Diseases on Christmas Trees in Southern Sweden and Western North Carolina

With Emphasis on Phytophthora Root Rot and Neonectria Canker

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Front cover photos: Fraser fir Christmas trees in North Carolina dying from Phytophthora root rot (left), Norway spruce Christmas trees with top-dieback where the fungus *Neonectria fuckeliana* was isolated from the margin between dead and live tissue (middle and right). Photos: Martin Pettersson

Back cover photo: Fraser fir (*Abies fraseri*) Christmas trees in the southern Appalachian Mountains. Photo: Martin Pettersson

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Diseases on Christmas Trees in Southern Sweden and Western North Carolina - With Emphasis on Phytophthora Root Rot and Neonectria Canker

Abstract

Surveys and inoculation experiments were conducted in Sweden and North Carolina to investigate diseases of Christmas trees, focusing on Phytophthora root rot and Neonectria canker. Christmas tree production is a significant business in North Carolina and for individual growers in Sweden.

In North Carolina, 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 the dominating species causing disease, but *P. cryptogea* also contributed significantly to Fraser fir loss. According to a questionnaire survey, 88% of Christmas tree growers had *Phytophthora* root rot in their fields. To combat *Phytophthora* root rot in North Carolina eastern white pine is planted on heavily infested sites as an alternative to Fraser fir. After screening eastern white pine families for *P. cinnamomi* tolerance, it is evident that families specifically selected for tolerance will reduce problems with *Phytophthora* root rot.

In Sweden, 16 disease-causing pathogens and six pests were discovered in a survey of Christmas tree plantations. Further studies focused on *Phytophthora* root rot and *Neonectria* canker. Five identified *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 *P. cryptogea*, *P. megasperma*, *P. plurivora* showed minor disease development. The *Phytophthora* species found were not widespread and it is currently a minor problem for Swedish Christmas tree growers.

From Norway spruce trees with top-dieback, *Neonectria fuckeliana* was commonly isolated. On Nordmann fir, *Neonectria neomacrospora* was found. Inoculation studies using *N. fuckeliana* and *N. neomacrospora* on Norway spruce and Nordmann fir, respectively, demonstrated that both pathogens caused disease, while a second *N. fuckeliana* inoculation study found symptom development to be minor.
For rapid and reliable identification of *N. fuckeliana* in northern Europe, a species-specific PCR-based test was developed.

Keywords: Phytophthora spp., *Neonectria* spp., fir (*Abies* spp.), spruce (*Picea* spp.), imported seedlings, disease-causing pathogens, plant symptoms and pathogen signs, real-time PCR, management tactics, biosecurity.

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To Tess

There is only one corner of the universe you can be certain of improving, and that's your own self.

Aldous Huxley

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I **Pettersson M.,** Frampton J., Rönnberg J., Shew H.D., Benson D.M., Kohlway W.H., Escanferla M.E. and Cubeta M.A. (2017). Increased diversity of *Phytophthora* species in Fraser fir Christmas tree plantations in the Southern Appalachians. *Scandinavian Journal of Forest Research* 32(5), 412-420.

II **Pettersson M.,** Frampton J. and Sidebottom J. (2017). Influence of Phytophthora Root Rot on Planting Trends of Fraser fir Christmas Trees in the Southern Appalachian Mountains. *Tree Planters' Notes* 60(1), 4–11. (Editor reviewed).

III Frampton J., **Pettersson M.** and Anne Margaret Braham. Genetic Variation for Resistance to Phytophthora Root Rot in Eastern White Pine Seedlings. (Submitted).

IV **Pettersson M.,** Frampton J., Rönnberg J., Brurberg M.B. and Talgø V. Presence of *Phytophthora* species in Swedish Christmas tree plantations. (Submitted).

V **Pettersson M.,** Frampton J., Rönnberg J. and Talgø V. (2016). Neonectria canker found on spruce and fir in Swedish Christmas tree plantations. *Plant Health Progress* 17(2), 202-205.

VI **Pettersson M.,** Talgø V., Frampton J., Karlsson B. and Rönnberg J. (2018). Pathogenicity of *Neonectria fuckeliana* on Norway spruce clones in Sweden and potential management strategies. *Forests* 9(105), doi:10.3390/f9030105.

VII **Pettersson M.,** Talgø V., Rönnberg J. and Brurberg M.B. Development and application of a real-time PCR assay for detection and identification of *Neonectria fuckeliana* from Norway spruce. (Manuscript).

The contribution of Martin Pettersson to the papers included in this thesis was as follows:

I Designed the study with co-authors, coordinated and conducted field and laboratory work, managed data analyses and wrote the manuscript.

II Designed the study with co-authors, designed the questionnaire for growers, applied and got approval from the institutional review board, managed data analyses, and wrote most of the manuscript.

III Collaborated in the statistical analyses, and wrote the first version of the manuscript.

IV Designed the study with co-authors, coordinated and conducted field and laboratory work, managed data analyses and wrote the manuscript.

V Designed the study with co-authors, coordinated and conducted field and laboratory work, managed data analyses and wrote the manuscript.

VI Designed the study with co-authors, coordinated and conducted field and laboratory work, managed data analyses and wrote the manuscript.

VII Developed the TaqMan real-time PCR-based assay for identification of *Neonectria fuckeliana*, tested the *N. fuckeliana* assay against a range of fungal DNA from both isolates and tissue samples, and wrote the manuscript in collaboration with the co-authors.

1 Introduction

1.1 Christmas trees

1.1.1 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 Christmas tree, O Christmas tree
how lovely are thy branches.
Not only green when summer's here
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 fir and spruce 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 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 (*Picea*) and fir (*Abies*) 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 density, 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.

1.1.2 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 dominating 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 drastically increased, while Scots pine 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* (Pancic) 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 the 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).

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

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. glauca* (Moench) Voss], eastern white pine (*P. strobus* 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. virginiana* Mill.), eastern red cedar (*Juniperus virginiana* L.), Leyland cypress (× *Cupressocyparis leylandii* 'Leighton Green'), 'Carolina Sapphire' Arizona cypress (*Cupressus arizonica* var. *glabra*), 'Blue Ice' Arizona cypress (*Cupressus glabra*), 'Green Giant' arborvitae (*Thuja* L. × 'Green Giant'),

Atlantic white cedar [*Chamaecyparis thyoides* (L.) Mills.] and various spruces (*Picea* spp.).

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)

1.1.3 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 the 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 turnover in Europe is approximately 1.5 billion EUR (1.76 billion USD) (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. For Sweden, no reliable statistics are available for the number of Christmas trees sold, land area cultivated, number of growers, or how many trees are imported annually. However, the consumption for 2016 was roughly estimated at 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 also 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 430 million EUR (506 million USD) (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

In the southern Appalachian Mountains (the mountainous area of North Carolina, Tennessee and Virginia), 5-6 million trees are harvested annually, with a wholesale value of over 100 million USD (Napier & Sidebottom, 2011).

1.1.4 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 wooly aphid (*Dreyfusia nordmannianae* Eckst.), the balsam woolly aphid (*D. piceae* Ratz.), the aphid *Aphrastasia 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, there was very little information 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 (*D. piceae* Ratz.), 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 for Fraser fir production.

For the insect and mite problems, there are pesticides 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.

1.2 *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).

1.2.1 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 towards 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 such as 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 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).

1.2.2 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 inherit new characteristics with the potential to develop into 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 are at risk, especially those that lack immunity (resistance) to *Phytophthora* species.

1.2.3 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 *Phytophthora* species. Limiting the introduction of *Phytophthora* through inspection of imported plants is therefore extremely important.

1.2.4 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% for any given field (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. 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 **Papers I**, **II and III**.

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 is planted in the Piedmont and Coastal Plain regions as an alternative, since 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 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.

