Improving apple quality by hot water treatment

PhD Thesis

by

Peter Maxin
Foreword

This Ph.D. dissertation has been submitted to Aarhus University in partial fulfillment of the requirements of the degree Doctor of Philosophy. Dr. Hanne Lindhard Pederson (Associate Professor) was my primary supervisor until she left the University on 31 August 2011, at which time Dr. Michelle Williams (Head of Department) took on this responsibility. Dr. Roland Weber (Esteburg, Fruit Research and Advisory Centre, Jork; Germany) and Prof. Susan Lurie (Department of Food Science, Volcani Center, Israel) were co-supervisors. This study was conducted from 3 September 2008 to 9 December 2011 at the Department of Food Science, Aarhus University, located at Aarslev, Denmark. The study had a short-term interruption due to paternity leave for a total of 14 weeks. In February to March 2011, I stayed at the Esteburg Fruit Research and Advisory Center to carry out an ad hoc Ph.D. course.

The primary focus of this Ph.D. project was apple fruit quality in relation to pre-storage hot-water treatments. The first year results showed neither an improvement nor any difference in physiological fruit quality following cold storage. Therefore, the main emphasis of the second and third project years was on fungal decay, the impact of hot water treatments, influences of pre-harvest factors on storage rot development and the introduction of a short term hot water treatment. Work on the latter aspect was initiated in October 2009 during a seven-day visit to the Volcani Center, Israel, where I worked with Professor Elazar Fallik.

I am indebted to Roland Weber for his input and for the time we spend identifying the different fungi associated with storage rots. I also thank him for his contribution to PCR identification of the fungi which provided the opportunity to produce high-impact results.

This thesis is presented in eight chapters. Chapter 1 is a general introduction on the Danish fruit growing sector and the connections to this study; Chapter 2 is a literature review on different fungi causing storage rots as well as commonly used chemical treatments and hot water treatments to reduce postharvest losses in apples. General methods that were used and developed in this Thesis are presented in Chapter 3. Chapter 4 contains a series of short trials that describe unpublished and preliminary results. It is the intention to repeat some of these findings for subsequent publication.
Chapters 5 to 7 contain the results of key experimental work. In Chapter 8 key general results from this thesis are discussed, brief conclusions and a perspective for this technology are provided. References are contained in Chapter 9.

I am very grateful for fruit donations generously made by Heinrich zum Felde (Jork), Alexander and Berbel Maxin (Jork), Peter Rolker (Jork), the Esteburg Centre and Michael Clever (Jork). This research needed a copious supply of plastic boxes, as used by many fruit farmers but not necessarily universities. I therefore extend my special thanks to Peter Rolker and to Bastian Benduhn (Esteburg Centre) for lending me the fruit storage boxes, and to Ørskov Frugt (Oure, Denmark) for shipping these boxes to Denmark and back to Germany. I also thank Berg GmbH and Jürgen Schacht (Jork) for their input of time and equipment in the first year trials on hot water rinsing.

Carsten Sørensesn, Innotheque APS, Middelfart, Denmark generous provided time and initiative in developing and creating the prototype of a hot-water rinsing machine that could, after modifications, run the second year hot water rinsing experiments.

Dr. Dirk Köpcke (Esteburg Centre) collaborated on both DCA and CA experiments, and stored hot water treated apples in his research facilities, therefore helping to clarify the behavior of pretreated fruit in these storage environments.

I am very grateful to the technical staff at the Department of Food Science, Aarhus University, Aarslev, and especially to Annette and Stig Sørensen. A special “thank you” also goes to the secretarial staff for their smooth and efficient help with administrative matters.

I am grateful for the support given to me by the Weber, Williams and Maxin families, and especially by my own family; Meike and Paul. This is in acknowledgement of the time my supervisors spent with me rather than their families, and of the time that I spent on the road instead of being at home. Thank you, Hanne, Michelle and Roland, for your critical advice in sharpening and polishing this Thesis and the various publications that form part of it. I thank my sister Uta Maxin for her input into improving the process figures in Chapter 4.
Thanks are also given to all the colleagues at the Department of Food Science, Aarhus University for providing a collaborative, welcoming and positive study environment.

Thank you!

Peter Maxin

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Summary

The Danish fruit industry has a focus on sustainable production systems. However, there is a need for new tools and technologies to enable orchardists to deliver quality fruit at competitive prices into the market. Research activities at Aarhus University aim to improve the availability and consumption of quality and healthy fruit. The use of postharvest hot-water treatments (HWT) can reduce storage losses of organically grown apples, as has been shown in several trials for different fungi.

