Resistance against fruit tree canker in apple

Evaluation of Disease Symptoms, Histopathological and RNA-Seq Analyses in Different Cultivars, Genetic Variation of *Neonectria ditissima*

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Cover: Apple flowers (photo: T. Larsen), Fruit tree canker symptoms on an apple tree (photo: H. Nybom), Fruit tree canker symptoms on main trunk, Sporodochium of *Neonectria ditissima*, Fungal hyphae of *N. ditissima* in the cortex, Fungal hyphae of *N. ditissima* under periderm (last four photos: M. Ghasemkhani).

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Abstract

Neonectria ditissima (formerly *Neonectria galligena*, anamorph *Cylindrocarpon heteronema*) is the causal agent of fruit tree canker, which is regarded as a serious economic problem in apple production. This fungus is closely associated with mild and wet conditions, and climate therefore has an important impact on its geographic distribution. Notable damage to apple trees is especially common in, e.g., North-Western Europe. Complete resistance has not yet been reported. In addition, no major genes have as yet been identified and resistance appears to be controlled mainly by additive gene action.

   Apple cultivars varied significantly in level of partial resistance towards apple canker after artificial inoculation of detached shoots and/or potted trees with macroconidia of *N. ditissima*. The inter-specific crabapple hybrid 'Prairifire' showed the least damage, indicating that valuable sources of resistance can be found in *Malus* species. *Neonectria ditissima* could be detected in artificially infected apple trees using a species-specific β-tubulin gene-based qPCR assay two months after inoculation. When quantified in different cultivars, a positive relationship between *N. ditissima* biomass and the size of canker-induced lesions was found. Interaction between plant and pathogen was studied using light microscopy of apple woody tissue; fungal hyphae were found in all cell types, and gel formation was observed in both a susceptible and a resistant cultivar but infection appears to progress more rapidly in the susceptible cultivar. Genetic variation among different *N. ditissima* isolates was investigated using SSR and AFLP markers. Comparatively low levels of genetic differentiation among orchards indicate that gene flow is prominent. Analyses of single-ascospore isolates from the same perithecia suggest that this fungus is heterothallic and therefore outcrossing. Furthermore, a focused genomics approach was applied in order to identify differentially expressed genes in response to fungal attack on apple trees. Obtained data indicate that apple cultivars inoculated with *N. ditissima* exhibit significant upregulation of defence-related genes and genes involved in detoxification, peroxidase-related reactions, phenylpropanoid metabolism and the lignification process.

Keywords: anatomy, β-tubulin gene, defence mechanism, genetic variation, mating system, qPCR, RNA-Seq, transcriptome

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Dedication

To my parents for their endless love, support and encouragement.
To my husband for his patience, love, friendship and humour.

Absence of understanding does not warrant absence of existence.
    Ibn Sina

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 **Ghasemkhani M.,** Liljeroth E., Sehic J., Zborowska A. and Nybom, H. (2015). Cut-off shoots method for estimation of partial resistance in apple cultivars to fruit tree canker caused by *Neonectria ditissima*. *Acta Agriculturae Scandinavica, Section B-Soil & Plant Science*, 65(5): 412-421.

II Garkava-Gustavsson L., **Ghasemkhani M.,** Zborowska A., Englund J.-E., Lateur M. and van de Weg E. Approaches for evaluation of resistance to European canker (*Neonectria ditissima*) in apple. *Acta Hortic*. (In press).

III **Ghasemkhani M.,** Holefors A., Marttila S., Zborowska A., Rur M., Rees-George J., Nybom H., Everett K., Scheper R. and Garkava-Gustavsson L. Detection and quantification of *Neonectria ditissima* in apple trees by real-time quantitative PCR and visualization by microscopy. (Submitted).

IV **Ghasemkhani M.,** Garkava-Gustavsson L., Liljeroth E. and Nybom H. Assessment of diversity and genetic relationships of *Neonectria ditissima*: the causal agent of fruit tree canker. (Manuscript).

V **Ghasemkhani M.,** Canbäck B., Alexandersson E., Nybom H. and Garkava-Gustavsson L. Identification of differentially expressed genes associated with response to fruit tree canker in apple using RNA-Seq analysis. (Manuscript).

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The contribution of Marjan Ghasemkhani to the papers included in this thesis was as follows:

I   Planned and performed some of the experimental work together with co-authors, performed all statistical analyses and wrote the manuscript.

II Performed the experimental work together with co-authors and participated in writing of the manuscript.

III Planned the experiments together with co-authors. Performed a large part of the experimental work, performed all statistical analyses and wrote the manuscript.

IV Planned the experiments together with co-authors. Performed all the experimental work, evaluated and analysed all data and wrote the manuscript.

V   Planned the experiments together with co-authors. Performed all the experimental work, evaluated and analysed the data together with one of the co-authors (bioinformatician) and wrote the manuscript.

Related articles by Marjan Ghasemkhani but not included in this thesis:

I. **Ghasemkhani M**., Sehic J., Ahmadi-Afzadi M., Nybom H. and L. Garkava-Gustavsson. Screening for partial resistance to fruit tree canker in apple cultivars. *Acta Hortic*. (In press).

II. **Ghasemkhani M**., Holefors A., Zborowska A., Scheper R., Everett K., Nybom H and Garkava-Gustavsson L. Development of a qPCR detection procedure of fruit tree canker caused by *Neonectria ditissima*. *Acta Hortic*. (In press).

III. Nybom H., Røen Dag., Karhu S., Garkava-Gustavsson L., Tahir I., Haikonen T., Røen K., Ahmadi-Afzadi M., **Ghasemkhani M**. and S. H. Hjeltnes. Prebreeding for future challenges in Nordic apples; susceptibility to fruit tree canker and storage diseases. *Acta Hortic*. (In press).

Abbreviations

AMOVA	Analysis of molecular variance
ANOVA	Analysis of variance
AUDPC	Area under the disease-progression curve
BC	Christian calendar concepts
CYPs	Cytochromes P450
dai	days after inoculation
DEGs	Differentially expressed genes
ELI3-1	Elicitor-activated gene 3-1
gDNA	Genomic DNA
ITS	Internal transcribed spacer
KEGG	Kyoto encyclopedia of genes and genomes
LOD	Limit of detection
LSD	Fisher's least significant difference
MEA	Malt extract agar
MLO12	Mildew resistance locus O 12
mRNA	Messenger RNA
PCA	Principal component analysis
PCR	Polymerase chain reaction
PR	Pathogenesis-related
qPCR	Quantitative real-time polymerase chain reaction
QTL	Quantitative trait loci
RNA-Seq	RNA sequencing

1   Background

1.1   Apple (*Malus × domestica*)

Apple, *Malus × domestica* (L.) Borkh., is a member of the Rosaceae family. It is the fourth most widely grown fruit in the world after citrus, grapes and banana (O'Rourke, 2003; Khachatourians, 2002), and is cultivated in temperate and subtropical areas. Due to its wide accessibility, apple is also one of the most popular and widely consumed deciduous fruits. Nearly half of the production is consumed as fresh fruit and most of the remainder is processed into apple juice, apple jam, apple butter and canned apple sauce. Charoset (apple relish) and dehydrated apple slices are other important commercial products. From a medicinal point of view, fresh apple has been reported to act as a purgative, and to reduce incidence of dental caries, prevent constipation, supply extra energy for heavy exercise and help to control obesity (Bakhru, 1995).

1.1.1   History and origin

Historical records and prehistoric remains indicate the existence of cultivation and dispersal of apple in Asia and Europe more than several thousand years ago. Apples were widely cultivated in the near East almost 3000 BC (e.g., Turkey, Syria, Iraq) (Hancock, 2008) and in the Persian Empire (e.g., Iran and beyond) by 500 BC (Hancock, 2008; Luby *et al.*, 2003). The cultivation of apple subsequently spread through Greece and the Roman Empire to Europe. Apple trees were soon planted in almost the whole of Europe and a large number of different cultivars were identified by the end of the 18th century (Hancock, 2008; Luby *et al.*, 2003; Juniper *et al.*, 1996).

Using molecular markers, Harris *et al.* (2002) showed that the wild apple *Malus sieversii,* located in Central Asia, is the major contributor to the domesticated apple. Other *Malus* species have also contributed to the gene pool, both in ancient times and more recently as donors of resistance to pests

and diseases. Almost all commonly grown apple cultivars are treated as *M. ×
domestica* but some crabapples (*Malus* spp.) are still grown, mainly for
ornamental purposes and as pollen donors in commercial orchards.

Today, the commercial apple production exceeds 70 million metric tons in
the world, with China, U.S., Turkey, Poland, India, Italy, Iran, Chile, France
and Russia being the leading producers (FAO, 2013; O'Rourke, 2003).

1.2   Fruit tree canker (*Neonectria ditissima*)

Several different fungi produce cankers on apple trees and reduce both yield
and growth, and may ultimately lead to death of the trees. Fruit tree canker,
caused by the fungus *Neonectria ditissima* (Tul. & C. Tul.) Samuels &
Rossman (*Neonectria galligena* (Bres.) Rossman & Samuels formerly known
as *Nectria galligena*), is one of the most important diseases of apple trees and
is regarded as a serious economic problem, especially in North-Western
Europe (Weber, 2014; Rossman & Palm-Hernandez, 2008; Rossman *et al.*,
1999).

1.2.1 History and origin

The first reports on *Neonectria ditissima* probably date from approximately
1880 when two studies of putative fruit tree canker were published; Goethe
(1880) and Hartig (1880) reported of a similar disease on a selection of broad-
leaved trees, especially copper beach. The early stages of canker formation on
apple trees were described by Wiltshire (1921), who reported that *N. ditissima*
can enter through cracks, which occur in the spring when the buds are swelling,
after leaf-fall in autumn and through lesions produced by the scab fungus
Venturia inaequalis. A detailed description of the disease and the anatomy of
the lesions was provided by Zeller (1926), while Cayley (1921) described the
life-history of *N. ditissima* in order to facilitate the use of fungicides during the
spore-producing periods.

