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Susceptibility of different apple cultivars to European canker

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Preface

The work presented in this thesis was carried out at the Department of Plant Science, the Norwegian University of Life Science, with Professor Arne Stensvand, Professor May Bente Brurberg, and PhD Jorunn Børve. All fieldwork was done at NIBIO Ullensvang. Laboratory work was carried out at both NIBIO stations Ås and Ullensvang.

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Abstract

European canker, caused by *Neonectria ditissima,* is a severe economic problem for apple producers in northern Europe. The fungus can cause dieback in mature and young trees. Apple cultivars vary in their level of susceptibility to *N. ditissima*. However, detailed information regarding cultivar susceptibility of the main cultivars grown in Norway is still missing. The present thesis aimed to assess possible cultivar differences in canker development and evaluate the effect of wound type on canker development under field conditions in western Norway.

The cvs. Discovery, Summerred, Red Aroma, Elstar, Red Gravenstein, and Rubinstep were planted in four experimental setups at experimental orchards at the Ullensvang fruit research station in western Norway. In two nursery experiments, trees were propagated with different methods, including grafting on rootstock M9 or cv. Golden Delicious interstem with rootstock M9 or T-budded on rootstock M9. Trees planted to assess wound type differences and for a green shoot inoculation experiment were grafted on M9 or B9. To provide entrance points for the pathogen, trees were either wounded by bending or cutting off side one year old side branches or pruning the top shoot on three different dates in 2019. Trees in all experiments were inoculated with spore suspensions of *N. ditissima* directly after wounding. Canker development in the different cultivars was regularly assessed visually up to 1.5 year after inoculation. In the fourth experiment, which took place in 2020, green shoots were inoculated by a map pin carrying mycelium of *N. ditissima* and visually assessed for four months.

After 1.5 year, one whole-tree experiment resulted in significant differences between cultivars, with a higher canker incidence for cvs. Red Gravenstein (38%) and Discovery (36%) compared to cvs. Rubinstep (18%), Red Aroma (17%) and Red Elstar (16%). Cv. Summerred was not significantly different from any other cultivar. Inoculation in wounds after bending side branches was more successful than inoculation in cut surfaces on side branches and main stem (33 and 19%, respectively). The propagation method did not influence canker development on the different cultivars. The development of new cankers was observed up to 16 months after inoculation. In the wound type experiment, between 4% and 21% of the total cankers developed in the second season after inoculation.

The green shoot inoculation experiment resulted in 78% infection, but no significant cultivar differences in lesion size or canker development were observed.

Bent branches might have been more favorable for canker development due to a larger and cracked surface than the cut surfaces. Results may suggest that at least two economically important cultivars, cvs. Discovery and Red Gravenstein, are highly susceptible to *N. ditissima,* and growers should pay additional attention to canker development on these potentially more susceptible cultivars. Cvs. Red Aroma, Red Elstar, and Rubinstep might be less susceptible to *N. ditissima,* and especially growers in high risk areas can benefit from growing these less susceptible apple cultivars. Even though the same cultivars were used in the described experiments, assessed over the same period and at the same site, the cultivar susceptibility varied between the experiments. Results of artificial inoculation experiments should, therefore, always be set in context to tree age, inoculation method, environmental factors, developmental time, and actual infection conditions in commercial apple orchards.

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1. Theoretical framework

1.1 Taxonomy

European canker, syn. fruit tree canker and apple canker, is caused by *Neonectria ditissima* (Tul. & C.Tul.) Rossman & Samuels, which is an ascomycete fungus in the class of Sordariomycetes. The majority of important plant pathogens of horticultural crops belong to this large class (Agrios, 2005).

Table.1.: Scientific classification of *Neonectria ditissima* (CABI, 2021)

Scientific Classification	
Kingdom	Fungi
Phylum	Ascomycota
Class	Sordariomycetes
Subclass	Hypocreomycetidae
Order	Hypocreales
Family	Nectriaceae
Genus	*Neonectria*
Species	*Neonectria ditissima*

Both the asexual and sexual reproduction stages of the fungus are known. The asexual state is named *Cylindrocarpon heteronema* (Berk. & Broome) Wollenw. (Castlebury et al., 2006). Throughout history, the name of the sexual stage has changed several times. During the 19th century, the pathogen belonged to the species, *Nectria ditissima* Tul. &C.Tul, which mainly consists of canker pathogens on forest trees (Hartig, 1889; Tulasne & Tulasne, 1865 cited after Weber, 2014). At the beginning of the 20th century, the pathogen was divided from *N. ditissima*. From then on, the new pathogen name was *Nectria galligena* Bres and was renamed to *Neonectria galligena* (Bres.) Rossman&Samuels in 1995. This name change was due to new knowledge regarding host range and microscopic details (Cayley, 1921).

The current knowledge is that *Neonectria galligena* (Bres.) Rossman & Samuels and *Neonectria ditissima* (Tul. & C.Tul.) Rossman & Samuels are the same species. This taxonomic decision was made by (Castlebury et al., 2006) based on the close relationship. The correct name for this species is now *Neonectria ditissima* (Tul. & C.Tul.) Rossman & Samuels (Rossman & Palm-Hernández, 2008).

1.2 Host range

The pathogen is known to have a wide host range. Besides apples (*Malus domestica*), the pathogen can infect pears (*Pyrus communis*). Compared to apple canker, pear canker is less of a problem in northwestern Europe (Weber, 2014). Due to the pathogens wide host range, it can damage hardwood forest trees such as maple, mountain-ash, quince, aspen, beech, black and yellow birch. Windbreaks consisting of poplar, hawthorn, and beech were found to be possible external sources of inoculum for apple orchards (Flack & Swinburne, 1977; Walter et al., 2015).

1.3 Geographical distribution

Today the disease is present in apple producing areas in Europe, North and South America, and New Zealand (Beresford & Kim, 2011). Suitable climatic conditions are a crucial factor for the pathogen. In parts of the world with a cool and humid climate the risk for losses due to apple canker is high (Beresford & Kim, 2011).

1.4 Symptoms

Canker

Symptoms of European canker have been described by many authors, e.g. (Amponsah et al., 2015; Ghasemkhani, 2012; Weber, 2014). Early disease symptoms are circular, brown discolored patches on various parts of the tree, such as the stem, branches, and fruit spurs. The epidermis starts to peel off, and the bark looks papery. The canker's inner part is sunken, black, and callus ridges are produced over the years. It becomes visible that the canker's edges protrude from the healthy bark, and cankers can be surrounded by swollen tissue. The severity of the symptoms varies with the tree age. Young trees are likely to develop cankers on the main stem or main branches and are expected to cause more significant losses than older trees with smaller infected branches(Obstabauberater des Beratungsrings, 2018).

Nevertheless, the main stem of older trees may also become infected, which will lead to a loss of productivity (Obstabauberater des Beratungsrings, 2018).

Fruit rot

Fruit infections can appear when spores are spread from cankers and land on fruits. Openings such as the calyx and wounds in general function as openings for spores to infect. Symptoms are brown rots on the calyx, the stalk end, or the cheek. The rots are slightly sunken, circular, and separated sharply from the healthy flesh (Saville & Olivieri, 2019; Sutton et al., 2013; Weber, 2014).

1.5 Infection process

It is common to observe new wood growth around the infected area, forming a wound callus. This prevents the spread of the pathogen into healthy tissue (Crowdy, 1949). *Neonectria ditissima* is known to invade all the tissue outside the xylem, as well as the xylem itself. The pathogen spreads in the xylem beyond the limits of the lesion, but its hyphae were found to be more frequently close to the canker than further away from the canker wound (Crowdy, 1949). A study of *N. ditissima* on *Fraxinus mandshurica* var. japonica revealed that yearly necrosis of phloem, xylem, and cambial cells forms characteristic canker rings (Sakamoto et al., 2004). The authors observed narrow annual rings on the lateral sides of the cankers and concluded that the ability of producing new xylem cells was lost due to the pathogen. Narrow vessels lead to decreased water conductivity. Therefore, branches and stems with cankers could not conduct enough water flow in spring until the current year's larger vessels developed. This reduced water flow causes the dieback of cankered trees.

Brown staining in the cortical wood, manifested through vertical lines, can be visible when cutting open wood close to a canker. This staining can be associated with the production of diffusible toxins that are not identified (Crowdy, 1949; Zeller, 1926). Vertical spread in the trunk was found to be more than 20 cm away from the canker wound. This observation was made in cv. Nicoter (Kanzi®) trees only six months after the first canker symptoms became visible (Weber, 2014).

Often more than one canker can be found on the same tree. This is why the theory of systemic spread in the tree arose (Hartig, 1889). It was believed that *N. ditissima* can cause infections from the inside of a tree. Where wood samples between two lesions were sampled, the pathogen's isolation was not successful. Therefore, the authors concluded that systemic spread is unlikely. They rather proposed that rain splash or water running down the trunk cause several cankers on the trunk. Spores and conidia then enter the bark through artificial or natural wounds and causing new infections. Crotches at the base of side branches on the apple trees are high-risk areas due to the presence of growth or stress cracks, the roughness, and finally the high ability to retain moisture (Weber & Hahn, 2013).

Importance of nursery infections

A critical question that arises regarding the infection process is the latent period of *N. ditissima*. According to McCracken et al. (2003), the latent period in nursery trees can be up to three years. This long latent period underlines the importance of healthy nursery material, especially regarding susceptible cultivars. A nursery inoculation trial identified the most susceptible infection stages across tree propagation methods. The authors inoculated M9 rootstocks and cv. Queen Cox budwood, with conidial suspension at different propagation phases: 1) after the removal of side shoots of rootstocks (defeathering) 2) after budding and 3) after heading back. The time the cankers took to develop in the nursery and after planting in the orchard varied between three months and three years. Most of the trees inoculated after defeathering showed canker symptoms already in the nursery 1.5 years after inoculation. Especially trees that were inoculated after budding and heading back developed more cankers when planted in the orchard. Trees inoculated after budding were planted in the orchard 1.5 years after inoculation, and headed back trees were planted one year after inoculation (McCracken et al., 2003). This study showed that due to the long latent period, symptoms of *N. ditissima* are often not visible during planting. This symptom delay causes various issues for growers. Orchard management can become more challenging because replaced trees cause a diverse tree size (Weber, 2014). Besides, after a long period, economic compensation from nurseries might be challenging (Weber & Børve, 2021).

A reliable molecular detection method for asymptomatic infections of *N. ditissima* in apple wood, especially for nursery trees, would therefore be highly desirable and even though much research was already done a commercially used method is not in use yet (Ghasemkhani et al., 2016; Harteveld et al., 2020; Scheper, 2020).

1.6 Epidemiology

The disease triangle is a commonly used model in plant pathology. If a susceptible host, a virulent pathogen, and a suitable environment are present simultaneously, the plant becomes diseased (Agrios, 2005). The three essential factors that lead to disease are presented in Fig. 1. Both pathogen factors and host factors are explained in more detail in the following chapter.

Fig.1.: Disease triangle. Adapted after (Saville & Olivieri, 2019)

Host Factors and cultivar differences

A crucial requirement for conidia and ascospores to infect the tree is a wound on the tree (Sutton et al., 2013). Examples are pruning wounds, frost cracks, and leaf scars (Weber, 2014). The relative importance of different wounds as entrance points varies between regions. For example, in Brazil, pruning wounds during the season are primary entrance wounds (Alves & Nunes, 2017). In New Zealand, picking scars are more significant (Amponsah et al., 2015). These regional differences can be explained by various factors, such as the local climatic conditions, availability of different plant protection products, and inoculum concentration where and when wounds are present (Saville & Olivieri, 2019). Important inoculum sources are lesions in trees and branches with cankers left in the orchard after pruning, infected fruit, and nursery plants (Børve et al., 2020a; Obstabauberater des Beratungsrings, 2018; Weber, 2014).

For an experiment in New Zealand, the spatial pattern and disease progression of *N. ditissima* were mapped using a Geographic Information System (Di Lorio et al., 2019). It was found that one common factor in regions with high canker incidence was the planted cultivar. The authors suggest that a difference in cultivar susceptibility to *N. ditissima*, among other things, could explain the higher canker incidence in some regions. Information about the level of susceptibility of available cultivars is an important decision-making tool for growers. For example, the organic production of modern and very susceptible cultivars such as cvs. Nicoter and Civni are economically not profitable in northern Germany (Weber, 2015). Various experiments have been conducted to identify the most susceptible and resistant apple cultivars. So far, none of the currently used cultivars are fully resistant against European canker (Garkava-Gustavsson et al., 2013). Many researchers found differences in susceptibility that can be used in breeding programs and give rise to potentially less susceptible genotypes (Garkava-Gustavsson et al., 2013; Ghasemkhani, 2015; Gómez-Cortecero et al., 2016; van de Weg, 1989). Recently progress was made to unravel molecular mechanisms of host-pathogen recognition (Bus et al., 2019) .

In a Swedish greenhouse experiment, one year old shoots of 55 different cultivars were inoculated with *N. ditissima* after removing buds or wounding the bark with a scalpel on the opposite side of a bud (Garkava-Gustavsson et al., 2013). In this experiment cv. Cox's Orange Pippin was identified as the most susceptible cultivar. The most resistant cultivars were, amongst others, cvs. Florina and Santana. Examples of cultivars with intermediate resistance levels were cvs. Rubinstar and Jonathan. This finding contrasts results from another shoot experiment in which cv. Jonathan was found to be highly resistant (van de Weg, 1989).

In an experiment with potted apple trees in New Zealand, a significant wood age effect and a significant difference in susceptibility of cultivars to *N. ditissima* were found (Amponsah et al., 2017a). Trees were wounded with a metallic file, and a conidial suspension was applied. Cv. Royal Gala trees had a significantly higher canker incidence than cvs. Braeburn, Golden Delicious, Fuji, Jonathan and Scilate (Envy™). Regarding the wood age, the results showed that three year old wood had more lesions than two and one year old wood (Amponsah et al., 2017a). Wound healing might be faster in younger wood compared to older wood (Saville & Olivieri, 2019). A different study with potted apple trees investigated the effects of wound age and apple cultivars. Three cultivars were inoculated with conidia of *N. ditissima* right after pruning and then three, six, and nine days after pruning in an additional experiment (Xu et al., 1998). It was shown that the infection incidence decreased with wound age, but the wound age effect varied among the cultivars. Trees of cv. Spartan apple trees did not show significant effects of wound age, while cv. Cox had a significantly higher incidence of wounds which were younger than nine days. Pruning wounds on cv. Fiesta showed a significantly higher canker incidence on wounds less than six days old. The authors suggested that the interaction between wound age and canker incidence points towards a cultivar difference in their wound healing rates. All studies mentioned above illustrate that it is crucial to evaluate all possible factors that could affect the canker development in experiments and orchards in general.

Pathogen factors

Not much is known about the pathogenicity factors and the worldwide level of diversity of *N. ditissima* (Gómez-Cortecero et al., 2016). Scheper et al. (2015) characterized the pathogenicity of three different isolates of *N. ditissima*. The authors inoculated one year old dormant potted cv. Royal Gala trees in a glasshouse. Disease incidence and lesion size was measured. These results showed that both almost non-pathogenic and pathogenic strains exist. However, it is not known whether the non-pathogenic isolates might have had higher pathogenicity on other cultivars. The lack of knowledge regarding the pathogenicity of different isolates makes it more challenging to plan and execute experiments

Both the asexual (conidia) and sexual (ascospores) spore stages of *N. ditissima* can cause infections. The anamorph stage consists of single-celled microconidia and two to four celled cylindrical macroconidia. Conidiophores that develop on the bark are visible as creamy white pustules (Ghasemkhani, 2012). Conidia can cause infection when spread by wind and rain. In 2-4 mm rain approximately 80% of spores are released after 30 min (Walter, 2020).

It is known that *N. ditissima* survives the winter as mycelium and perithecia in cankered tissue. Two-celled sexual ascospores are produced in perithecia. Ascospores can be forcibly discharged from the perithecium and carried by the wind, or ascospores can be washed off by rain (Weber, 2014). The colors of perithecia are a helpful indicator of the development stage. Unripe perithecia are usually orange, and mature perithecia have a characteristic red color and can have a white cirrus of ascospores on top. Perithecia get darker as they get older until the perithecia become black and empty (Ghasemkhani, 2012). In Norway, ripe perithecia can be found the whole year round but mainly during winter (Børve et al., 2020b). Worldwide, differences in spore production and dispersal have been reported (Amponsah et al., 2017b; Munson, 1939; Weber & Børve, 2021), but research about regional spore availability, especially the role of ascospores is still ongoing (Weber & Børve, 2021). In New Zealand, ascospore discharge was observed all year round (Amponsah et al., 2017b), whereas in the United Kingdom, a peak discharge of ascospores was found in January and February. The lowest ascospore discharge was reported during the summer months (Munson, 1939).

Ongoing spore trap experiments in northern Germany revealed that ascospore discharge is highest in spring. Ascospores were not released during the leaf fall period in autumn (Weber & Børve, 2021). In Norway, Ascospore release was observed from late August to mid-December (Børve et al., 2020a).

Environmental factors for infection of *N. ditissima*

Besides the interaction between host and pathogen population, the environment's influence is a crucial part of disease development. A worldwide review of canker incidence associated with climate conditions indicates that *N. ditissima* requires approximately 30% rain days per month and temperatures between 11 and 16°C for more than 8 hours per day to be a problematic pathogen in a region (Beresford & Kim, 2011). The authors observed a reduced occurrence of peripheral cankers in regions where the percentage of days with rain was less than 30% and less than 8 hours in a temperature range at 11 to 16°C. This review shows that *N. ditissima* infections can occur over a range of temperature and wetness conditions. In Chile, the germination of conidia and ascospores was almost nonexistent at 5°C, and a prolonged wetness period did not lead to leaf scar infections either (Latorre et al., 2001). However, an experiment in the Netherlands showed that conidia could germinate and infect pruning wounds at low temperatures, such as 1°C (de Jong & van der Steeg, 2012).

The optimal germination temperature is expected to be around 20-25°C (Latorre et al., 2001). At 20°C, the germination frequency was more than two times faster for ascospores than for conidia. The pathogen activity is declining at temperatures of 30°C and above. There are different limits for definite conditions and spread regarding the required wetness period published. At 20°C, a two-hour period was sufficient to promote a significant infection, but a more extended moisture period was required (Latorre et al., 2001). This finding contrasts with other experiments that show at least six hours of free water were necessary to infect leaf scars of cv. Gravenstein trees with conidia. Canker infections increased with longer moisture periods (Dubin & Englisch, 1974). Xu et al. (1998) inoculated pruning wounds in potted trees with *N. ditissima* and tested the effect of wetness duration on canker incidence. The authors found no increase in incidence with increased duration of wetness.

These different findings might be due to the different wound types used in the experiments. Therefore, the influence of surface wetness period on canker incidence may vary with the entry point.

2. Research topics

Most of the research regarding cultivar differences in susceptibility to *N. ditissima* has been carried out on detached shoots and potted trees in controlled environments (Garkava-Gustavsson et al., 2013; Ghasemkhani et al., 2015a; van de Weg, 1989). Detached shoot experiments can be performed with a higher number of cultivars and require less time and funding, when compared to experiments with whole trees (Garkava-Gustavsson et al., 2013; Scheper et al., 2018). Van de Weg (1989) laid the foundation for international research on canker development on detached shoots. In a glasshouse, shoots were wounded with two different wound types. For the first wound type, one mm of the upper layer of leaf marks together with the corresponding axillary bud was cut away. For the second wound type only the upper layer of leaf marks was cut away. In addition to the two wound types, leaves on the shoot were either removed or retained. After two months, significant cultivar differences were found with both described wounding methods when measuring the lesion length and calculating the incubation period and percentage of infected shoots.

Experiments in Sweden with detached shoots and potted trees (Garkava-Gustavsson et al., 2013) based their artificial wound experiments on the wound types used by van de Weg (1989). Detached shoots were inoculated in a greenhouse, and potted trees were inoculated underneath a plastic roof. In both experiments, the lesion size was measured regularly for up to three months, and the disease development was calculated from the mean lesion length per cultivar and assessment date. When comparing lesion length in detached shoots and potted trees, five apple cultivars showed similar lesion length development across the two experiment types.
A different experiment in Sweden, conducted in two following year, had the objective to obtain information about the susceptibility of apple cultivars and at the same time compare detached shoot assays with whole tree assays (Garkava-Gustavsson et al., 2016).