Preliminary results are presented as a follow up on different research activities in this Thesis. Seven short studies are aligned to three themes: pre harvest effect on the developing storage rots, effect of hot water treatments on fruit quality and physiology and effect of hot water treatments on pathology. The distribution of storage rot fungi in apples over two years was investigated in one orchard. The single-tree infection rate influenced by local inoculum and changing between years is reported. The influence of root pruning of different rootstocks on inoculum and decay rate in apples was investigated. The effect of crop load and nutrition level on fruit quality parameters (firmness, colour, sugar content, heat scald index) was also examined. Neither an improvement nor a quality decrease could be found following moderate hot water treatments. Hot water dipping (HWD) temperatures exceeding 54°C for 3 min resulted in severe heat scald. The influence of hot water rinsing (HWR), hot water dipping and 1-methycyclopropene (1-MCP) on the incidence of 'Elstar' skin spots under different atmosphere storage was evaluated. Evaporation of water through the fruit skin of HWR, HWD and cold water dipped control fruit was evaluated during a 36 h stress treatment. After 36 h, HWR treated fruit showed a trend towards a reduced loss of water through the cuticle relative to control fruit. The efficacy of HWD in limiting the further development of Sooty-blotch disease during storage was examined. Following HWD, Sooty-blotch fungi neither showed any further development in storage, nor was there any reduction in the existing mycelium.

Hot water dipping (HWD) of spore suspensions of different storage rots was carried out to determine the effective temperature causing a 50% inhibition of spore viability (ET50) and the mortality curve in response to dipping temperature. Spore suspensions of Penicillium expansum, Neonectria galligena and Botrytis cinerea were used to inoculate apple fruit and to evaluate HWD efficacies in a wide range of
temperatures. Spores of \textit{P. expansum} were inoculated both pre- and post-HWD. Similar developments of \textit{P. expansum} rot in fruit inoculated pre- and post-HWD in the temperature range of 44-53°C led to a new perspective on the mode of action of HWD. An indirect effect of HWD as a heat-shock which induces the immune response of the fruit is contrasted with its direct inhibitory effect on fungal inoculum.

A new storage rot, rubbery rot caused by the fungus \textit{Phacidiopycnis washingtonensis}, was reported for the first time from Denmark. A description of the new rot, the orchard where it was detected and the incidence of decay were documented. The response of \textit{P. washingtonensis} spores to HWD and the response of artificially inoculated fruit towards HWD were investigated. Naturally infected fruit with \textit{P. washingtonensis} were subjected to HWD and HWR, and compared with fruit subjected to 1-MCP in both controlled atmosphere (CA) and dynamically controlled atmosphere (DCA) storage. The efficacies of HWD and HWR towards \textit{P. washingtonensis} were comparable to Neofabraea spp. The influence of ripening inhibition caused by 1-MCP, CA or DCA did not influence the incidence of \textit{P. washingtonensis}.

Combinations in time and temperature for rinsing and dipping of naturally infected apples in hot water were tested against natural infections of \textit{Neofabraea perennans}, \textit{N. alba}, \textit{N. galligena}, \textit{B. cinerea}, \textit{P. expansum}, \textit{Monilinia fructigena}, \textit{Colletotrichum acutatum}, \textit{P. washingtonensis}, \textit{Phoma exigua}, \textit{Gibberella avenacea}, \textit{Mucor} spp. and \textit{Cladosporium} spp. The influence of heat scald on the incidence of storage infections by \textit{P. expansum} and a higher disease incidence by several pathogens following elevated temperatures were discussed. HWR showed comparable efficacies to HWD. Several advantages of HWR were discussed.

The findings of this study are compared to previously reported and recent research results within the area of hot water dipping, fruit quality and fungal storage rots in apples. An overview on storage rots and postharvest control means is given in the context of organic farming. The general mode of action of HWT is evaluated for different fungi and apple varieties. Possible implementation strategies and perspectives of HWR for the apple industry are discussed.
Resumé

Den danske frugtbranche og Aarhus Universitet har i fællesskab sat fokus på bæredygtige produktionssystemer, som har til formål at forbedre adgangen til og forbruget af sund kvalitetsfrugt. I den forbindelse er der behov for at udvikle nye værktøjer og teknologier for at frugtavlerne kan producere kvalitetsfrugt til konkurrencedygtige priser. Brugen af varmtvands-behandlinger (HWT) efter høst er en sådan ny teknologi, som allerede har vist sig effektiv til bekæmpelse af flere forskellige rådsvampe i økologisk æbleproduktion.


hæmmer modningsprocesserne i frugten, ingen indflydelse på forekomsten af P. washingtonensis.