Apple canker, Neonectria canker, and European canker are common names
of fruit tree canker. A previous study using RAPD and ribosomal DNA
polymorphisms has shown that this pathogen originated in North America
(Plante *et al.*, 2002). *Neonectria ditissima* belongs to the phylum *Ascomycota*,
class *Sordariomycetes*, subclass *Hypocreomycetidae* and order *Hypocreales*.
According to evidence from molecular phylogenetic studies and information
about the asexual and sexual reproduction of *N. ditissima*, this pathogen
belongs to family *Nectriaceae,* and *Neonectria* is the only identified and
proven teleomorph of *Cylindrocarpon heteronema* (Chaverri *et al.*, 2011;
Castlebury *et al.*, 2006). During the past 150 years, the scientific name of this

disease has changed frequently and currently, *N. ditissima* (Tul. & C. Tul.) Samuels & Rossman has taken precedence over *N. galligena* (Rossman & Palm-Hernandez, 2008; Castlebury *et al.*, 2006).

1.2.2 Geographic distribution

Fruit tree canker has been reported from Australia, Chile, Europe, South Africa, New Zealand and Japan (Weber, 2014; Beresford & Kim, 2011; Xu & Robinson, 2010; Grove, 1990). A minimum number of days with rainfall (> 30 %) and five months per year with specific temperatures (> 8 h at 11–16 °C) are required for this pathogen to cause severe canker (Beresford & Kim, 2011). Local climatic factors have a perceptible impact on the distribution and severity of canker. Severe canker frequently occurs on trees growing on poorly drained soils, on soils that have pockets that are poorly drained at lower altitudes and on infertile soils at higher altitudes (Weber, 2014; Brandt, 1964). *Neonectria ditissima* infects apple, pear (usually less severely than apple), and many hardwood forest trees such as beech, birch, maple, aspen, quince and hickory (Braun & Craig, 2008; Farr *et al.*, 1989; Flack & Swinburne, 1977).

1.2.3 Morphology

Morphological characteristics of *N. ditissima* are observed on asexual reproduction organs, i.e., sporodochia, conidiophores and conidia, and sexual reproduction organs, i.e., perithecia, asci and ascospores. Sporodochia consist of conidiophores (Figure 1), which are usually branched and arise from the white mycelium on the surface of the bark (Figure 2) (Agrios, 1997).

Figure 1. Sporodochia of *Neonectria ditissima.*

The asexual stage of this pathogen, *Cylindrocarpon heteronema*, produces two kinds of conidia; macroconidia and microconidia. Fully developed mycelia produce sporodochia on the surface of infected and dead bark (Agrios, 1997). Macroconidia are ellipsoidal or cylindrical with rounded ends, and are produced at the end of the conidiophores of these sporodochia (Figure 3). Microconidia are instead single-celled and vary in shape and size (Ghasemkhani, 2012). Macroconidia are extremely infectious, while the epidemiological role of microconidia is unclear (Weber, 2014; Saure, 1961; Zeller, 1926).

Perithecia, the sexual fruiting body of this fungus, are produced on infected tissue. They are bright red and vary in size, depending on environmental conditions and position on the bark. Perithecia can be found much later in the year than sporodochia (Figure 4) (Weber, 2014; Lortie, 1964).
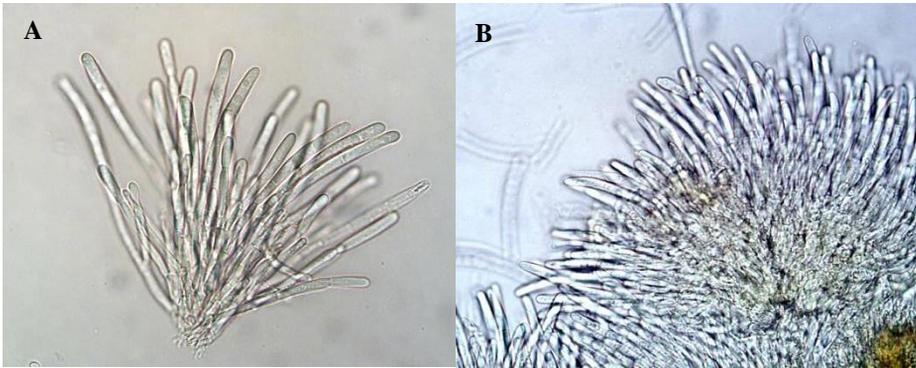
Figure 2. Conidiophores from a sporodochium produced on apple bark, A: part of conidiophore, B: densely aggregated mass of conidiophores.

The asci of *N. ditissima* are cylindrical and contain eight ascospores (Figure 5A) (Ainsworth & Bisby, 2011; Hanlin, 1971). Ascospores are two-celled, one-septate and ellipsoidal, and vary moderately in shape and size (Figure 5B). They are released in spring and early summer but there is also a short period of discharge in the autumn, and can spread over long distances (Swinburne, 1971). Weather conditions, especially humidity, have a significant impact and it has been reported that ascospore discharge does not occur unless the leaves are wet (Wessel, 1979; Lortie & Kuntz, 1963; Wiltshire, 1921).
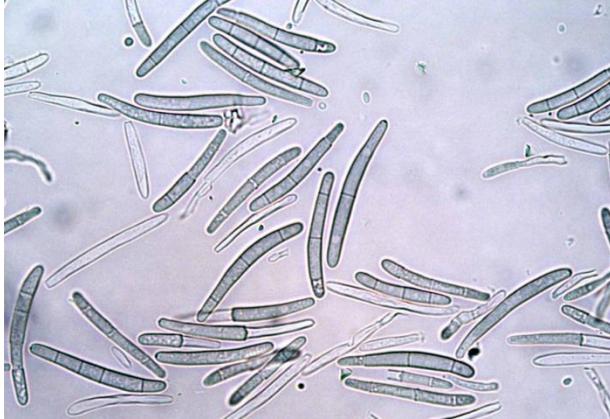
Figure 3. Macroconidia of *Neonectria ditissima* collected on the bark of an apple tree.

*Figure 4.* Perithecia of *Neonectria ditissima,* produced during winter. Photo: Bruce A. Watt.
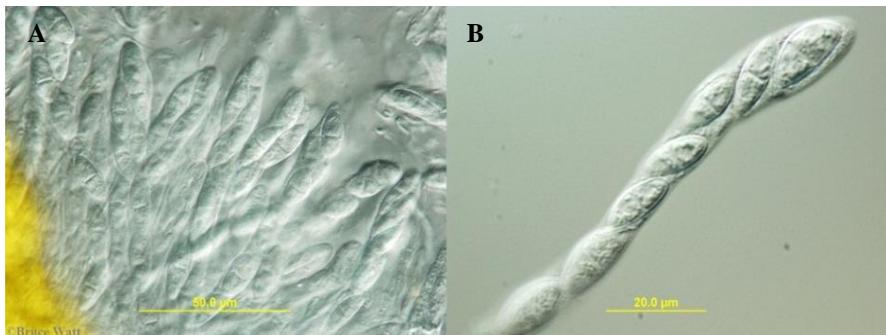
Figure 5. Neonectria ditissima produces asci in perithecia, A: two-celled ascospores, B: one ascus with 8 two-celled ascospores. Photo: Bruce A. Watt.

1.2.4 Growth of *N. ditissima* in apple tree tissues

According to Crowdy (1949), hyphae of *N. ditissima* attack xylem vessels, tracheids and fibers, and have also been detected inside xylem vessels (Langrell, 2000). The fungal hyphae are abundant close to the infection sites and become less frequent further away from these sites (Sakamoto *et al.*, 2004). The fungus also penetrates to the soft tissues outside the xylem. Peripheral tissue can be stimulated to form a phellogen barrier, which blocks the vascular tissue and helps to restrict further infection (Clatterbuck, 2006). This barrier can break down if attacked by a large aggregate of mycelia, possibly due to mechanical pressure or to a high concentration of toxins produced by the fungus. However, the host soon forms a new phellogen barrier and this process is continuously repeated (Zeller, 1926). The fungus can also penetrate to the phloem fibers after first killing the living cells by secretion of toxin (Crowdy, 1949). The mycelia grow within cells at the early stage of infection and move between cells through pits. Later, the mycelia develop intercellularly and hyphae grow spirally around small groups of fibers within the bundles (Crowdy, 1949; Zeller, 1926).

1.2.5 Life cycle and infection

Neonectria ditissima survives in cankered tissues as mycelium and perithecia during the winter and/or during other detrimental conditions. Primary infection usually originates from ascospores in the spring (McCracken *et al.*, 2003; Welch, 1934). When the temperature is well above freezing and there is sufficient moisture, sporodochia are generally produced in the first year of new canker wounds. Perithecia usually develop and appear in late summer and autumn (Figure 6). Therefore, inoculum that can cause infection is available the whole year around except for during freezing conditions in winter or extremely dry periods in summer (Weber, 2014; Xu & Butt, 1994). The spores produced by the fungus are dispersed within the same tree and to neighbouring trees, with peak production of both kinds of spores at 10–16 °C in the autumn (Beresford & Kim, 2011; Latorre *et al.*, 2002).

There is, however, considerable climate-dependent variation in the reproduction and spread of this pathogen. In Chile and United States perithecia are rarely formed, and the principal source of inoculum is probably conidia (Dubin & English, 1970; Crowdy, 1952). In northern Germany, severe infection may result when the perithecia are sufficiently mature to release ascospores during leaf fall in the autumn (Weber, 2014). In the British Isles, most of the ascospores are instead discharged during spring (Weber, 2014; Swinburne, 1971; Munson, 1939).
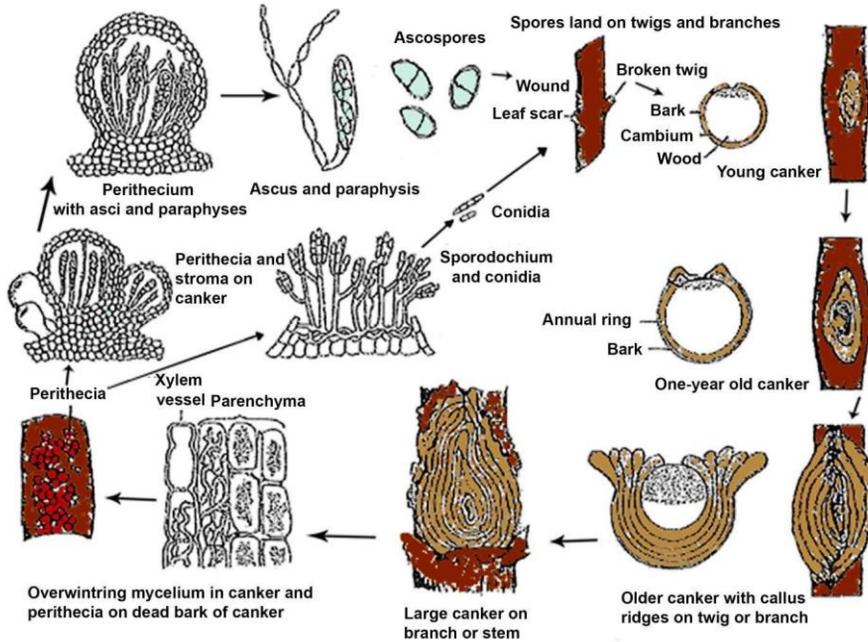
Figure 6. The life cycle of fruit tree canker caused by *N. ditissima*. Figure modified from Agrios (1997).