Detached shoots were inoculated after wounding, according to Garkava-Gustavsson et al. (2016). Potted trees were inoculated after cutting off buds, and trees in the field were inoculated naturally by placing cankered wood above the trees. Information about cultivar differences was obtained by calculating disease percentage, disease development, and incubation time. The relationship between colonization rates in detached shoots and potted trees did not always show dependable results. The authors point out that even though the assessment of colonization rate in potted trees had more resolution power, detached shoot experiments are suitable for initial screening of cultivars and obtaining knowledge about infection development (Garkava-Gustavsson et al., 2016). An experiment in New Zealand compared the susceptibility of different apple cultivars and phenotyping methods (Scheper et al., 2018). Potted cv. Braeburn trees had a high canker incidence (64%), while none of the detached shoots showed canker symptoms. The authors suggested that detached shoot experiments might not be reliable to identify susceptible apple cultivars to *N. ditissima*.

The overall objective of the present field experiments was to gain knowledge about the susceptibility to *N. ditissima* of six frequently grown and important cultivars in Norway. Obtained results will be put in context with published literature to evaluate if results from the conducted field experiments, where trees were inoculated after wounding, correspond to existing knowledge from artificial inoculation experiments on detached shoots or potted trees.

The specific objectives were to:

- Study the cultivar difference in development of canker symptoms on trees inoculated with *N. ditissima* under field conditions.
- Evaluate the effect of wound type, i.e., bended branches or cuts, on disease development of *N. ditissima.*
- Assess if three different grafting methods influence canker development in different cultivars.
- Study the symptom occurrence of *N. ditissima* in map pin inoculated shoots of five different cultivars over a four-month period and asses possible cultivar differences in lesion length and inner staining under field conditions.
- Investigate if *N. ditissima* can be detected in branches without visible staining by real-time PCR four to five centimeters away from the canker wound and assess possible cultivar differences

3. Materials and Methods

Three different experiments were performed to assess cultivar differences at NIBIO Ullensvang in 2019-2020.

3.1 Wound type experiment

3.1.1 Experimental setup

In total, 240 trees of the cvs. Discovery, Summerred, Red Aroma Fagravoll (Red Aroma), Elstar Boerekamp Exellent Star (Red Elstar), Red Gravenstein, and Rubinstep Pirouette (Rubinstep) were planted at NIBIO Ullensvang in May 2019. Except for cv. Red Aroma, all trees were grafted on a dwarfing rootstock Malling 9 (M9). Cv. Red Aroma was grown on Budagovsky 9 (B9) rootstock. All trees were delivered from a commercial supplier and planted in a nursery plot at distance 0.8m*1.5m. During the 2019 and 2020 seasons, some trees showed symptoms and signs of powdery mildew, apple scab and aphid outbreaks. On 14 June 2019 1.12 L/ha Movento (Spirotetramat, Bayer AG) and 0.375 L/ha Topas 100 EC (Penconazole, Syngenta) were sprayed against aphids and powdery mildew, respectively. On June 12 and 9 July 2020, 0.37 L/ha Topas 100 EC and 0.5 kg/ha Delan (Dithianon, BASF) were applied against powdery mildew and apple scab. None of the planted trees showed visible canker symptoms before the start of the experiment.

Experimental design

Before planting, all trees were split into three treatment groups. Within each group, two trees of the same cultivar were randomly planted together in three replicates. These two trees were either inoculated with *N. ditissima* or used as control trees. All trees were planted on 8 May 2019 and inoculated on 13 May 2019.
In total, three different inoculation methods were used:

1) Non-wounded: The main stem of the non-wounded trees was sprayed with a spore suspension (10^4 ascospores per ml) until runoff.
2) Bent: All one year old side branches of the trees were bent close to the breaking point. Then a 20 µl drop of spore suspension (10^4 ascospores per ml) was directly placed on the wound. The number of bent branches varied from four to 14 per tree.
3) Cut: All one year old side branches were cut off and the trees were the top shoot was pruned (headed back) with a garden pruning shear. A 20 µl drop of spore suspension (10^4 ascospores per ml) was immediately applied to the cuts. The number of cuts varied from four to 14 per tree.

The corresponding number of trees were given the same treatments, but autoclaved water was used instead of inoculation with *N. ditissima.*

3.1.2 Inoculum

A canker found on a cv. Red Gravenstein from a commercial orchard in Western Norway that had been underneath a spore trap at NIBIO Ullensvang since May 2018 was brought into the laboratory. Perithecia were then transferred to a microscope slide, and a drop of autoclaved water was added. A cover glass was put on top of the perithecia. To ensure that ascospores were released, a light pressure was applied to the cover glass. Then ascospores were transferred into a beaker with autoclaved water. With the help of a hemocytometer, ascospores of *N. ditissima* were counted. The final concentration was adjusted to 10^4 ascospores per ml. This suspension was immediately used for inoculation. Drops of the spore suspension were placed on microscope slides and incubated at 20°C for 24 hours before counting 100 spores per drop. The microscope slides were kept in a plastic box with a moist cloth to ensure water saturated air. A spore was regarded as germinated when having a germination tube longer than the spore width. For this experiment, the germination rate was approximately 95%.

3.1.3 Fieldwork

During the growing season of 2019, all trees were visually assessed for canker symptoms at two to four weeks intervals. During each assessment, the number of wounds that developed canker were counted. The first assessment took place 2.5 months after inoculation, and the last assessment in 2019 at the beginning of October, after 4.5 months. In 2020, the trees were assessed for the first time in February. During the following months until May, assessments took place once a month. From 3 June 2020 onwards, visual assessments were conducted regularly every second week.
Assessments in the period from May 2019 until June 2020 were performed by personnel at NIBIO Ullensvang. The author did the assessments from June 2020 onwards.

3.1.4 Final assessment

The final assessments of the trees were done over a longer period in autumn 2020. Non-wounded trees were assessed from 10 to 13 September. The cut and bent treatments, and corresponding control trees were assessed in the period from 6 to 12 November 2020. All trees were cut at soil surface and removed from the orchard to ensure a thorough examination. Further examination of the whole tree including the above soil surface part of the rootstock were performed. Cuts and bent branches showing symptoms of *N. ditissima* were counted for each tree and growths of different wood ages was measured. Also, the number of branch angles was counted in the unwounded treatment group and trees were examined for cankers. Simultaneously, cankers were examined for brown wood staining, starting from the canker, and going in the main stem above and underneath the canker. The branches were examined for staining as well. All wood staining was measured roughly with a folding meter stick.

To confirm that cankers were caused by *N. ditissima* following criteria had to be filled; typical symptoms of European canker on the bark, such as discoloration, flaking off the epidermis layer, and cracked and swollen tissue around the canker. To ensure the identity of cankers they were brought into the laboratory and examined for sporodochia and perithecia. Because not all sporodochia and perithecia were visible with the naked eye, cankers were examined through a stereomicroscope. If no perithecia or sporodochia were found, symptomatic wounds were examined for necrotic tissue in the wood and dark brown lesions in the bark surrounded by swollen tissue.

When the latter symptoms were present, the wound was classified as Neonectria canker, even though no spore structures were found.

3.1.5 Method for isolation from symptomatic plant tissue

Isolations on artificial growth media were made from wood tissue of one canker per cultivar, regardless the wounding type, on 14 November 2020 according to the protocol below.

In a laminar flow cabinet, a canker was cut into small pieces of 2-4 cm length, for 10 seconds immersed in 70% Ethanol and thereafter in 0.5% Sodium hypochlorite solution for 90 seconds. The pieces were placed on absorbent paper, and after drying, they were transferred to agar plates amended with tartaric acid (0.21 g Tartaric acid per 100 ml Potato Dextrose Agar) under sterile conditions. Agar plates were incubated at 20°C and steady light for 24 hours. The plates were checked daily for fungal growth. The sections showing growth of *N. ditissima* transferred to standard Potato Dextrose Agar (PDA) plates five days after isolation.

3.1.6 Real-time PCR

Sample collection and preparation

Samples from both the cut and bent treatment methods were collected for the real time PCR. One sample from a canker per cultivar was taken as a positive control. Four wood samples per cultivar at four to five cm from the canker were selected (Fig. 2). Only cankers without staining were chosen for real-time PCR sampling. For each cultivar, a negative control from a healthy non-inoculated tree was sampled. The control was taken four to five cm away from the cut or wound on the bent branch. All wood samples were stored in the fridge for four to seven days until the DNA was extracted.

Fig. 2: Samples for real-time PCR were taken directly from the canker (circle) and four to five cm away from the wound (rectangle). Photo: Theresa Weigl

On 17 November 2020, all samples were crushed in liquid nitrogen using a mortar and pestle. All samples were stored at -80°C for either one or two days until the DNA was extracted according to DNeasy® Plant Mini Kit. The preparation of samples for real-time PCR analysis took place at NIBIO Ullensvang. All samples were stored at -18°C until they were analyzed at NIBIO Ås.

Real-time PCR method

The real-time PCR analysis took place at NIBIO Ås on 17 February 2021 and was performed using CFX96™ Real-Time System (Bio-Rad, Hercules, CA; USA) in a 96-well reaction plate. Duplicates of each sample were used. The *N. ditissima* assay is based on Taqman technology and was designed at NIBIO (Brurberg M.B, unpublished results). To address the issue of false-negative results from real-time PCR detection, an amplification control targeting the 18S ribosomal RNA was included. The reaction mix for *N. ditissima* was 12.5 µl Sso Advanced Universal Probes Supermix 2x, 2.25 µl Primer forward/reverse pmol/10 µl, 0.9 µl Probe 10 pmol/µl and 4.1 µl Nuclease free H_2O, in total 20 µl. Corresponding for 18S, the reaction mix consisted of 12.5 µl Sso Advanced Universal Probes Supermix 2x, 0.6 µl Primer forward/reverse 5 pmol/µl, 0.8 µl probe 5 pmol/µl and 7.5 µl Nuclease free H_2O, in total 22 µl.

For each reaction 22 μl of master mix was pipetted into each well in the reaction plate and then 3 μl extracted DNA was added. The thermal cycling conditions for the real-time PCR were as follows:

N. ditissima: Initial denaturation at 95°C for 3 min followed by 40 cycles of 10 sec denaturation at 95°C and primer annealing/extension at 60°C for 30 sec.

18S: Initial denaturation at 95°C for 3 min followed by 45 cycles of 5 sec denaturation at 95°C and primer annealing/extension at 60°C for 20 sec.

3.2 Nursery experiments

3.2.1 Experimental setup

For this experiment, three different grafting methods and six cultivars were combined. The grafting took place at NIBIO Ullensvang while T-budded trees were purchased.

1) The cvs. Discovery, Red Aroma, Red Gravenstein, Rubinstep and Summerred were T-budded in July 2017 at the commercial nursery Fjeld Hagebruk in Ringerike, Norway. The scion wood used was obtained from the Elite plant station at Sagaplant AS in Midt-Telemark, Norway and M9 rootstock were obtained from a commercial producer.
1) In winter 2018 budwood of the cvs. Discovery, Elstar, Red Aroma, Red Gravenstein, Summerred, Rubinstep, were received from the Elite plant station. In March 2018, the cultivars were grafted on rootstock M9 at NIBIO Ullensvang.
2) Scion wood of the cvs. Elstar, Discovery, Red Gravenstein, Rubinstep, and Summerred was grafted on 1-year old cv. Golden delicious interstem with M9 rootstock. Grafting was done in March 2018. Cv. Golden Delicious trees, used as interstem, were stored in cold storage before grafting. Bud wood was purchased from the Elite plant station, and cv. Golden Delicious trees used as interstem were obtained from a commercial supplier importing from the Netherlands.

All grafts were tied with Flexiband A, which is a type of rubber band. The grafts were potted in 4.4 L plastic pots and placed in a plastic ventilated tunnel (size 4 × 20 m and 2.5 m heigh) in the end of April. Ten pots were placed together in open polystyrene boxes. Watering was done by drip irrigation, and fertilizer was given manually. All healthy appearing trees were then planted in a nursery plot at NIBIO Ullensvang in July 2018. The planting distance was 0.5m*1.5m.

The different grafting methods were grouped together, and all trees of the same cultivar were planted together in a nursery plot. Dead trees were removed from the nursery plot on in autumn 2019 and assessed for canker development.
Inoculation was done on two or three trees together, randomly distributed within the grafting method and cultivar group. The experiment consisted of five replicates per cultivar, grafting and inoculation method, including two (grafted) or three (T-budded) trees.

On 14 June 2019, 1.12 L/ha Movento and 0.375 L/ha Topas 100 EC were applied to all trees to prevent aphids and powdery mildew. In the following month 0.14 kg/ha Teppeki (Flonicamid, Belchim) was applied on 22 July 2019 against aphids. On the 12 June 2020, 0.37 L/ha Topas 100 EC and 0.5 kg/ha Delan WG were applied. The latter treatment with Topas 100 EC and Delan WG was repeated 9 July 2020. None of the planted trees showed visible canker symptoms before the start of the experiment, but some trees had symptoms of bacterial canker (*Pseudomonas syringae*).

Experimental design

All trees were equally divided into three groups and headed back at 70 cm on 4 April 2019. Two out of the three groups received separate inoculation treatments while the third group was for the uninoculated control.

1) Headed back: Trees were inoculated with a 20 µl drop of ascospore suspension (10^3 ascospores per ml) right after heading back one year old wood on 4 April 2019. This inoculation method simulated how *N. ditissima* invades a heading cut.
2) Bent: In the second treatment group, angles of one year old branches were bent down on 28 June 2019, and a 20 µl drop of with conidia suspension (10^5 conidia per ml) was applied to the wound. This treatment intended to imitate the adjusting of branches to 90-degree angles, which is done in nurseries. The number of bent branches varied from one to nine per tree.

A corresponding number of trees were not inoculated with *N. ditissima*. A 20 µl drop distilled water was applied on the headed back control trees on 4 April 2019. Branches were bent on 28 June 2019, and a 20 µl drop of distilled water was applied on the wound. These trees were used as control trees for both treatments.

3.2.2 Inoculum

Inoculum for headed back trees

A canker found on a cv. Red Gravenstein tree from a commercial orchard in eastern Norway that had been underneath a spore trap at NIBIO Ullensvang since June 2018 was brought into the laboratory. The ascospore suspension was prepared according to 3.1.2 and the final concentration was adjusted to 10^3 ascospores per ml. This suspension was used for inoculation of the headed back trees right after. Drops of the spore suspension were placed on microscope slides and incubated at 20°C for 24 hours before counting 100 spores per drop. The microscope slides were kept in a plastic box with moist clothes to ensure water saturated air. The germination rate was as defined above (see 3.1.2) and was approximately 80%.

Inoculum for bent branches

A canker obtained from a commercial orchard in the Hardanger region was used as source material for the conidia suspension. The canker was found on a B9 rootstock of a cv. Discovery tree. Sporodochia were transferred into a beaker with autoclaved water. The suspension was adjusted to 10^5 conidia per ml with a haemocytometer. Directly after adjusting the concentration, the bent branches were inoculated with the conidial suspension. The conidial germination rate was determined after incubating in a for 24 hours at room temperature. Drops of the spore suspension were placed on microscope slides and incubated, in a plastic box with water saturated air, at 20°C for 24 hours before counting 100 spores per drop. The germination rate was defined as above (see 3.1.2) and was approximately 83%.

3.2.3 Fieldwork

During the season of 2019, all trees were first visually assessed for canker symptoms on 28 August 2019, i.e., two months after the inoculation of the bent branches and 4.5 months after the inoculation of the headed back trees. From then on, trees were assessed biweekly, with the last field assessment in 2019 on 10 October. In each field assessment trees with canker were noted, the number of cankers per trees were not counted. During winter 2019/20, no field assessments took place.

On 4 March 2020, all trees were assessed, and thereafter trees were assessed once a month for canker symptoms. The last field assessment was in October. Assessments in the period from June 2019 until June 2020 were performed by personnel at NIBIO Ullensvang. The author did the assessment from June 2020 onwards.

3.2.4 Final Assessment

The final assessment of all trees took place from 21 October to 4 November 2020. All trees, including their rootstock, were removed from the orchard to ensure a thorough examination. The number of cankers were counted on trees that were inoculated with *N. ditissima* after bending the branches and trees were examined for cankers on the main stem and rootstock. Trees that were headed back and inoculated with *N. ditissima* were examined for cankers in the top and for cankers on the main stem and the rootstock. The same methods and criteria for canker detection were applied as in 3.1.4.

3.3 Map pin inoculation of green shoots

3.3.1 Experimental setup

In total, 100 apple trees from cold storage were placed in water 20 June 2020 and planted five days later at NIBIO Ullensvang. Twenty trees of each of the cvs. Discovery, Elstar Eke (Red Elstar), Red Aroma, Red Gravenstein and Summerred were planted in two rows, 4.5 m apart. The planting distance was 100 cm. All trees were grafted on M9. Five trees of the same cultivar were planted together in order to create four replicates. The cultivars were planted in a randomized design.
Because some trees showed symptoms of aphid damage 0.3 l/ha Calypso (Thiacloprid, Bayer AG) was applied 4 August 2020.

3.3.2 Inoculum

On 16 June 2020, a cankered cv. Holsteiner Cox tree from an organic orchard at NIBIO Ullensvang was taken into the laboratory. On the same day, a canker wound on that tree was cut into 2-4 cm long pieces. In a sterile bench, these pieces were immersed for 10 seconds in 70% Ethanol and then put in 0.5% Sodium hypochlorite solution for 90 seconds. The sections were placed on bench paper, and after drying, they were transferred to agar plates amended with APDA.

Agar plates were incubated at 20°C and checked daily for fungal growth. After three days, sections showing growths of *N. ditissima* were transferred to PDA. When the agar plates were covered with mycelium, they were stored in the fridge until the day of inoculation. After inoculation, a piece of the remaining mycelium was re-transferred to PDA. After three days sections showing growth of *N. ditissima* were transformed to PDA and stored in the fridge after mycelium growth was observed. On 31 July, mycelium was again transferred to PDA and after mycelium growth was visible, the PDA plate was stored in the fridge. After 54 days, the mycelia from this agar-plate was crushed, DNA was extracted and sequenced at NIBIO Ås on 23 September 2020. The identification of the isolates was confirmed by sequencing of amplicons of the internal transcribed spacer region (ITS) rDNA using universal primers ITS4 and ITS4 (White et al., 1990). Sequencing was done by personnel of NIBIO Ås. The sequence length was 527 base pairs. Up to a 100% sequence match to *N. ditissima* was detected.

3.3.3 Experimental design

On 1 July 2020, one shoot per replicate was inoculated with *N. ditissima.* A standard map pin was used to infect the shoots with *N. ditissima* (Talgø & Stensvand, 2013). Shoots with a minimum length of one cm were chosen either for inoculation with *N. ditissima* or as a control. For the cultivar Summerred, it was only possible to inoculate two replicates, because trees in other replicates did not show any growth. One agar plate with fungal growth of *N. ditissima* was brought to the experimental orchard (Fig. 3A). In the orchard, a small amount of the mycelium was taken up with the tip of a map pin. The pin was then inserted in the middle of the shoot between the base and its end (Fig 3 A, B). Per replicate, a clean map pin was stuck into one control shoot.