Yderligere resultater præsenteres mere kortfattet. Det blev undersøgt om HWD kunne begrænse den videre udvikling af sodplet under lagring, og der kunne ikke konstateres nyvækst af svampen på lageret, og eksisterende infektioner ikke kunne dræbes ved HWD.


Det blev undersøgt om frugterne mistede spændstighed (fordampning gennem frugtskrællen) efter behandling med HWD og HWR. Efter 36 timers varimestress tenderede de HWR behandlede frugter til at have mistet mindre vand end frugter som var behandlet med HWD eller kontrolfrugter, som var dyppet i koldt vand.

Chapter 1: Introduction

The fruit industry in Denmark is challenged by global competitors. Denmark’s economy is one of the strongest in the world in terms of per capita income (Einhorn and Logue 2010) and, therefore, it is an attractive market for all types of highly priced food products. Despite this competition in the market place, Danish apples are sold at high prices in the supermarkets. However, relatively little revenue is returned to Danish orchardists; in the years 2009 (reflecting the season 2008/9), 2010 and 2011 the average payment to growers was 2.75 DKK (=0.21€) per kg apples (pers. communication Ejvin Straaup, Langeland, DK). This return to the growers did not cover the production costs in established orchards, which were equivalent to 3 DKK (=0.23€) per kg apples (Pedersen 2006). Danish regulations currently disadvantage Danish orchardists in the market place. Danish growers must meet tight Danish regulations and yet they compete on their own market with imported fruit produced under different environmental and social requirements. For example, in 2009 the only registered active compounds for organically managed Danish orchards were wettable sulphur and Bacillus thuringiensis, whereas a comparable orchardist in Germany would have had access to more than 20 registered compounds (based on Annex II of the organic EU directive 2092/91 from 1991, subsequently replaced by EU directive 834/2007).

In addition, production costs in Denmark are comparatively high as a result of high salary costs and taxation on pesticides and fertilizers. Previously, a reasonable income for orchardists in the years 2002 to 2005 (ca. 4-5 DKK/kg) led to enhanced planting activities in 2005 as well as 2006, thereby increasing the apple growing area. In Denmark 1,267 ha apples were cultivated in 2005, increasing to 1,609 ha in 2010. These numbers include organic apple orchards which increased from 158 ha in 2005 to 300 ha in 2010. To limit overhead in growers’ organizations (GO) and to reduce sales competition, restructuring and merging of some GO started in 2008 and has not yet been completed in 2012.

The annual Danish apple season starts with the summer apple ‘Transparente’ which is harvested at the end of July in average years. Due to climate limitations, ‘Golden Delicious’ marks the latest possible variety with an approximate harvest time from
mid-October until early November. The average Danish apple consumption is 15 kg fresh apple per person per annum. The local production has a market share of 30%, the remaining demands being met by imports (Danmarks Statistik 2007). Danish fruits are marketed until March (Dansk Kernefrugt, 2011).


Many research projects focused on delivering research-based innovation for implementation into the fruit industry are being carried out in an attempt to secure the long-term future of the Danish fruit industry. The EU ‘ISAFRUIT’ project (2007-2010) was coordinated in Denmark with research activities at Aarhus University (AU-Aarslev), focusing on increasing consumption of fruit from sustainable production and thereby improving public health. The Danish ‘Fruit Pack’ project aims to develop sustainable solutions (especially water and nutrient use) in both organic and conventional fruit production systems. These research activities are aimed at positioning the Danish industry to compete more successfully against imported fruit and to be able to differentiate their products on the Danish and European fruit market.
Research-based evaluation, development and implementation of new methods to control postharvest diseases are a key focus of research in Denmark, especially if they provide alternatives to fungicide applications. One such technology is hot water dipping (HWD). In 1964, HWD was first reported to reduce storage rots in apples (Burchill 1964). At the same time, the registration of highly effective chemicals, such as Benomyl, meant that there was no urgency to continue this research on non-chemical alternatives in North Western (NW) Europe. However, more recently there has been a renewed interest in this topic due to the high storage losses in organic production systems, a change in public awareness (Fallik et al. 2001), and assumed (Palm 1997) or reported (Weber & Palm 2010) fungicide resistance of key fungal storage-rot pathogens (Chapter 2.6). Recent studies have indicated the potential of this technology, for example HWD has been shown to reduce storage decay caused by ‘Gloeosporium’ rot (chiefly Neofabraea alba and N. perennans) and brown rot (Monilinia fructigena) by more than 80% (Maxin et al. 2005). However, the economic profitability of HWD is highly dependent on fruit prices, meaning that this technology is of interest mainly to organic production systems (Maxin and Klopp 2004) where it has been implemented in Germany in recent years (Maxin et al. 2006).

