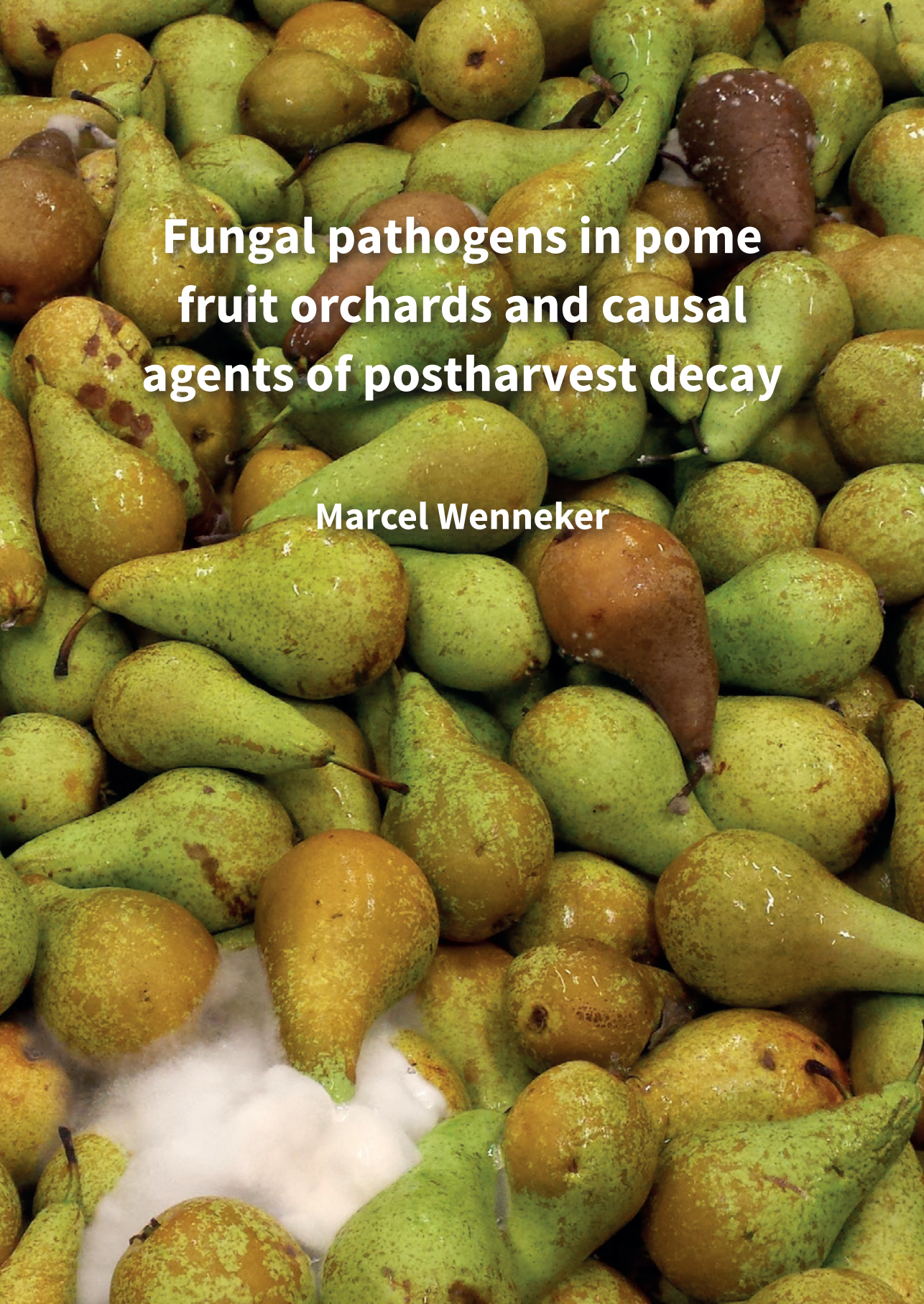

Fungal pathogens in pome fruit orchards and causal agents of postharvest decay

Marcel Wenneker

Propositions

1. Postharvest diseases should be regarded as complex problems that require systems intervention approaches for their control.
   (this thesis)

2. Adequate molecular detection of latent infections depends more on adequate sampling protocols than on the sensitivity of the detection technique.
   (this thesis)

3. Preventing inappropriate data analysis, such as occurs in p-hacking, is important to prevent obstruction of scientific progress.

4. Augmentative biological control conflicts with the objective to stimulate biodiversity.

5. If retailers and food producers are truly concerned about consumer health, they should focus on reducing salt and sugar content of food rather than on pesticide residues and GMOs.

6. The replacement of natural grass by synthetic turf deteriorates defending skills in soccer, especially concerning sliding tackles.

Propositions belonging to the thesis, entitled:

"Fungal pathogens in pome fruit orchards and causal agents of postharvest decay"

Marcel Wenneker
Wageningen, 25 February 2019

Fungal pathogens in pome fruit orchards and causal agents of postharvest decay

Marcel Wenneker

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Fungal pathogens in pome fruit orchards and causal agents of postharvest decay

Marcel Wenneker

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Contents

1.

General introduction and outline of the thesis

Introduction

The terms 'pome' fruit describes a major group of deciduous fruit-bearing tree species originating from the temperate zone of the northern hemisphere. In Europe, apple represents about 50% of total fruit production. Apple (*Malus domestica*) and pear (*Pyrus communis*) are the most common species of the pome fruit group in the Netherlands, with a total production of 353,000 and 349,000 tons, respectively, in 2014. The main apple and pear cultivars are Elstar and Conference, comprising 40 and 75% of the respective production areas (CBS, 2016).

There are a number of major diseases of pome fruit worldwide. Apple scab (*Venturia inequalis*), powdery mildew (*Podosphaera leucotricha*), European fruit tree canker (*Neonectria ditissima*), fire blight (*Erwinia amylovora*), and fruit rots are the main diseases of apple (McHardy, 1996; Sutton et al., 2014). The main diseases on pears are pear scab (*Venturia pirina*) and brown spot (*Stemphylium vesicarium*) (Llorente and Montesinos, 2006; Villalta et al., 2004).

Control of fungal diseases in commercial orchards currently depends on multiple applications of various fungicides. The routine fungicide programme used by fruit growers is mainly devoted to the control of apple scab and powdery mildew on apples, and brown spot of pears. Spray schedules with alternating use of active ingredients from several fungicidal groups are common (Manktelow et al., 1996). Several weather-based decision support systems are available for growers to support their decisions on the timing of specific fungicide applications (Holb et al., 2003; Llorente et al., 2000). However, governmental regulations restrict the use of fungicides (European Union, 2009). The increasing demand for fruit without, or with only low, pesticide residues is a reason why alternatives for synthetic fungicides are needed.

Integrated Pest Management (IPM) is a pest and disease control strategy that promotes the use of a variety of techniques and methods, including pest and disease resistant cultivars and biological, cultural, and physical control methods. Pesticides are employed in IPM, but only if monitoring, economic thresholds, or disease forecasts indicate a need. Thus, IPM programs in apple and pear are using pesticides more efficiently, but potential problems which cannot be overlooked include pesticide resistance build-up and the continued use of pesticides that are harmful to beneficial arthropods and insects (Desneux et al., 2007; Roubos et al., 2014). The use of specific pesticides instead of broad pesticides, and the reduced pesticide use for control of major pests may lead to an increased importance of minor pests (Damos et al., 2015).

Postharvest diseases of pome fruit

Fruit are stored in in regular atmosphere (RA) for short-term storage and in controlled atmosphere (CA) for long-term storage until packing. Pome fruit may remain for up to 12 months in storage, during which time fruit rot diseases may develop (Fig. 1). Despite the

use of fungicides and improved storage technologies, postharvest fruit rot diseases still remain an important limiting factor for the long-term storage of apples and pears.

Figure 1. Postharvest decay of pears.

Postharvest diseases of apple and pear result in significant economic losses during storage, and are caused by a range of fungal pathogens (Sutton et al., 2014). Multiple fungicide treatments before harvest are common to reduce the risk of postharvest diseases (Palm and Kruse, 2012). Owing to the earlier mentioned demand for fruit with a restricted number of chemical residues and low total residue levels, as well as for residue-free fruit (Boccaletti and Nardella, 2000; Magnusson and Cranfield, 2005; Ott, 1990), it is expected that risks of losses by postharvest diseases will further increase.

Classification of postharvest diseases of pome fruit

Postharvest diseases of pome fruit generally originate from two sources: infections through wounds at harvest or during the fruit handling and packing process, and latent fungal infections that occur in the orchards before harvest. Therefore, the classification of pome fruit diseases due to fungal pathogens is based on their mode of penetration in the fruit: (i) wound pathogens and (ii) latent infections.

Wound pathogens

All postharvest pathogens on pome fruit are potential wound pathogens. Wounds caused by insects and birds, as well as by physical damage before or during harvest, are an important entrance site for pathogens such as *Botrytis cinerea* (grey mold), *Penicillium expansum* (blue mold), *Mucor piriformis*, and *Monilinia fructigena* (brown rot) (Snowdon,

1990; Sutton et al., 2014). These pathogens typically cause a rapid decay of fruit in the pre- and postharvest stage. Fungicide applications shortly before harvest and careful handling of fruits during harvest are effective measures to reduce losses by wound pathogens.

Latent pathogens

Postharvest rots caused by latent pathogens result from infections that occur in the orchard but remain quiescent during the growing phase and remain unnoticed at harvest. Development and symptom expression takes place during storage. Common pathogens causing such late postharvest losses are able to infect fruits through lenticels, such as the *Colletotrichum acutatum* species complex (bitter rot) (Spolti et al., 2012), *Neofabraea alba* (Soto-Alvear et al., 2013), and *Neofabraea perennans* (Bull's eye rot) (Weber, 2009). Other pathogens infect apple cultivars that have an open blossom end (calyx) into the core and cause dry or wet core rot; mainly *Alternaria* spp. and *Fusarium* spp. (Niem et al., 2007; Sever et al., 2012). Eye rot and calyx end rot are caused by *Neonectria ditissima* and *Fusarium* spp. (Sever et al., 2012; Weber and Dralle, 2013).

Occurrence of postharvest diseases in Europe and the USA

Postharvest rot of pome fruit can be caused by a large number of fungi. The importance of each postharvest pathogen can vary from one country to another. According to a report published in 1931, 90 species of fungi are associated with fruit rots in stored apples, and more than 40 species in 22 genera are responsible for them in Washington State in the USA (Heald and Ruehle, 1931). Additional pathogens have been reported since that time (Edney, 1983). Incidences of different causal agents may vary depending on cultivar, climate during growing season and agricultural practices. This is further influenced by storage conditions, handling of products, and registered fungicides. Apple rot incidence may vary depending on cultivar (Sever et al., 2012; Weber, 2011) and harvest time (Børve et al., 2013).

Detailed surveys of postharvest diseases in pome fruit are not often conducted. In most cases postharvest losses are registered as 'rot', without specifications of the causal agent. Therefore, it is often unknown whether specific postharvest pathogens are recently introduced or have been present in a region for a long time (Kim and Xiao, 2008).

When surveys on postharvest rots are performed, different pathogens are detected. In a survey during storage in February and March in Latvia the total percentage of rotten apples in various cultivars varied from 3.6-58.9% (Grantina-Ievena, 2015). All major postharvest pathogens described in Northern Europe were detected. The most common apple fruit rot causing agents were *Neofabraea alba*, *Neofabraea malicorticis, Fusarium* spp*., Penicillium* spp., *Colletotrichum* spp., *Botrytis cinerea, Monilinia fructigena*. The species *Cadophora luteo-olivacea, Phomopsis velata* and *Alternaria alternata* were considered of minor importance based on the low incidences observed (Grantina-Ievena, 2015). In Norway, the most important storage diseases in organically grown apples were

caused by *Colletotrichum acutatum* (bitter rot) and *Neofabraea* spp. (Bull's eye rot), up to 64% and 30% respectively, from all rotten apples. Grey mould caused by *Botrytis cinerea, Fusarium* rot caused by several *Fusarium* species, brown rot caused by *Monilinia fructigena* and blue mold decay caused by *P. expansum* were found more rarely (Børve et al., 2013). Apple rot studies in Denmark and Germany in organic orchards or orchards not treated with fungicides after petal fall have shown that *Neofabraea alba* and *Neofabraea perennans* were the most common storage-rot fungi (up to 62%). Other fungi, such as *Neonectria galligena, M. fructigena, Cladosporium* spp., *P. expansum, Phacidiopycnis washingtonensis, C. acutatum, Gibberella avenacea, B. cinerea* were present in up to 5% of the samples (Maxin et al., 2012a). *Fusarium* rot was detected on 9 to 30% of apples depending on cultivar stored in Ultra Low Oxygen (ULO) conditions in Croatia (Sever et al., 2012). Blue mold decay caused by *P. expansum* was found on 30 to 60% of cold-stored apples in France, an important disease not only in other European countries but also in the USA (Morales et al., 2010).

New and emerging postharvest diseases in (Northern) Europe and the USA

During the past 50 years, substantial changes in the relative importance of different storage rot fungi have been recorded (Weber, 2011). Climate change may favour the development in more temperate areas of pathogens better known from warmer regions, such as *Glomerella acutata* and *Neofabraea alba* (Weber, 2009). Examples of new and emerging pathogens and diseaes are: *Diplodia seriata*, the cause of a preharvest rot of apples in northern Germany since 2007 (Weber, 2009; Weber and Quast, 2009); rubbery rot caused by *P. washingtonensis* in Northern Europe (Weber, 2011), *N. kienholzii* (de Jong et al., 2001; Michalecka et al., 2016), *Colletotrichum* spp. (Baroncelli et al., 2014; Ivic et al., 2013; Mari et al., 2012), *Phacidium lacerum* (Wiseman et al., 2016), and *Sphaeropsis pyriputrescens* (Xiao et al., 2004) in Europe and the USA. Besides climate change, international trading activities are an important factor contributing to the spread of new diseases (Anderson et al., 2004), as are changes in the use of fungicides and storage duration (Russell, 2005).

Isolation and identification of fungal fruit pathogens

Control of postharvest diseases is a challenging task because multiple diseases need to be targeted in order to produce decay-free or decay-limited fruit (Kim and Xiao, 2008). Robust identification of postharvest pathogens is the first necessary step for development and implementation of relevant measures for disease control. Postharvest pathogens that cause visible symptoms are often isolated on artificial media, such as potato dextrose agar (PDA). Their identification is typically based on characteristic morphological structures such as spore morphology, sporulation, and mycelial growth and morphology. This type of work needs specialists and requires considerable experience. Moreover, the identification of pathogens based on cultural characteristics can require a long time. An additional complication is that slowly growing pathogens might be overgrown by more

quickly growing saprophytic fungi on artificial media. Molecular techniques, which are used more often as a diagnostic tool, can provide results more rapidly and with greater accuracy when compared with conventional methods.

Identification of closely related species

Until relatively recently the taxonomy of plant pathogenic fungi relied exclusively on morphological characteristics. Within the past decade, the use of molecular techniques to sequence various gene regions has revolutionised fungal systematics. DNA sequence analysis has usually aligned with conventional taxonomy and has simplified the identification of closely related species that were difficult to identify using conventional techniques (Everett, 2014). For example, initially the *Colletotrichum* species complex was classified solely on morphological characteristics, which was considered unreliable (Damm et al., 2012). More recently multi-locus DNA sequence typing was used to define *Colletotrichum* species (Damm et al., 2012). With taxon-specific primers analysis isolates from different hosts, or different isolates from the same host, were grouped within different species complexes, e.g. *C. gloeosporioides* or *C. acutatum* (Damm et al., 2012). Based on multi-locus DNA sequence *Colletotrichum* species causing bitter rot or anthracnose on fruits have been identified and characterised: e.g. anthracnose disease of mango in Italy (Ismail et al., 2015), bitter rot of apple and pear in Croatia (Ivic et al., 2013), and *Colletotrichum* species associated with apple diseases in southern Brazil and Uruguay (Velho et al., 2014). Based on partial sequence analysis of the β-tubulin gene, fungal isolates causing bull's eye rot on apple in Poland could be classified into three species: *Neofabraea alba, N. perennans* and *N. kienholzii* (Michalecka et al., 2016).

Application of Koch's postulates

To establish a causative relationship between the isolated pathogen and the postharvest disease Koch's postulates have to be fulfilled. In most cases, Koch's postulates are applied using the basic principles of the methodology, i.e. inoculation is performed on surface-sterilized fruit with 20 µl of a spore suspension (10^5 conidia ml^{-1}) or mycelium prepared from 14-day-old PDA cultures after wounding the fruit with a needle or a manual cut with a sterile scalpel. Inoculated fruits are sealed in a plastic bag or box and incubated at temperatures optimal for symptom development. Symptoms are recorded every two days and compared with mock-inoculated controls. Fungal colonies are re-isolated from the lesions, cultured on PDA, and compared with the morphological characteristics of the original isolates. The identity of the re-isolations is also typically confirmed by sequencing. This methodology is described in a large number of first reports that concern novel postharvest pathogens (Garibaldi et al., 2010; Mari et al., 2012; Xiao and Rogers, 2004).

Control and management of postharvest disease

Postharvest diseases of pome fruit are currently largely controlled by pre- and postharvest handling practices and the application of synthetic fungicides. However, the deregistration of effective and widely used fungicides, the development of fungicide-resistant strains of postharvest pathogens, and the increase of Integrated Pest Management (IPM) and organic culture increased the demand to develop alternative control methods (Russell, 2005). That need is strengthened by consumer reluctance to chemical residues in food and public concern about synthetic fungicides.

Preharvest control

For preharvest treatment of postharvest diseases the strategy consists of several applications of fungicides. The number of active ingredients may vary per country depending on its legislation. The number of specific pre-harvest fungicide treatments against postharvest diseases is around two to four, mostly applied shortly before harvest. Groups of active ingredients with a dominant role in the control of postharvest diseases of apples and pears, such as tolylfluanide and benzimidazole (carbendazim, thiophanate-methyl), are now banned in many countries.

Postharvest control

It is crucial to operate carefully during harvest and postharvest handling in order to limit mechanical injuries that might act as entrance courts for wound pathogens. Alternative methods to pre- and postharvest fungicide treatments have been studied in order to prevent fruit losses in the postharvest phase, including biological control agents (BCA's), application of natural biocides, induction of natural defence mechanisms of harvested products, and genetic resistance (Jijakli and Lepoivre, 2004; Spadaro et al., 2003). Also the use of heat, ionising irradiation, ultraviolet C irradiation or CO_2 in physical treatments to control postharvest diseases has acquired increasing over recent years (Jijakli and Lepoivre, 2004; Tian, 2007).

Mari et al. (2007) reported that despite the substantial progress obtained with biological control agents (BCAs), these are still not routinely applied in the postharvest phase. The main drawbacks are insufficient and inconsistent performance of BCAs, difficulty in obtaining an adequate formulation and difficulty in controlling rot caused by latent infections. The use of plant bioactive compounds has shown that the treatment conditions (concentration, form of application, formulation, exposure time, time of treatment, etc.) should be established not only in relation to active substance and fungal pathogen, but also to fruit and vegetable response to treatment. Elicitors showed fungicidal activity that is sometimes inconsistent with fungistatic effects only, and related to treatment timing and the developmental stage of the plant.

Epidemiology and population dynamics of postharvest diseases

There is considerable knowledge on the epidemiology of the wound pathogens *Botrytis cinerea, Penicillium expansum* and *Monilinia fructigena*. In contrast, knowledge on the occurrence of the different postharvest diseases caused after quiescent infections during long-term storage and their epidemiology is very limited. There are different reasons for this lack of knowledge: (i) less attention was paid to several 'minor' pathogens as long as multiple broad spectrum fungicide applications controlled the build-up of pathogen populations in orchards; (ii) the differentiation of symptoms of the different diseases is not as clear as for the wound pathogens, resulting in limited data on the relative abundance of the different pathogens; and (iii) the detection of the pathogens in the orchard is difficult because they tend to grow slow on culture media in comparison to other pathogens, making that studies based on isolation and culturing techniques are laborious.

Quantitative real-time qPCR makes an accurate, reliable and high throughput quantification of target fungal DNA possible in various environmental samples, including host tissues, soil, water and air, thus opening new research opportunities for the study of diagnosis, inoculum threshold levels, epidemiology and host-pathogen interactions (Schena et al., 2004). Quantification of fungal populations in environmental samples using species-specific TaqMan PCR assays is a powerful tool to gain new insights in populations dynamics of pathogens (Sanzani et al., 2014). Research on disease epidemiology is needed to understand the relationships between the build-up of pathogen inoculum on the various substrates during time and infection periods for developing fruits in the orchard. This knowledge will allow estimating the relative importance of different substrates as inoculum sources for fruit infections. Recently, a specific TaqMan PCR technique has been developed to detect and quantify pear-pathogenic inoculum of *Stemphylium vesicarium* in pear orchards (Köhl et al., 2013). The assessment of inoculum potential in the orchard environment increases the efficacy of disease control methods. It can be assumed that disease control of postharvest diseases will improve with sanitation methods that reduce inoculum loads in the orchard; like apple scab and brown spot of pears. This knowledge can be used for the development of focussed sanitation measures (Holb, 2006; Gomez et al., 2007; Llorente et al., 2010). Understanding the role of microbial colonizers in competitive substrate colonization (Köhl et al., 2015) will allow the development of measures to stimulate beneficial components of microbiomes or to apply beneficial antagonistic strains to the relevant plant residues aiming at the suppression of pathogen colonization, survival and sporulation (Carisse and Rolland, 2004; Llorente et al., 2006; Rossi and Pattori, 2009).

Management of orchard diseases – case study European fruit tree canker

As stated before, European fruit tree canker is one of the most important diseases on apples. Fruit tree canker caused by *Neonectria ditissima* is a serious problem in apple-producing regions with moderate temperatures and high rainfall throughout the year;

especially in northwestern Europe, Chile, and New Zealand. The fungus produces two spore types: conidiospores and ascospores. Both spore types enter through wounds, either natural ones such as leaf scars and fruit scars, or artificial ones such as pruning wounds. Inoculum and points of entry on the tree are available all year around (Amponsah et al., 2015). The disease is most destructive in young trees infected with canker, as latent infections appear as systemic infections and trunk cankers several years after planting (McCracken et al., 2003) (Fig. 2).

In contrast to the orchard, the epidemiology of *N. ditissima* in the nursery is not understood and infected trees are rarely seen in nursery production, so it is assumed that the disease is present as latent infections (McCracken et al., 2003). Control measures are applied to protect primary infection sites, mainly leaf scars, from invasion by external inoculum. However, latent infections may occur when young apple trees are infected symptomless during propagation. Several molecular tools have been developed to detect *N. ditissima* (Ghasemkhani et al., 2016; Langrell, 2002; Langrell and Barbara, 2001). However, considering the wealth of possible infection sites within a single tree, these are not suitable for detecting latent infections in whole trees. The availability of a fast and reliable screening method for the occurrence of latent *N. ditissima* infections in apple and pear trees that can be used prior to planting in the orchard would contribute in developing strategies for the control of European fruit tree canker.

Figure 2. Fruit tree canker of apple caused by *Neonectria ditissima.*

Apple cultivars differ in their susceptibility to *N. ditissima*, though most modern cultivars are susceptible (Weber, 2014). Variation in resistance has been observed among apple cultivars (Garkava-Gustavsson et al., 2013; Kemp et al., 1999; Van de Weg et al., 1992). Although *N. ditissima* is mainly described as apple tree pathogen, also pear (*Pyrus communis*) occasionally suffers from severe incidences (Goos, 1975; Scheer, 1980). Control of *N. ditissima* is achieved through autumn and spring applications of fungicides to protect leaf scars and pruning cuts from infection (Cooke, 1999; Weber, 2014). Pruning of cankers, covering wounds with paint and cutting out of diseased wood are also important practices for disease control. However, despite these control measures the occurrence of epidemics cannot be prevented (Weber, 2014), partly because the most effective fungicides have been banned, and furthermore due to the introduction of recently developed very susceptible apple cultivars such as Kanzi and Rubens (Weber and Hahn, 2013). Recently, considerable attention is given to screening of apple cultivars for resistance to European canker (Ghasemkhani et al., 2015; Garkava-Gustavsson et al., 2016; Gómez-Cortecero et al., 2016). Breeding new cultivars with a high level of resistance to *N. ditissima* infection would be of great help towards a more sustainable apple production. Reliable methods for evaluating resistance across a substantial set of genotypes are required to enable directed breeding for resistance to this disease (Gómez-Cortecero et al., 2016). In order to perform in-depth studies of the genetic background to fruit tree canker resistance, reliable phenotypic data are needed for a substantial set of different genotypes. Therefore, suitable methods for scoring resistance should be developed.

Outline of this thesis

As fruit may be stored for an extended period, up to 12 months after harvest, postharvest diseases caused by various fungal pathogens can be a limiting factor for long-term storage.

In **Chapter 2** I present the results of packinghouse surveys of postharvest diseases on stored apples and pears conducted from 2012 to 2018. Decayed apple and pear fruits were sampled from commercial packinghouses, representing orchards of various apple and pear producing areas and cultivars in the Netherlands, and several novel postharvest pathogens were identified. This chapter is presented as a series of "first disease reports".

In **Chapter 3** I describe an important lenticel spot disease of pome fruit, caused by *Fibulorhizoctonia psychrophila*, in more detail. Typically, the causal pathogens of postharvest diseases that infect fruits during growing seasons and remain quiescent until disease symptoms occur after several months in storage. Epidemiological knowledge of these diseases is limited. However, knowledge on population dynamics is essential for the development of preventative measures to reduce risks of fruit infections during the growing season.

Chapter 4 describes Taqman PCR assays for quantification of *N. alba, N. perennans, C. malorum* and *C. luteo-olivacea* in environmental samples. Various host tissues, dead weeds and grasses, soil and applied composts were collected in ten apple and ten pear orchards and assessed for the presence of these fungi. The temporal dynamics of pathogens was followed in four apple orchards and four pear orchards.

Chapter 5 describes a novel method for screening of apple and pear trees at the nursery stage for latent fruit tree canker infections caused by *N. ditissima* to be used prior to planting in orchards. The method may also contribute in developing strategies for the control of European fruit tree canker.

As apple cultivars differ in their levels of susceptibility to *N. ditissima*, **Chapter 6** describes the appropriateness of two resistance parameters, i.e. infection frequency and lesion growth. Important criteria for such parameters are: (1) consistency across experiments, (2) sufficient resolution to reveal genetic differences between apple genotypes, (3) insensitivity to particular disease-specific artefacts, and (4) representation of distinctive components of resistance. Both parameters were evaluated in parallel tests using ten apple cultivars in three experimental years, applying semi-natural infection of leaf scars (infection frequency) or inoculation of artificial wounds (lesion growth). We compared six parameters for lesion growth, and present methods that can be used to develop strategies for the control of European fruit tree canker: e.g. in the breeding of new apple cultivars with high levels of resistance to *N. ditissima*.

The occurrence of dead dormant flower buds is a common phenomenon of economic importance in the major pear production areas of Europe. In **Chapter 7** I describe the research that was carried out to identify the causal agent of dead flower buds of pears and to develop and evaluate possible control strategies.

Finally, in **Chapter 8** I discuss the major results from the research that is described in this thesis and put these in a wider context. Eventually, I argue that postharvest diseases should be approached as complex problems that require multiple interventions at different stages of the disease process in a systems intervention approach for their control.

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2.

New and emerging postharvest diseases of pome fruit in the Netherlands

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Wenneker, M., Pham, K.T.K., Woudenberg, J.H.C., Thomma, B.P.H.J., 2018. First report of *Alternaria arborescens* species complex causing leaf blotch and associated premature leaf drop of 'Golden Delicious' apple trees in the Netherlands. Plant Disease 102: 1654.

Introduction

Apple (*Malus domestica*) and pear (*Pyrus communis*) are important pome fruit crops in the Netherlands, with a total production of 353,000 and 349,000 tons, respectively, in 2014. The main apple and pear cultivars are Elstar and Conference, comprising 40 and 75% of the respective production areas (CBS, 2016). Upon harvest, fruit are stored under specific controlled atmosphere (CA) conditions for up to 11 months, depending on the cultivar and volume to be marketed. Storage conditions are always a balance between the required quality demands and the prevention of physiological disorders (Van Schaik and Verschoor, 2003). Initially, CA storage technology was restricted to standard or traditional CA storage in which O_2 levels were maintained at about 2-3%. However, improvements in gas monitoring equipment and storage room structure have resulted in the development of several additional CA-based methods to improve quality maintenance.

Postharvest diseases caused by various fungal pathogens can be a limiting factor to long-term storage. The major postharvest diseases of pears and apples are caused by fungi. Postharvest diseases are the result of either latent infections that occur in the field during the growing season, or infections from wounds that occur during harvest and handling operations. Consequently, fungal pathogens associated with postharvest rots of pears and apples can be separated into two main groups: “latent” pathogens (e.g. *Neofabraea* spp.) and “wound” pathogens (e.g. *Botrytis* spp., *Penicillium* spp., and *Monilia* spp.).

The objective of the present study is to identify the causal agents and incidence of postharvest fruit rot diseases of apple and pear in the Netherlands. Therefore, packinghouse surveys of postharvest diseases in stored apples and pears were conducted from 2012 to 2018. Decayed apple and pear fruits were sampled from commercial packinghouses, representing orchards in various apple and pear producing areas and cultivars in the Netherlands. In total, approximately 350 samples were analyzed during the storage seasons from 2012 to 2018. In the following paragraphs the reports of new and emerging postharvest diseases are described.

First report of *Cadophora luteo-olivacea* causing side rot on ‘Conference’ pears in the Netherlands

Pear (*Pyrus communis*) is an important fruit crop in the Netherlands. Symptoms of side rot disease of pear fruits were first observed in 2008 on cv. Conference in storage in the Netherlands. Typical round to oval, dark-brown, and slightly sunken spots (size 0.5 to 1.0 cm in diameter) appeared after six or more months of cold storage under controlled atmosphere (Fig. 1). Lesions of rinsed pears were sprayed with 70% ethanol and tissue under the lesion was placed onto potato dextrose agar (PDA) at 20°C in the dark. Colonies obtained from single spores produced on PDA were flat, felty and cottony in the middle,

with smooth margins, an even edge, and varying in color from white turning to gray/black-olivaceous. Under UV light, ellipsoid or elongate conidia were produced (2.2 to 2.3 × 4.9 to 6.5 µm). Both cultural and morphological characteristics of the pathogen were similar to those described for *Cadophora* sp. (Spadaro et al., 2011). Three representative isolates (PPO 11-1228, PPO 24-1234, and PPO 107-1267) were sequenced using primers ITS1/ITS4 and EF1-728F and EF1-986R (Carbone and Kohn, 1999). MegaBLAST analysis revealed that the *ITS* sequences (GenBank accession nos. KT350591, KT350592, and KT350593) matched with 99.8 to 100% identity to *Cadophora luteo-olivacea* in GenBank (KU141394 and KU141395). The *TEF1* sequences (KT350597, KT350598, and KT350599) were 100% identical with many other culture collection *C. luteo-olivacea* sequences in GenBank (HQ661071 and KF764576) and only 71 to 80% to other *Cadophora* species isolated from pear (KT350601 and KT350602). Alcohol surface sterilized fruits were inoculated in pathogenicity tests in two ways: i) with an agar disk (10 mm diameter) with actively growing mycelium of *C. luteo-olivacea* prepared from a 14-day-old culture grown on PDA (isolates PPO 11-1228, PPO 24 1234, and PPO 107-1267); and ii) with 20 µl of a spore suspension (10^5 conidia ml^{-1}) prepared from a 21-day-old PDA culture after wounding with a needle (isolates PPO 11-1228 and PPO 107-1267). Both experiments were performed at 5 and 15°C, on 10 'Conference' pears per isolate-temperature combination. Inoculated fruits were sealed in plastic bags and were incubated in darkness. Typical symptoms appeared 7 to 14 days and 4 to 6 weeks later, for fruits incubated at 15 and 5°C, respectively. Mock-inoculated controls with water and PDA only controls remained symptomless. Fungi isolated from the lesions had morphological characteristics that resembled the original isolates from infected pears. The identity of the reisolations

Figure 1. Side rot of 'Conference' pears caused by *Cadophora luteo-olivacea.*

was confirmed as *C. luteo-olivacea* by sequencing, thus completing Koch's postulates. Side rot of long-term stored pears has first been reported in Oregon, United States (Bertrand et al., 1977). The primary causal fungus was identified as *C. malorum* (syn. *Phialophora malorum*) (Sugar and Spotts, 1992). Recently, a skin pitting disease of kiwifruit caused by *C. luteo-olivacea* has been reported from Italy (Spadaro et al., 2010). To our knowledge, this is the first report of side rot disease of pear fruits caused by *C. luteo-olivacea*.

First report of *Colletotrichum godetiae* causing bitter rot on 'Golden Delicious' apples in the Netherlands

Apple (*Malus domestica*) is an important fruit crop in the Netherlands, with a total production of 418,000 tons in 2011. Symptoms of apple bitter rot were observed on 'Golden Delicious' apples in the Netherlands in July 2013 after 9 months of storage in a packing house at controlled atmosphere. Lesions were round, 1 to 5 cm in diameter, gray and dry with acervuli, producing orange spore masses in concentric rings (Fig. 2). Fruit were rinsed with sterile water, and lesions were sprayed with 70% ethanol until droplet runoff. The skin was removed aseptically with a scalpel, and tissue under the lesion was isolated and placed onto Potato Dextrose Agar (PDA). The PDA plates were incubated at 20°C in the dark, and single-spore isolates were propagated on PDA. The isolates were identified as *Colletotrichum* sp. based on culture morphology, having light gray to pale orange mycelium and, when viewed from the reverse side, ranged from pink to reddish orange. The cultures carried yellowish spore masses and dark melanized structures similar to acervuli that oozed orange conidia. Conidia were cylindrical to fusiform, pointed at one or both ends, and measured 8.0 to 17.0 µm × 3.5 to 5.0 µm. Both cultural and morphological characteristics of the pathogen were similar to those described for *C. acutatum*, causal agent of bitter rot of apple. A representative isolate (PPO 44377) was used for multilocus gene sequencing (Damm et al., 2012). Genomic DNA was extracted using the LGC Mag Plant Kit (Berlin, Germany) in combination with the KingFisher method (Waltham, USA) and six loci were amplified and sequenced. Primer pairs ACT-512F + ACT-783R, CHS-354R + CHS-79F, GDF1 + GDR1, CYLH3F + CYLH3R, BT2Fd + BT4R, and ITS1 and ITS4 (White et al., 1990) were used for amplification of parts of the actin (*ACT*), chitin synthase (*CHS-1*) gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), histone H3 (*HIS3*), beta-tubulin (*TUB2*) and *ITS* region of the rDNA gene, respectively. One sequence for each locus has been deposited in GenBank under Accession Nos. KR180290 (*ACT*), KR180292 (*CHS-1*), KR180293 (*GAPDH*), KR180294 (*HIS3*), KR180295 (*TUB2*), and KR180296 (*ITS*). MegaBLAST analysis revealed that the *ITS* sequences matched with 98.9 to 100% identity to *Colletotrichum* spp. belonging to *C. acutatum* species complex (including *C. godetiae*). The phylogenetic trees constructed using sequences of *ACT*, *CHS-1*, *GAPDH*, *HIS3*, and *TUB2* of our strain (PPO-44377), and

available sequences from GenBank confirmed the identity of this strain as *C. godetiae*. Koch's postulates were performed on 15 'Golden Delicious' apples. Surface-sterilized fruit were inoculated with 20 µl of a spore suspension (10^5 conidia/ml) prepared from a 15-day-old PDA culture after wounding with a needle. Inoculated fruits were sealed in a plastic bag and were incubated in darkness at 20°C. Symptoms appeared after 4 to 6 days on 80% of the fruits while mock-inoculated controls with water remained symptomless. Fungal colonies isolated from the lesions and cultured on PDA had morphological characteristics that resembled the original isolate from the infected apples. There are few reports of symptoms associated with *C. godetiae* on apple in Europe (Baroncelli et al., 2014; Ivic et al., 2013; Munda, 2014). This is the first report of bitter rot caused by *C. godetiae* on apple fruit in the Netherlands. Currently, bitter rot is not an important disease in apples in the Netherlands. However, it is worldwide spread and is considered one of the most important diseases, causing considerable crop losses, and may become an emerging problem in the Netherlands in the near future.

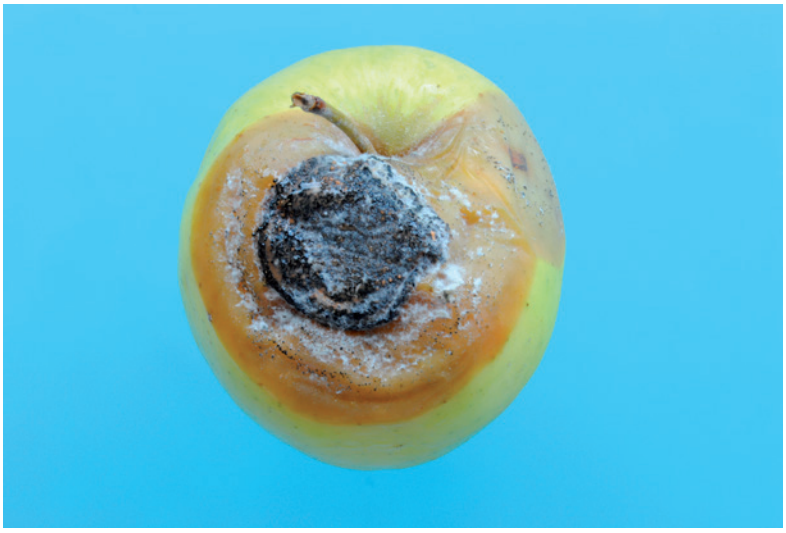

Figure 2. Bitter rot on 'Golden Delicious' apple caused by *Colletotrichum godetiae.*

First report of *Neofabraea kienholzii* causing bull's eye rot on pear (*Pyrus communis*) in the Netherlands

Pear (*Pyrus communis* L.) is an important fruit crop in the Netherlands, with a total production of 349,000 tons in 2014, and 'Conference' is the main cultivar. In the Netherlands, pears are kept in controlled atmosphere cold storage up to 11 months after harvest. Symptoms of bull's eye rot were observed in 2015 on 'Conference' pears in storage in the Netherlands. Bull's eye lesions

on apple and pear fruits are generally caused by four *Neofabraea* species: *N. alba* Jacks, *N. malicorticis* Guthrie, *N. perennans* Kienholz, and *N. kienholzii* Seifert, Spotts & Lévesque (Gariepy et al., 2005). *N. alba* is the major pathogen causing bull's eye rot on pear fruits in the Netherlands. Independent of the species, the symptoms appear as flat or slightly sunken lesions, which are brown, often lighter brown in the center (Spotts et al., 2009). To isolate the causal agent, fruit were rinsed with sterile water, lesions were sprayed with 70% ethanol until droplet runoff, the skin was removed aseptically with a scalpel, and tissue under the lesion was isolated and placed onto potato dextrose agar (PDA). PDA plates were incubated at 20°C in the dark, and single spores were transferred to fresh PDA plates. The isolates produced colonies with white-yellowish to brownish mycelium. Microconidia were produced on feathery fascicles of aerial mycelium, with a white, powdery, or sugary appearance on the surface of the agar colony. Microconidia were 2.5 to 6.5 × 1.5 to 2.5 µm, ellipsoidal, slightly asymmetrical to a curved form. The identity of a representative isolate (PPO 45010) was confirmed by means of multilocus gene sequencing. To this end, genomic DNA was extracted using the LGC Mag Plant Kit (Berlin) in combination with the Kingfisher method (Waltham, MA). Segments of the internal transcribed spacer region (*ITS*), 28S ribosomal RNA (*28S rRNA*) and beta-tubulin (*TUB2*) loci were amplified, sequenced with primers ITS1/ITS4, LR0R/LR5, and Btub2Fd/Btub4Rd (Chen et al., 2016), and deposited in GenBank under accession nos. KX424942 (*ITS*), KX424941 (*28S rRNA*), and KX424940 (*TUB2*). MegaBLAST analysis revealed that the *ITS*, *28S rRNA*, and *TUB2* sequences matched with 99 to 100% identity to *N. kienholzii* isolates in GenBank (KR859082 and KR859083 [*ITS*], KR858873 and KR858874 [*28S rRNA*], KR859288 and KR859289 [*TUB2*]). Alcohol surface sterilized fruits were inoculated in pathogenicity tests in two ways: (i) with an agar disk (10 mm diameter) with actively growing mycelium of *N. kienholzii* prepared from a 14-day-old culture grown on PDA; and (ii) with 20 µl of a spore suspension (10^5 conidia ml^{-1}) prepared from a 21-day-old PDA culture after wounding with a needle. Both experiments were performed on 10 'Conference' pears. Inoculated fruits were sealed in plastic bags and were incubated in darkness at 20°C. Typical symptoms appeared between 7 and 14 days. Mock inoculated controls with water and PDA-only controls remained symptomless. Fungi isolated from the lesions had morphological characteristics that resembled the original isolates from infected pears. The identity of these isolates was confirmed as *N. kienholzii* by sequencing, thus completing Koch's postulates. Bull's eye rot of apple and pear is an important postharvest disease, occurring in major fruit growing areas of North America, Chile, Australia, and Europe (Henriquez et al., 2004; Spotts et al., 2009). *N. kienholzii* was reported twice on apple in Europe (Michalecka et al., 2016). To the best of our knowledge, this is the first report of *N. kienholzii* causing bull's eye rot of pear in Europe.

First report of *Fusarium avenaceum* causing wet core rot of 'Elstar' apples in the Netherlands

Apple (*Malus domestica*) is an important fruit crop in the Netherlands. 'Elstar', the main cultivar, occupies 40% of the apple production area. Symptoms of apple wet core rot were observed on Elstar in January to March 2013 after 4 to 6 months storage in different packing houses at controlled atmosphere. The disease was present in a large number of lots harvested at orchards from different locations across the Netherlands, and incidences up to 25% were recorded. Apples exhibited light-brown wet rot, initially developing in the core and subsequently spreading into the surrounding cortex, often with a white to rose-reddish mycelium (Fig. 3). Apples from five lots with infections (four apples per lot; 20 in total) were rinsed with sterile water, sprayed with 70% ethanol until droplet runoff and halved aseptically with a scalpel. The tissue next to the core rot was isolated and placed onto potato dextrose agar (PDA). The PDA plates were incubated at 20°C in the dark, and single-spore isolates were propagated on fresh PDA plates. All cultures formed abundant white aerial mycelium with yellow to rose pigment and a dark pink to red reverse. Macroconidia were slightly falcate, thin-walled, usually 5 septate, with a tapering apical

Figure 3. Wet core of 'Elstar' apples caused by *Fusarium avenaceum.*

cell, and 40 to 80×3.5 to 5 µm. Pathogen characteristics were similar to those described for *F. avenaceum*, causal agent of wet core rot of apple (Sanzani et al., 2013; Sørenson et al., 2009). The identity of two representative isolates (Fu1-44145 and Fu2-44145) from different apple lots was confirmed by means of multilocus gene sequencing. Genomic DNA was extracted and sequences of *ITS* region, *TEF1* α, and histone H3 loci were amplified and

sequenced. The sequences have been deposited in GenBank (Accession Nos. KT350586 and KT350587 [*ITS*], KT350603 and KT350604 [*TEF1*], KT935567 and KT935568 [*HIS3*]). MegaBLAST analysis revealed 99.8 to 100% identity to *Fusarium* spp. belonging to the *F. avenaceum-F. tricinctum* species complex. The *TEF1* and *HIS3* sequences of both isolates were 100% identical with *F. avenaceum* sequences JQ429374, GQ915502, JQ435857, and GQ915469, confirming their identity as *F. avenaceum*. Koch's postulates were satisfied in two experiments, with 15 Elstar apples per isolate. In the first experiment, surface-sterilized fruits were inoculated with 20 µl of a suspension of 10^5 conidiospores/ml prepared from a 15-day-old PDA culture after wounding with a needle. Inoculated fruits were sealed in a plastic bag and incubated in darkness at 20°C. In the second experiment, apples were surface sterilized, cut in half longitudinally, and one half was inoculated with 20 µl of the conidiospore suspension into the core of the apple. Control apple halves were inoculated with sterilized water. After inoculation, the halves were covered with plastic foil and incubated in darkness at 20°C. In both experiments, symptoms appeared after 4 to 6 days on 100% of the fruits; controls remained symptomless. Fungal colonies isolated from the lesions and cultured on PDA plates morphologically resembled the original isolates. *Fusarium avenaceum* is a wound pathogen that has been isolated from apple fruit in Croatia and in the United States (Kou et al., 2014; Sever et al., 2012). Only few reports describe wet core symptoms associated with *F. avenaceum* on apple (Sanzani et al., 2013; Sørenson et al., 2009). This is the first report of wet core rot caused by *F. avenaceum* on apple fruit in the Netherlands. As wet core of apple is undetectable until the fruit is cut or consumed, it affects consumer confidence. Due to potential mycotoxin production during infection, *F. avenaceum* infections potentially pose a safety issue (Sørenson et al., 2009).