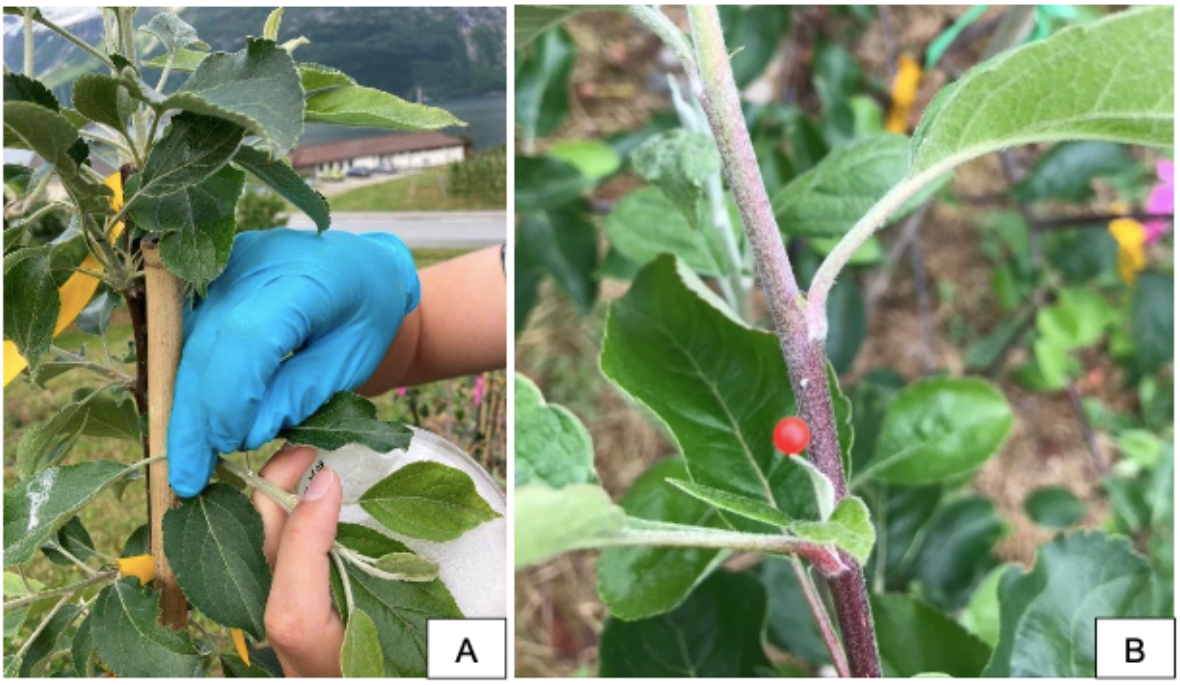

Fig. 3.: (A) The map pin was first contaminated with mycelium of *Neonectria ditissima* and then placed in the green shoot. (B) A map pin, contaminated with *Neonectria ditissima* on a cv. Discovery shoot. This picture was taken at the day of the inoculation. Photos: A: Kurab Benjamas Røen B: Theresa Weigl

3.3.4 Fieldwork

After inoculation, the first visual assessment took place on 13 July 2020. From then on, notes about new canker symptoms were made biweekly. The last field assessment took place on 10 October 2020.

3.3.5 Final assessment

On 26 October 2020, all shoots were cut off at their base and brought into the laboratory. First, the total length of the shoots was measured. Then, the area around the map pin was inspected for canker symptoms, and the length of lesions were measured. With a garden pruning shear, the shoot was cut ten cm away from the pin. Possible staining now became visible at the cutting edges. Another cut was made five cm away from the pin at both ends and examined for discoloration. The last cut was made in 2.5 cm distance in each direction from the map pin (point of inoculation), so the total length of the shoot piece was five cm. This piece was split in the middle to look for any disease development in the wood around the inoculation point. Staining going out from the pin was measured.

Symptomatic areas of shoots were isolated on 26 and 27 October 2020. The method for isolation from symptomatic plant tissue can be found in chapter 3.1.5 Mycelium was not transferred to regular PDA.

3.4 Data analysis

All statistical analyses were performed with SAS University Edition (SAS Institute, Cary, NC, USA). Data were analyzed with the Generalized Linear Model (GLM), and the Student-Newman-Keuls method separated mean values at P = 0.05. Letters are used to show significant differences in figures and tables. Means with the same letter are not significantly different from each other. The percentage of trees with cankers was calculated per replicate. Canker incidence per tree (cankers developed/wounds inoculated) was calculated and arcsin-square root transformed before statistical analysis. Numbers presented in figures and tables are re-transformed. Incidence of inoculated wounds containing sporodochia and perithecia were calculated per tree [(sporodochia or perithecia/inoculated wounds) *100]. Trees without cankers were excluded. The experiment with trees planted in 2018 was not fully replicated because young trees died both in the nursery and in the orchard

The area under the disease progress curve (AUDPC) was calculated per replicate in Microsoft® Excel for Mac 2016 (Version 16.48). Data was analyzed with the GLM and mean values were separated at P = 0.05 with the Student-Newman-Keuls method. All diagrams were created with Excel.

In the wound type experiment one canker on each cvs. Red Elstar and Rubinstep tree were overlooked during the field assessments in 2019 and 2020, most likely due to the foliage in the summer months. The cankers were discovered at the final assessment in autumn 2020. Since it is not possible to reconstruct when the cankers first appeared, those cankers were not included in the results describing the analysis of the field canker development.

Cankers in the field were overlooked in the nursery experiment as well. In total nine cankers on cvs. Elstar, Red Aroma, Red Gravenstein, Rubinstep and Summerred were overlooked. They were discovered at the final assessment in autumn 2020. The apple trees with those cankers were excluded in the results describing the canker development but included in the results for the final assessment.

The mean lesion length and inner staining in the green shoot experiment was calculated per cultivar and potential differences and data was analyzed with the GLM and mean values were separated at P = 0.05 with the Student-Newman-Keuls method as well.

4. Results

4.1 Climatic conditions

Trees planted for the nursery experiment in 2018 were headed back and inoculated with *N. ditissima* in the morning of 4 April 2019. At 11:00 that day the recorded temperature was 6.7°C, and air humidity was 76%. The precipitation measured on the day of inoculation was 0.3 mm. This was the first inoculation done in 2019. The average temperature on 4 April 2019 was 8.7°C (Fig. 4). No rain was recorded one day before and 24 days after inoculation (Fig. 4).

Both bent and cut branches of the wound type experiment were inoculated on the morning of 13 May 2019. The average temperature of 8.3°C on that day was slightly lower than the average temperature on the 4 April (Fig. 4). On 13 May 2019 at 11:00, the weather station in Lofthus, Ullensvang, measured 8.7°C, an air humidity of 52% and precipitation of 0.4 mm. The next rain event was nine days after inoculation, on 23 May 2019. Compared to the measured temperatures on the morning of the inoculation of the headed back trees planted in 2018 the temperature at 11:00 in the morning was 2°C higher.

The branches of trees planted for the nursery experiment in 2018 were bent and inoculated in the morning of 28 June 2019. The average temperature of 16.6°C was much higher compared to the two other inoculation days. At 11:00, the temperature was 16.5°C, and the daily mean temperature was 16.6°C. No precipitation was measured that day (Fig. 4) and the air humidity was 57%. Precipitation was measured two days after inoculation.

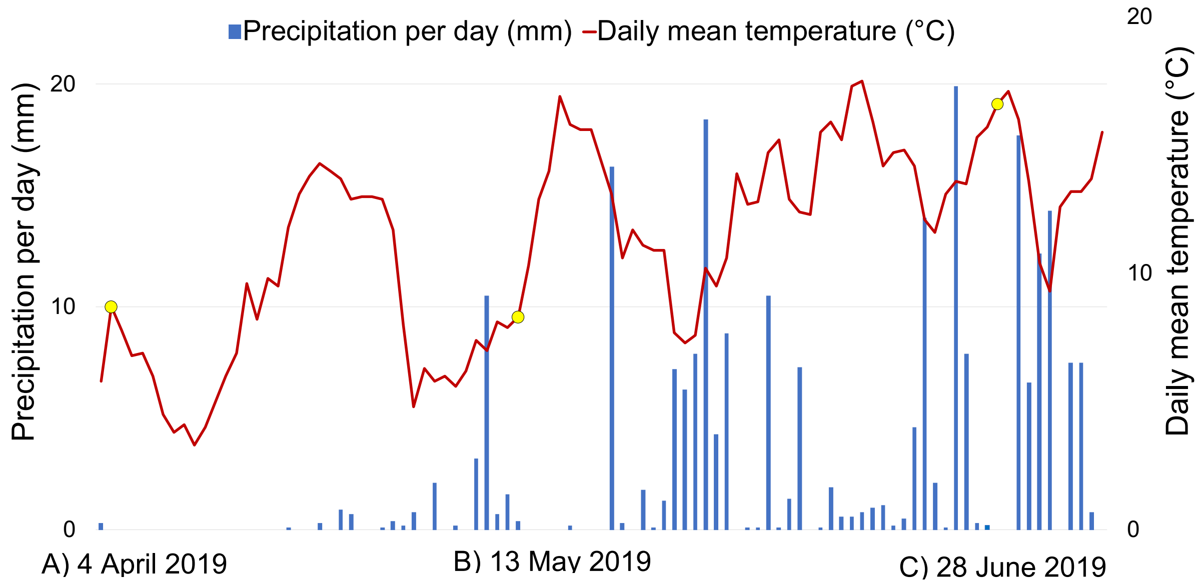

Fig. 4.: The average temperature (°C) and precipitation (mm) from 3 April to 8 July 2019 recorded at NIBIO Ullensvang. Trees were inoculated with *Neonectria ditissima* (yellow circle) after: A) Heading back in the nursery experiment B) Bending and cutting off branches and heading back in the wound type experiment, and C) Bending branches in the nursery experiment.

The green shoots of trees planted in 2020 were inoculated in the morning of 1 July 2020. At 11:00 on the day of inoculation, the temperature was 15.6°C, and the measured air humidity was 46%. In the afternoon, the temperature decreased to 14°C at 16:00. It started raining in the evening and the total precipitation was 9.9 mm (Fig. 5). The average temperature on the day of inoculation was 15.3°C.

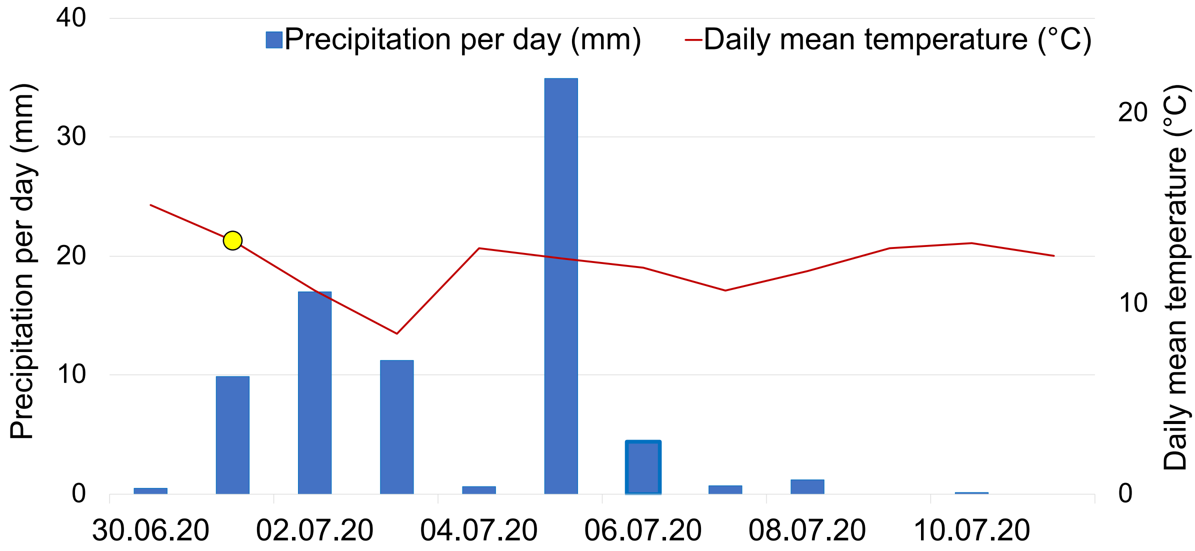

Fig. 5.: The average temperature (°C) and precipitation (mm) the day before inoculation with *Neonectria ditissima* on green shoots of newly planted apple trees, the day of inoculation (yellow circle) and ten following days recorded at NIBIO Ullensvang.

4.2 Symptoms of *Neonectria ditissima*

In all wood inoculation experiments, the observed European canker symptoms were discoloration of the bark, swelling at the inoculation site, and girdling. Discolored bark around the wound was usually the first visible symptom of *N. ditissima* (Fig. 6A,B). On some canker wounds the epidermis became blistery, and the bark was easy to peel off (Fig. 6A,B). On cuts, typical discoloration appeared (Fig. 6B). Swelling around the inoculation site was observed with progression of the disease development (Fig. 6C,D). Deep, sunken areas at the inoculation site (Fig. 6E) and girdling on bent branches (Fig. 6F) were observed after 16 and 18.5 months, respectively.

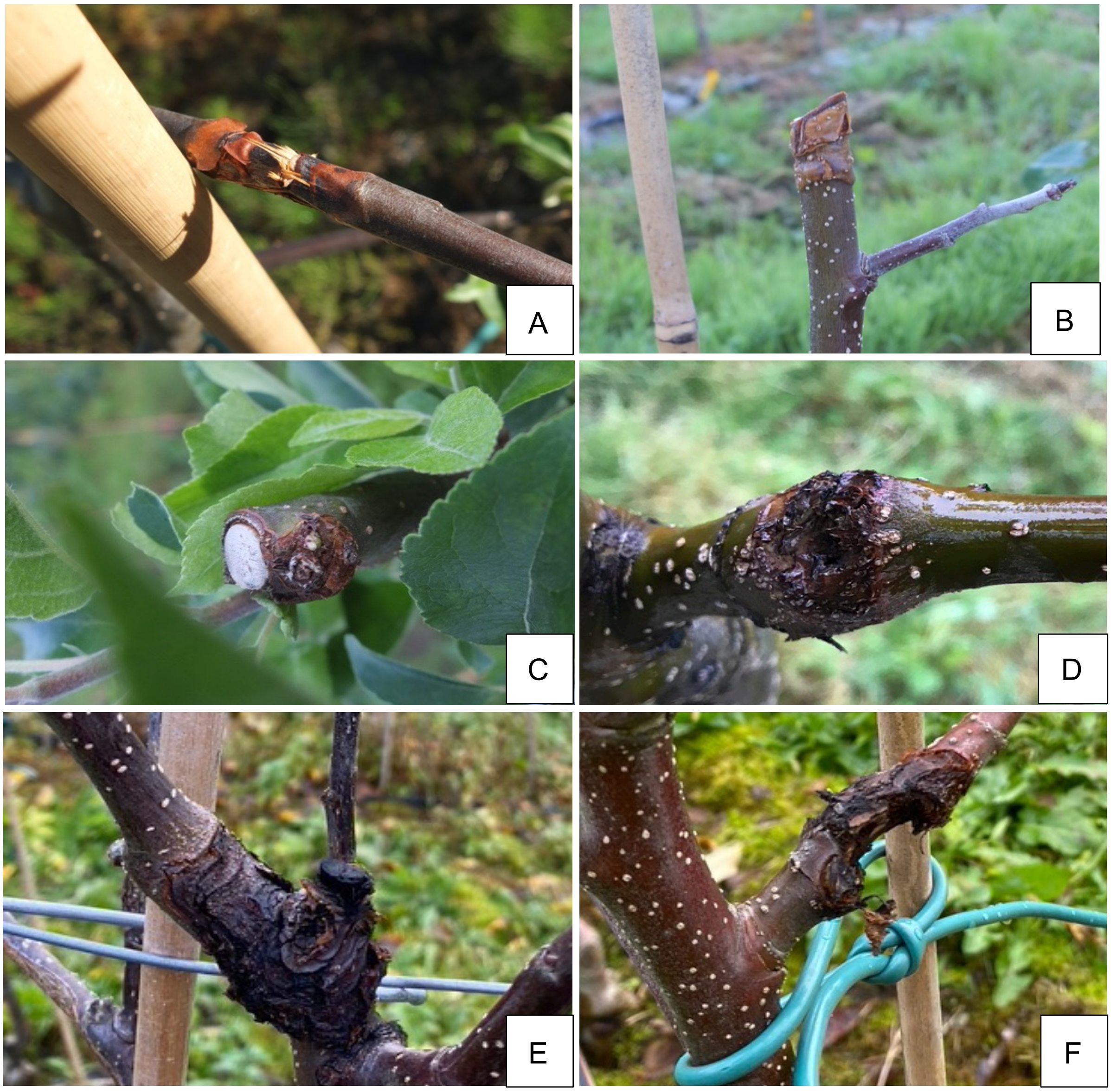

Fig. 6.: Trees of apple cultivars inoculated with *Neonectria ditissima*. (A) A bent and inoculated bent branch on a cv. Red Gravenstein tree, one month after inoculation (B) Headed back cv. Rubinstep tree, two months after inoculation (C) An inoculated cut on a cv. Discovery tree two months after inoculation, the bark next to the cut is discolored and swollen. (D) A bent and inoculated cv. Discovery branch 13 months after inoculation. Swelling around the inoculation site was visible. (E) A headed back cv. Summerred tree 18 months after inoculation, swelling and dark brown concentric rings are visible. (F) Deep sunken area in the inoculation site of a cv. Rubinstep tree. The branches were bent and inoculated in May 2019 and photographed 16 months afterwards. Photos A-C: Jorunn Børve, D-F: Theresa Weigl

4.3 Wound type experiment

4.3.1 Method comparison

Incidence of trees with canker

Inoculation of both bent branches and cut surfaces resulted in higher number of trees with canker after 18 months than on the non-inoculated control trees (P = 0.0001). In contrast, the number of trees with canker of inoculated but unwounded trees and their corresponding control trees were identical (P = 1.0). No further statistical analysis was carried out for the non-wounded inoculation method because only one cv. Summerred tree developed canker.

Inoculation methods resulted in significantly different numbers of cankered trees after 18 months (P = 0.0002, Fig. 7).

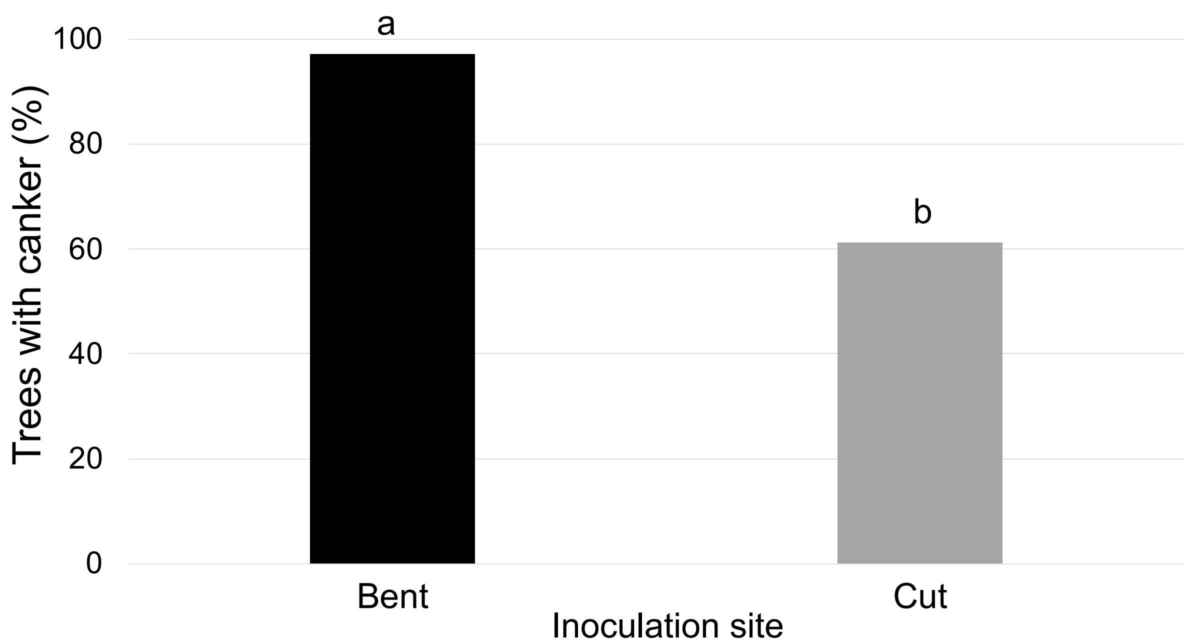

Fig. 7.: Mean percentage of trees that had developed cankers on six apple cultivars by November 2020. Trees were wounded by bending side branches (Bent) or by cutting tops and side branches (Cut) at time of inoculation with *Neonectria ditissima* in May 2019. Different letters above the bars indicate significant differences according to Student Newman Keuls method at P = 0.05. Means of three replicates per cultivar and inoculation method.