This thesis is based on eight hypotheses concerning the mode of action of HWD, its efficacies against different storage-rot fungi, and the influence of hot water treatments (HWT) on apple fruit quality. Hypotheses 1-3 were established at the time of writing the initial proposal and grant application and Hypothesis 4 was developed within the first 6 months of the project. While setting up experiments to accept or reject Hypothesis 2, a cascade of four additional hypotheses was developed and addressed experimentally (Hypothesis 5-8). Together these hypotheses are conceptionally related in presenting an evolving chain of thoughts elaborated in the General Discussion (Chapter 8).
The eight hypotheses underlying this Thesis were as follows:

1: HWD is able to improve apple fruit quality.

2: HWD is effective in controlling all major current storage rots in Danish apples.

3: HWD is able to control existing minor storage rots on Danish apples.

4: Hot water rinsing (HWR) is an alternative to HWD.

5: The mode of action of HWT is based on killing fungal inoculum.

6: HWD is able to reduce storage-rot on artificially inoculated apples.

7: Penicillium rot increases on fruit damaged by excessive HWD temperature.

8: The reduction of Penicillium rot in artificially inoculated apples is due to an effect of HWD on the fruit, not on the fungus.

The global objectives of this study were to develop the HWD system for application in apple production systems; to optimize HWT in order to minimize risk; to quantify the efficacy of the treatment on as wide a range of storage rots as possible; and to evaluate the response of apple varieties grown in the Danish climate to HWT. In order to identify risks and understand the mode of action of HWD, excessive heat treatments were a valuable tool. A careful survey of fungi causing fruit rots of apples in NW Europe and after different types of HWT was performed.

The outcome of this research was the delivery of new knowledge that deepens our understanding of HWD. It is hoped that this knowledge will contribute to the future sustainability of fruit production (especially organic production) in Denmark.
Chapter 2: Literature review – current management strategies for postharvest decay on apples and prospects for hot water treatments

2.1 Scale of postharvest losses

Postharvest diseases make a major contribution towards the economic losses incurred during apple production. Damage resulting from bruising at harvest, insect feeding or physiological disorders reduces the acceptability of the product to the consumer, and fruit infected with fungi may also be contaminated with mycotoxins (Morales et al. 2010). These fruit cannot be sold either fresh or processed, and therefore have to be disposed of. The proportion of fruit wasted due to postharvest diseases depends on several factors, e.g. apple variety, management practice, age of the orchard, and climate during the growing season.

Figure 2.1: Fruit mummies on ‘Elstar’
Chapter 2: Literature review

The four apple varieties ‘Elstar’, ‘Ingrid Marie’, ‘Discovery’ and ‘Aroma’ together comprise a large share (44%) of the Danish harvest (see Figure 1.1). They are of particular interest in the context of this Thesis because they share the attribute of retaining fruit mummies on the tree for 12 months or more (Figure 2.1). Mummies arise from fruit naturally aborted during their normal course of development. On some apple varieties these aborted fruit remain on their position and mummify, instead of falling onto the ground. Fruit mummies may be formed at various times during the season, most frequently during the month of June. They may become colonized by several storage-rot fungi throughout the season, providing a source of inoculum for apples prior to harvest (Sutton 1981; Schulte 1997; Weber 2011). Mummy-retaining varieties such as ‘Elstar’ are known to be at an elevated risk from post-harvest diseases, especially in fruit from orchards under reduced-fungicide or organic management (Knoche et al. 2000; Holb 2008).