First report of *Fusarium avenaceum* causing postharvest decay on 'Conference' pears in the Netherlands

Pear (*Pyrus communis*) is an important fruit crop in the Netherlands, with a total production of 349,000 tons in 2014. 'Conference' is the main pear cultivar that occupies 75% of the total pear production area in the Netherlands. In the Netherlands, pears are kept in controlled atmosphere cold storage for 9 to 11 months after harvest. Lesions were observed on pears of the cultivar Conference in a survey carried out from 2012 to 2014 in packing houses in the Netherlands. In general, low incidences of 1 to 5% were recorded. Lesions showed brown and watery circular necrosis, were slightly sunken, often with visible whitish, yellowish, or pink mycelia covering the lesions (Fig. 4). Fruits were rinsed with sterile water, and lesions were sprayed with 70% ethanol until droplet runoff. The skin was removed aseptically with a scalpel, and tissue under the lesion was isolated and placed onto potato dextrose agar (PDA). The PDA plates were incubated at 20°C in the dark, and single spore isolates were propagated on fresh PDA plates. These isolates produced fast-growing colonies with extensive aerial mycelium which was initially white,

then turning yellow to pink and a dark pink to red reverse. Macroconidia were slightly falcate, thin-walled, usually 5 septate, with a tapering apical cell, and measured 40 to 80 × 3.5 to 5 µm. Both cultural and morphological characteristics of the pathogen were similar to those described for *Fusarium* spp.. The identity of two representative isolates (Fu5-44261 and Fu6-44280) from different pear lots was confirmed by means of multilocus gene sequencing. Genomic DNA was extracted using the LGC Mag Plant Kit (Berlin) in combination with the Kingfisher method (Waltham, MA). Sequences of *ITS* region, translation elongation factor 1-alpha (*TEF1*), and histone H3 (*HIS3*) loci were amplified and sequenced. The sequences have been deposited in GenBank under accession numbers KT350588 and 89 (*ITS*), KT350605 and 06 (*TEF1*), and KT935569 and 70 (*HIS3*). MegaBLAST analysis revealed that our *ITS* sequences matched with 99.8 to 100% identity to *Fusarium* spp. belonging to the *Fusarium tricinctum* species complex. The *TEF1* sequences of both isolates were 99 to 100% identical with *F. avenaceum* culture collection sequences in GenBank (JQ429374 and GQ915502). The *HIS3* sequences of both isolates were 97 to 99% identical with the sequences of accessions JQ435857 and GQ915469, confirming the identity of these isolates as *F. avenaceum*. Koch's postulates were performed on 15 'Conference' pears per isolate. Surface sterilized fruit were inoculated with 20 µl of a suspension of 10^5 macroconidia ml^{-1} prepared from a 15-day-old PDA culture, after wounding with a needle. Inoculated fruits were sealed in a plastic bag and incubated in darkness at 20°C. Symptoms appeared after 4 to 6 days on 100% of the fruits while mock-inoculated controls with water remained symptomless. Fungal colonies isolated from the lesions and cultured on PDA morphologically resembled the original isolate from the infected pears. Symptoms observed on artificially inoculated 'Conference' pear fruit were identical to the

Figure 4. Postharvest decay on 'Conference' pears caused by *Fusarium avenaceum.*

decay observed on 'Conference' pears that were obtained from cold storage. Only few reports describe symptoms associated with *F. avenaceum* on apple in Europe and the United States (Kou et al., 2014; Sever et al., 2012; Sørensen et al., 2009), while *F. avenaceum* infections of pears have not been reported. Thus, this is the first report of storage decay caused by *F. avenaceum* on pear. *F. avenaceum* infections may constitute a safety issue due to the potential production of mycotoxins such as moniliformin (Sørensen et al., 2009).

First report of *Neonectria candida* causing postharvest decay on 'Conference' pears in the Netherlands

Pear (*Pyrus communis*) is an important fruit crop in the Netherlands, with a total production of 349,000 tons in 2014, and 'Conference' is the main pear cultivar that occupies 75% of the total pear production area. In the Netherlands, pears are kept in controlled atmosphere cold storage up to 11 months after harvest. Occasionally, storage rots are observed when storage crates are contaminated with orchard soil. In a storage trial (2012 to 2013), boxes with 'Conference' pears were amended with soil particles from the same orchard from which the pears were harvested (four orchards), and stored for 11 months. Boxes without amended soil were included as controls. In contrast to the control boxes, up to 15% of the pears stored in boxes with soil particles showed typical rot symptoms (lesions) of an unknown causal agent. The lesions showed brown and watery circular necrosis, were slightly sunken, and displayed whitish to yellowish mycelia covering the lesions (Fig. 5). To isolate the causal agent, fruit were rinsed with

Figure 5. Storage rot caused by *Neonectria candida* on a 'Conference' pear.

sterile water, lesions were sprayed with 70% ethanol until droplet runoff, the skin was removed aseptically with a scalpel, and tissue under the lesion was isolated and placed onto potato dextrose agar (PDA). The PDA plates were incubated at 20°C in the dark, and single spore isolates were transferred to fresh PDA plates. These isolates produced fast-growing colonies with white-yellowish mycelium. Conidia were hyaline, cylindrical, 1 to 3 septate, and 15.8 to 26.4 × 5.3 to 7.9 µm. The fungus was morphologically identical to *Neonectria candida* (syn. *N. ramulariae;* anamorph *Cylindrocarpon obtusiusculum*) (Lombard et al., 2015). The identity of a representative isolate (VTN10Bs3) was confirmed by means of multilocus gene sequencing. To this end, genomic DNA was extracted using the LGC Mag Plant Kit (Berlin, Germany) in combination with the Kingfisher method (Waltham, USA). Sequences of the *ITS* region, translation elongation factor 1-alpha (*TEF1*), and actin (*ACT2*) loci were amplified, sequenced, and deposited in GenBank under accessions KU588183 (*ITS*), KU588186 (*TEF1*), and KU588184 (*ACT2*). MegaBLAST analysis revealed that our *ITS*, *TEF1*, and *ACT2* sequences matched with >99 to 100% identity to *N. candida* isolates in GenBank (KM249079 and JF735314 [*ITS*], JF735791 and HM054091 [*TEF1*], and KM231146 [*ACT2*]). Subsequently, Koch's postulates were performed on 15 'Conference' pears. Surface sterilized fruits were inoculated with 20 µl of a suspension of 10^5 conidiospores ml^{-1} water, prepared from a 15-day-old PDA culture, after wounding with a needle. Inoculated fruits were sealed in a plastic bag and incubated in darkness at 20°C. Symptoms appeared after 7 days on 100% of the fruits while mock-inoculated controls with water remained symptomless. Fungal colonies isolated from the lesions and cultured on PDA morphologically resembled the original isolate from the infected pears. Moreover, symptoms observed on artificially inoculated 'Conference' pear fruit were identical to the decay observed on 'Conference' pears that were obtained from the cold storage experiment. The identity of the reisolations was confirmed as *N. candida* by sequencing. *N. candida* (syn. *N. ramulariae*) is known as a globally distributed soilborne fungus (Domsch et al., 2007), but only few studies have identified the fungus as plant pathogen (Hirooka, 2012). This is the first report of *N. candida* causing storage rot of pears. Importantly, we note that the occurrence of storage rots may be enhanced by contamination of storage crates or fruit with orchard soil.

First Report of *Truncatella angustata* causing postharvest rot on 'Topaz' apples in the Netherlands

In the Netherlands, about 30% of the organic apple (*Malus domestica* Borkh.) production consists of apple scab resistant cultivars, such as Topaz and Santana. However, organic 'Topaz' apples show a high incidence of fungal rot after storage. Hot-water treatment (HWT) of freshly harvested apple fruit prior to long-term storage is an important strategy for the control of postharvest diseases, especially in the organic production sector (Maxin et al., 2012). The recommended treatment temperatures and times vary according to the

cultivar because of the risk of heat damage to the fruit peel. In January 2016, light peel damage caused by HWT was observed on 'Topaz' apples from an organic orchard. Also, up to 15% of the 'Topaz' apples showed typical rot lesions of an unknown causal agent. The lesions showed brown, irregular necrosis and were slightly sunken. To isolate the causal agent, fruits were rinsed with sterile water, lesions were sprayed with 70% ethanol until droplet runoff, the skin was removed aseptically with a scalpel, and tissue under the lesion was placed onto potato dextrose agar (PDA). The PDA plates were incubated at 20°C in the dark, and single spore isolates were transferred to fresh PDA plates. The colonies that appeared on PDA were cottony to woolly, dull white to brown in color, with black acervuli mainly in the center of the PDA plates. The isolates produced four-celled conidia, 16 to 19 × 7 to 9 µm, straight to slightly curved, with two brown to dark-brown median cells that had thick walls. More than one hyaline apical appendage, variable in size and branched dichotomically, were observed and a basal appendage was absent. The fungus was morphologically identical to *Truncatella angustata* (Pers.) S. Hughes (Sutton, 1980). The identity of two representative isolates (PPO 45246 and PPO-45321) was confirmed by means of gene sequencing. To this end, DNA was extracted using the LGC Mag Plant Kit (Berlin, Germany) in combination with the Kingfisher method (Waltham, MA). Sequences of the *ITS* region were amplified using primers ITS1/ITS4, sequenced, and deposited in GenBank under accession numbers KX085227 and KX085228. MegaBLAST analysis revealed that both of our *ITS* sequences matched 99% with *T. angustata* isolates in GenBank (EU342216, JX390614, and KF646105). Koch's postulates were fulfilled using 10 'Topaz' apples. Surface sterilized fruits were inoculated with 20 µl of 10^5 conidiospores ml^{-1} in water, prepared from a 15-day-old PDA culture of the isolate PPO 45246, after wounding with a needle. Inoculated fruits were sealed in a plastic bag and incubated in darkness at 20°C. Symptoms appeared after 7 days on 100% of the fruits while mock-inoculated controls with water remained symptomless. Fungal colonies isolated from the lesions and cultured on PDA morphologically resembled the inoculated isolates. The identity of the reisolations was confirmed as *T. angustata* by sequencing. *T. angustata* has a worldwide distribution and has also been reported to cause leaf spot on *Rosa canina* (Eken et al., 2009), canker and twig dieback on blueberry (*Vaccinium* spp.) (Espinoza et al., 2008), and fruit rot of olive (*Olea europaea*) (Arzanlou et al., 2012). To the best of our knowledge, this is the first report of *T. angustata* causing fruit rot of apples. Importantly, we note that the occurrence of this fruit rot may be enhanced by wounding, in this case as a result of hot water treatment.

First report of *Rosellinia quercina* causing postharvest decay on 'Conference' pears in the Netherlands

Pear (*Pyrus communis*) is an important fruit crop in the Netherlands, with a total production of 374,000 tons in 2016. 'Conference' is the main pear cultivar representing 75% of the total pear production area. In the Netherlands, pears are kept in controlled atmosphere cold storage up to 11 months after harvest. In 2017, symptoms of an unknown cause were observed in low incidences (<1%) on 'Conference' pears in storage from 4 different locations across the Netherlands. The symptoms appeared as yellow-brown circular lesions with distinct borders and a whitish centre, and were slightly sunken (Fig. 6). To isolate the causal agent, fruit were rinsed with sterile water, lesions were sprayed with 70% ethanol until runoff, the skin was removed aseptically, and tissue under the lesion was isolated and placed onto Potato Dextrose Agar (PDA). The PDA plates were incubated at 20°C in the dark. Pure cultures were obtained by transfer of hyphal tips onto fresh PDA plates. These isolates produced fast-growing colonies with white mycelium. Cultures did not produce any specialized structures associated with sexual or asexual reproduction. However, they presented the typical pear-shaped swelling immediately above the septum of mycelia, which is characteristic of the genus *Rosellinia* (Castro et al., 2013). The identity of 2 representative isolates (KP00109 and KP00113) was confirmed by means of gene sequencing. Genomic DNA was extracted using the LGC Mag Plant Kit (Berlin) in combination with the Kingfisher method (Waltham, MA). Segments of the internal transcribed spacer region (*ITS*) were amplified using ITS1/ITS4 primers (White et al., 1990) and deposited in GenBank, accession MG775691 and MG775692. MegaBLAST analysis revealed that the *ITS* sequences matched with 100% identity to reference *Rosellinia*

Figure 6. Symptoms of natural infections on 'Conference' pears caused by *Rosellinia quercina.*

quercina isolate ATCC36702 in GenBank (AB017661), 99% identity to *Rosellinia desmazieri* (AY805591), and only 89% with several isolates of *Rosellinia necatrix* (KF719201, AY909001, EF592568). Subsequently, Koch's postulates were performed on 15 detached 'Conference' pears. Experiments were carried out on surface-sterilized fruit. One side of the fruit was wounded using a sterile cork borer before inserting a mycelial plug (5 mm diameter) of an actively growing 7-day-old culture of *R. quercina* on PDA. Control inoculations were performed using plugs without mycelia. The inoculated fruit were covered in plastic bags and incubated in darkness at 20°C. Symptoms appeared after 7 days on 100% of the fruits while mock-inoculated controls remained symptomless. Fungal colonies isolated from the lesions on PDA morphologically resembled the original isolates. Moreover, symptoms observed on artificially inoculated fruit were identical to the decay observed on the fruit that were obtained from cold storage. The identity of the re-isolations was confirmed as *R. quercina* by sequencing. *Rosellinia* is a large and complex genus (Peláez et al., 2008). Among the best known root pathogens is the white root rot caused by *R. necatrix*, destructive to many fruit tree species including apple, cherry, peach, plum, pear, olive, and avocado (Pérez-Jiménez, 2006; Sun et al., 2008). *R. quercina* is known to cause root damage in young oaks (*Quercus pubescens*) (Peláez et al., 2008). This is the first report of *R. quercina* causing storage rot of pears. Possibly, fruits got contaminated through rain splash via the orchard soil or contaminated bins at harvest. Currently, the occurrence of *Rosellinia* spp. in the Netherlands is largely unknown. However, due to its ability to affect fruit crops attention is needed.

First report of *Alternaria arborescens* species complex causing leaf blotch and associated premature leaf drop of 'Golden Delicious' apple trees in the Netherlands

Apple (*Malus domestica*) is an important fruit crop in the Netherlands, with a total production area of 7,600 ha in 2016, and 1,600 ha of fruit tree nurseries. For decades, fruit growers and nurseries have reported a leaf blotch, characterized by irregular light brown spots, bordered by a dark brown to purple margin, and premature leaf drop, mainly on 'Golden Delicious'. The disease causes severe, sometimes almost complete defoliation. Leaf blotch typically appears in early July and is most severe in the latter part of the growing season. In a survey in August 2014, affected 'Golden Delicious' leaves were collected from different orchards and taken to the laboratory. Next, 20 leaves per location were rinsed with sterile water, sprayed with 70% ethanol until droplet runoff and rinsed again with sterile water, and tissue sections were excised from lesions using a sterile scalpel and placed onto potato dextrose agar (PDA). The PDA plates were incubated at 20°C in the dark, and single spore isolates were transferred to fresh PDA plates. These isolates produced fast-growing colonies of irregular shape, tan brown to black and felty. Sporulation patterns showed long conidiophores with extensive terminal

branching. Conidia were ovoid with a tapering apical beak and a size range of 10 to 30 × 5 to 10 µm, with 1 to 5 septa. The isolated fungi were morphologically identical to small spored *Alternaria* spp. (Simmons, 2007). However, small spored *Alternaria* spp. cannot be identified based on morphological characteristics (Woudenberg et al., 2015). The identity of 12 representative isolates from two different orchards in their 4th growing season, located in the central part of the Netherlands, was determined by multilocus gene sequencing. To this end, genomic DNA was extracted using the UltraClean Microbial DNA isolation kit (MoBio Laboratories, Carlsbad, CA). Sequences of the *ITS* region, the *endoPG* gene, and the anonymous region *OPA10-2* locus were amplified and sequenced as described in Woudenberg et al. (2013, 2015) and deposited under GenBank accession numbers MG744449–MG744460 (*ITS*), MG744473–MG744484 (*endoPG*), and MG744461–MG744472 (*OPA10-2*). MegaBLAST analysis revealed that our *ITS*, *endoPG*, and *OPA10-2* sequences matched with >99 to 100% identity to *Alternaria arborescens* species complex (SC) isolates in GenBank (AF347033 and KP124400 *[ITS]*, AY295028 and KP124104 [*endoPG*], and KP124712 and KP124714 [*OPA10-2*]). Subsequently, Koch's postulates for three *A. arborescens* SC isolates were performed in the laboratory on 'Golden Delicious' leaves. Surface sterilized leaves were inoculated on the abaxial side with 10 µl of a suspension of 10^5 conidiospores ml^{-1} water, prepared from a 14-day-old PDA culture, after wounding with a needle, with four inoculations per leaf. Inoculated leaves were sealed in a plastic box and incubated in darkness at 20°C. The experiment was carried out in five replicates. Symptoms appeared within 7 days on 100% of the leaves, while mock-inoculated controls with water remained symptomless. Fungal colonies isolated from the lesions cultured on PDA morphologically resembled the original isolate from the infected leaves. The identity of the reisolations was confirmed as *A. arborescens* SC by sequencing. To our knowledge, this is the first report of *A. arborescens* SC causing leaf blotch and subsequent premature leaf drop of apple cultivars in the Netherlands. Multiple *Alternaria* species groups are associated with leaf blotch diseases of apple in Australia and Italy (Harteveld et al., 2013; Rotondo et al., 2012). *A. arborescens*-like isolates are most prevalent in Australia and are mostly associated with leaf blotch symptoms (Harteveld et al., 2013).

Discussion

Postharvest diseases of apple and pear are caused by a range of fungal pathogens, and often result in significant economic losses during storage. In general, this group of pathogens infects developing fruits during the growing season and remains quiescent without causing symptoms during this season and even after harvest during the first weeks in storage. Typically, symptoms of disease occur after several months in cold storage under controlled atmosphere. Common pathogens causing such late postharvest losses are *Neofabraea* spp. (lenticel rot or bull eye's rot), *Neonectria galligena* (Nectria rot;

blossom-end rot), *Colletotrichum acutatum* species complex (bitter rot), *Phytophthora* spp., *Alternaria* spp., and *Stemphylium vesicarium*.

Our survey of apple and pear fruit lots in the Netherlands revealed a number of new and emerging postharvest diseases. The most important pathogens were *Cadophora luteo-olivacea* causing side rot on pears, and *Fibulorhizoctonia psychrophila* (see chapter 3 of this thesis) as the causal agent of lenticel spot on apples and pears. Also new problems were observed that are caused by several pathogens that were not earlier described to occur on apple or pear in the Netherlands, such as *Fusarium avenaceum* on pear and apple, *Neonectria candida* and *Neofabraea kienholzii* on pear, and *Colletotrichum godetiae* and *Truncatella angustata* on apple. These pathogen species seem to occur as emerging problems in the Netherlands, and it requires investigation of management practices and storage conditions to control them. These pathogens have in common that the observed rots start from a latent infection: the infection occurs in the orchard, but the pathogen lives quiescently in fruits for several months after harvest before symptoms of disease start to occur. *Fusarium avenaceum* is also a wound pathogen that has been shown to account for the majority of Fusarium rot on apple fruit in Croatia (Sever et al., 2012).

The epidemiology of the wound pathogens Bo*trytis cinerea, Penicillium expansum* and *Monilinia fructigena* is well known (e.g. Amiri and Bompeix, 2005; Elmer and Michailides, 2007; Xu and Robinson, 2000). In contrast, knowledge on the occurrence of the different postharvest diseases that occur after latent infections during long-term storage and their epidemiology is limited. The control of this complex of very diverse pathogens is difficult because infections may occur during the entire period from flowering until harvest (Aguilar et al., 2017; Weber and Dralle, 2013; Xu and Robinson, 2010). Even the biology of *Neofabraea* spp., the prevalent cause of bull's eye rot and one of the main postharvest diseases of apple, has not been studied in detail (Aguilar et al., 2017; Cameldi et al., 2016). Mummified fruits and the formation of cankers on wood has been described as be important inoculum sources of several of the postharvest pathogens (Beer et al., 2015; Gariépy et al., 2005; Henriquez et al., 2006; Sugar and Spotts, 1992; Weber, 2012). Preventative measures mostly aim at the reduction of the disease pressure by sanitation, including the removal of mummified fruit and the pruning of cankers.

Research on the epidemiology of new and emerging postharvest pathogens is needed to understand the relationships between the build-up of pathogen inoculum on the various substrates during time, and infection periods for developing fruits in the orchard. The development of TaqMan PCR to monitor pathogen populations, combined with careful and strategic collecting of environmental samples, will facilitate detailed studies of their epidemiology. With these tools the niches for survival and the sources for inoculum production of the pathogen can be identified, as well as the relative importance of different inoculum sources such as fallen leaves and fruit, but also herb plants in orchard lawns. This knowledge will allow estimating the relative importance of different

substrates as inoculum sources for fruit infections (Köhl et al., 2009). In a next step, this knowledge can be used for the development of focussed sanitation measures (Holb, 2006; Llorente et al., 2010). Data on spore flights and weather conditions for infection are essential for development of disease-forecasting models (Rossi et al., 2005).

After infection has occurred and the fruits are stored, the latent pathogens switch to an active infection strategy and cause decay provided that a suitable environment occurs concerning the ripening fruits (Prusky et al., 2013). The transition from quiescent to necrotrophic colonization of the pathogens is probably influenced by physiological changes in the fruit which occur during ripening, such as cell wall disassembly, and altered defence responses, but also the decline of active antifungal compounds (Prusky et al., 2007). Climacteric fruits, such as apple and pear, continue to undergo respiration and transpiration after harvest. These processes continue during cold storage at a slower rate, depending on storage conditions (Thompson, 1998). During quiescence, fungal growth and disease development are initially arrested, but will resume after certain physiological or biochemical cues in the host have been satisfied (Coates and Johnson, 1997; Lattanzio et al., 2001). However, the exact timing and cues causing this transition are unknown and should be the subject of future investigations.

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3.

Fibulorhizoctonia psychrophila is the causal agent of lenticel spot on apple and pear fruit in the Netherlands

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Abstract

In a survey for postharvest diseases of apples and pears in the Netherlands, an unknown postharvest fruit rot was observed. The disease appeared to originate from infected lenticels. A fungus was consistently isolated from the decayed fruits. The fungal pathogen was isolated on potato dextrose agar, and at low temperatures development of a fast-growing whitish mycelium was observed. Growth of this fungus was observed between 1 and 20°C with an optimum at 15°C, while incubation of mycelium at 25°C resulted in no growth. The isolates did not produce asexual or sexual spores. The isolates were characterized and identified by morphology and molecular phylogenetic analysis. Genomic DNA was isolated and amplified using ITS1-ITS4, EF1 and RPB2 primers, and BLAST searches in GenBank placed the fungus taxonomically in the genus *Fibulorhizoctonia*, with the highest matches to *F. psychrophila*. Pathogenicity of representative isolates from apple and pear fruit was confirmed under laboratory conditions. To the best of our knowledge this is the first report of *F. psychrophila* causing lenticel spot on apple and pear, and also the cause of a whitish mould on storage bins.

Introduction

Apple (*Malus domestica*) and pear (*Pyrus communis*) are important fruit crops in the Netherlands, with a total production of 353,000 and 349,000 tons, respectively, in 2014. The main apple and pear cultivars are Elstar and Conference, comprising 40 and 75% of the respective production areas (CBS, 2016).

Fruit are stored under specific controlled atmosphere (CA) conditions for up to 11 months, depending on the cultivar and volume to be marketed. Storage conditions are always a balance between the required quality demands and prevention of physiological disorders (Van Schaik and Verschoor, 2003). Initially CA storage technology was restricted to standard or traditional CA storage in which O_2 levels were maintained at about 2-3%. However, improvements in gas monitoring equipment and storage room structure have resulted in the development of several additional CA-based methods to improve quality maintenance. One of these methods is Dynamic Controlled Atmosphere (DCA) storage, which maintains O_2 levels below 1% for apples of cv. Elstar.

As fruit may be stored for an extended period, postharvest diseases caused by various fungal pathogens can be a limiting factor to long-term storage (Gariépy et al., 2005; Snowdon, 1990). In this study, packinghouse surveys of postharvest diseases on stored apple and pear fruit were conducted from 2012 to 2015. Decayed apple and pear fruit were sampled from commercial packinghouses, representing orchards of various apple and pear producing areas and cultivars in the Netherlands. The aim of the study was to determine the main causal agents of postharvest diseases of apple and pear fruit in the Netherlands.

Symptoms of lenticel spot were observed on fruit of apple cv. Elstar and pear cv. Conference during these surveys. Disease incidences ranged from very low to >25%. The symptoms started as small brown to black spots (1-5 mm^2) that originated from lenticels. The spots enlarged in a circular fashion and became sunken as the disorder progressed. The centre of the lesion was depressed, often with cracks and mycelium in the centre. One fruit could have several to many lesions. In prolonged cold storage the disease developed further, and gradually decay of the whole fruit occurred (Fig. 1a, b). In particular cases the lenticel spot was accompanied with mycelial or fungal growth on fruit and overgrowth of the (wooden) storage bins (Fig. 2a, b). The symptoms were normally expressed after a prolonged period of storage of at least 5-6 months at controlled atmosphere (CA). In previous reports from packinghouses, lenticel spot was observed, but at that time it was considered a physiological disorder and often related to DCA storage conditions (Wenneker et al., unpublished data). The experiments described were performed in order to reveal the identity of the causal agent of lenticel spots on apples and pears.

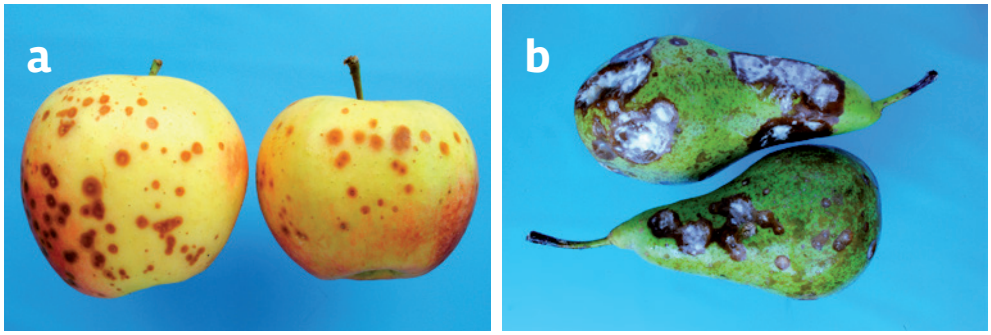

Figure 1. Lenticel spot symptoms on apple cv. Elstar (a) and pear cv. Conference (b).

Figure 2. A whitish mould on apples (a) and wooden storage bins (b) associated with lenticel spot.

In total 7, 1, 9, and 5 lots of apple fruit and 7, 0, 1, and 4 lots of pear fruit with symptoms of lenticel spot were analysed in 2012, 2013, 2014, and 2015, respectively. Mycelium samples were taken from wooden bins, with lenticel spot affected fruits, and covered with a whitish mould at three different storage locations.

Representative samples of 20 fruits were collected and used for isolation and further studies. Symptomatic fruit were transported to the laboratory, rinsed with sterile water, and sprayed with 70% ethanol until runoff. Small pieces of the fruit flesh from the border between healthy appearing and diseased tissue were placed onto potato dextrose agar (PDA). The PDA plates were incubated at 5 and 20°C in the dark.

Mycelial growth on PDA of two apple isolates (44640, 44656) and one pear isolate (44747) was evaluated at different temperatures. For that, a 5-mm diameter mycelial plug from the margin of 10-day-old cultures was placed in the centre of PDA plates. Inoculated plates were incubated in the dark at 1, 5, 10, 15, 20, and 25°C. The colony diameter

was measured after 4, 7, and 11 days on each of the plates. Three replicates for each isolate were used.

The identities of a representative isolate from apple (44640) and pear (44747) were confirmed by means of multi-locus gene sequencing. Genomic DNA was extracted using the LGC Mag Plant Kit (Berlin, Germany) in combination with the Kingfisher method (Waltham, USA). The ITS1-5.8S-ITS2 region, translation elongation factor 1-alpha (EF1) and DNA-directed RNA polymerase II subunit two (RPB2) loci were amplified and sequenced. Mycelium samples taken from the whitish mould covering storage bins containing infected apple or pear fruit were sequenced directly (ITS1-5.8S-ITS2 region), as the mycelium showed no growth after placing it on PDA.

Experiments were conducted to test pathogenicity of the fungus on apple and pear fruits. Three isolates of *Fibulorhizoctonia psychrophila*, two from apple (44640 and 44656) and one from pear (44747) were selected. Experiments were carried out on surface-sterilized fruit. One side of the fruit was wounded using a sterile cork bore before inserting a mycelium plug (10 mm diameter) of actively growing mycelium of *F. psychrophila* prepared from a 7-day-old culture grown on PDA. All three isolates were tested on apple, while the isolates 44640 (from apple) and 44747 (from pear) were tested on pear. The inoculated fruit and controls treated with PDA agar plugs only, were sealed in plastic bags and incubated in the dark at 10°C.

After placing the apple and pear tissue onto PDA at 5°C, white, rapidly growing colonies were observed, while incubation at 20°C resulted in no growth (Fig. 3). All 22 apple and 12 pear samples revealed the same fungus. Pure cultures were obtained by transfer of hyphal tips onto fresh PDA and incubating them at 5°C. Plates completely covered with the fungus were incubated at 5° and 20°C with 12/12 h photoperiod and near-UV light (Philips TL-D, 18W BLB). The isolates did not produce asexual or sexual spores. Optimal mycelial growth of the three isolates tested occurred at 15°C, and growth was absent at 25°C (Fig. 4).

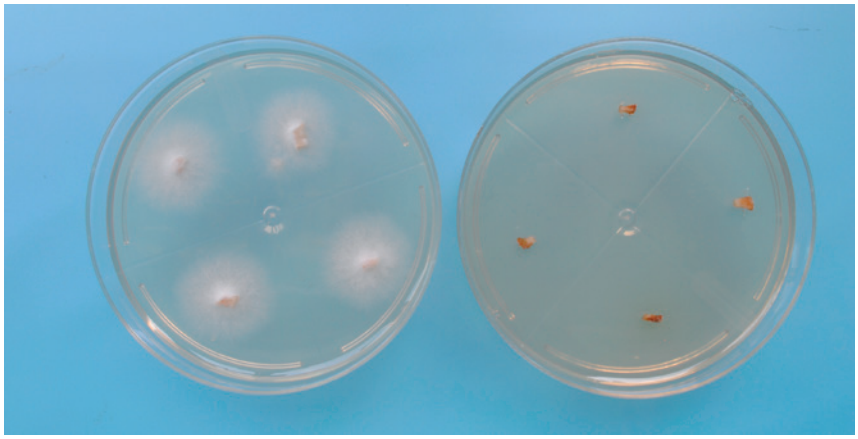

Figure 3. Isolations of lenticel spot from apple tissue after 7 days, incubated at 5°C (left) and 20°C (right).

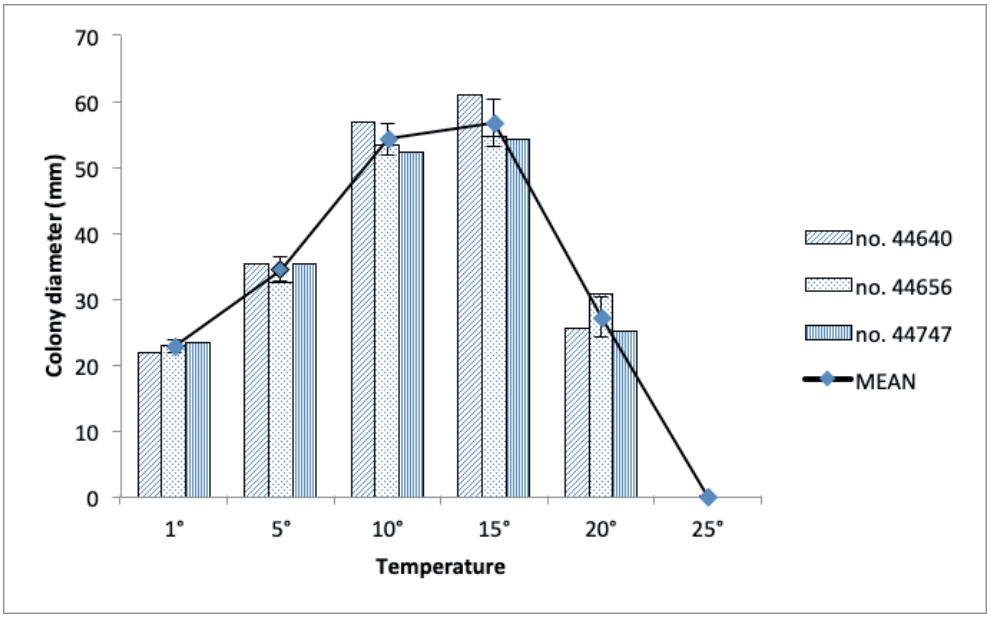

Figure 4. Colony size of three isolates of *Fibulorhizoctonia psychrophila* after 11 days at different temperatures grown on potato dextrose agar (PDA). Colony diameters are the average of three PDA plates for each isolate. The mean value is the average of the nine PDA plates. Error bars indicate the standard deviation.

The sequences of the isolate from apple (44640) and pear (44747) were deposited in GenBank under accession numbers KT223398 & KT223399 (ITS), KT962188 & KT962189 (EF1) and KT962186 & KT962187 (RPB2). MegaBLAST analysis revealed that the ITS sequences matched with *Fibulorhizoctonia* spp. (teleomorph: *Athelia* spp.), and more specifically with 99.3% identity to the *F. psychrophila* reference isolate of the CBS: 109695 from carrot (De Vries et al., 2008), confirming the identity of the isolates as *F. psychrophila*. ITS sequencing of *F. psychrophila* revealed 97.2%, 95.4 %, and 92.5% match to the ITS sequence of *Athelia arachnoidea* CBS:418.72 (GU187504), *Athelia epiphylla* CFMR:FP-100564 (GU187501), and *Fibulorhizoctonia* centrifuga CFMR 580 (U85790), respectively, indicating that *F. psychrophila* is closely related to these three species. EF1 sequencing of *F. psychrophila* revealed 86.6% and 86.4% match to the EF1 sequence of *A. arachnoidea* CBS:418.72 (GU187504), and *A. epiphylla* CFMR:FP-100564 (GU187501), respectively. RPB2 sequencing of *F. psychrophila* revealed 83.4% and 82.4% match to the RPB2 sequence of *A. arachnoidea* CBS:418.72 (GU187504), and *A. epiphylla* CFMR:FP-100564 (GU187501), respectively. The EF1 and RPB2 sequences of *F. centrifuga* CFMR 580 (U85790) were not available in GenBank. Sequencing of the whitish mould covering storage bins with lenticel spot affected apples and pears also confirmed the identity of this mould as *F. psychrophila*.

All apple and pear fruits inoculated with the fungus developed symptoms of decay 3 weeks after inoculation. Re-isolations from lesions consistently displayed growth and morphological characteristics identical to those previously described. ITS sequencing of the three isolates confirmed the identity of the causal agents. This indicates that apple and pear isolates of *F. psychrophila* are pathogenic to apple and pear fruit.

It may be concluded that the lenticel spot disease on apple cv. Elstar and pear cv. Conference was caused by the basidiomycete *Fibulorhizoctonia psychrophila*. Originally, this disease was classified as a physiological disorder, and sometimes directly related to new methods of long storage such as DCA. It is more likely that the expression of symptoms is related to the storage period, humidity and low temperatures. *F. psychrophila* also appears to be the whitish mould that may cause dense coverage of fruit and crates in storage. The identification of the causal agent might previously have been hampered due to the low optimal grow temperature of the fungus. It was not possible to isolate the fungus from infected apple material at 20°C as no fungal growth occurred. The decrease in growth of *F. psychrophila* at temperatures above 15°C suggests a strong adaptation of this fungus to cold biotopes. Currently, lenticel spot is considered one of the most serious postharvest diseases of cv. Elstar in the Netherlands.

There are a number of basidiomycete species reported from cold stored apple and pear fruit causing so-called fisheye rot or lenticel spot. Coprinus rot in cold storage affected several apple cultivars and cv. d'Anjou pears in British Columbia and Oregon (Meheriuk and McPhee, 1984; Spotts et al., 1981). The symptoms consisted of dry, dark brown lesions with tan centres, and importantly in all cases, advanced stages of decay were marked by masses of cottony, white mycelium that covered the surface of infected fruit and packing materials (Meheriuk and McPhee, 1984). Eventually, the fungus was identified as a new species, *Coprinus psychromorbidus* (Traquair, 1987). In The Netherlands, the appearance of a whitish mould in storage is often automatically associated with *C. psychromorbidus*, however the fungus was never found in our study.

Another type of fisheye rot of stored apple fruit has been reported from North America (Butler, 1930; Eustace, 1903), Canada (Weresub and Illman, 1980), and Poland (Bielenin, 1986). Eustace (1903) and Butler (1930) described the pathogenic species under the name *Corticium centrifugum*, which is now known as *Butlerelfia eustacei* (Weresub and Illman, 1980). This fungus was also reported as the causal agent of fish eye rot on pear fruit in The Netherlands (Stalpers and Loerakker, 1984). *Butlerelfia* is a fungal genus in the family Atheliaceae. *Athelia* species are well known as cold-tolerant spoilage fungi in other crops that are stored at low temperatures. According to Blumenfeld and Dobra (1986), *A. epiphylla* was isolated from pear fruit in Argentina.

Our study revealed that the isolated *F. psychrophila* from apple and pear fruit is closely related to the isolate of de Vries et al. (2008) that was described as a new carrot-spoilage fungus, detected in refrigerated storage facilities for carrots in The Netherlands. ITS sequencing of *F. psychrophila* revealed 95% identity to the ITS sequence of *A.*

arachnoidea and *A. carotae*, indicating that *F. psychrophila* is closely related to these two species. According to de Vries et al. (2008), the teleomorphs have not been observed in *F. carotae* and *F. psychrophila*. The optimal growth of *F. carotae* and *A. arachnoidea* was between 18 and 21°C. For *F. psychrophila* growth was observed between 0 and 20°C, with an optimum between 9 and 12°C, while 15 to 32°C resulted in no growth.

Currently, information about the epidemiology, the teleomorph, infection routes, and conditions of infection of apple and pear fruit by *F. psychrophila* is lacking. In general, *Athelia* spp. such as *A. arachnoidea, A. bombacina*, and *A. epiphylla* are described as active colonisers and decomposers of deciduous leaves and conifer needle residue, and thus their primary ecological role is as nutrient recycling agents (Adams and Kropp, 1996). Apparently, at some stage these species are also able to infect apple and pear fruit. Orchard litter, such as dead roots, dead leaves and dead stems of grasses and clovers from the orchard floor, were considered as potential sources of inoculum for *C. psychromorbidus* in postharvest rot of apple and pear fruit stored at low temperature in British Columbia (Sholberg and Gaudet, 1992). Possibly, orchard litter could also serve as a source of inoculum for lenticel spot of apples and pears.

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4.

Dynamics of postharvest pathogens *Neofabraea* spp. and *Cadophora* spp. in plant residues in Dutch apple and pear orchards

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[a] These authors contributed equally to this work.

Abstract

Postharvest diseases of apple and pear cause significant losses. *Neofabraea* spp. and *Cadophora* spp. infect fruits during growing seasons and remain quiescent until disease symptoms occur after several months in storage. Epidemiological knowledge of these diseases is limited. TaqMan PCR assays were developed for quantification of *N. alba*, *N. perennans, C. malorum* and *C. luteo-olivacea* in environmental samples. Various host tissues, dead weeds and grasses, soil and applied composts were collected in 10 apple and 10 pear orchards in May 2012. *N. alba* was detected in 73% of samples from apple orchards and 58% of samples from pear orchards. *N. perennans* was present in few samples. *C. luteo-olivacea* was detected in 99% of samples from apple orchards and 95% of samples from pear orchards. *C. malorum* was not detected in any sample. In apple orchards, highest concentrations of *N. alba* were found in apple leaf litter, cankers, and mummies, and of *C. luteo-olivacea* in apple leaf litter, mummies and dead weeds. In pear orchards, *N. alba* and *C. luteo-olivacea* were found in highest concentrations in pear leaf litter and in dead weeds. Substrate colonization varied considerably between orchards. The temporal dynamics of pathogens was followed in four apple orchards and four pear orchards. In apple orchards the colonization by pathogens decreased from April until August and increased after August until December. This pattern was less pronounced in pear. Knowledge on population dynamics is essential for the development of preventative measures to reduce risks of fruit infections during the growing season.

Introduction

Postharvest diseases of apple and pear result in significant economic losses during storage. Multiple fungicide treatments before harvest are common to reduce the risk of postharvest diseases (Palm and Kruse, 2012). There is a growing market demand for fruit with a restricted number of various residues and low total residue levels, as well as for residue-free fruit (Ott, 1990; Boccaletti and Nardella, 2000; Magnusson and Cranfield, 2005). In this situation it is expected that risks of losses by postharvest diseases will further increase and that more pathogen species may occur.

Postharvest diseases of apple and pear are caused by a range of fungal pathogens. Wounds caused by insects and birds, as well as by physical damage before or during harvest are an important entrance for several pathogens such as *Botrytis cinerea* (grey mould), *Penicillium expansum* (blue mould) and *Monilinia fructigena* (brown rot) (Snowdon, 1990). These pathogens typically cause a rapid decay of fruit in the pre- and postharvest stage. Fungicide applications shortly before harvest and careful handling of fruits during harvest are effective measures to reduce losses by wound pathogens.

Another group of pathogens infects developing fruits during the growing season and remains quiescent without causing symptoms during the growing season and during the first weeks in postharvest storage. Typically, symptoms of disease occur after several months in cold storage under controlled atmosphere. Common pathogens causing such late postharvest losses are *Neofabraea alba* (syn. *Phlyctema vagabunda*; lenticel rot disease; often also named bull's eye rot; Chen et al., 2016; Soto-Alvear et al., 2013), *Neofabraea perennans* (bull's eye rot; Weber, 2009), *Neonectria ditissima* (nectria rot, blossom-end rot; Weber and Dralle, 2013), *Colletotrichum acutatum* species complex (bitter rot; Spolti et al., 2012), *Phytophthora* spp., *Alternaria* spp., *Fusarium* spp. (Sever et al., 2012), *Cadophora malorum* (syn. *Phialophora malorum*; Sugar and Spotts, 1992) and *Stemphylium vesicarium* (Weber and Dralle, 2013).

Bull's eye rot of apple and pear is an important postharvest disease, occurring in major fruit growing areas of North America, Chile, Australia, and Europe (Henriquez et al., 2004; Spotts et al., 2009). Bull's eye lesions on apple and pear fruits are generally caused by four *Neofabraea* species: *N. alba, N. malicorticis, N. perennans* and *N. kienholzii* (Gariepy et al., 2005). *N. alba* is the major pathogen causing bull's eye rot on pome fruits in continental Europe (Henriquez et al., 2004; Michalecka et al., 2016). *N. perennans* is most frequently associated with bull's eye rot occurring on the west coast of the USA and Canada (Henriquez et al., 2004). In Europe its presence has been detected in the Netherlands, Germany and the UK (De Jong et al., 2001). The occurrence of *N. malicorticis* is associated with the disease symptoms on the west coast of the USA and Canada, and occasionally in Europe: Denmark, the Netherlands, Portugal (Verkley, 1999). The fourth causal agent of bull's eye rot disease, *N. kienholzii*, was detected in samples from Nova Scotia, Canada, and Portugal (Henriquez et al., 2004). *N. kienholzii* was reported twice on apple in Europe (Michalecka et al., 2016),

and recently for the time first to cause bull's eye rot of pear in Europe (Wenneker et al., 2017b).

C. luteo-olivacea is associated with a trunk disease of grapes (Halleen et al., 2007; Manning and Mundy, 2009; Gramaje et al., 2011), and is reported as the causal agent of skin pitting of kiwifruit (Spadaro et al., 2010). The occurrence of *C. luteo-olivacea* in the Netherlands causing side rot on pear has recently been reported (Wenneker et al., 2016f) whereas *C. malorum* (syn. *Phialophora malorum*) has been reported earlier as a side rot of pears in North America (Sugar and Spotts, 1992).

A survey of apple and pear fruit lots in the Netherlands in 2011-2013 revealed that one of the most important pathogens was *Neofabraea alba*, whereas *N. perennans* was found only occasionally (Wenneker et al., 2016a). The other most common pathogen was *Cadophora luteo-olivacea* that was mainly seen in pears (Wenneker et al., 2016f). Besides *C. luteo-olivacea*, a group of genetically distinct isolates of *Cadophora* was found consistently at low incidence which do not belong to a described *Cadophora* species (Wenneker, unpublished). In this study, *Cadophora* X is used to refer to this group of isolates. *C. malorum* was not found during this Dutch survey. Other pathogens such as *Fusarium* spp., *Alternaria* spp., and *Cladosporium* spp. were isolated at low frequencies and are considered of minor importance. New problems with sooty blotch and lenticel spot of apple were also noticed (Wenneker et al., 2017a) as well as several pathogens not earlier described in the Netherlands, such as *F. avenaceum* on pear and apple (Wenneker et al., 2016c, 2016e), *Neonectria candida* on pear (Wenneker et al., 2016d), and *Colletotrichum godetiae* on apple (Wenneker et al., 2016b). The survey conducted between 2011 and 2013 revealed also strong seasonal effects with different incidences and severities of the various postharvest rot causing pathogens in different years.