Incidence of cankers per wound

Analyzing the incidence of cankers per wound showed that significantly more bent branches developed canker than cut surfaces (P = 0.0001). In total, 33.2% of wounds caused by bending branches and subsequent inoculation developed canker after 18 months. Inoculation of cut surfaces resulted in canker on 19.1% of the wounds.

4.3.2 Cultivar differences in the final assessment

Incidence of trees with canker

In mean of bent and cut inoculation methods, no cultivar differences were found in the incidence of trees with cankers (%) (P = 0.0771). Neither were any cultivar differences found when analyzing the number of trees developing cankers inoculated after either the cut or bent treatments separately (P = 0.1481 and P = 0.4651, respectively).

Incidence of cankers per wound

When combining the bent and cut treatment methods, significant differences in canker incidence were found between the six cvs (P = 0.0021). Cv. Red Gravenstein (37.9%) and Discovery (36.3%) had significantly more cankers on the cut and bent wounds than cvs. Rubinstep (18.0%), Red Aroma (17.4%) and Red Elstar (16.4%). Cv. Summerred (29.7%) was not significantly different from the other five cultivars (Fig. 8).

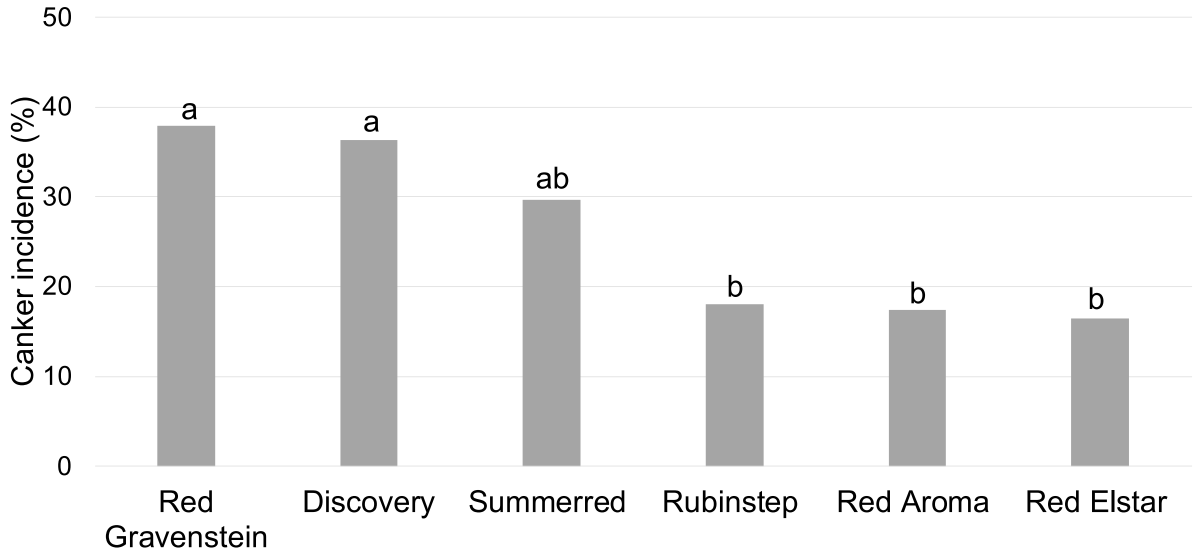

Fig. 8.: Mean canker incidence (%) per apple cultivar in the final assessment in November 2020. Branches of trees were either bent or cut and headed back at the time of inoculation with *Neonectria ditissima* in May 2019. Trees were inoculated on four to 14 wounds per tree. Canker incidence was calculated per tree across the bent and cut inoculation methods. Bars with different letters indicate a significant difference according to Student Newman Keuls method at P = 0.05.

The statistical analysis did not show a significant difference in canker incidence between the six cvs. inoculated after bending branches alone (P = 0.6879). However, significant differences were found between the cultivars when analyzing the cut wounds separately (P = 0.0085, Fig. 9). Cv. Red Gravenstein had a higher canker incidence (38.8%), than cvs. Red Aroma (6.8%), Red Elstar (6.7%) and Rubinstep (5.7%). Cvs. Discovery (29.6%) and Summerred (28.9%, Fig. 9) were not significantly different from the other cultivars.

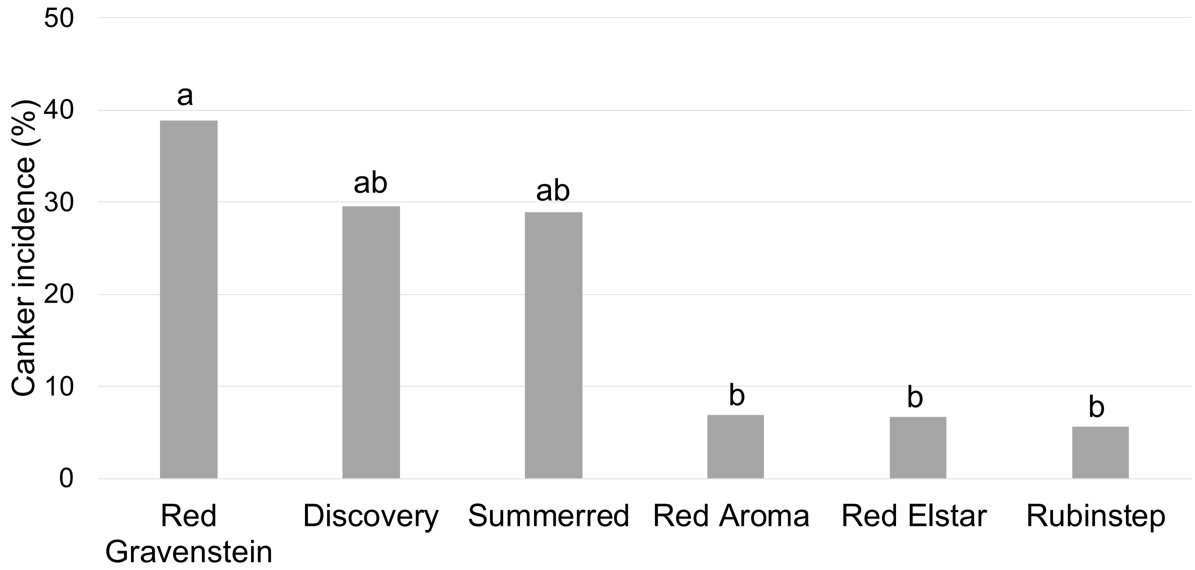

Fig. 9.: Mean canker incidence (%) in the final assessment in November 2020 per cultivar for cut surfaces. Trees were headed back and branches were cut off at the time of inoculation with *Neonectria ditissima* in May 2019. Trees were inoculated on four to 14 wounds per tree. In the final assessment, 18 months later, cut branches and tops with canker symptoms were counted per tree, and canker incidence per tree was calculated. Bars with different letters indicate a significant difference according to Student Newman Keuls method at P = 0.05.

4.3.3 Canker development in the field

None of the trees developed cankers from the last field assessment, on 2 October 2020, until the final assessment in November 2020. All cultivars developed cankers in the first 4.5 months after inoculation of bent branches in May 2019. No cankers developed in the following dormancy period. Only the canker incidence of cvs. Red Elstar and Summerred increased during the season 2020 (Fig. 10). Only 3.5% of the total cankers developed in 2020, the second season after inoculation with *N. ditissima.* The calculated area under the disease progress curves were not significantly different from each other (P = 0.7776, Table 2).

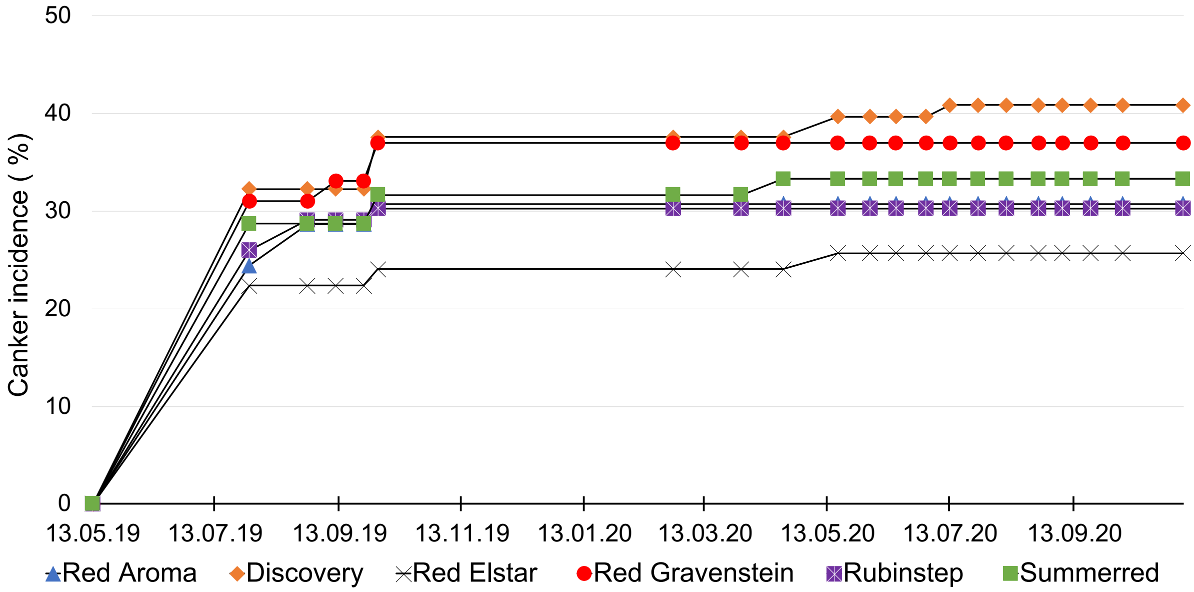

Fig. 10.: Development of canker incidence (%) of six apple cultivars over an 18-month period. Branches were bent and the resulting wounds were inoculated with *Neonectria ditissima* in May 2019. Numbers presented are means of three replicates per cultivar. One replicate consisted of two trees. Trees were inoculated on four to 14 wounds per tree.

Figure 11 illustrates the average canker incidence of the proportion of inoculated cut branches and headed back wounds that developed canker symptoms per tree. Cv. Red Gravenstein and cv. Discovery had the most rapid disease development and with an AUDPC value significantly higher than cvs. Red Aroma, Red Elstar and Rubinstep (Table 2). After the first assessment in July 2019, cv. Red Aroma did not develop new cankers during 2019 and 2020 (Fig. 11). Cv. Red Elstar had the lowest canker incidence 2.5 months after inoculation, but during 2020 the incidence more than doubled from 3% to 6.7%. A similar development was seen for cv. Summerred. Canker incidence was 10.7% at the first assessment but increased almost threefold until the last assessment in 2020, to 28.8% (Fig. 11). The total proportion of cankers that developed in the second season was 20.4%.

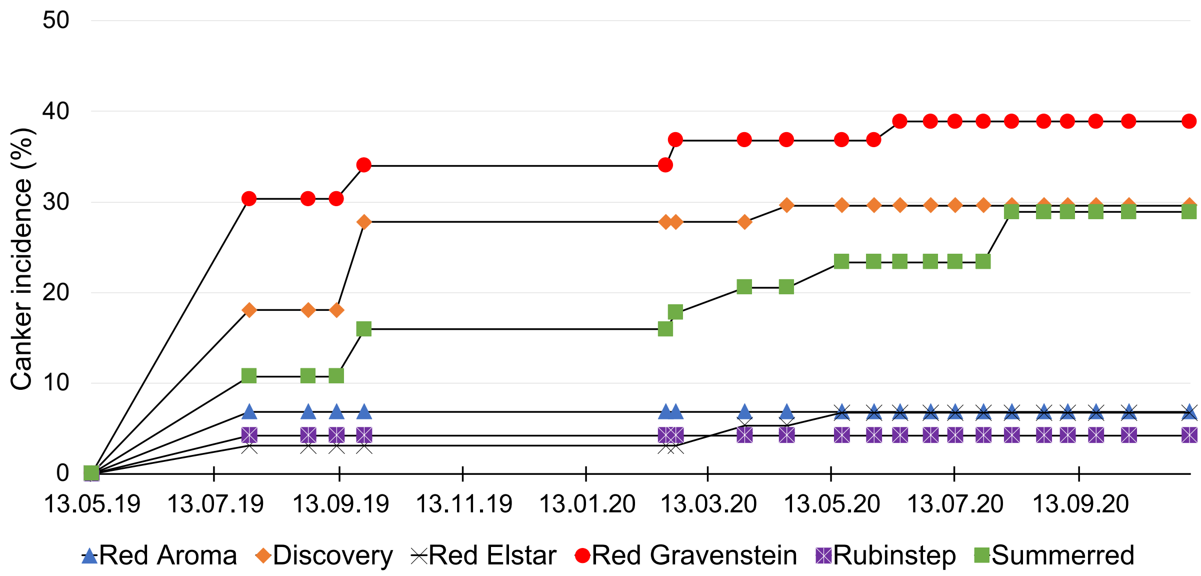

Fig. 11.: Development of canker incidence in 18 months from the inoculation of six apple cultivars. Trees were both headed back, and side branches were cut before inoculation with *Neonectria ditissima* in May 2019. Numbers presented are means of three replicates per cultivar. One replicate consisted of two trees. Trees were inoculated on four to 14 wounds per tree.

Table 2.: Area under the disease progress curve (AUDPC)[a] calculated per replicate for six apple cultivars over an 18-month period, cut surfaces or bent branches were inoculated with *Neonectria ditissima* in May 2019.

	AUDPC	
Cultivar	**Cut surfaces**	**Bent branches**
Red Gravenstein	180.0 a[b]	180.8 a
Discovery	135.9 ab	189.1 a
Summerred	99.7 bc	159.5 a
Red Aroma	34.2 c	152.0 a
Red Elstar	23.7 c	122.1 a
Rubinstep	21.1 c	149.3 a
P-value	0.0006	0.7776

[a]Mean of incidence values of three replicates per cultivar each including two trees.
[b]Different letters behind the values are significantly different according to Student Newman Keuls test at P= 0.05.

4.3.4 Wood staining

No significant differences in staining incidence were found between the cut and bent inoculation methods across all cultivars (P = 0.0772). In 20% of the cankers caused by bent branches and inoculation, staining was visible in at least one direction and in only 7.9% of the cankers, resulted from cut surfaces and subsequent inoculation, staining was found (Fig. 12).

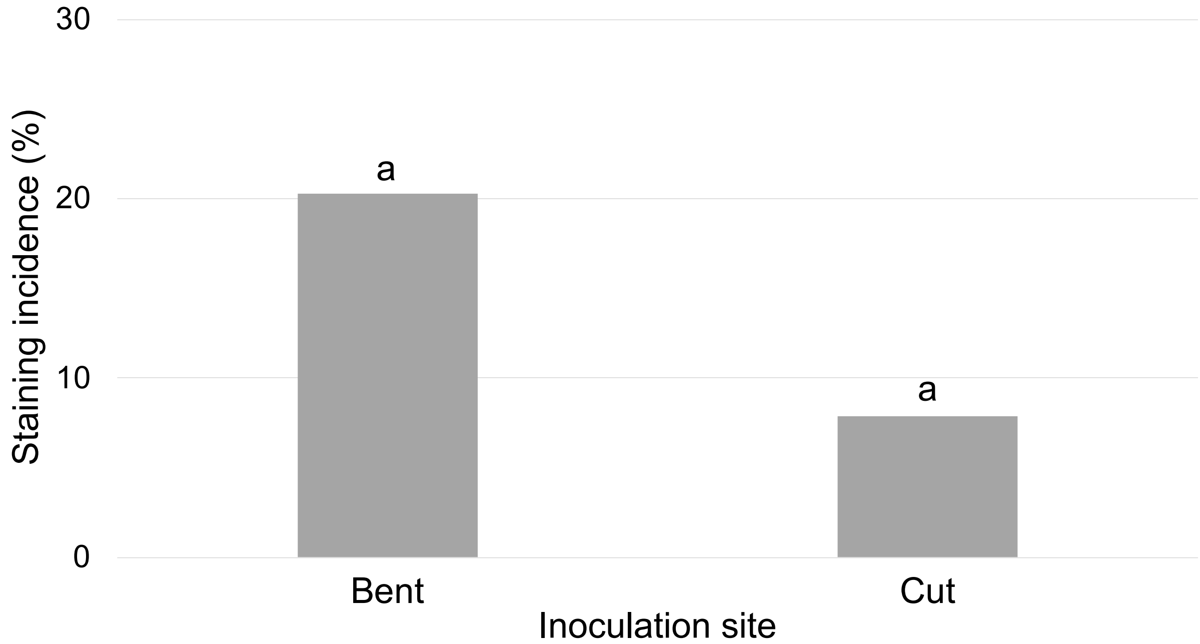

Fig. 12.: Staining incidence (%) in the cut and bent inoculation methods across all apple cultivars. Trees were either wounded by bending side branches or by heading back and cutting off side branches at the time of inoculation in May 2019. Staining per canker was measured in the final assessment, 18 months after inoculation, both underneath and above the canker. Staining incidence was calculated per tree. Means of three replicates per cultivar. One replicate consisted of two trees. Bars with different letters indicate a significant difference according to Student Newman Keul method at P = 0.05.

Average staining starting in cankered wounds caused by bent branches was 14.1 cm underneath the canker and 7.5 cm above the canker (Fig. 13). Staining up to 50 cm underneath one canker was observed in a cv. Red Aroma tree.

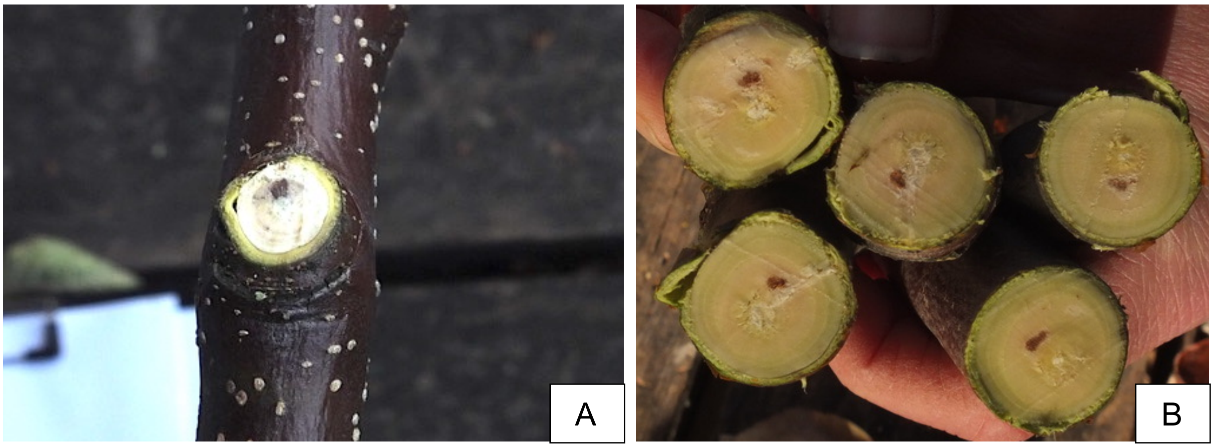

Fig. 13: (A) Visible staining going into the main stem, in a cv. Red Gravenstein tree. The canker was caused by bending a side branch and subsequent inoculation with *Neonectria ditissima* in May 2019. (B) Staining in the main stem below the same canker of a cv. Red Gravenstein tree (see A). The picture shows staining (total 36 cm) in different parts of the main stem and was taken 18 months after inoculation. Photos: Jorunn Børve

On average, staining was 37.8 cm underneath and 4.5 cm above the canker. One branch growing shortly below a cankered top of a cv. Red Elstar tree had staining of 67 cm in the branch. No significant cultivar differences were found across the cut and bent inoculation methods when analyzed together (P = 0.8223).

4.3.5 Cankers beyond the inoculation site

Additional cankers which were not related to the inoculation point were observed in the final assessment. Overall, two cankers, most likely caused by chafing from the wire, which supports the trees, were found on cvs. Red Gravenstein trees. Around the grafting region, two cankers on cv. Summerred trees and one canker on a cv. Red Gravenstein tree was found. Only three trees in all control groups had main stem cankers.