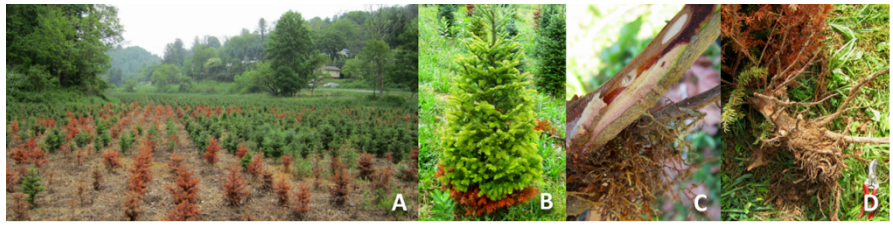

Figure 3. Phytophthora root rot causing losses of Fraser fir in the southern Appalachian Mountains, North Carolina. Characteristic symptoms are tree mortality in the field (A), flagging of basal branches (B), cambial stem lesion with distinct borders between healthy and diseased tissue (C), and heavily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

1.2.5 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 **Paper IV**. 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 a risk for introducing and spreading *Phytophthora* in Sweden.

1.2.6 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 **Paper I**). 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 firs (*A. magnifica var. shastensis* Lemmon) are among the most susceptible species (Frampton & Benson, 2012; Chastagner & Benson, 2000). Less susceptible species are eastern white pine, Nordmann, Turkish and momi firs (*A. firma* Sieb. et Zucc.) (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 the base 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 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 in combination with genetic engineering (**Paper II**). 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.

1.3 *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).

1.3.1 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* is naturally occurring 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).

1.3.2 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 tees (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 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 (**Paper V**).

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

Neonectria neomacrospora is an aggressive pathogen on fir species (*Abies* spp.) 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 (**Paper V**). Currently, there is limited information available about *N. neomacrospora*.

1.3.4 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*.

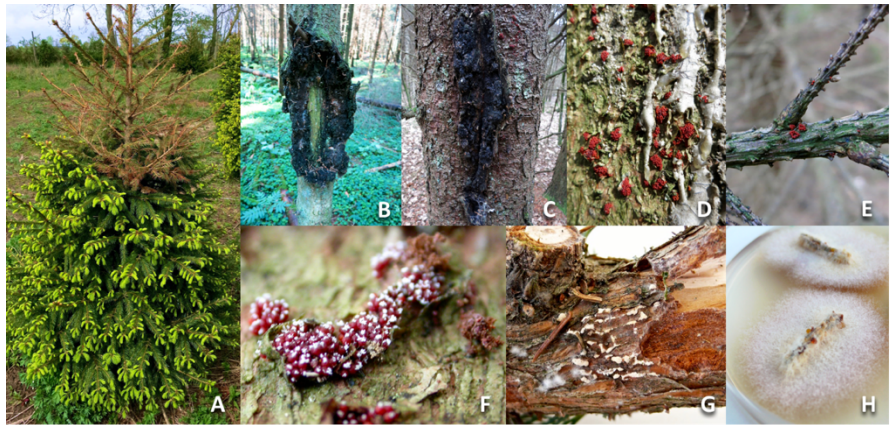

Figure 4. Plant symptoms and pathogen signs of Neonectria canker caused by the fungus *Neonectria fuckeliana*. Norway spruce Christmas tree with top-dieback where the fungus *N. fuckeliana* was isolated from the margin between dead and live tissue (A). Canker wound (B-C), resin flow (D), perithecia (sexual 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

1.4 Scope, aim and research questions

A large amount of knowledge about Christmas tree diseases and pests is now available from other parts of the world, the majority from North America, 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.

As mentioned in 1.2 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 the first project was to investigate which *Phytophthora* species were associated with and contribute to Fraser fir Christmas tree losses in North Carolina (**Paper I**). Such information is important when providing management recommendations to growers in order to restrict and minimize the spread and effect of alien *Phytophthora* species.

Paper II 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.

In the eastern white pine Christmas tree production in the Piedmont and Coastal Plain regions of North Carolina, where Phytophthora root rot caused by *P. cinnamomi* has increased due to contaminated nursery stock, less susceptible eastern white pine seedlings are needed to minimize losses. The aim of **Paper III** was to determine whether there were variations in susceptibility to *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 *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 this thesis 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.

Thus, the main scope of the thesis 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.

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). The aim was therefore to investigate whether *Phytophthora* species were present in or near Swedish Christmas trees fields (**Paper IV**).

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 (**Paper V**). In **Paper VI**, the focus is on Neonectria canker caused by *N. fuckeliana* on Norway spruce, and **Paper VII** 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.

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):

- 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 alien *Phytophthora* species in the future through advanced recommendations to growers (**Paper I**). The **hypothesis** was that several new-to-the-region *Phytophthora* species contribute to the losses in Fraser fir.

- Investigate the history and influence of Phytophthora root rot on Fraser fir planting trends in western North Carolina (**Paper II**).

- Investigate variation of resistance between 83 eastern white pine families to *P. cinnamomi* and demonstrate whether 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 (**Paper III**). 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 (Pettersson *et al*., 2015). This was done to get an overview of the situation and thereby enable the selection of focus areas for the thesis. 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 (**Paper IV**). 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 (**Paper V**). The **hypothesis** was that *N. neomacrospora* and *N. fuckeliana* were present on fir and spruce, respectively, in Swedish Christmas tree plantations.

- Determine the ability of *N. fuckeliana* to cause disease on Norway spruce cuttings (**Paper VI**). More specifically to:
  - 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).
  - 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 (**Paper VII**). The **hypothesis** was that a species-specific primer pair could be found and become a useful tool for detection of *N. fuckeliana*.

2 Materials and Methods

2.1 Study areas

2.1.1 Study areas in North Carolina

The study areas for **Papers I**, **II and III** (Fig. 5) were situated in North Carolina. For the *Phytophthora* species survey (**Paper I**), 103 Christmas tree fields in 14 counties in North Carolina, Tennessee and Virginia (Fig. 6) were sampled in 2014. **Paper II** investigated the history and influence of Phytophthora root rot on Fraser fir planting trends in the southern Appalachian Mountains. For the eastern white pine inoculation (**Paper III**), the experiment was conducted in 2015 and 2016 in an outdoor shade house (40% shade) at the NCSU Horticulture Field Laboratory in Raleigh during the summer and fall, and a greenhouse during the winter.

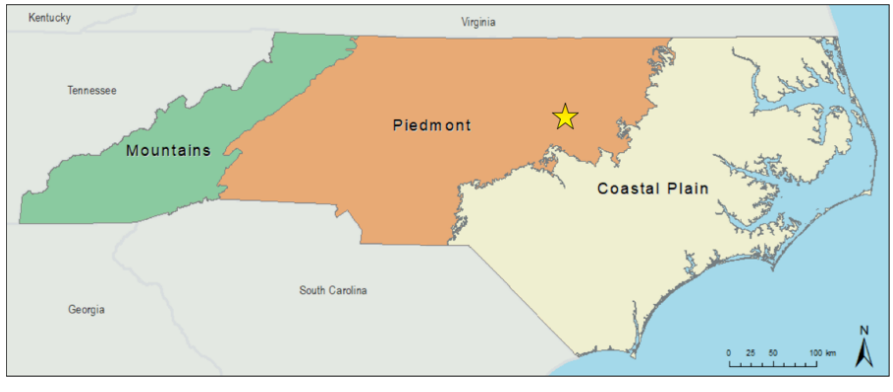

Figure 5. Map of North Carolina. The study area for **Papers I and II** was situated in the southern Appalachian Mountains (green) where the majority of Fraser fir Christmas trees are produced. Eastern white pine is the most common Christmas tree species in the Piedmont (orange) and Coastal Plain regions (beige). The eastern white pine inoculation experiment (**Paper III**) was conducted at the NCSU Horticulture Field Laboratory in Raleigh (star).