There is considerable knowledge on the epidemiology of the wound pathogens *Botrytis cinerea, Penicillium expansum* and *Monilinia fructigena*. In contrast, knowledge on the occurrence and epidemiology of the different postharvest diseases that generally only show symptoms after long-term storage is limited. There may be different reasons for this lack of knowledge: (i) less attention was paid to several 'minor' pathogens as long as multiple broad spectrum fungicide applications controlled the build-up of pathogen populations in orchards; (ii) the differentiation of symptoms of the different diseases is not as clear as for the wound pathogens, resulting in limited data on the relative abundance of the different pathogens; and (iii) the detection of the pathogens in the orchard is difficult because they tend to grow slowly on culture media in comparison to other pathogens, making studies based on isolation and culturing techniques laborious.

The control of this complex of very diverse pathogens is difficult because infections may occur during the entire period from flowering until harvest. Infection periods are often not clearly known and may differ between pathogens. Preventative measures aim at reduction of disease pressure by sanitation. Mummified fruits are considered as a main inoculum source and should be removed (Weber, 2012; Beer et al.,

2015). Cankers on wood can be important inoculum sources of several of the postharvest pathogens (Sugar and Spotts, 1992; Gariépy et al., 2005; Henriquez et al., 2006).

The objectives of the study were: (i) to develop tools for the quantitative species-specific detection of *N. alba, N. perennans, C. malorum* and *C. luteo-olivacea*, including the above mentioned *Cadophora* X (which is closely related but genetically distinct from *C. luteo-olivacea*), in environmental samples; (ii) to study the population dynamics of pathogens in orchards; and (iii) to identify major inoculum sources of the different pathogens. This knowledge on the disease epidemiology is essential for the development of preventative measures to reduce the risk of fruit infections during the growing season. Various types of necrotic plant tissues and soil were sampled during the growing season 2012 in 10 apple orchards and 10 pear orchards in The Netherlands. The colonization of the samples by the pathogens was quantified using the four newly developed pathogen-specific quantitative TaqMan PCR assays.

Materials and methods

Fungal isolates and development of TaqMan PCR assays

Fungal isolates of *Cadophora* and *Neofabraea* species were obtained from naturally infected apple and pear fruit. To isolate the causal agents, fruit were rinsed with sterile water, lesions were sprayed with 70% ethanol until droplet runoff, the skin was removed aseptically with a scalpel, and tissue under the lesion was isolated and placed onto potato dextrose agar (PDA; Oxoid). The PDA plates were incubated at 18°C in the dark, and single spore isolates were propagated on PDA.

Fungal isolates (Table 1) were grown at 18°C on PDA. Mycelium and spores of cultured isolates were scraped from the agar surface, freeze-dried and macerated by bead-beating for 10 s at 5000 beats per minute (bpm) in a ribolyser (Hybaid). Subsequently, DNA was extracted automatically on a KingFisher 96 Instrument (Thermolabsystems) using the sbeadex maxi plant kit (AGOWA; LGC genomics). DNA concentration was measured using PicoGreen in a fluorescence plate reader (TECAN). The isolates were identified by sequence analyses of the *ITS* region using the primer set ITS1 and ITS4 (White et al., 1990). The PCR products were purified and sequenced in both directions by Macrogen Europe. Sequence searches were performed in Genbank using *BLASTN* function. Four TaqMan-based real-time PCR assays were developed on internal transcribed spacer (*ITS*) or small subunit (*18S rRNA*) regions. The benefit of these multi copy genes is a lower detection level, in general 100 times, compared to a single copy gene. Primers and probes, locked nucleic acid -(LNA) or miner groove binder- (MGB) probe for optimum detection, for an ITS1 assay were designed for the simultaneous quantification of *C. luteo-olivacea* and *Cadophora* X and the species-specific quantification of *C. malorum*. For *Neofabraea* the ITS1 region shows too few differences to discriminate between the different species of *Neofabraea*, also the large subunit (LSU), RNA polymerase II subunit (*rpb2*) and tubulin (*tub*) could not be used (Chen et al., 2016), therefore

an 18S ribosomal RNA assay was designed for the species-specific quantification of *N. alba* and *N. perennans*. Primer Express 3.0.1 (Thermo Fisher Scientific) and visual OMP software 1.0 (Dnasoftware) were used for the design of primers and probes.

The specificity of the four developed TaqMan PCR assays was tested using 1 ng DNA of non-target fungi listed in Table 1. The test was considered as specific if, for the non-target fungi, no reactions or C_t values were measured that were higher than the C_t value for the target fungus at the lowest concentration of the dynamic range of the TaqMan PCR.

Table 1. Fungal isolates used in this study and their origin.

Fungal species	Isolate number	Host	Country	Year
Alternaria mali	T3e	*Lamium purpureum*	Netherlands	2014
A. mali	T6b	Unknown grass	Netherlands	2014
Aureobasidium pullulans	H2	Apple cv. Golden Delicious	Netherlands	2006
Botrytis cinerea	700	*Gerbera* sp.	Netherlands	2005
B. cinerea	1066	Unknown	Netherlands	2008
B. cinerea	T48a	*Ranunculus* sp.	Netherlands	2014
Cadophora luteo-olivacea	1228	Pear cv. Conference	Netherlands	2013
C. luteo-olivacea	1234	Apple cv. Elstar	Netherlands	2013
C. luteo-olivacea	1236	Apple cv. Elstar	Netherlands	2013
C. luteo-olivacea	1237	Pear cv. Conference	Netherlands	2013
C. luteo-olivacea	1241	Pear cv. Conference	Netherlands	2013
C. luteo-olivacea	1245	Pear cv. Conference	Netherlands	2013
C. luteo-olivacea	1246	Pear cv. Conference	Netherlands	2013
C. luteo-olivacea	1248	Pear cv. Conference	Netherlands	2013
C. luteo-olivacea	1249	Pear cv. Conference	Netherlands	2013
C. luteo-olivacea	1250	Apple cv. Elstar	Netherlands	2013
C. luteo-olivacea	1251	Apple cv. Elstar	Netherlands	2013
C. luteo-olivacea	1260	Apple cv. Elstar	Netherlands	2013
C. luteo-olivacea	1264	Pear cv. Conference	Netherlands	2013
C. luteo-olivacea	1265	Pear cv. Conference	Netherlands	2013
C. luteo-olivacea	1267	Pear cv. Conference	Netherlands	2013
C. luteo-olivacea	CBS 357.51	Apple	Italy	1950
Cadophora X	1225	Pear cv. Conference	Netherlands	2013
Cadophora X	1227	Pear cv. Conference	Netherlands	2013
Cadophora X	1240	Pear cv. Conference	Netherlands	2013
Cadophora X	1243	Pear cv. Conference	Netherlands	2013

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Cadophora X	1261	Pear cv. Conference	Netherlands	2013
Cadophora malorum	CBS 687.96	*Fagus sylvatica*	Netherlands	Unknown
C. malorum	CBS 864.69	Wood	Germany	1962
C. malorum	CBS 866.69	*Cyclamen* sp.	Netherlands	1964
C. malorum	CBS 858.69	PVC tubing	Germany	1969
C. malorum	CBS 165.42	*Amblystoma mexicanum*	Netherlands	Unknown
C. malorum	CBS 100591	Ornithogenic soil	Antartica	1996
C. malorum	1282	Apple cv. Elstar	Netherlands	2013
Cladosporium cladosporioides	H26	Apple cv. Jonagold	Netherlands	2006
Cladosporium herbarum	H27	Apple	Germany	2006
Coniothyrium cereale	H33	Apple	Germany	2006
Cryptococcus victoriae	H43	Apple	Netherlands	2006
Epicoccum nigrum	L45x	*Galium aparine*	Netherlands	2014
E. nigrum	T5d	Unknown grass	Netherlands	2014
Fibulorhizoctonia psychrophila	1235	Apple cv. Elstar	Netherlands	2013
F. psychrophila	1242	Pear cv. Conference	Netherlands	2013
F. psychrophila	1262	Apple cv. Elstar	Netherlands	2013
F. psychrophila	1268	Pear cv. Conference	Netherlands	2013
Fusarium avenaceum	IPO 92-3	*Triticum aestivum*	Germany	Unknown
Fusarium culmorum	IPO 90-283	*Hordeum vulgare*	Finland	Unknown
Kabatiella sp.	1269	*Zea mays*	Germany	2013
Neofabraea alba	1239	Pear cv. Conference	Netherlands	2013
N. alba	1252	Pear cv. Conference	Netherlands	2013
N. alba	1253	Pear cv. Conference	Netherlands	2013
N. alba	1255	Pear cv. Conference	Netherlands	2013
N. alba	1256	Pear cv. Conference	Netherlands	2013
N. alba	1259	Apple cv. Wellant	Netherlands	2013
N. alba	1263	Pear cv. Conference	Netherlands	2013
N. alba	1266	Apple cv. Pinova	Netherlands	2013
Neofabraea perennans	1231	Pear cv. Conference	Netherlands	2013
N. perennans	1232	Pear cv. Conference	Netherlands	2013
N. perennans	1238	Pear cv. Conference	Netherlands	2013
N. perennans	1247	Pear cv. Conference	Netherlands	2013
N. perennans	1257	Pear cv. Conference	Netherlands	2013
N. perennans	CBS 207-57	Apple	Netherlands	Unknown
Neonectria ditissima	H58	Apple	Netherlands	2006

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Penicillium expansum	P8b	*Triticum aestivum*	Netherlands	2014
Pezicula corticola	CBS 259.31	Unknown	Denmark	Unknown
Phialophora gregata	1230	Pear cv. Conference	Netherlands	2013
Phoma exiqua	T1a	*Taraxacon* sp.	Netherlands	2014
Phoma herbarum	J33a	Unknown grass	Germany	2014
Phoma macrostoma	L44d	*Papaver* sp.	Netherlands	2014
Phoma pinodella	H33	Apple	Germany	2006
Stagonospora sp.	H38	Apple	Germany	2006
Stemphylium vesicarium	933	Pear cv. Conference	Netherlands	2003
Trichothecium roseum	H17	Apple cv. Cox OP	Netherlands	2006
Venturia inequalis	CBS 813.69	Apple cv. Boskoop	Netherlands	1969

a The fungal strains starting with CBS were obtained from the Westerdijk Fungal Biodiversity Institute (CBS-KNAW, Utrecht, Netherlands). The other strains are from collections of Wageningen University & Research.

Orchards and sampling of plant tissues

Ten apple orchards and 10 pear orchards were selected in the main fruit growing areas of The Netherlands (Table 2). All orchards were managed commercially with applications of fungicides for disease control and herbicides for weed control.

Samples of various necrotic plant residues and tree parts were collected in the 20 orchards during the growing season of 2012 to investigate their role as potential inoculum sources for the fruit rot pathogens *N. alba, N. perennans, C. malorum* and *C. luteo-olivacea* including *Cadophora* X. In four apple orchards (nos. 1, 3, 7 and 8) and four pear orchards (nos. 13, 18, 19 and 20), samples were taken monthly from May until September and in December 2012. In all other orchards samples were taken only in May 2012. In the Netherlands, meteorological spring begins on 1 March, summer on 1 June, autumn on 1 September and winter on 1 December. The flowering period of apples (cv. Elstar) and pears (cv. Conference) is from Mid-April to Mid-May and the main harvest period of these cultivars is in September.

In each orchard, four plots were sampled. Plots in apple orchards consisted of a grass alley and two neighbouring tree strips with approximately 50 trees that were 25 m long and 3 m wide. In pear orchards, plots with approximately 40 trees had a width of 3.25 m. The following substrates were collected from five randomly chosen trees per row within the plots (if present in the orchard at the sampling date): 10 mummies, 10 cankers on twigs and 10 fruit spurs. From five randomly chosen sites within a sampling plot, the following substrates were sampled from the orchard floor into a 150 ml jar filled to the top: 10 segments (each 5–10 cm long) of prunings, residues of fallen apple or pear leaves from tree strips and grass alleys, residues of dead leaves of grasses from tree strips and grass alleys, residues of dead weeds from tree strips, soil of the 1 cm top layer from five sites in each tree strip, and compost ('champost', pasteurized compost used for mushroom production applied for soil improvement; approximately 100 ml) from five sites in each tree strip. For each type of substrate, samples taken within the same sampling plot were pooled so that four replicate samples for each orchard, substrate type and sampling date were available. Samples were stored at -18°C until processing.

Table 2. Origin of plant samples assessed for colonization by postharvest pathogens.

Orchard number	Place	Location	Cultivar
Apple orchards			
1	Dreumel	51°50'11"N 5°25'50"E	Pinova
2	Dreumel	51°50'11"N 5°25'50"E	Elstar
3	't Goy	52°00'05"N 5°13'18"E	Elstar
4	Noordeloos	51°54'14"N 4°56'40"E	Elstar
5	Wijk bij Duurstede	51°58'51"N 5°18'1"E	Elstar
6	Oosterhout	51°38'37"N 4°50'27"E	Kanzi
7	Jaarsveld	51°58'11"N 4°58'36"E	Elstar
8	Noordeloos	51°54'14"N 4°56'40"E	Pinova
9	Dronten	52°32'07"N 5°42'57"E	Topaz
10	Dronten	52°32'07"N 5°42'57"E	Elstar
Pear orchards			
11	Dreumel	51°50'11"N 5°25'50"E	Conference
12	Noordeloos	51°54'14"N 4°56'40"E	Conference
13	Werkhoven	52°00'11"N 5°16'27"E	Conference
14	Oosterhout	51°38'37"N 4°50'27"E	Conference
15	't Goy	52°00'05"N 5°13'18"E	Conference
16	Jaarsveld	51°58'11"N 4°58'36"E	Conference
17	Dronten	52°32'7"N 5°42'57"E	Conference
18	Ressen	51°59'20"N 5°16'47"E	Conference
19	Jaarsveld	51°96'93"N 4°97'50"E	Conference
20	Wijk bij Duurstede	51°58'51"N 5°18'01"E	Conference

Sample processing and TaqMan PCR assays

After storage, frozen samples were shredded. A subsample of approximately 150 ml was weighed and freeze-dried to assess dry matter content. Then the dried material was pulverized in a laboratory mill with a 1-mm mesh sieve (Cyclotec 1093 Sample Mill). Powdered subsamples were stored at -18°C until DNA extraction. DNA was extracted from subsamples of approximately 50 mg using the sbeadex maxi plant kit and the Kingfisher 96 Instrument with the following modifications. Lysis was at 65°C for one hour with 500 µl lysis buffer. After centrifuging 100 µl supernatant was used in the further protocol. Separate PCR reactions were performed in a 384 well format in CFX 384 (Biorad) Real-Time PCR Detection System to quantify *N. alba, N. perennans, C. malorum, C. luteo-olivacea/Cadophora* X and green fluorescent protein (GFP) serving as an amplification control (AC) (Klerks et al., 2004). For each TaqMan-PCR reaction of *N. alba* and *N. perennans*, a 0.8 µl sample was mixed with 9.2 µl reaction mix containing 5 µl Premix Ex Taq (Takara Bio Inc.), 200 nM fluorescein (FAM)-labelled probe and 300 nM of each forward and reverse primer (Table 3). For *C. malorum* a 0.8 µl sample was mixed with 9.2 µl reaction mix containing 5µl Premix Ex Taq, 100 nM FAM labelled probe with A and 50 nM FAM labelled probe with C and 300 nM of each forward and reverse primer (Table 3). For *C. luteo-olivacea*, a 0.8 µl sample was mixed with 9.2 µl reaction mix containing 5 µl Premix Ex Taq, 100 nM FAM labelled probe and 50 nM of forward and 900 nM reverse primer (Table 3). A 10-fold serial dilution ranging from 400 pg to 40 fg of DNA of *N. alba* isolate 1259, *N. perennans* isolate 1247, *C. malorum* isolate 866.69 and *C. luteo-olivacea* isolate 1251 was included in each 384-well plate for reference. If measurements of the AC amplification indicated inhibition of TaqMan-PCRs, measurements were repeated after 2- and 10-fold dilution of the sample. The concentrations of extracted pathogen DNA in the samples were calculated from the derivative cycle threshold values (C_t values) of TaqMan-PCRs for the DNA dilution series and for DNA extracts of plant samples, and expressed as pg DNA of pathogen DNA per mg plant residue (dry weight).

Table 3. TaqMan PCR primers and probes used for species-specific quantification of *Neofabraea alba, N. perennans, Cadophora luteo-olivacea/Cadophora* X and *C. malorum* in environmental samples.

Fungal species	Forward primer (5'-3')	Reverse primer (5'-3')	TaqMan probe (5'-3')
Cadophora malorum	accgaccttctccgaa	cccaccgaagcttgcca	acggctcga-ZEN-aaccccacgad
C. luteo-olivacea/ Cadophora X	ggktcgacgrctctaaaccb	gccccacagragcttgctb	taccgaagtagggtagcce
Neofabraea alba	acgattaaactagagttatatatgagaaagtc	ggttgctttcgcctttaccac	tAaattCGAtgataccaTgf
N. perennans	tcctcgtggtatcacagaatttcab	ccaaagtaggatacggttagactab	aaatactcaccttcttaaatataace

[a] Primers for *C. malorum* from Integrated DNA Technologies.
[b] Primers for *C. luteo-olivacea/Cadophora* X and *N. perennans* from Applied Biosystems.
[c] Primers for *N. alba* from Biolegio.
[d] Probe with an internal quencher (ZEN) labelled at the 5' with a fluorescein label, 6-FAM and at the 3' with Iowa black FQ (Integrated DNA Technologies).
[e] Labelled at the 5' with a fluorescein label, 6-FAM, and at the 3' with MGB (Applied Biosystems).
[f] Labelled at the 5' with a fluorescein label, 6-FAM and at the 3' with BHQ1 (Biolegio); LNA nucleotides in uppercase letters.

Results

Development of TaqMan PCR assays

Primers and probes for the species-specific quantification of *Neofabraea alba, N. perennans* and *C. malorum*, and the quantification of *Cadophora luteo-olivacea/Cadophora* X are given in Table 3. The specificity of the TaqMan PCR assays developed for the detection of *C. luteo-olivacea/Cadophora* X, *C. malorum, Neofabraea alba* and *N. perennans* was first evaluated in silico. BLAST search in Genbank (NCBI) with the species-specific probes for *C. luteo-olivacea* or *C. malorum* gave a 100% match with the target and no match with seven other closely related *Cadophora* species (Travadon et al., 2015). Blast search with the *N. alba* specific probe showed a 100% match with the target and no match with the closest related *Neofabraea* species. In contrast, the *N. perennans* probe has a 100% match with sequences of *N. malicorticis* and *N. populi*. In the entire *N. perennans* amplicon there was only one nucleotide difference in the reverse (Rv) primer area with *N. populi*, and the *N. malicorticis* amplicon is identical, so that this TaqMan PCR detects *N. perennans, N. malicorticis* and *N. populi*. Blast search with the species-specific probes for the four developed targets gave no match with the whole genome of *Malus domestica* (biosample SAMN02981243). The in silico validation was confirmed by a TaqMan PCR for *Cadophora luteo-olivacea/ Cadophora* X using a test panel of 16 *C. luteo-olivacea* isolates and five isolates considered as *Cadophora* X originating from different locations (Table 1) and all isolates of non-target fungal species including *Cadophora malorum* listed in Table 1. A positive TaqMan PCR result was found with all 16 *C. luteo-olivacea* isolates including the five isolates of *Cadophora* X. Calibration curves of the TaqMan PCR developed for detection of *C. luteo-olivacea/Cadophora* X were obtained for *C. luteo-olivacea* isolate 1251 (Table 4). No TaqMan PCR reaction or Ct values higher than the Ct value for *C. luteo-olivacea/ Cadophora* X at 40 fg (Table 4) were obtained for the all non-target isolates. The specificity of the TaqMan PCR developed for the detection of *C. malorum* was confirmed by testing a panel of seven *C. malorum* isolates originating from different locations (Table 1) and all isolates of the non-target fungal species including 16 isolates of *C. luteo-olivacea* and five isolates of *Cadophora* X listed in Table 1. A positive TaqMan PCR result was found with all seven *C. malorum* isolates. Calibration curves of the TaqMan PCR developed for detection of *C. malorum* were obtained for *C. malorum* isolate 866.69 (Table 4). No TaqMan PCR reaction or C_t values higher than the C_t value for *C. malorum* at 4 fg were obtained for all non-target isolates. The specificity of the TaqMan PCR developed for the detection of *N. alba* was confirmed by testing a panel of eight *N. alba* isolates originating from different locations (Table 1) and all isolates of the non-target fungal species including six isolates of *N. perennans* listed in Table 1. A positive TaqMan PCR result was found with all eight *N. alba* isolates. Calibration curves of the TaqMan PCR developed for detection of *N. alba* were obtained for *N. alba* isolate 1259 (Table 4). No TaqMan PCR reaction or C_t values higher than the C_t value for *N. alba* at 40 fg were obtained for all non-target isolates. The

specificity of the TaqMan-PCR developed for the detection of *N. perennans* was confirmed by testing a panel of six *N. perennans* isolates originating from different locations (Table 1) and all isolates of non-target fungal species including *N. alba* (eight isolates) listed in Table 1. A positive TaqMan PCR result was found with all six *N. perennans* isolates. Calibration curves of the TaqMan PCR developed for detection of *N. perennans* were obtained for *N. perennans* isolate 1247 (Table 4). No TaqMan PCR reaction or C_t values higher than the C_t value for *N. perennans* at 40 fg were obtained for all non-target isolates.

Table 4. Calibration curve characteristic of TaqMan PCR assays for the detection of *Cadophora luteo-olivacea, C. malorum, Neofabraea alba* and *N. perennans*.

Species	Isolate	Dynamic range	C_t value for lowest concentration	Slope	R^2
C. luteo-olivacea	1251	40 fg-400 pg	34	3.7	0.95
C. malorum	CBS 866.69	4 fg-400 pg	36	3.5	0.99
N. alba	1259	40 fg-400 pg	34	3.4	0.98
N. perennans	1247	40 fg-400 pg	35	3.5	0.98

Colonization of different substrates by postharvest pathogens

Cankers, prunings, fruit spurs and soil samples were collected in four replicates in the 10 apple and 10 pear orchards (Table 5). The other substrates were not present in the sampled plots of all orchards, so fewer samples were available. Fruit mummies were abundant in all apple orchards, except in orchard 6 ('Kanzi'). This was in contrast to pear orchards, where mummies were found only in two orchards. Pear leaf litter was collected in nearly all pear orchards, but apple leaf litter was available from only four apple orchards. Dead grass was collected in half of the apple and pear orchards. Dead weeds were present in the majority of the sampled plots. Champost had only been used in two apple orchards and three pear orchards.

N. alba was detected in 73% of the samples from apple orchards and 58% of the samples from pear orchards (Table 5). *N. perennans* was not present in any of the substrate samples collected in pear orchards. In apple orchards, *N. perennans* was detected in only six out of 264 samples, from dead apple leaves, cankers, mummies and prunings, all at low concentrations. *C. luteo-olivacea/Cadophora* X was detected in 99% of the samples from apple orchards and 95% of the samples from pear orchards. *C. malorum* was not detected in any of the samples.

In apple orchards, the highest concentrations of *N. alba* were found in apple leaf litter, cankers, and mummies (Table 5; Fig. 1). Lowest concentrations were present in soil, champost, and dead grass. In pear orchards, *N. alba* was most frequently found in dead weeds, pear leaf litter, dead grass, and cankers, with highest concentrations in dead weeds and pear leaf litter. *C. luteo-olivacea/Cadophora* X was present in apple orchards

Table 5. *Neofabraea alba* (Na), *N. perennans* (Np) and *Cadophora luteo-olivacea* (Clo)/ *Cadophora* X colonization of different substrates sampled in Dutch apple and pear orchards in May 2012.

		Na			Np			Clo/*Cadophora* X		
			pg DNA of Na mg^{-1}			pg DNA of Np mg^{-1}			pg DNA of Clo mg^{-1}	
Substrate	Total no. of samples	No. of samples with Na	Mean[a]	Maximum	No. of samples with Np	Mean[a]	Maximum	No. of samples with Clo	Mean[a]	Maximum
Apple										
Cankers	40	37	223	2322	2	0.4	1	40	654	7293
Apple leaf litter	13	9	62	209	1	0.7	1	13	5810	12526
Mummies	36	35	338	3227	1	0.3	0	36	2449	8046
Prunings	40	35	10	74	2	3.2	6	40	161	1801
Fruit spurs	40	35	17	217	0	-	0	40	252	2795
Dead weed	27	21	23	254	0	-	0	27	17082	68320
Dead grass	20	14	2	13	0	-	0	20	615	3506
Champost	8	3	1	4	0	-	0	8	17	42
Soil	40	5	0.10	0.24	0	-	0	37	3	14
Total	264	194			6			261		
Pear										
Cankers	40	26	50	420	0	-	0	38	11	98
Pear leaf litter	36	31	118	1445	0	-	0	36	1708	7446
Mummies	6	3	5	12	0	-	0	6	262	548
Pruning	40	23	5	35	0	-	0	40	21	103
Fruit spurs	40	19	4	15	0	-	0	37	4	21
Dead weed	32	29	277	5899	0	-	0	32	5054	20185
Dead grass	20	15	3	12	0	-	0	20	400	3393
Champost	12	4	1	1	0	-	0	12	6	25
Soil	40	5	0.10	0.16	0	-	0	33	2	12
Total	266	155			0			254		

[a] For samples with pathogen present.

in all nine different types of substrates and was absent only in a few soil samples. Large differences were observed in concentrations of *C. luteo-olivacea/Cadophora* X between the different substrate types. Highest concentrations were present in apple leaf litter and mummies as well as in dead weeds (Table 5; Fig. 1). Low concentrations were measured in soil and champost. In pear orchards, *C. luteo-olivacea/Cadophora* X was also present in nearly all samples of the nine different substrates. Highest concentrations of *C. luteo-olivacea/Cadophora* X were measured in pear leaf litter and dead weeds, and very low concentrations were found in fruit spurs, soil and champost.

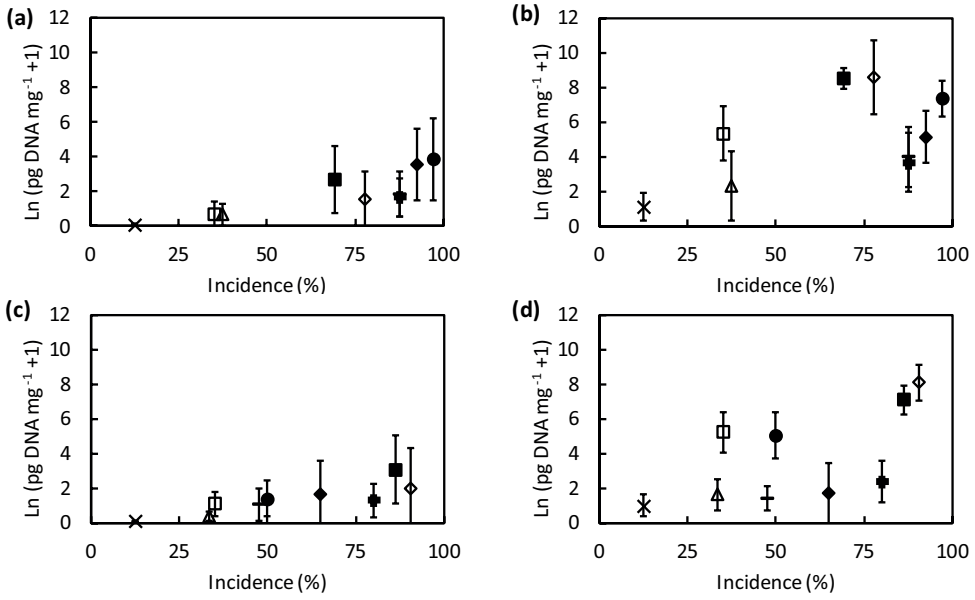

Figure 1. Mean incidence (percentage positive samples of the assessed samples) and mean concentration (pg DNA per mg plant residue (dry weight)) of *Neofabraea alba* and *Cadophora luteo-olivacea*/*Cadophora* X in various substrates sampled in orchards in May 2012. Host tissue: Cankers ♦, leaf litter ▪, mummies ●, prunings —, fruit spurs +; non-host tissues: dead weeds ◇, dead grasses □, champost Δ, soil x. (a) *N. alba* in apple orchards; (b) *C. luteo-olivacea/Cadophora* X in apple orchards; (c) *N. alba* in pear orchards; (d) *C. luteo-olivacea/Cadophora* X in pear orchards. See Table 5 for numbers of samples assessed; bars indicate standard deviation.

Variation in substrate colonization between orchards

In apple orchards, cankers, apple leaf litter, mummies and dead weeds generally showed high colonization by *N. alba* and *C. luteo-olivacea/Cadophora* X. The colonization of these substrates by the pathogens varied strongly between orchards. A large variation was observed in the concentration of *N. alba* in canker samples of the 10 apple orchards, ranging from 2 to 631 pg DNA of *N. alba* per mg canker tissue (dry weight) (backtransformed values; Fig. 2). High variation was also present in the concentration of *C. luteo-olivacea/Cadophora* X in these cankers, ranging from 45 to 2466 pg DNA mg^{-1}. No correlation was found for the concentration of *N. alba* or *C. luteo-olivacea/*

Cadophora X for canker samples within an orchard. The highest concentration of *N. alba* was measured in cankers of orchard 1 ('Pinova'), 5 ('Elstar'), 8 ('Pinova'), 9 ('Topaz') and 10 ('Elstar'). Canker samples with low concentrations of *C. luteo-olivacea/Cadophora* X and high concentrations of *N. alba* were not observed. Apple leaf litter was only present in three apple orchards, with low concentrations of *N. alba* with 0-58 pg DNA mg^{-1}, and high concentrations of *C. luteo-olivacea/Cadophora* X above 2654 pg DNA mg^{-1}. Highest concentrations of *N. alba* were measured in fruit mummies from orchards 4 ('Elstar'), 5 ('Elstar'), 8 ('Pinova'), and 10 ('Elstar'), ranging from 149 to 552 pg DNA mg^{-1}. Very low concentrations were measured in mummies from orchard 6 ('Kanzi') with 2 pg DNA mg^{-1}. High concentrations of *C. luteo-olivacea/Cadophora* X with 510 to 4921 pg DNA mg^{-1} were measured in cankers, with considerable differences in concentrations of *C. luteo-olivacea/Cadophora* X from the different orchards. Dead weeds were sampled in seven apple orchards. Low concentrations of *N. alba* below 13 pg DNA mg^{-1} were detected in dead weed samples of all orchards. However, high concentrations of *C. luteo-olivacea/Cadophora* X between 652 and 41,900 pg DNA mg^{-1} were measured in dead weed samples.

Cankers, pear leaf litter and dead weeds were the most colonized substrates in pear orchards (Fig. 3), whereas fruit mummies were almost absent. Cankers sampled in the pear orchards contained relatively low concentrations of *N. alba*, below 6 pg DNA mg^{-1} for nine orchards, and an exceptional higher value with 279 pg DNA mg^{-1} for orchard 17. *Cadophora luteo-olivacea/Cadophora* X was found at low levels of below 23 pg DNA mg^{-1}. The concentration of *N. alba* in pear leaf litter differed considerably between orchards, ranging from 1 to 324 pg DNA mg^{-1}. The lowest concentrations of *N. alba* were measured in orchards 14 and 20 and the highest in orchards 11 and 18. Values for *C. luteo-olivacea/Cadophora* X were above 687 pg DNA mg^{-1}, with little variation between orchards. A similar pattern was found for dead weeds. The concentration of *N. alba* varied considerably between orchards ranging from 1 to 564 pg DNA mg^{-1}, whereas *C. luteo-olivacea/Cadophora* X was detected more consistently at high levels ranging from 1014 to 11,890 pg DNA mg^{-1}.

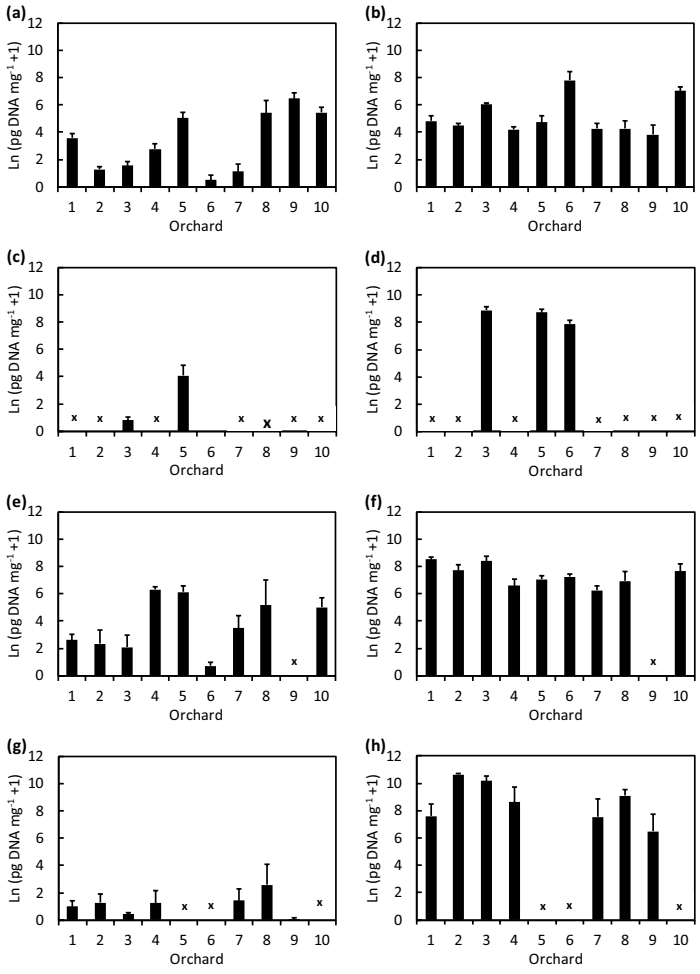

Figure 2. Colonization of different plant residues from 10 apple orchards by postharvest pathogens. (a) *Neofabraea alba* in cankers; (b) *Cadophora luteo-olivacea/ Cadophora* X in cankers; (c) *N. alba* in apple leaf litter; (d) *C. luteo-olivacea/ Cadophora* X in apple leaf litter; (e) *N. alba* in mummies; (f) *C. luteo-olivacea/ Cadophora* X in mummies; (g) *N. alba* in dead weeds; (h) *C. luteo-olivacea/ Cadophora* X in dead weeds. Mean concentration (pg DNA per mg plant residue (dry weight)) of 10 cankers, 10 mummies or 100 ml of apple leaf litter or dead weeds sampled in May 2012 from four replicates plots per orchard. Bars indicate standard error of the mean; 'x' indicate orchards where plant tissue was not available.

Temporal dynamics of *N. alba* and *C. luteo–olivacea/Cadophora* X in necrotic plant tissues

The temporal dynamics of colonization of *N. alba* and *C. luteo-olivacea/Cadophora* X was quantified in four apple orchards, planted with two different apple cultivars (orchard 1 and 8: Pinova; orchard 3 and 7: Elstar) and four pear orchards, all planted with Conference, in five substrates: leaf litter, mummies, prunings, dead weeds and dead grass, sampled monthly from May until September and in December. These substrates were selected based on results obtained in May because they generally had the highest incidence and concentration of *N. alba* and *C. luteo-olivacea/Cadophora* X.

The different types of apple residues were not all present on the orchard floors during the monitored months in all four orchards. Leaf litter was mainly present from August until December. The other substrates were present during all months that were monitored, although old mummies disappeared during the growing season,

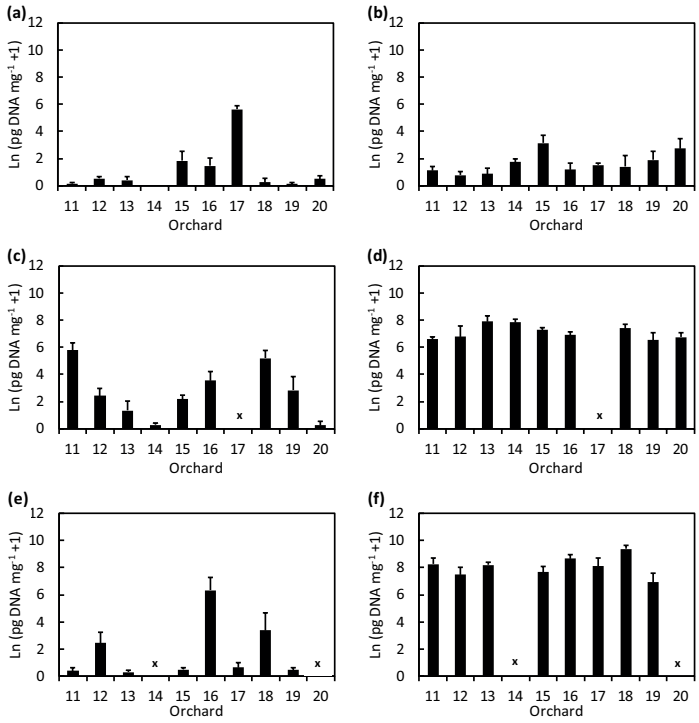

Figure 3. Colonization of different plant residues from 10 pear orchards by postharvest pathogens. (a) *Neofabraea* alba in cankers; (b) *Cadophora luteo-olivacea/ Cadophora* X in cankers; (c) *N. alba* in pear leaf litter; (d) *C. luteo-olivacea/ Cadophora* X in pear leaf litter; (e) *N. alba* in dead weeds; (f) *C. luteo-olivacea/ Cadophora* X in dead weeds. Mean concentration (pg DNA per mg plant residue (dry weight)) of 10 cankers, 100 ml of apple leaf litter or dead weeds sampled in May 2012 from four replicates plots per orchard. Bars indicate standard error of the mean; 'x' indicate orchards where plant tissue was not available.

and new mummies were formed during the season. In apple orchards the dynamics of detection of different pathogens in the substrates generally showed the same trends. Both pathogens decreased from April until August and increased after August until December, with the exception of dead grasses. The level of detection in dead grasses was relatively constant throughout the season (Fig. 4). However, for mummies, prunings and dead weeds orchard differences were found. For example, the concentration of *N. alba* in mummies was highest in orchard 8 ('Pinova') in June with 25,698 pg DNA mg^{-1} but less than 204 pg DNA mg^{-1} in June in mummies originating from the other orchards. The concentration of *C. luteo-olivacea/Cadophora* X in dead weeds decreased earlier in orchard 7 ('Elstar') compared to the other three orchards, but increased again rapidly in July. In prunings, concentrations of *C. luteo-olivacea/Cadophora* X increased from May to August in orchards 1 ('Pinova') and 3 ('Elstar') but decreased during this period in orchards 7 ('Elstar') and 8 ('Pinova'). A similar trend was found for *N. alba* in the same samples of prunings.

In pear orchards a decrease in colonisation by both pathogens in early summer followed by an increase in colonization after August until December was measured in pear leaf litter (Fig. 5). In the other substrates, populations generally were more constant and at lower levels compared to apple orchards. The temporal dynamics in substrate colonization differed between orchards. For example, *N. alba* in prunings increased in orchard 18 in June

to 37 pg DNA mg^{-1} whereas in the other orchards the concentration of *N. alba* was below 2 pg DNA mg^{-1}. In the same orchard, *N. alba* was also higher in early summer in dead weeds, with 34 pg DNA mg^{-1} in June, compared to 2 pg DNA mg^{-1} or lower in the other orchards. Measurements of *C. luteo-olivacea/Cadophora* X were more consistent for orchards for most substrates except for dead weeds. Moderate to high concentrations were found in June in orchards 13 (175 pg DNA mg^{-1}), 18 (7596 pg DNA mg^{-1}) and 19 (106 pg DNA mg^{-1}), but in orchard 20 only 3 pg DNA mg^{-1} were detected. In this orchard the concentration of *C. luteo-olivacea/Cadophora* X in dead weeds increased during summer, whereas this pathogen decreased in the other orchards.

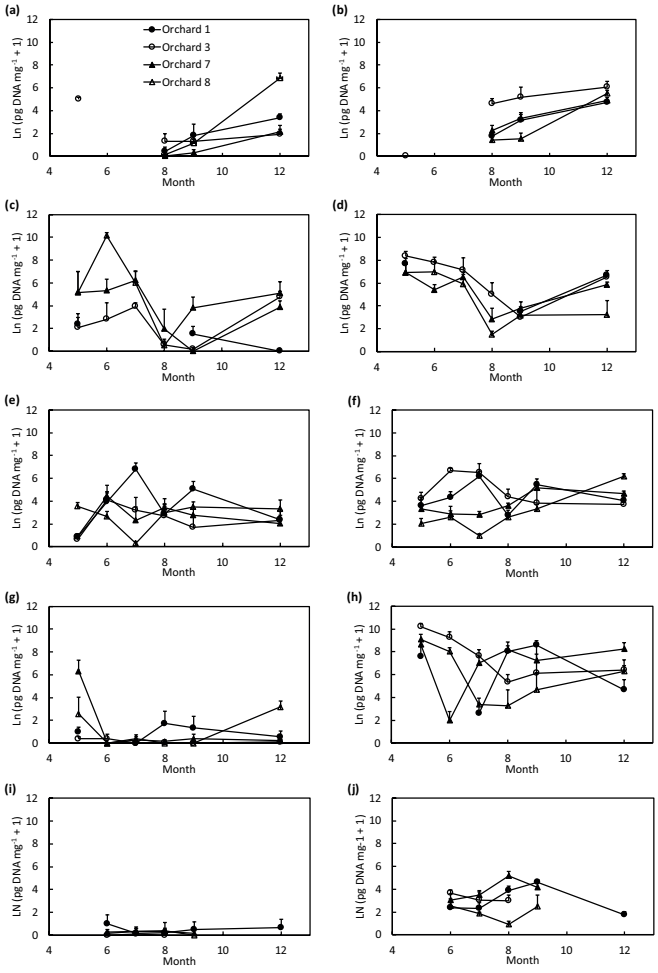

Figure 4. Population dynamics of postharvest pathogens on various plant tissues in apple orchards 1, 3, 7 and 8. (a) *Neofabraea alba* on apple leaf litter on orchard floor; (b) *Cadophora luteo-olivacea/Cadophora* X on apple leaf litter on orchard floor; (c) *N. alba* on mummies; (d) *C. luteo-olivacea/Cadophora* X on mummies; (e) *N. alba* on prunings; (f) *C. luteo-olivacea/Cadophora* X on prunings; (g) *N. alba* on dead weeds; (h) *C. luteo-olivacea/Cadophora* X on dead weeds; (i) *N. alba* on dead grasses; (j) *C. luteo-olivacea/Cadophora* X on dead grasses. Mean concentration (pg DNA per mg plant residue (dry weight)) of four replicated samples each consisting of 10 mummies, 10 segments of prunings 5-10 cm long, or 100 ml of apple leaf litter, dead weeds or dead grasses. Bars indicate standard error of the mean.

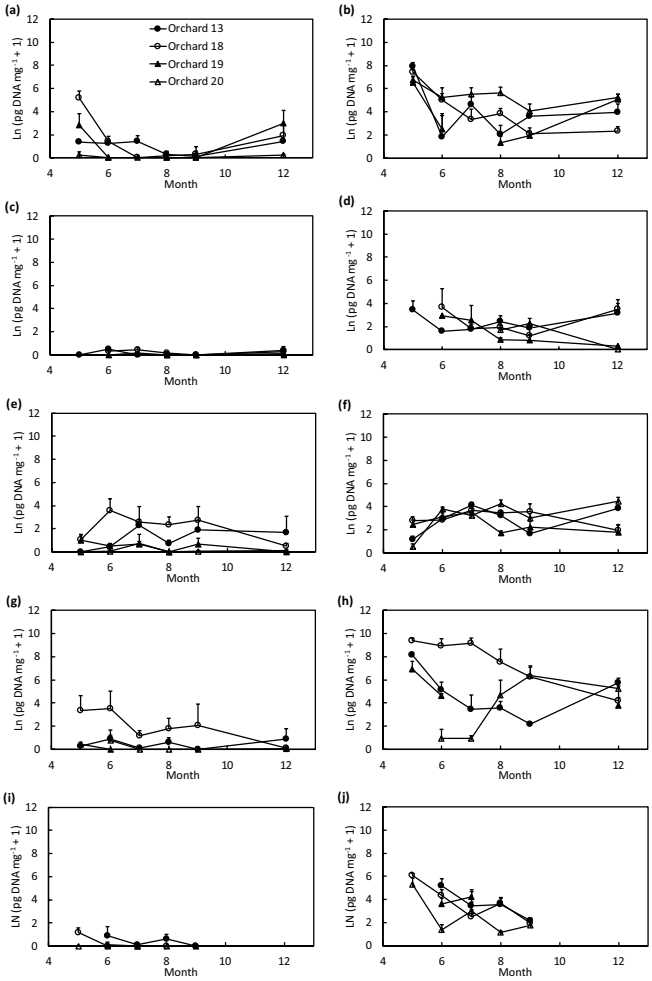

Figure 5. Population dynamics of postharvest pathogens on various plant tissues in pear orchards 13, 18, 19 and 20. (a) *Neofabraea alba* on pear leaf litter on orchard floor; (b) *Cadophora luteo-olivacea/ Cadophora* X on pear leaf litter on orchard floor; (c) *N. alba* on mummies; (d) *C. luteo-olivacea/ Cadophora* X on mummies; (e) *N. alba* on prunings; (f) *C. luteo-olivacea/Cadophora* X on prunings; (g) *N. alba* on dead weeds; (h) *C. luteo-olivacea/ Cadophora* X on dead weeds; (i) *N. alba* on dead grasses; (j) *C. luteo-olivacea/ Cadophora* X on dead grasses. Mean concentration (pg DNA per mg plant residue (dry weight)) of four replicated samples each consisting of 10 mummies, 10 segments of prunings 5-10 cm long, or 100 ml of pear leaf litter, dead weeds or dead grasses. Bars indicate standard error of the mean.