Even if crop protection measures have been taken and suitable storage conditions chosen, in North Western (NW) Europe losses due to postharvest diseases may be in the order of 5% to 10% in integrated production (IP), and approximately twice as high in organic production (Palm and Kruse 2005; Maxin et al. 2006; Vorstermans and Creemers 2007). In Germany, a 1% storage loss is equivalent to 9,000 t fruit with a value of approximately €3,000,000 per year, in Denmark such a loss would be equivalent to 250 t and €75,000.

2.2 Incidence of storage-rot fungi

The contribution of each fungal pathogen to the total postharvest loss varies between regions and seasons. Inoculum development in the orchard depends on local climatic conditions (temperature, precipitation, humidity and wind), fungicides used, management practices and, as discussed above, apple varieties grown.

Decay development and the incidence of different fungi are influenced by the maturity stage of the fruit when it is placed in storage (Somia and Palm 1997), and by the ripening process during storage. Ripening can be delayed by lowering the storage temperature, which may also reduce mycelial growth during the storage phase (Berrie et al. 2011). The speed of ripening can also be influenced by manipulating the gas concentration during storage. Current storage strategies are
controlled atmosphere (CA), ultra-low oxygen (ULO) and dynamically controlled atmosphere (DCA) (Lafer 2010). Yet another way to retard the fruit ripening process and decay development is the pre-storage application of the ethylene blocker 1-methylcyclopropene (1-MCP) (Rizzoli and Acler 2009). The humidity level in the storage environment also influences decay development of some fungi, whereby increased humidity tends to increase the incidence of decay (Xu and Robinson 2000; Amiri and Bompeix 2005b).

Long-term experimental storage trials (5 months) of apples from German IP orchards resulted in storage-rot infection rates of 5-15% (Palm and Kruse 2005). However, in organic production systems similar studies showed storage losses of 30-80% in the apple cultivar ‘Pinova’ (Zimmer et al. 2011) and 10-40% with different cultivars in Europe (Trapman and Jansonius 2008). No recent and comparable studies are available from Denmark. In general, by the end of a storage trial the maturity stage of the fruit is beyond that acceptable for consumption. Therefore, observed fruit losses reported in experimental trials cannot be used as a basis for predicting decay during commercial distribution and consumption phases. In addition, when considering losses from pack-houses it should be noted that only a proportion of the total fruit losses are reported; diseases that are only expressed during the subsequent shelf-life phase of the distribution chain, i.e. apples wasted during the retail period or prior to consumption in private households, are not included in the estimation of fruit loss.

An entirely different factor influencing the reported incidence of the different fungal pathogens is the way in which fruit rots have been assessed. The total quantity of rotted fruit increased when a warm storage period (e.g. 7 or 14 days) was included in the assessment to simulate retail/consumer conditions (Lafer 2010). The accuracy of identification of different fungi is highly variable across the literature. Some studies merely compared decayed and non-decayed fruit (Trierweiler et al. 2003), whereas other studies attempted to differentiate decayed fruit by macroscopic features such as e.g. lenticel rot, brown rot, Penicillium rot and Botrytis rot (Delele et al. 2012). In the most detailed studies, infected apples were stored separately until sporulation of the different fungi so that species identification with a microscope was possible (Tahir et al. 2009). *In vitro* studies of pure-culture isolates on petri dishes with subsequent identification by PCR analysis is a standard technique for reporting the identification
of new diseases (Henriquez 2005; Weber 2011). A combination of the latter two approaches (microscopical and PCR analysis) was adopted in this Thesis because of the high variety of different diseases encountered (Figure 2.2).

Figure 2.2: Different storage rots encountered in this Thesis, including Neofabraea alba (labels 229, 231, 240, 241, 242, 247, 251), N. perennans (224, 225, 230), Phacidioptenus washingtonensis (236), Botrytis cinerea (235), Monilinia fructigena (246, 252, 253) and Neonectria galligena (247).

Storage diseases in apples may be grouped according to their mode of penetration of the fruit and features of disease development (Table 2.1). Only a few fungi such as Neonectria galligena, Neofabraea and Colletotrichum spp. are able to invade an intact fruit; such infections result in lenticel rot. Spores of these fungi are splash-dispersed and washed into the lenticels of apple fruit. During storage these spores germinate and the mycelium grows into the fruit flesh. Core rot and eye rot of apples are the result of early infections during the growing season. Shortly after blossom, spores splashed by rain can invade the inner part of the young fruit through the blossom end which at this early stage still points upwards. Most frequently these rots
are caused by *Botrytis cinerea*, *Neonectria galligena* and *Fusarium* or *Alternaria* species (Jijakli and Lepoivre 2004).