Dissemination of conidia and ascospores depends on e.g., climate conditions such as rainfall during the season and rain splash, wind, and the activity of insects, all of which are considered as carriers. Leaf scars in the autumn constitute the most important entry sites for infection (Swinburne *et al.*, 1975; Saure, 1961; Wiltshire, 1921). Other wounds e.g., cracks, frost injuries, pruning wounds in winter and summer, wounds caused by insects or by the picking of fruits, and apple scab lesions can also act as entry sites for infection (Weber, 2014; Pijut, 2006; Mols & Boers, 2001; Dewey & Swinburne, 1995; Swinburne, 1971; Dubin & English, 1970; Kennel, 1963; Saure, 1961). Different types of wounds, i.e. natural or artificial, are available on trees throughout the whole year, and the spores can grow and germinate instantly, and establish in the bark tissue within 3 to 4 hours (Xu *et al.*, 1998; Brandt, 1964). Conidia can spread during the growing season to other trees and this cycle is repeated several times.

A previous study showed that 50 and 5000 macroconidia per leaf scar, caused 20% and 90% infection, respectively (Dubin & English, 1970) while 100 macroconida on fresh pruning wounds are needed to cause 32% infection (Xu *et al.*, 1998). Sometimes, wounds are not sufficiently deep for successful

penetration by the fungus, and the infection is limited to the cortex (Wiltshire, 1921). After initial entry of *N. ditissima* and establishment of infection, wound callus grows around the infected area in order to prevent the spread of the infection into new tissue.

1.2.6 Symptoms

Neonectria ditissima can infect trees of all ages. The first symptoms of infection on apple branches and stems consist of small dark reddish spots in an area surrounding the infection site. Sunken areas can develop around the infected sites, e.g., on shoot bases, leaf scars and wounds (Figure 7A) (Crowdy, 1949).

Large lesions on main branches or stems usually originate from infection at the base of side shoots that have extended into the larger branches. The host forms phellogen barriers around the infected sites in response to infection resulting in swelling area (Beltra *et al.*, 1969). Concentric ridges are observed in exposed wood of old cankers, caused by differences in the seasonal growth rate of the fungus and the host (Figure 7B) (Crowdy, 1949). Cross-sections of infected branches mostly show a browning area within the wood, and hyphae of *N. ditissima* can be detected in the lumen of xylem vessels (Weber, 2014; Ventura *et al.*, 2004; Dewey & Swinburne, 1995). Conidial spore masses, looking like white fruiting bodies, can be identified on young cankered shoots in the summer and early autumn, while perithecia are recognized as red fruiting bodies in spring, autumn and winter (Weber, 2014; Crowdy, 1949).

Neonectria ditissima can also cause fruit rotting, but symptoms are seldom observed before harvest. During storage, symptoms can develop on all parts of the fruit, and the skin over the rotted area becomes dark brown (Weber, 2014).

1.3   Management of the fruit tree canker

Control of fruit tree canker is difficult since the fungus produces spores the whole year and there are always suitable entry sites on apple trees for infection. A combination of methods, including orchard management procedures, chemical treatments and choice of comparatively resistant cultivars, is required to keep the disease under at least partial control. These procedures include cutting out all infected tissue and covering the wounds with disinfectant as soon as an infected tree has been detected during winter pruning. A number of chemicals like Bordeaux paste, Copper-Count-N, and Cuprofix Ultra 40 have been used on the cankers for damage control (Cooke, 1999; English *et al.*, 1979; Swinburne *et al.*, 1975), as also spraying of the trees with e.g., copper oxychloride (Cooke *et al.*, 1993) but these chemicals are not permitted any

longer in an increasing number of countries. Scaniavital Kambium, which is a Swedish product made from natural sea bottom-derived paste containing silica, calcium and various organic components, has been commercialized for treatment of fruit tree canker (www.nordiskalkali.se). Unfortunately, none of these protectant procedures are sufficient, and it becomes especially difficult to control the disease if it is established already in young trees in newly planted orchards (Cooke, 1999; Swinburne *et al.*, 1975).

Figure 7. A: Sunken area around initial symptoms on young branches infected by *N. ditissima*, B: concentric rings typical of an older canker infection.

1.3.1 Development of healthy apple cultivars

It is well known that both *Malus* species and apple cultivars show variable levels of resistance to fruit tree canker (Sasnauskas *et al.*, 2006; Kozlovskaya *et al.*, 1999; Lateur & Populer, 1994; van de Weg, 1989), and some of the traditional cider apple cultivars are comparatively resistant as also some rootstocks like 'M.1' and 'M.12' (Moore, 1960). However, complete resistance has not yet been reported (Ghasemkhani *et al.*, 2015; Garkava-Gustavsson *et al.*, 2013; van de Weg, 1989; van de Weg, 1987) and there are few plant breeding programs focused on developing apple genotypes with high levels of resistance to this pathogen.

Level of resistance is generally associated with the mode of inheritance. Single-gene or oligogenic resistance is controlled by one or a few major-effect genes (also known as qualitative or vertical resistance) while polygenic resistance is controlled by many genes each of which have a restricted phenotypic effect (quantitative or horizontal resistance) (Balconi *et al.*, 2012;

Baker & Cook, 1974). In qualitative resistance, most of the determined R gene(s) are dominantly inherited but this can vary with pathogen isolate (Roelfs, 1988), disease phenotype (Johnson, 1992), genetic background or environment (Balconi *et al.*, 2012). These genes are often race-specific, providing full protection until there is a breakdown in resistance, as has happened with the *Vf*-gene against apple scab (Bénaouf & Parisi, 2000; Parisi *et al.*, 1993). However, specific R-gene resistance has not been observed against fruit tree canker.

By contrast, quantitative resistance usually provides only partial resistance (Yang *et al.*, 1997). The roles of specific loci in such traits can be investigated by identification and genetic mapping of QTL (quantitative trait loci) (Balconi *et al.*, 2012; Talukder *et al.*, 2004). Possibly a limited number of major QTLs is involved in the resistance to apple canker (van de Weg & Jansen, 1989), and a more recent study has demonstrated that resistance to apple canker is controlled predominantly by additive gene action (Gelvonauskiene *et al.*, 2007).

2 Aim and objectives

Most of the studies have been conducted on the epidemiology of this disease and there are few studies on host plant resistance while other aspects of the resistance remain unclear. In addition, the mechanism of resistance in apple cultivars against fruit tree canker is poorly understood. In this thesis work, susceptibility of different apple cultivars to fruit tree canker was investigated. In addition, studies on detection, quantification of this fungus and plant-pathogen interaction in artificially infected trees and genetic variation of the pathogen, were performed. The last step was to elucidate how the apple defence is affected by this pathogen on a molecular level. The more specific objectives were to:

➢ Determine levels of resistance to fruit tree canker in an apple germplasm collection using *N. ditissima* spore inoculation with two different plant models: detached shoots and potted trees, respectively.

➢ Detect and quantify biomass of *N. ditissima* in cankered apple cultivars and analyse the association between pathogen DNA accumulation in different apple cultivars and levels of disease severity.

➢ Investigate the ultrastructure of plant-pathogen interaction by microscopy to improve understanding of partial resistance to *N. ditissima*.

➢ Evaluate genetic variation of *N. ditissima* among and within apple orchards and investigate the mating system using SSR and AFLP markers.

➤ Apply RNA-Seq analysis to identify differentially expressed genes that can help to elucidate the mechanisms behind resistance towards *N. ditissima* in apple.

3 Material and methods

3.1 Plant and fungal materials

Apple cultivars, which are commonly grown in the Nordic countries and have a potential interest in breeding programs, have been used together with a few international and/or in other ways special cultivars.

For the detached-shoots spore-inoculation experiments, dormant shoots were collected from trees in the apple germplasm orchard at Balsgård (N 56°6′, E 14°9′), Swedish University of Agricultural Sciences, in southern Sweden, and from one cultivar in Finland. The shoots were kept in cold storage (+4 °C) from collection to four days prior to inoculation and no fungicide was applied.

For the potted-trees spore-inoculation experiments, one-year-old apple trees grafted on the rootstock M9, were produced in a Swedish nursery using budwood from Balsgård. The potted trees were kept in an unheated greenhouse and watered weekly. No fungicides and fertilizers were used.

For qPCR experiments, histopathology and gene expression study, one-year-old trees were produced and handled as previously described.

For the genetic variation study, single-ascospore isolates were produced from perithecia taken from naturally occurring cankers on apple branches collected in seven orchards in southern Sweden and in one orchard in Belgium (Table 1).

In order to prepare the spore suspension for spore-inoculation experiments, sporodochia of *N. ditissima* from naturally occurring cankers on apple branches were collected in the orchards at Balsgård. Macroconidia of *N. ditissima* were distinguished based on morphological characters, and a macroconidial suspension was obtained. The concentration was adjusted to 1×10^5 conidia mL^{-1}.

For the qPCR and gene expression studies, the macroconidial suspension, as previously described, was grown on agar media (1.5%). Next day, single

macroconidia were cultured on agar media (1.5%) to produce sporodochia. Later, produced sporodochia were used for spore suspensions.

Table 1. Geographic origin of isolates of *N. ditissima* used in this study

Number of isolates	Orchard	Latitude and longitude	Year
3	Jonstorp, Sweden	(N 56°13', E 12°40')	2013
2	Julita, Sweden	(N 59°11', E 16°1')	2013
2	Jönköping, Sweden	(N 57°46' N, E 14°9')	2013
2	Kivik, Sweden	(N 55°41', E 14°13')	2013
26	Balsgård, Sweden	(N 56°6', E 14°9')	2013
3	Bjärred, Sweden	(N 55°43' , E 13°1')	2013
4	Gembloux, Belgium	(N 54°34' , E 4°42')	2014
2	Stockholm, Sweden	(N 59°19', E 18°4')	2014

Isolator: Marjan Ghasemkhani

3.2 Estimation of resistance towards *N. ditissima* in apple cultivars

In the first study (Paper I), genetic differences in resistance to fruit tree canker was investigated in 30 apple cultivars, which were screened using wound-inoculated cut shoots in a standard greenhouse across two years (2012 and 2013). Eleven of these apple cultivars were also inoculated in a greenhouse with advanced climate control (biotron) in order to evaluate the robustness of the method. In the second study (Paper II), the correspondence of the results on resistance/susceptibility between years and between two plant models (cut shoots and one year-old trees) was verified on 15 cultivars.