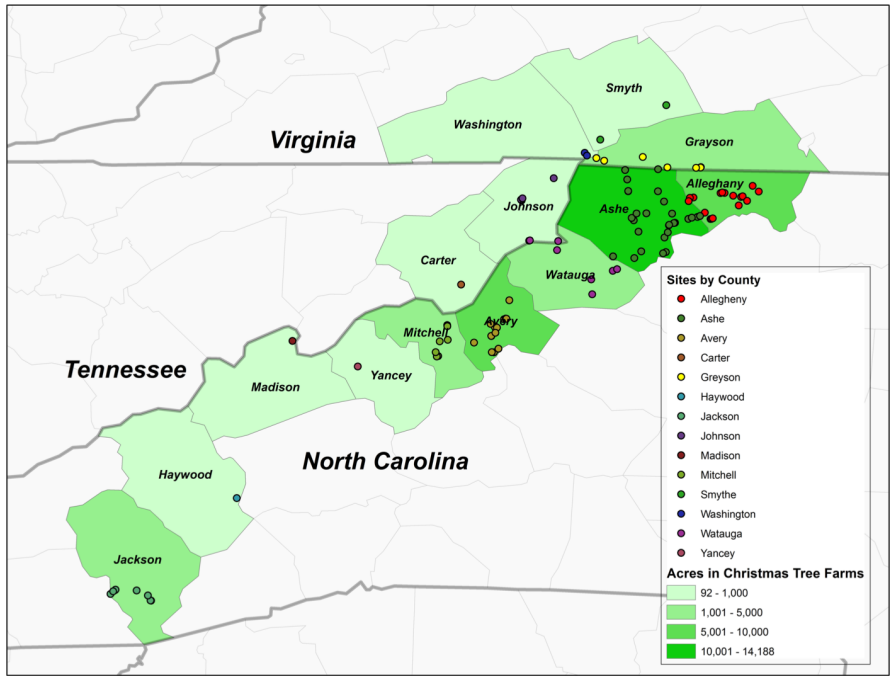

Figure 6. Map showing the locations of Christmas tree fields where Fraser fir trees were displaying Phytophthora root rot symptoms. These sites were sampled in a regional *Phytophthora* species survey in the southern Appalachian Mountains in 2014. In total, 103 field sites (spread over 14 counties according to a weighted distribution based on the proportion of Christmas tree acreage per county) were sampled in North Carolina, Tennessee and Virginia.

2.1.2 Study areas in Sweden

Most Christmas tree production (and almost all fir production) is based in the southern part of the country (Fig. 7). 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 study areas for **Papers IV and V** were in southern Sweden, and experiments for **Paper VI** took place at the Forestry Research Institute of Sweden (Skogforsk) in Svalöv, and **Paper VII** at the Norwegian Institute of Bioeconomy Research (NIBIO) in Ås, Norway.

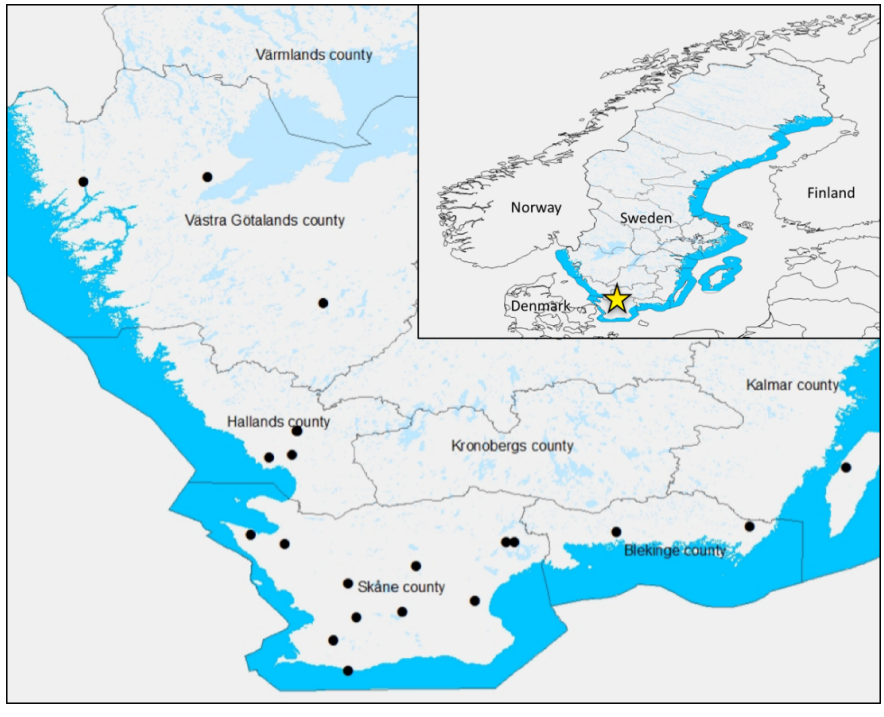

Figure 7. Map of southern Sweden with the 21 Christmas tree farms (indicated as black dots) included in a disease and pest survey in spring 2015. The star on the smaller-scale map of the Nordic countries (upper right hand corner) indicates the area where the majority of Swedish Christmas tree production is based and where the studies were conducted.

2.2 Sampling procedures in the fields in North Carolina and Sweden

At all study locations, samples were taken from Christmas trees with disease symptoms, with GPS coordinates recorded for all sites. For Phytophthora root rot, the symptoms included branch flagging, chlorosis or new growth wilting (Fig. 3). For Neonectria canker, symptoms included top-dieback, canker or resin flow (Fig. 4A-D). Measurements (stem diameter, height, symptom severity, % dieback, etc.) and/or pictures (whole tree, close-up of diseased tissue, etc.) were taken. For *Phytophthora*, tissue samples were collected in the form of roots by uprooting the symptomatic tree, investigating the roots and

excising a part or all of root system (Fig. 8A-B). For *Neonectria,* stem sections including the margin between dead and live tissue were taken. Non-symptomatic trees were sampled as controls. Tissue samples were placed in resealable plastic bags, stored in a cooler and transported to the laboratory to be examined within a week.

In addition to sampling symptomatic trees in Swedish Christmas tree fields, two other methods for detecting *Phytophthora* species were used:

- Detection by baiting with *Rhododendron* 'Cunningham's White' leaves submerged in waterways receiving runoff from the Christmas tree fields (Fig. 8D). The leaves, which are known to be sensitive to *Phytophthora* infection, were placed in mesh bags with styrofoam floaters and anchored to a tree. The bait bag floated near the surface for approximately one week before being retrieved and shipped to the laboratory
- Detection by baiting soil samples for *Phytophthora*. Soil samples from wet areas in several fields were excavated, placed in plastic boxes and transported to the laboratory

2.3 Detection and identification of *Phytophthora* and *Neonectria* species

2.3.1 *Phytophthora*

Detection from roots

Phytophthora species attack the fine roots of a tree first. Therefore, when there were fine roots available, they were sampled. When fine roots were not available, larger roots, including the margin between dead and live tissue, were sampled. In the laboratory, the root samples were all washed clean under running tap water. The roots were also surface-sterilized by dipping them in a 10% bleach solution for 30 seconds followed by rinsing in deionized water. The roots were dried on filter paper and cut with sterile scissors into 5-10 mm segments. Samples of 5-10 fine root segments or individual larger root segments were placed in Petri dishes containing *Phytophthora*-selective media using sterile forceps (Fig. 8C). The media used were V8 juice agar (V8A), clarified V8 juice agar (cV8A), corn meal agar (CMA) and potato dextrose agar (PDA) enhanced with selective chemicals such as pimaricin, ampicillin, rifampicin, pentachloronitrobenzene (PCNB) and hymexazol (PARPH) (Telfer *et al.*, 2015; Jeffers, 2006). The PARPH media is specially designed to allow *Phytophthora* species to grow while inhibiting bacteria by using ampicillin and rifampicin (antibiotics), soil-borne fungi by using pimaricin (antibiotic) and

PCNB (fungicide), and soil-borne *Pythium* species by using hymexazol (fungicide).

Detection from Rhododendron leaf baits

Rhododendron leaf baits from waterways with dark or water-soaked lesions were washed under running tap water (Fig. 8F-I). The leaves were then sectioned into approximately 1 cm^2 pieces with a scalpel, capturing the margin between dead and live tissue. Three to five leaf sections were plated onto PARPH media using the same method as for the roots.

Detection from soil samples

Deionized water was added to the plastic boxes the soil had been collected in from the fields until the soil was completely submerged. The soil and water were then mixed and left over night until the soil had settled to the bottom and the water was clear. Five *Rhododendron* leaves were placed on the water surface to bait for possible *Phytophthora* zoospores (Fig. 8E). After approximately one week, leaves displaying dark water-soaked lesions were investigated as described above for the presence of *Phytophthora*.