Discussion

Detection of postharvest pathogens by TaqMan PCR in various samples obtained from 20 representative Dutch orchards confirmed results of isolations from postharvest rots in stored fruit lots of apple and pear (Wenneker et al., 2016a). In both studies *N. alba* and *C. luteo-olivacea/Cadophora* X were identified as the most common pathogens, whereas *N. perennans* was detected only occasionally and *C. malorum* could not be detected. *N. alba* was found as a major postharvest pathogen causing lenticel rot (bull's eye rot) on both apple and pear fruit during the Dutch surveys in 2011-2013 and has now been detected consistently in the monitored apple and pear orchards. In contrast, *C. luteo-olivacea/ Cadophora* X causing side rot was found to be a major postharvest pathogen of pear fruit

lots, but was less abundant on stored apple fruit lots. Interestingly, the pathogen was consistently detected at high concentrations in both apple and pear orchards.

In this study, the concentration of the postharvest pathogens was quantified in various substrates. In the apple orchards, *N. alba* was detected in almost all samples of cankers and mummies at high concentrations. The pathogen was also consistently present at lower concentrations on apple leaf litter, fruit spurs, and prunings as well as on dead grasses and weeds. In contrast to apple orchards, leaf litter and dead weeds appeared to be the substrates with the highest incidences and concentration of *N. alba* in pear orchards. *Cadophora luteo-olivacea/Cadophora* X was commonly present in the assessed substrates in apple and pear orchards. Highest concentrations of *C. luteo-olivacea/ Cadophora* X were found on apple and pear litter, mummies (present in apple orchards) and dead weeds, but only low concentrations in soil samples. In this study, the amount of substrate present in the orchards and the dynamics of these substrates during time has not been assessed. Consequently, it is not possible to estimate the relative importance of the different types of substrates for the development of pathogen populations as done earlier for *Stemphylium vescarium* populations in pear orchards (Köhl et al., 2009, 2013). Mummies are generally observed frequently in Dutch apple orchards, but rarely in pear orchards. Possibly, mummies are an important inoculum source of *N. alba* in Dutch apple orchards, as has also been found for German apple orchards (Weber, 2012).

In this study, both *N. alba* and *C. luteo-olivacea* have consistently been detected in leaf litter of apple and pear and in necrotic tissues of dead weeds and grasses. In many cases high concentrations of the pathogens were quantified. These are important new findings which may help to better understand how complex population dynamics of these necrotrophic pathogens depend on the availability of various necrotic host and non-host tissues for survival and multiplication. Obviously, necrotic tissues of non-hosts potentially can play an important role, as found earlier for pear-pathogenic *S. vesicarium* populations in pear orchards (Köhl et al., 2013). Until now, *Neofabraea* spp. and *Cadophora* spp. causing postharvest fruit rots have been primarily described as colonizers of woody tissue including cankers (Henriquez et al., 2004; Gramaje et al., 2011).

For *N. alba* there was a considerable fluctuation in substrate colonization over time. This was less pronounced for *C. luteo-olivacea*. Detailed information on infection periods of the pathogens is lacking. Latent infections may occur from flowering onwards during the entire growing period or fruits may differ in susceptibility depending on their physiological stages, so that the majority of infections may be caused during specific developmental stages. A better understanding of the critical infection periods will help to highlight the relative importance of pathogen colonization of different substrates and their dynamics during a growing season.

The colonization of substrates by *N. alba* and *C. luteo-olivacea/Cadophora* X varied markedly between orchards. For example, a large variation between apple orchards was observed in the concentration of *N. alba* and *C. luteo-olivacea/Cadophora* X in cankers

and mummies and between pear orchards for the concentration of *N. alba* in pear leaf litter and dead weeds. The design of the sampling does not allow conclusive results on the reason for the observed variation. Possibly, differences in orchard management play a role as well as factors such as cultivars, tree age or local climatic conditions. The observed variation between orchards indicates that pathogen populations and consequently infection risks depend on still unknown factors. The identification of the major factors affecting population development of *N. alba* and *C. luteo-olivacea/Cadophora* X may allow the development of new preventative measures to manage the risk of postharvest losses in the orchard.

Quantification of fungal pathogens in environmental samples using species-specific TaqMan PCR assays is a powerful tool to give new insights into pathogen population dynamics. However, DNA-based quantitative detection methods need further improvements because the efficiency of DNA extraction may differ between samples and part of the detected DNA may represent dead cells. Sequence differences between *Neofabraea* malicorticis and *N. perennans* appear to be small, only 0.4% for the combined data set of ß-tubulin, *ITS* rDNA and mitochondrial rDNA (De Jong et al, 2001). The small genetic variation between the species might indicate a separation at the subspecific level (Verkley, 1999). There is a cross-reaction of the *N. perennans* probe with *N. populi*, causing bark lesions on Populus trees. Therefore, possible cross-reactions might occur, e.g. when poplar windbreaks are surrounding the orchard. However, in this survey the orchards were surrounded by *Alnus* (alder) windbreaks. Moreover, *N. perennans* was only detected occasionally.

Further research on disease epidemiology is needed to understand the relationships between the build-up of pathogen inoculum on the various substrates during time and infection periods for developing fruits in the orchard. This knowledge will enable estimates of the relative importance of different substrates as inoculum sources for fruit infections. Further, this knowledge could be used for the development of focussed sanitation measures (Holb, 2006; Gomez et al., 2007; Llorente et al., 2010). Understanding the role of microbial colonizers in competitive substrate colonization (Köhl et al., 2015) will allow the development of measures to stimulate beneficial components of microbiomes or to apply beneficial antagonistic strains to the relevant plant residues, with the aim suppressing pathogen colonization, survival and sporulation (Carisse and Rolland, 2004; Llorente et al., 2006; Rossi and Pattori, 2009).

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5.

Development of a method for detection of latent European fruit tree canker (*Neonectria ditissima*) infections in apple and pear nurseries

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Abstract

Fruit tree canker caused by *Neonectria ditissima* is a serious problem in apple-producing regions with moderate temperatures and high rainfall throughout the year; especially in northwestern Europe, Chile, and New Zealand. Control measures are applied to protect primary infection sites, mainly leaf scars, from invasion by external inoculum. However, latent infections may occur when young apple trees are infected symptomlessly during propagation. This study aimed to develop a method for detection of latent fruit tree canker infections. Inoculations with conidiospore suspensions of *N. ditissima* were carried out in tree nurseries on the main stems of two-year-old trees of three apple cultivars and one pear cultivar. The inoculations were carried out during the natural abscission period in the autumn. No visible lesion or canker formations were present at the time when the inoculated trees were uprooted. It appeared that the infections may remain latent during the period from infection to uprooting (2 months) and during the subsequent 4 months of cold storage of the trees. Nevertheless, symptoms were generally induced within eight weeks after transfer of infecting planting material from the nursery field into a climate chamber with high temperature and high relative humidity. The methodology presented is developed to detect latent infections of *N. ditissima* in nursery trees, prior to planting in the orchards, and it may contribute to reducing the problem with European fruit tree canker in commercial production.

Introduction

European canker of apple and pear trees is caused by the fungal pathogen *Neonectria ditissima* (syn. *Nectria galligena;* anamorph *Cylindrocarpon heteronema*). The fungus typically induces cankers on side shoots, minor branches and the main stem of infected trees (Cooke, 1999). N. ditissima is a wound parasite (Swinburne, 1975; Xu et al., 1998), and leaf scars formed during abscission are considered to be the most important site of infection (Crowdy, 1952; Dubin and English, 1974). These infections are caused during prolonged periods of rainy weather by sexual ascospores that are produced in perithecia, as well as by asexual conidiospores that are produced in sporodochia (Swinburne, 1975; Beresford and Kim, 2011).

Apple cultivars differ in their susceptibility to *N. ditissima*. For instance, whereas cv. Jonathan is considered as fairly resistant, cvs. Elstar and Jonagold are considered as moderately susceptible, and cvs. Kanzi and Gala as highly susceptible (Pedersen et al., 1994; Van de Weg et al., 1992; Palm et al., 2011; Garkava-Gustavsson et al., 2013; Weber, 2014). Although *N. ditissima* is mainly described as an apple tree pathogen, also pear (*Pyrus communis*) occasionally suffers from severe incidences (Goos, 1975; Scheer, 1980).

Control of *N. ditissima* is achieved through autumn and spring applications of fungicides to protect leaf scars and pruning cuts from infection (Cooke, 1999; Weber, 2014). Pruning of cankers, covering wounds with paint and removal of diseased wood are also important practices for disease control. However, despite these control measures, the occurrence of epidemics cannot be prevented (Weber, 2014).

Recently, severe canker outbreaks have been reported in young orchards in the Netherlands and other Northwestern European countries, particularly on some of the more recently introduced apple cultivars such as Kanzi and Rubens (Weber and Hahn, 2013). It is suggested that the major source of infection by *N. ditissima* in newly planted orchards was brought in with the introduction of trees from nurseries, because significant numbers of young trees developed large cankers along the main stem. Since the trees did not show symptoms at the time of planting in the orchards, they likely became infected during propagation without showing symptom development (Brown et al., 1994; McCracken et al., 2003; Weber, 2014). Several molecular tools have been developed to detect *N. ditissima* (Langrell and Barbara, 2001; Langrell, 2002; Ghasemkhani et al., 2016). However, considering the wealth of possible infection sites within a single tree, these are not suitable for detecting latent infections in whole trees. Therefore, the objective of this work was to develop a fast and reliable screening method for the occurrence of latent infections of *N. ditissima* in apple and pear trees that can be used prior to planting in the orchard.

Results and discussion

Inoculum of *N. ditissima* was obtained by collecting fresh cankers from the apple cvs. Topaz and Schone van Boskoop, that were placed in plastic bags in a climate chamber for 24 to 48 hours at 20˚C, during which sporodochia formed. On the day of inoculation, sporodochia were washed with sterile distilled water, the conidiospore suspension was filtered through cheese cloth, the concentration was determined using a haemocytometer and adjusted to 1×10^5 conidiospores mL^{-1}. The final conidiospore concentration was serially diluted with sterile distilled water to obtain a range of concentrations: 1×10^5; 1×10^4; 1×10^3; 1×10^2; 1×10^1 conidiospores mL^{-1}. Sterile distilled water was used as control. For all trials, viability of conidiospores was confirmed to be >95% by counting the number of germinated conidiospores upon plating of 50 µl of the conidiospore suspension for 24 h at 20°C on water agar.

Inoculations were carried out in a tree nursery on the main stems of two-year-old trees of the following apple cultivars (year of planting in parentheses) Elstar (2004), Santana (2004) and Pinova (2005), and on the pear cultivar Conference (2007) during the natural abscission period in the autumn. Leaves were gently removed from the main stems of the trees to generate a fresh leaf scar wound. Within five minutes after removal of the leaves the scars were inoculated with 10 µl of a 10-fold dilution series of conidiospore suspensions with a micropipette, resulting in 0; 0.1; 1; 10; 100 or 1000 macroconidia per leaf scar, followed by coverage with Vaseline (petroleum jelly) after droplet absorption to prevent desiccation of conidiospores (Van de Weg, 1989). Seven days after inoculation the Vaseline was removed using Cleanex paper.

For each inoculum density, 15 trees were used and four inoculations on the main stem with the same inoculum density were performed on each tree. The inoculations were carried out in late October/early November at two times (two inoculated leaf scars each time) that were separated by one week, and with 4 or 5 buds between adjacent inoculation sites. Leaves were removed just prior to abscission. The first removed leaf was approximately number 15 from the apex. After inoculation, the trees were left in the nursery field for another two months, until the period of commercial uprooting in late December/early January, by which time they were completely defoliated. Importantly, at the time of commercial uprooting, the trees had not yet received the required chilling period to break their dormancy. Visual inspection revealed the absence of symptoms of *N. ditissima*, i.e. cankers or lesions present on the inoculated leaf scars, at the moment of collecting the trees from the nursery field. Therefore, all inoculations were considered as potential latent infections.

Upon arrival at the laboratory, 90 trees per experiment (i.e. 6 inoculum densities × 15 trees) were randomly divided into two batches. Thirty trees (i.e. 6 inoculum densities × 5 trees) were directly placed into a climate chamber at 18˚C and 90% relative humidity (RH). These trees were placed in 5 containers (= replicates), consisting of 1 tree per inoculum

density. The remaining 60 trees (i.e. 6 inoculum densities × 10 trees) were placed in a cold storage facility for four months at 5˚C, and treated according to commercial storage conditions, in order to break dormancy.

After four months, these 60 trees were transferred from the cold storage facility and 30 trees were transferred into a climate chamber at 18˚C and 90% RH (same conditions as the trees that were directly transferred after collecting from the nursery field); in 5 replicates (containers) with 1 tree per inoculum density/container. The remaining 30 trees were individually potted and placed in an outdoor field and exposed to natural conditions in a randomized block design with 1 tree per inoculum density/block (5 replicates). Importantly, upon placing these trees in the climate room or under semi-field conditions, normal tree development took place with leaf growth and flowering. Visual inspection confirmed that the trees were still without lesions caused by *N. ditissima* after storage at 5˚C, and thus the inoculated leaf scars could still be considered as containing potential latent infections.

The trees in the climate chambers were placed in wet sand in the dark at 90% RH and 18˚C. The trees in the outdoor field were potted in standard potting soil, and received fertigation according to standard practices.The trees were assessed weekly for the occurrence of cankers, starting in the first week after transfer, for up to 12 weeks. Growing lesions larger than 5 mm were recorded as active lesions. Logistic regression was used to relate the fraction of lesion incidence to the log10 of the inoculum density for each cultivar. Before log transformation 1 was added to the inoculum density to avoid taking the logarithm of 0. The logistic regression model was then $logit\ (\pi)=\alpha_i+\beta_i\ Log10\ (density+1)$ in which π denotes the fraction of lesions, and the subscript i denotes the three different treatments. For the apple and pear cultivars the effect β_i of the log density was not significantly different among treatments and therefore a common effect β was assumed. Treatment effects were summarized by pairwise testing of the intercept parameter α_i at the 5% significance level.

The evaluation of different methods for assessing latent infections of *N. ditissima* was done by scoring the development of lesions on the inoculated leaf scars (Fig. 1). For all apple cultivars, the first lesions were observed when 10 conidia were used per leaf scar, while pear cv. Conference developed lesions already when 1 spore was used. The lowest lesion incidences were recorded in the outdoor fields. For apple cv. Elstar, lesion incidences increased at densities of 100 and 1,000 conidiospores per leaf scar. The total percentage of lesions remained relatively low, with approximately 20 to 30% of the inoculations resulting in lesions for both conidiospore densities. When trees of apple cv. Santana were placed in the climate chamber directly after uprooting, the lesion incidences increased at higher conidiospore densities. However, when trees were kept in cold storage the incidences did not increase with spore densities from 100 to 1,000 spores per leaf scar. For apple cv. Pinova, at higher inoculum densities the lesion incidences rapidly increased when trees were placed in the climate room, leading to very high lesion incidences. However, in the outdoor field after cold storage no increase in lesion incidence was observed from 100 to 1,000 conidiospores per leaf scar. For pear cv. Conference for 100 conidiospores per leaf scar over 80% incidence was recorded.

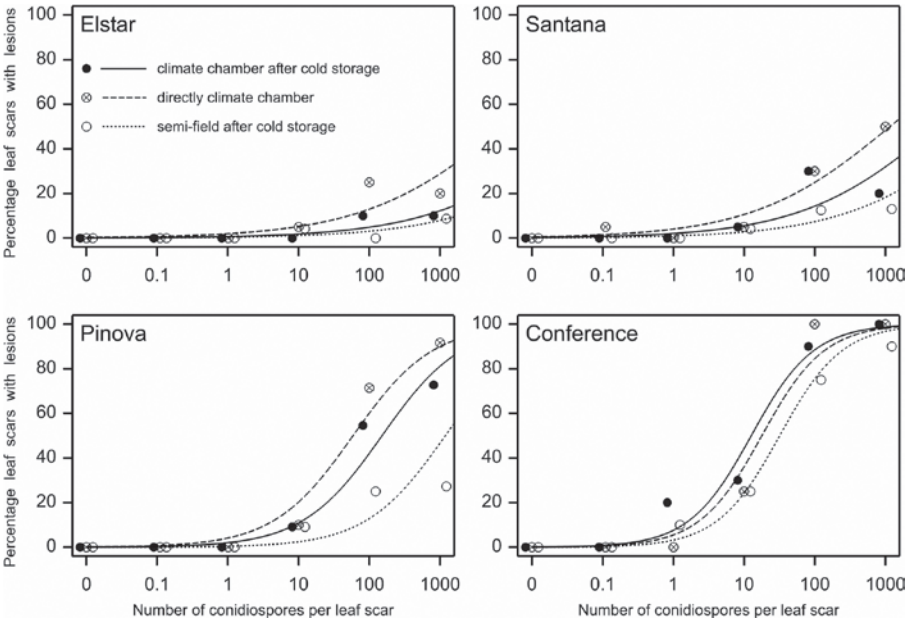

Figure 1. Percentage of leaf scars showing lesions after inoculation with different concentrations of conidiospores of *N. ditissima.*

There were no significant differences in lesion incidences among apple cultivars placed in the climate chamber directly after uprooting compared to after a 4-month period in cold store (Table 1). However, trees of cvs. Elstar and Santana placed into the climate chamber directly after uprooting from the nursery field showed higher lesion incidences when compared with trees placed in the outdoor field after cold storage. For cv. Pinova both methods showed significantly higher lesion incidences compared to the trees that were placed under outdoor conditions. For pear cv. Conference no significant differences were observed in the lesion incidences between the three methods applied. We further confirmed that inoculum dose is an important parameter for establishing infections, as documented earlier (Dubin and English, 1974; Van de Weg, 1989; Weber, 2014).

Table 1. Estimate of the intercept parameter α_i for each treatment in the logistic regression model and the results of pairwise testing of the treatments.

Treatment after uprooting	Elstar		Santana		Pinova		Conference	
Climate chamber throughout	-5.904	b*	-5.235	b	-6.720	b	-7.393	a
Cold storage (4 months) then climate chamber	-6.972	ab	-5.924	ab	-7.451	b	-7.052	a
Cold storage (4 months) then semi-field	-7.450	a	-6.665	a	-9.046	a	-7.966	a

* Different letter labels within a column indicate significant differences at the 5% significance level.

An important outcome of the experiments was that the climate chamber method revealed at least the same, but often higher percentages of lesions compared to planting of trees under natural conditions. Moreover, placing dormant trees in the climate chamber directly after uprooting did not negatively affect lesion incidences. It can therefore be concluded that this method may be suitable to detect latent infections of *N. ditissima* prior to planting trees of various cultivars of apple and pear in the orchard.

Another important observation was that no visible lesion or canker formations were present at the time when the inoculated trees were uprooted; i.e. approximately 2 months after inoculation with *N. ditissima* spores. Also, after four months of cold storage of the trees no visible lesions were present. This shows that infections during leaf fall in the nurseries remain quiescent until after planting in the orchard. Even at a high inoculum pressure these infections may remain latent during the period from infection to uprooting (2 months) and during the subsequent 4 months of cold storage of the trees. As a consequence, these infections remain unnoticed in the nurseries.

For the assessment of infections of *N. ditissima* in batches of commercial planting material, the sample size and sampling strategy is important. Assuming a random distribution of the disease, 300 trees are required to detect 1% incidence of latently infected trees with 95% probability, and 200 trees are required to detect 1.5% incidence with 95% probability (Janse and Wenneker, 2002). The total number of observed affected trees within the sample would reflect the total percentage of infected trees within the population.

The methodology presented here is developed to detect latent infections of *N. ditissima* in nursery trees, prior to planting in the orchards. Molecular tools could be used to verify the presence of *N. ditissima* as the causal agent when lesions are observed (Ghasemkhani et al., 2016). The method may contribute in reducing the problem with European fruit tree canker in commercial production.

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6.

Methods for the quantification of resistance of apple genotypes to European fruit tree canker caused by *Neonectria ditissima*

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Abstract

European fruit tree canker, caused by *Neonectria ditissima*, is an important disease of pome fruit worldwide. Apple cultivars differ in their levels of susceptibility to *N. ditissima*. In order to design an effective plant resistance test, we examined the effectiveness of two resistance parameters, i.e. infection frequency and lesion growth. Both parameters were evaluated in parallel tests using ten apple cultivars in three experimental years, applying semi-natural infection of leaf scars (infection frequency) or inoculation of artificial wounds (lesion growth). We compared six parameters for lesion growth, of which a new parameter, Lesion Growth Rate (LGR), appeared the best with respect to reproducibility and statistical significance. LGR is defined as the slope of the regression of lesion size versus time. The slope was estimated for each lesion, employing a common start date, and a lesion specific end date determined by the girdling of the lesion. The two parameters infection frequency and LGR were examined in separate experiments and in three successive years, and provided complementary information and resulted in reproducible conclusions on the relative resistance levels to *N. ditissima* of the tested cultivars. The presented methods can be used to develop strategies for the control of European fruit tree canker.

Introduction

European canker is an important disease of pome fruit worldwide, which may cause severe losses mainly in temperate regions (Weber, 2014). The disease is caused by the fungal pathogen *Neonectria ditissima* (*Neonectria galligena*, formerly *Nectria galligena*; anamorph *Cylindrocarpon heteronema*). The formation of cankers on woody tissue is the most obvious symptom of *N. ditissima*. Cankers can be formed on twigs, branches, and the main stem (Cooke, 1999). Growing cankers often girdle twigs and branches, and eventually kill parts of a tree. When cankers are formed on minor branches, growers will prune them out. However, cankers formed on major branches or on the main stem may lead to the loss of whole trees (McCracken et al., 2003). *N. ditissima* also causes fruit rotting of apple, which may develop already in the orchard or later, during storage (Berrie, 1989; Xu and Robinson, 2010).

N. ditissima is a wound pathogen, and all kinds of natural and artificial wounds can be infected (Swinburne, 1975; Xu et al., 1998). Leaf scars formed during leaf fall are considered to be the major site of infection (Dubin and English, 1975). Warm and humid weather with frequent rainfall is favourable for infections (Beresford and Kim, 2011). The fungus overwinters as mycelium in twigs and callus tissue of cankers, or as perithecia in cankered wood. Infection may be caused by conidia and ascospores (Swinburne, 1975). *N. ditissima* produces ascospores in perithecia and conidia in sporodochia. Ascospores are released at the end of winter and during spring. On old cankers, conidia and ascospores can be seen year round but their production and germination depend on climate conditions. Rain is an important vector of the pathogen by aerial rain splash from tree to tree and by rain splash and runoff within infected trees (Swinburne, 1975). Sporulation, spore dispersal and infection of *N. ditissima* are favoured by rainfall (McCracken et al., 2003). Frequency, duration of rainfall, and surface wetness are considered to be more important than amount of rainfall (Beresford and Kim, 2011; Dubin and English, 1975; Latorre et al., 2002; Swinburne, 1975; Xu et al., 1998).

Apple cultivars differ in their level of susceptibility to *N. ditissima*. The cultivar Jonathan is considered as fairly resistant, 'Elstar' and 'Jonagold' as moderate to fairly resistant, and 'Kanzi' and 'Gala' as highly susceptible (Garkava-Gustavsson et al., 2013, 2016; Gómez-Cortecero et al., 2016; Grabowski, 1992; Krähmer and Schmidle, 1979; Krüger, 1983; Palm et al., 2011; Pedersen et al., 1994; Van de Weg, 1989 a,b; Van de Weg et al., 1992). However, these reports sometimes disagree in the levels of resistances for these cultivars. Xu et al. (1998) listed a number of causes that might explain these differences. In some studies canker size was used as a resistance criterion, whereas in other studies canker incidence or incubation period were recorded. The relationships between canker incidence, incubation period, and canker size may also depend on cultivars and experimental conditions.

The objective of this study was to evaluate the complementarity or redundancy of two components of resistance (infection frequency and lesion growth) and the usefulness of a series of alternative parameters for lesion growth. Infection frequency and lesion size were quantified for a number of commercially available apple cultivars in three experimental years.

Materials and methods

Plant material

Ten commercially available apple cultivars differing in levels of resistance to *N. ditissima* were evaluated (i.e., the three highly susceptible cultivars Braeburn, Discovery and Gala, the two moderately susceptible cultivars Cox's O.P. and Topaz, and the five moderately to fairly resistant cultivars Golden Delicious, Elstar, Honeycrisp, Jonathan, and Santana) (Kemp et al., 1999).

Six experiments were performed at the Experimental Station of Wageningen University & Research, Randwijk, The Netherlands. They were conducted with apple trees that were in their second growing season after grafting on rootstock M9. Trees were approximately 1.5 m in height at the time of the start of the experiments. The potted trees were grown outdoors, in 10-liter pots without any training system (unpruned trees), and were fertilized and watered using drip irrigation.

Components of resistance to European canker

Two components of resistance were examined: (i) infection frequency, which represents the resistance to onset of new lesions, and (ii) lesion growth after artificial inoculation of wounded leaf scars. They were examined in separate experiments, here indexed as 1 and 2 respectively, and in three successive seasons – 2003 to 2004, 2004 to 2005, and 2006 to 2007 – here referred to as experiments A, B and C, respectively. Each of the six experiments (A1 to C2) started with new trees.

Infection frequency (natural infections)

Experimental design. In the autumn 2003 (experiment A1), 2004 (experiment B1), and 2006 (experiment C1), potted trees of the 10 cultivars were used for the infection frequency experiments. The trees stayed outdoors until the following spring. The experimental design was a randomized complete block design with eight (experiment A1) or four (experiments B1 and C1) blocks, whereby each block contained one tree of each cultivar.

Inoculation. The trees were subjected to a high infection pressure by hanging prunings with sporulating cankers above the trees in October (i.e., before the first leaf drops occurred) and removing them after complete defoliation of the trees in January (A1 and B1) or February (C1). These prunings came from various apple cultivars from unsprayed orchards. In experiment A, prunings of 'Jonagold' and 'Schone van Boskoop'

were used; in experiment B, from 'Santana', 'Topaz', and 'Schone van Boskoop'; and in experiment C, from 'Alkmene' and 'Discovery'. Prunings from the different cultivars were placed alternating between two neighbouring trees.

Assessment of infection frequency. Infection frequency was defined as the percentage infected leaf scars on the main stem of a tree. In experiment A1, all leaf scars of the main stem of each tree were counted, as well as the number of leaf scars with cankers. This was performed in mid-April 2004 when cankers were still individually visible. The infection frequency of a tree was calculated as the number of infected leaf scars divided by the total number of scars. This was very laborious and, therefore, in the same experiment A1, the percentage infected scars was also directly scored by a visual assessment. The observed percentage and the visual percentage were highly correlated (R^2 = 0.94) and therefore, in experiments B1 and C1, the trees were only visually assessed. This was performed in mid-May, because individual cankers were still visible at that time.

Data analysis. A logistic regression model which employs the binomial distribution was used to analyse the number of cankers on the stem for experiment A1. Overdispersion relative to the binomial distribution was accounted for by inflating the binomial variance with a so-called overdispersion factor (McCullagh and Nelder, 1989). In experiments B1 and C1, percentages rather than counts were observed, and these were analysed with the same statistical model now with binomial totals equal to 100. Pairwise differences between cultivars were tested at the 5% significance level after correcting for differences between blocks.

Lesion growth

Experimental design. A randomized block design was used, in which each apple cultivar occurred once per block, employing eight blocks in experiment A2 and four blocks in experiments B2 and C2.

Preparation of inoculum. The inoculum was obtained from the same collection of cankers that was used for the natural infection experiments. The cankers were placed in plastic bags 24 to 48 hours before inoculation. During this period, sporodochia were produced. On the day of inoculation, the newly formed sporodochia were washed with sterile distilled water and the conidia were collected. The spore suspension was filtered through cheesecloth. Macroconidia density was determined using a haemocytometer, and adjusted to 2 x 10^5 spores ml^{-1}. Germination of macroconidia was assessed by plating 50 µl of the spore suspension on water agar for 24 h incubation at 20°C, after which germinated and non-germinated macroconidia were counted.

Inoculation of artificial leaf scar wounds. In autumn, just before leaf fall, the potted trees were transferred to a plot equipped with rain protected roofing to avoid secondary infection during the course of the experiment. Next, inoculation was performed during the natural leaf fall period on the main stems. The inoculations were performed according to Van de Weg (1989 a,b), with several slight modifications.

Stems were wounded by removal of a leaf and by cutting away one millimetre from the upper layer of leaf marks together with the corresponding axillary bud using a scalpel. In experiment A2, each tree was inoculated at six positions, numbered from bottom to top, with at least three leaves between the wounds. The two top inoculations in experiment A2 girdled relatively often and quickly; therefore, in experiments B2 and C2 only the lower four positions were used. This implies that, effectively, four inoculation positions at similar heights were used. The inoculation sites were tagged with paint on the reverse site of the inoculation position, in order to facilitate tracing these sites during inoculation and disease assessments. Within five minutes after wounding, 10 µl of the conidial suspension was placed per leaf mark, using an automatic micropipette. After complete absorption of the suspension into the tissue, wounds were covered with white, acid free petroleum jelly (Vaseline). The Vaseline was removed four days after inoculation using tissue paper. After inoculation, the trees were kept in the unheated greenhouse for nearly a year.

It is known that disease incidence and lesion growth may vary between and within experiments, probably because of sensitivity to experimental conditions such as the temperature and humidity at the time of infection and the quality of the inoculum (Garkava-Gustavsson et al., 2013, 2016). Therefore, inoculations were spread over multiple days. A further division in time was needed due to the amount of labor, because the performed experiments were part of larger experiments involving hundreds of trees. For experiment A2, inoculations were carried out during two days (13 and 19 November 2003), and these dates were more or less randomly distributed over blocks, genotypes and positions. In experiment B2, eight inoculation days were systematically used: first, the lowest positions were inoculated block by block, and on subsequent days, higher positions were treated. This was done in such a way that all wounds with the same block and position were inoculated on the same day (Table 1). In experiment C2, three inoculation days were used. On the first day, positions 1 and 2 for all trees in blocks 1 and 2 were treated; on the second day all 4 positions in blocks 3 and 4; and, on the third day, positions 3 and 4 in block 1 and 2 were inoculated (Table 1).

Table 1. Inoculation dates in experiment B (2004) and experiment C (2006).

	Inoculation dates per block[a]							
Inoculation Position	Experiment B (2004)				Experiment C (2006)			
	1	2	3	4	1	2	3	4
1	3 Nov	4 Nov	4 Nov	5 Nov	31 Oct	31 Oct	9 Nov	9 Nov
2	8 Nov	8 Nov	9 Nov	9 Nov	31 Oct	31 Oct	9 Nov	9 Nov
3	10 Nov	10 Nov	10 Nov	11 Nov	10 Nov	10 Nov	9 Nov	9 Nov
4	11 Nov	11 Nov	15 Nov	15 Nov	10 Nov	10 Nov	9 Nov	9 Nov

[a] Oct = October and Nov = November.

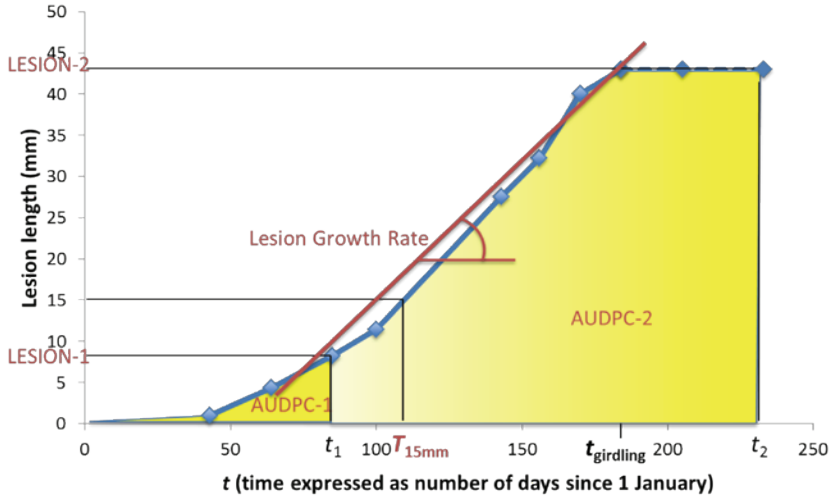


Figure 1. Six parameters used to characterize lesion growth. The line marked with diamonds represents a typical development of lesion (canker). The observation time points were taken in this example from the growing season of 2004. The six parameters are defined as follows. LESION-1 refers to the lesion size (in millimeters) when still very few lesions had caused girdling in the cultivars yet, thus preventing artefacts by girdling. For all genotypes, the same t_1 value was used within a year but t_1 varied between years, because the time when girdling started varied between years. LESION-2 refers to lesion size (in millimeters) during the last observation of the year, at t_2. When girdling by that particular lesion or by a lesion below had occurred earlier in the season, the lesion size was taken just before girdling, as indicated by the dashed line. AUDPC-1 refers to the area under the disease progress curve, starting from the first of January till t_1. AUDPC-2 is the AUDPC until t_2, using the dashed line. The parameter T15mm (in days) represents the period since the first of January until the lesion reached the length of 15 mm. Lesion Growth Rate (millimeters per day) is the slope of the regression line. For estimation of the lesion growth rate, we ignored the lesion sizes after girdling or stem death.

Assessment of lesion size. After inoculation, the trees were kept at the same plot with rain-protected roofing for nearly a year. Measurements of lesion sizes began when the first lesions were visible, and were repeated every 4 to 5 weeks until it was no longer possible to take accurate measurements due to lesion coalescence or tree death. In experiment A2, lesion length was measured on 12 occasions (12 February to 20 August 2004); in experiment B2 on 9 occasions (26 January to 21 October 2005), and in experiment C2 on 5 occasions (29 January to 21 May 2007). When lesions girdled the stem, thus causing death of the stem above the lesion, the measurement of that lesion and the lesions above were terminated. Sizes were measured with a digital calliper, accurate to 1 mm. Inoculations that failed were not used in the statistical analysis. In total, six parameters for lesion growth were compared (Fig. 1), as follows:

1. LESION-1: Lesion size (in millimeters) early in spring, when the first girdling lesions were observed.
2. LESION-2: Lesion size (in millimeters) at the end of the experiment.
3. AUDPC-1: Area under disease progress curve, from 1 January until the time point at which lesion size LESION-1 was assessed.

4. AUDPC-2: Area under disease progress curve, from 1 January until the end of the experiment.
5. T15mm (days): The time point (number of days since 1 January) at which the lesion reached the length of 15 mm.
6. LGR: Lesion Growth Rate (in millimeters per day). The estimated slope of the linear regression line of lesion size versus time, using a single start date specific for each experiment, and a lesion specific end date determined by girdling of the lesion (Fig. 1).

The AUDPC was calculated using the trapezium rule. The LGR was estimated by the slope of the linear regression line through the data points. The data series used varied between lesions. The start date was common to all lesions of the same experiment and was deduced from the data; it was the last date of observation at which, as yet, no lesion growth was observed. The end date was lesion specific; it was taken as the date at which the lesion stopped growing resulting in an almost flat part of the lesion growth curve. This date usually coincides with girdling of the stem at the lesion position or a lower position. Individual growth curves for each lesion and the estimated linear regression line are shown in Supplementary Figures S1, S2, and S3.

Data analysis. The six parameters for lesion size were initially statistically analysed for each experiment and position separately. Parameters were analysed according to the randomized block design, implying that genotype differences were tested after correcting for differences between blocks. This was done by means of linear regression rather than analysis of variance because, occasionally, observations were missing, causing unbalance in the design. In all three experiments trees were inoculated at various dates. In experiments B2 and C2, inoculation dates coincided with blocks within each position and, therefore, correcting for blocks also corrected for additive differences in inoculation dates. However, in experiment A2 the two inoculation dates did not coincide with blocks and, therefore, an additional correction was made for possible differences between inoculation dates. To stabilize the variance, all parameters were log transformed prior to statistical analysis. Pairwise differences between genotypes were tested at the 5% significance level.

It was also investigated whether a combined analysis would be possible for the four different lesion positions within each experiment. To this end, Bartlett's test for homogeneity of variance was employed to test whether the four residual variances for each position were similar. This was generally the case and, therefore, a combined analysis of the four positions was employed, still separately for each experiment. The combined analysis was a split-plot analysis in which differences between cultivars were tested at the tree level, whereas lesion position and the interaction between cultivar and position were tested at the position level within trees. In these combined analyses,

possible differences between inoculation dates were accounted for. The model was fitted by means of REML, instead of analysis of variance, with 'block/tree/position' as random model and 'inoculationDate + cultivar*position' as fixed model, where date of inoculation is considered as a correction factor.

Consistency across lesion positions for a parameter was quantified by examining the two-way table of means classified by cultivar and position, which was obtained from the separate analyses. If the differences between cultivars were identical for each position we concluded that lesion position was irrelevant and thus that the parameter was consistent with respect to position. In that case, an additive regression model with terms cultivar and position, which is fitted to the two-way table, would give a perfect fit. Thus, discrepancies could be measured by the adjusted R^2 of the additive model when fitted to the two-way table, where a large R^2 implies more consistency than a low R^2. This was done for each experiment separately. In the same way, consistency across experiments could be measured by fitting a model with terms experiment and cultivar to the two-way table of means classified by experiment and cultivar, which was obtained from the three combined analyses. Note that these means are obtained from the combined model without the interaction between positions and cultivar as this interaction was never significant (see Results). By comparing R^2 values among the six different parameters for lesion size, we were able to select the parameter with the highest consistency.

The parameters for lesion growth were further evaluated for statistical significance (i.e., their ability to reveal significant differences between cultivars). This was assessed by the mean of the *F* value for statistical testing of differences between cultivar, where the mean is taken over the separate analyses of the four inoculation positions.

Results

Components of resistance to European canker

Apple cultivars differ in their levels of susceptibility to *N. ditissima*, the causal agent of European canker of apple trees. Canker incidence and canker size are mostly used as components of resistance. Most likely, they represent two different resistance mechanisms. Therefore, we assessed both components in two types of experiments carried out in parallel; the first series for measuring the frequency of infections per main tree stem during natural leaf drop, and the second series for measuring lesion growth after artificial inoculation of wounds. For lesion growth, we aimed at finding a summarizing parameter that is not sensitive to artefacts, is consistent across lesion positions and across experiments, and that is able to reveal significant differences between apple cultivars according to their well-documented reputation.

Infection frequency

One of the parameters that describes disease incidence is infection frequency, which represents the resistance to onset of new lesions. In three experimental periods, apple cultivars were exposed to sporulating cankers during the natural leaf drop period. The overall mean infection percentage across the tested cultivars was 45%, 41% and 52% for the experiments A1, B1 and C1 respectively. Apparently, there was not a predominant effect of the year on the infection frequency.

Cultivar differences in infection percentages. The cultivars showed a wide range in infection percentage (from 8 to 90%). Significant differences between cultivars were observed (Fig. 2; Supplementary Table 1). Jonathan and Topaz were least affected. In contrast, Gala and Discovery appeared to be highly diseased and, thus, seem to have little resistance to becoming infected. These results were the same for the three experimental periods. The other six cultivars were intermediate and somewhat more variable in their infection frequencies. For instance, Golden Delicious in experiment A1 and Honeycrisp in experiment B1 showed relatively high infection percentages compared to the other experiments and other tested apple cultivars.

Consistency of results from year to year. An important feature of a resistance parameter is consistency of results from year to year. This consistency was generally good: 'Jonathan' and 'Topaz' always had the lowest and 2nd lowest percentage respectively, and 'Discovery' and 'Gala' always had the highest infection percentage. The ranking order of the cultivars with intermediate percentages varied among them (Golden Delicious, Santana, Honeycrisp, Elstar) (Fig. 2). The good consistency is also reflected by the high R^2 value, which was 75%.

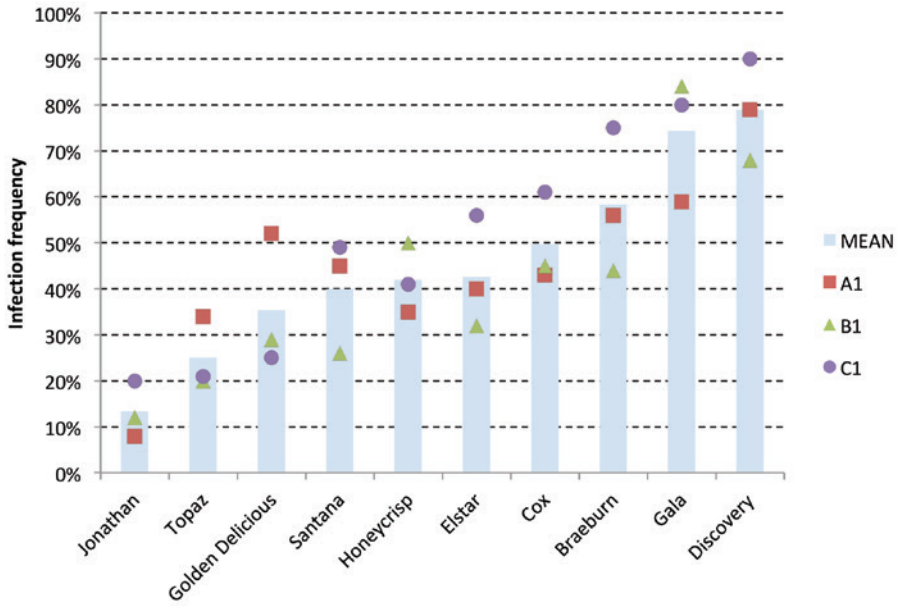


Figure 2. Percentage of leaf scars showing European canker symptoms due to natural infection on the main stem for the 10 evaluated apple cultivars in three independent experiments (A1, B1, and C1).

Lesion growth

Lesion growth after infection is the second *N. ditissima* resistance component considered for apple cultivars in our experiments. For the assessment of lesion growth, artificial wounds were made during leaf fall, and inoculated with a spore suspension. Necrotic lesions were first observed at 8 to 10 weeks following inoculation. There were no indications of cultivar resistance to infections via artificial inoculation: 96 to 100% of the inoculations led to growing lesions in all 3 years with our inoculation method. Lesions were similar in appearance as cankers observed in the field. The morphological characteristics of the cankers differed among the cultivars. In the more resistant cultivars the lesion progressed slowly and developed as cankers (i.e., callus formation) whereas, in the more susceptible cultivars the lesions progressed quickly, without signs of callus formation.

In general, the early lesion symptoms consisted of a depressed and discoloured area of bark near the point of inoculation. In a later stage of development, lesions progressed primarily vertically across the stem. However, in experiment A2 and C2 lesions frequently girdled the stem, leading to death of the stem above the lesion. The last measurement before the moment of girdling or stem death was used as the final measurement of that lesion.

Parameters for lesion growth. The lengths of the individual lesions were plotted over time. A typical example of lesion growth is depicted in Figure 1. This figure illustrates that factors such as the length of the latency period and girdling of the stem might influence final length of the lesions. For this reason we evaluated six different parameters for lesion size: LESION-1, LESION-2, AUDPC-1, AUDPC-2, T15mm, and LGR.

Separate analysis per inoculation position and the combined analysis. The six logarithmically transformed resistance parameters were statistically analysed separately for each experiment and position. In addition a combined analysis, also separately for each experiment, was performed on the four positions within each experiment. Resulting pairwise differences between cultivars, along with simple ranking of the cultivars, are given in Supplementary Table 2. The interaction between cultivar and position of inoculation was never significant in the combined analysis, while significant differences between positions are found for some parameters in some experiments. This implies that lesion size parameters may be influenced by the position of the inoculation on the main stem, but that differences in lesion size parameters between cultivars are similar for all four positions.

Consistency of parameters. Consistency across lesion positions was assessed by means of the adjusted R^2 of the additive regression model applied to the cultivar by position table of means (columns Position 1 to Position 4 in Supplementary Table 2), separately for each experiment. The parameters LESION-1 and AUDPC-1 had low R^2 values (Table 2), indicating low consistency, whereas LESION-2, AUDPC-2, T15mm and LGR had higher R^2 values. The growth rate parameter (LGR) had the largest consistency in experiment A2 and B2 whereas, in experiment C2, parameter T15mm was more consistent. Overall, LGR had

the largest consistency across lesion positions within experiments. Consistency across experiments was also assessed by the adjusted R^2 value now for the cultivar by experiment table of means resulting from the combined analysis of all four positions (columns Combined 1-4 in Supplementary Table 2). The two LESION parameters had the lowest consistency (Table 2) and LGR had the largest consistency.