Rootstock cankers

Apple rootstocks were not inoculated with *N. ditissima* but in the final assessment many trees with rootstock cankers were observed. Therefore, the author decided to analyze this unexpected result. Trees that were wounded by bending branches and inoculated with *N. ditissima* were significantly different from the corresponding control trees (P = 0.0002). In the mean of all cultivars, half of the trees inoculated after bending the branches had rootstock cankers. In comparison, only 5.6% trees in the corresponding non-inoculated control group had rootstock cankers (Fig. 14). A similar observation was made for trees with cut and inoculated surfaces (P = 0.0104). One-quarter of trees with surface cut inoculated with *N. ditissima* had rootstock cankers. The corresponding control group had significantly fewer cankers (2.8%, Fig. 14). No significant difference in cankered rootstocks was found between the non-wounded trees and their corresponding control group (P = 0.1779).

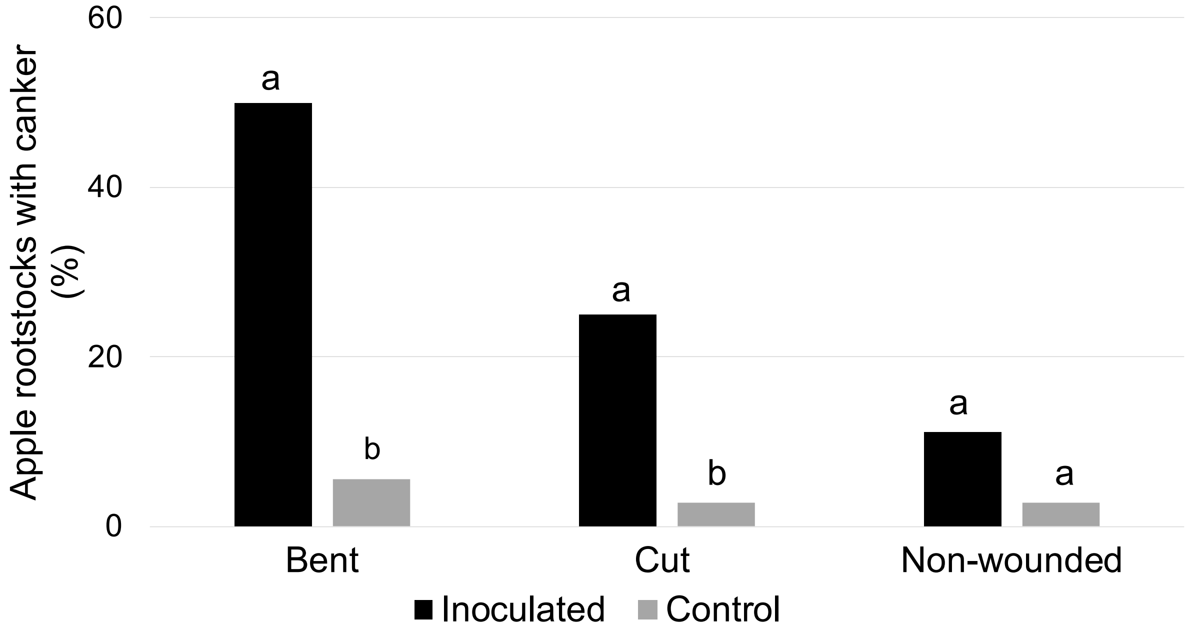

Fig. 14.: Rootstock cankers (%) on apple trees inoculated with *Neonectria ditissima* or water in wounds by bending side branches (Bent) or heading back and cutting side branches (Cut), or remained non-wounded (Non-wounded), assessed after 18 months. Mean of three replicates with two trees per cultivar and inoculation methods. Different letters above the bars indicate significant differences according to Student Newman Keuls method at P = 0.05.

In the mean of the three inoculation methods significant cultivar differences were found (P = 0.0479). In total, 50% of the cv. Red Gravenstein trees had rootstock cankers. Both cvs. Discovery and Summerred trees were found with 38.6% rootstock cankers. Cvs. Red Aroma and Red Elstar had both 16.7% trees with rootstock cankers and cv. Rubinstep had 11.1% rootstock cankers. Student-Newman Keuls method (P = 0.05) did not show significant differences between any of the cultivars.

4.3.6 Incidence of sporodochia and perithecia

Cankers that were a result of wounding and subsequent inoculation, and other cankers found on trees, were examined for sporodochia and perithecia in November 2020 (Fig. 15). Sporodochia on cankers were observed with the naked eye and the help of a stereomicroscope (Fig. 15 A,B). On all cankers found on rootstocks, red perithecia were present. On cankers caused by wounding and inoculation with *N. ditissima,* the majority of perithecia found were red (Fig. 15C). On only six cankers orange perithecia, the early stage, were found (Fig. 15D). In total four cankers had brown and black degenerated perithecia.

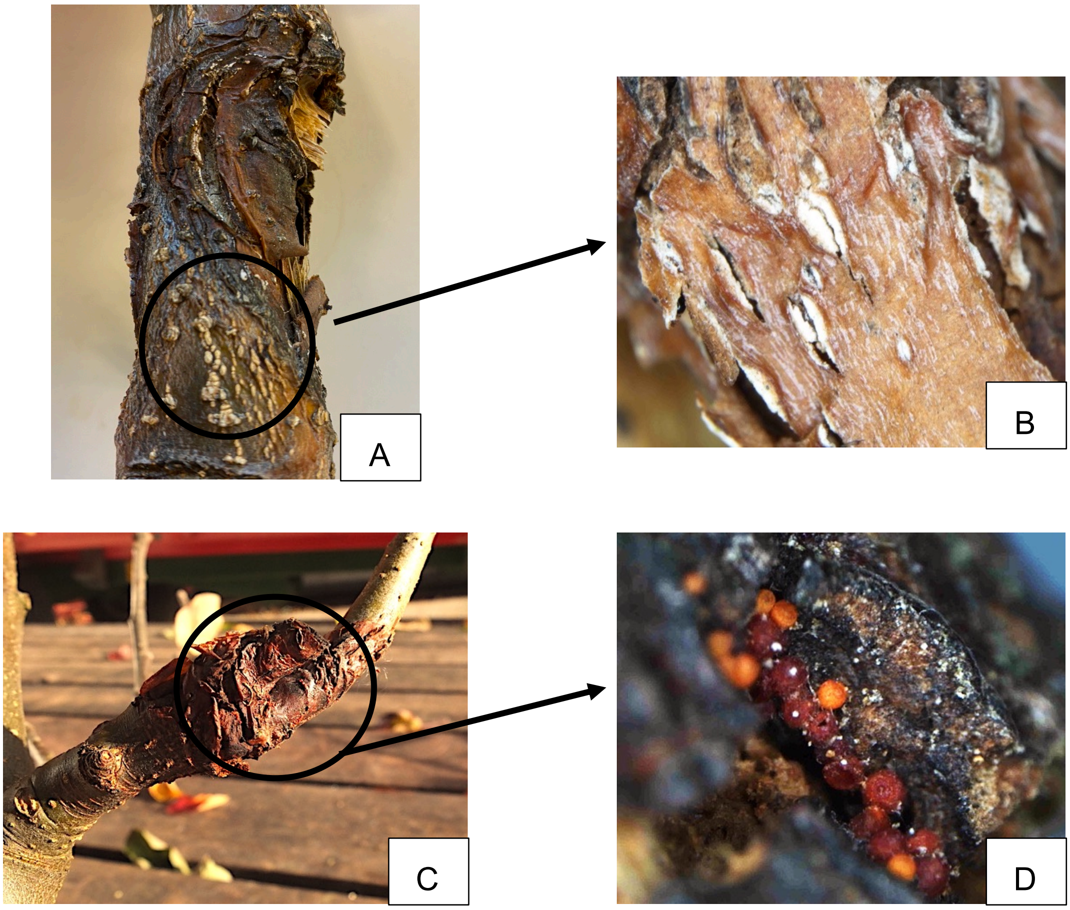

Fig. 15: (A) A detached canker from a cv. Red Elstar tree with visible sporodochia caused by bending branches and subsequent inoculation with *N. ditissima*. (B). The picture was taken with the stereomicroscope, and sporodochia in a higher magnification can be seen. (C) Canker of a cv. Discovery tree. The canker was caused by cutting off a side branch and subsequent inoculation with *Neonectria ditissima*. Red perithecia can be seen with the naked eye. (D) The picture on the right was taken with a stereomicroscope and both orange and red perithecia with white cirrus are visible. Photos A,B,D: Theresa Weigl, C: Jorunn Børve

A significant difference in incidence of sporodochia was found between the bent (51.6%) and cut (25.0%) inoculation methods (P = 0.0066, Fig. 16). Perithecia were found on 39.0% and 42.1% of the cankers that developed after the bent and cut treatments, respectively. There was no significant difference in incidence of perithecia between the two inoculation methods (P = 0.7151, Fig. 16).

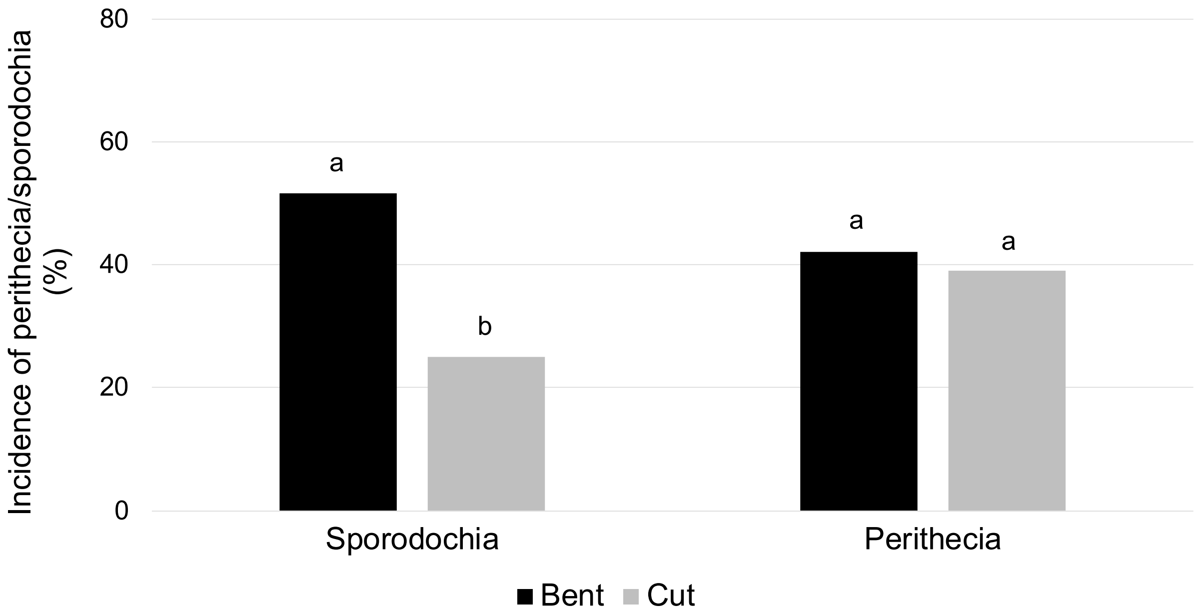

Fig.16.: Incidence (%) of cankers with sporodochia and/or perithecia on wounds caused by bending down branches (bent) and by cutting off side branches and heading back (cut) after 18 months. Means of three replicates and two trees per replicate. Bars with different letters indicate a significant difference according to Student Newman Keuls method at P = 0.05 per spore type.

No significant cultivar differences were found in sporodochia incidence when analyzing the bent and cut inoculation methods together (P = 0.2426). Furthermore, no significant cultivar differences in sporodochia incidence were found when analyzing cankers developed on either bent branches and cut surfaces (P = 0.1493 and P = 0.6395, respectively).

Significant cultivar differences in the incidence of perithecia were found when analyzing the bent and cut inoculation methods together (P = 0.0001, Fig.17). Cvs. Discovery, Summerred and Red Gravenstein were significantly different from cvs. Red Elstar and Red Aroma (Fig. 17).

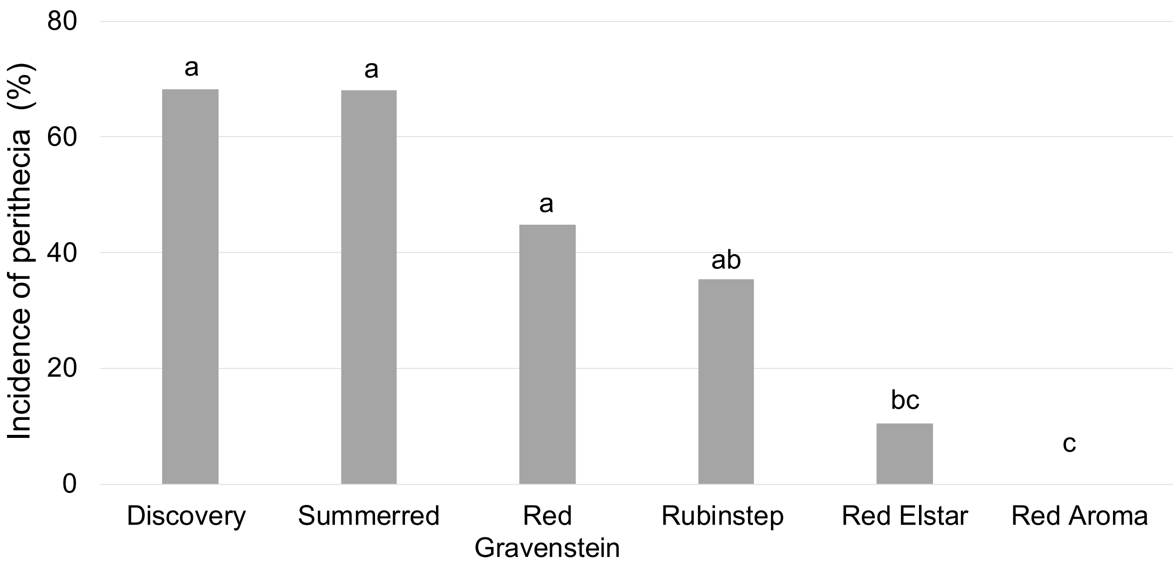

Fig.17.: Mean incidence (%) of cankers with perithecia in six cultivars across both inoculation methods (bent and cut). Trees were wounded by bending side branches or by heading back and cutting off side branches at the time of inoculation in May 2019. Cankers with perithecia and sporodochia were counted per tree and incidence per tree was calculated. Bars with different letters indicate a significant difference according to Student Newman Keuls method at P = 0.05. Means of three replicates and two trees per replicate.

When analyzing the bent branch treatments separately, significant cultivar differences in incidence of perithecia (P = 0.0010). Cv. Discovery had the highest incidence of perithecia (79.4%). This incidence of perithecia was significantly higher for cvs. Rubinstep (30.6%), Red Elstar (17.5%), and Red Aroma (0%). Cv. Summerred (71.7%) was the cultivar with the second-highest incidence of perithecia and was significantly different from cvs. Red Aroma and Red Elstar. Cv. Red Gravenstein (43.3%) was not significantly different from any other cultivar. The third lowest perithecia incidence was found in cv. Rubinstep (30.6%). The latter cultivar had a significantly lower perithecia incidence than cv. Discovery. No significant cultivar differences in perithecia incidence were found when analyzing cankers developed on cut surfaces separately (P = 0.1244).

4.3.7 Real-time PCR

For the asymptomatic wood samples taken 4-5 cm away from the canker wounds, the mean Cq values for *N. ditissima* real time PCR differed from 31.6 (cv. Rubinstep) to 35 (cv. Red Elstar, Fig. 18A). In both samples taken from a cankered wound of the cvs. Red Gravenstein and Summered no signal for the 18S amplification control was detected (Fig. 18B).

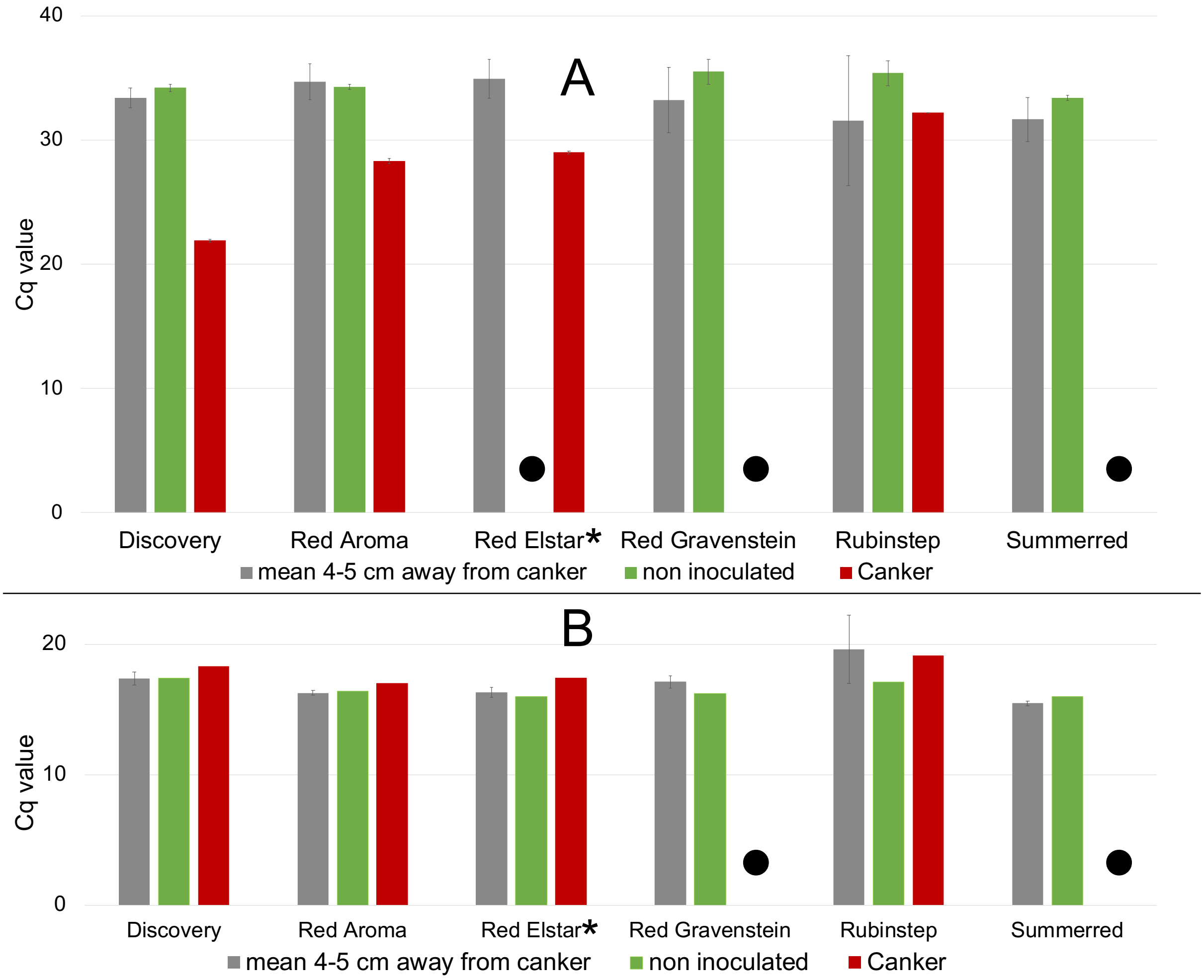

Fig. 18.: Cq real-time PCR values for wood of six apple cultivars. Asymptomatic wood samples (grey), wood samples from non-inoculated trees (green) and wood samples from a canker showing clear symptoms of *Neonectria ditissima* (red).
A) Cq values for *Neonectria ditissima* real-time PCR. Values for the canker and non-inoculated tissue sample are means of technical duplicates and samples taken four to five cm away from the canker are means of duplicates of four different trees.
B) Cq values for the amplification control (18SRNA) Real-time PCR. No technical duplicates were utilized. Samples taken four to five cm away from the canker are means of four different trees.

● Not detected

* Only three samples from trees were taken from cv. Red Elstar because not enough cankers without staining were found in the final assessment

4.4 Nursery experiment with bent branches

In mean of all cultivars and propagation methods, 27.9% of trees inoculated after bending branches developed canker. This was significantly different from the non-inoculated control trees where no canker developed after 16 months (P = 0.001).