PARPH plates containing plant tissue were incubated in a dark cabinet at room temperature (~20°C) for up to one week, and examined daily using an inverted microscope for the presence of mycelium resembling *Phytophthora*. Mycelium of *Phytophthora* species is often irregular in size and shape, generally slow growing compared to *Pythium* species, and has coenocytic hyphae (hyphae without septa). Mycelium resembling *Phytophthora* species was transferred to new culture plates. The transfer was done under a sterile hood, where small plugs with single hypha from the edge of the cultures were cut using a sterile scalpel. If the first plate was contaminated with bacteria, it was transferred to selective media; if there was no contamination, it was transferred to unamended media such as PDA or V8A.

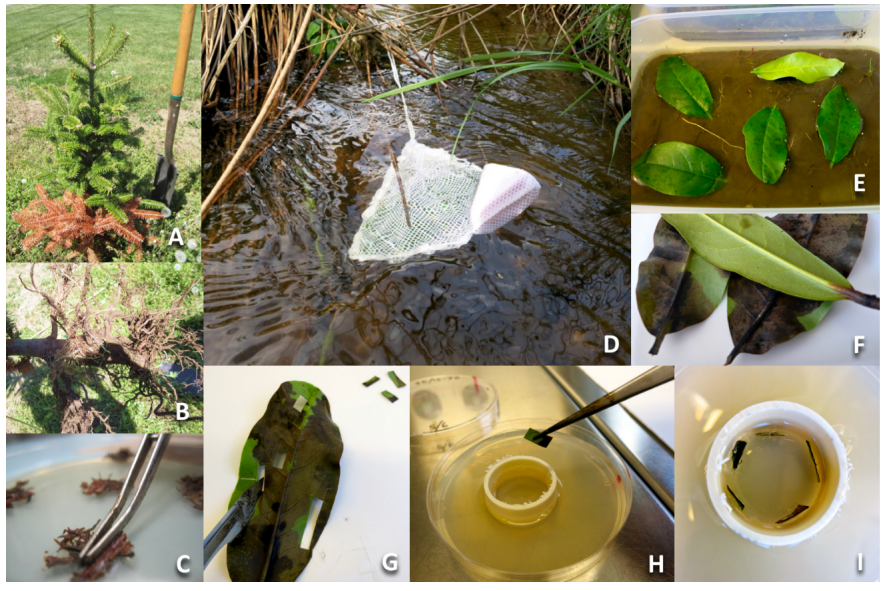

Figure 8. Isolation and detection of *Phytophthora* from roots, soil samples and *Rhododendron* leaf baits from waterways. Roots taken from a symptomatic Fraser fir plant were washed clean under running tap water and surface-sterilized before plating out onto *Phytophthora* selective agar plates (A-C). *Rhododendron* leaf baits inside a net bag floating in water (D). Soil sample from the field baited with *Rhododendron* leaves (E). *Rhododendron* leaf baits with dark patches indicating *Phytophthora* infections, washed clean under running tap water before being plated onto *Phytophthora* selective agar plates (F-I). Photos: Martin Pettersson

2.3.2 *Neonectria*

Detection by isolation

The common method used to isolate *Neonectria* from diseased trees was to dissect wood samples that included the margin between dead and live tissue. In the laboratory, the wood samples were submerged for 10 seconds in 70% ethanol and 90 seconds in a 0.5% sodium hypochlorite (NaOCl) bleach bath to kill microorganisms living on the surface. After surface sterilization, wood samples were sectioned into smaller pieces with a sterile scalpel or plated directly onto PDA. This enabled the fungi inside the wood to grow out onto the medium, without having to compete with surface microorganisms. However, there can still be other fungi living inside the wood and contamination by secondary fungi is therefore common. There is no selective media available for *Neonectria* species. The cultures were checked daily for seven days for mycelium outgrowth resembling *Neonectria*.

Detection by incubation

Neonectria was also detected by incubating stem sections (10-20 cm) containing the margin between dead and live tissue in moist chambers. The samples were surface-sterilized and larger stem sections were split in half before incubation. A wet sheet of paper was placed on netting at the bottom of the moist chambers to create high humidity and stimulate fungal growth. Depending on the length of incubation, this method yielded either *Neonectria* mycelium with microconidia emerging from the wood, sporodochia (asexual fruiting structures) or early development of perithecia (sexual fruiting bodies). Mycelium with conidia often emerged within 14 days, whereas perithecia development took more than a month. The samples were examined under a dissection microscope. Mycelium with conidia were transferred using a sterile needle from the wood sample to PDA supplemented with 0.5 mg/ml of streptomycin sulfate (PDAS) to supress bacterial growth.

2.3.3 Morphological identification

The isolation of *Phytophthora* and *Neonectria* species on growth medium was followed by morphological identification. The mycelial outgrowth was compared with reference cultures or pictures of *Phytophthora* or *Neonectria* species.

For *Phytophthora*, the colony morphology was assessed on V8A, cV8A or PDA plates incubated at room temperature (~20°C) for 1-2 weeks. Other morphological features, e.g. sporangia and oospores, were also compared to species descriptions and illustrations in databases such as Q-bank (www.q-bank.eu), and in the literature, such as Gallegly and Hong (2008); Erwin and Ribeiro (1996). Temperature-growth relationships for three *Phytophthora* species detected in Sweden (**Paper IV**) were investigated by measuring radial growth rates in millimeters per day on PDA at 6 different temperatures: 5, 10, 15, 20, 25, 30, and 35°C.

Neonectria fuckeliana and *N. neomacrospora* have culture morphologies that are easy to recognize (see Figure 3 in **Paper V**) (Booth & Samuels, 1981; Booth, 1979). However, isolation of *N. fuckeliana* from infected plant tissue proved to be difficult. This was due to fast-growing secondary fungi emerging from the wood samples. It was hard to isolate *N. fuckeliana*, even when plants were inoculated and showed *N. fuckeliana* disease symptoms. Therefore, several attempts on the same diseased plant were sometimes necessary to isolate the fungus. This was difficult with small plants, and time-consuming. It was not always possible to obtain clean cultures due to competing fungi and/or bacteria. Temperature-growth relationships for the two *Neonectria* species

(**Paper V**) were studied in the same manner as described for *Phytophthora* above.

2.3.4 Molecular identification

Background

In the 1990s, nucleic acid-based detection methods, such as end-point polymerase chain reaction (PCR) assays, became a complement to morphological identification (Gardes & Bruns, 1993). PCR assays revolutionized diagnostics of plant pathogens by providing rapid and accurate detection of many species, including pathogens that could not be cultured (e.g. rust fungi). This DNA barcoding of fungal species uses primers that bind to specific sites on the DNA that are commonly present in most fungi. However, the regions between the primers are highly variable between different species, i.e. they contain unique DNA sequences for each species. One such region is the internal transcribed spacer (ITS) region containing ribosomal DNA (rDNA). Sequences of 500- to 800-base pairs (bp) are produced using different primers. The standard primers used by most labs are ITS1 and ITS4. The ITS region is the universal DNA barcode marker for fungi (Schoch *et al.*, 2012). Species-specific primer PCR assays for certain fungi are developed for faster identification directly from infected plant tissue, which excludes morphological identification.

Today, more powerful new molecular technologies, such as quantitative real-time PCR assays, have been developed and are currently replacing the conventional end-point PCR. Real-time PCR is faster and more sensitive and, most importantly, can quantify the amount of a pathogen (Schaad & Frederick, 2002). One of the most widely used real-time PCR methods is the TaqMan system. In a TaqMan real-time PCR assay, a sequence-specific oligonucleotide probe labelled with a fluorescent reporter is used. Detection only occurs if the complementary target sequence is present in the DNA of the pathogen, then a fluorescence signal is emitted and it is quantified by the real-timer PCR machine. For many important plant pathogens, such as *Phytophthora sojae* and *Fusarium oxysporum*, species-specific TaqMan assays have been developed (Catal et al., 2013; Haegi et al., 2013) and are commonly used for diagnostics in disease clinics and research laboratories around the world.

The species-specific real-time PCR-based test presented in **Paper VII** was developed because we wanted a rapid, reliable and sensitive method for identification of *N. fuckeliana* causing canker disease on Norway spruce trees.

Phytophthora

Mycelia from approximately one-week-old pure cultures resembling *Phytophthora* were scraped off the agar with a sterile scalpel. The cells of the

mycelia were then lysed in liquid nitrogen using a mortar and pestle. Approximately 0.1 gram of wet ground tissue was transferred into 2 ml safe-lock microcentrifuge tubes, and DNA was extracted from the tissue using DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions.