3.2.1 Inoculation procedure

In the first experiment, shoots were transferred to a standard greenhouse at Balsgård and a greenhouse with advanced climate control at Alnarp (biotron) four days before inoculation, and were placed in 1L glass bottles. The glass bottles were kept under a plastic tent at Balsgård (Figure 8A) and in a chamber at Alnarp. Three axillary buds on each shoot were inoculated with spore suspension. A white petroleum jelly was used to cover the wounds for protection immediately after inoculation (Figure 8B), and was removed with tissue paper four days later.

In a second study, one-year-old trees and cut shoots were kept in an unheated greenhouse and in the biotron at Alnarp, respectively. They were inoculated as previously described. For evaluation of infection percentage,

another set of one-year-old trees were kept in the field during natural leaf fall. These trees were subjected to overhead irrigation at a precipitation regime normal for a Swedish autumn (20 mm/day). More details on the procedures are provided in Papers I and II.

3.2.2 Data evaluation and statistical analysis

In the first study (Paper I), lesion length was measured with a digital calliper at five time points at specified time intervals: five days in both years in the standard greenhouse and in the biotron 2013; and seven days in the biotron in 2012. In total, 90 and 120 evaluations were obtained in the standard greenhouse and in the biotron (3 and 4 shoots × 2 replicates in time × 3 leaf scars/shoot × 5 assessments), respectively, for each cultivar in each year.
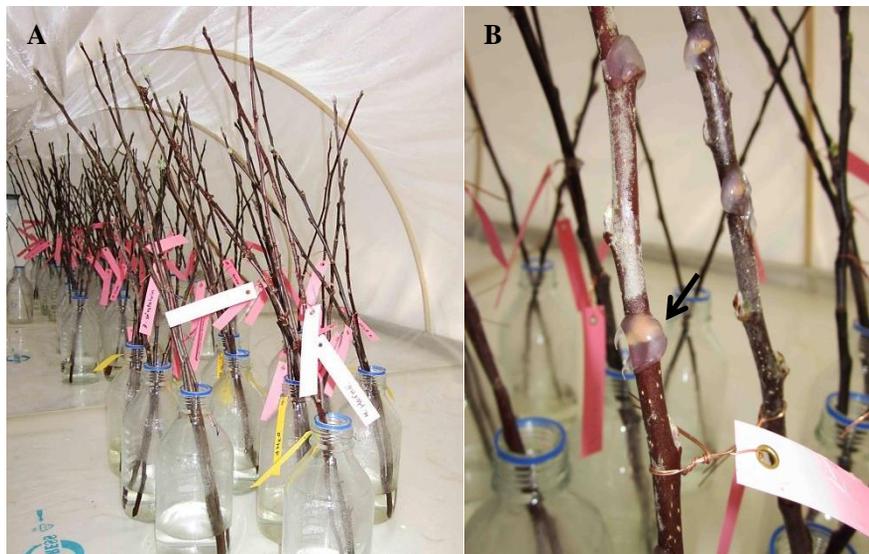
Figure 8. A: Cut-off apple shoots under a plastic tent in a standard greenhouse at Balsgård, B: The wounds were covered with white petroleum jelly just after inoculation.

Level of partial resistance was quantified in three different ways for the 11 cultivars assessed in both facilities: infection percentage, incubation period and area under the disease-progression curve (AUDPC). Separate two-way ANOVAs were calculated for these three variables with effects attributed to cultivar, year, replicate and facility. In order to investigate correspondence between replicates in both facilities, Pearson correlation tests were performed. A set of one-way ANOVAs was calculated for the 30 cultivars in both years in the standard greenhouse using AUDPC values, and an LSD post-hoc test was

used to compare the cultivars. In addition, Pearson correlation tests were applied to examine correspondence of the results between years.

In the second study (Paper II) one-way ANOVA was calculated for each plant model (cut shoots and potted trees) separately. A two-way ANOVA was performed with AUDPC as a dependent factor, and cultivar and year as independent variables for both plant models, and a Tukey's test was used to compare the cultivars. Pearson correlation tests were applied to investigate relationships between two plant models across two years with AUDPC values. Infection percentage of one-year-old trees was evaluated by dividing the proportion of infected scars to the total number of leaf scars. Spearman's rank correlation test was carried out to investigate possible relationships between the two years for each model, and between AUDPC and infection percentage. Calculations were conducted using SAS version 9.3, Minitab version 16 and Excel.

3.3 Detection and quantification of *N. ditissima* with quantitative real-time PCR

In this part of the thesis (Paper III), specificity of newly designed primer pairs was investigated. One primer pair, which passed several validation tests, was subsequently used to quantify the biomass of *N. ditissima* in cankered trees of artificially infected apple cultivars.

3.3.1 Primer design and validation of primer specificity

The primer pairs were designed from a β-tubulin gene by aligning regions conserved within *N. ditissima* with sequence information of different fungi that were deposited in GeneBank. Several primer combinations were compared using *in silico* analysis. Two primer sets were used for future evaluation.

Genomic DNA of *N. ditissima* was amplified with the designed primer pairs and resulting PCR products were purified, sequenced and assembled. Assembled sequences were aligned to gDNA sequences of *N. ditissima*. In the next step, extracted DNAs of *N. ditissima* from different isolates (Table 2 in Paper III) and different fungal pathogens that have been found on apple wood (Gomori, 1955) (Table 1 in Paper III), were used to evaluate primer specificity evidenced by presence or absence of a single PCR product of gDNA of *N. ditissima*. And finally, the melting curves with a single high peak during qPCR assay were evaluated. This method was also used for detection of *N. ditissima* and in investigations of the association between pathogen DNA accumulation and lesion size of infected apple cultivars.

The slope of a standard curve was used to estimate the amplification efficiency of the best primer pair using 5-fold serial dilution of gDNA of *N. ditissima,* and limit of detection (LOD) of *N. ditissima* was determined by running a total of 60 samples.

3.3.2 Experimental procedures

For quantification of *N. ditissima* in infected apple cultivars, one-year-old apple trees representing six cultivars, both susceptible and partially resistant , with three replications (trees) of each cultivar, in total 18 trees, were kept in an unheated greenhouse and inoculated with a macroconidial suspension. The samples were taken at two different time points; one month after inoculation and two months after inoculation, both at the point of infection and in the area surrounding the point of infection. DNAs were extracted and a qPCR assay was carried out. The reactions were run in 20 μL using 0.2 ml 96-well PCR plates. For amplification of *N. ditissima* and apple DNA, designed primer pairs and ubiquitin gene primers described by Botton *et al.* (2008) were used, respectively. More details on the procedures are provided in Paper III.

3.3.3 Data evaluation and statistical analysis

Analysis of qPCR data was carried out by Bio-Rad CFX manager Software v. 3.0.1. Pearson correlation was used to assess the relationship between lesion size and relative *N. ditissima* DNA quantity across the six cultivars. Statistical analyses were performed using the software R (R Development Core Team, 2013).

3.4   Histopathology of canker on *Malus* caused by *N. ditissima*

In order to understand how apple cultivars may defend themselves against fruit tree canker, plant-pathogen interaction in one susceptible and one partially resistant cultivar was compared.

3.4.1 Experimental procedures

A light microscopy study was undertaken on 'Cox's Orange Pippin' chosen as a susceptible cultivar, and on 'Santana' chosen as a partially resistant cultivar. One-year-old potted trees and cut shoots were inoculated and sampled at the infection site, at 110 days after inoculation (dai) (trees), and at 5, 18, 29, and 36 dai (cut shoots).

Specimens were soaked in fixative (2% (w/v) paraformaldehyde, 2.5% (v/v) glutaraldehyde in 0.1 M Na-phosphate buffer, pH 7.2) during gentle agitation. The fixed samples were dehydrated and embedded in Spurr's resin. Semithin

sections were cut and placed on object slides for light microscopy. Toluidine blue staining was used to observe the general structure and fungal hyphae. More details on the technical procedures are provided in Paper III.

3.4.2 Data evaluation

Sections were examined by a Leica DMLB light microscope and after determining the location of interest, photographs were taken with a Leica DFC450 digital camera.

3.5 Genetic variation in *N. ditissima*

In this part of the thesis, genetic diversity of *N. ditissima* was evaluated using single-ascospore isolates from different orchards (Paper IV). Single-ascospore isolates derived from the same perithecium were used for conclusions about mating system of this pathogen.

3.5.1 DNA extraction and amplification

Single-ascospore isolates were obtained from perithecia on pieces of bark and wood collected from different orchards in Sweden and Belgium (Table 2). In order to extract DNA of these isolates, fungal cultures on MEA 2% (Figure 9) were transferred into malt extract/glucose/peptone (MGP) liquid medium (Shaner & Finney, 1977) and incubated for 10 days (Figure 10). The mycelium was harvested, washed with dH_2O and freeze-dried. DNAs were extracted and quantity and quality checked.

Seven SSR primer pairs (Marra & Corwin, 2009), were used for SSR analysis. Genomic DNA was amplified using PCR.

AFLP analysis of DNA was carried out with an AFLP Microbial Fingerprinting kit. Eleven primer combinations were used. Reproducibility of AFLP amplification was checked by adding 5–8 randomly taken samples to each 96-well plate.

PCR products were separated on an ABI 3130xl DNA analyser and software package Gene-Marker v 1.85 was used for the fragment analysis. More details about experimental procedures are provided in Paper IV.