There was no difference in canker development between propagation methods (P = 0.3299), 16 months after inoculation of the trees. Of the T-budded trees, 31.6% developed canker and similar numbers for trees grafted on cv. Golden Delicious interstem (24.0%) and on M9 (23.4%) had canker. No significant difference in canker development among the six cultivars was found (P = 0.4036). The percentage of trees with canker varied from 14.6% in cv. Discovery to 41.1% in cv. Summerred (Fig. 19).

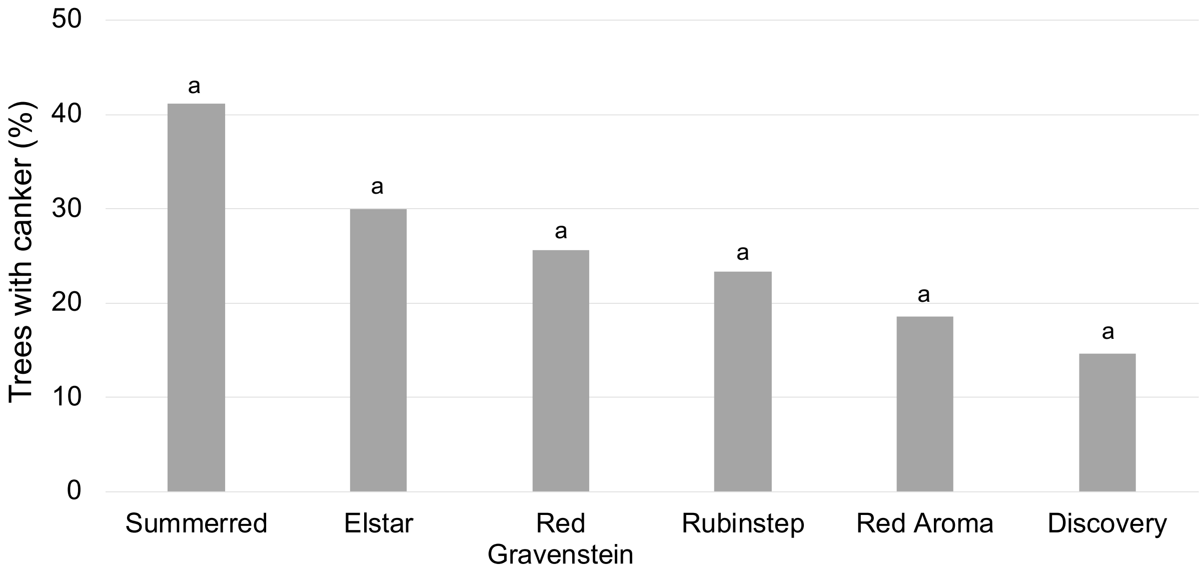

Fig.19.: Trees with canker (%) inoculated with *Neonectria ditissima* after bending the branches inoculated with *Neonectria ditissima* and assessed after 16 months. Mean of five replicates of two or three trees and three propagation methods. Bars with different letters indicate a significant difference according to Student Newman Keuls method at P = 0.05.

Canker incidence per wound

In mean of six cultivars, there were no significant differences in wounds with canker (%) among cultivars 16 months after inoculation (P = 0.0661). The canker incidence for all cultivars grafted on cv. Golden Delicious interstem a with M9 rootstock was 10.8% and for T-budded cultivars the incidence was 10.1%. Cultivars grafted directly on M9 had a canker incidence of 6.7%. In mean of the three propagation methods, no significant difference between cultivars were found (Fig. 20, P = 0.3664).

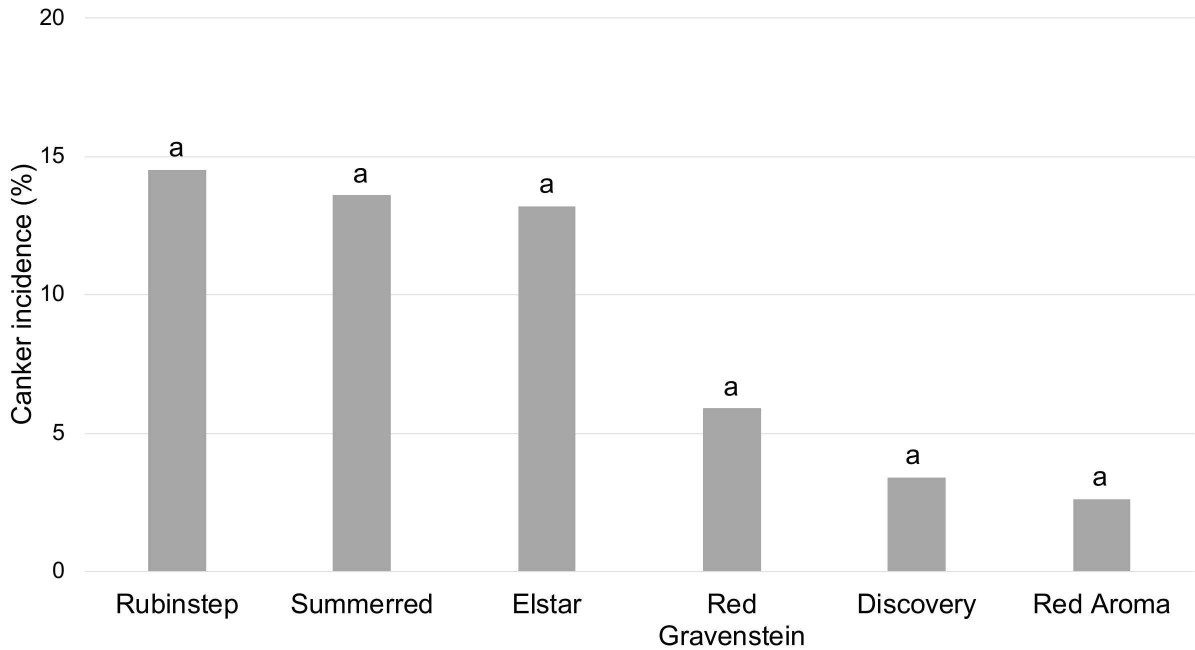

Fig. 20.: Canker incidence (%) in wounds on bended branches inoculated with *Neonectria ditissima* and assessed after 16 months. Trees were inoculated on one to nine wounds per tree. Mean of three propagation methods and five replicates per cultivar with two or three trees in each replicate were calculated. Bars with different letters indicate a significant difference according to Student Newman Keuls method at P = 0.05.

Canker development in the field

Cankers developed in the season of 2019, but no new cankers developed from October 2019 until March 2020 (Fig. 21). In the season of 2020, five out of six cvs. developed new cankers. Across all cultivars, 45.5% of the trees developed canker in the season of 2020. In total, two cankers on cv. Red Aroma trees were overlooked in the field assessments. No significant difference in canker development between the different cultivars, expressed as AUDPC, was found (P = 0.3695, Table 3).

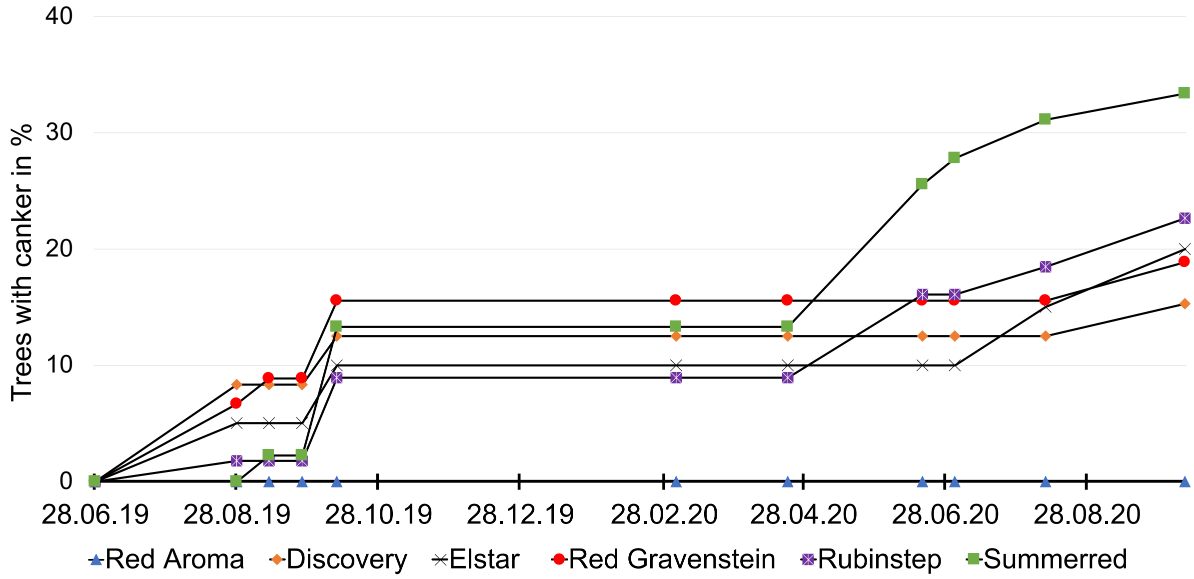

Fig. 21: Development of cankers after inoculation with *Neonectria ditissima* in wounds after bending branches in June 2019. Mean of three propagation methods and five replicates per cultivar with two or three trees in each replicate were calculated.

Table 3.: Area under the disease progress curve (AUDPC)[a] calculated per replicate for the six apple cultivars over a 16 to 18.5 months period. Bent branches and heading back wounds were inoculated with *Neonectria ditissima*.

	AUDPC	
Cultivar	**Bent branches**	**Heading back cut**
Summerred	117.7 a[b]	43.4 a
Red Gravenstein	76.2 a	49.3 a
Elstar	67.9 a	48.8 a
Discovery	62.0 a	44.4 a
Rubinstep	55.2 a	11.1 a
Red Aroma	0.0 a	0.0 a
P-value	0.3695	0.7776

[a] Mean of five replicates of three propagation methods (grafted on M9, grafted on Golden Delicious interstem, and T-budded on M9). One replicate consisted of two or three trees.
[b]Different letters behind the values are significantly different according to Student Newman Keuls test at P= 0.05.

Wood Staining

In mean of all cultivars and propagation methods, 26 cankers (43.6%) had staining starting from the canker on the bent branches. On average, the staining underneath the cankers in was 6.9 cm and 5 cm above the cankers. Staining going out in branches was on average 0.8 cm. The longest staining underneath a canker (42 cm) was found in a cv. Summerred tree, and the longest staining above a canker (74cm) was found in another cv. Summerred tree.

Cankers beyond the inoculation site

Two out of 34 cv. Summerred trees had cankers on the rootstock in the final assessment, and one canker on the top of a cv. Summerred tree inoculated after bending side branches was found in the final assessment.

Incidence of sporodochia and perithecia

In total, four cankers had sporodochia (6.9%), and seven cankers (12.1%) had perithecia 16 months after inoculation. No statistical analysis regarding cultivars and propagation methods was done. The maturity of the perithecia was not assessed.

4.5 Nursery experiment with headed back trees

Trees with canker

Significantly more cankers developed on inoculated headed back trees (7.7%) than on non-inoculated control trees, where no cankers were found after 18.5 months (P = 0.0022). In the mean of all cultivars, there was no significant difference between propagation methods in the final assessment (P = 0.7086). Trees grafted on cv. Golden Delicious interstem had 10.4% cankered trees and trees grafted on M9 had 5% cankered trees. Within the T-budded propagation method, 8.3% trees had canker.
No significant cultivar difference across all propagation methods was observed (P = 0.7383).

Canker development in the field

In the dormancy period, no new cankers developed in any of the cultivars. None of the cv. Red Aroma developed cankers in the inoculation wounds over the whole experiment period (Fig. 22). Trees of cvs. Elstar, Discovery, Red Gravenstein and Rubinstep developed cankers only in the first season. Cv. Summerred developed cankers in both seasons 2019 and 2020. Across all cultivars 8.3% of trees developed canker in the season 2020. No difference between cultivars were observed in mean of the propagation methods (P = 0.7776, Table 3)

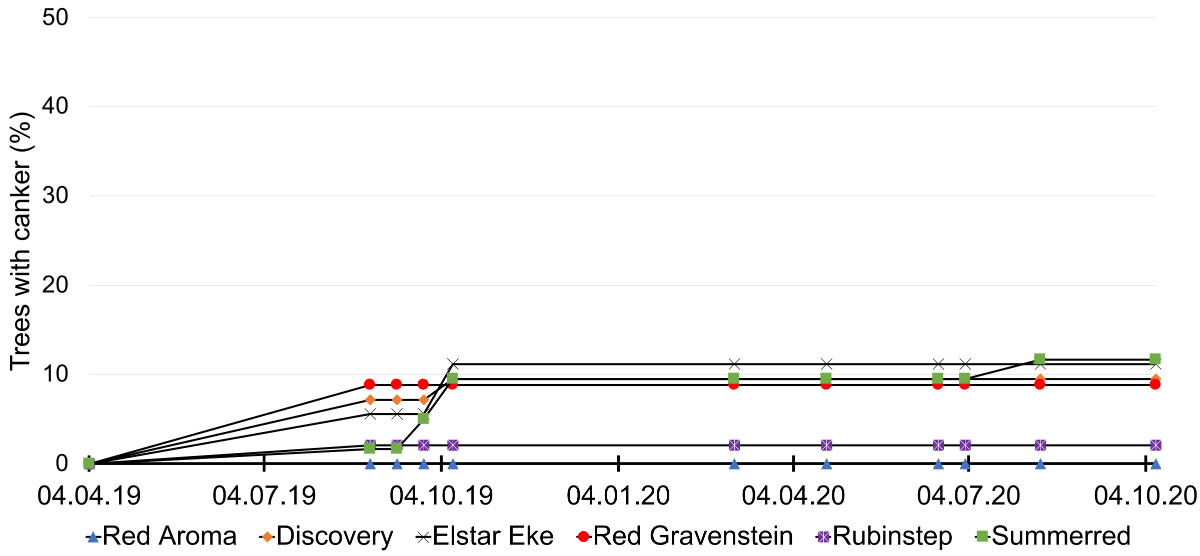

Fig. 22: Development of cankered trees, inoculated with *Neonectria ditissima* after heading back in April 2019. Mean of three propagation methods and five replicates per cultivar with two or three trees in each replicate were calculated.

Wood Staining

Staining from cankers, caused by heading back and inoculation, was observed in seven cankers (58.3%). The average staining in the main stem was 7.4 cm and the longest staining was observed in a cv. Summerred tree (40 cm). One canker on a cv. Discovery tree had a two cm long staining going out in the branch below the headed back wound.

Cankers beyond the inoculation site

In total, one out of 30 cv. Summerred trees had rootstock cankers in the final assessment. On six trees cankers on the main stem were found in addition to the canker on the headed back wound.

Incidence of sporodochia and perithecia

In total, eight cankers (66.7%), developed in headed back wounds, had sporodochia and three cankers (25%) had perithecia at the final assessment in autumn 2020. No statistical analysis regarding cultivars and propagation methods was done. The maturity of the perithecia was not assessed.

4.6 Map pin inoculation of green shoots

No canker symptoms around the map pins were found in the field assessment two weeks after inoculation on 1 July 2020. On 12 August 2020, 17.6% of the inoculated shoots showed canker symptoms. Two weeks later, 58.8% of the shoots had symptomatic infections around the map pin. At the end of the assessment period 77.8% of the shoots were found with symptomatic infections around the map pin. None of the non-inoculated control shoots developed canker infections.

On 29 July 2020, first canker symptoms in cvs. Red Elstar and Discovery became visible. Already two weeks later all inoculated shoots of cv. Summerred showed canker symptoms. All cultivars did not develop new canker symptoms two months after inoculation (Fig. 23). No significant difference in canker development between the different cultivars, expressed as AUDPC, was found (P=0.9707, Fig. 23).

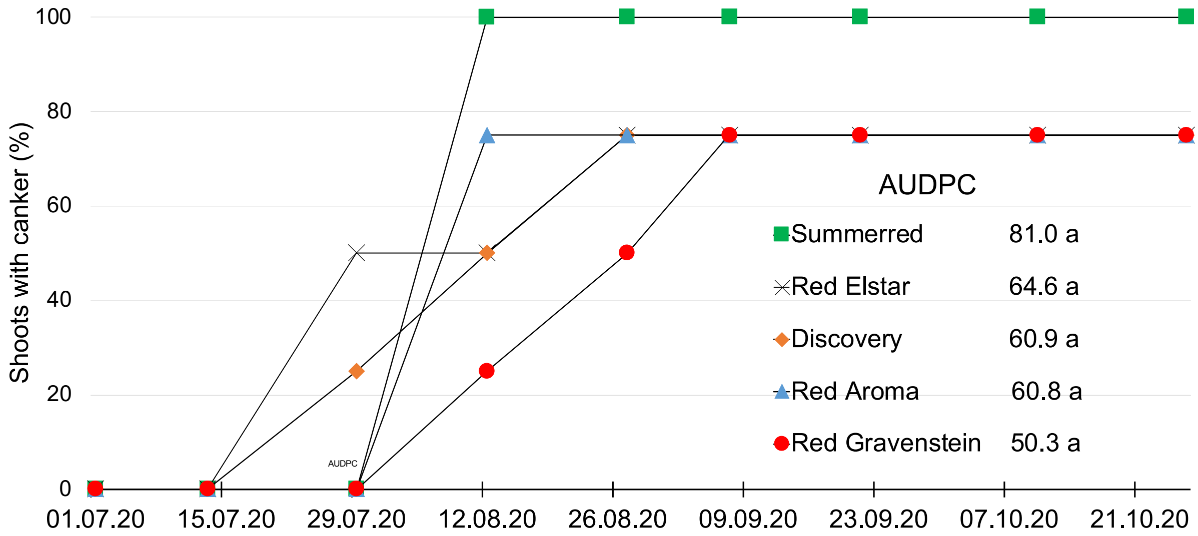

Fig. 23.: Development of canker incidence in four months from the inoculation of five apple cultivars. Green shoots were inoculated with map pins, containing *Neonectria ditissima* in July 2020. Mean of four (two for cv. Summerred) replicates (one shoot per replicate) per replicate. Area under the disease progress curve (AUDPC) calculated per replicate for five apple cultivars.

[a] Different letters behind the values are significantly different according to Student Newman Keuls test at P= 0.05.

In the final assessment, the average length of the outer lesion was 18.6 mm, and the length of the staining inside was 24.1 mm. On average, the inner staining was 5.5 mm longer compared to the outer lesion, but no significant difference was found between the length of the outer lesion and the staining inside (P = 0.4772). No significant cultivar differences between neither the inner staining (P = 0.7680, Fig. 23) nor the outer (P = 0.6889, Fig. 23) lesion were found. The average outer lesion length varied from 8.3 mm (cv. Red Aroma) to 30.5 mm (cv. Summerred). The average length of the inner staining differed from 15.6 mm (cv. Red Gravenstein) to 38.7 mm (cv. Red Elstar).

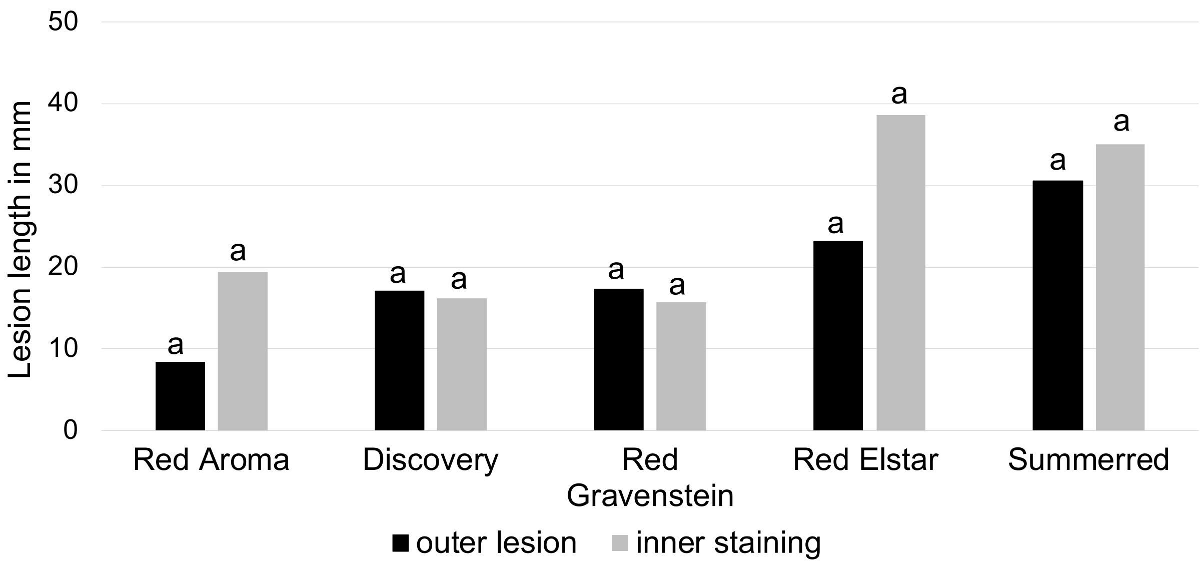

Fig. 23.: Lesion length and inner staining in green shoots four months after inoculation with map pins, containing *Neonectria ditissima.* Mean of four (two of cv. Summerred) replicates with one shoot per replicate. Bars with different letters indicate a significant difference according to Student Newman Keuls method at P = 0.05 per outer lesion or staining.