Amplification of the internal transcribed spacer region of nuclear rDNA was performed using the universal forward primers ITS1, ITS5 or ITS6 and reverse primer ITS4 (Grünwald *et al.*, 2013; White *et al.*, 1990). Other loci used for identification of some isolates from North Carolina (**Paper I**) were the β-tubulin gene with TubuF2 and TubuR1 primers, and the mitochondrial gene, cytochrome c oxidase subunit 1 (COI) with COIF-1 and COIR-1 primers. Both of these genes are regions of the fungal DNA with large variations between species, but the primers bind to flanking regions found in most fungal species.

The amplification conditions for the PCR thermocycler for ITS, β-tubulin and COI were modified from White *et al.* (1990), Kroon *et al.* (2004) and Robideau *et al.* (2011), respectively (see **Papers I and IV**). The amplified DNA products were then examined using gel electrophoresis in agarose gel to ensure correct DNA amplification. When DNA amplification was successful, a single band of DNA was present in the gel.

For **Paper I**, the majority of amplified DNA was sent to the NCSU Genomic Sciences Laboratory for Sanger sequencing. However, more information was required on five different isolates so a small portion was also sent to GENEWIZ (South Plainfield, New Jersey). For **Paper IV**, the PCR products were sent to GATC in Germany for sequencing. All of the laboratories returned the Sanger sequences by e-mail. The sequences were then examined with the software Geneious version 8.1.4, software for molecular biology and next-generation sequencing (NGS) analysis (Kearse *et al.*, 2012). The raw sequences were trimmed, assembled and manually controlled for errors, and the sequences were compared with reference sequences in GenBank using the basic local alignment search tool (BLAST). This gives a comparison of how similar our sequence is to other sequences posted online. If the sequence was at least 99% similar to a certain *Phytophthora* species, and much less similar to all other species in the database, it was assumed to be correctly identified to the species level. Once the species level was confirmed, the sequence was given an accession number and deposited in GenBank.

Neonectria

After disease symptoms were identified, the pathogen isolated onto PDA and the morphological examination conducted, the molecular detection method for *Neonectria* was the same as for *Phytophthora* described above, i.e. the ITS region was sequenced and a BLAST search was conducted in GenBank. As

noted, however, isolation of *N. fuckeliana* from spruce proved difficult due to secondary fungi, limiting the morphological and molecular identifications.

Species-specific PCR assay for N. fuckeliana

In the past, several species-specific PCR assays were designed and used for *N. ditissima* (Ghasemkhani *et al.*, 2016; Langrell, 2002; Langrell & Barbara, 2001) and one for *N. fuckeliana* (Langrell, 2005), the majority of which were end-point PCR assays, including the one for *N. fuckeliana*. While the application of end-point PCR assay is more efficient compared to identification by culturing and morphological detection, end-point PCR assays are less sensitive than real-time PCR and cannot quantify the amount of pathogen.

In **Paper VII**, the work presented was conducted to develop a species-specific TaqMan real-time PCR assay for rapid identification of *N. fuckeliana*. This assay identifies the fungus directly from infected plant tissue. A species-specific primer pair and probe were designed for *N. fuckeliana* from the ITS-1 region. The primer pair selected was based on multiple-sequence alignments of *N. fuckeliana*, other *Neonectria* species (such as the closely related *N. ditissima* and *N. neomacrospora*) and fungi from other genera (such as *Nectria cinnabarina*) obtained from GenBank (see Table 2 in **Paper VII**). The *N. fuckeliana* assay was validated by testing *N. fuckeliana* DNA. The species-specificity for the assay was then verified using DNA from different *Neonectria* species and closely related fungi from other genera as non-target controls. Testing was done using tissue from cultures (see Table 1 in **Paper VII**), perithecia and infected plant material. Sterile MilliQ water and DNA from disease-free host tissues were used as negative controls. The detection limit for the TaqMan assay was determined through a six 1:10 dilution series, and a standard curve analysis was conducted.

2.4 Inoculation tests

Inoculation tests were included in **Papers I, III, IV, V and VI**. Mycelium and/or spores of the pathogens cultured on agar were used to inoculate artificially wounded and/or non-wounded seedlings, and inoculation tests conducted to obtain information about pathogenicity. Information about the sensitivity of the host plant to the pathogen was obtained. Duration of the inoculation experiments varied depending on how quickly symptoms developed.

Inoculation tests were conducted to meet Koch's proof of pathogenicity (Koch's postulates). Koch's postulates are four criteria that must be fulfilled in order to establish a causal relationship between a microorganism and a disease. The pathogen must be:

1. Associated consistently with the occurrence of the disease
2. Isolated from diseased tissue into pure culture
3. Inoculated onto a healthy plant of the same species and reproduce the same symptoms of disease
4. Reisolated from the diseased tissue of the inoculated plant into pure culture

Koch's postulates are used when:

- A new pathogen is suspected to cause a disease on a host plant
- A known pathogen is described on a new host
- A known pathogen is found on a known host in a new environment

Koch's postulates were used in the work presented in **Paper I** to demonstrate pathogenicity of *P. citrophthora* (R.E. Sm. & E.H. Sm.) Leonian on Fraser fir. *Phytophthora citrophthora* that had colonized rice grains were used to inoculate seedling roots according to Frampton and Benson (2012); Benson *et al.* (1997).

For the work presented in **Paper IV**, both the rice grain inoculation method presented in **Paper I** and a mycelium plug inoculation method of stem and branch wounds were used. Both methods were used to demonstrate pathogenicity of three *Phytophthora* species found in association with Swedish Christmas tree plantations. The stem/branch wound was made by removing one needle and placing a mycelial plug onto the needle scar. A wet cotton pad was used to cover the plug and ParaFilm was wrapped around the inoculation point to keep the plug in position.

In **Paper V**, the use of Koch's postulates to demonstrate the pathogenicity of two *Neonectria* species (*N. fuckeliana* and *N. neomacrospora*) was described. The two pathogens had been isolated in Sweden from Norway spruce and Nordmann fir Christmas trees, respectively. *Neonectria fuckeliana* and *N. neomacrospora* were inoculated onto their respective host plants. Map pins were used to scrape mycelia off PDA plates and the contaminated map pins were inserted into stems and branches of the seedlings as described by Talgø and Stensvand (2013).

Inoculation tests are also used to assess the aggressiveness of plant pathogens or the susceptibility of hosts. **Paper III** reports on the results of an inoculation study investigating the resistance of eastern white pine families to *P. cinnamomi*. The long-term goal here was to reduce losses due to Phytophthora root rot through planting more *Phytophthora*-resistant eastern white pine families. As mentioned in the specific objectives, 83 eastern white pine families were inoculated using *P. cinnamomi* isolate (23ss04) originally derived from Fraser fir. This isolate had been used in several studies (Frampton *et al.*, 2013; Frampton & Benson, 2012), and results from the work presented

in **Paper III** could therefore be compared to results obtained in previous tests. In a supplementary study using 20 of the 83 eastern white pine families, another *P. cinnamomi* isolate (2334) originally derived from eastern white pine was inoculated using the rice grain method described above. This was done to determine possible differences in aggressiveness between an isolate from Fraser fir and an isolate from eastern white pine.

In **Paper VI**, several different inoculation tests were presented. These included:

- A pilot study examining the usefulness of microconidia as an inoculation source for *N. fuckeliana* on Norway spruce seedlings
- A larger greenhouse inoculation study examining the pathogenicity/aggressiveness of *N. fuckeliana* on actively growing versus dormant Norway spruce plants, as well as the differences between different wound types
- A field study done on 7-year-old Norway spruce plants investigating whether stem inoculation would result in similar top-dieback symptoms as previously observed on Norway spruce in Finnish and Norwegian forest stands and in Swedish Christmas tree fields

For these inoculation tests, three Swedish isolates of *N. fuckeliana* were used. Here, microconidia were added to artificially created wounds, except for the field study where mycelial plugs were used as inoculum.