Table 2.1: Postharvest diseases in stored apples (modified from Jijakli and Lepoivre 2004).

<table>
<thead>
<tr>
<th>Origin of infection</th>
<th>Disease name</th>
<th>Causal agent</th>
<th>Source of inoculum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lenticel rot</td>
<td>Bitter rot</td>
<td><em>Colletotrichum</em> spp.</td>
<td>Mummified fruit and cankers</td>
</tr>
<tr>
<td></td>
<td>Bull’s eye rot</td>
<td><em>Neofabraea perennans</em></td>
<td>Mummified fruit and cankers</td>
</tr>
<tr>
<td></td>
<td>Gloeosporium rot</td>
<td><em>Neofabraea alba</em></td>
<td>Mummified fruit, cankers and leaves</td>
</tr>
<tr>
<td></td>
<td>Nectria rot</td>
<td><em>Neonectria galligena</em></td>
<td>Mummified fruit and cankers</td>
</tr>
<tr>
<td></td>
<td>Speck rot or rubbery rot</td>
<td><em>Phacidiopycnis washingtonensis</em></td>
<td>Mummified fruit</td>
</tr>
<tr>
<td>Core rot</td>
<td>Mouldy core rot and dry core rot</td>
<td><em>Alternaria</em> spp.</td>
<td>Dry organs</td>
</tr>
<tr>
<td></td>
<td>Wet apple core rot</td>
<td><em>Fusarium</em> spp.</td>
<td>Various debris</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Fusarium avenaceum</em></td>
<td></td>
</tr>
<tr>
<td>Eye rot</td>
<td>Dry eye rot or blossom-end rot</td>
<td><em>Botrytis cinerea</em></td>
<td>Various debris and cankers</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Neonectria galligena</em></td>
<td></td>
</tr>
<tr>
<td>Wound pathogens</td>
<td>Blue mould</td>
<td><em>Penicillium</em> spp.</td>
<td>Various debris, storage bins and walls or soil</td>
</tr>
<tr>
<td></td>
<td>Grey mould</td>
<td><em>Botrytis cinerea</em></td>
<td>Various debris</td>
</tr>
<tr>
<td></td>
<td>Brown rot</td>
<td><em>Monilinia fructigena</em></td>
<td>Mummified fruit and cankers</td>
</tr>
<tr>
<td></td>
<td>Mucor soft rot</td>
<td><em>Mucor piriformis</em></td>
<td>Organic matter</td>
</tr>
<tr>
<td>Other fruitrots</td>
<td>Phytophthora rot</td>
<td><em>Phytophthora syringae</em> <em>P. cactorum</em></td>
<td>Soil and cankers</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Fusarium</em> spp.</td>
<td>Organic matter</td>
</tr>
<tr>
<td></td>
<td>Rhizopus rot</td>
<td><em>Rhizopus stolonifer</em></td>
<td>Various debris</td>
</tr>
</tbody>
</table>

Many fungi are not able to attack an intact fruit; these fungi require wounds on the fruit as a point of entry. In the orchard, wounds on fruit are caused by mammals, birds or insects as well as passing machinery, wind damage, hail or careless handling during picking.
2.3 Postharvest fruit rots of apples

The taxonomy of many fungal postharvest pathogens has been controversial which is reflected in the common usage of several different Latin names for some of them. All names in widespread circulation are listed in Table 2.2, those adopted for the remainder of this Thesis are underlined.

2.3.1. Gloeosporium rot or bull’s eye rot caused by *Neofabraea* spp.

In NW Europe the most common storage rots are caused by *Neofabraea alba* and its sister species *Neofabraea perennans*. Both species produce conidiomata on the surface of decayed areas of the fruit after prolonged storage (Figure 2.3A,C). These are readily distinguished by microscopic features of their conidia which are asymmetrically elongated in *N. perennans* (Figure 2.3D) but strongly curved in *N. alba* (Figure 2.3B).