5. Discussion

The present study provides information about the susceptibility to European canker of six commercially important apple cultivars in Norway. The five cvs. Discovery, Summerred, Red Aroma, Red Elstar and Red Gravenstein constitute 90% of the apple volume produced in Norway (Øie, 2020). The sixth assessed cultivar was cv. Rubinstep which is a rather new cultivar in Norway (Frøynes et al., 2020). The general outlook is that the cultivar has a good storing property and therefore can be profitable for the Norwegian apple industry (Røen, 2007).

Information about canker development in the six apple cultivars was obtained by wounding of trees (bent or cut one year old branches and/or headed back) and subsequent inoculation with spore suspension of *N. ditissima*. Additionally, map pins were used to inoculate green shoots with mycelium of *N. ditissima* to assess possible cultivar differences regarding lesion length, inner staining, and symptom development. All the experiments were conducted under field conditions and experimental duration varied from four to 18.5 months. This allowed long term observations which provide practical and valuable results for Norwegian growers and the fruit industry.

The major findings from the wound type experiment were a significantly higher canker incidence for bent branches (33%) than cut surfaces (19%) and a significant cultivar difference in mean of both inoculation methods. After 18 months, Cvs. Red Gravenstein (38%) and Discovery (36%) had a significantly higher canker incidence than cvs. Rubinstep (18%), Red Aroma (17%) and Red Elstar (16%). A significant cultivar difference in disease development was found when analyzing the trees that were cut and headed back in the wound type experiment. No significant cultivar differences were found in either of the two nursery experiments (bent branches or headed back trees) and the three different propagation methods did not result in differences in canker susceptibility. In all whole tree field experiments, cankers on the inoculation site developed in the second season after inoculation. This result shows the possibly long latent period of *N. ditissima.* At the final assessment in autumn 2020, wood staining starting from some cankers on the inoculation site was found in all whole tree experiment, but no cultivar differences were found. In November 2020 sporodochia and perithecia were found on some cankers developed on the inoculation site. No significant cultivar differences in canker development, lesion size, and inner staining were found when inoculating green shoots with map pins contaminated with *N. ditissima*.

Wound type difference

The current study found that significantly more trees developed canker when inoculated after bending side branches than inoculated after heading back and cutting side branches. There are several possible explanations for this result. Firstly, wounds due to bending have a larger surface area than straight cut wounds and may offer *N. ditissima* a larger area to infect the tissue. This finding agrees with those obtained by (Amponsah et al., 2015) in New Zealand. Smaller wounds, such as leaf scars, were less susceptible to *N. ditissima* than larger wounds, like pruning cuts. An explanation approach for the different susceptibility of pruning cuts and leaf scars that goes behind the wound size was made by Xu et al. (1998) and Ghasemkhani et al. (2015). The authors suggested that in leaf scars, a rapid host defense response is likely to have evolved. This might be due to natural selection since many leaf scars form naturally during the leaf fall period. In contrast, pruning wounds do not occur naturally and may lack efficient defense mechanisms.

In a field experiment in New Zealand branches were inoculated with a misting bottle containing spore suspension of *N. ditissima*, after artificial wounding. Branches were either wounded by inserting injection needles, map pins, or using a metallic file to cause rasp wounds (Walter et al., 2016). After two months, only a few inoculations with needles and pins resulted in symptoms. In contrast, up to 70% of the inoculations in rasp wounds showed symptoms. Interestingly, isolation showed that tissue colonization by *N. ditissima* had occurred in all three inoculation methods, even though no lesions were visible. Therefore, the wound size and type might have an impact on symptom expression.

Another possible explanation for the higher canker incidence in bent branches might be that wounds caused by bending might be able to contain moisture better compared to cut surfaces. Water, and moisture in general, are crucial requirements for germination of *N. ditissima* (Dubin & Englisch, 1974; Latorre et al., 2001). The higher moisture-holding ability due to the cracked surface might have stored water better, providing a better germination environment for spores of *N. ditissima* and finally led to a higher infection success. Trees in the wound type experiment were inoculated in the morning of 13 May 2019 with only 0.4 mm precipitation during the day and 8.3°C average temperature and humidity of 52% at 11:00 AM. Due to the low precipitation and relatively low air humidity, the larger and cracked surface and the bent branches might have retained more moisture than the cut surfaces. The little available precipitation might have just run down on cut surfaces. As previously mentioned, Latorre et al. (2001) found that an extended moisture period was required at lower temperatures compared to 20°C to develop canker symptoms. On the day of inoculation, the average temperature was below 10°C and, therefore, lower than the proposed optimal germination temperature of 20-25°C (Latorre et al., 2001). This low temperature may explain why water availability was even more critical for disease development and the higher percentage of cankered trees across all cultivars inoculated after bending branches.

In the present study, only one main stem canker was found when trees remained non-wounded before sprayed with a *N. ditissima* spore suspension. The comparison to the corresponding control resulted in identical mean values. This shows that without artificial wounding (bent branches or cut surfaces), the pathogen might not have found suitable entrance points to infect the trees. This assumption agrees with the results of a field experiment in New Zealand (Walter et al., 2016). Non-wounded cv. Scilate branches were inoculated with a misting bottle containing spore suspension of *N. ditissima*. No canker symptoms developed on the branches after five months, and the isolation of the asymptomatic inoculation site was not successful.

Development over time

The two inoculation methods in the wound type experiment resulted in canker symptoms, especially discoloration of the wood, that was already visible 47 days after inoculation with *N. ditissima*. This observation was valid for all cultivars. In the nursery experiment, in which trees were inoculated after bending side branches, four out of six cultivars showed canker symptoms two months after inoculation. In an experiment in New Zealand, the bark of apple trees was wounded with a rasp and subsequently inoculated with a conidial suspension of *N. ditissima* (Amponsah et al., 2017a*),* and canker symptoms were visible already 46 days after inoculation. Even though the described experiment took place in a glasshouse with high humidity conditions, the time needed for symptoms to appear was somehow comparable to inoculation under field conditions.

In the wound type experiment, most cankers developed in the first 4.5 months after inoculation with *N. ditissima* in wounds caused by bending of side branches. In the second growing season, only cvs. Discovery, Red Elstar, and Summerred developed more cankers in the bent branches. When trees headed back and side branches were cut off, four out of six cultivars developed cankers in the second season. A significant cultivar difference in disease developed, expressed through AUDPC, was found. Cv. Discovery had a significantly higher AUDPC value than cvs. Red Aroma, Red Elstar, and Rubinstep. A newly developed parameter representing the lesion growth rate in mm per day was developed by Wenneker et al. (2017), and this parameter showed high consistency across different years in which the experiments were conducted. Leaf scars of different cultivars were inoculated, and lesion size was measured.

Wenneker et al. (2017) found that cv. Discovery had a higher lesion growth rate compared to cvs. Elstar and Golden Delicious. In the present study, disease development was calculated based on infection percentage and cv. Discovery was identified as a susceptible cultivar as well. Therefore, different experimental approaches might be used to gain similar results for the susceptibility of various cultivars.

The nursery experiment inoculated after heading back trees showed that most of the cankers developed in the first six months after inoculation. Cv. Summerred was the only cultivar that developed more cankers in the second season. In the nursery experiment in which trees were inoculated after branches were bent, especially cvs. Summerred, Rubinstep, and Elstar showed a steady and comparatively sharp increase in cankered trees from circa 11 months after inoculation onwards. Since cankers on cv. Red Aroma were overlooked in the field; no statement about canker development for this cultivar can be made. A difference in canker development over time was also observed in England by Xu et al. (1998) and varied with cultivars. Potted trees in greenhouses were pruned and, after that, inoculated with a conidial suspension of *N. ditissima*. The rate of increase in lesion numbers was more gradual for cvs. Bramley and McIntosh than for cvs. Cox and Golden Delicious. The latter two cvs. had the highest increase in numbers of lesions about 40 to 70 days after inoculation.

Regardless of the inoculation methods and experiment type (nursery or wound type experiment) some trees developed new canker symptoms more than one year after inoculation. This observation indicates that the pathogen can infect wood and with no symptom development for a long time. Such latent infections were also reported by Amponsah et al. (2017a). More than 50% of asymptotic wood pieces yielded *N. ditissima* when isolating from the wounds four months following inoculation, clearly indicating that the pathogen remained latent throughout the whole assessment period. These findings are consistent with experiments in nurseries and orchards, which have shown that the pathogen can stay latent for up to three years (McCracken et al., 2003). Because of the possible long latent period, more cankers could have developed in the present field experiments if the trees had been kept for a longer time.

A similar speculation was made by Børve et al. (2019). Scion wood of the cvs. Discovery and Summerred was inoculated with *N. ditissima* at the time of propagation and assessed for canker development up to 38 months after inoculation. The authors assumed that trees might have developed more canker infections if the trees remained in the orchard for a longer time. More research is needed to determine biotic and abiotic factors that determine the latent period and symptom development.

Inoculation success and cultivar difference

No significant cultivar differences were found in the two nursery experiments where inoculation wounds were made either by bending side branches or heading back. In the mean of all cultivars and propagation methods, 29% of trees inoculated after bending branches and 8% of trees inoculated after heading back developed canker after 16 and 18.5 months, respectively. The relatively low inoculation success and subsequently low number of diseased trees in both nursery experiments may explain the missing cultivar differences. The low inoculation success contrasts with artificial inoculation after wounding potted trees, in Sweden, where all inoculations resulted in canker symptoms (Garkava-Gustavsson et al., 2013). Potted trees were wounded by removing a bud or wounding the bark. A different experiment in Sweden, tested the suitability of potted trees and detached shoots, for evaluation of resistance to *N. ditissima,* and a significant year effect was found (Garkava-Gustavsson et al., 2016). Both the detached shoot experiment and potted trees were inoculated with *N. ditissima* and placed in a climate chamber or an unheated glasshouse, respectively. In the second year, the infection progressed faster. The temperature and humidity in the climate chamber were higher in the second year. The temperature in the greenhouse was higher in the second year as well. The authors suggest that the different climate conditions could have led to a faster infection process. In the present study, all trees in the three different experiments were inoculated in the same year but at varying time points. Differences in temperature, wetness period, and humidity might have influenced the infection success.

Artificial rasp wound inoculation experiments with potted apple trees in different parts of New Zealand resulted in different disease progress depending on the weather conditions (Scheper et al., 2019). Symptom development of *N. ditissima* advanced most rapidly with increasing daily hours of 11-16°C and air humidity between 74.6–87.2%. The temperature in one of the nursery experiments (inoculation after bending side branches) was slightly higher than the optimal, but the relative air humidity was much lower than the optimal. Trees in the second nursery experiment were headed back in April 2019, and the average temperature was 8.7°C. The average temperature decreased steadily in the days after inoculation and reached a low of 3.3°C, on the 8th day after inoculation. The trees planted for the wound type experiment were inoculated and wounded on 13 May 2019 with an average temperature of 8.3%, and the temperature increased the days after inoculation with a maximum average temperature of 16.9°C the 4th day after inoculation. The inoculation success might have been influenced by possibly not optimal climate conditions for *N. ditissima* and may have led to a low overall inoculation success. Nevertheless, Scheper et al. (2019) point out that more research in controlled climate conditions is needed to separate the effects of temperature, air humidity and, rainfall frequency on disease development. Weber (2014) points out that tree physiology might influence the susceptibility to canker as well. Especially, fast-growing trees are more susceptible to *N. ditissima*. Headed back branches were inoculated on 4 April 2020 and since it was early in the season, trees were still growing slowly, and might have been less susceptible to *N. ditissima*.

The wound type experiment was the only experiment that resulted in significant cultivar differences. When analyzing the two wound types (bent branches and cut surfaces) together, cvs. Red Gravenstein and Discovery had a higher canker incidence than cvs. Rubinstep, Red Aroma and Red Elstar. Cv. Summerred was not significantly different from any cultivar. The present wound type experiment revealed that the wounding type might influence the results of artificial inoculation experiments. While cultivars, inoculated after heading back and cutting side branches, were significantly different in canker incidence, no significant differences in incidence were found for trees inoculated after bending and inoculation of side branches. The incidence for cankered trees inoculated after bending branches was significantly higher than the incidence for trees inoculated after heading back and cutting branches.

The inoculation success was high so that almost all trees inoculated after bending branches had canker in autumn, 2020 but only 33% of the cut surfaces resulted in canker, after 18 months. These numbers show that the overall infection success for wounds was not very high, but cankers were distributed across many trees. This wide distribution but low inoculation success may have led to a misinterpretation of susceptibility and may explain why no significant cultivar differences were found in trees inoculated after bending branches. For further research regarding the susceptibility to *N. ditissima* of apple cultivars, it is crucial to establish a connection between the wounding type and the canker susceptibility. To provide more reliabale data, the following discussion of cultivar differences will focus on the results for the statistical analysis when combining the bent and cut treatment methods

In the present study, the cvs. Red Gravenstein and Discovery were the cultivars with the highest canker incidence and significantly different from Red Aroma, Red Elstar, and Rubinstep. This finding is consistent with Weber (2014), who classified cvs. Gravenstein and Discovery as a highly susceptible cultivar based on literature review and his own observations. In contrast, a detached shoot experiment in Sweden found no significant difference in disease development between the cvs. Red Gravenstein and Discovery and cv. Aroma (Garkava-Gustavsson et al., 2013). In an overview about the canker situation in Norway from 2015, the authors had observed less canker on cv. Gravenstein than on cv. Discovery. At that time, European canker was relatively new as a serious disease in some parts of Norway (Børve et al., 2015). This observation is in line with the obtained results for cv. Discovery but cv. Red Gravenstein might be more susceptible under Norwegian growing conditions than previously expected. From the present results, it can be concluded that the cvs. Red Gravenstein and Discovery are potentially highly susceptible, and extra inspection of trees and removal of cankers might be necessary when growing those cultivars.

When combining both inoculation methods, cv. Summerred was the only cultivar who was not significantly different from any cultivar. In experiments, assessing the development of canker on trees propagated with naturally infected or artificially infected scion wood, no difference in susceptibility between cvs. Discovery and Summerred was identified at the final assessment, 31 to 38 months after propagation (Børve et al., 2019).

This finding agrees with the results from the present wounding experiment. In general, Norwegian growers classify cv. Summerred as a very susceptible cultivar (Børve et al., 2019). A possible explanation for the discrepancy between the presented results and the observations of Norwegian growers could be the difference between natural infection and the artificial infection that took place in this experiment. Spore trap experiments have shown that cv. Summerred dropped its leaves during the peak of ascospore release in September and October, whereas the less susceptible cv. Aroma dropped its leaves towards the end and after the high season for ascospore release (Børve et al., 2020a). In addition, the earlier leaf fall of cv. Summerred was associated with higher mean temperatures, which can be favorable for *N. ditissima*. Therefore, the infection pressure, under natural field conditions, might be higher for cv. Summerred than for other cultivars, and because artificial inoculation does not always represent natural infection conditions, this might have led to an experimental bias for cv. Summerred.

Based the wound type experiment results, cv. Aroma was found to be a less susceptible cultivar and Norwegian growers consider cv. Red Aroma as a cultivar with low canker susceptibility. The results of this study agree with observations made previously on both detached shoot and whole trees (Garkava-Gustavsson et al., 2016; Ghasemkhani et al., 2015a). In Norway, cv. Red Aroma was the cultivar that was planted most frequently in the period 2011-2019 (Milford et al., 2021). From a plant health point of view, it is beneficial that most apple trees planted from 2011-2019 were cv. Red Aroma trees, considering the low susceptibility to *N. ditissima* of this cultivar.

Another cultivar that showed a low canker incidence in the present wound type experiment was cv. Rubinstep, a relatively new cultivar in Norway. In a Danish field experiment, various apple cultivars were evaluated for susceptibility to *N. ditissima* for up to seven years (Falk Kühn, 2004). Cv. Rubinstep showed relatively low susceptibility to European canker. The results of the danish field experiment are in agreement with the rather low susceptibility found in the present study. In Norway, cv. Rubinstep is a relatively new cultivar (Frøynes et al., 2020) and might be a promising cultivar for fruit growing in Norway regarding its possibly low canker susceptibility.

Cv. Red Elstar had a significantly lower canker incidence than cvs. Red Gravenstein and Discovery as well. In an artificial inoculation experiment with potted apple trees cv. Elstar was classified as a cultivar with intermediate resistance (Wenneker et al., 2017). Furthermore, Weber (2014) classified cv Elstar as a cultivar with low susceptibility based on observations and literature. In field trials with various naturally infected apple cultivars, conducted in northern Germany, cv. Elstar was found to be quite resistant compared to cv. Nicoter. However, Weber & Børve (2021) pointed out that trees of different cultivars were obtained from different nurseries, which might have led to a distortion of the results. In an experiment in western Norway, cvs. Red Aroma, Discovery, Rubinstep, Santana, Elstar, and Holsteiner Cox were planted in an ecological orchard. The trees were not inoculated with *N. ditissima* but assessed for symptoms of European canker over three years. Cv. Elstar was the cultivar with the second highest percentage of removed trees (5%). This observation shows that even if an apple cultivar is classified as less susceptible, environmental factors, managing practices, and planting material influence on canker development in the orchard (Milford et al., 2021). Again, this demonstrates how challenging it is to make statements about the susceptibility of apple cultivars to *N. ditissima.*

Comparing the obtained results from the present study to already published research shows that most of the findings regarding canker susceptibility agreed with previously published research on whole trees and partly as well for detached shoot experiments. This suggests that detached shoot experiments might provide a sufficient starting point for further investigation. Detached shoot experiments can usually only be carried out in a limited period up to six weeks. In contrast, whole tree experiments can last, as demonstrated, for more than a year and possibly even longer. Ghasemkhani et al. (2016) suggest that the difference in the level of resistance found between the different cultivars in detached shoot experiments and whole tree assessments might be due to different aspects in cultivar response highlighted through the two experimental approaches. In artificial inoculation experiments with detached shoots and potted trees, the first canker symptoms on detached shoots were already visible after ten days, and potted inoculated trees showed first symptoms about one month after inoculation. The developing lesions were measured, and the disease progression (AUDPC) was calculated.

The authors suggested that because of the slower disease development in whole trees, more information about the tolerance to *N. ditissima* can be obtained. On the other hand, detached shoot experiments might give more information about the susceptibility of a cultivar. Different results between detached shoot experiments and whole trees assays regarding cultivar susceptibility were also observed by Scheper et al. (2018). If a detached shoot experiment or a whole tree experiment is more informative depends highly on the specific research questions. Preliminary screening of many cultivars might be better obtained with detached shoot experiments because they take up less space and are appropriate for a restricted budget and require less time.