3 Results and discussion

3.1 Phytophthora root rot in Christmas tree fields in North Carolina (**Papers I, II and III**)

In the study presented in **Paper I**, where the specific objective was to identify the *Phytophthora* species associated with symptomatic and diseased Fraser fir Christmas trees sampled in the southern Appalachian Mountains, *Phytophthora* was recovered from the roots of about 54% of the symptomatic Fraser fir trees (167 of 309 trees) and 9.4% of the non-symptomatic control trees sampled (3 of 32 trees). This indicates that we were either not very effective in obtaining isolates from symptomatic trees or that the disease symptoms were due to other factors such as drought or other diseases. It also tells us that many other non-symptomatic trees were likely damaged by Phytophthora root rot.

Based on morphology and molecular identification, six different *Phytophthora* species were isolated and identified: *P. cinnamomi*, *P. cryptogea*, *P. pini* Leonian, *P. sansomeana* E.M. Hansen & Reeser, *P. europaea* E.M. Hansen & T. Jung, and *P. citrophthora*. This represents an increase in the number of species previously found in surveys done on Fraser fir Christmas tree fields in the southern Appalachian Mountains. Previous surveys, almost exclusively found *P. cinnamomi* responsible for the Phytophthora root rot (Benson & Grand, 2000; Grand & Lapp, 1974). In our survey, *P. cinnamomi* did account for most of the isolates retained, but the proportion (70.3%) was lower than in previous studies. *Phytophthora cryptogea* was found to be the second most commonly appearing *Phytophthora* species (23.1%). It is known as an aggressive pathogen on fir species (McKeever & Chastagner, 2016; Grand & Lapp, 1974), and causes Phytophthora root rot on Christmas trees in the states of Washington, Oregon, Wisconsin, New York, Connecticut and Pennsylvania (McKeever & Chastagner, 2016; Hoover & Bates, 2013; Chastagner *et al.*, 1995; Pratt *et al.*,

1976). This corresponds well with our findings where *P. cryptogea*, in addition to *P. cinnamomi*, contributed to Fraser fir losses.

Also other *Phytophthora* species we detected in North Carolina, i.e. *Phytophthora pini* (1.1%), *P. sansomeana* (1.1%) and *P. europaea* (2.2%), are known to cause Phytophthora root rot in states far away from the southern Appalachian Mountains (McKeever & Chastagner, 2016; Chastagner & Benson, 2000; Chastagner *et al.*, 1995; McCain & Scharpf, 1986). *Phytophthora europaea* has been associated mostly with oak trees (*Quercus* spp.) (Balci *et al.*, 2007; Balci *et al.*, 2006), and it is unusual to find it on fir species.

Phytophthora citrophthora (2.2%) had never been isolated from roots of Fraser fir before and proved pathogenic on this host. Approximately 50% of the inoculated Fraser fir seedlings died within three months. It is not as aggressive as *P. cinnamomi,* however, which killed 100% of the inoculated plants in three months. Nevertheless, *P. citrophthora* can clearly contribute to losses of Fraser fir.

Three of the *Phytophthora* species (*P. europaea*, *P. citrophthora*, and *P. sansomeana*) were new to the region. We could therefore confirm our hypothesis that the diversity of *Phytophthora* species that contribute to the loss of Fraser fir in the southern Appalachian Mountains has increased. We were also surprised by the amount of *P. cryptogea* detected. However, there was no evidence that these *Phytophthora* species, together with *P. cryptogea*, could be attributed to the import of seedlings from out-of-state nurseries. In order to establish such a link, seedling samples would need to be tested before they are planted.

In **Paper II**, where the specific objective was to investigate the history of Phytophthora root rot on planting trends of Fraser fir in western North Carolina, results from a questionnaire completed by 89 local Christmas tree growers revealed that by 2013, 64% of the growers had shifted from using locally produced bare-root seedlings to out-of-state-grown planting stock. Growers with more land planted, were more likely to have shifted to imported seedlings than small-scale growers. Approximately 80% of the land included in the survey was thus planted with out-of-state planting stock in 2013, and approximately 88% of the growers reported that they had Phytophthora root rot causing mortality in their Christmas tree fields. Hence, the survey showed that Phytophthora root rot continues to have a major impact in Fraser fir plantations.

In **Paper III**, where the objective was to investigate the variation of resistance to *P. cinnamomi* among 83 eastern white pine families and to determine the

degree to which resistance was controlled by genetics, the mortality of seedlings among the inoculated families ranged from 13-81%, thus confirming our hypothesis that there is large variation in susceptibility to *Phytophthora* between different eastern white pine families. Furthermore, there was a relatively high degree of genetic control of disease resistance as the family mean heritability (h_f^2), in 2015, was estimated at 0.85 (i.e. the difference in resistance was 85% attributable to family genetic differences). Mortality began at week six and slowly increased over time. This was radically different from experiments using the same *P. cinnamomi* isolate (23ss04) to inoculate Fraser fir, where 100% mortality was observed after 16 weeks (Frampton & Benson, 2012). After 16 weeks, only 19% mortality was observed in eastern white pine. It therefore seems that eastern white pine is moderately resistant to *P. cinnamomi*.

Another objective of the study was to test for differences in aggressiveness between one *P. cinnamomi* isolate (23ss04) derived from Fraser fir and one isolate (2334) derived from eastern white pine. There was a difference in disease mortality between the 20 families inoculated with *P. cinnamomi* isolate no. 2334 in the supplemental study and isolate no. 23ss04 used in the main study. At 16 weeks, 40% mortality was observed for *P. cinnamomi* isolate no. 2334 compared to 26% for isolate no. 23ss04. At 69 weeks after the first inoculation, 58% mortality was observed for *P. cinnamomi* isolate no. 2334 compared to 52% for isolate no. 23ss04. The higher mortality for *P. cinnamomi* isolate no. 2334 (isolated from eastern white pine) suggests that this isolate has evolved to become a more aggressive pathogen on eastern white pine. This demonstrates the adaptability of *P. cinnamomi* to infect different hosts.

Results from this study confirm that a good management tactic for Christmas tree growers is to plant more resistant families of eastern white pine as an alternative to Fraser fir on *Phytophthora*-infested land. However, our results are not in line with an earlier study by Kirby and Grand (1975). In their experiments, 87% of the eastern white pine seedlings died 21.4 weeks after *P. cinnamomi* inoculation. Seedlings grown in saturated soil died faster than seedlings in a non-saturated soil, leading them to conclude that eastern white pine was not suitable as an alternative species to Fraser fir on wet sites. One reason why our results are not completely in line with Kirby and Grand is likely due to the amount of inoculum used. Kirby and Grand (1975) used two different inoculation techniques, both of which are likely more infectious than placing, in total, eight *P. cinnamomi* infested rice grains close to the plants (**Paper III**). One of the techniques Kirby and Grand (1975) used was to mix 40-50 ml of *P. cinnamomi*-infested oat grains into the soil of the eastern white pine pots. The other technique was to dip the root system of the seedlings into

a suspension with high concentration of hyphal fragments, sporangia, and chlamydospores.

3.2 Diseases, pests and nutrient deficiencies of Swedish Christmas trees (**2015 disease survey**)

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 seem to be the largest potential 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 **Paper V**. *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 (**Paper V and VI**). 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 (**Paper IV**). 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. 9A-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. 9C). The fungus has a two-year life cycle where basidio-spores from the bird cherry attack young spruce shoots and cause them to bend. If the alternative host (bird cherry) is removed, the fungus cannot complete its life cycle.

- **Chrysomyxa needle rust** [*Chrysomyxa abietis* (Wallr.) Unger] attacks needles on new shoots on 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. 9D-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. 9F). 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. 9G-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 and a distinctive black band around the base of infected needles can be seen with the naked eye (Fig. 9I-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. 9K). 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. 9L-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 in the text.