Table 2. Consistency (adjusted R^2 values) for the six lesion growth parameters across inoculation positions, separately for each experiment and across experiments[a].

Consistency across inoculation position						
Experiment	**LESION-1**	**LESION-2**	**AUDPC-1**	**AUDPC-2**	**T15mm**	**LGR**
A2	0.35	0.75	0.24	0.79	0.82	0.85
B2	0.43	0.73	0.33	0.71	0.67	0.85
C2	0.59	0.61	0.53	0.62	0.67	0.59
MEAN	0.46	0.70	0.37	0.71	0.72	0.76
Consistency across experiments combined						
	LESION-1	**LESION-2**	**AUDPC-1**	**AUDPC-2**	**T15mm**	**LGR**
	0.45	0.46	0.73	0.71	0.68	0.81

[a] Larger values indicate higher consistency.

Statistical significance. The six parameters were further evaluated for their suitability to reveal significant differences between cultivars. The statistical significance of the parameters was assessed by the *F* values for statistical differences between cultivars. The LGR parameter had the highest statistical significance across all experiments, as well as for each of the single experiments (Table 3). Only in one experiment (C2), one other parameter (AUDPC-2) performed equally well. Therefore, LGR appears to be the best parameter for revealing statistically significant differences in lesion size among cultivars.

Table 3. Statistical significances: F values for cultivar for the six resistance parameters based on lesion growth[a].

Experiment	LESION-1	LESION-2	AUDPC-1	AUDPC-2	T15mm	LGR
A2	1.19	2.68	1.38	3.29	4.09	5.52
B2	2.08	2.90	1.25	2.92	2.37	3.21
C2	3.16	4.09	3.31	3.74	3.22	4.04
MEAN	2.14	3.22	1.98	3.32	3.23	4.26

[a] Average F values over the four inoculation positions on the stem are given in the bottom line. A high *F* value implies a high statistical significance.

Evaluation of lesion growth of the tested apple cultivars. Because the LGR was the best-performing parameter regarding consistency and statistical significance, we discuss the LGR for the tested cultivars in more detail. The average LGR among the tested cultivars was 0.27, 0.13, and 0.34 mm per day in experiment A2, B2, and C2, respectively, clearly indicating a strong effect of experimental conditions on lesion growth. Many significant differences were found between the cultivars (Supplementary Table 2). In general, the largest LGR values were observed for the cultivars Gala, Discovery, and Braeburn, and the smallest LGR values for the cultivars Jonathan, Topaz, Santana, Golden Delicious, Elstar, and Honeycrisp (Fig. 3), with intermediate LGR values for Cox Orange Pippin.

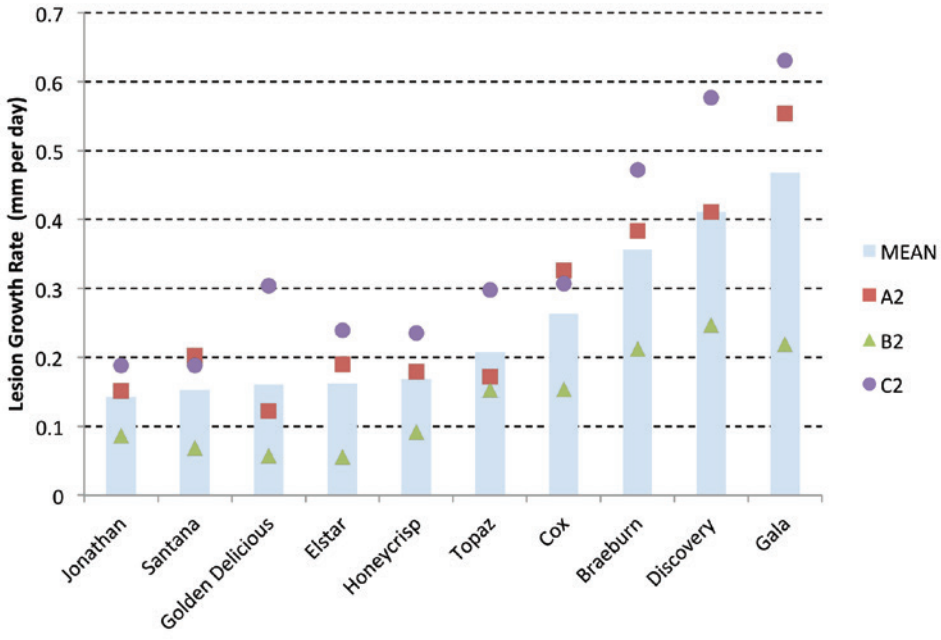
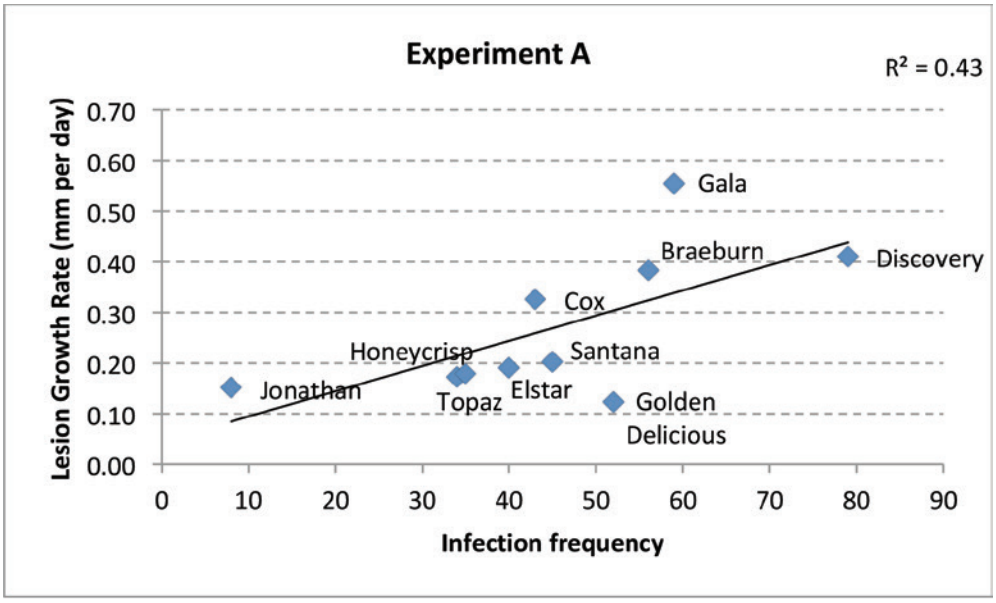
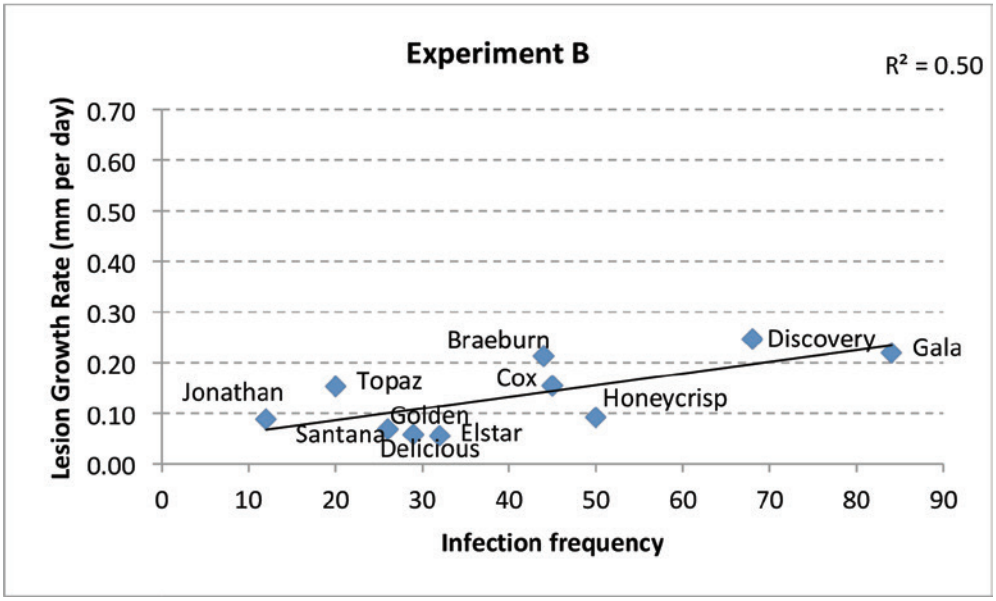


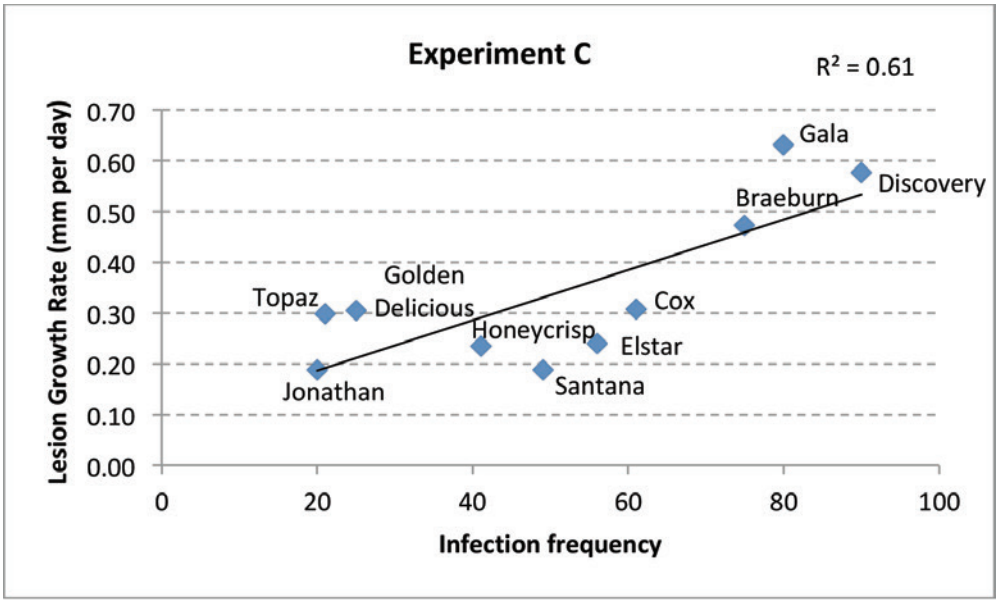
Figure 3. Lesion growth rate for the 10 evaluated apple cultivars in the three experiments A2 to C2.

Correlations between components of resistance

The correlation between infection frequency and Lesion Growth Rate was also investigated (Fig. 4). In all experiments, a positive correlation between LGR and infection frequency was found, with R^2 values of 0.43, 0.50, and 0.61 for the experiments A, B, and C, respectively. A higher R^2 value of 0.77 was obtained when using mean values calculated over the three experiments.


Experiment A
R² = 0.43
Lesion Growth Rate (mm per day)
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
10
20
30
40
50
60
70
80
90
Infection frequency
Gala
Braeburn
Discovery
Cox
Honeycrisp
Santana
Jonathan
Topaz
Elstar
Golden
Delicious


Experiment B
R² = 0.50
Lesion Growth Rate (mm per day)
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
10
20
30
40
50
60
70
80
90
Infection frequency
Braeburn
Discovery
Gala
Jonathan
Topaz
Cox
Honeycrisp
Golden
Santana
Elstar
Delicious



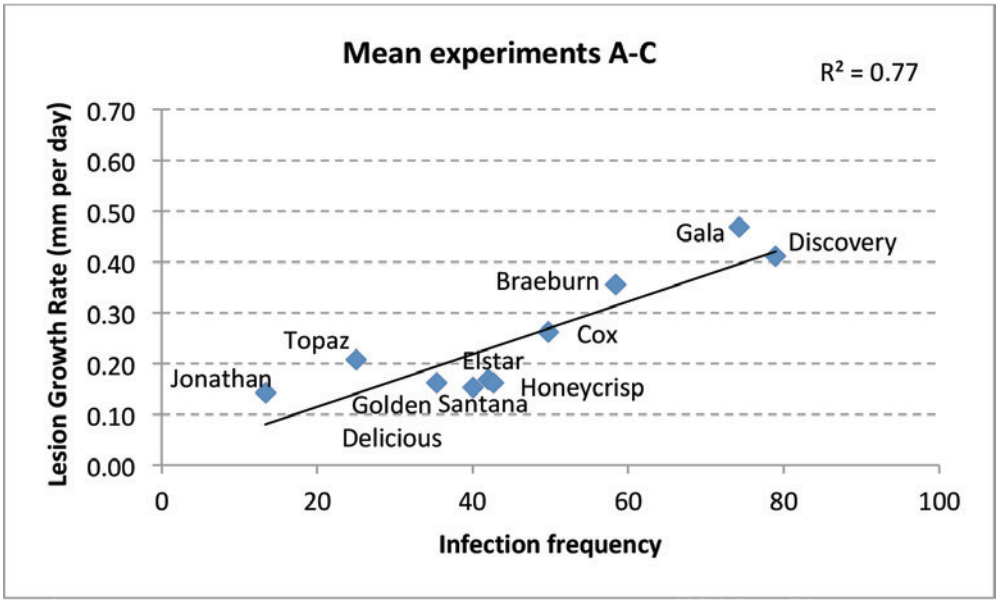


Figure 4. Relationships between infection frequency and lesion growth rate for the experiments A, B, C, and the mean values of A to C.

Discussion

The goal of this research was to find reliable, quantitative parameters for components of resistance in apple to European fruit tree canker caused by the fungal pathogen *N. ditissima*. Important criteria for such parameters are (i) consistency across experiments, (ii) sufficient resolution to reveal genetic differences between apple genotypes, (iii) insensitivity to particular disease-specific artefacts, and (iv) representation of distinctive components of resistance. To this end, we evaluated infection frequency and lesion growth for ten commercially available apple cultivars, and examined six alternative parameters for lesion growth.

In apple, leaf scars that arise during leaf fall in autumn are a main port of entrance for *N. ditissima*. Therefore, our experiments on infection frequency were carried out with naturally formed leaf scars under outdoor weather conditions. In these experiments we used potted trees that were exposed to inoculum sources (cankered prunings) during the entire leaf drop period. The results obtained over three experimental years showed that the infection frequency as a parameter for resistance to *N. ditissima* was reproducible, with sufficient statistical significance differences between cultivars. However, some genotypes with an intermediate level of resistance showed variable results over the experimental years. Thus, for a reliable evaluation of the genotypes with intermediate levels of resistance for leaf scar infection, multiple experiments are necessary.

Infection percentage as a parameter for resistance was successfully applied by Alston (1970), Garkava-Gustavsson et al. (2016) and Van de Weg (1989a). Our experimental conditions seemed more conducive for infection than those of Garkava-Gustavsson et al. (2016), given that their infection percentages ranged from 0 to 35% whereas ours ranged from 13 to 79%, despite following similar experimental approaches. Our range was only slightly less wide than those obtained by Alston (1970) (0-86%) and Van de Weg et al. (1989a) (3 to 100%), who both performed inoculation of artificial wounds. Moreover, classifications of cultivar susceptibility to infection were consistent among all studies for the (few) commonly tested cultivars. Apparently, various experimental approaches can be used to obtain similar information on the relative resistance of apple cultivars to infection.

Under natural conditions, *N. galligena* requires an appropriate wetness period to infect, which is also true for leaf scars (Latorre et al., 2002). Evaluation of leaf drop periods and weather conditions, especially precipitation, revealed that leaf scars of all cultivars were frequently exposed to possible infections from the first leaf drop until the last leaf drop. Also, the majority of cultivars had their main leaf drop during the same period (often after some days of low temperature). This makes it unlikely that specific cultivars escaped from possible *N. ditissima* infections due to weather conditions. Thus, under our conditions, there was no need for water showers (overhead irrigation), as used by Garkava-Gustavsson et al. (2016).

The second parameter we evaluated was lesion growth after inoculation of artificial wounds. In three lesion growth experiments, we successfully aimed for high infection frequencies (close to 100%) in order to get a statistically meaningful number of informative lesions with the

least possible numbers of trees. This approach was followed in view of cost efficiency and test-capacity by maximizing the number of individuals that can be tested within a single experiment, which is of importance for large germplasm screening experiments as for genetic studies on segregating quantitative trait loci (QTL) mapping populations. We circumvented the need for a wetness period by temporally sealing inoculated wounds with Vaseline. The required moisture probably comes from the wounded plant tissues, including xylem vessels. This approach showed to be highly efficient as it resulted in infection percentages across cultivars from 97% to 100% in the current experiments, 95 to 100% in Van de Weg (1989a), and 90% in Van de Weg (1989b).

A complicating factor in measuring lesion size was girdling of inoculated shoots or young stems when the lesions grew wider. As a result of girdling, the lesions stopped expanding, suggesting a final lesion length and, thus, creating a kind of artefact. Moreover, lesions above the site of girdling were affected even more dramatically. In those cases, lesion size measurements are uninformative. Consequently, such values for lesion size will lead to an underestimation of the levels of susceptibility for some cultivars. This is a common problem for this method (Garkava-Gustavsson et al., 2013; Ghasemkhani et al., 2015; Gómez-Cortecero et al., 2016). In view of this problem, these lesions as well as lesions above the one that girdled the stem were given the constant value of the last measurement before girdling by Garkava-Gustavsson et al. (2013), Ghasemkhani et al. (2015) and Van de Weg (1989a). To overcome this problem, lesion progress could be estimated by extrapolation but the cultivar-specific patterns of disease progression suggest that such extrapolation would have limited value (Garkava-Gustavsson et al., 2013). Therefore, Van de Weg (1989b) ranked cultivars at different stages of disease development; at early stages, the most susceptible cultivars could be distinguished from the less susceptible ones while, at the later stages, highly and moderately resistant genotypes could be distinguished.

In our experiments, we compared six different parameters for quantification of lesion growth (Fig. 1). Of these, LGR appeared the best performing parameter for consistency among lesion positions within a stem (Table 2), for consistency among experiments (Table 2), as well as for statistical significance (Table 3). LGR equals the estimated slope of the regression line of lesion size versus time (Fig. 1). It does not use data after girdling, thus preventing the girdling artefacts that are present in LESION-2 and AUDPC-2. Also, the more resistant individuals have the opportunity to develop lesions for a long time period compared to LESION-1 and AUDPC-1, where disease assessments are halted as soon as the first lesions (usually the most susceptible individuals) girdled the stem.

The advantage of LESION-1 and AUDPC-1 is that the artefact of girdling is prevented. At this time, the lesions of the more resistant individuals are usually still small or even not yet macroscopically visible, thereby possibly giving a poor signal to noise ratio and not allowing differentiation between the more resistant individuals. This explains the rather low reproducibility and statistical significance of LESION-1 and AUDPC-1. Further, LGR uses multiple measurements, thus increasing accuracy compared to LESION-1, LESION-2, and T15mm (which is based on the extrapolation between just two data points). Finally, the possibility to monitor disease development over a relatively long time period gives LGR the ability to reflect the interaction

between plant and pathogen across a wider range of physiological and developmental stages and weather conditions. This may give the best prospects to differentiate individuals for their resistance to N. ditissima, as observed in the orchard under standard cultural practices. In view of these qualities, we recommend LGR as the parameter of choice.

The LGR values were estimated using linear regression. A prerequisite of LGR is an approximately linear growth of the lesions. This condition of linear growth was generally met in the three experimental years (Supplementary Figures 1, 2, and 3) and will generally be the best approach when lesion size is assessed for a number of months rather than weeks.

The mean LGR appeared to vary strongly among the three experimental years (0.27, 0.13, and 0.34 mm per day in experiment A2, B2 and C2, respectively). The cause for this is not clear and might be due to differences in temperature and relative humidity in the growing season as well as during the first days after inoculation (Van de Weg et al., 1992). However, the relative differences in LGR-values of resistant and susceptible cultivars appeared to be consistent for the three experiments.

The parameter T15mm was an interesting solution for referring to larger lesions for the more resistant individuals while the assessment of the most susceptible individuals is not affected by girdling. Overall, this parameter outperformed LESION-1, AUDPC-1 and LESION-2 with respect to reproducibility among years and statistical significance, and performed similarly to AUDPC-2. Parameter T15mm also has the advantage that the time needed for disease assessment drops more rapidly during the course of an experiment, as trees can be excluded from further evaluation once the defined size limit has been reached. Thus, parameter T15mm may be considered the second best parameter following LGR.

For artificial inoculation we made four wounds per stem just below the abscission layer of leaves. The lowest wound appeared to lead to the lowest lesion growth rates for all cultivars. In view of this, it is advisable to use comparable inoculation positions, counting from the apex of the tree, when comparing genotypes or treatments.

Infected leaf scars need a certain period before symptoms become visible after inoculation and lesion size can be measured. This latency period was used as a parameter for measuring resistance in several cut shoots experiments (Garkava-Gustavsson et al., 2013; Ghasemkhani et al., 2015; Van de Weg, 1989a). They found latency a useful parameter for this type of short running experiments, where the plant material used would not last for long. However, in these experiments some cultivars showed only a minor sinking in or blackening of the stem. This could hamper correct assessment of the latency period. Different cultivars also showed different kinds of symptoms which also complicates assessment of the latency period. Both aspects (i.e., necrosis and variation in symptom type) could be accounted for by the use of a modified latency period defined as the time needed after inoculation to reach a certain small lesion size such as 5 mm (W. E. van de Weg, personal communication). In addition, the infection sometimes developed quickly, which required numerous data recordings within a short time window. This could be achievable with small-sized cut-shoot experiments, but not with large scale experiments on trees. Moreover, latency period reflects the resistance of an individual

colonization rate only for a very short time interval and for a specific stage in the interaction between plant and pathogen, whereas lesion size parameters as used in this research allow long term evaluations. For these reasons, we did not include latency period in our experiments.

Initial infection and subsequent lesion growth are different stages in disease development in which different components of resistance might be involved. Therefore, we investigated both aspects in independent experiments. Infection frequency and LGR showed quite a high correlation for their mean values of cultivars over the three experimental periods (R^2 of 0.77; Fig. 4D). This high correlation was due to small deviations from the regression line along the entire scale of observations rather than the presence of a major group of clustered cultivars and one or two outliers. This implies that the two parameters (i.e., infection frequency and LGR) are associated to one another, although they refer to different phases in the pathogenesis. Practically, this may mean that, when resources and labour are limited, one may choose for one parameter only. The statistical significance for infection frequency was better than for LGR. Apparently, this implies that, with infection frequency as a parameter it is easier to find significant differences between cultivars. Moreover, this method required far less labour input compared to LGR. Consequently, Infection frequency can be regarded as the preferred method compared to Lesion growth. However, in view of variability between years and the low correlation within single year experiments, we recommend to repeat these measurements during different years. Indeed, single year results may lead to misleading conclusions with both parameters. For instance, in experiment A1, the low susceptible cultivar Golden Delicious was more infected than the moderately susceptible cultivar Cox (Fig. 4A), whereas in experiment C2 both cultivars showed the same LGR (Fig. 4C) while they could clearly be distinguished when using the 3-year data (Fig. 4D). The high variability among years might indicate that the lower correlations for the individual experiments were caused by environmental factors (noise), rather than by different resistance mechanisms.

To the best of our knowledge, the relationship between infection percentage and colonization rate (lesion growth) have been studied only once before in independent experiments (Garkava-Gustavsson et al., 2016). In that research, infection percentage and colonization rate (evaluated as AUDPC) seemed to provide complementary information. For example, Gala was second highest for AUDPC but had intermediate infection percentages for both experimental years. These different findings might be due to differences in experimental approaches. Garkava-Gustavsson et al. (2016) mentioned to need for further optimization of their approach for assessing infection percentages.

The methods described in this article for the quantification of resistance of apple genotypes to European fruit tree canker may contribute in developing strategies for the control of European fruit tree canker; for example, breeding of new apple cultivars with high levels of resistance to *N. ditissima* infection would be helpful toward more sustainable apple production.

Acknowledgements

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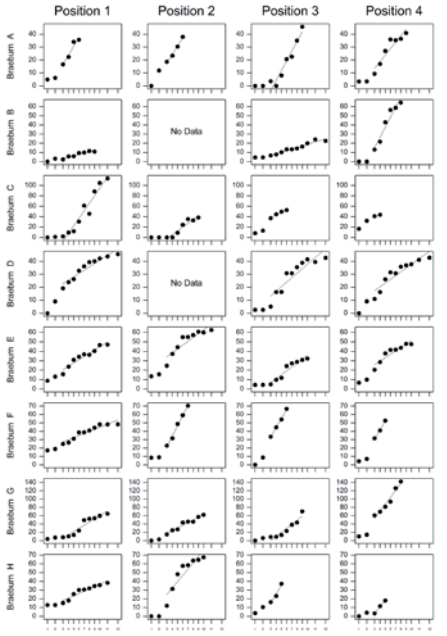
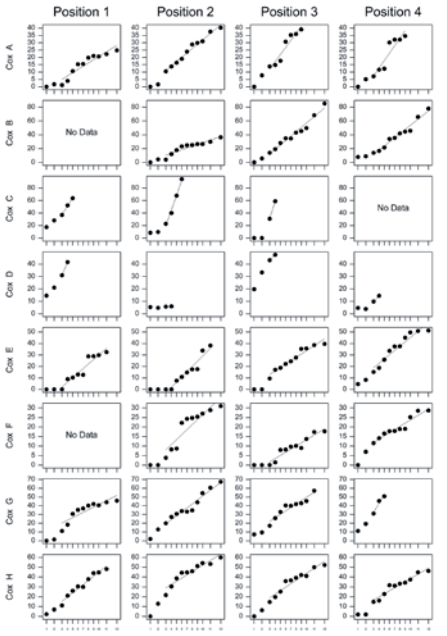
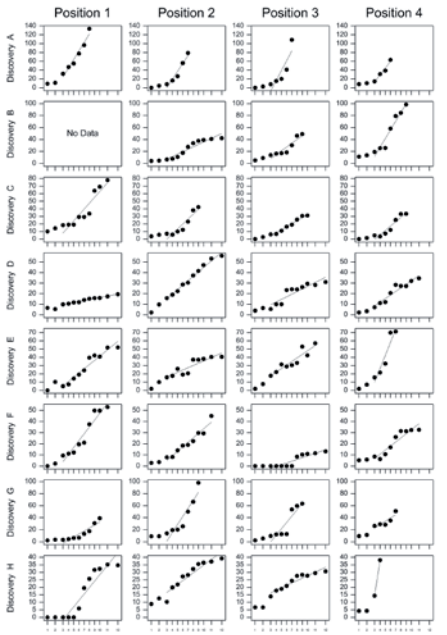
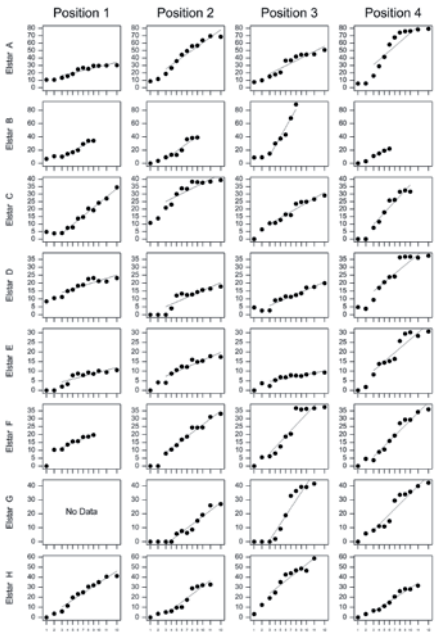
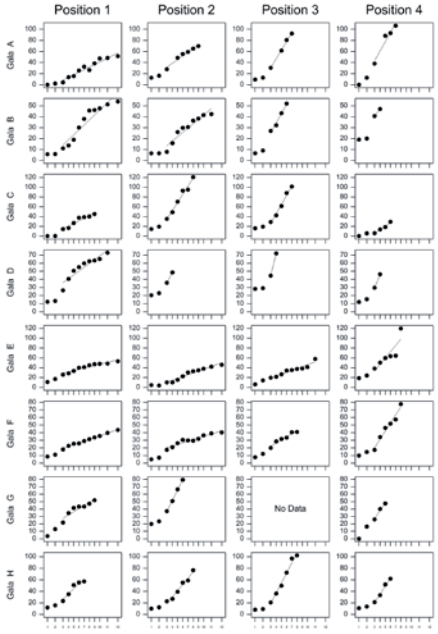
Supplemental material







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Gala G
Gala H
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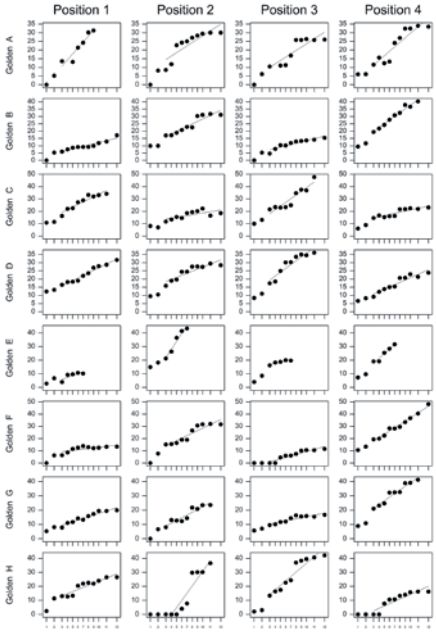
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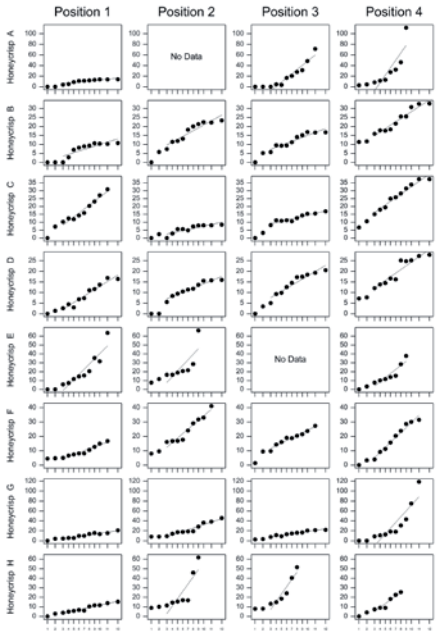
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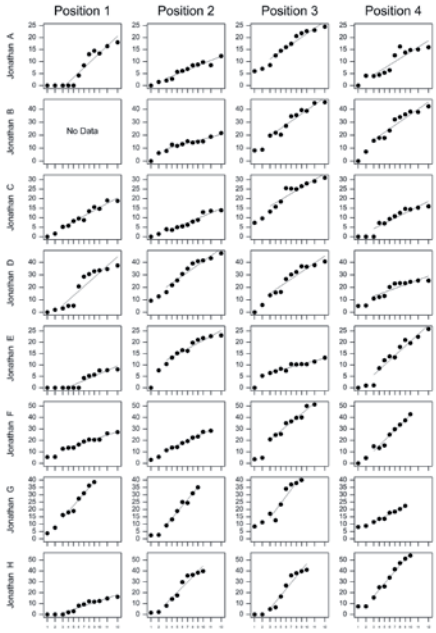
No Data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Jonathan H
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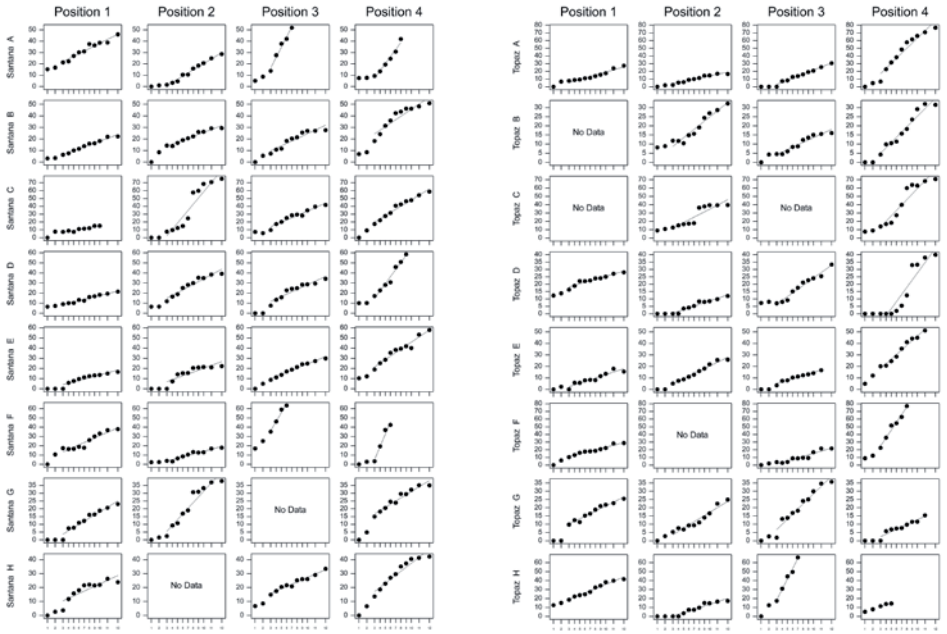
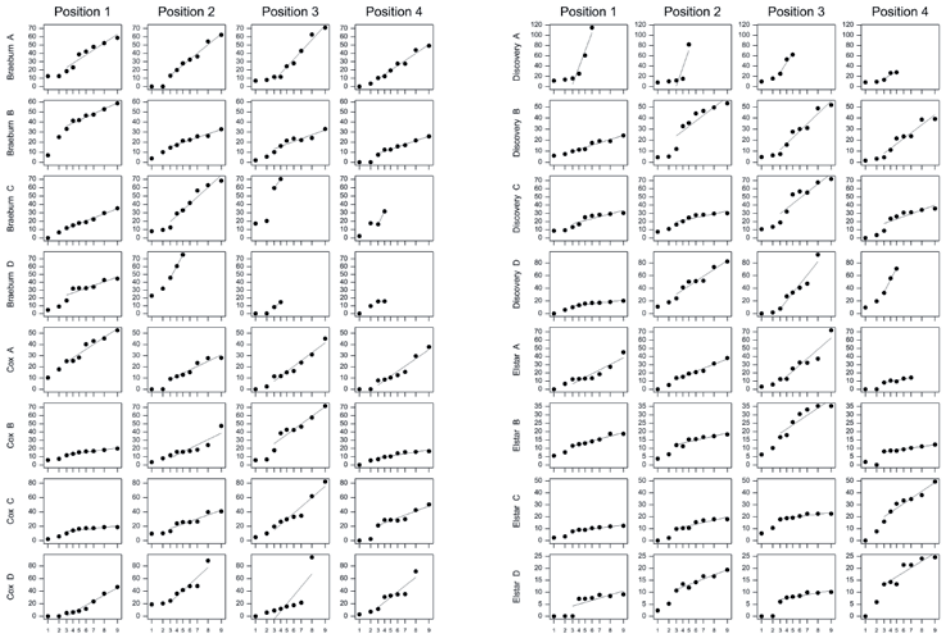
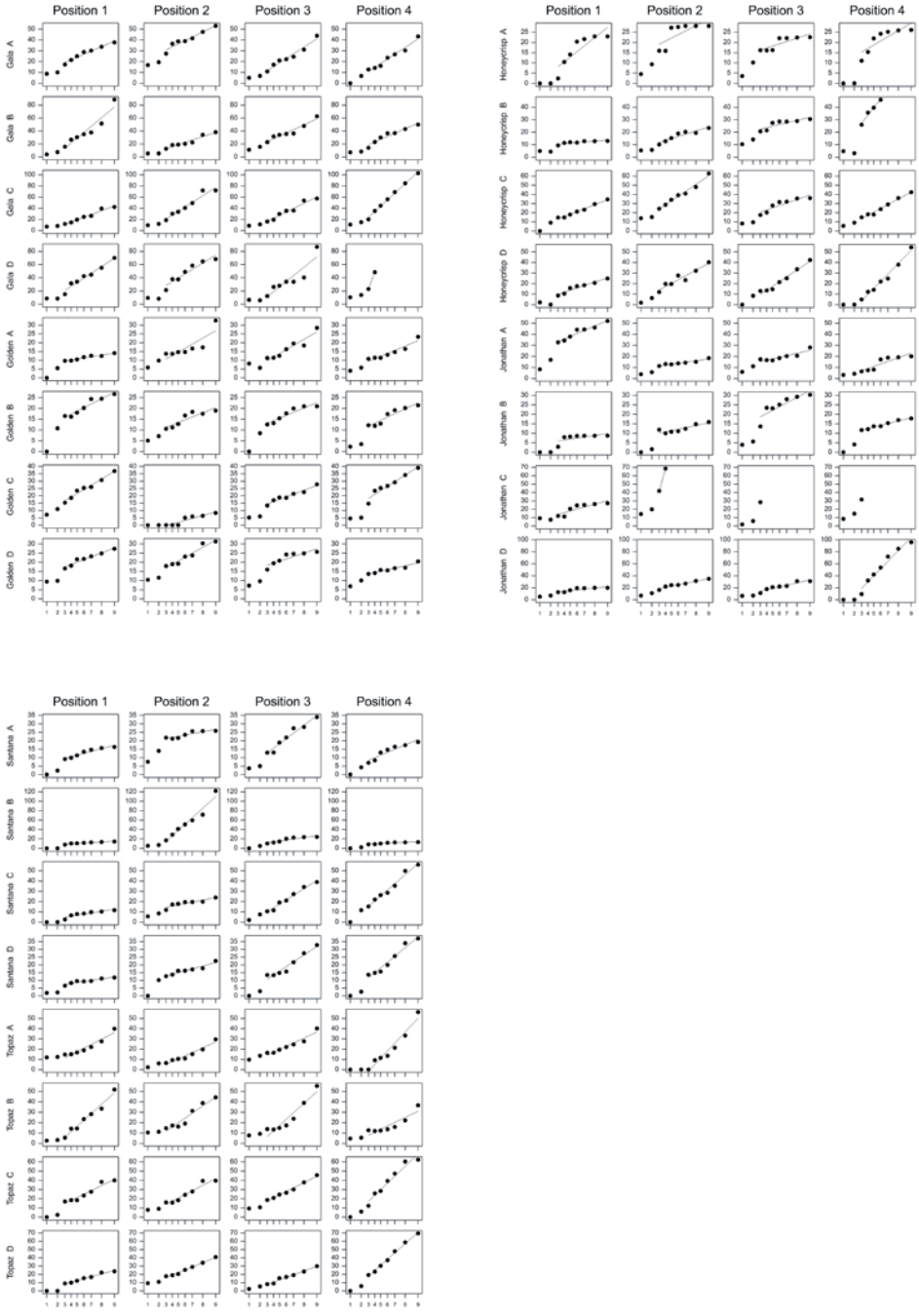
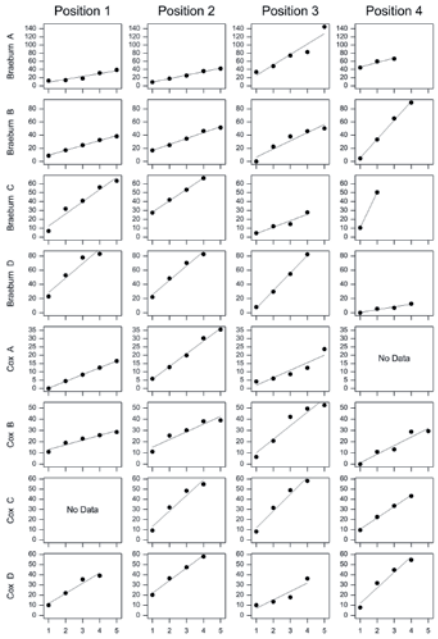


Supplementary figure S1. Individual growth curves for each inoculation and the estimated linear regression lines for experiment A1.





Supplementary figure S2. Individual growth curves for each inoculation and the estimated linear regression lines for experiment B1.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Braeburn B
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Cox A
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Cox C
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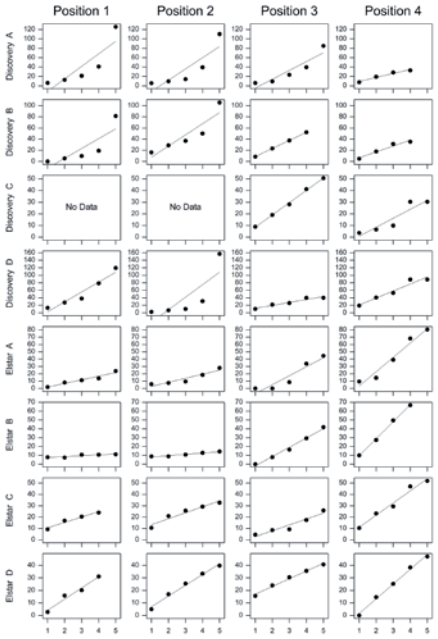
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Elstar C
Elstar D
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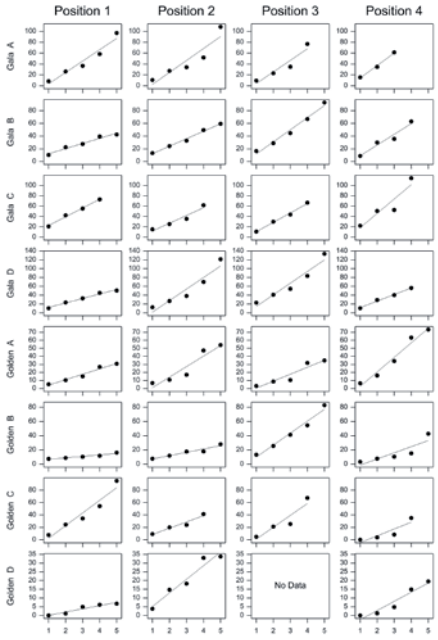
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Golden D
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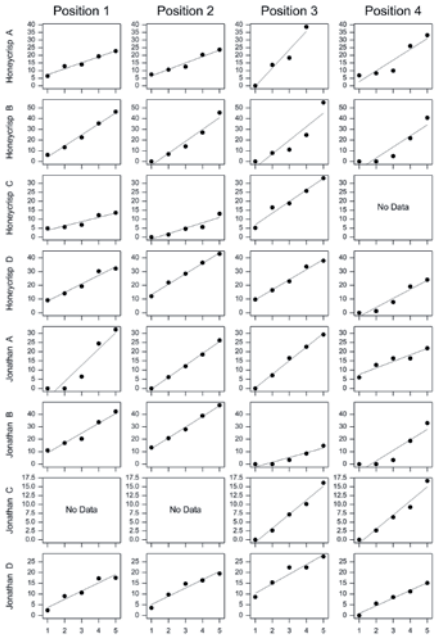
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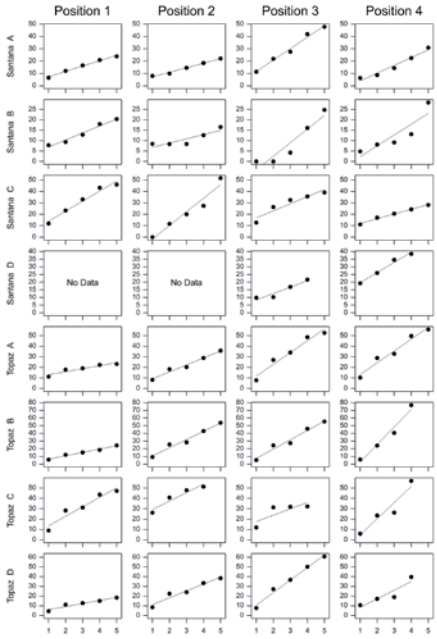

Supplementary figure S3. Individual growth curves for each inoculation and the estimated linear regression lines for experiment C1.

Supplementary Table 1.

Infection frequency

A logistic regression model, which employs the binomial distribution and a logit link, was used to analyse the number of cankers on the stem with binomial totals equals to the total number of leaf scars on the stem. Overdispersion relative to the binomial distribution was accounted for by inflating the binomial variance with a so-called overdispersion factor (McCullagh and Nelder, 1989). The experiments were performed three times, i.e. in 2003-2004, 2004-2005, and 2006-2007, are referred as the experiments A, B and C, respectively. In experiments B and C percentages rather than counts were observed and these were analysed by the same statistical model now with binomial totals equal to 100. Differences between cultivars were tested after allowing for differences between blocks. Pairwise testing of cultivars was done at the 5% level and cultivars which are not significantly different received the same letter (Table 1A).

Table 1A. Percentages of infected leaf scars for the tested apple cultivars.

Cultivar	A		B		C	
Jonathan	8	a....	12	a....	20	a.....
Topaz	34	.b...	20	ab...	21	a.....
Golden Delicious	52	..cd.	29	abc..	25	a.....
Honeycrisp	35	.b...	50	..cd.	41	.b....
Santana	45	.bcd.	26	abc..	49	.bc...
Elstar	40	.bc..	32	abc..	56	.bc...
Cox	43	.bcd.	45	.bcd.	61	..cd..
Braeburn	56	...d.	44	.bcd.	75	...de.
Gala	59	...d.	84	....e	80	....ef
Discovery	79	....e	68	...de	90	.....f

Ranking of cultivars based on the mean percentage infection is given in Table 1B.

Table 1B. Ranking of the tested apple cultivars for the three experiments based on the mean percentage infection.