3.5.2 Data analysis

Genetic diversity parameters i.e., number of polymorphic loci (NPL), Nei's gene diversity (H), percentage of polymorphic loci (PPL) and Shannon's information index (I) were evaluated. Analysis of molecular variance (AMOVA) was performed in order to partition genetic variation into within- and among population components. Pairwise genetic similarities between

single-ascospore isolates obtained from the same or from different perithecia were estimated using Jaccard's similarity coefficients (Weising *et al.*, 2005). Similarity among isolates from the same or from different orchards was illustrated with a dendrogram constructed with UPGMA and a principal component analysis (PCoA). Mantel tests (Mantel, 1967) were performed in order to check the goodness-of-fit of the cluster analysis with the matrix on which it was based, and to investigate possible correlation between two genetic distance matrices based on SSR and AFLP markers, respectively. Calculations were conducted using POPGENE v 1.32 (Yeh *et al.*, 1997), NTSYS 2.02 (Tamura *et al.*, 2007) and Arlequin 3.11 (Excoffier *et al.*, 2005). More details about statistical calculations are provided in Paper IV.
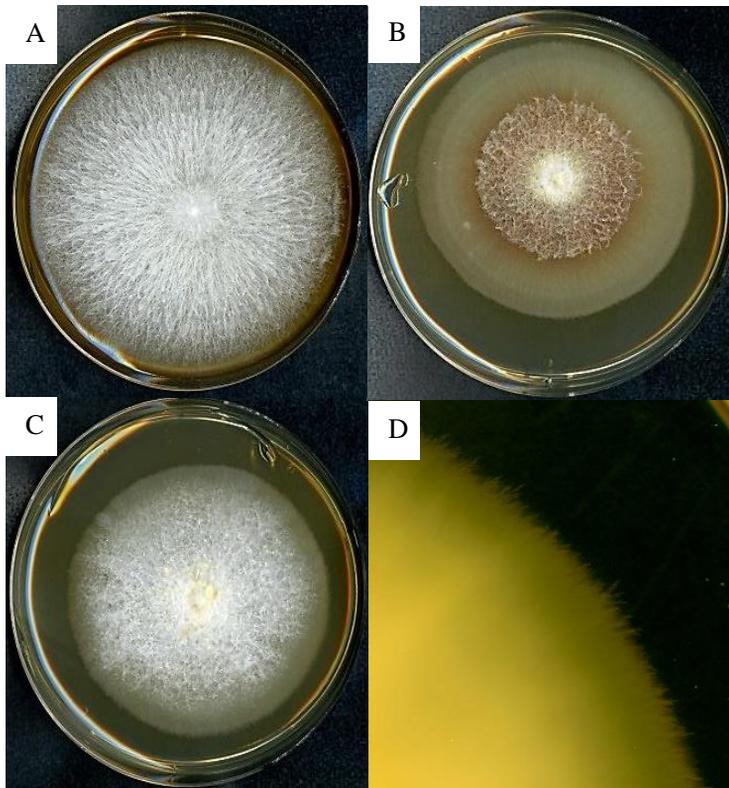

Figure 9. Neonectria ditissima, culture on malt extract agar 2%. A, B, C: Fungal cultures with a circular mycelium. D: Erose margin of a fungal culture.

Figure 10. Different isolates of *Neonectria ditissima* growing in liquid media.

3.6 Gene expression analysis using RNA-sequencing data

Differentially expressed genes, that could be relevant for resistance to fruit tree canker in apple, were investigated using RNA-Seq analysis (Paper V).

3.6.1 Experimental procedures

Trees of two cultivars, the partially resistant 'Jonathan' and the susceptible 'Prima', were inoculated with *N. ditissima* as previously described (Paper III) and with water as a control. Tissue samples were taken at three different time points, i.e., 5, 15, and 30 dai. In total, 36 tissue samples from the two different apple cultivars at three different time points, each with two inoculations (*N. ditissima* and water) in three replicates (tree), were used for RNA extraction. Concentration, purity and integrity of RNAs were determined, and libraries were generated with 1 µg of total RNA using Illumina TruSeq Stranded mRNA, multiplexed and sequenced in two lanes on Illumina HiSeq High Output mode.

3.6.2 Data analysis

Quality of the reads was determined with the fastQC software version 0.11.2 (Andrews, 2010). The adapter sequences were removed with Trimmomatic, version 0.32 (Bolger & Giorgi, 2014) together with low quality sequences. In order to remove the fungal reads, the reads were mapped to the reference genome of apple, assembly version 1 (Velasco *et al.*, 2010), as well as to the reference genome of the fungus *Nectria haematococca* (Coleman *et al.*, 2009) using TopHat2, version 2.0.13 (Trapnell *et al.*, 2009). A very limited

number of identified fungal reads were removed. Then the filtered reads were used for further analysis. HTseq-count, version 0.6.1 (Anders *et al.*, 2014), was used for calculation of read counts per gene. Normalization and differential expression analysis were performed according to the DESeq2 model and package (Love *et al.*, 2013; Anders & Huber, 2010). A threshold for identification of significantly differentially expressed genes in each cultivar was based on setting the false discovery rate (FDR) to ≤ 0.05. More details about data analysis are provided in Paper V.

4 Results and discussion

4.1 Variation among apple cultivars inoculated with spores of *N. ditissima*

In the first study (Paper I), damage caused by canker lesions on cut shoots inoculated with *N. ditissima*, was quantified using three different variables: incubation period, infection percentage and AUDPC. Variation among cultivars for each of these variables was determined with two-way ANOVA (Table 1 in Paper I). Significant differentiation among cultivars was obtained with infection percentage and AUDPC, while incubation period produced less informative data and was not used for further analyses. When different replicates were compared, AUDPC values produced the most consistent data (Figure 1 and Table 2 in Paper I) and were therefore used in subsequent analyses.

Significant differences among the 30 inoculated cultivars were found in both years (2012; $P < 0.001$, and 2013; $P < 0.001$). The most resistant cultivar was 'Prairifire' in both years while 'Jonathan' and 'Rödluvan' showed the highest susceptibility in 2012 and 2013, respectively (Figure 11). Additionally, 'Aroma', 'Golden Delicious', 'Santana' and 'Frösåker' were found to be relatively resistant while 'Elise' and 'Hornö' were relatively susceptible in both years. A significant correlation was found across years, 2012 and 2013 ($r = 0.57$, $P < 0.001$). The results for the more resistant cultivars were less varied between years compared to results for the more susceptible cultivars.

In the second study (Paper II), significant differences among cultivars were observed in both sets of experiments, i.e. cut-off shoots and one-year-old potted trees. There was no significant interaction of cultivar × year for either cut-off shoots or one-year-old trees. Instead a significant correlation was found between data for the two years for each set of experiments, indicating repeatability over years.
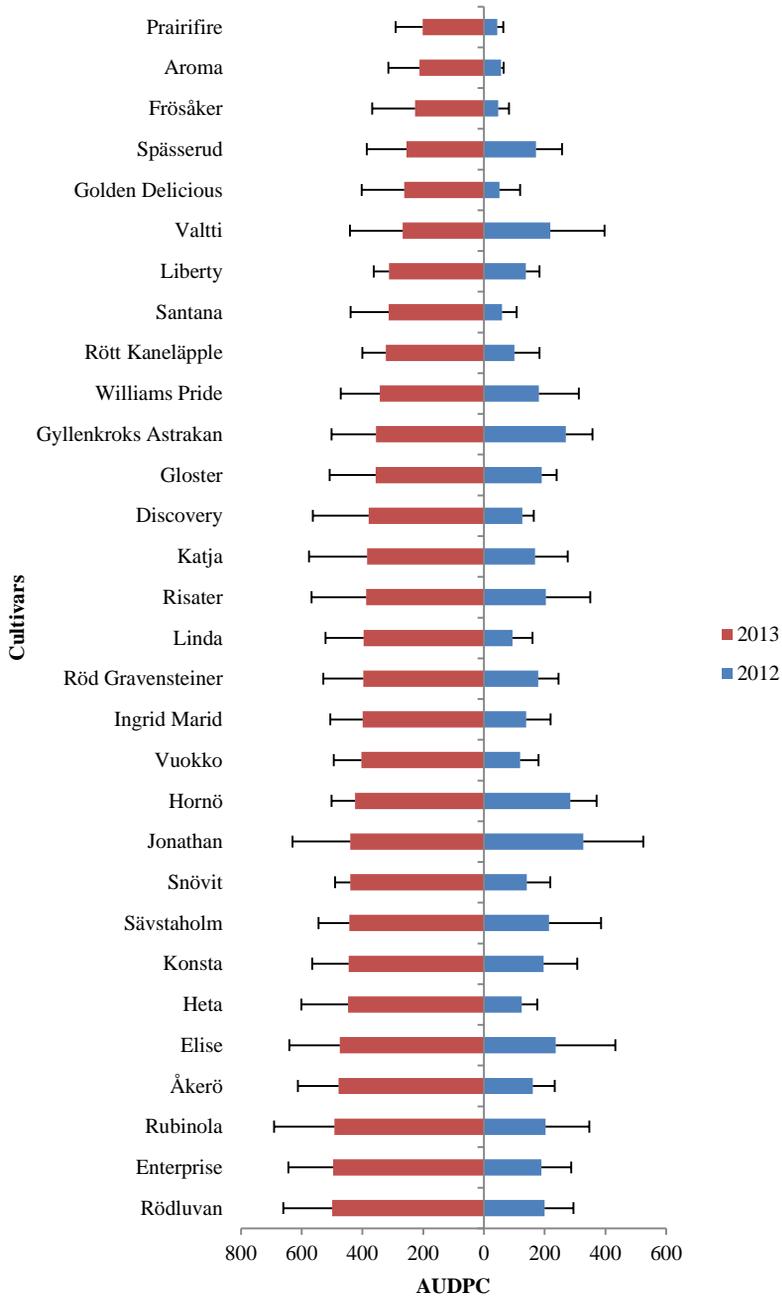
Figure 11. Average value for AUDPC for 30 apple cultivars inoculated with *N. ditissima* in the standard greenhouse in 2012 and 2013.

In the cut-off shoots experiment in the biotron, 'Cox's Orange Pippin' was the most susceptible cultivar in both years. The most resistant cultivars were instead 'Santana', 'Golden Delicious' and 'Aroma', and these also had low values for infection percentage. In the one-year-old trees experiment, 'Golden Delicious' was the most resistant cultivar in both years, while also 'Santana' and 'Aroma' were considered as resistant in 2012 and 2013, respectively. 'Charlamovsky' was the most susceptible cultivar and also showed the highest value for infection percentage. Additionally 'Gala', 'Cox's Orange Pippin', 'Elise' and 'Prima' were found to be relatively susceptible in both years.

While the cut-off shoots method can produce rough estimates of resistance levels within a short timeframe, inoculation of one year-old trees produces data with superior resolution power and repeatability over years. This method is, however, more expensive and time-consuming and further development of phenotyping procedures is needed. Assessment of infection percentage under 'natural' conditions (dispersal of spores onto unwounded potted trees) can be a valuable complement to the wound inoculations, since different components of resistance may be targeted.