The reason for the different levels of canker susceptibility in various apple cultivars is not apparent yet (Ghasemkhani, 2015; Ghasemkhani et al., 2016; Weber, 2014). The histopathology might vary between susceptible and partially resistant cultivars. In an artificial inoculation experiment woody tissue from infected cvs. Cox's Orange Pippin and Santana were studied using a light microscope (Ghasemkhani, 2015). Cv. Cox's Orange Pippin is known to be a susceptible cultivar, whereas cv. Santana has proven to be partially resistant (Ghasemkhani, 2015). However, the authors did not find histopathological differences between the two cultivars that explain the difference in susceptibility. Nevertheless, a difference in disease development was observed between the two cultivars. In the susceptible cultivar, hyphae were observed earlier than in the partially resistant cultivar. In cortex parenchyma cells, a large number of hyphae and collapsed cells were found in the susceptible cultivar. These observations indicate a difference in the degree of colonization between the cultivars. The authors suggest that phytoalexins might impact the defense reaction against the pathogen and that the level of resistance might not be related to the anatomy of the woody tissue. These phytoalexins might be responsible for reducing the rate of colonization and disrupting pathogen metabolism (Ghasemkhani, 2015; Ghasemkhani et al., 2016). Phytoalexins are low weight antimicrobial compounds that are produces by plants and can act as toxins to pathogens. These compounds can belong to various chemical families such as phenolics, terpenoids and indoles (Jeandet, 2015). Research on the apple scab pathogen, *Venturia inaequalis*, showed that the resistant apple cultivar Liberty produces a phytoalexin (Hrazdine et al., 2007). This phytoalexin was named malusfuran and is toxic to *V. inaequalis*, at minimal concentrations.

More research is needed, to examine to what extend phytoalexins contribute to the defense reaction to *N. ditissima*. It may be speculated that cultivars that had a higher canker incidence in the present experiments contained fewer phytoalexins and therefore have a higher degree of colonization by *N. ditissima*.

In addition, a difference in expressed genes is likely to impact disease response and explain differences between susceptibility and partially resistant cultivars (Garkava-Gustavsson, 2020; Ghasemkhani, 2012; Gómez-Cortecero et al., 2016). With the knowledge of cultivars with low susceptibility such as cv. Aroma (Garkava-Gustavsson et al., 2016; Ghasemkhani et al., 2015a) and the unraveling of important genome sequences and large effect quantitative trait locus (QTLs) (Bus et al., 2019; Gómez-Cortecero et al., 2016; Karlstrom et al., 2020), the basis for identifying and understanding the genetic basis has already been laid.

Overall, the presented results increase the knowledge about canker susceptibility of economically important cultivars in Norway and about one potentially economically important cultivar (cv. Rubinstep) in the future. If Norwegian growers can produce more apples due to decreased tree dieback, fewer apples need to be imported, and this can have a positive effect on the Norwegian economy and the carbon footprint. The interest of Norwegian consumers in local food is continuously growing, showing that there is a market for local products (Pentz, 2017). Furthermore, positive consequences about the use of less susceptible apple cultivars (potentially cvs. Rubinstep, Red Aroma, and Red Elstar) could lead to a decrease in the use of plant protection products which is favorable for the environment (La Torre et al., 2018; Simon et al., 2011).

Wood Staining

Wood staining was observed in all experiments and wound types. However, no significant differences between cultivars or wounding methods were found in each of the three experiments. In the final assessment, it became apparent that the presence of staining did not only vary among trees, but also among cankers on the same tree.
No evident factors which might influence the staining were identified. Across all experiments and wounding types, the length of staining, starting from the inoculation site, varied between less than five to 74 cm in the main stem and one year old wood of the trees.

In a previous report, approximately similar lengths of wood staining were observed from inoculated graft unions in cvs. Discovery and Summerred (Børve et al., 2019). The wood staining was measured up to 50 cm into the scion wood and 15 cm into the rootstock. The staining was measured up to 36 months after planting. In northern Germany, naturally infected cv. Nicoter trees showed wood staining up to 20 cm underneath and above cankers (Weber & Hahn, 2013). The staining was measured only six months after the first canker symptoms became visible.

In contrast, the staining in the present field experiments was measured circa 16 months after symptoms of *N. ditissima* were detected, and individual cankers had longer wood staining compared to the maximum staining length of 20 cm observed by Weber & Hahn, (2013). The reason for the longer observed staining in the present study is not clear, but older cankers might lead to longer staining in the main stem. However, it is also important to underline that shorter staining was observed as well, and no conclusion about the staining occurrence in the different cultivars can be made.

The general advice provided by Weber & Hahn (2013), which early removal of cankered tissue is crucial to prevent the growing penetration of *N. ditissima* into the wood, is still highly valid. The presented results confirm that *N. ditissima* can penetrate far into the wood when cankers are not removed. Therefore, canker removal is crucial for growers. In New Zealand, the presence of *N. ditissima* in stained and unstained wood in potted trees, one year after artificial inoculation of leaf scars, was investigated (Scheper, 2020). The inoculated shoots were cut into one cm segments, starting at the inoculation point and going towards the base of the shoot, and real-time PCR (Ghasemkhani et al., 2016) was used for canker detection. In potted trees with canker, 25% of the segments where no wood staining was visible, the real-time PCR detected the pathogen. This was particularly valid for the cvs. Golden delicious and Scilate. In 10% of the segments, wood staining was observed, but the real-time PCR did not detect the pathogen. The results show that growers cannot rely on internal staining when removing cankers and the authors recommend removing at least 20 cm of wood below a canker. Furthermore, this recommended distance might depend on the cultivar and the canker age.

Susceptibility of different propagation methods to *Neonectria ditissima*

In the nursery experiments (both with bent branches and headed back trees), no significant difference was found among the three used propagation methods, i.e., scion grafted on either a cv. Golden Delicious Interstem grafted on M9 rootstock, or directly on M9 rootstock, or T-budded on M9 rootstock. As mentioned above, the inoculation success was relatively low in both nursery experiments. This circumstance could have led to non-significant differences between the three propagation methods. Previous artificial inoculation experiments in which infected scion wood was T-budded and grafted on different rootstocks (Antonovka, B9, M9) resulted in faster disease development and higher incidence on T-budded trees (Børve et al., 2019).

Incidence of sporodochia and perithecia

In both the wound type and nursery experiments, sporodochia and perithecia were found in autumn 2020. However, statistical analysis was only conducted for the wound type experiment because a small number of cankers with sporodochia and perithecia were found in both nursery experiments and the inoculation success in those experiments was generally low.

In the wound type experiment, no significant cultivar differences in the incidence of sporodochia were found across both inoculation experiments. A significant difference in wound type was found across all cultivars. Significantly more cankers had sporodochia when inoculated after bending side branches than cut surfaces. Børve et al. (2020) found that the presence and development of spore structures can depend on the location of cankers. Naturally occurred cankers on trees, and detached cankers were observed from May 2018 until December 2019. No ripe perithecia were found on rootstocks, while perithecia on both attached and detached cankers were present in 2019. In New Zealand, rainfall frequency was found to be an essential factor for increased conidial production (Scheper et al., 2019). Furthermore, wetness and high humidity are required for sporodochia production (Swinburne, 1975). The assessment of the spore structures in the present study took place at the beginning of November 2020. On 18 out of 30 days before the final assessment, rainfall was measured. The high number of rainy days could have led to an increase in conidia production, especially on cankers developed after bending branches.

Those cankers on the bent branches may have been more exposed to rain than cankers developed on cut branches because of the more horizontal position. Rain might have been accumulated in the rough structures of the more horizontal canker wounds, and this could have been favorable for the development of sporodochia. In contrast, branches were cut, and cankers developed on their end. More water runoff might have occurred on cankers on the end cut branches, and those cankers had a shorter wetness period. Further research is needed to determine factors influencing sporodochia development. Furthermore, a cultivar difference in conidia release was found in New Zealand (Walter et al., 2017). In a commercial orchard, canker lesions were selected, and spore release was measured using plain glass slides. The number of conidia found on glass slides underneath cankers of cv. Royal Gala was higher than for cv. Granny Smith. This interesting observation could be the basis of more research concerning spore production in different apple cultivars.

In the present wound type experiment, between 42.8% (bent) and 39.0% (cut) of the cankers developed on the inoculation site, had perithecia at the beginning of November 2020. The majority of the perithecia were ripe. It is essential to underline that the cankers in the present study were only examined for spore structures once in the final assessment in autumn 2020. Therefore, no statement can be made when mature perithecia were present for the first time. Highly relevant research about perithecia and sporodochia presence and development was recently conducted in Norway. In a systematic survey of European canker in western Norway, ripe perithecia were present all year round but mainly during the winter (Børve et al., 2020b). In northern Germany, the presence of mature perithecia from October onwards, after a wet summer, was observed by Weber & Børve (2021). From autumn onwards, a selection of naturally infected cankers was weekly assessed for mature perithecia. Approximately half of the perithecia were mature in November after a wet summer. In general, the presence of mature perithecia increased during winter and peaked in March. Many researchers have reported regional differences in ascospore production and ripening (Amponsah et al., 2017b; Munson, 1939; Weber & Børve, 2021). More research is still needed regarding the development of perithecia and ascospore distribution.

In the wound type experiment, significant cultivar differences in the incidence of perithecia were found when analyzing both wounding methods together. Cvs. Discovery, Summerred, and Red Gravenstein had a significantly higher incidence of perithecia than cvs. Red Elstar and Red Aroma. When analyzing the bent branch treatment separately, significant cultivar differences in incidence of perithecia were found as well. Cultivars, such as cvs. Discovery and Red Gravenstein, which seem to be highly susceptible, might have a higher perithecia incidence as well. However, the canker incidence of cv. Summerred was not significantly different from the other cultivars but a significantly higher incidence of perithecia compared to the cvs. Red Elstar and Red Aroma was found. In an experiment in Norway, grafted apple trees were inoculated with *N. ditissima* at the time of propagation, and perithecia on cv. Discovery trees were first observed eight months after inoculation. On cv. Summerred perithecia were found 17 months after inoculation (Børve et al., 2019). To increase the understanding of perithecia development in different cultivars more research is needed. Cultivar related knowledge about perithecia development is crucial to identify potential sources of inoculum that could infect other trees already in the first growing season.

Rootstock cankers in the wound type experiment

In the final assessment of the wound type experiment, it became apparent that many trees had canker in the rootstock, although these were never artificially inoculated. In all the rootstock cankers, red perithecia were found. Statistical analysis revealed that trees inoculated with the bent and cut method had significantly more rootstock canker than their corresponding control trees. Rootstocks can get infected with *N. ditissima* in the nursery without showing symptoms at the time of planting (Børve et al., 2018). Rootstock cankers will then develop in the orchard and can cause severe losses. Therefore, rootstock cankers are a challenge for nurseries and growers. A study with organic nursery trees in the Netherlands resulted in 7% rootstock cankers (Jansonius et al., 2004). The used rootstocks were M9 and MM106. Most of the rootstock cankers became visible between two and six months after planting. The authors suggested that removing shoots on the lower part of the tree in the first year may have caused entrance sides (Jansonius et al., 2004).

In the presented wound type experiment, the rootstocks may have been infected with *N. ditissima* in the nursery, and infections became visible after planting. However, this explanatory approach does not explain why non-inoculated trees had significantly fewer rootstock cankers. Therefore, an explanation might be that inoculum was spread to the rootstocks during inoculation or was washed down from the inoculation points further up during rain events. The rootstocks had numerous root primordia, and they might have been possible entrance points for *N. ditissima*. Such root primordia are known for their fast growth, and they can easily crack, making them vulnerable to infections of the fungus. Furthermore, their presence prolongs the wetness period at the tree base (Swinburne, 1975).

Real-time PCR

The aim of using the real-time PCR method in this thesis was to increase the general understanding of disease development in different apple cultivars and the presence of *N. ditissima* in asymptotic wood, four to five cm away from a canker wound.

No signal was detected for the sample taken directly from the canker wound of cvs. Red Gravenstein and Summerred. The negative control, taken from non-inoculated trees, of cv. Red Elstar did not show a signal for the *N. ditissima* real-time PCR. In contrast, the amplification control showed a Cq value of 16. The possible reasons for the lack of signals are diverse. The extracted DNA may have been of poor quality, or a technical mistake might have happened executing the real-time PCR. The inhibition of amplification is a common problem when detecting plant pathogens with real-time PCR. Since woody tissue contains known inhibitory substances such as lignin, a complex aromatic polymer linked with polysaccharides and celluloses, the real-time PCR might have been inhibited by those contaminated substances (Bickley & Hopkins, 1999; Langrell & Barbara, 2001). For cvs. Red Elstar, Red Gravenstein, and Summerred, it is not possible to make a statement about the occurrence of *N. ditissima* in the samples taken four to five cm away from the canker wounds due to the missing negative or positive control. Therefore, the underlying context for the interpretation of the *N. ditissima* real-time PCR is missing.

For cvs. Discovery and Re Aroma the mean Cq values for the samples taken four to five cm away from the wound were higher than the Cq value for the samples taken directly from the canker wound. It can be concluded that higher amounts of *N. ditissima* genes were detected in the canker wound. However, cv. Rubinstep was an exception to this observation. Cv. Rubinstep was the only cultivar that had a lower mean Cq value for the samples taken four to five cm away from the canker wound than both the canker and the non-inoculated control sample.

Since most of the negative controls showed similar Cq values compared to the samples taken four to five cm away from the canker wound, *N. ditissima* genes were not detected in asymptotic tissue. This observation could be because *N. ditissima* was not present in samples taken at a distance from the canker or because of for technical reasons. It is possible that asymptotic infection in wood tissue samples, taken in four to five cm distance, was not detected because the level of fungus present was below the limit of detection of the developed real-time PCR method. Real-time PCR methods developed by a Swedish research group (Garkava-Gustavsson et al., 2013; Ghasemkhani et al., 2016) proposed to improve their developed methods in regards to asymptotic *N. ditissima* detection. One Swedish real-time PCR method was developed based on primers specific to a variable region of the *Neonectria* genome (Garkava-Gustavsson et al., 2013). This method was able to reliably detect the presence of *N. ditissima* in infected and symptomatic tissue. However, the pathogen could not be detected in asymptomatic tissue. The authors point out that further improvement of the developed method is needed before it can be used as a tool to replace phenotyping of *N. ditissima.* Ghasemkhani et al. (2016) developed a real-time PCR method based on the ß-tubulin gene. The developed methods produced reliable results for symptomatic tissue.

Additionally, Scheper et al. (2020) detected *N. ditissima* in asymptomatic applewood with the Real-time method developed by Ghasemkhani et al. (2016). In a series of orchard experiments in New Zealand, asymptomatic wood tissue in was sampled in various distances to cankers. The sensitivity of pathogen isolation (Amponsah et al., 2014), and real-time PCR were compared and in two out of three experiments, pathogen isolation was proven to be more sensitive.

The authors found that many segments tested negative by real-time PCR for the presence of *N. ditissima,* while the pathogen was successfully isolated from the same segments. However, the results were inverted for other segments, with a positive real-time PCR result and a negative result for the pathogen isolation. For example, in cvs. Braeburn and Jonathan, real-time PCR was positive in asymptomatic wood 10 cm away from the canker wound, but pathogen isolation was unsuccessful.

A Dutch research group developed a promising real-time PCR method based on the ITS gene to detect and quantify *N. ditissima* (Harteveld et al., 2020). The method was not published before handing in this master thesis, but a conference presentation offered first insight in the potential of the method. Cvs. Elstar and Gala were inoculated on fresh pruning wounds, and samples for the real-time PCR were taken two mm, one, and three cm away from the pruning wound. The wood samples were taken at different time points after inoculation. The results showed that two, four, and eight weeks post-inoculation, the pathogen was present in all asymptotic samples taken two mm, one, and three cm away from the inoculated pruning cut. The developed real-time PCR method was proven to be more sensitive than isolation. In addition, the comparison of the used cvs. Gala and Elstar revealed cultivar differences. Cv. Gala had a higher incidence of *N. ditissima* than cv. Elstar. The pathogen moved faster in cv. Gala and was found earlier in wood that was further away from the inoculation site compared to cv. Elstar (Harteveld et al., 2020). This observation is in line with literature where cv. Gala is classified as a highly susceptible cultivar, whereas cv. Elstar is known to be less susceptible (Weber, 2014). Additionally, the presented results from the wound type experiment of this thesis showed that cv. Elstar had a relatively low susceptibility to *N. ditissima*. Besides, Ghasemkhani et al. (2016) found different levels of *N. ditissima* biomass in different cultivars. Cv. Elise, which is known to be susceptible, had the highest amount of *N. ditissima* while cv. Aroma had the lowest amount. This finding shows again that differences in the level of colonization can be related to cultivar differences. In the presented wound type experiment, cv. Red Aroma was found to be a less susceptible cultivar, and this observation was made by other researchers before (Ghasemkhani, 2015; Weber, 2014).

No reliable statements about cultivar differences can be made based on the presented results for the real-time PCR (Brurberg M.B, unpublished results). This is due to missing values for the different cultivars and due to similar Cq values for samples taken four to five cm away from the canker wound and their corresponding negative control samples.

Map pin inoculation of green shoots

The map pin inoculation of green shoots did not result in significant cultivar differences between outer lesion length and inner staining. In a previous experiment in New Zealand, where detached cv. Scilate shoots were inoculated with conidia suspension and covered with plastic bags, lesion size varied in length from 3.7 to 6.7 mm (Walter et al., 2016). Outer lesion size was measured two months after wounding (injection needle, pin, and rasp wound,) and inoculation with *N. ditissima* was carried out in a controlled environment. In a glasshouse experiment, bud scars on potted trees were inoculated with a conidial suspension (Scheper et al., 2018). Lesion length was measured after 12 weeks, and mean lesion length varied between 10 and 27 mm in potted trees. In the present experiment, lesion sizes four months after inoculation were larger and varied from 7 to 50 mm. Compared to the trials in New Zealand, this difference in maximal lesion length may partly be due to a longer development period and lesion length increasing over time.

Furthermore, the present experiment has proven that inoculation with map pins and mycelia of *N. ditissima* is a time-saving and efficient method for inoculation of shoots. The efficiency of the map pin inoculation method was already demonstrated in rootstock experiments (Børve et al., 2018).

In the present experiment, the first canker symptoms were visible 29 days after inoculation. This was an earlier symptom development than was shown in a trial in the Netherlands, where the first necrotic lesions were observed eight to ten weeks after inoculation (Wenneker et al., 2017). Potted apple trees were wounded by removing leaves on the main stem followed inoculation with a spore suspension of *N. ditissima* underneath a roof. The physiological condition of trees might have an impact on the susceptibility to canker.

According to Weber (2014), especially young trees with more vegetatively growing shoots are likely to be more affected than older and slower-growing trees. In the present experiment, green shoots were inoculated on the first of July 2020, and therefore shoots were still growing. They might have had more vegetatively growth on the inoculation site compared to the potted trees used in the artificial inoculation experiment by Wenneker et al. (2017), which were inoculated on the main stem.

In a detached shoot experiment, first signs of girdling already became visible between 20 and 25 days after inoculation (Garkava-Gustavsson et al., 2013). Shoots were inoculated with conidial suspensions after the removal of buds and were kept in plastic tents to ensure high humidity. In a greenhouse experiment lesion were first observed after 10-14 days after removing buds and subsequent inoculation (Ghasemkhani et al., 2015a). The reason for the difference in disease development is not clear but might be due to a difference in inoculation method, climate conditions, and a difference between experimental types. A slower disease progression in one year old trees than detached shoots was also found by Garkava-Gustavsson et al. (2016).

All shoots of cv. Summerred had canker symptoms after 1.5 months of inoculation. However, it is important to keep in mind, that only two out of four shoots were inoculated in July 2020 because not enough shoots of cv. Summerred were present. The disease progression of some other cultivars, such as cvs. Red Elstar and Gravenstein, was more gradual. This shows that disease expression in some cultivars takes longer than in others. However, differences in disease progression, presented through AUDPC, were not significant. Therefore, no cultivar differences were observed, neither for lesion length nor for disease development.

Researchers suggested using the latency period as a parameter for measuring cultivar resistance to *N. ditissima* (Garkava-Gustavsson et al., 2013; Ghasemkhani et al., 2015a; van de Weg, 1989). However, using the latency period in the present experiment as an indicator for susceptibility to *N. ditissima* is problematic because of the missing replicates for cv. Summerred and because the period in between each field was two weeks. It is known that symptoms can develop quickly, and assessments every couple of days would be more suitable to obtain reliable information about the latency period (Garkava-Gustavsson et al., 2013; Wenneker et al., 2017).

Combining different assessment methods, such as latency period, lesion size measurements, disease development over time and infection incidence can provide complementary information and are crucial to obtain reliable information about the susceptibility to *N. ditissima* of different apple cultivars (Garkava-Gustavsson et al., 2016; Wenneker et al., 2017).

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