Cultivar	A	B	C
Jonathan	1	1	1
Topaz	2	2	2
Golden Delicious	7	4	3
Honeycrisp	3	8	4
Santana	6	3	5
Elstar	4	5	6
Cox	5	7	7
Braeburn	8	6	8
Gala	9	10	9
Discovery	10	9	10

Supplementary Table 2.

The different parameters for evaluating lesion growth and hence for assessing resistance against *N. ditissima*; i.e. LESION-1, LESION-2, AUDPC-1, AUDPC-2, T15mm, and Lesion Growth Rate (LGR) were analysed with ANOVA. The experiments were performed three times, i.e. in 2003-2004, 2004-2005, and 2006-2007, and are referred as the experiments A, B and C, respectively. The results were analysed for the individual positions of inoculation on the stem (P1, P2, P3, P4) and averaged among all inoculation positions. To facilitate interpretation of the results, ranking numbers were added from 1 to 10. (R1, R2, R3, and R4 corresponding with the points of inoculation, and R5 corresponding with the average value among all inoculation positions). The six parameters were first statistically analysed for each year and each position separately. The experiment was designed as a randomized block experiment but due to non-negligible number of missing observations analysis of variance was replaced by linear regression which allows for difference between blocks and differences between treatments. Pairwise differences were tested at the 5% significance level and results were presented by giving cultivars the same letter when not significantly different. For the 2003 data analyses was carried out after allowing for tInoc differences. Residual plots indicated that for all the six parameters the variation increases with the mean which violates the assumption of homoscedasticity in the statistical analysis. This was resolved by performing a log transform prior to analysis.

The different parameters for evaluating lesion growth:

1. LESION-1 (mm). Lesion size (mm) early in spring when the first girdling lesions were observed.
2. LESION-2 (mm). Lesion size (mm) at the end of the experiment.
3. AUDPC-1. Area under disease progress curve, from 1 January till the time point at which lesion size 'LESION-1' was assessed.
4. AUDPC-2. Area under disease progress curve, from 1 January till the end of the experiment.
5. T15mm (days). The time point (number of days since 1 January) at which the lesion reached the length of 15 mm.
6. LGR: Lesion Growth Rate (mm day^{-1}). The estimated slope of the linear regression line of lesion size versus time, using a single start date specific for each experiment, and a lesion specific end date determined by girdling of the lesion.

Exp. A-LESION 1	Position 1	Position 2	Position 3	Position 4	Combined 1 - 4	R1	R2	R3	R4	R5
Braeburn	1.92 .bc	0.75 a	2.31 a	2.84 .bc	1.94 .bc	8	5	7	9	9
Cox	-0.10 ab.	1.11 a	2.07 a	1.75 abc	1.33 ab.	2	6	6	4	4
Discovery	0.56 abc	2.05 a	1.54 a	2.36 abc	1.64 abc	4	9	4	7	7
Elstar	1.35 abc	0.59 a	1.26 a	2.24 abc	1.36 ab.	7	3	3	6	5
Gala	2.94 ..c	2.29 a	2.66 a	3.03 ..c	2.80 ..c	10	10	10	10	10
Golden	2.40 .bc	1.64 a	1.65 a	1.61 abc	1.85 .bc	9	7	5	3	8
Honeycrisp	1.04 abc	0.67 a	1.00 a	2.21 abc	1.27 ab.	6	4	2	5	3
Jonathan	-1.22 a..	2.00 a	2.46 a	1.01 ab.	1.16 ab.	1	8	9	2	2
Santana	0.51 abc	0.20 a	2.43 a	2.40 abc	1.44 ab.	3	1	8	8	6
Topaz	0.61 abc	0.45 a	0.81 a	0.60 a..	0.53 a..	5	2	1	1	1

Exp. A-LESION 2	Position 1	Position 2	Position 3	Position 4	Combined 1 - 4	R1	R2	R3	R4	R5
Braeburn	3.69 .bcd	3.81 ..cd	3.78 abc	3.89 .bc	3.78 ..cd	8	8	8	9	8
Cox	3.41 abc.	3.74 .bcd	3.83 .bc	3.42 ab.	3.62 .bc.	7	7	9	3	7
Discovery	3.74 ..cd	3.87 ..cd	3.71 abc	3.86 .bc	3.79 ..cd	9	9	7	8	9
Elstar	3.09 a...	3.50 abc.	3.59 abc	3.62 abc	3.45 ab..	3	6	5	4	5
Gala	4.03 ...d	3.98 ...d	4.00 ..c	4.07 ..c	4.02 ...d	10	10	10	10	10
Golden	3.13 a...	3.33 ab..	3.25 a..	3.40 ab.	3.28 a...	4	4	1	2	1
Honeycrisp	3.05 a...	3.29 ab..	3.26 ab.	3.78 abc	3.38 ab..	2	3	2	6	4
Jonathan	2.99 a...	3.24 a...	3.53 abc	3.30 a..	3.29 a...	1	2	4	1	2
Santana	3.22 ab..	3.43 abc.	3.69 abc	3.83 .bc	3.54 abc.	6	5	6	7	6
Topaz	3.16 a...	3.13 a...	3.37 ab.	3.63 abc	3.33 ab..	5	1	3	5	3

Exp. A-AUDPC 1	Position 1	Position 2	Position 3	Position 4	Combined 1 - 4	R1	R2	R3	R4	R5
Braeburn	5.34 .bc	3.53 a	5.44 ab	5.92 .bc	5.04 ab	8	4	7	9	8
Cox	2.91 ab.	3.99 a	4.92 ab	5.50 .bc	4.48 a.	2	5	6	6	3
Discovery	3.66 abc	5.44 a	4.63 ab	5.72 .bc	4.87 ab	4	9	4	8	7
Elstar	4.76 .bc	3.51 a	4.60 ab	5.23 abc	4.51 a.	6	3	3	4	4
Gala	6.07 ..c	5.66 a	6.49 .b	6.31 ..c	6.19 .b	10	10	10	10	10
Golden	5.86 ..c	4.90 a	4.81 ab	4.85 abc	5.12 ab	9	7	5	3	9
Honeycrisp	3.96 abc	4.87 a	4.11 a.	5.37 abc	4.59 a.	5	6	2	5	6
Jonathan	1.52 a..	5.16 a	5.59 ab	4.15 ab.	4.21 a.	1	8	8	2	2
Santana	3.61 abc	3.20 a	5.68 ab	5.64 .bc	4.58 a.	3	1	9	7	5
Topaz	4.87 .bc	3.34 a	3.94 a.	3.56 a..	3.75 a.	7	2	1	1	1

Exp. A-AUDPC 2	Position 1	Position 2	Position 3	Position 4	Combined 1 - 4	R1	R2	R3	R4	R5
Braeburn	8.49 ...de	8.58 ..cde	8.59 .bc	8.82 ..cd	8.61 ...de	9	8	8	9	9
Cox	8.19 .bcd.	8.47 .bcde	8.59 .bc	8.25 ab..	8.40 .bcd.	7	7	9	3	7
Discovery	8.36 ..cd.	8.59 ...de	8.38 abc	8.69 .bcd	8.50 ..cd.	8	9	6	8	8
Elstar	7.86 abc..	8.20 abcd.	8.35 abc	8.39 abc.	8.20 abc..	3	6	5	5	5
Gala	8.88 ....e	8.82 ....e	8.91 ..c	9.04 ...d	8.91 ....e	10	10	10	10	10
Golden	8.03 abcd.	8.20 abcd.	8.08 ab.	8.23 ab..	8.13 ab...	6	5	3	2	4
Honeycrisp	7.71 ab...	7.97 ab...	7.98 a..	8.45 abc.	8.05 a....	2	2	1	6	3
Jonathan	7.60 a....	7.98 ab...	8.33 abc	8.06 a...	8.02 a....	1	3	4	1	2
Santana	7.98 abcd.	8.04 abc..	8.49 abc	8.63 .bcd	8.28 abc..	5	4	7	7	6
Topaz	7.86 abc..	7.71 a....	8.01 ab.	8.32 ab..	7.98 a....	4	1	2	4	1

Exp. A-T15mm	Position 1	Position 2	Position 3	Position 4	Combined 1 - 4	R1	R2	R3	R4	R5
Braeburn	4.52 ab..	4.54 ab..	4.60 .bc	4.34 ab..	4.50 .b..	2	2	4	2	2
Cox	4.67 abc.	4.59 ab..	4.44 .b.	4.47 .bc.	4.50 .b..	3	4	2	5	3
Discovery	4.79 .bcd	4.65 .bc.	4.75 .bc	4.37 ab..	4.65 .bc.	5	5	6	3	4
Elstar	5.07 ..cd	4.75 .bcd	4.78 .bc	4.69 ..cd	4.80 ..cd	8	6	8	7	7
Gala	4.28 a...	4.24 a...	3.92 a..	4.09 a...	4.15 a...	1	1	1	1	1
Golden	4.91 .bcd	4.58 ab..	4.77 .bc	4.58 .bcd	4.72 .bcd	6	3	7	6	6
Honeycrisp	5.15 ...d	4.00 ..cd	4.97 ..c	4.71 ..cd	4.95 ...d	10	9	10	8	10
Jonathan	5.10 ..cd	4.91 .bcd	4.66 .bc	4.82 ...d	4.86 ..cd	9	7	5	10	8
Santana	4.73 .bc.	4.94 .bcd	4.45 .b.	4.46 .bc.	4.66 .bc.	4	8	3	4	5
Topaz	4.92 .bcd	5.08 ...d	4.90 ..c	4.74 ..cd	4.91 ...d	7	10	9	9	9

Exp. A-LGR	Position 1	Position 2	Position 3	Position 4	Combined 1 - 4	R1	R2	R3	R4	R5
Braeburn	-1.34 .b	-0.79 ...d	-0.91 ...d.	-0.84 ..cd.	-0.99 ..cd	7	9	9	8	8
Cox	-1.13 .b	-1.20 .bcd	-1.10 ..cd.	-1.24 .bc..	-1.13 ..c.	9	7	8	5	7
Discovery	-1.00 .b	-0.96 ..cd	-1.14 ..cd.	-0.50 ...de	-0.91 ..cd	10	8	7	9	9
Elstar	-2.13 a.	-1.66 ab..	-1.49 abcd.	-1.39 abc..	-1.65 ab..	3	5	5	3	5
Gala	-1.14 .b	-0.72 ...d	-0.10 ....e	-0.24 ....e	-0.61 ...d	8	10	10	10	10
Golden	-2.29 a.	-1.93 a...	-2.15 a....	-1.92 a....	-2.90 a...	1	3	1	1	1
Honeycrisp	-2.07 a.	-1.760 ab..	-2.02 ab...	-1.23 ..c..	-1.73 ab..	5	4	2	6	4
Jonathan	-1.97 a.	-2.00 a...	-1.76 abc..	-1.83 ab...	-1.90 ab..	6	2	3	2	2
Santana	-2.08 a.	-1.63 abc.	-1.38 .bcd.	-1.17 ..c..	-1.62 .b..	4	6	6	7	6
Topaz	-2.25 a.	-2.01 a...	-1.63 abc..	-1.29 .bc..	-1.77 ab..	2	1	4	4	3

Exp. B-LESION 1	Position 1	Position 2	Position 3	Position 4	Combined 1 - 4	R1	R2	R3	R4	R5
Braeburn	3.26 ...d	3.33 .b	3.05 abc	2.93 abc	3.11 ..cd	10	9	7	8	8
Cox	2.58 abc.	2.99 .b	2.96 abc	2.80 abc	2.84 .bc.	4	6	6	5	6
Discovery	2.76 .bcd	3.24 .b	3.38 ..c	3.21 .bc	3.15 ..cd	8	8	10	9	9
Elstar	2.31 ab..	2.52 ab	2.60 ab.	2.58 a..	2.51 ab..	2	2	2	2	2
Gala	3.12 ..cd	3.39 .b	3.13 .bc	3.30 ..c	3.24 ...d	9	10	9	10	10
Golden	2.72 .bcd	1.29 a.	2.71 ab.	2.67 ab.	2.35 a...	7	1	4	3	1
Honeycrisp	2.46 ab..	2.91 .b	2.86 abc	2.92 abc	2.79 .bc.	3	4	5	7	5
Jonathan	2.64 abc.	3.05 .b	3.05 abc	2.85 abc	2.90 .bcd	5	7	8	6	7
Santana	2.16 a...	2.97 .b	2.53 a..	2.51 a..	2.54 ab..	1	5	1	1	3
Topaz	2.65 abc.	2.70 ab	2.66 ab.	2.77 abc	2.69 ab..	6	3	3	4	4

Exp. B-LESION 2	Position 1	Position 2	Position 3	Position 4	Combined 1 - 4	R1	R2	R3	R4	R5
Braeburn	3.88 ...de	4.04 ..c	3.68 ab	3.63 abcd	3.78 ..cd	9	9	6	5	7
Cox	3.43 .bcde	3.85 .bc	4.26 .b	3.65 .bcd	3.80 ..cd	6	7	10	6	8
Discovery	3.59 .bcde	4.05 ..c	4.22 .b	3.70 .bcd	3.89 ...d	7	10	9	7	9
Elstar	2.87 ab...	3.10 ab.	3.32 a.	3.07 a...	3.09 a...	2	2	2	1	1
Gala	4.03 ....e	4.03 ..c	4.11 .b	4.08 ...d	4.06 ...d	10	8	8	10	10
Golden	3.21 abcd.	3.00 a..	3.24 a.	3.22 ab..	3.17 ab..	5	1	1	2	2
Honeycrisp	3.11 abc..	3.58 abc	3.47 a.	3.70 .bcd	3.47 .bc.	4	4	4	8	5
Jonathan	3.10 abc..	3.38 abc	3.38 a.	3.46 abc.	3.33 ab..	3	3	3	4	4
Santana	2.59 a....	3.59 abc	3.47 a.	3.28 ab..	3.24 ab..	1	5	5	3	3
Topaz	3.62 ..cde	3.64 abc	3.73 ab	3.99 ..cd	3.75 ..cd	8	6	7	9	6

Exp. B-AUDPC 1	Position 1	Position 2	Position 3	Position 4	Combined 1 - 4	R1	R2	R3	R4	R5
Braeburn	7.22 ...d	7.17 .b	6.90 a	6.58 ab	6.95 .bc	10	9	5	6	8
Cox	6.56 abcd	6.90 ab	6.77 a	6.46 ab	6.67 abc	5	3	3	4	6
Discovery	6.86 .bcd	7.16 .b	7.01 a	6.85 ab	6.97 .bc	7	8	8	9	9
Elstar	6.03 ab..	6.50 ab	6.60 a	6.32 a.	6.36 a..	2	2	2	2	1
Gala	7.05 ..cd	7.30 .b	7.08 a	7.17 .b	7.15 ..c	9	10	10	10	10
Golden	6.88 .bcd	5.59 a.	6.82 a	6.72 ab	6.51 ab.	8	1	4	8	3
Honeycrisp	6.16 abc.	6.96 ab	7.05 a	6.51 ab	6.67 abc	3	5	9	5	5
Jonathan	6.61 abcd	7.00 ab	7.00 a	6.66 ab	6.82 abc	6	6	7	7	7
Santana	5.74 a...	7.01 ab	6.53 a	6.40 a.	6.42 a..	1	7	1	3	2
Topaz	6.49 abcd	6.91 ab	6.93 a	6.28 a.	6.65 abc	4	4	6	1	4

Exp. B-AUDPC 2	Position 1	Position 2	Position 3	Position 4	Combined 1 - 4	R1	R2	R3	R4	R5
Braeburn	8.98 ....e	9.12 ..c	8.80 abcd	8.68 abcd	8.87 ...de	10	9	7	6	8
Cox	8.49 .bcde	8.77 .bc	9.00 ..cd	8.57 abc.	8.71 ..cd.	6	7	9	4	7
Discovery	8.74 ..cde	9.20 ..c	9.25 ...d	8.88 ..cd	9.02 ....e	8	10	10	9	9
Elstar	7.93 ab...	8.23 ab.	8.42 a...	8.19 a...	8.19 a....	2	2	2	1	1
Gala	8.95 ...de	9.10 ..c	8.95 .bcd	9.10 ...d	9.02 ....e	9	8	8	10	10
Golden	8.41 .bcde	7.99 a..	8.40 a...	8.33 ab..	8.29 ab...	5	1	1	2	2
Honeycrisp	8.22 abc..	8.68 abc	8.66 abc.	8.71 .bcd	8.57 .bcd.	3	6	6	7	5
Jonathan	8.35 .bcd.	8.59 abc	8.60 abc.	8.62 abc.	8.54 .bc..	4	3	4	5	4
Santana	7.73 a....	8.68 abc	8.47 ab..	8.33 ab..	8.31 ab...	1	5	3	3	3
Topaz	8.51 .bcde	8.60 abc	8.63 abc.	8.74 .bcd	8.62 ..cd.	7	4	5	8	6

Exp. B-T15mm	Position 1	Position 2	Position 3	Position 4	Combined 1 - 4	R1	R2	R3	R4	R5
Braeburn	4.38 a..	4.19 a.	4.33 a.	4.64 ab..	4.39 a....	1	2	1	3	1
Cox	4.75 ab.	4.39 ab	4.71 ab	4.92 abcd	4.69 .bcd.	4	3	7	7	5
Discovery	4.79 ab.	4.46 ab	4.49 ab	4.53 a...	4.57 abc..	5	4	2	2	3
Elstar	5.57 ..c	5.02 ab	4.92 .b	5.28 ...d	5.20 ....e	9	9	9	10	10
Gala	4.61 ab.	4.16 a.	4.50 ab	4.51 a...	4.45 ab...	2	1	3	1	2
Golden	4.87 ab.	5.15 .b	4.81 ab	4.98 .bcd	4.95 ...de	7	10	8	8	8
Honeycrisp	5.17 .bc	4.58 ab	4.57 ab	4.71 ab..	4.76 ..cd.	8	5	5	4	6
Jonathan	4.64 ab.	4.74 ab	4.54 ab	4.83 abc.	4.69 abcd.	3	8	4	5	4
Santana	5.74 ..c	4.61 ab	4.94 .b	5.13 ..cd	5.10 ....e	10	6	10	9	9
Topaz	4.86 ab.	4.73 ab	4.70 ab	4.89 abcd	4.80 ..cd.	6	7	6	6	7

Exp. B-LGR	Position 1	Position 2	Position 3	Position 4	Combined 1 - 4	R1	R2	R3	R4	R5
Braeburn	-2.00 .bc	-1.39 .b	-1.41 .bcd	-1.32 ..cd	-1.55 ..c	8	9	8	9	8
Cox	-2.47 abc	-1.77 ab	-1.27 ..cd	-2.00 abcd	-1.87 .bc	6	7	9	5	7
Discovery	-1.97 .bc	-1.34 .b	-0.95 ...d	-1.35 ..cd	-1.40 ..c	9	10	10	8	10
Elstar	-3.05 ab.	-2.90 a.	-2.79 a...	-2.85 a...	-2.89 a..	2	1	2	2	1
Gala	-1.65 ..c	-1.73 ab	-1.57 .bcd	-1.15 ...d	-1.52 ..c	10	8	7	10	9
Golden	-2.96 ab.	-2.79 a.	-2.83 a...	-2.86 a...	-2.86 a..	4	2	1	1	2
Honeycrisp	-2.82 ab.	-2.32 ab	-2.61 a...	-1.85 .bcd	-2.39 ab.	5	5	4	6	5
Jonathan	-2.98 ab.	-2.36 ab	-2.64 a...	-2.17 abc.	-2.44 ab.	3	4	3	4	4
Santana	-3.43 a..	-2.61 ab	-2.20 ab..	-2.51 ab..	-2.68 a..	1	3	5	3	3
Topaz	-2.03 .bc	-2.00 ab	-1.96 abc.	-1.55 ..cd	-1.88 .bc	7	6	6	7	6

Exp. C-LESION 1	Position 1	Position 2	Position 3	Position 4	Combined 1 - 4	R1	R2	R3	R4	R5
Braeburn	3.59 .bc	3.78 ...d	3.70 ..cd	3.64 ...de	3.69 ...de	9	10	9	9	9
Cox	2.40 a..	3.57 ..cd	3.25 abcd	3.47 ...de	3.15 .bc..	1	8	5	7	6
Discovery	3.08 abc	3.00 abc.	3.40 .bcd	3.33 ..cde	3.20 .bc..	7	5	7	5	7
Elstar	2.81 abc	2.88 ab..	2.77 ab..	3.58 ...de	3.02 .bc..	5	3	2	8	5
Gala	3.64 ..c	3.59 ..cd	3.81 ...d	3.86 ....e	3.74 ....e	10	9	10	10	10
Golden	2.69 abc	3.02 abc.	3.25 abcd	2.56 abc..	2.87 ab...	3	6	6	3	3
Honeycrisp	2.77 abc	2.65 a...	2.93 abc.	2.06 a....	2.61 a....	4	1	4	1	2
Jonathan	2.56 ab.	2.97 abc.	2.46 a...	2.22 ab...	2.55 a....	2	4	1	2	1
Santana	3.10 abc	2.75 a...	2.90 ab..	2.95 .bcd.	2.90 ab...	8	2	3	4	4
Topaz	3.00 abc	3.40 .bcd	3.52 .bcd	3.40 ...de	3.34 ..cd.	6	7	8	6	8

Exp. C-LESION 2	Position 1	Position 2	Position 3	Position 4	Combined 1 - 4	R1	R2	R3	R4	R5
Braeburn	3.96 .bcd	4.07 ..cd.	4.15 ..cde	3.81 .bc	4.00 ..cd	8	8	8	7	8
Cox	2.98 a...	3.86 .bcd.	3.70 .bc..	3.78 abc	3.56 ab..	1	7	4	6	5
Discovery	4.72 ...d	4.81 ....e	4.38 ...de	3.74 abc	4.38 ...d	10	10	9	5	10
Elstar	3.05 a...	3.29 a....	3.62 abc..	4.09 .bc	3.52 ab..	2	1	3	9	4
Gala	4.13 ..cd	4.42 ...de	4.49 ....e	4.27 ..c	4.33 ...d	9	9	10	10	9
Golden	3.16 ab..	3.64 abc..	4.11 ..cde	3.64 abc	3.61 abc.	3	5	7	4	6
Honeycrisp	3.25 ab..	3.32 ab...	3.71 .bc..	3.02 a..	3.33 a...	4	2	5	1	2
Jonathan	3.41 abc.	3.37 ab...	3.04 a....	3.02 a..	3.19 a...	7	4	1	2	1
Santana	3.31 abc.	3.33 ab...	3.46 ab...	3.44 ab.	3.38 ab..	6	3	2	3	3
Topaz	3.27 abc.	3.78 abc..	3.89 .bcd.	4.03 .bc	3.75 .bc.	5	6	6	8	7

Exp. C-AUDPC 1	Position 1	Position 2	Position 3	Position 4	Combined 1 - 4	R1	R2	R3	R4	R5
Braeburn	7.24 .bc	7.45 ....e	7.23 ..cd	7.25 ..c	7.30 ....e	9	10	9	9	9
Cox	5.70 a..	7.20 ..cde	6.79 .bcd	7.07 ..c	6.66 ..cd.	1	8	5	8	6
Discovery	6.56 abc	6.58 abcd.	6.93 .bcd	6.88 ..c	6.74 ..cd.	6	5	7	5	7
Elstar	6.43 abc	6.56 abcd.	6.10 ab..	7.03 ..c	6.54 ..c..	5	4	2	6	5
Gala	7.30 ..c	7.25 ...de	7.42 ...d	7.51 ..c	7.38 ....e	10	9	10	10	10
Golden	6.15 abc	6.65 abcd.	6.91 .bcd	5.83 ab.	6.36 abc..	3	6	6	3	3
Honeycrisp	6.43 abc	6.09 a....	6.50 abcd	5.03 a..	6.02 ab...	4	1	4	1	2
Jonathan	5.89 ab.	6.45 abc..	5.60 a...	5.42 a..	5.84 a....	2	3	1	2	1
Santana	6.75 abc	6.39 ab...	6.31 abc.	6.63 .bc	6.50 .bc..	8	2	3	4	4
Topaz	6.73 abc	7.16 .bcde	7.20 ..cd	7.07 ..c	7.05 ...de	7	7	8	7	8

Exp. C-AUDPC 2	Position 1	Position 2	Position 3	Position 4	Combined 1 - 4	R1	R2	R3	R4	R5
Braeburn	8.26 ..cd	8.44 ...d	8.37 ..cd	8.25 .bc	8.34 .....fg	9	9	9	7	9
Cox	7.14 a...	8.21 ..cd	7.91 .bc.	8.13 .bc	7.82 ..cde..	1	7	5	6	6
Discovery	8.24 .bcd	8.29 ..cd	8.26 .bcd	8.05 .bc	8.19 ....efg	8	8	8	5	8
Elstar	7.39 abc.	7.55 ab..	7.62 ab..	8.28 .bc	7.72 .bcd...	3	3	2	9	5
Gala	8.37 ...d	8.45 ...d	8.62 ...d	8.61 ..c	8.52 ......g	10	10	10	10	10
Golden	7.29 ab..	7.82 abc.	8.20 .bcd	7.49 ab.	7.66 abcd...	2	5	6	3	4
Honeycrisp	7.48 abcd	7.32 a...	7.76 .bc.	6.86 a..	7.36 ab.....	5	1	4	1	2
Jonathan	7.46 abcd	7.56 ab..	7.02 a...	6.95 a..	7.23 a.....	4	4	1	2	1
Santana	7.69 abcd	7.46 a...	7.64 ab..	7.64 ab.	7.58 abc....	7	2	3	4	3
Topaz	7.63 abcd	8.12 .bcd	8.22 .bcd	8.26 .bc	8.06 ...def.	6	6	7	8	7

Exp. C-T15mm	Position 1	Position 2	Position 3	Position 4	Combined 1 - 4	R1	R2	R3	R4	R5
Braeburn	3.68 ab.	3.23 a..	3.63 ab.	3.11 a.....	3.40 a...	2	1	2	1	1
Cox	4.41 .bc	3.62 ab.	4.11 .bc	3.88 .bcd..	4.03 .bc.	7	4	7	6	5
Discovery	4.03 abc	4.02 .bc	3.87 ab.	3.86 .bcd..	3.98 .bc.	3	5	4	5	4
Elstar	4.53 ..c	4.34 ..c	4.23 .bc	3.86 .bcd..	4.23 ..cd	9	8	9	4	7
Gala	3.53 a..	3.51 ab.	3.38 a..	3.39 ab....	3.44 a...	1	2	1	2	2
Golden	4.66 ..c	4.13 .bc	3.88 abc	4.50 ...def	4.31 ..cd	10	6	5	8	8
Honeycrisp	4.46 .bc	4.45 ..c	4.16 .bc	4.86 .....f	4.47 ...d	8	9	8	10	10
Jonathan	4.31 abc	4.15 .bc	4.58 ..c	4.71 ....ef	4.47 ...d	6	7	10	9	9
Santana	4.15 abc	4.51 ..c	4.06 abc	4.06 ..cde.	4.18 .bcd	4	10	6	7	6
Topaz	4.16 abc	3.54 ab.	3.66 ab.	3.75 abc...	3.77 ab..	5	3	3	3	3

Exp. C-LGR	Position 1	Position 2	Position 3	Position 4	Combined 1 - 4	R1	R2	R3	R4	R5
Braeburn	-0.96 .bcd	-0.87 ..cd.	-0.59 ...de	-0.63 ..cd	-0.75 ..cd	8	8	9	9	8
Cox	-1.52 abc.	-1.04 .bcd.	-1.10 .bcd.	-0.94 .bcd	-1.18 .bc.	6	7	5	6	7
Discovery	-0.09 ...d	-0.08 ....e	-0.83 ..cde	-0.97 .bcd	-0.55 ...d	10	10	7	5	9
Elstar	-2.02 a...	-1.86 a....	-1.25 abc..	-0.64 ..cd	-1.43 ab..	1	1	3	8	4
Gala	-0.79 ..cd	-0.46 ...de	-0.28 ....e	-0.34 ...d	-0.46 ...d	9	9	10	10	10
Golden	-1.80 ab..	-1.22 abc..	-0.71 ..cde	-1.06 .bc.	-1.19 .bc.	3	6	8	4	6
Honeycrisp	-1.76 ab..	-1.60 ab...	-1.11 .bcd.	-1.26 abc.	-1.45 ab..	4	3	4	3	3
Jonathan	-1.37 abc.	-1.51 abc..	-1.78 a....	-1.82 a...	-1.67 a...	7	4	1	1	1
Santana	-1.75 abc.	-1.79 ab...	-1.49 ab...	-1.63 ab..	-1.67 a...	5	2	2	2	2
Topaz	-1.89 a...	-1.26 abc..	-1.01 .bcd.	-0.71 ..cd	-1.21 .b..	2	5	6	7	5

7.

Identification of *Alternaria* spp. as causal agent of Dead Flower Buds Disease of Pear (*Pyrus communis*) in the Netherlands and methods for disease control

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Abstract

The occurrence of dead dormant flower buds is a common phenomenon of economic importance in the major pear production areas of Europe. Thus far, the cause of dead flower buds disease remained unknown. Several causes have been proposed, including insufficient tree chilling, unmet dormancy requirements, incompatibility between scion and cultivar, but also various biotic stress agents such as pathogens and pests. In this study, we tested the relationship between reduction of tree growth and dead flower bud incidences, but found no indication that growth regulation can prevent the occurrence of dead flower buds. It has been proposed that the bacterium *Pseudomonas syringae* pv. *syringae* may be the causal agent of dead flower buds of pear. However, although we found the bacterium as epiphyte and even as endophyte on and in flower buds, our findings argue that *P. syringae* pv. *syringae* is not the causal agent of dead flower buds disease in the Netherlands. In our research, *Alternaria* spp. were consistently found in diseased flower buds, and strong correlations between dead dormant flower buds and infection rates of flower buds with *Alternaria* spp. were recorded. The isolated *Alternaria* species were identified as *A. arborescens* SC and *A. alternata* SC. Field experiments for disease control showed that the disease may be controlled by specific fungicide applications. Thus, we propose that dead flower buds of pear in the Netherlands should be regarded as a fungal disease caused by *A. alternata* SC and potentially also *A. arborescens* SC.

Introduction

Dead dormant flower buds of pears (*Pyrus communis*) are a common phenomenon in pear cultivation in the Netherlands, Belgium and Mediterranean countries, and may cause significant (financial) losses due to low harvests (Deckers and Schoofs, 2001; Deckers et al., 2008; Montesinos and Vilardell, 1991, 2001; Wenneker et al., 2004, 2006). Similarly, further cases of flower bud abortion and floral primordia necrosis are reported from South America (e.g. Uruguay) and South Africa (Pinto de Arruda and Camelatto, 1999; Verissimo et al., 2004; Yamamoto et al., 2010). The impact varies from reduced numbers of flowers per bud, to buds that are completely killed, and can reduce the productivity of pear trees substantially (Deckers and Schoofs, 2001).

Flower bud formation of pear is the period from the start of the flowering process with flower induction in spring or summer, until flower expression with anthesis occurring in the following spring. After flower induction, successive physiological steps occur, leading to morphological differentiation when the apex becomes organized into a flower primordium or inflorescence. By leaf fall in autumn all parts of the flowers are present in a large percentage of flower buds, and the development of the tissues starts with the sepals, followed by petals and anthers, to finalize with the ovary. In pear, the lower flowers in the cluster develop first and the apical flower develops last, which also is the order of flowering in spring (Faust et al., 1997; Marafon et al., 2010).

Flower bud break in perennial trees is affected by two temperature-dependent processes: I) accumulation of chilling temperatures to the level required to break dormancy, and II) accumulation of heat units required for the buds to bloom and foliate (Naor et al., 2003). Inadequate chilling results in several physiological and anatomical abnormalities, including deformation and abscission of flowers, leading to yield reduction (Stushnoff et al., 1984). In temperate climates, significant damage on deciduous fruit trees are produced in buds, flowers and developing fruits after dormancy due to frosts during bloom, which is typically more destructive than low winter temperatures (Rodrigo, 2000). Besides damage to the internal tissue of the flower buds, frost damage allows explosive development of epiphytic bacteria in the buds which results in total decay of these buds (Montesinos and Vilardell, 1991).

In Southern Brazil it was observed that, regardless of origin or chilling requirements, different cultivars of *Pyrus communis* and *Pyrus pyrifolia* produced aborted flower buds (Nakasu et al., 1995). The pear flower bud abortion occurred during the pre-bloom stage following winter dormancy. The aborted buds had dry protector bracts, and dry and necrotic internal flower primordia.

Nakasu et al. (1995) proposed that fluctuations of high temperatures followed by low temperatures are responsible for flower bud abortion. Nevertheless, the impact (if any) of pathogens on flower bud abortion is completely unknown and an area research that deserves attention (Deckers et al., 2008; Montesinos and Vilardell, 2001; Nakasu et al.,

1995). The bacterium *Pseudomonas syringae* pv. *syringae* is the causal agent of blossom blast of pear, characterized by blast of blossom and leaves, which occurs in periods of cool wet weather during bloom and post-bloom stages (Mansvelt and Hattingh, 1986; Qiu et al., 2008; Whitesides and Spotts, 1991). In Europe it has been suggested that *P. syringae* pv. *syringae* could be a causal agent of dead flower buds (Deckers et al., 2008; Montesinos and Vilardell, 1991). However, symptoms of blossom blast differ from the symptoms of dead flower buds that are characterized by partial or complete necrosis of flower buds during dormancy or at bud break. This dead flower bud necrosis may affect primordial flowers, leaves and scales, and usually starts at the tip part of the bud and progresses to the base. Depending on disease severity, flowers per cluster may be reduced, buds may produce abnormal flowers, or buds may be completely inactive (Fig. 1). When disease incidence is high, vegetative growth of trees is delayed, and trees may become alternate bearers annually.

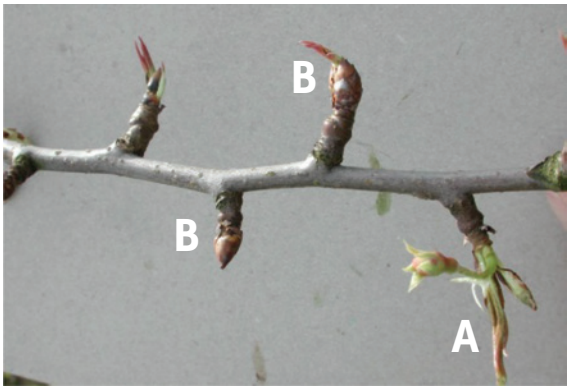

Figure 1. Symptoms of affected flower buds of pear, the number of flowers per cluster may be reduced (A), or buds may be completely inactive (B).

It has also been suggested that the occurrence of dead flower buds is related to vigorous tree growth (Deckers et al., 2008). Insufficient reduction of shoot growth eventually causes lack of light within the canopy which may negatively affect flower bud development (Maas, 2005). High density pear planting systems with 2,500 to 3,000 trees per hectare in the Netherlands are maintained by the use of dwarfing rootstocks, like quince MC and quince Adams, and additional growth retarding practices. Since the banned use of chlormequat (CCC) in pear orchards from 2001 onwards, root pruning and incision of tree trunks have become major growth-retarding methods. In addition to the mechanical methods for controlling tree vigour, Regalis (prohexadione-calcium) and ethephon (Ethrel-A) were tested as alternative chemical growth regulators (Maas, 2005, 2008).

Due to its economic impact, pear growers desire control methods to restrict dead flower buds. Therefore, the aims of this research are (i) to identify the cause of dead flower buds of pears, and (ii) to develop and evaluate possible control strategies.

Materials and methods

Tree growth regulation and Resistim application

All trials were performed from 2002-2005 in an orchard with spindle shaped pear trees (cultivar Conference) on Quince MC rootstock that were planted in 1999 in a single row planting system (3.5 m x 1.5 m) at the experimental station in Randwijk, the Netherlands. The following treatments were performed on the same trees in three consecutive years:

1. Luxan ethephon (active ingredient: ethephon 48%): four applications with seven to ten days intervals with the first application at two weeks after bloom. The first application was 250 ml ha^{-1}, the second application 150 ml ha^{-1}, and the third and fourth applications 100 ml ha^{-1}.
2. Regalis (active ingredient: prohexadione-calcium, 10%): three applications of 1 kg ha^{-1} with tree week intervals and the first application starting at three to five leaf stage.
3. Root pruning: two-sided pruning at 35 cm from the trunk at the east side of the trees at the end of May and the west side of the trees at the beginning of June.
4. Resistim (potassium phosphonate: potassium 139 g l^{-1}; phosphorus 75 g l^{-l}): seven weekly applications of two liters ha^{-1} from the beginning of May onwards.
5. Non-treated control (no growth regulation).

Ethephon, Regalis, and Resistim treatments were carried out as spray applications with a handheld spray gun (manufacturer EMPAS, Veenendaal, the Netherlands) with a 1.2 mm ceramic hollow cone nozzle at 1.1 – 1.2 Mpa and a spraying volume of 1000 l ha^{-1}. The experiment was done in a randomized block design with five replicates. Each replicate consisted of five trees. Observations were made on the middle three trees.

Dead flower bud assessment and statistical analyses

Dead flower bud incidences were assessed in April in the year after the treatments were carried out. Dead flower bud incidence was measured as the percentage of dead flower buds per tree. All flower buds per tree were counted and the disease incidence per tree was calculated from the overall count. In 2004 and 2005 assessments were carried out for different bud types with specific observations for end buds, buds on 1-year old twigs, and on >1-year old twigs. Mean dead flower bud incidence of all trees for each replicate was used for statistical analysis. The effect of the treatments was determined with ANOVA at a 0.05 probability level.

Assessment of bacterial pathogen presence

Three pear orchards were sampled in 2002 (August 29, September 25, November 1 and 29), and 2003 (February 14, November 12 and December 3) to determine *Pseudomonas* spp. population levels. A total of 29 bulk samples were taken, each composed of 50 buds from

randomly chosen trees, and transported in plastic bags to the laboratory of the National Plant Protection Service (NPPS, Wageningen, The Netherlands). All samples were divided in two sub-samples of 25 buds each, one of which was processed non-sterilized while the other was surface sterilized in 70% ethanol for 30 seconds, rinsed three times with distilled water and dried on paper tissue.

The sub-samples were then macerated with mortar and pestle in 5 ml 0.05 M sterile phosphate buffered saline (PBS), left for 30 minutes and again macerated. Suspension aliquots of 100 µl were plated onto 3 modified sucrose peptone (MSP) agar plates (Mohan and Schaad, 1987) and 20 µl was plated onto King's B agar (King et al., 1954) and Levan medium (Lelliott and Stead, 1987) and incubated for 4 days at 28°C. Typical colonies were transferred to nutrient agar (NA) for purification and subsequent identification (Janse, 1991).

On November 29 2002, December 20 2002, March 13 2003 and February 3 2004 additional bulk samples were collected. In the laboratory these buds were cut in half and examined with a stereo microscope for necrotic flower primordia. Symptomatic buds were processed individually for assessing *Pseudomonas* spp. presence by macerating in 0.5 ml 0.05 M sterile PBS and subsequent plating as described above.

For *Pseudomonas* inoculation assays, four replicates of 3 twigs (approximately 100 flowers buds in total) were injected into the flower buds with a *Pseudomonas syringae* pv. *syringae* (strain PD2873) bacterial suspension (10^6 CFU ml^{-1}). To this end, the needle was carefully introduced into the bud through the 'distal' end until half of its longitudinal axis was reached. Control buds were mock inoculated with sterile water.

Assessment of fungal pathogen presence

Eight commercial pear orchards (cultivar Conference) were sampled in January and February 2004 to determine the presence of fungal pathogens. Random samples of 100 flower buds per orchard were taken. From these samples, 50 buds were cut in half and examined with a stereo microscope for necrotic flower primordia, and 50 buds were individually tested for infection with *Alternaria* spp.. The buds used for determination of infections were surface sterilized by immersing them for 30 minutes in 2.5% formaldehyde-solution (active ingredient 40%) and then thoroughly washed in sterile demineralized water to remove sterilizing agent. Subsequently the buds were cut into two pieces, and the flower primordia of each bud were plated onto Potato Dextrose Agar (PDA). Plates were incubated at 20°C in the dark for 5-7 days and assessed for the presence of *Alternaria* spp..

From 2006-2013 (except 2010) in 13 commercial pear orchards (cultivar Conference) random samples of 50 flower buds per orchard were individually tested for *Alternaria* spp. infections in February. In addition, dead flower bud incidences per orchard were determined in April and this was measured as the percentage of dead flower buds per tree. All flower buds of 15 random trees per orchard were assessed.

Identification of the *Alternaria* species

A total of 63 single spore isolates were used in this study. Isolates were selected from diseased flower buds of different pear cultivars (Conference, 25 isolates; Doyenné du Comice, 10 isolates; Xenia, 10 isolates) and 18 isolates from apple cultivar Golden Delicious with leaf blotch symptoms (Wenneker et al., 2018). Each single spore isolate was grown on potato dextrose agar (PDA; Difco Laboratories Inc.) and incubated for 10–14 days at 20°C in the dark. The isolates were identified to the genus level by morphological characteristics. The identity of the isolates was confirmed by means of multi-locus gene sequencing. To this end, genomic DNA was extracted using the UltraClean™ Microbial DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA). Sequences of the *ITS* region, the *endoPG* gene and the anonymous region *OPA10-2* locus were amplified and sequenced as described by Woudenberg et al. (2013, 2015) and subsequently deposited in GenBank.

Pathogenicity trials

Dormant pear flower buds

Shoots were cut from one year-old wood of 'Conference' trees in April, when flower buds were just beginning to swell. Four replicates of 3 twigs (approximately 100 flowers buds in total) were placed in a climate chamber in transparent 300 ml bottles filled with water to which 'white Chrysal' (15 g l^{-1}) was added. Water was refreshed weekly, and a few millimetres from the basal part of each shoot were cut away every other day. Flower buds were injected with 10 µl of an *Alternaria alternata* spore suspension (10^5 spores ml^{-1}) prepared from 14-day-old culture. The needle was carefully introduced into the bud through the 'distal' end until half of its longitudinal axis was reached. Control buds were mock inoculated in the same way with sterile water. The shoots were incubated at 15°C and a 10-h photoperiod. After inoculation the relative air humidity was maintained at 100% during the first day by covering the shoots with a plastic bag. Viability of the *Alternaria* spores was confirmed to be >90% by counting the number of germinated spores upon plating of 50 µl of the spore suspension for 24 h at 20°C on water agar.

Pathogenicity *Alternaria* spp. on detached apple and pear leaves

Ten isolates of the two *Alternaria* species groups were selected randomly from the set of 63 isolates as described previously; i.e. 3 *Alternaria arborescens* SC isolates from 'Golden delicious' apple leaves and 3 *A. arborescens* SC isolates from dormant pear flower buds (2 isolates of 'Conference' and of 1 'Doyenné du Comice'), and 4 *Alternaria alternata* isolates from 'Conference' flower buds. Surface sterilized leaves were inoculated on the abaxial side with 10 µl of a suspension of 10^5 conidiospores ml^{-1}, prepared from a 14-day-old PDA culture, after wounding with a needle, with four inoculations per leaf. Control leaves were mock inoculated with sterile water. Inoculated leaves were sealed in a plastic box, to maintain the humidity, and incubated in darkness at 20°C. Pathogenicity was assessed after 3, 5, and 7 days. The experiment was carried out in five replicates.

Fungicide trials

Efficacy of products

This experiment was performed in a nine-year-old pear orchard with spindle shaped pear trees (cultivar Conference) on Quince MC rootstock planted in a single row planting system (3.5 m x 1.5 m) at the experimental station at Randwijk, the Netherlands. The following treatments were performed in twelve weekly spray applications with the first application starting at the end of May and the last application at end of August (shortly before harvest).

1. Thiram (active ingredient: thiram, 80%): applications of 2 kg ha^{-1}.
2. Switch (active ingredient: 37.5% cyprodinil + 25% fludioxonil): applications of 0.8 kg ha^{-1}.
3. Eupareen (active ingredient: tolylfluanid, 50%): applications of 2.25 kg ha^{-1}.
4. Saponin (active ingredient: *Yucca schidigera* extract, 90%): applications of 7.5 l ha^{-1}.
5. Malvin (active ingredient: captan, 80%): applications of 2.25 kg ha^{-1}.
6. Untreated control (no fungicide applications).

The spray applications were carried out with a cross flow sprayer (Homeco Urgent, Dieren, the Netherlands) with Albuz lilac hollow cone nozzles at 5 Mpa and a spray volume of 320 l ha^{-1}. The experiment was done in a randomized block design with four replicates. Each replicate consisted of seven trees. Observations were made on the middle five trees. All flower buds per tree were counted and the disease incidence per tree was calculated from the overall count. Mean disease incidence of all trees for each replicate was used for statistical analysis. Effect of treatments was determined with ANOVA at a 0.05 probability level.

Timing of application

This experiment was carried out in a six-year-old pear orchard located at the experimental station at Randwijk, the Netherlands. The orchard was of spindle shaped pear trees (cultivar Conference) on Quince MC rootstock. Trees were planted a single row planting system (3.5 m x 1.5 m). The efficacy of the *Alternaria*-specific fungicide Rovral aquaflow (active ingredient: iprodione 50%) was tested at a dose rate of 1500 ml per 1000 liter (0.15% v/v).