The resistance mechanism of apple against fruit tree canker is still not adequately known. Necrotrophic fungi like *N. ditissima,* are usually classified either as host-specific pathogens or as broad host-range pathogens (Mengiste, 2012). The ability of broad-host-range necrotrophs, as *N. ditissima*, to cause disease is usually attributed to the production of a diversity of phytotoxic metabolites, cell wall-degrading enzymes, and cell death elicitors that kill host cells and cause necrosis, whereas host-specific necrotrophs generally produce toxins that have activity on only a limited number of plant species (Laluk & Mengiste, 2010; Friesen *et al.*, 2008). Resistance to necrotrophic fungi is quantitative, and usually requires many genes for full resistance (Laluk & Mengiste, 2010). By contrast, the causal agent of apple scab, *Venturia inaequalis,* is a hemibiotrophic fungus (Jha et al., 2009), and qualitative, race-specific resistance is involved in the defence response of apple to this pathogen including detoxification of fungal toxins, transcriptional regulation and modulation of the defence response (Bastiaanse *et al.*, 2014; Poland *et al.*, 2009).

Previous studies have shown that there is large variation among apple cultivars in level of resistance to *N. ditissima* (Braun, 1997; Pedersen *et al.*, 1994), and different apple cultivars can probably utilize various defence responses to suppress spread and growth of this pathogen. In the current study 'Prairifire', which is an ornamental crab apple with a pedigree including *M. atrosanguinea*, *M. zumi calocarpa*, *M. niedzwetskyana* and *M. moerlandsii* 'Liset', showed the highest level of resistance to apple canker, which is likely

to be connected with its unique genetic background. Valuable resistance genes have thus been found also in many different *Malus* species. Resistance to the bacterial disease fire blight caused by *Erwinia amylovora*, is controlled by several QTLs with restricted effects in common apple cultivars (Gardiner *et al.*, 2012; Parravicini *et al.*, 2011; Civetta *et al.*, 2009; Flachowsky *et al.*, 2007; Khan *et al.*, 2006), but some major fire blight resistance genes have recently been found in material originating from wild apple species, e.g., *Malus robusta* (Kellerhals *et al.*, 2014; Peil *et al.*, 2007). Several major genes for scab resistance have also been obtained from wild *Malus* species such as *M. floribunda* (Würdig *et al.*, 2015; Belfanti *et al.*, 2004). In keeping with these results, there may be one or several resistance genes in 'Prairifire' that could potentially play an important role in future breeding against fruit tree canker.

4.2 Quantitative real-time PCR for detection and quantification of *N. ditissima* in susceptible and resistant apple cultivars

Validation of one primer pair, Bt–fw135/Bt–rw284, showed that a single amplicon (150 bp), representing a specific, targeted sequence of *N. ditissima* gDNA was obtained from *N. ditissima* isolates (Figure 1 in Paper III). Moreover, this primer pair amplified a fragment only in DNA samples of *N. ditissima* and not in samples of any other fungal pathogen (Table 1 in Paper III). Amplification specificity was confirmed by detection of a single specific peak in the qPCR assay. Amplification efficiency of this primer pair was determined to be 99.6% using the standard curve method. An analysis of limit of detection (LOD) showed that the lowest amount of *N. ditissima* gDNA that can be detected by qPCR assay was 1 pg (Figure 12).

Results of the qPCR assay showed that the fungus was not detected in the sampled tissue until two months after inoculation, and then only when samples were taken at the point of infection. The fungus was not detected in the area surrounding the infection site in any of the six studied cultivars.

Variation in the ability to withstand or suppress the development of fungus was observed by qPCR assay in the six cultivars. Samples of the susceptible cultivar 'Elise' contained the highest amount of fungal biomass while samples of the partially resistant 'Aroma' had the lowest.

In our study, availability of a species-specific primer pair, Bt–fw135/Bt–rw284, derived from the β-tubulin region, allowed detection and quantification of gDNA of *N. ditissima* in the infected tissues. Similarly, qPCR assays with primers designed from the internal transcribed spacer (ITS) regions proved successful in the study of differences between resistant and susceptible

varieties of soybean in terms of accumulation of *Phytophthora sojae* (Catal *et al.*, 2013).

Specific primers have been developed previously from the ITS regions with the aim to detect *N. ditissima* (Langrell, 2002; Langrell & Barbara, 2001), but these primers were not suitable for use in qPCR due to large size of the amplified fragment (412 bp) and cross reactions with apple DNA.

In order to increase the sensitivity of our primer, other approaches like TaqMan-based qPCR can be investigated. As previously shown, the application of SYBR Green primers and TaqMan probe can increase the sensitivity of primers substantially (Garces *et al.*, 2014). It has also been reported that the simultaneous use of two TaqMan probes labeled with two different reporter dyes can increase the assay sensitivity and reproducibility at the low detection range (Yip *et al.*, 2005).
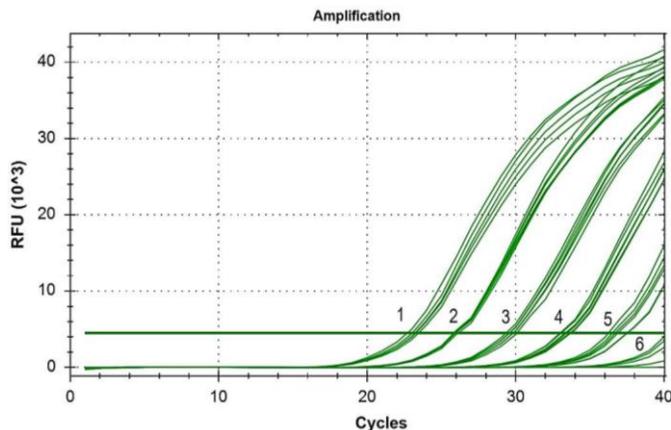

Figure 12. Detection limit of qPCR. Signals 1-6, the results of qPCR for 10 ng, 1 ng, 0.1 ng, 0.01 ng, 1 pg and 0.1 pg DNA per reaction, respectively.

4.3 Histopathology of infection and colonization of susceptible and resistant apple cultivars by *N. ditissima*

For this study, potted trees and cut shoots of the susceptible 'Cox's Orange Pippin' and the partially resistant 'Santana', were inoculated with spores of *N. ditissima*. Woody tissue from the affected area was then examined to identify possible differences that could be related to level of susceptibility. Plastic embedding with long incubation times of the specimens resulted in good quality sections from both infected and non-infected samples.

The structural organization of the bark, wood and pith was clearly seen in the transverse, healthy stem sections stained with toluidine blue (Figure 13) and fungal hyphae were visualized by dark purple colour. In one-year-old trees, a tangled mass of fungal hyphae was seen under the periderm of both cultivars at 110 dai, which may be the first step towards the production of sporodochia (Figure 14A). This finding is in agreement with a previous study on the canker of aspen caused by *N. ditissima* (Zalasky, 1968). A larger number of hyphae was observed in cortex parenchyma cells of the susceptible cultivar compared to the partially resistant cultivar, and these cells were completely collapsed in the susceptible cultivar (Figure 14B). Primary and secondary phloem (Figure 14C), primary and secondary xylem, xylem rays and pith cells were affected in the infected tissues of both cultivars (Figure 4 in Paper III). These findings are in agreement with previous studies on apple canker caused by *N. ditissima* (Crowdy, 1949) and infection of *Fraxinus* by *N. ditissima* (Sakamoto et al., 2004). The hyphae could clearly penetrate the secondary cell walls in the phloem fibers, xylem cells and lignified walls, as also reported in a study using 'Cox's Orange Pippin' and 'Worcester Pearmain' (Crowdy, 1949). Gel formation was observed in both of our cultivars with no difference in extent or frequency. In summary, 'Cox's Orange Pippin' (susceptible cultivar) was more heavily colonized with *N. ditissima* compared to 'Santana', which is a partially resistant cultivar.
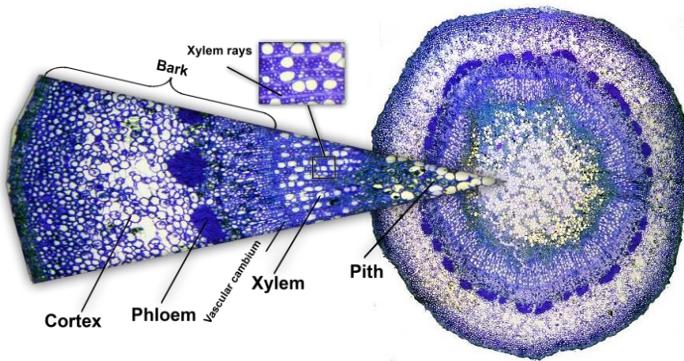
*Figure13.* Transverse section of healthy stem with toluidine blue staining. Overview of the stem showing cortex, phloem, xylem, xylem ray and pith cells. The diameter of the section is 3.5 mm.