Both these pathogens are typically prevalent in mild humid climates with high precipitation during the summer months. *N. perennans* has been reported to cause significant storage losses all over the world. It is the most common cause of storage rot in the Lower Elbe region of Northern Germany (Palm and Kruse 2005). Taken together, *N. perennans* and *N. alba* contribute approximately 75% of the total storage decay in that region (Weber 2009b). In the Pacific NW of the US, high apple losses were attributed to *N. perennans* (Gariepy et al. 2003; Gariepy et al. 2005; Spotts et al. 2009), and this fungus was also reported in Brazil (Henriquez et al. 2006). Cunnington (2004) revised Australian reports of *N. malicorticis*, concluding that all herbarium specimens collected after 1970 were, in fact, *N. perennans*. In Germany, long-term evaluations carried out from 1950 showed that the incidence of *N. perennans* and *N. alba* followed a North to South gradient; while the ratio of these two species was 3:1 in Northern Germany, the majority of fruit at Lake Constance in the deep south of Germany were infected by *N. alba*, a 1:1 ratio for these two rots being found in central regions of the country (Kennel 1988; Weber 2009b). A share of both rots in the same orchard has been reported in the USA on pears, an alternative host, where the ratio of *N. alba* to *N. perennans* was 3:1 (Henriquez et al. 2004). In Sweden, surveys on apple storage rots described *N. alba* as being the predominant rot (Tahir et al. 2009). *Neofabraea* spp. were able to cause postharvest crop losses of 10% in IP produce (Palm and Kruse 2005) and 40% in fruit from organically managed orchards.
(Zimmer et al. 2011). Fruit in these experimental trials were stored for an extremely long time period, therefore it is likely that commercial fruit losses from these orchards would have been lower. Assuming a price of €0.33 kg\(^{-1}\) and an average annual harvest of 330,000 t for the Lower Elbe region, an incidence of Gloeosporium rot of only 1\% of the total fruit in storage would be equivalent to a loss of approximately €1,000,000.

Table 2.2: Anamorph and teleomorph states of different storage-rot fungi (partly adapted from Weber 2009b). Currently valid taxonomic names are printed in **bold**; names used in this Thesis are underlined.

<table>
<thead>
<tr>
<th>Anamorph</th>
<th>Teleomorph</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Phlyctema vagabunda</em></td>
<td><em>Neofabraea alba</em></td>
</tr>
<tr>
<td>Gloeosporium album</td>
<td><em>Pezicula alba</em></td>
</tr>
<tr>
<td><em>Cryptosporiopsis perenanns</em></td>
<td><em>Neofabraea perenanns</em></td>
</tr>
<tr>
<td>Gloeosporium perenanns</td>
<td><em>Pezicula malicorticis</em></td>
</tr>
<tr>
<td><em>Colletotrichum acutatum</em></td>
<td><em>Glomerella acutata</em></td>
</tr>
<tr>
<td>Colletotrichum xanthii</td>
<td></td>
</tr>
<tr>
<td><em>Cylindrocarpon mali</em></td>
<td><em>Neonectria galligena</em></td>
</tr>
<tr>
<td>Cylindrocarpon heteronema</td>
<td><em>Nectria galligena</em></td>
</tr>
<tr>
<td><em>Botrytis cinerea</em></td>
<td><em>Botryotinia fuckeliana</em></td>
</tr>
<tr>
<td><em>Monilia fructigena</em></td>
<td><em>Monilinia fructigena</em></td>
</tr>
<tr>
<td><em>Fusarium avenaceum</em></td>
<td><em>Gibberella avenacea</em></td>
</tr>
<tr>
<td><em>Phacidiopycnis</em></td>
<td>(unknown)</td>
</tr>
<tr>
<td><em>washingtonensis</em></td>
<td></td>
</tr>
<tr>
<td><em>Mucor piriformis</em></td>
<td><em>Mucor piriformis</em></td>
</tr>
<tr>
<td><em>Boerema exigua</em></td>
<td>(unknown)</td>
</tr>
<tr>
<td><em>Phoma exigua</em></td>
<td></td>
</tr>
</tbody>
</table>

Hot water dipping (HWD) for various times at temperatures of 45-53°C has given high efficacies in controlling Gloeosporium rots on apples (Burchill 1964; Trieweiler 2003; Maxin et al. 2005). As yet, no results for hot water rinsing (HWR) against this important storage rot have been published.
2.3.2 Blossom-end and lenticel rots caused by *Neonectria galligena*

*Neonectria galligena* is present in most orchards in NW Europe and is responsible for causing fruit tree canker. In freshly planted orchards the source of primary inoculum is often introduced with the trees directly from the nursery (McCracken et al. 2003), or it invades as airborne ascospores from established neighboring orchards (Swinburne 1975). Infection of twigs and branches occurs all year round if wetness periods and temperatures are suitable (Swinburne 1975; Xu and Robinson 2010). Globally, the presence of *N. galligena* in orchards has been correlated to months with >30% of the days having rainfall and an average temperature of 11-16°C for more than 8 h per day (Beresford and Kim 2011). Sporulation, spore dispersal and infections of *N. galligena* are directly linked with rainfall (McCracken et al. 2003).