Spray applications were carried out with a handheld spray gun (manufacturer EMPAS, Veenendaal, the Netherlands) with a 1.2 mm ceramic hollow cone nozzle at 1.1 – 1.2 Mpa and a spraying volume of 1000 l ha^{-1}. The experiment was done in a randomized block design with four replicates. Each replicate consisted of 7 trees. Observations were made on the middle five trees.

The experiment consisted of the following treatments:

1. Untreated control (no fungicide applications) (T1).
2. Fourteen spray applications with two weeks interval with the first application at end of May until harvest (nine applications), after harvest (two applications) and before bloom the following season (three applications) (T2).
3. Nine spray applications with two weeks interval with the first application at end of May and until harvest (T3).
4. Three spray applications with two weeks interval with the first application at the end of May (T4).
5. Three spray applications with two weeks interval with the first application at the beginning of July (T5).
6. Three spray applications with two weeks interval with the first application at the beginning of August (T6).
7. Two spray applications with two weeks interval with the first application at the beginning of October (T7).
8. Three spray applications with two weeks interval with the first application at the beginning of March of the new growing season (T8).

Before bloom (February) 50 dormant flower buds per treatment (randomly taken over replicates) were individually assessed for infection with *Alternaria* spp., as described previously. Disease incidence was assessed at the beginning of bloom (April). All flower buds per tree were counted and the disease incidence per tree was calculated from the overall count. Mean disease incidence of all trees for each replicate was used for statistical analysis. Effect of treatments was determined with ANOVA at a 0.05 probability level.

Results

Effect of tree growth regulation and Resistim application on dead dormant flower buds

To investigate to what extent the occurrence of dead flower buds on pear trees in the Netherlands are a physiological disorder, the effects of tree growth regulation on the occurrence of dead dormant flower buds was assessed. To this end, tree growth regulation by application of ethephon, Regalis (prohexadione-Ca) or by root-pruning was performed annually on the same trees, between 2002 and 2004, and the occurrence of dead flower buds was monitored in the following year. The incidence of dead flower buds in the control treatment that did not receive any means of growth regulation was 35%, 20%, and 17% in 2003, 2004, and 2005, respectively. No effect of any of the treatments was observed on the incidence of dead flower buds in 2003 (Fig. 2). For ethephon no effect was observed in 2004 and 2005 as well. In contrast, Regalis application increased dead flower bud incidences in 2004 and 2005, as did root pruning in 2004. However, overall we did not find any indication that growth regulation can prevent the occurrence of dead flower buds.

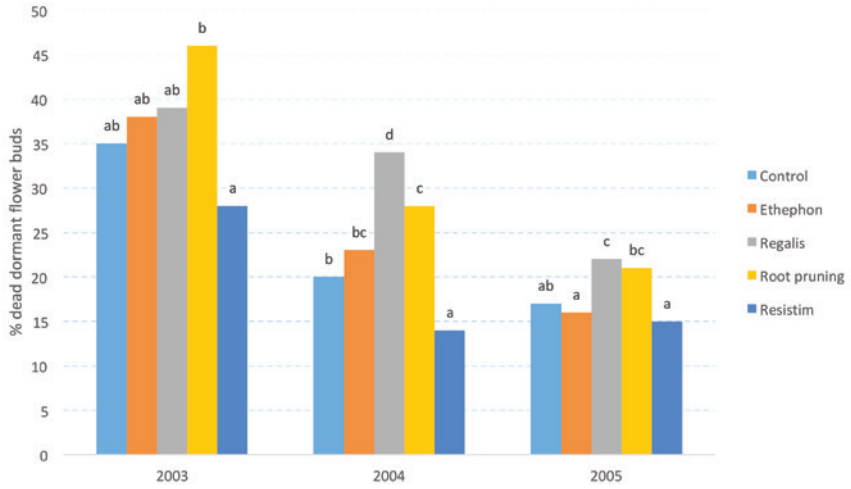

Figure 2. Effect of growth regulation and Resistim treatments on dead dormant flower buds.

Besides growth regulators, we also tested the ability of Resistim (potassium phosphite), a phosphonate derivate that is based on phosphorous acid that is used in crop protection, to affect dead flower buds incidence. Interestingly, Resistim application reduced this incidence in 2004. Remarkably, the reduction concerned end buds only and not buds of one-year-old or older twigs (Fig. 3). The fact that Resistim reduced dead flower buds incidence hinted towards the possible involvement of microbial pathogens in the occurrence of dead flower buds disease.

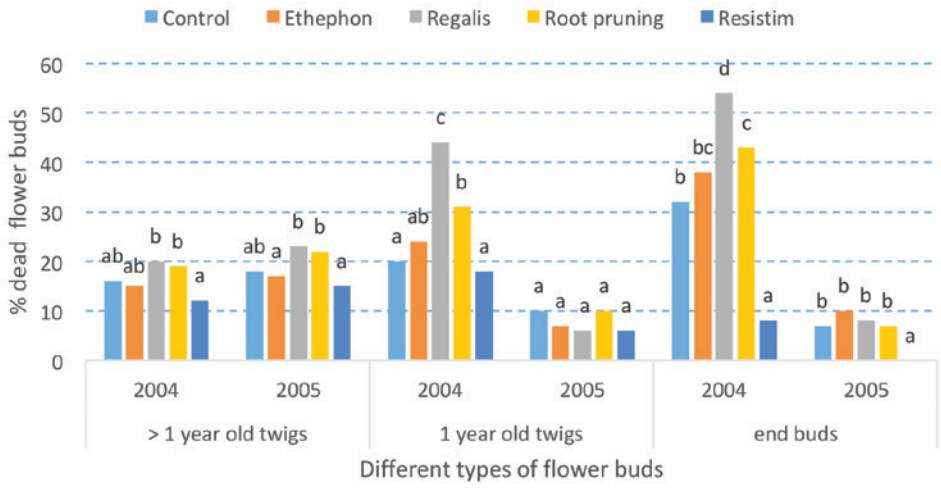

Figure 3. Effect of growth regulation and Resistim application on death of specific flower buds in 2004 and 2005.

Assessment of possible bacterial pathogen involvement in the occurrence of dead flower buds disease

It has previously been suggested that *Pseudomonas syringae* pv. *syringae* may be a causal agent of dead flower buds (Montesinos and Vilardell, 1991; Deckers et al., 2008). To investigate this hypothesis, three pear orchards with a history of dead flower buds in Randwijk, the Netherlands, were sampled. To this end, 50 buds per bulk sample per orchard were collected in August, September and November of 2002, and in February of 2003. The inspection of individual flower buds revealed that approximately 50% of the buds collected in February 2003 showed visual symptoms of internal necrosis in these three orchards. Analysis of surface-sterilized as well as non-sterilized bud samples revealed that *P. syringae* pv. *syringae* could not consistently be isolated from the buds. Of the 15 surface sterilized samples that were composed of 25 dead buds each, the bacterium was only found in five samples. Similarly, of the 12 surface-sterilized samples of 25 dead buds, the bacterium was found in six samples. Moreover, in the samples where the bacterium was found, only low densities were monitored of maximum 5 colony forming units in 100 µL bud extract.

Besides random bud samples, symptomatic dormant 'Conference' buds were collected from another orchard that showed severe symptoms of dead flower buds disease with >50% affected flower buds and individually analyzed in 2003, 2004, and 2006. Only six of 41 buds (15%) in 2003, 1 of 15 buds (7%) in 2004, and 3 of 20 buds (15%) in 2006 carried P. syringae pv. syringae.

Despite the lack of correlation between *P. syringae* pv. *syringae* colonization and dead flower buds occurrence, an inoculation experiment was carried out in the laboratory on cut shoots with dormant 'Conference' pear flower buds. Although the inoculation of dormant flower buds with *P. syringae* pv. *syringae* resulted in significantly more dead flower buds than among non-inoculated buds, mock-inoculation with buffer resulted in similarly increased numbers of dead flower buds.

Collectively, our findings suggest that *P. syringae* pv. *syringae* may be present as epiphyte and even as endophyte on and in flower buds, but is not the causal agent of dead flower buds disease.

Assessment of possible fungal pathogen involvement in the occurrence of dead flower buds disease

For a number of years, dormant pear flower buds from various orchards were assessed for the presence of disease symptoms (Fig. 4). In general, from August to November no visual disease symptoms were observed. However, from November onwards the first necrotic spots were observed on the flower primordia as well as necrosis of individual flower primordia. Subsequently, the symptoms progressed and often resulted in total decay of the dormant flower bud (Fig. 4C). However, in other cases necrosis of only the apical flower was observed (Fig. 4B).

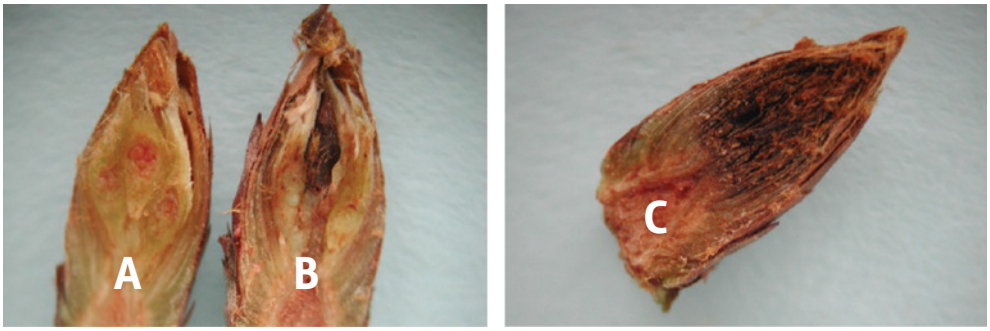

Figure 4. Typical symptoms of dead flower buds disease. Cross section through a healthy flower bud (A); a diseased flower bud with complete necrosis of the apical flower (B); and total decay of a dormant flower bud (C).

An extended assessment for the occurrence of internal symptoms, comprising necrotic spots and dead flower primordia, in dormant flower buds collected in eight commercial 'Conference' orchards was carried out in February 2004. The incidence of affected dormant flower buds ranged from 2 to 50% between the orchards. Isolations from symptomatic flower primordia generally yielded only one type of fungus. All isolates produced fast-growing colonies of irregular shape, tan brown to black and felty. Sporulation patterns showed long conidiophores with extensive terminal branching. Conidia were ovoid with a tapering apical beak and a size range of 10 to 30 × 5 to 10 µm, with 1 to 5 septa. The isolated fungi were morphologically identical to small spored *Alternaria* spp. (Simmons, 2007). Importantly, small spored *Alternaria* spp. cannot be classified further based on morphological characteristics (Woudenberg et al., 2015).

The *Alternaria* spp. was found in almost all diseased flower buds, but also frequently occurred in asymptomatic flower buds (Table 1). The infection rate of flower buds (i.e. symptomatic and asymptomatic flower buds) with *Alternaria* spp. ranged from 10 to 85%. However, the occurrence of visible flower bud symptoms and infection with *Alternaria* spp. correlated highly in these orchards (Fig. 5).

Table 1. Percentage of symptomatic and a-symptomatic dormant flower buds (assessed in January/February) infected with *Alternaria* spp.. Mean value of 500 buds (i.e. 50 buds of 10 commercial orchards were assessed annually 2007-2009).

	Symptomatic buds		A-symptomatic buds	
	No. of buds	% infected with *Alternaria* spp.	No. of buds	% infected with *Alternaria* spp.
2007	240	95	260	70
2008	91	88	409	30
2009	109	87	381	29

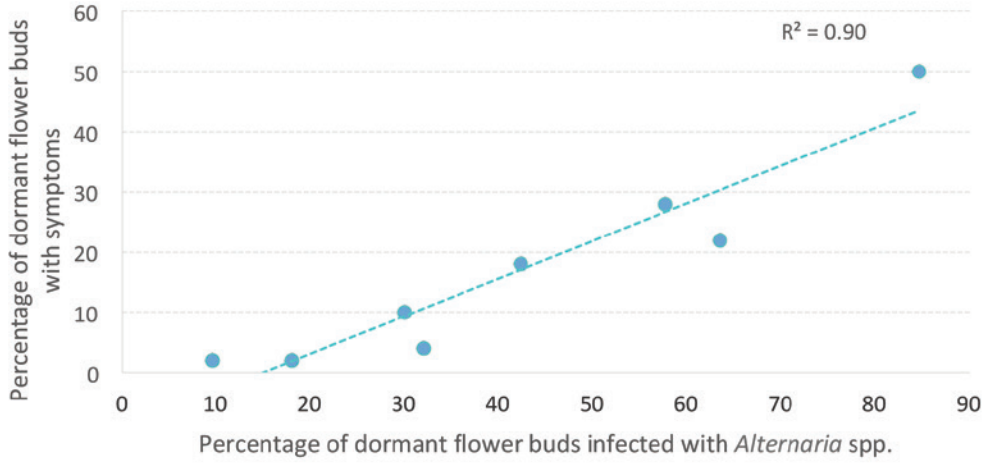

Figure 5. Relation between symptoms and infection of *Alternaria* spp. in dormant flower buds of commercial orchards in 2004. Each dot represents a commercial orchard.

Further implication of *Alternaria* spp. in dead flower buds disease of pear

Yearly assessments were carried out between 2006 and 2013 (except for 2010) in commercial 'Conference' pear orchards to evaluate incidences of dead flower buds due to *Alternaria* spp.. To this end, *Alternaria* infections were determined in February on 50 randomly collected dormant buds per orchard, and the dead flower bud incidences were determined at bloom on 10-15 random trees per orchard. The infection levels of dormant flower buds with *Alternaria* spp. varied considerably between years and orchards. Very high *Alternaria* infection levels were observed in 2006 and 2007, when >60% of the dormant flower buds were infected. In 2007 two orchards even showed infections in 100% of the dormant flowers buds. In 2008, 2009 and 2012 a wide range of dormant flower bud infections by *Alternaria* spp. were recorded, varying from 5 to 90% between the orchards. In contrast, in 2011 and 2013 overall relatively low infection levels were observed. In the years 2007, 2008, 2009 and 2012 a high correlation was found between infection with *Alternaria* spp. and the occurrence of dead flower buds. Nevertheless, in 2006, 2011 and 2013 low correlations were found (Supplemental material Figure S1).

Between 2006 and 2011 it was determined that 33 to 55% of the *Alternaria* infections resulted into dead flower buds, whereas in 2012 all infections led to dead buds. In contrast, in 2013 only on average 13% of the infections resulted in dead flower buds (Table 2). In conclusion, the *Alternaria* infection rates vary between years and orchards. Likewise, also the severity of symptom expression varies considerably, with a dead flower bud as the most severe symptom.

Table 2. Average percentage of infected flower buds, percentage of dead flower buds, and percentage of infections that resulted into dead flower buds in commercial 'Conference' orchards*.

Year	Average % infected flower buds**	% of dead flower buds**	% of infections resulting into dead flower buds**
2006	78	33	42
2007	84	46	55
2008	39	21	55
2009	43	14	33
2011	27	10	37
2012	43	45	101
2013	21	3	13

*: see supplemental figure S1 for the results of the individual orchards.

**: average of all flower buds of the assessed commercial orchards.

Identification of the *Alternaria* species found in dead flower buds of pear

Five mono-spore isolates of the *Alternaria* spp. were prepared, sent to the Westerdijk Fungal Biodiversity Institute (The Netherlands), and identified as belonging to the *Alternaria alternata* species complex (SC) in 2004. We subsequently characterized a collection of 45 mono-spore *Alternaria* spp. isolates that were collected from symptomatic pear flower buds of different pear cultivars (Conference, Doyenné du Comice, Xenia). All isolates were morphologically identical to small spored *Alternaria* spp. (Simmons, 2007). To determine the species level, sequences of the *ITS* region, the *endoPG* gene and the anonymous region *OPA10-2* locus were amplified and sequenced as described in Woudenberg et al. (2013, 2015) and deposited under GenBank accession numbers for the *A. arborescens* species complex (34 isolates) MH975070-MH975103 (*ITS*), MH975104-MH975137 (*endoPG*), and MH975104-MH975137 (*OPA10-2*), and for the *A. alternata* species complex (11 isolates) MH975172-MH975182 (*ITS*), MH975194-MH975204 (*endoPG*), and MH975183-MH975193 (*OPA10-2*). MegaBLAST analysis revealed that our *ITS*, *endoPG* and *OPA10-2* sequences matched with >99%-100% identity either to the *A. arborescens* species complex (34 isolates) and the *A. alternata* species complex (11 isolates) in GenBank (*A. arborescens:* AF347033 & KP124400 (*ITS*), AY295028 & KP124104 (*endoPG*) and KP124712 & KP124714 (*OPA10-2*); *A. alternata*: KP124298 & KP124305 (*ITS*), AY295020 & KP124005 (*endoPG*) and JQ800620 & KP124613 (*OPA10-2*)) (Table 3).

Table 3. Identification of *Alternaria* species linked to dead flower buds of different pear cultivars.

Pear cultivar	# isolates	# *A. alternata* SC	# *A. arborescens* SC
Conference	25	10	15
Doyenné du Comice	10	1	9
Xenia	10	0	10
Total	45	11	34

Pathogenicity trial

To confirm pathogenicity of isolates that were obtained, an inoculation experiment on dormant 'Conference' pear flower buds was carried out on cut shoots in the laboratory with a single spore isolate of *A. alternata* SC from a symptomatic pear bud. Whereas an increase in the number of dead buds was observed upon mock-inoculation with buffer, presumably due to damage by the needle, inoculation with *A. alternata* resulted in sharply increased dead flower bud incidence. The fungus was successfully re-isolated from symptomatic buds and the identity was confirmed by morphological characteristics. These data suggest that *A. alternata* SC is capable of causing dead flower buds.

We further generated a collection of 18 single spore isolates from apple leaves ('Golden Delicious') with Alternaria leaf blotch symptoms (Wenneker et al., 2018) and determined the species based on *ITS, endoPG* and the *OPA10-2* sequences. Also in this case, we found that the isolates belonged to the *A. arborescens* (13 isolates) or the *A. alternata* species complex (5 isolates). Next, a pathogenicity assay on detached apple and pear leaves was carried out, assuming that this would indicate the possibility of *A. arborescens* and/or the *A. alternata* species complex to cause necrotic flower bud primordia in pear flowers and leaf blotch on apple. To this end, the pathogenicity of 6 *A. arborescens* SC isolates (3 isolates from apple leaf blotch and 3 isolates from diseased pear buds) and 4 *A. alternata* SC isolates (from pear) was tested on wounded apple and pear leaves in a cross-inoculation experiment. Symptoms appeared within 7 days on all of the inoculated apple and pear leaves, while mock-inoculated controls remained symptomless. Fungal colonies isolated from the lesions cultured on PDA morphologically resembled the original isolates and the identity of the re-isolations was confirmed as *A. arborescens* SC or *A. alternata* SC by sequencing. This finding indicates that both *Alternaria* species are cross-pathogenic between apple and pear.

Fungicide trials

Since we concluded that *Alternaria* spp. are the causal agents of dead flower buds of 'Conference' pears, field experiments for disease control were conducted in which various numbers of fungicide sprays and products were applied. The efficacy of a number of widely used general fungicides was tested in a weekly spraying program with 12 applications during the growing season. Dead dormant flower bud incidences ranged from 27 to 79% between the different treatments (Fig. 6). The lowest dead flower bud incidences were observed for the Switch (active ingredient: cyprodinil + fludioxonil) applications. Interestingly, no effect on dead flowers buds incidence was observed upon twelve applications of thiram, saponin or tolylfluanid, whereas captan applications even increased of dead flower buds numbers.

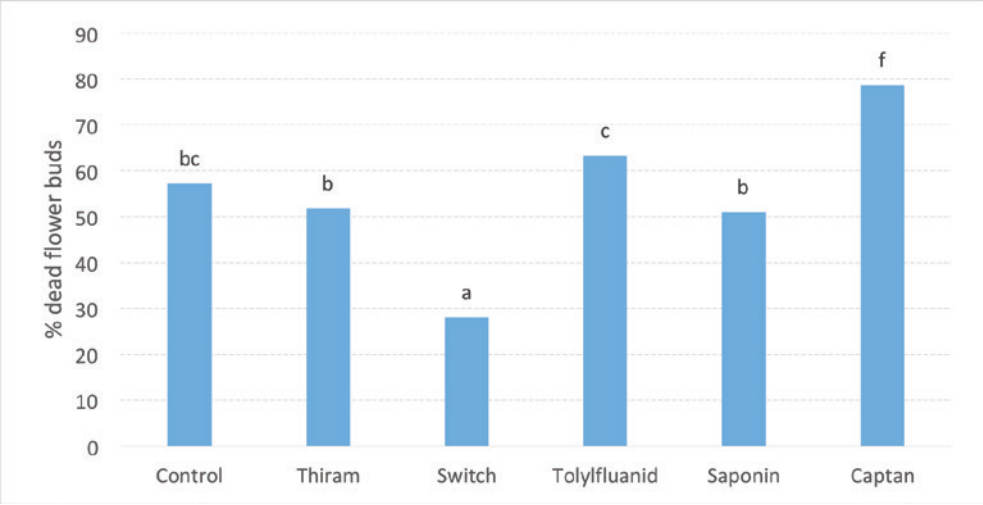

Figure 6. Efficacy of different products to control dead dormant flower bud incidences.

In addition, we tested the timing of application of the *Alternaria* specific fungicide Rovral with the active ingredient Iprodione by spraying in distinct periods, with different numbers of sprayings. Dead dormant flower bud incidences ranged from 14 to 48% in this experiment (Fig. 7). The lowest dead flower bud incidences were observed for the most frequently sprayed treatments (i.e. 9 and 14 applications; treatments 3 and 2, respectively), while treatments with less spray applications resulted in higher dead flower bud incidences. Spray applications shortly before bloom (treatments 7 and 8) had little to no effect on dead flower buds incidence.

Assessments before bloom revealed 4 to 64% of the dormant flower buds carried *Alternaria* spp. infections (Fig. 8). The lowest infection rates were observed in the most frequently sprayed treatments (9-14 spray applications; treatments 3 and 2, respectively), while less spray applications resulted in higher infection rates. The infection rates of dormant flower buds that were sprayed shortly before bloom (treatments 7 and 8) were comparable to the untreated control, conforming that spraying shortly before bloom is not effective.

A strong correlation between the treatment, infection rate of flower buds with *Alternaria* spp. and the occurrence of dead flower buds was observed, indicating that control of *Alternaria* spp. reduced dead flower buds incidences significantly (Fig. 9).

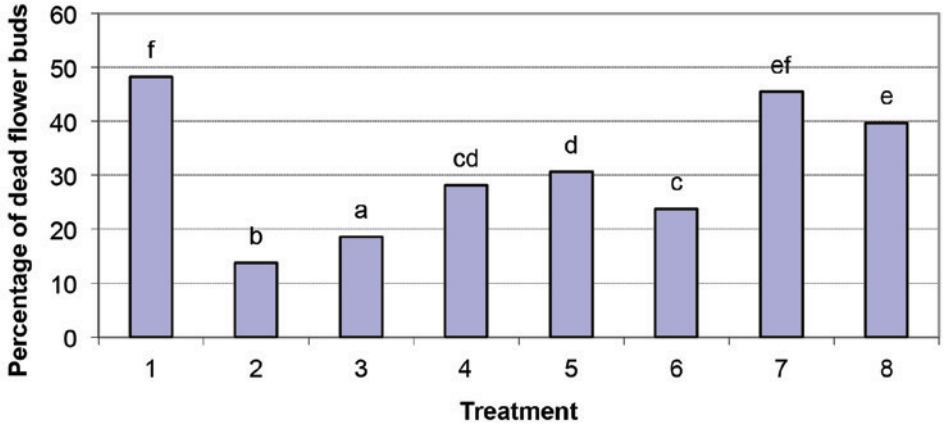

Figure 7. Efficacy of treatments against the occurrence of dead dormant flower buds.

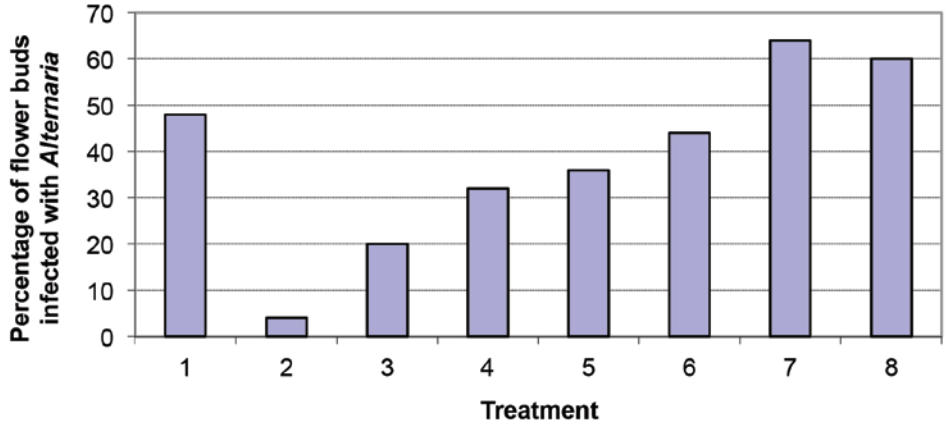

Figure 8. Efficacy of treatments against *Alternaria* spp. infections. The assessments were performed on 50 flower buds, randomly taken from each of the different treatments.

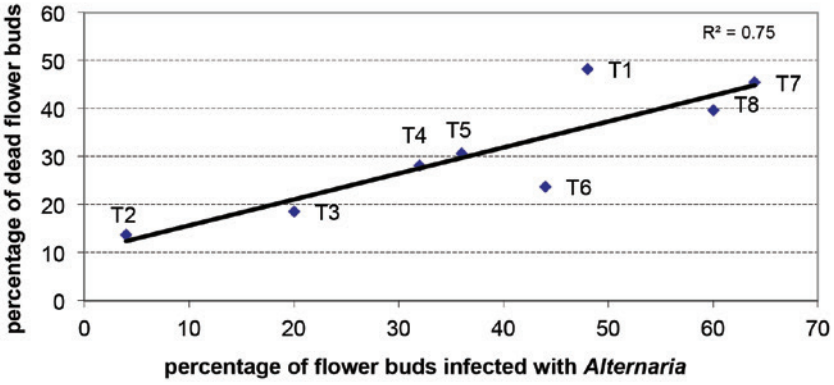

Figure 9. Correlation between infection rate with *Alternaria* spp. and dead flower buds (T1 to T8 are treatment numbers).

Discussion

Dead flower bud disease of pear is a phenomenon of economic importance in the major 'Conference' pear production areas of Europe (Deckers et al., 2008; Montesinos and Vilardell, 1991, 2001). Especially in years with low bud numbers per tree, the disease causes significant (financial) losses due to low harvests. Thus far, the cause of dead flower buds disease remained unknown. Several causes have been proposed including insufficient tree chilling, unmet dormancy requirements, incompatibility between scion and cultivar, and various biotic stress agents such as pathogens and pests (Montesinos and Vilardell, 2001).

In this study it was tested whether insufficient reduction of tree growth leads to increase dead flower buds incidences. Therefore various treatments to control tree growth were carried out. However, we did not find any indication that growth regulation can prevent the occurrence of dead flower buds. In contrast, several of the treatments led to increased dead flower bud incidences, such as Regalis application in 2004 and 2005, and root pruning in 2004. Thus, tree growth control is not suitable for reducing dead flower buds incidence on pear.

To provide evidence for the hypothesis that microbial pathogens may be involved in dead flower bud disease, Resistim applications were tested. Resistim (potassium phosphate) is a phosphonate derivative that is based on phosphorous acid and that affects plant production and productivity, but can also act as a biocide (Gómez-Merino, 2015). More specifically, phosphite can be used for chemical control of various species of pathogenic bacteria, fungi and oomycetes (Amiri and Bompeix, 2011; Burra et al., 2014; Groves et al., 2015; Lobato et al., 2008, 2011; Silva et al., 2011; Yogev et al., 2006). Thus, the observation that Resistim reduced the occurrence of dead flower buds on young twigs and dead terminal buds suggests an involvement of bacterial or fungal pathogens in the occurrence of dead flower buds of pears.

For many years it was commonly believed that the bacterium *Pseudomonas syringae* pv. *syringae* may be the causal agent of dead flower buds of pear (Montesinos and Vilardell, 1987). This was partly due to the fact that *P. syringae* pv. *syringae* was proven to be the causal agent of blossom blast (Mansvelt and Hattingh, 1990; Whitesides and Spotts, 1991), the symptoms of which are characterized by blast of blossom and leaves in periods of cool wet weather during bloom and post-bloom stages (Jones and Aldwinckle, 1990). However, blossom blast symptoms differ from the symptoms of dead flower bud disease as the latter is characterized by partial or complete necrosis of flower buds during dormancy or bud break as confirmed by our research.

P. syringae pv. *syringae* and associated ice nucleation active bacteria have been related to symptom development of blast of dormant flower buds in cold years, and Koch's postulates have been performed to demonstrate its involvement as causal agent (Montesinos and Vilardell, 1987; 1991). However, extensive follow-up research in Spain did not

reveal a significant relation between dead flower bud incidences and *Pseudomonas* levels (Montesinos and Vilardell, 2001). Also, antibacterial treatments (copper and kasugamycin) did not affect the occurrence of dead flower buds (Montesinos and Vilardell, 2001). Finally, also in our study a clear correlation between *P. syringae* pv. *syringae* colonization and dead flower buds occurrence could not be established. Collectively, these findings argue that *P. syringae* pv. *syringae* is not the causal agent of dead flower buds disease in the Netherlands, although the bacteria may be present as epiphyte and even as endophyte on and in flower buds.

In our research, *Alternaria* spp. were consistently found in diseased flower buds, and also frequently in asymptomatic flower buds. Moreover, strong correlations between dead dormant flower buds and infection rates of flower buds with *Alternaria* spp. were observed. From some affected flower buds no *Alternaria* spp. or other fungi were isolated, possibly due to a stringed disinfection. Surveys in commercial pear orchards in the Netherlands revealed high incidences of dead flower buds (up to 80%) in certain years, that correlated with high infection rates (up to 100%) of dormant flower buds with *Alternaria* spp.. Apparently, *Alternaria* spp. are capable of penetrating flower buds during the growing season, and during winter these infections result in necrotic flower tissues and dead flower buds in spring. Specific *Alternaria* fungicides could control dead flower buds significantly. General fungicides were not effective in controlling this disease.

The isolated *Alternaria* species were identified as *A. arborescens* SC and *A. alternata* SC and in laboratory tests the pathogenicity of *A. alternata* SC was proven on flower buds of detached pear twigs, while pathogenicity of *A. arborescens* SC on pear flower buds has still be proven. These results confirm *Alternaria* spp. as causal agents of dead flower buds of pear. Further research is needed to understand the infection process and pathogenicity of the different *Alternaria* species related to dead flower buds.

The genus *Alternaria* encompasses both nonpathogenic and pathogenic species. Although most *Alternaria* species are saprophytes (Thomma, 2003), the genus also harbors well known (opportunistic) plant pathogens that cause a range of diseases on cereals, ornamentals, vegetables and fruits (Basim et al., 2017; Thomma, 2003). *Alternaria alternata* is known to cause late blight in pistachio (Pryor and Michailides, 2002; Evans et al., 1999) and several diseases in fruit crops such as moldy-core in apple (Reuveni et al., 2002), and brown rot in citrus (Timmer et al., 1998). Interestingly, *Alternaria* alternata was recently identified as the causal agent of bud and blossom blight in olive (*Olea europaea*) trees (Lagogianni et al., 2017).

Multiple *Alternaria* species have been implicated to cause leaf blotch and fruit spot of apple in many parts of the world (Filajdic and Sutton, 1991; Gur et al., 2017; Harteveld et al., 2013; Rotondo et al., 2012). In Australia, four species groups were found to be associated with these diseases, including the *Alternaria arborescens*, *Alternaria tenuissima/Alternaria mali, Alternaria alternata/A. tenuissima* intermediate and *Alternaria longipes* species groups (Harteveld et al., 2013). Three *Alternaria* species groups were

similarly proposed to cause the diseases in Italy, including *A. arborescens, A. alternata* and *A. tenuissima* (Rotondo et al., 2012). To date, however, fruit spot of apple caused by *Alternaria* spp. has not been reported in the Netherlands.

Pathogenicity of *Alternaria* spp. is often correlated with to toxin production. *Alternaria alternata* includes both saprophytic and pathogenic isolates. Some isolates are known to produce host-specific toxins (HSTs) and are pathogenic to specific hosts, including apple, as well as European and Japanese pears (Tsuge et al., 2012). Other isolates produce non-HSTs and cause cell damage to several hosts of different genera (Meena et al., 2016). Approximately 30 nHST secondary metabolites are known and characterized, of which alternariol (AOH), alternuene (ALT), and alterotoxin (ATX) are some of the known toxins produced by different *Alternaria* species (Andersen et al., 2015; Lee et al., 2015). These toxins often target basic cellular processes and are regarded as potent mycotoxins.

In our analyses we identified *A. arborescens* SC and *A. alternata* SC as pathogens that may cause leaf spots on apple and pear in the Netherlands. Presumably, these *Alternaria* species are cross pathogenic and both capable in causing dead flower buds of pear. These findings are supported by studies by Harteveld et al. (2014) and Rotondo et al. (2012) that concluded that pathogenicity on apple is not an exclusive character of a specific *Alternaria* species group but is acquired by isolates independently. PCR detection of the HST AM-toxin gene in some isolates us supports the hypothesis that an HST may be involved in pathogenicity of some isolates (Rotondo et al., 2012). However, the AM-toxin genes were not detected in all isolates, suggesting that other mechanisms of pathogenesis may be involved as well (Rotondo et al., 2012). These studies support the conclusion that pathogenicity of *Alternaria* species and isolates affecting leaves and fruit of apple and flower buds of pears may be acquired independently. The reasons for different *Alternaria* species causing the same disease on a particular host are not understood and need attention.

Upon identifying *Alternaria* spp. as the causal agent of dead flower bud disease of pear, an effective fungicide based control strategy was developed. The choice of fungicides is important for achieving appropriate control of dead flower bud disease. It was previously noted that cultural measures may help to control *Alternaria* brown spot in citrus, but fungicide applications are essential to produce blemish free fruit (Timmer et al., 2000). Nevertheless, attempts to control *Alternaria* and moldy-core in apple by using foliar sprays of several fungicides, including benomyl, captan, dodine, mancozeb or some of their combinations, have been unsuccessful in the past, probably due to low efficacy (Reuveni, 2006). Our experiments similarly showed that a number of standard fungicides, such as thiram, tolylfluanid and captan, were not effective in controlling dead flower buds disease. Moreover, the captan applications even appeared to increase disease incidence. Possibly, this fungicide affects antagonistic fungi of *Alternaria* spp., leading to improved conditions for massive growth of *Alternaria* spp. on pear buds. In contrast, spray applications with iprodione (Rovral) and a combination of cyprodinil and

fludioxonil (Switch) reduced *Alternaria* spp. infections and the occurrence of dead flower buds significantly if they were applied during the (summer) growing season as spraying shortly before bloom was not effective.

In conclusion, dead flower buds of pear in the Netherlands should be regarded as a fungal disease caused by *A. alternata* SC and potentially also *A. arborescens* SC which may be controlled by specific fungicide applications.

Acknowledgements

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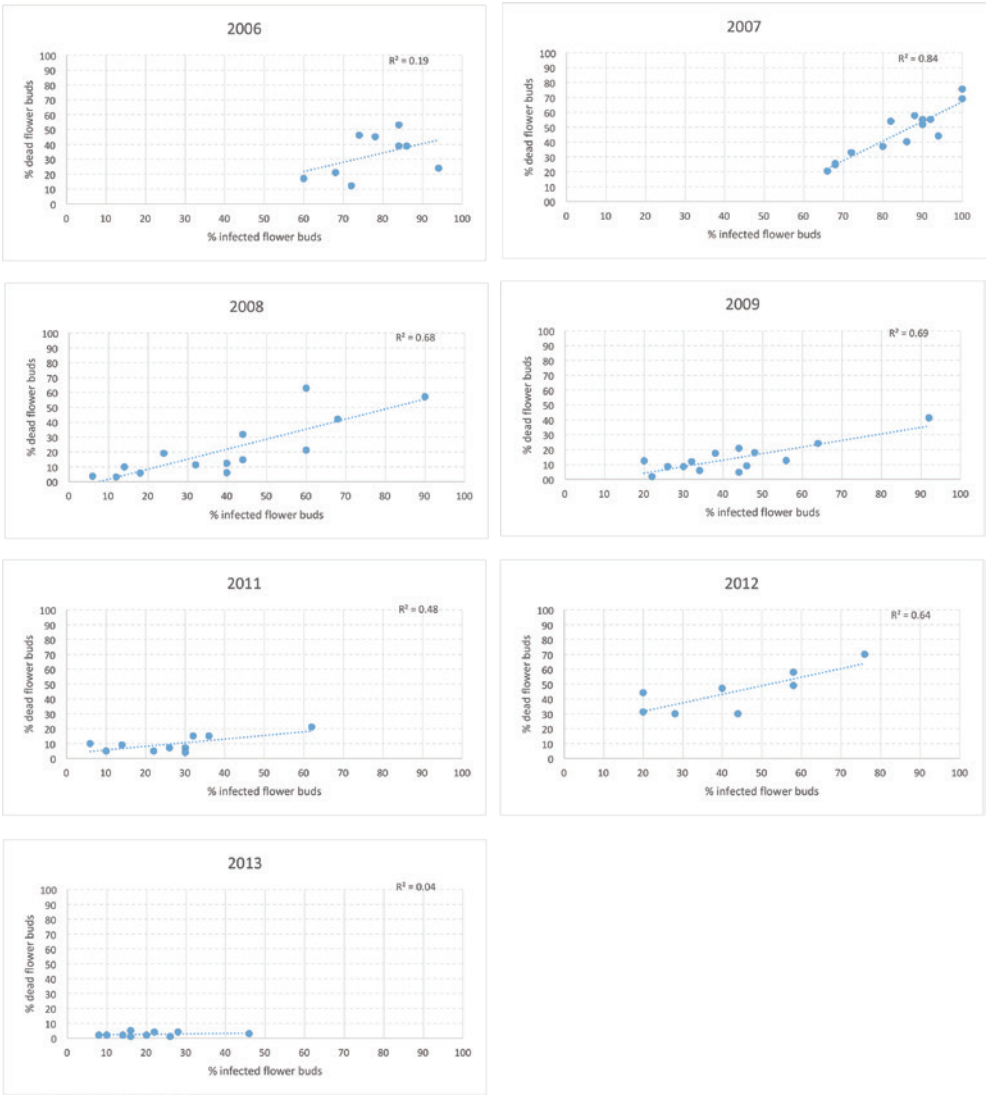
Supplemental material



Supplementary Figure S1. Relation between dead flower buds and infection of *Alternaria* spp. in dormant flower buds of commercial orchards (2006 to 2009 and 2011 to 2013). Each dot represents a commercial orchard.

8.

General discussion

Latent postharvest pathogens and their management: from single measures to a systems intervention approach

Abstract

The studies presented in this thesis deal with important fungal diseases of pome fruit. While one part of the studies focused on postharvest diseases, the other part focused on *Neonectria ditissima* as the causal agent of European fruit tree canker, and *Alternaria* spp. that are causal agents of dead flower buds of pear. In this chapter I will focus on fungal postharvest pathogens and their control. Postharvest diseases of pome fruit are typically caused by a wide diversity of fungal pathogens, and the list of confirmed causal agents is still growing. Well-known pathogens causing postharvest losses are *Neofabraea* spp. and *Colletotrichum* spp., but in many cases the causal agents that occur in a specific region remain unknown and their control relies on the routine use of fungicide applications. However, due to the growing concern over the use of synthetic fungicides, alternative control measures are highly desired. Over the past years the use of physical treatments, natural compounds, and biocontrol agents have been investigated as alternatives. However, no single method has emerged that can robustly and reliably control postharvest diseases of pome fruit in practice. Here, I argue to approach postharvest diseases as complex problems that require multiple interventions at different stages of the disease process in a systems intervention approach for their control. Such approach requires a deep understanding of the epidemiology the causal agents in the orchard, fruit defense mechanisms against pathogens, and the molecular biology of host-pathogen interactions in order to develop novel disease control methods in which the deployment of resistant cultivars can be a cornerstone.

General introduction

Production and storage of pome fruit

Apple (*Malus domestica*) and pear (*Pyrus communis*) are important fruit crops that are cultivated in the Netherlands, with a total production of 353,000 and 349,000 tons, respectively, in 2014. The main apple cultivar is Elstar that is grown on 40% of the total apple production area, while the main pear cultivar is Conference that is grown on 75% of the production area (CBS, 2016). After harvest, fruit are stored under specific controlled atmosphere (CA) conditions for up to 11 months, depending on the cultivar and volume to be marketed. Storage conditions are always a balance between the required quality demands and prevention of physiological disorders (Van Schaik and Verschoor, 2003). Initially, CA storage technology was restricted to standard or traditional storage at low temperatures in which O_2 levels were maintained at about 2-3%. However, improvements in gas monitoring equipment and storage room structure have resulted in the development of several additional CA-based methods to improve quality maintenance, such as ultralow oxygen (ULO) and dynamic controlled atmosphere (DCA) (Thewes et al., 2015). However, as fruit are typically stored for extended periods of time, postharvest diseases have become a limiting factor of significant concern.

Postharvest diseases of pome fruit

Postharvest diseases of pome fruit result in significant economic losses during storage worldwide every year. They are typically caused by a variety of fungal pathogens, although also bacterial and oomycete postharvest pathogens occur. Despite technological advances in postharvest handling of fresh fruit, postharvest fruit losses range from 5 to 20% with upwards of 50% on susceptible cultivars (Janisiewicz and Korsten, 2002; Jones and Aldwinckle, 1991; Jurick et al., 2011). For example, bull's eye rot is the main disease of stored apples in Poland, causing up to 30–40% of postharvest losses on susceptible apple cultivars (Michalecka et al., 2015). Similarly, bulls eye rot, lenticel rot and bitter rot have been reported to cause up to 30% decay during storage of organically grown apples in northern Germany (Maxim et al., 2005).

Postharvest diseases of apple and pear are caused by a range of fungal pathogens (Sutton et al., 2014). Wounds caused by insects and birds, as well as by physical damage that is inflicted before or during harvest, are an important entrance site for pathogens such as *Botrytis cinerea* (grey mould), *Penicillium expansum* (blue mould) and *Monilinia fructigena* (brown rot) (Snowdon, 1990). These pathogens typically cause rapid decay of fruit in the pre- and postharvest stage. Fungicide applications shortly before harvest and careful handling of fruits during harvest are effective measures to significantly reduce losses by these wound pathogens.

Another group of pathogens infects developing, intact, fruits during the growing season and remain quiescent, without causing symptoms, during the growing season

and often even during the first months of postharvest storage. Only when yet unknown physiological or biochemical cues in the host have been satisfied, typically only after several months in CA storage, symptoms start to appear (Coates and Johnson, 1997; Lattanzio et al., 2001). Common pathogens causing such late postharvest losses are *Neofabraea alba* (syn. *Phlyctema vagabunda*; Chen et al., 2016; Soto-Alvear et al., 2013), *Neonectria ditissima* (Weber and Dralle, 2013), the *Colletotrichum acutatum* species complex (Spolti et al., 2012), *Phytophthora* spp., *Alternaria* spp., *Fusarium* spp. (Sever et al., 2012), *Cadophora malorum* (syn. *Phialophora malorum;* Sugar and Spotts, 1992) and *Stemphylium vesicarium* (Weber and Dralle, 2013). Moreover, novel latent postharvest pathogens are described continuously. Postharvest pathogens are able to pass or overcome the natural defense systems that operate in fruit (Alkan et al., 2015). They infect through wounds, direct penetration of intact tissue, or colonization of natural openings such as lenticels, stems, and pedicels (Prusky and Lichter, 2007). Fruit maturity has been implicated in the susceptibility of apples to particular fruit rot diseases. Brook (1977) observed that apples did not show symptoms of apple bitter rot caused by *C. gloeosporioides* until fruit were approaching maturity. Similarly, increasing maturity in apples resulted in higher incidences of bull's eye rot caused by *Neofabraea alba* (Edney, 1964) and also blue mould caused by *Penicillium expansum* (Vilanova et al., 2014). The increased disease incidence towards the end of a growing season has been hypothesized to be due to changes in the availability of natural openings in response to fruit maturity (Alguilar et al., 2017). For instance, during fruit maturation changes in mineral content but also environmental factors may affect the breakdown of lenticels (Turketti et al., 2012). Alternatively, the increased susceptibility could be due to fruit maturation-related degradation of phenolic compounds that inhibit fungal growth during fruit maturation (Edney, 1964). Interestingly, a recent study of Everett et al. (2018) has shown that the incidence of infection of 'Royal Gala' apples by *C. acutatum* was related to temperature rather than to maturity of the fruit. However, in this case, only late in the 'Royal Gala' cultivation season the mean daily temperatures exceeded 15°C, so temperatures that are permissive for infection only occurred when fruit were more mature. Also high nitrogen (N) content in fruit has been implicated in the incidence of bull's eye rot, blue mould and brown rot on apple fruit (Lysiak, 2013; Sharples, 1985), potentially due to weaker cell walls and thus greater sensitivity to fungal pectolytic enzymes (Bateman and Basham, 1976).