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

Damaging agent	**Host**
Pathogen (disease)	
Armillaria spp. (Armillaria root rot)	*A. nordmanniana*, *P. pungens*
Camarosporium sp.	*P. pungens*
Chrysomyxa abietis (Chrysomyxa needle rust)	*P. abies, P. pungens*
Delphinella abietis (Delphinella shoot blight)	*A. nordmanniana*
Gemmamyces piceae (Gemmamyces bud blight)	*P. pungens*
Herpotrichia juniperi (black snow mould)	*P. pungens*
Lirula macrospora (Lirula needlecast)	*P. abies, P. pungens*
Lophoderminum piceae (Lophodermium needle cast)	*P. pungens*
Neonectria fuckeliana (Neonectria canker)	*P. abies*
Neonectria neomacrospora (Neonectria canker)	*A. nordmanniana*
Phytophthora spp. (Phytophthora root rot)	*P. abies* (soil and water)
Rhizosphaera kalkhoffii (Rhizosphaera needles cast)	*P. pungens*
Sirococcus strobilinus (Sirococcus blight)	*P. pungens*
Sydowia polyspora (Current season needle necrosis)	*A. nordmanniana, A. procera*
Sydowia polyspora (Sclerophoma shoot dieback)	*A. nordmanniana, P. abies*
Thekopsora areolata (Cherry spruce rust)	*P. abies*
Insect and mite (pest)	
Adelges abietis (pineapple gall woolly aphid)	*P. abies*
Adelges viridis (spruce pineapple gall woolly aphid)	*P. abies*
Aphrastasia pectinatae	*A. nordmanniana*
Dreyfusia nordmannianae (silver fir woolly aphid)	*A. nordmanniana*
Dreyfusia piceae (balsam woolly aphid)	*A. nordmanniana*
Nalepella species (gall mites)	*A. nordmanniana*
Nutrient deficiencies + wildlife damage	
Magnesium (Mg) deficiency	*A. nordmanniana*
Manganese (Mn) deficiency	*A. nordmanniana*
Damage by wildlife (deer, vole, etc.)	*A. nordmanniana, P. abies, P. pungens*

Figure 9. 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

3.3 Phytophthora root rot in Christmas tree fields in Sweden (**Paper IV**)

Paper IV presents the *Phytophthora* survey, where three symptomatic seedlings, nine soil samples and 30 bait samples were collected from 14 Swedish Christmas tree farms. From the material collected, 26 *Phytophthora* isolates were obtained from one symptomatic seedling, seven soil samples and 18 baits. Five known *Phytophthora* species were identified: *P. cryptogea*, *P. gonapodyides* (H.E. Petersen) Buisman, *P. lacustris* Brasier, Cacciola, Nechw., T. Jung & Bakonyi, *P. megasperma* and *P. plurivora* T. Jung & T.I. Burgess; as well as one unknown *Phytophthora* species (the ITS and COI sequences of

which were most similar to *P. inundata* and *P. humicola*, respectively). Of these species, *P. gonapodyides*, *P. lacustris*, *P. megasperma* and *P. plurivora* were present in several locations. Therefore, the hypothesis that several *Phytophthora* species are present in Swedish Christmas tree plantations was confirmed. This poses a potential risk to Swedish Christmas tree production, as well as forestry and agricultural production. *Phytophthora cryptogea*, *P. megasperma* and *P. plurivora* pose the greatest threat, since they are known to cause damage and mortality to trees and other plants of economic and ecological value. *P. cryptogea*, for example, is an aggressive, soil-borne pathogen of fir species used as Christmas trees (Shafizadeh & Kavanagh, 2005; Chastagner & Benson, 2000; Chastagner *et al.*, 1990; Hamm & Hansen, 1982). It is also pathogenic to a wide range of vegetable crops (Larsson & Gerhardson, 1992; Larsson & Gerhardson, 1990).

In the pathogenicity test of *P. cryptogea*, *P. megasperma*, and *P. plurivora* on Norway spruce and Nordmann fir seedlings, we were able to reisolate all three species from both tree species. However, no extensive root rot or dieback occurred under our experimental conditions. Only the inoculation with *P. cryptogea* and *P. megasperma* resulted in minor symptoms. These symptoms included reduced number of fine roots following inoculation with colonized rice grains, and canker formation or branch dieback after mycelium plug inoculation of stem and branches.

P. megasperma was the only *Phytophthora* isolated directly from the roots of a symptomatic Norway spruce seedling under field conditions. None of the other *Phytophthora* isolations were directly connected to the Christmas trees in the field. If the *Phytophthora* species are not highly aggressive on Nordmann fir and Norway spruce, they may not greatly affect Christmas tree production as the rotation times are short compared to forest production. However, it may have a larger effect on forest production. This is because soil-borne *Phytophthora* species attack the fine roots of trees, reducing tree growth and increasing tree stress. This results in a higher susceptibility to other diseases and stressful weather conditions such as drought.

It is evident from the literature that international seedling trade is a major pathway for introducing invasive *Phytophthora* species (Jung *et al.*, 2016). Since most of the Christmas tree planting stock of fir and spruce in Sweden is imported from European nurseries, the risk of introducing new and more aggressive *Phytophthora* species is high. In Norway, the Christmas tree industry has expanded rapidly and relies in part on seedlings from European tree nurseries. High mortality of newly imported bare-root seedlings due to *Phytophthora* was reported from Norway (Talgø *et al.*, 2007). Swedish agricultural and forestry authorities need be aware of this risk, and help to

prevent more *Phytophthora* species from becoming established in Swedish Christmas tree fields. Once *Phytophthora* has been introduced, it is almost impossible to eradicate.

3.4 Neonectria canker in Christmas tree fields in Sweden (**Paper V**)

As seen in **Paper V**, the survey of 21 Christmas tree farms in southern Sweden (Fig. 7) resulted in isolation of *N. fuckeliana* and *N. neomacrospora* from spruce and fir, respectively. This confirmed the hypothesis that both Neonectria species were present in Swedish Christmas tree fields, similar to the situation in Norway and Denmark. Visual inspection of the fields found Norway spruce trees with top-dieback (up to three-four top branch whorls diseased and dead) (Fig. 4A) on 12 out of the 21 farms. The trees with top-dieback commonly had a scattered distribution in the fields. *Neonectria fuckeliana* was identified by isolation or incubation in 7 of the 12 samples. Dead shoots were also found on eight Nordmann and noble fir trees, where *N. neomacrospora* was identified through isolation in three samples.

Koch's postulates were demonstrated for *N. fuckeliana* on spruce and *N. neomacrospora* on fir. All inoculated plants developed canker wounds resulting in dead shoots and top-dieback similar to what was observed in the fields. Both species could be reisolated. The control plants remained healthy.

N. fuckeliana commonly occurs on Norway spruce in Europe, where it has been regarded as a weak pathogen (Vasiliauskas & Stenlid, 1998; Vasiliauskas & Stenlid, 1997; Vasiliauskas *et al.*, 1996; Roll-Hansen & Roll-Hansen, 1979; Bazzigher, 1973). However, more recent studies show severe damage of Norway spruce in Finland (Uimari *et al.*, 2018; Lilja *et al.*, 2012). Similar damage has also been reported in Norway and Denmark (Thomsen *et al.*, 2016; Talgø *et al.*, 2015a). The findings from Sweden are in line with the latter. *N. fuckeliana* should therefore be viewed as a potential threat to Norway spruce production also in forest sites (Uimari *et al.*, 2018; Lilja *et al.*, 2012).

A similar situation exists for *N. neomacrospora*. Older studies have shown that *N. neomacrospora* causes damage to fir species in both North America and Norway (Booth, 1979). Epidemic outbreaks, however, had not been reported until recently. Since 2008, *N. neomacrospora* has caused epidemic outbreaks and mortality, and has been isolated from approximately 20 fir species in Norway, Denmark, Belgium, the UK and US (Nielsen *et al.*, 2017; Schmitz *et al.*, 2017; Skulason *et al.*, 2017; Pérez-Sierra *et al.*, 2016; Chastagner *et al.*, 2014). No epidemic outbreak was found in the Swedish fir Christmas tree

plantations, but the distribution and development of *N. neomacrospora* in Sweden needs to be followed closely.

3.5 Pathogenicity of *Neonectria fuckeliana* on Norway spruce clones in Sweden and potential management strategies (**Paper VI**)

In **Paper VI**, the main objective was to determine the ability of *N. fuckeliana* to cause disease on Norway spruce cuttings. Additional objectives were to determine how different types of wounds impact the occurrence and severity of *N. fuckeliana* infections, to describe symptom development of *N. fuckeliana* infections on the cuttings, and to determine whether symptom development correlates with field observations.