In cut shoots, fungal hyphae were seen in cortex, phloem and xylem cells, and phloem cells had collapsed in 'Cox's Orange Pippin' by 18 dai. Cortex and phloem cells had deformed and gel formation was observed in both cultivars at

29 dai. In the resistant cultivar 'Santana', fungal hyphae were detected by 36 dai (Figure 5 in Paper III). The pace of infection development differed between the cultivars; fungal hyphae were observed earlier in the susceptible cultivar compared to in the partially resistant one. However, more research is needed to determine the nature of defence mechanisms used against the pathogen in the resistant cultivar.
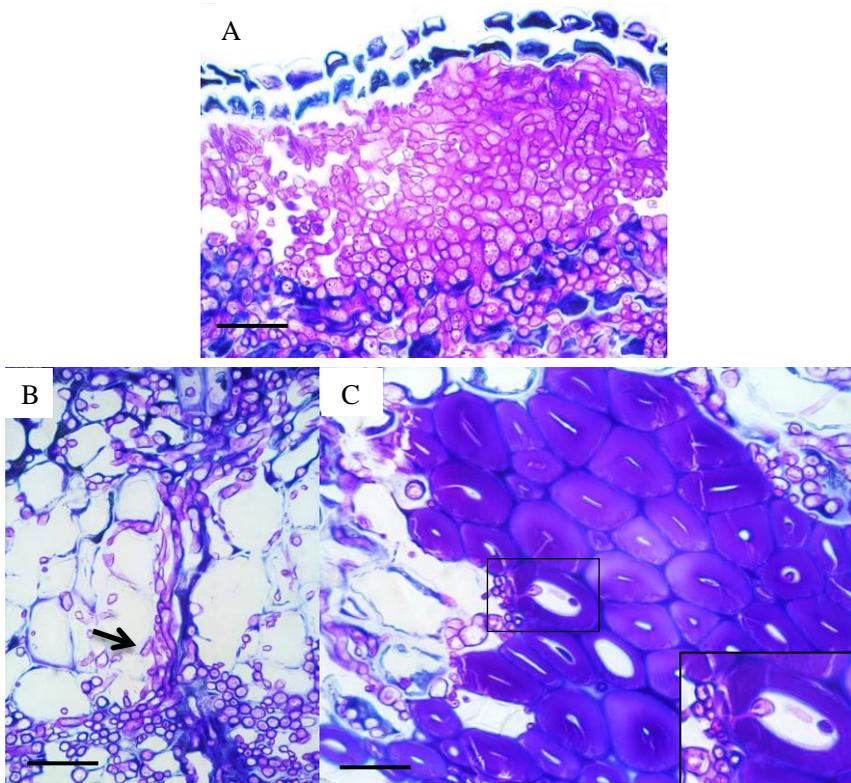
Figure 14. Transverse section of one-year-old tree 110 days after inoculation in infected cultivar, 'Cox's Orange Pippin'. A: A hyphal aggregation that possibly shows an early stage of sporodochial formation. B: Fungal hyphae in cortex parenchyma cells, which were deformed and collapsed. C: Fungal hyphae in secondary phloem, they penetrate through the thick cell wall of fiber cells in the secondary phloem. Scale bar: 20 μm.

Disease progression was slower in one-year-old trees compared with cut shoots. Hyphae were less frequent on the side opposite to the necrotic tissue, in accordance with previous studies (Sakamoto *et al.*, 2004; Crowdy, 1949) where the infection was reported to be relatively local. Some occasional gel formation

was found in both cultivars but no tyloses were detected. To sum up, no significant structural differences were found between resistant and susceptible cultivars, but the spreading of the infection was slower in the partially resistant cultivar. This finding is consistent with previous studies on grapes (Baccari & Lindow, 2011; Chatelet *et al.*, 2011) where no significant differences were found in overall stem anatomy between resistant and susceptible varieties infected by *Xylella fastidiosa*.

The major difference between the susceptible and the resistant cultivar was the degree of colonization by the pathogen. Possibly, a differential release of inhibitors such as phytoalexins that are natural plant antibiotics could be involved in restricting the spread of the pathogen more efficiently in resistant cultivars. Previous studies have shown that degree of host resistance and the level of phytoalexin accumulation are often strongly correlated (Hall *et al.*, 2011; Shi *et al.*, 1991). Therefore, more research is needed to determine whether phytoalexins influence the defence reaction against *Neonectria*, possibly reducing the rate of colonization and disrupting pathogen metabolism.

4.4 Genetic diversity and genetic relationships of *N. ditissima*

SSR primers produced a higher level of band polymorphism across the studied isolates than AFLP primers. This is likely due to detection of multiple alleles at a given locus by SSR markers, whereas AFLP markers mainly detect single alleles at multiple loci randomly distributed in the genome. An AFLP-based analysis of molecular variation (AMOVA) among 44 isolates, obtained from eight different orchards, revealed relatively low genetic differentiation among orchards (11% compared to 89% within-orchard variation).

A principal coordinate analysis (PCoA) was performed with SSR data for 28 isolates (Figure 1 in Paper IV). The isolates were loosely grouped and there was no association with the geographic origin. Similar results were obtained with a PCoA using AFLP data for the same 28 isolates, and with a UPGMA-based cluster analysis using AFLP data for 44 isolates (Figure 2 in Paper IV). In the latter analysis, isolates from the same tree clustered together while isolates from different trees in the same or different orchards did not show any spatial pattern. Gene flow between populations (orchards) may prevent geographic subdivision (Lenormand, 2002), either due to relatively high levels of historical gene flow or high levels of spontaneous mutations and/or to the movement of plant material from one region to another (Carlile *et al.*, 2001).

To study the mating system of this pathogen, single-ascospore isolates from the same perithecium or from different perithecia on the same or different trees, were analysed with both SSR and AFLP markers. The SSR results

suggested that all single-ascospores from a single tree were identical. Considerably more variation was detected with AFLP markers, even among isolates from the same perithecium (Figure 15). Obviously, the fungus is heterothallic allowing outcrossing, but still selfing cannot be ruled out. Previous studies based on spore morphology of *N. ditissima* have reported heterothallic reproduction (Krüger, 1974) as well as homothallic (El-Gholl *et al.*, 1986). The congeneric species *N. haematococca* has both homothallic and heterothallic populations (Coleman, 2008), and this may be true also for *N. ditissima*.

Management of sexually reproducing pathogens is more difficult due to the constant appearance of new genotypes, which increase the variation of features like higher aggressiveness, fungicide resistance, and better fitness in the population. An outcrossing mating system in *N. ditissima* can thus be expected
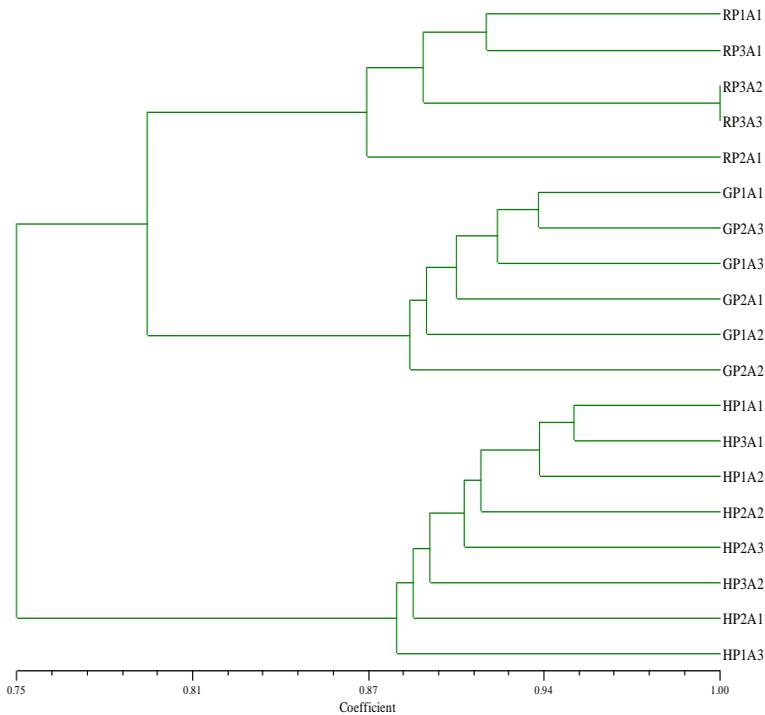

Figure 15. Dendrogram derived from AFLP band patterns of single-ascospore isolates arising from different perithecia of *N. ditissima*. R: Red Delicious, G: Gravensteiner, H: Holsteiner Cox, P1-3: different perithecia, A1-3: different single-ascospores.

to speed up the evolution of adaptation to resistant hosts and commonly applied fungicides (McDonald & McDermott, 1993).

When the two marker types were compared with a Mantel test, genetic distances among isolates showed no correspondence, possibly due mainly to insufficient number of bands detected with the SSR primers. AFLP markers therefore seem to have a better potential for evaluating genetic diversity and relationships of this pathogen.

4.5   Identification of differentially expressed genes in partially resistant and susceptible cultivars in response to inoculation with *N. ditissima*

In order to identify genes that are differentially expressed in response to the pathogen attack, one partially resistant ('Jonathan') and one susceptible cultivar ('Prima') were investigated, at three different time points after inoculating one-year-old potted trees with spores of *N. ditissima*. A total of 36 RNA samples were collected for RNA-Seq. Approximately 392 million paired end reads were obtained. A total of 345 million Illumina reads passed quality filtering. The majority of reads were mapped to the apple reference genome sequence (Velasco et al., 2010).

A principal component analysis (PCA) of DESeq2 normalized counts for 46157 genes (out of total of 63541) is shown in Figure 16. The lowest number of differentially expressed genes (DEGs) was obtained at 5 dai whereas the highest number was found at 30 dai (Table 1 in Paper V).

A total of 1055 genes, which were differently regulated in the resistant cultivar compared to in the susceptible cultivar, were selected for further analysis. Blasting these DEGs against the KEGG (Kyoto Encyclopedia of Genes and Genomes) database indicate that they are involved in 72 metabolic pathways (Table 3 in Paper V).

A functional classification was carried out first for each cultivar separately using the DEGs (Figure 3 in Paper V) and then with the GO terms of the 1055 gene list (Figure 17). Based on these results, the molecular function group was divided into 11 subcategories, the cellular components into 6 subcategories and the biological processes into 12 subcategories in both analyses.

Several genes were associated with apoptosis processes such as *RPM1*, and some were involved in defense response, e.g., *MLO12* (mildew resistance locus O 12), *Bet v I* (pathogenesis-related proteins), *NPR3* (involved in response to wounding), *ACT7* (involved in response to wounding) and *BAK1* (Leu-rich receptor serine/threonine protein kinase). All of these genes were up-regulated in the partially resistant cultivar 'Jonathan'. The *Bet v I* gene belongs to family

10 of plant pathogenesis-related proteins (PR-10). Pathogenesis-related (PR) proteins have been reported to be induced in plants in the event of a pathogen attack (Linthorst & Van Loon, 1991; Van Loon, 1985).

Several genes involved in the electron transport pathway were also up-regulated in the partially resistant cultivar, e.g., *CYP71A25* (cytochrome P450), *CYP71A26* (encoding oxygen binding) and *ATBCB* (copper binding protein). Cytochromes P450 (CYPs) belong to a superfamily family of proteins and are involved in catabolism of secondary metabolites e.g., hormone and signaling molecules, antioxidants, allelochemicals and defense compounds (Bak *et al.*, 2011; Morant *et al.*, 2003). In this study, the expression level of *CYP71A25* and *CYP71A26* were up-regulated in response to *N. ditissima* inoculation of the partially resistant cultivar. Trognitz *et al.* (2002) showed that cytochromes P450 play a role in the defense response in plants.