Because of their complicated biology that involves an enigmatic switch from a quiescent to a symptomatic stage latent postharvest pathogens are poorly understood and their control is challenging. In this discussion chapter, I will focus on latent postharvest pathogens that are responsible for late postharvest losses of pome fruit and discuss how these pathogens can be controlled.

Specific latent postharvest pathogens

As stated, a growing list of fungi is reported to be associated with postharvest fruit rots of pome fruit. In order to develop effective control strategies it is necessary to assess which are the most important postharvest pathogens that occur in a specific region on the crop. Based on the current literature the economically most important postharvest pathogens in most apple and pear growing areas are *Colletotrichum* spp. and *Neofabraea* spp..

***Colletotrichum* spp.**

Colletotrichum species are considered as major pathogens associated with pre- and postharvest fruit diseases, besides causing anthracnose on vegetables, wheat, fruit and ornamental plants worldwide (Alaniz et al., 2015; Cannon et al., 2012; Dean et al., 2012; Phoulivong et al., 2010). Apple bitter rot caused by *Colletotrichum* spp. is a widespread fruit disease occurring in most countries where apples are cultivated (Shi et al., 1996). *C. acutatum* species complex (SC) infections on apples in Europe are frequently reported with increasing numbers of recent reports from Italy, Belgium, Slovenia, England, Norway and France (Børve and Stensvand, 2015; Grammen et al., 2018; Mari et al., 2012; Munda, 2014; Munir et al., 2016; Nodet et al., 2016). Studies from Germany and Sweden describe postharvest losses of apple fruits of 10 and 25%, respectively, by *C. acutatum* SC (Børve and Stensvand, 2017; Weber and Palm, 2010).

In warmer climates, *C. acutatum* SC infections lead to symptoms on apple during the summer period while the apples are still on the trees. However, in northern areas the fungus is more commonly observed as a storage pathogen (Everett et al., 2018). Disease symptoms of bitter rot start with the development of small dark brown spots expanding to light brown sunken lesions. Afterwards conidia are formed in acervuli concentrically in the centre of the lesion (Damm et al., 2012). All apple cultivars are susceptible to bitter rot, and in particular those belonging to the late-harvest group, such as Granny Smith, Pink Lady, and Fuji (Velho et al., 2015). Apple bitter rot has a higher destructive potential than other apple rots and can result in losses up to 50% at pre- and postharvest stages (Everett et al., 2015; Velho et al., 2015).

Besides *Colletotrichum acutatum* SC, also the *Colletotrichum gloeosporioides* SC has been implicated in bitter rot. In Japan, bitter rot is one of the most severe diseases in apple production in general (Yokosawa et al., 2017). In countries such as in Brazil (Crusius et al., 2002) and the USA (Shi et al., 1996; Gonzales et al., 2006) both *C. gloesporioides* SC and species within the *C. acutatum* complex occur together. Historically, in New Zealand apple bitter rot was reported to be caused by the *Colletotrichum gloeosporioides* SC, but more recently the most frequently isolated causal organism has been *C. acutatum* SC (Everett et al., 2015).

Both *C. acutatum* SC and *C. gloeosporioides* SC are considered as hemibiotrophs that first have a biotrophic infection stage in which they retrieve their nutrients from living plant cells, followed by a necrotrophic stage in which they kill host tissue to obtain their

nutrition (Peres et al., 2005). The fungus overwinters on infected peach and blueberry buds and twigs, but on apple the source of inoculum is not obvious, as the presence of the pathogen is only apparent when it causes disease on fruit (Peres et al., 2005). Recently, a few studies of the aetiology and epidemiology on apples have been published (Børve and Stensvand, 2013, 2017). Also, a disease cycle for *C. acutatum* SC infecting apples and causing bitter rot in New Zealand was proposed (Kerry et al., 2018), suggesting that inoculum is most commonly rain-splashed from decaying petals, bud scales, twigs and infected fruitlets that have fallen to the ground since spring, with some inoculum also released from twig cankers and mummified fruit in the canopy. Infection is proposed to occur after conidiospore deposition on fruit, leaves and buds if they formed, in the presence of sufficient moisture and temperatures above 15°C when the spores germinate and form appressoria to establish quiescent infections (Peres et al., 2005). Infections of buds and leaves are symptomless, because symptoms are not observed on leaves on the tree in New Zealand and buds do not seem to be negatively affected in the following spring. In spring, buds open and the cycle can begin again (Kerry et al., 2018).

The penetration and infection is well described for *Colletotrichum* spp.. For instance, penetrating hyphae of *Colletotrichum* appressoria develop within the cuticle and uppermost epidermal cell layers of unripe fruit without eliciting visible host reactions, suggesting that fungal effectors that are secreted to support host colonization may interfere host response mechanisms (Giraldo and Valent, 2013; Kleemann et al., 2012). The appressoria of *Colletotrichum* spp. are highly polarized cells from which a needle-like penetration hypha emerges in order to puncture the cuticle and epidermal cell wall (Howard and Valent, 1996; Latunde-Dada, 2001). At this stage, *Colletotrichum* is noted for its ability to maintain itself in an extended quiescent state until fruit ripening (Prusky et al., 2013).

Timely applications of fungicides are presumed to reduce infections of buds during summer, thus disrupting the disease cycle and more effectively controlling the disease (Everett et al., 2015). This may provide a considerable improvement in reducing the number of applications over the currently recommended practice of calendar spraying throughout the season (Sutton, 2014). Over the past few years, resistance of *Colletotrichum* spp. to the quinone-outside inhibitors (QoI) group of fungicides have appeared (Forcelini et al., 2018) and QoI resistant *Colletotrichum* isolates have been recovered from apples (Munir et al., 2016).

Neofabraea spp.

Bull's eye rot of apple and pear is an important postharvest disease, occurring in major fruit-growing areas of North America, Chile, Australia and Europe (Henriquez et al., 2004, 2008; Soto-Alvear et al., 2013; Spotts et al., 2009). The disease commonly occurs in most apple cultivars with an incidence of 10–20%, and may exceed 40% in years that are favourable to pathogen infection (Cameldi et al., 2016; Soto-Alvear et al., 2013). In

Europe, 'Golden Delicious' and several late maturing apple cultivars, such as Pink Lady, are particularly susceptible to the disease (Cameldi et al., 2016; Neri et al., 2009). Bull's eye lesions on apple and pear fruits are generally caused by *Neofabraea* species, with *N. vagabunda* (syn. *N. alba*) as the main causal agent. However, also *N. malicorticis, N. perennans,* and *N. kienholzii* have been described to cause the disease (Gariépy et al., 2005; Michalecka et al., 2016; Pešicová et al., 2017; Soto-Alvear et al., 2013; Spotts et al., 2009).

Besides symptoms on stored fruit, *Neofabraea* spp. cause cankers on branches or develop saprophytically on pruning stubs and dead tree branches (Henriquez et al., 2006; Verkley, 1999). The pathogen spreads by asexual sporulation on fruit mummies and bark cankers (Spotts, 1990; Weber, 2012). Conidiospores are produced throughout the year, but the highest sporulation levels occur during autumn (Henriquez et al., 2006). Although rain splash is considered the principal mechanism for conidial dispersal, conidia can also be splash-dispersed by over-tree irrigation practices (Grove et al., 1992). Infections typically occur in the orchard throughout the growing season, anytime between petal fall and harvest, when unripe fruits are penetrated through the lenticels. Fruit susceptibility increases gradually during the season (Aguilar et al., 2017; Cameldi et al., 2016; Spotts, 1990). After infection, the pathogen arrests its growth and remains quiescent until the fruit reaches a certain stage of ripeness when it can invade fruit tissues. Typically, bull's eye rot symptoms appear only after 3–4 months in cold storage when numerous lesions may develop on a single fruit (Neri et al., 2009). Fruit lesions are circular, flat to slightly sunken, brown and often with a lighter brown center (Snowdon, 1990).

Current management practices to control *Neofabraea* spp. in the orchard include pruning of cankers from infected trees to minimize the buildup of inoculum during the fruit growing season, removal of fallen fruit and dead tree branches from the orchard floor, and reduced use of over-tree irrigation systems that may promote splash dispersal of conidia from sporulating cankers onto developing fruit (Creemers, 2014). Furthermore, fungicide application is a common component of bull's eye rot management (Aguilar et al., 2018).

Postharvest pathogens of pome fruit in the Netherlands and their control

Postharvest disease caused by *Colletotrichum* spp. and *Neofabraea* spp. are generally not causing severe problems in the Netherlands, most likely because our main apple cultivar Elstar and pear cultivar Conference are not susceptible to these pathogens. However, more susceptible apple cultivars, such as Pinova and Topaz, are frequently affected by *Neofabraea* spp. also in the Netherlands.

Until recently, it was unknown what the main causal agents of postharvest decay of pome fruit in the Netherlands were. In order to determine this, decayed apple and pear fruit were sampled from commercial CA storage facilities. In total, approximately 350 samples, derived from orchards with various apple and pear cultivars and from various

production areas in the Netherlands, were analyzed between 2012 and 2018. These surveys revealed the presence of common postharvest pathogens, such as *Botrytis cinerea* and *Neofabraea alba*, but also a number of new and emerging postharvest pathogens, such as *Fusarium avenaceum* on pear and apple, *Neonectria candida* and *Neofabraea kienholzii* on pear, and *Colletotrichum godetiae* and *Truncatella angustata* on apple (Chapter 2). In most cases these newly described postharvest pathogens were isolated at low incidences only. In contrast, two latent postharvest pathogens more frequently appeared: *Cadophora luteo-olivacea* causing side rot on pears, and *Fibulorhizoctonia psychrophila* as the causal agent of lenticel spot on apples and pears (Chapter 3). For both diseases incidences range from very low to nearly 100% of stored fruits. Thus, these latter two fungal species are presently considered as the most important postharvest pathogens on pome fruit in the Netherlands.

The use of synthetic fungicides is currently the main means to control side rot and lenticel spot diseases. However, despite the routine use of fungicide applications fruit infections during the orchard phase are a growing problem. This may be due to the use of non-effective chemicals, ineffective spray application technologies or inadequate timing of the applications. Basically, robust knowledge on how to control these diseases with fungicide applications is lacking and current management is largely practiced in an empirical fashion. This requires urgent attention in order to ensure the deposition of sufficient quantities of active ingredients on fruits for disease protection during the entire storage period. However, the growing public concern over the health and environmental risks associated with high levels of fungicide residues on fruits, as well as the development of fungicide resistance in fungal pathogens, has resulted in the urge for developing alternative methods for disease control (Wisniewski et al., 2016).

Alternatives to chemical fungicides for controlling latent postharvest diseases

Over the past decades the use of physical treatments, natural compounds, and biocontrol agents have been investigated as alternatives for the use of fungicides. More recently, the fruit microbiome is considered as an important factor for controlling latent postharvest diseases

Physical treatments

Physical treatments, like hot water and hot air treatments, radio frequencies and microwaves, hypobaric and hyperbaric pressures and far ultraviolet radiation (UV-C light) are considered as promising control means to reduce or delay the development of postharvest pathogens (Maxin et al., 2012; Usall et al., 2016). In Europe hot water dips are used for organic apples (Maxin et al., 2012). However, there are several disadvantages of hot water dipping that include high investment costs, relatively low throughput, additional labor during harvest time, high running costs and negative CO_2 footprint due to the energy requirement (Maxin et al., 2014). Consequently, hot water dipping is not

implemented on larger scales in the fruit industry. As reduction in application time of the heat treatment could increase the interest in commercial use, research efforts have focused on short hot water treatments (rinsing) and expanding machine capacities (Maxin et al., 2012).

Radio frequency and microwave heating may provide effective alternative means to control postharvest diseases. The time required for microwave treatment is more favorable for commercial application, but the design and production cost for an equipment currently still obstructs its widespread application (Usall et al., 2016).

Among the remaining physical means, ultraviolet-C light (UV-C) treatment was considered to be interesting due to the simultaneous combination of direct activity against pathogens through germicidal effects on fungal spores with resistance induction through stimulation of defense mechanisms in several postharvest commodities including stone, pome and citrus fruit (Nigro et al., 1998; Stevens et al., 1996; Valero et al., 2007; Wenneker et al., 2013). Although UV-C irradiation does not completely inhibit mycelial growth *in vitro*, a reduction in growth and sporulation was recorded for most tested fungal species (Wenneker et al., 2013). However, UV-C has a superficial effect only due to the limited penetrating capacities of the waves. Thus, the potential for controlling latent infections will eventually be limited. Also, control of wound infections is not possible due to shielding effects by pores and irregularities on the fruit surface (Lagunas-Solar et al., 2006).

Presently, short hypobaric and hyperbaric pre-storage treatments with low and high ambient air pressure, respectively, are considered as promising alternative treatments for postharvest disease control, although their use remains largely unexploited to date (Usall et al., 2016).

Natural compounds

The application of microbial and plant volatile organic compounds (VOCs) to control postharvest decay have recently been reviewed by Mari et al. (2016). Plant-produced volatiles including, among others, aldehydes such as acetaldehyde, 2-E-hexenal and benzaldehyde, alcohols such as ethanol and acetic acid, essential oils, isothiocyanates and microbial volatile organic compounds have been shown to preventing pathogenic infections in many horticultural commodities (Mari et al., 2011; Sivakumar and Bautista-Baños, 2014). The main concerns with respect to their use are related to the registration process, but also VOC degradation and residues in fruit, formulation and impact on taste and smell of fruits (Mari et al., 2016).

Biological control agents

Biological control agents have been the focus of considerable research efforts in academia as well as of commercial companies worldwide for decades (Droby et al., 2016). Various antagonists of postharvest pathogens have been tested under laboratory, semi-commercial, and commercial conditions, and some of them were even developed

into commercial products. Nevertheless, the commercial deployment of postharvest biocontrol agents has met little success, which has been attributed to various problems, including inconsistent performance, high cost relative to synthetic fungicides, registration hurdles, difficulties in mass production and formulation of the antagonist, and lack of industry acceptance (Droby et al., 2009; Droby et al., 2016). Thus far, research on biocontrol of postharvest diseases has mainly focused on identifying microorganisms that are antagonistic to wound pathogens and the effects of biocontrol agents on latent postharvest pathogens of pome fruit have hardy received attention in these studies (Droby et al., 2009; Sharma et al., 2009).

The fruit microbiome

Microbial communities living on the surface of fruit have been the source of most of biocontrol agents. The commonly-used approach to identify novel biocontrol agents involves the identification of a single antagonist that can develop rapidly in wounded fruit tissue, thus preventing pathogens from becoming established. This approach, however, neglects interactions of antagonists with other microbes that occupy the same, or surrounding, niches as part of a microbial network and as a component of a complete biological system with the host (Droby et al., 2016).

Thus far, the overall diversity and composition of microbial communities on harvested produce, how they vary across produce types, and the factors that influence their composition after harvest and during storage, has been poorly studied (Droby and Wisniewski, 2018). Recently, massive sequencing of PCR amplicons of specific barcode genes in amplicon metagenomics or metabarcoding approaches have revealed microbial diversities and relative quantities of community members in environmental samples (Abdelfattah et al., 2015). Such technology can similarly be used to characterize the composition of microbial communities on fruit. For example, Abdelfattah et al. (2016) demonstrated that the diversity of the fungal microflora of harvested apples differed significantly between fruit parts. Whereas *Penicillium* was dominant in peel samples, *Alternaria* was dominant in calyx- and stem-end samples. This type of information needs to be considered when designing biocontrol systems for the management of postharvest diseases. For mango it was recently shown based on microbiome comparisons of stem ends that are resistant and susceptible to stem end rot in red and green fruit, respectively, that fungal and bacterial community change with fruit peel color, storage duration, and storage temperature (Diskin et al., 2017). Currently, *Neofabraea* spp. infection levels on apples at the time of harvest and the microbial dynamics on the apple skin during storage are characterized using a metagenomics approach (Bühlmann, pers. comm.). Ultimately, this type of research may lead to the synthetic design of microbial communities that can be used for postharvest disease management.

Conclusion

Some of the alternative methods to chemical fungicides for controlling latent postharvest diseases seem to hold promise for future application if the remaining challenges are met. After all, significant gaps still exists between the basic research that led to the discovery of these methods and their implementation under commercially relevant conditions. In order for such method to be applicable in practice, it must perform effectively and reliably, and be profitable to the company that has invested in its development, registration, and marketing. The results of the search for alternatives to chemical fungicides over the past thirty years show that, although several novel approaches have been identified as potential alternatives, no single method has emerged to robustly and reliably control postharvest diseases of pome fruit in practice. Thus, it may be advisable to move the focus from finding a single 'silver bullet' intervention that can be used to effectively control disease to composing and integrated systems approach by selecting the right set of control measures from a wide array of alternatives (Wisniewski et al., 2016). However, this view implies that latent postharvest diseases are complex problems that require multiple interventions at different stages of the disease process. Consequently, understanding the epidemiology of latent postharvest pathogens in the orchard, fruit defense mechanisms against pathogens, and the molecular biology of their interactions is required in order to develop novel disease control methods (Droby et al., 2009; Tian et al., 2016). Such control methods should focus on reduction of the inoculum pressure of postharvest pathogens, interference of the typical latent stage of late postharvest pathogens and maximum exploitation of the plant's own immune system.

The inoculum pressure of postharvest pathogens

Control of the complex diversity of postharvest pathogens in orchards is difficult because infections may occur during the entire period from flowering until harvest. Exact infection periods are often not known and may differ between the various pathogens. Although considerable knowledge exists on the epidemiology of the typical wound pathogens *B. cinerea, P. expansum* and *M. fructigena,* knowledge on epidemiology of the causal agents of latent postharvest diseases is limited.

In this thesis (Chapter 4) we showed that both *N. alba* and *C. luteo-olivacea* were consistently detected in leaf litter of apple and pear and in necrotic tissues of dead weeds and grasses, and in many cases high concentrations of the pathogens were quantified. These are important new findings that may help to better understand how complex population dynamics of these necrotrophic pathogens depend on the availability of various necrotic host and non-host tissues for survival and multiplication. Further research is needed to understand the relationships between the accumulation of pathogen inoculum on the various substrates over time and infection periods on developing fruits in the orchard. This knowledge will enable estimations of the relative importance of different substrates as inoculum sources for fruit infections. Further, this

knowledge can be used for the development of focused sanitation measures (Holb, 2006; Llorente et al., 2010), or the development of measures to stimulate beneficial microbiome inhabitants that can antagonize pathogen colonization, survival and sporulation on those substrates (Carisse and Rolland, 2004; Llorente et al., 2006, 2010; Rossi and Pattori, 2009).

The quiescent stage of postharvest pathogens

The latent phase, also called quiescent phase, is a dynamic equilibrium among host, pathogen, and environment, which does not result in any visible symptoms on the host (Jarvis, 1994; Prusky et al., 2013). During this stage, the fungal pathogens reside in the cuticular wax or in the intercellular space until the fruits ripen (Adaskaveg et al., 2000; Prins et al., 2000; Prusky et al., 1981). Apparently, at a particular moment physiological and biochemical responses of the host trigger changes in that equilibrium that activate the pathogen that is kept at a low metabolic level during the quiescent stage to activate pathogenicity mechanisms, resulting in active parasitic development in the host tissues (Prusky, 1996). It has been proposed that the termination of the quiescent stage is the result of (i) induced accessibility of disassembled cell wall substrates during fruit softening and ethylene induction; (ii) a decline in preformed antifungal compounds, such as polyphenols, phytoalexins, and other fungitoxic substances; (iii) a decline in inducible host-defense responses; and (iv) more favourable pH conditions in the host tissue. The pH in the fruit may change either naturally during fruit ripening or through induction by the pathogen that secretes pH modulators such as ammonia and organic acids as one of the first waves in their attack (Prusky et al., 2013; Yakoby et al., 2000). Both increases and decreases of ambient pH, for instance by secretion of ammonia and organic acids, respectively, have been recorded depending on pathogen and host characteristics (Alkan et al., 2013). For example, *Penicillium expansum* acidifies the ambient pH by the secretion of gluconic acid (Prusky et al., 2004), while *Botrytis cinerea* (Manteau et al., 2003) and *Sclerotinia sclerotiorum* (Cessna et al., 2000) secrete oxalic acid to acidify the pH while enhancing their polygalacturonase gene expression and other cell-wall-degrading enzymes involved in tissue maceration (Misaghi, 1982; Prusky and Lichter, 2007). In contrast, *Colletotrichum* spp. were found to alkalinize the infection court by the secretion of ammonia to stimulate pathogenicity and necrotrophic colonization through the activation of host NADPH oxidases to generate reactive oxygen species, thereby accelerating host cell death (Miyara et al., 2010; Prusky et al., 2001).

Besides ammonia and organic acids, fungal effector proteins may have also been proposed to act as pH modulators in host tissue. It was recently demonstrated that the root-infecting fungus *Fusarium oxysporum* uses a functional homologue of the plant regulatory rapid alkalinization factor (RALF) peptide RALF to induce alkalinization and cause disease (Fernandes et al., 2017; Masachis et al., 2016). In Arabidopsis, the cell surface-localized receptor-like kinase FERONIA mediates the RALF-triggered alkalinization response, presumably through inactivation of a plasma membrane H+-ATPase.

Interestingly, RALF homologues are found in many plant-pathogenic fungi, suggesting the widespread exploitation of these peptides to modulate host tissue pH levels (Fernandes et al., 2017; Masachis et al., 2016).

Although not much is known for latent postharvest pathogens specifically, recently a pH increase was recorded in apple tissue infected by *N. vagabunda* (Cameldi et al., 2017). However, further research is necessary to clarify the nature and the origin of the alkalizing compounds and to understand the effects of the pH modulation on *N. vagabunda* pathogenicity.

Breeding for resistant cultivars to postharvest diseases

Plants have an innate immune system that comprises a wide variety of constitutive and inducible defense mechanisms to protect themselves against pests and pathogens (Cook et al., 2015; De Wit, 2007). Constitutive or preformed defenses include physical barriers such as cell walls and epidermal cuticles, but also chemicals such are antimicrobial phytoanticipins and some pathogenesis-related (PR) proteins. In addition to these preformed barriers, plant cells have the ability to detect invading pathogens and respond with inducible defenses (Cook et al., 2015; De Wit, 2007). These can be triggered when plant cells recognize microbe-associated molecular patterns (MAMPs), including structural proteins, lipopolysaccharides, and cell wall components commonly found in microbes, through a set of cell surface receptors, also referred to as pattern recognition receptors (Nürnberger et al., 2004) or invasion pattern receptors (Cook et al., 2015). Upon recognition, various defense responses are induced such as cell wall alterations, deposition of callose and the accumulation of PR proteins that include chitinases, glucanases and proteases that all negatively affect microbial colonization (Van Loon et al., 2006). Typically, also an oxidative burst occurs that involves the release of highly reactive oxygen molecules that damage the cells of invading organisms , cross-links host cell-wall components and acts as a signaling molecule to further enhance host immunity (Pitzschke et al., 2006).

Compatible pathogens can overcome the activation of host immunity by the secretion of effectors that perturb such responses (Cook et al., 2015). Thus, pathogen effectors are crucial molecules for disease establishment (Rovenich et al., 2014). However, in turn plants have evolved receptors to recognize effectors or effector-mediated perturbations of host targets (Chisholm et al., 2006; Jones and Dangl, 2006). These receptors may reside on the cell surface, but also inside the cytoplasm to detect (the activity of) cytoplasmically-delivered pathogen effectors (Cook et al., 2015). Often, the recognition of effectors has been associated with the occurrence of a hypersensitive response (HR); a localized programmed cell death response that may limit pathogen access to water and nutrients, and thus block further growth of the pathogen (De Wit, 2007). However, necrotrophic pathogens may actually benefit from such cell death response, and have evolved in some cases to deliberately activate this host immune response to their benefit (Cook et al., 2015; Lorang et al., 2012).

In tissues that are distal from the infection site, plants are protected by so-called systemic acquired resistance (SAR) (Grant and Lamb, 2006). SAR is effective against a broad range of pathogens and is dependent on various plant hormones, including salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (Grant and Lamb, 2006). More recently, however, also various other hormones, such as auxin, abscisic acid (ABA), cytokinins (CKs), and brassinosteroids have been implicated in the activation of defense responses (Robert-Seilaniantz et al., 2011).

Although relatively little research has investigated the occurrence of the various defense mechanisms described here in fruit trees and fruits, it can safely be anticipated that the majority of these mechanisms operates in these plants too, considering the evolutionary ancient origin of these mechanisms in the plant kingdom. Consequently, breeding for enhanced pathogen resistance is a feasible approach.

Current apple breeding objectives include high fruit quality, good agronomic performance and durable disease resistance, mainly towards apple scab (*Venturia inaequalis*), powdery mildew (*Podosphaera leucotricha*), and fire blight (*Erwinia amylovora*) (Baumgartner et al., 2015). It should be emphasized that classic pome fruit breeding is a long-term and labor-intensive approach. The first fruits can usually be expected at the earliest in the fourth year after crossing. However, usually the first fruit quality selection step is carried out at the fifth to the seventh year after crossing. The success of newly developed disease resistant apple varieties is largely dependent on their fruit quality (Baumgartner et al., 2015). Currently, cultivated apples have often no resistance to fungi causing fruit decay as breeders seldom evaluate for resistance to postharvest diseases (Ahmadi-Afzadi et al., 2013; Volk et al., 2015).

Fruit cultivars may show a large variation in susceptibility to (latent) postharvest diseases (Tian et al., 2016), as was demonstrated among apple cultivars for *Colletotrichum* spp. (Biggs and Miller, 2001; Grammen et al., 2018) and bull's eye rot caused by *Neofabraea* spp. (Blazek et al., 2003; Hortova et al., 2014; Soto-Alvear et al., 2013). Unravelling resistance mechanisms in immature and mature fruit can be helpful to make progress in breeding programs.

New methods to allow for more precise selection of tree and fruit characters in breeding programs were developed in recent years (Laurens et al., 2018). Marker assisted selection (MAS) allows to speed up and facilitate the selection of novel cultivars. While some traits are determined by major genes, others are determined by the additive effect of several genes that are called quantitative trait loci (QTLs). For traits that are primarily controlled by single major genes, a strategy called genome scanning approach (GSA) can be used to identify linked molecular markers without generating a complete genetic map (Patocchi et al., 2005). For traits controlled by multiple genes, QTL mapping is generally applied (Tian et al., 2016; Wisniewski et al., 2016). In peach breeding programs, Pacheco et al. (2014) and Martínez-García et al. (2013) have identified QTLs for brown rot response traits have been

identified (Martínez-García et al., 2013; Pacheco et al., 2014), while preliminary results from apple breeding programs have identified QTLs for blue mould resistance in *Malus sieversii* (Norelli et al., 2014) and a mapping population of 'Royal Gala' × *M. sieversii* PI613981 (Wisniewski et al., 2016).

In order to understand mechanisms involved in apple resistance to postharvest pathogens an approach involving temporal and spatial regulation of the transcriptome, proteome and metabolome combined with pathological analysis must be undertaken (Abdelfattah et al., 2015, 2016; Prusky et al., 2013). In this respect, sequencing of the genome of *Colletotrichum* species and transcriptome analysis of fungal–fruit interactions has revealed genes and key enzymes that are involved in the biosynthesis of fungal secondary metabolites that are important for pathogenicity and fruit defense responses (Alkan et al., 2015; Moraga et al., 2018). Nevertheless, typically, annotation processes and gene functional analyses are tedious and complicated. Nevertheless, significant progress has been made in the determination of transcriptomic and proteomic factors that may lead to resistance in cultivated apples (Buron-Moles et al., 2015a,b; Vilanova et al., 2014), and several studies have provided data on genetically determined levels of resistance to *P. expansum* in apple cultivars (Ahmadi-Afzadi et al., 2015; Tahir et al., 2015), and wild apples (Janisiewicz et al., 2016; Norelli et al., 2013).

Recently, a number of fruit crop genomes has been sequenced, including those of grapevine (Jaillon et al., 2007), apple (Velasco et al., 2010), banana (D'Hont et al., 2012), citrus (Xu et al., 2013), peach (Verde et al., 2013), and pear (Chagné et al., 2014). Also, the genomes of several postharvest pathogens have been sequenced, including those of *Botrytis cinerea* (Amselem et al., 2011), several species of *Alternaria* (Dang et al., 2015), *Colletotrichum* (Gan et al., 2013), *P. expansum* and *P. italicum* (Ballester et al., 2015; Li et al., 2015). The genetic information that has been disclosed by these projects will provide insights in the virulence factors of these important postharvest pathogens, which can again be used in breeding and selection programs.

Concluding remarks and future perspectives

Losses due to the postharvest decay of pome fruits still represent a major concern from an economic point of view. However, it should be realized that fruit decay is a natural process to release seeds from mature fruit in order to start a new generation of the plant genotype. Currently, chemical fungicides represent the main tool for controlling the major postharvest pathogens as well as the deployment of optimal storage conditions. Interestingly, the synthetic cyclic olefin 1-methylcyclopropene (1-MCP) that blocks ethylene receptors and that is used to extend fruit firmness during storage and marketing (Köpcke, 2015) has also been shown to delay of onset of storage rots (McArtney et al., 2011) such as bull's eye rot on pears (Spotts et al., 2007) and on apples (Maxim and Weber, 2011; Cameldi et al., 2016). Due to the growing concern over the use of synthetic fungicides, alternative measures to control postharvest diseases are sought. However, most of the alternative treatments

developed so far have limitations that impede their effectiveness as single treatments. Combining different treatments within an integrated postharvest disease management strategy needs further development. First of all, this requires a deeper knowledge of the fruit-pathogen-environment interactions at the physiological, biochemical and molecular level. Considering that combining plant genomics with classical breeding is a challenge for molecular biologists as well as for traditional breeders, an increased understanding of the basis of effective resistance mechanisms against the causal agents of postharvest pathogens is required. Eventually, such resistance mechanisms can be introduced into breeding programs to obtain postharvest disease resistant cultivars.

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Summary

Apples and pears (pome fruit) are important deciduous fruit species cultivated on a worldwide scale. Mild and humid climatic conditions, such as those prevalent in North Western Europe, favour fungal diseases on pome fruit, such as apple scab (*Venturia inequalis*), brown spot of pear (*Stemphylium vesicarium*), European fruit tree canker (*Neonectria ditissima*), and postharvest fruit rots. Pome fruit may remain for up to 12 months in storage, during which time fruit rot diseases may develop. Postharvest diseases of apple and pear are caused by a range of fungal pathogens.

Chapter 2 presents the results of packinghouse surveys of postharvest diseases on stored apples and pears conducted from 2012 to 2018 in the Netherlands. This survey revealed a number of new and emerging postharvest diseases. The most important pathogens were *Cadophora luteo-olivacea* causing side rot on pears, and *Fibulorhizoctonia psychrophila* as the causal agent of lenticel spot on apples and pears. Also new problems were observed, caused by several pathogens not earlier described in the Netherlands on apple or pear, such as *Fusarium avenaceum* on pear and apple, *Neonectria candida* and *Neofabraea kienholzii* on pear, and *Colletotrichum godetiae* and *Truncatella angustata* on apple.

Chapter 3 describes the important lenticel spot disease of pome fruit, caused by *Fibulorhizoctonia psychrophila*, in more detail. Growth of this fungus was observed between 1°C and 20°C with an optimum between 10° and 15°C, while incubation of mycelium grown at 25°C resulted in absence of growth. The isolates did not produce asexual or sexual spores. Pathogenicity of representative isolates from apple and pear fruits was tested under laboratory conditions. Furthermore, isolates were characterized and identified by morphology and molecular phylogenetic analysis. Currently, information about the epidemiology, teleomorph, infection routes, and conditions of infection of apples and pears by *F. psychrophila* is lacking.

Typically, the causal pathogens of postharvest diseases infect fruits during growing seasons and remain quiescent until disease symptoms occur after several months in storage. Epidemiological knowledge of these diseases is limited. However, knowledge on population dynamics is essential for the development of preventative measures to reduce risks of fruit infections during the growing season. **Chapter 4** describes Taqman-PCR assays for quantification of *N. alba, N. perennans, C. malorum* and *C. luteo-olivacea* in environmental samples. Various host tissues, dead weeds and grasses, soil and applied composts were collected in ten apple and ten pear orchards. In apple orchards, highest concentrations of *N. alba* were found in apple leaf litter, cankers, and mummies, and of *C. luteo-olivacea* in apple leaf litter, mummies and dead weeds. In pear orchards, *N. alba* and *C. luteo-olivacea* were found in highest concentrations in pear leaf litter and in dead weeds. *C. malorum* was not detected in any sample. Substrate colonization varied considerably between orchards. The temporal dynamics of pathogens was followed

in four apple orchards and four pear orchards. In apple orchards the colonization by pathogens decreased from April until August and increased after August until December. This pattern was less pronounced in pear. Knowledge on population dynamics is essential for the development of preventative measures to reduce risks of fruit infections during the growing season.

Fruit tree canker caused by *Neonectria ditissima* is a serious problem in regions were apple production takes place in climates with moderate temperatures and high rainfall throughout the year. Control measures are applied to protect primary infection sites, mainly leaf scars, from invasion by external inoculum. However, latent infections may occur when young apple trees are infected symptomless during propagation. **Chapter 5** describes a novel method for screening of apple and pear trees at the nursery stage for latent fruit tree canker infections caused by *N. ditissima* to be used prior to planting in orchards. The method may also contribute in developing strategies for the control of European fruit tree canker.

As apple cultivars differ in their levels of susceptibility to *N. ditissima*, **Chapter 6** examines the appropriateness of two resistance parameters, i.e. infection frequency and lesion growth. Important criteria for such parameters are: (1) consistency across experiments, (2) sufficient resolution to reveal genetic differences between apple genotypes, (3) insensitivity to particular disease-specific artefacts, and (4) representation of distinctive components of resistance. Both parameters were evaluated in parallel tests using ten apple cultivars in three experimental years, applying semi-natural infection of leaf scars (infection frequency) or inoculation of artificial wounds (lesion growth). We compared six parameters for lesion growth, of which a new parameter, Lesion Growth Rate (LGR), appeared the best with respect to reproducibility and statistical significance. LGR is defined as the slope of the regression of lesion size versus time. The slope was estimated for each lesion, employing a common start date, and a lesion specific end date determined by the girdling of the lesion. Infection frequency and LGR were examined in separate experiments and in three successive years, and provided complementary information and resulted in reproducible conclusions on the relative resistance levels to *N. ditissima* of the tested cultivars. The presented methods can be used to develop strategies for the control of European fruit tree canker: e.g. in the breeding of new apple cultivars with high levels of resistance to *N. ditissima.*

Chapter 7 reports on dead dormant flower buds of pear as a common phenomenon of economic importance in the major pear production areas of Europe. We examined the effect of insufficient reduction of tree growth to dead flower bud incidences. However, no indication was found that growth regulation can prevent the occurrence of dead flower buds. Our findings furthermore argue that *P. syringae* pv. *syringae* is not the causal agent of dead flower buds disease in the Netherlands, as was commonly believed, although the bacteria may be present as epiphyte and even as endophyte on and in flower buds. Our research revealed strong correlations between dead dormant flower buds

and infection rates of flower buds with *Alternaria* spp.. We concluded from this research that dead flower buds of pear in the Netherlands should be regarded as a fungal disease caused by *A. alternata* SC and potentially also *A. arborescens* SC which may be controlled by specific fungicide applications.

Finally, **Chapter 8** discusses the major results described in this thesis and puts these in a wider context, with a focus on fungal postharvest pathogens and their control. The use of physical treatments, natural compounds, and biocontrol agents as alternative measures to fungicide treatments are discussed. I argue that postharvest diseases should be regarded as complex problems that require multiple interventions at different stages of the disease process in a systems intervention approach for their control. Such approach requires a deep understanding of the epidemiology of the causal agents in the orchard, fruit defense mechanisms, and the molecular biology of host-pathogen interactions in order to develop novel disease control methods.

Samenvatting

Appels en peren (hard fruit) zijn belangrijke fruitsoorten die wereldwijd geteeld worden. Milde en vochtige klimatologische omstandigheden, zoals die in Noordwest Europa voorkomen, zijn gunstig voor de ontwikkeling van schimmelziekten op appels en peren. Voorbeelden hiervan zijn appelschurft (veroorzaakt door *Venturia inequalis*), zwartvruchtrot (*Stemphylium vesicarium*) bij peer, Europese vruchtboomkanker (*Neonectria ditissima*) en vruchtrot tijdens de bewaring. Appels en peren worden tot 12 maanden bewaard, en gedurende deze periode kunnen zich allerlei vruchtrotsoorten ontwikkelen die door een groot aantal verschillende schimmelsoorten veroorzaakt worden.

In **hoofdstuk 2** worden de resultaten gepresenteerd van inventarisaties van bewaarrotziekten die zijn uitgevoerd tussen 2012 en 2018. Dit onderzoek bracht een aantal nieuwe en opkomende bewaarziekten aan het licht. De belangrijkste ziekteverwekkers waren *Cadophora luteo-olivacea*, de veroorzaker van visogen bij peren, en *Fibulorhizoctonia psychrophila,* de veroorzaker van lenticel spot bij appels en peren. Ook een aantal nieuwe vruchtrotveroorzakers werden aangetroffen, zoals *Fusarium avenaceum* bij appel en peer, *Neonectria candida* en *Neofabraea kienholzii* bij peer, en *Colletotrichum godetiae* en *Truncatella angustata* bij appel.

Hoofdstuk 3 beschrijft lenticel spot bij appel en peer, een belangrijke bewaarziekte veroorzaakt door *Fibulorhizoctonia psychrophila,* in meer detail. Myceliumgroei van deze schimmelsoort werd vastgesteld bij een temperatuur tussen 1°C en 20°C, en met een optimum tussen 10° en 15°C. Bij temperaturen hoger dan 25°C vond geen myceliumgroei meer plaats. De schimmelisolaten produceerden geen geslachtelijke of ongeslachtelijke sporen op de voedingsbodems. De pathogeniciteit van verschillende isolaten werd bevestigd in het laboratorium. Daarnaast werden de isolaten gekarakteriseerd via morfologische en moleculair fylogenetische analyses. Op dit moment ontbreekt kennis over de epidemiologie, de teleomorf of geslachtelijke verschijningsvorm, infectie-routes, en de omstandigheden die infecties door *F. psychrophila* van appels en peren stimuleren.

De veroorzakers van bewaarziekten bij fruit hebben als bijzonder kenmerk dat ze de vruchten tijdens het groeiseizoen infecteren, om daarna in een rustfase te gaan, en pas na enkele maanden in bewaring symptomen te veroorzaken. Kennis van de epidemiologie van deze bewaarrotveroorzakers is beperkt. Kennis van de populatiedynamica van deze schimmels is essentieel om preventieve maatregelen te ontwikkelen en om hiermee het risico op vruchtinfecties tijdens het groeiseizoen te verminderen.

In **hoofdstuk 4** worden Taqman-PCR assays beschreven voor het kwantificeren van *N. alba, N. perennans, C. malorum* en *C. luteo-olivacea* in boomgaardmonsters. Verschillende waardplantweefsels, dode onkruiden en grassen, grond en compost werden verzameld in tien appel- en tien perenboomgaarden. In de appelboomgaarden werden de hoogste concentraties van *N. alba* gevonden in appelbladresten, kankers

en vruchtmummies. De hoogste concentraties van *C. luteo-olivacea* werden gevonden in appelbladresten, vruchtmummies en dode onkruiden. In perenboomgaarden werden de hoogste concentraties van zowel *N. alba* als *C. luteo-olivacea* aangetroffen in perenbladresten en dode onkruiden. *C. malorum* werd in geen enkel monster aangetroffen. De concentratie van schimmelpathogenen in en op de verschillende substraten varieerde aanzienlijk tussen de boomgaarden. De aanwezigheid van de ziekteverwekkers werd in vier appel- en vier perenboomgaarden tijdens het seizoen gevolgd. In de appelboomgaarden verminderde de kolonisatie van de substraten door de ziekteverwekkers van april tot augustus, en nam toe van augustus tot december. Deze trend was minder duidelijk in perenboomgaarden.

Vruchtboomkanker, veroorzaakt door *Neonectria ditissima*, is een belangrijk probleem in de appelteelt onder milde en vochtige klimatologische omstandigheden. Beheersing van vruchtboomkanker vindt plaats door bescherming van met name bladlittekens tegen infectie door de schimmelsporen. Latente infecties kunnen optreden als jonge appelbomen tijdens de vermeerderingsfase in de kwekerij symptoomloos geïnfecteerd raken. **Hoofdstuk 5** beschrijft een nieuwe methode om appel- en perenboompjes in de kwekerij te onderzoeken op aanwezigheid van latente infecties met *N. ditissima*, nog voordat de boompjes in de boomgaard worden geplant. Deze methode kan bijdragen aan de ontwikkeling van strategieën voor de beheersing van Europese vruchtboomkanker.

Appelrassen verschillen in vatbaarheid voor *N. ditissima.* **Hoofdstuk 6** beschrijft de toepasbaarheid van twee resistentieparameters: infectie frequentie en laesiegroei. Belangrijke criteria voor de bruikbaarheid van dergelijke parameters zijn: (1) consistentie tussen verschillende experimenten, (2) voldoende resolutie om genetische verschillen tussen appelgenotypen aan te kunnen tonen, (3) ongevoeligheid voor bepaalde ziekte-specifieke artefacten en (4) representatie van verschillende resistentiecomponenten. Beide parameters werden gedurende drie jaar in parallelle experimenten met tien appelrassen geëvalueerd. In deze experimenten werden bladlittekens geïnfecteerd (infectie frequentie) of kunstmatig aangebrachte wondjes geïnfecteerd (laesiegroei). In totaal werden zes parameters voor laesiegroei vergeleken, waarbij de Laesie Groei Snelheid (LGS) het beste bleek op basis van reproduceerbaarheid en statistische significantie. De LGS wordt gedefinieerd als de hellingshoek van de regressielijn van de laesiegrootte in de tijd. Deze hellingshoek werd bepaald voor iedere laesie afzonderlijk, waarbij een gemeenschappelijke startdatum en een specifieke einddatum gebruikt werd. Deze einddatum was afhankelijk of ringen van de stam door de laesie plaats vond. De infectie frequentie en LGS werd gedurende drie jaar in verschillende experimenten onderzocht en leverde complementaire informatie op. Het onderzoek resulteerde in consistent bevestigde conclusies over de relatieve resistentie-niveaus van de getoetste appelrassen tegen *N. ditissima.* De onderzochte parameters kunnen gebruikt worden om strategieën

te ontwikkelen voor de beheersing van Europese vruchtboomkanker, bijvoorbeeld in het veredelingsonderzoek van appelrassen met een hoog resistentieniveau tegen *N. ditissima.*

Hoofdstuk 7 behandelt dode bloemknoppen bij peer. Dit is een wijdverbreid probleem in de belangrijkste perenproductiegebieden in Europa. In het onderzoek werd onder meer het effect van groeibeheersing van perenbomen op het optreden van dode bloemknoppen bestudeerd. Maar groeibeheersing van perenbomen leidde niet tot minder dode bloemknoppen. Ook toonden we aan dat de bacterie *P. syringae* pv. *syringae,* hoewel die epifytisch en endofytisch in de bloemknoppen aanwezig kan zijn, niet de veroorzaker is van dode bloemknoppen in Nederland, zoals verondersteld werd. Het onderzoek toonde wel een sterke correlatie aan tussen dode bloemknoppen en infectie met *Alternaria* spp.. We concluderen dat dode bloemknoppen bij peer gezien moet worden als een schimmelziekte is die veroorzaakt wordt door *A. alternata* SC en mogelijk *A. arborescens* SC en die met specifieke fungicidenbespuitingen beheerst kan worden.

Tenslotte worden in **hoofdstuk 8** de belangrijkste resultaten van dit proefschrift in een bredere context beschreven, met nadruk op pathogenen die bewaarziekten veroorzaken en de beheersing van deze pathogenen. De toepassing van fysische methoden, natuurlijke stoffen, en biologische middelen worden besproken als alternatieven voor fungicidentoepassingen. Ik stel daarbij dat bewaarziekten van fruit gezien moeten worden als complexe problemen, waarbij meerdere acties ondernomen moeten worden in een systeembenadering om deze bewaarziekten te kunnen beheersen. Een dergelijke benadering vereist een goed begrip van de epidemiologie van de ziekteverwekkers in de boomgaard, de afweermechanismen van vruchten en de moleculaire biologie van de waardplant-pathogeen interactie.

Nawoord

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Het is af!

De oogst is binnen!

Marcel

Curriculum vitae

Marcel Wenneker was born on 21 July 1964 in Doetinchem, the Netherlands. He attended the Atheneum at the Ulenhofcollege in Doetinchem, from which he graduated in 1984, and subsequently studied Plant Sciences at Wageningen University from which he graduated in 1991. He worked, amongst others, as a bacteriology researcher at the National Plant Protection Service. He was appointed as a researcher in fruit crops at Wageningen University & Research in Randwijk in 2000. At this research station he conducted the research described in this thesis. Currently he is appointed as senior researcher at Wageningen University & Research, Business Unit Field Crops, in Randwijk.

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