Chile. *Mycological Progress*, 16(11-12), pp. 1015-1027.


NCTA (2017a). National Christmas Tree Association - “Quick Tree Facts”. Available from:
 http://www.realchristmastrees.org/ [27 Feb 2017].

NCTA (2017b). National Christmas Tree Association. Available from:
 http://www.realchristmastrees.org/ [27 Feb 2017].

Newhouse, A.E., Polin-McGuigan, L.D., Baier, K.A., Valletta, K.E., Rottmann, W.H.,
chestnuts show enhanced blight resistance and transmit the trait to T1 progeny. Plant
Science, 228, pp. 88-97.

variation in susceptibility to the fungus Neonectria neomacrospora in the genus Abies.


Orlikowski, L.B., Duda, B. & Szkuta, G. (2004). Phytophthora citricola on European beech and

community structure analyses in Oregon nurseries inform systems approaches to disease

Advisory Note, 16, pp. 1-6.

*Phytophthora: A Global Perspective*, pp. 166-177.


*Phytopathology*, 66(6), pp. 710-714.


(Abies lasiocarpa) in Denmark. *Forest Pathology*, 47(3),
https://doi.org/10.1111/efp.12326.