The pilot study examining the usefulness of microconidia as an inoculation source for Norway spruce seedlings proved that microconidia functions as inoculum. After two months, all the inoculated seedlings displayed swollen, non-healing and spreading cambium wounds with resin flow. From each inoculated seedling, *N. fuckeliana* could be isolated onto PDA. It seems likely that microconidia can be successfully used as an inoculant. This is beneficial as harvesting ascospores from perithecia is more difficult and time-consuming than harvesting the microconidia. Although this pilot study used a 15 µl (one large drop) of undiluted microconidial solution (a high dose of microconidia), it is likely that a diluted standardized microconidial concentration would be a good tool for conducting inoculation studies.

For the greenhouse inoculation study, it was evident that *N. fuckeliana* infections had taken place during both active and dormant growth stages. There was not a large difference in lesion length under bark between active and dormant cuttings for each of the wound treatments. However, the fungus was more frequently reisolated and molecularly detected from actively growing cuttings. The reason for this is unclear. All cuttings received the same spore concentration adjusted to approximately 5 x 10^5 conidia/ml (5000 spores per 10 µl). It could be due to the fungus colonizing softer juvenile tissue of the actively growing cuttings more easily. However, actively growing trees have a more immediate defense reaction to wounding compared to dormant trees (Gärtner & Heinrich, 2009). The results with regard to reisolation were nevertheless unexpected, as it was assumed that dormant seedlings would have been easier to colonize and infect by the fungus.

When compared to the removed-needle treatment (small wounds) and the non-wounded treatment, inoculated seedlings that received the shoot-topped and shoot-wounded treatments (large wounds) had increased:

- number of lesion lengths that were measurable
- lesion lengths under the bark
- number of reisolates on PDA
- number of detections by the TaqMan assay

As none of the non-wounded cuttings developed symptoms and the fungus could not be reisolated, it is clear from this greenhouse inoculation study that *N. fuckeliana* requires an open wound as an entry point. The shoot-topped and shoot-wounded treatments exposed the cambium and sapwood, whereas the removed-needle treatment only provided an opening through the bark, exposing the green cambium. The results were as expected, since *N. fuckeliana* has been described as a pathogen that infects trees through pruning wounds, dead branches, branch stubs or cracks due to wind or frost (Crane *et al.*, 2009; Roll-Hansen & Roll-Hansen, 1979; Vasiliauskas & Stenlid, 1997)

The greenhouse experiment also shows, however, that the small and shallow wounds from removing needles constituted a large enough entry point for *N. fuckeliana* infection, and the pathogen was reisolated after 8-10 months. However, symptoms of disease did not appear within this time-frame.

The wounds on the control cuttings inoculated with sterile water had healed smoothly. *Neonectria fuckeliana* could not be detected on PDA or by the TaqMan assay.

In the field study, none of the stem-inoculated 7-year-old Norway spruce trees developed symptoms such as stem cankers or top-dieback as observed in Norway spruce stands in Finland (Uimari *et al.*, 2018; Lilja *et al.*, 2012) or Christmas tree fields in Sweden (**Paper V**). Furthermore, no signs of the pathogen (such as perithecia and/or sporodochia) were detected within the 11-month time-frame. There was a difference in total lesion length under bark, where inoculated trees had slightly larger lesion lengths compared with control trees. We were able to reisolate the fungus from all 24 inoculated trees and 11 out of 12 control trees. Using the TaqMan assay, it was also detected molecularly from 6 out of 6 inoculated trees and 6 out of 6 control trees. These results were surprising, as mycelial plugs from *N. fuckeliana* cultures with hypha and microconidia were placed inside the wound to give a fast and assured infection. It seems likely that the trees were already infected, without displaying symptoms, before our trial started. Some background level of *N. fuckeliana* infection could be expected in outdoor trials (Hopkins *et al.*, 2012), but the degree of infection was unexpectedly high. However, if the trees in the field trial had the same background inoculum level, then the larger lesion length under bark (approximately 12 mm) for the inoculated trees was likely due to the additional inoculum of *N. fuckeliana* mycelial plugs.

The results from both the greenhouse study and the outdoor inoculation study resulted in a low degree of symptom development. This suggests that *N. fuckeliana* is a weak pathogen or an endophyte able to colonize the wood without causing too much damage, as described by Roll-Hansen and Roll-Hansen (1979). This is contradicted, however, by the rapid symptom development in the pilot inoculation study and the map-pin inoculation study described in **Paper V**. Therefore, another possible explanation is that our inoculation methods did not function well in the greenhouse study or the outdoor inoculation study.

One hypothesis, that inoculation of Norway spruce with *N. fuckeliana* would result in similar top-dieback development as seen in Christmas tree fields, was confirmed by the small pilot inoculation study but contradicted by the larger greenhouse and outdoor inoculation studies. Another hypothesis was that seedlings with larger wounds would develop symptoms faster. This was confirmed in the greenhouse inoculation experiment.

3.6 Development and application of a real-time PCR assay for detection and identification of *Neonectria fuckeliana* from Norway spruce (**Paper VII**)

In **Paper VII**, the focus was to provide a better detection tool for Neonectria canker caused by *N. fuckeliana* on Norway spruce.

The developed primer pair and probe successfully amplified the ITS-1 region of all the *N. fuckeliana* isolates. No cross-reaction occurred with closely related *Neonectria* species, or other related fungal genera tested (see Table 1 in **Paper VII**). Thus, the *N. fuckeliana* TaqMan assay will save time in the laboratory as it is not required to take precautions to avoid contamination or obtain pure cultures of the pathogen. The TaqMan assay successfully detected DNA from *N. fuckeliana* cultures, perithecia and infected plant tissue (wood samples). PCR inhibitors found in wood and bark tissue did not inhibit the assay.

The *N. fuckeliana* TaqMan assay proved sensitive and efficient for *N. fuckeliana* detection and quantification as the standard curve showed high efficiency and reproducibility (see Fig. 3. in **Paper VII**). Therefore, the real-time PCR assay developed in this study is a suitable tool for rapid identification and detection of the pathogen from diseased tissue samples. Another valuable feature with the *N. fuckeliana* assay is that the amount of DNA in samples can be compared to each other through relative quantification; a trait that may be useful in screening different Norway spruce families for potential resistance against *N. fuckeliana* infections.

The *N. fuckeliana* TaqMan assay was used in **Paper VI** to complement the commonly used culture-based diagnostic method, which is sometimes difficult and unreliable. However, since the *N. fuckeliana* TaqMan assay does not differentiate between dead and live DNA, the TaqMan assay is best used in combination with the culture-based diagnostic method. To prove that *N. fuckeliana* was alive and active and thereby the likely disease-causing agent in **Paper VI**, it was essential to obtain *N. fuckeliana* isolates. Using the *N. fuckeliana* TaqMan assay in **Paper VI** also demonstrated that the assay can be used to detect the pathogen in non-symptomatic tissue samples.

4 General discussion, practical implications and future research

4.1 A comparison of the 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 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 (**Paper II**). 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 (**Paper IV**).

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 **Paper IV** 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 the dominating problem seen in Christmas trees fields (**Papers I, II and III**). 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 (**Paper I),** 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 (**Paper I**). 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. 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 Pyntegröntodlarförening), 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. 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).

4.2 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 (Fig. 9G-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 **Papers IV and V** 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 (**Paper V**), 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 (**Paper IV**). 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 (**Paper V**), and wounds from shearing Norway spruce Christmas trees are large enough for *N. fuckeliana* to cause disease (**Paper VI**).

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. *Phytophthora* species, 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 demonstrated in **Paper V**, 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 (**Paper VII**).

4.3 Management tactics to protect Christmas tree production

The organisms causing the diseases and pests found in the **2015 disease survey** (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 (**Paper III**)
- 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 should be based on general disease and pest activity
- Using biological treatments such as stimulating naturally occurring beneficial arthropods (ladybugs, predatory mites, etc.) is 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 (**Paper VI**)

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 aphids 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 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 (**Paper V**), and likely comes from nearby forests, efficient management strategies are needed to avoid further top-dieback. We learned from **Paper VI**, 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.

4.4 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 (**Papers IV and V**) 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 (**Papers I, II and III**) or Neonectria 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 (**Papers I and II**).

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 dominating Christmas tree 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 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 aphids 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.

4.5 Future research

4.5.1 *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, **Paper III** 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 **Paper III** 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 (**Paper I**).

4.5.2 *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 **Paper VII** 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 the 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) analysis 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.

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