Some genes were involved in the flavonoids biosynthesis pathway e.g., *DMR6* (encoding oxidoreductase activity) and *TT10* (Laccase-like polyphenol oxidases). Some genes involved in metabolic processes e.g., *ELI3*-1 (elicitor-activated gene 3-1), *GT/UGT74F2* (UDP-glucosyltransferase 74F2), *ATGSTF13* (glutathione transferase) and *CAD1* (glutathione gamma-glutamylcysteinyltransferase 1) were differentially regulated between resistant and susceptible cultivars (Table 2 in Paper V). The *PAL1* and *PAL2* genes, which were up-regulated in the partially resistant cultivar, participate in the defense of plants against pathogens and catalyzes the first step in the biosynthesis of phenylpropanoids, which is responsible for the production of lignin (Dixon *et al.*, 2002). Since an increased activity of the *PAL* gene has been observed in apple leaves infected by *Venturia inaequalis* (Petkovsek *et al.*, 2011; Schovankova & Opatova, 2011), increased *PAL* activity will most likely contribute to reduce also infection by *N. ditissima.*

Expression of some genes such as *WRKY72*, *WRKY70*, *WRKY40*, *MYB2* and transcription factor genes, were induced in response to *N. ditissima* inoculation. The WRKY proteins are a large family of transcription factors (Eulgem *et al.*, 2000). WRKY family members appear to be involved in the regulation of different physiological programs in plants such as pathogen defense (Eulgem & Somssich, 2007). Eulgem *et al.* (2000) also reported that infection with viruses, bacteria and fungi increased the level of WRKY mRNA.

Cytochromes P450 (CYPs) belong to the largest family of plant proteins and are involved in detoxification and biosynthesis of secondary metabolites (Morant *et al.*, 2003), and can play a role in the defense response of plants (Trognitz *et al.*, 2002). The current study demonstrated that expression levels of *CYP71A25* and *CYP71A26* were up-regulated in response to *N. ditissima* inoculation in the partially resistant cultivar.
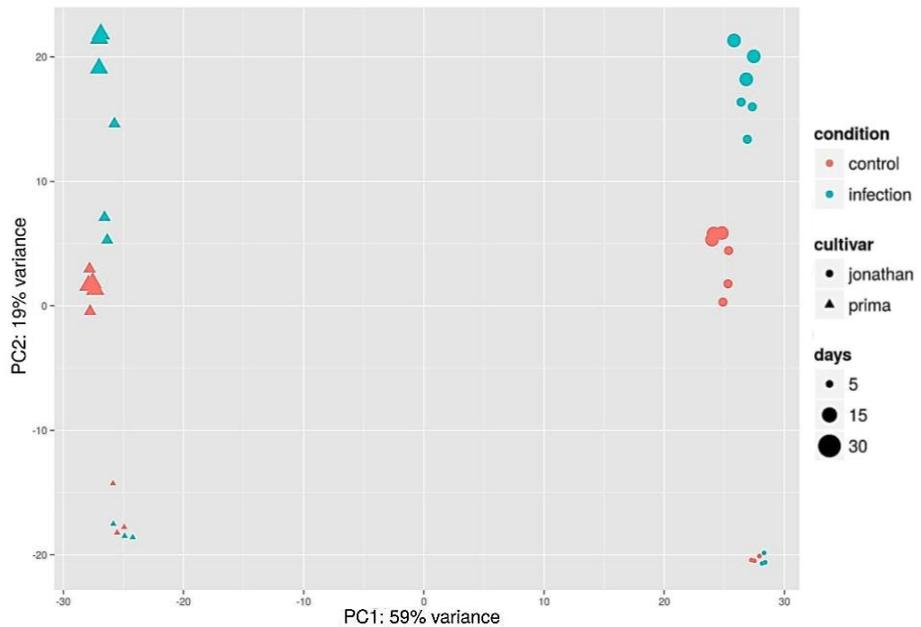
Figure 16. Principal component analysis of 36 samples using DESeq2 package, which normalized counts for 46157 genes (out of total of 63541).

The response of apple cultivars inoculated with *N. ditissima* also involves the up-regulation of genes involved in fortification of cell walls, detoxification, phosphorylation and pathogen defense.

The results of this study revealed differentially expressed genes at transcription level between a susceptible and a resistant apple cultivar, infected by *N. ditissima*, by utilizing RNA-Seq technology. In addition, it provided a wealth of information for further investigation in order to understand the pathogenicity mechanisms of different apple cultivars against fruit tree canker.
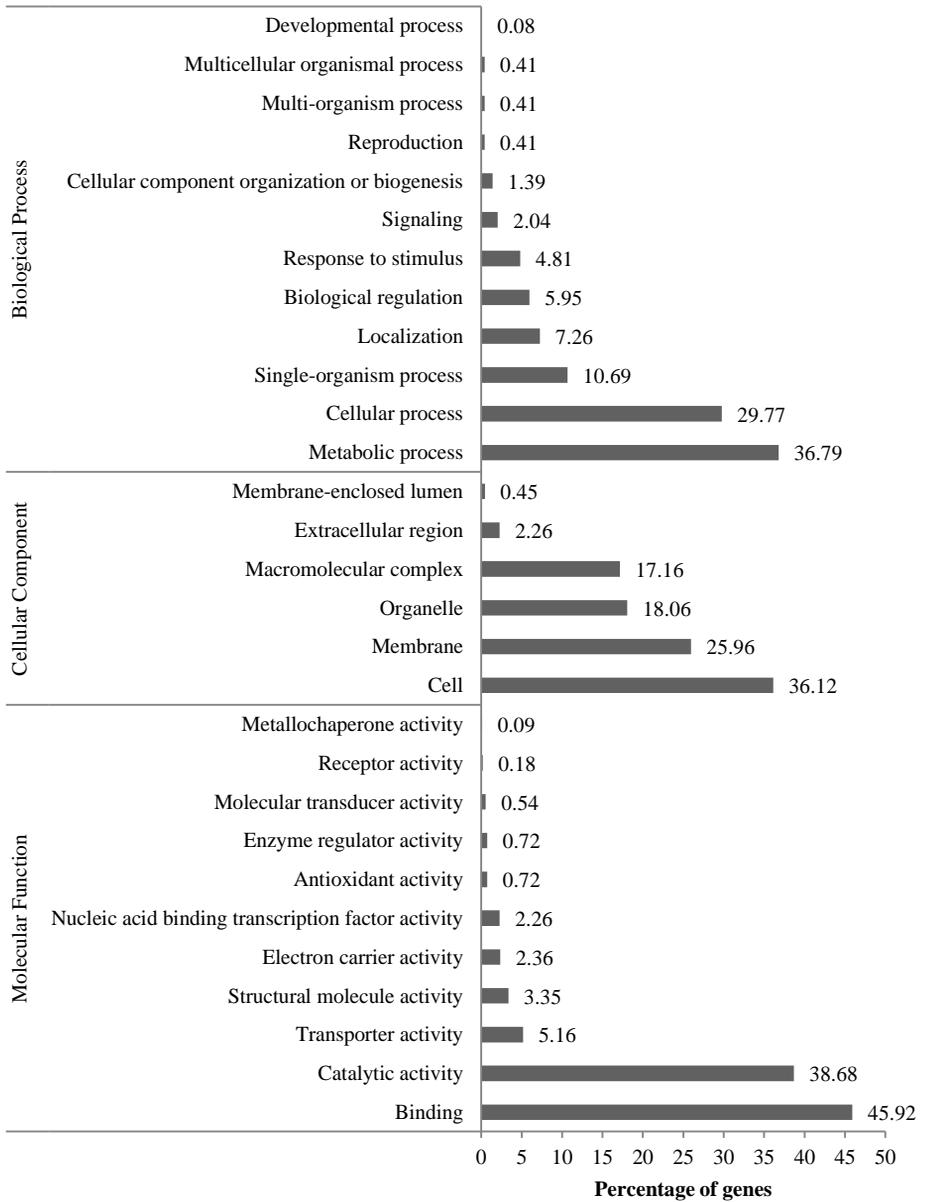
*Figure 17.* Gene ontology classification with cut off level 2 of differentially expressed genes.

5   Conclusions and future perspectives

The studies conducted within the framework of this thesis can be summarized in the following conclusions:

> Variation in levels of resistance to canker was observed among the investigated apple cultivars. This variation was estimated by different parameters: area under the disease-progression curve (AUDPC), incubation period and infection percentage. Two types of plant models, cut-off shoot and one-year-old tree were used to verify the results. Cultivar differences in resistance found in this study can be exploited in breeding programs.

> A qPCR assay was successfully developed, making it possible to detect and quantify *Neonectria ditissima* in apple cultivars using a species-specific primer pair. However, this assay is not sufficiently sensitive to detect the fungus in asymptomatic tissue. Since there was a relationship between the *N. ditissima* biomass and lesion size in different cultivars, this qPCR assay may be useful for investigating differences between resistant and susceptible cultivars.

> A light microscopy analysis of two infected apple cultivars showed that the fungal hyphae can penetrate into all cell types i.e., cortex, phloem, xylem and pith cells but the infection proceeds faster in susceptible cultivars resulting in a higher amount of fungal hyphae within a given time period. This indicates that level of resistance is not related to the anatomy of the apple woody tissue; instead other mechanisms, e.g., chemical defence, are more likely to be involved in canker resistance.

➤ The mating system of *N. ditissima* was investigated by application of AFLP analysis of single-ascospore isolates derived from the same perithecium. The results indicate that this fungus is heterothallic, i.e. outcrossing. However, a few isolates were identical, suggesting that homothallic reproduction may also occur.

➤ Neither SSR nor AFLP markers could detect a spatial pattern of genetic variation among single-ascospore isolates of *N. ditissima* collected in eight orchards, and differentiation between orchards was also quite restricted. Possibly high levels of gene flow due to long-distance transfer of plant material is one reason for the low differentiation and lack of an isolation-by-distance pattern, but high mutation rates can also play a role.

➤ A gene expression analysis with RNA-Seq data suggests that many genes involved in detoxification, lignification, phosphorylation and pathogen defense are differentially expressed in resistant and susceptible apple cultivars. Some genes with defense-related function, *e.g. Bet v I, MLO12*, *ACT7*, *NPR3* and *BAK1*, were induced in the partially resistant apple cultivar during infection.

➤ The observed changes in gene expression between the susceptible and resistant cultivars highlight possible candidate genes that may play a role in the partial resistance mechanisms of apple and increase our understanding about the molecular mechanisms that are involved against fruit tree canker.

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