Fruit infections by *N. galligena* can be categorized into two groups, i.e. eye rot (blossom-end rot) and lenticel rot (Table 2.1). The former develops from infections during blossom time, and the infection becomes clearly visible at the blossom end of the fruit by harvest time (Xu and Robinson 2010). Lenticel rot (Figure 2.3e) occurs during storage after a latency interval of several weeks as a result of infections originating from the field (Xu and Robinson 2010). During further storage, pale brown sporodochia of *N. galligena* are formed; these produce the highly diagnostic, elongated, 1- to 5-septate macroconidia of the *Cylindrocarpon* anamorph (Figure 2.3f).

Lenticel rot caused by *N. galligena* has not so far been reported to be controlled by HWD. In fact, Maxin et al. (2005) found that its incidence increased after 1 min HWD at 49, 51 and 53°C compared to an untreated control, whereas there was no positive or negative influence on decay by 2 and 3 min HWD treatments.

2.3.3 Rubbery rot caused by *Phacidiopycnis washingtonensis*

*Phacidiopycnis washingtonensis* has only recently been identified as the cause of a fruit rot in American (Kim and Xiao 2006) and European (Weber 2011) apple production. From 2003–2006, in approximately 25% of the surveyed orchards of Washington State (USA), 1-4% of the apples were infected with *P. washingtonensis* (Kim and Xiao 2006). Following 9 months’ storage up to 24% losses caused by *P. washingtonensis* were observed in ‘Red Delicious’ apples. Depending on the apple variety, invasion of *P. washingtonensis* occurred through stem end, calyx end,
lenticels or wounds (Kim and Xiao 2006; Weber 2012). The first report of \textit{P. washingtonensis} on apples in Europe was from the Lower Elbe region in Northern Germany, where fruit losses were below 1\% in most orchards (Weber 2011). Mummified fruits of ‘Golden Hornet’ crabapples commonly planted as pollinators were identified as a major source of inoculum; fruit mummies of dessert apple varieties such as ‘Estar’ were also infected, albeit to a lesser degree (Weber 2011). In Europe, cankers caused by \textit{P. washingtonensis} have not yet been found. Mycelium of \textit{P. washingtonensis} may spread from an infected fruit to an adjacent intact fruit, causing clusters of rubbery rot (Weber 2012).

Rubbery rot is a highly variable disease, appearing as a very pale but firm rot when fruit are removed from long-term storage under an atmosphere of reduced oxygen content, but rapidly undergoing a browning if sufficient oxygen becomes available during subsequent cold storage or at room temperature (Weber 2012). Conidiomata (pycnidia) are produced only on blackened fruit (Figure 2.3g). These extrude pale grey or yellow tendrils or drops of conidia which are hyaline, ellipsoid with pointed ends in outline, and contain two large or several smaller lipid droplets (Figure 2.3h). To date there are no reports on the efficacy of HWD on rubbery rot.

\subsection*{2.3.4 Bitter rot (anthracnose) caused by \textit{Colletotrichum acutatum}}

\textit{Colletotrichum acutatum} has a wide range of hosts; both trees and fruit are infected by this pathogen. In Northern Europe, high pre- and postharvest fruit losses in strawberries, blueberries and cherries were attributed to \textit{C. acutatum} (Sundelin et al. 2005; Børve and Stensvand 2006; Stensvand et al. 2006). \textit{C. acutatum} has been reported as the most prevalent storage pathogen in Norway (Weber 2009b; Børve et al. 2010). In the rest of NW Europe, the relative importance of this fungus in stored apples is low, although losses may be on the increase (Weber 2009a). In subtropical and tropical areas, \textit{C. acutatum} as well as \textit{C. gloeosporioides} are well known pathogens (Peres et al. 2005). In apple orchards, conidia are splash-dispersed from buds formed during the previous growing season, and these may infect young leaves and fruit (Børve and Stensvand 2007). Fruit mummies and twig cankers cause infections on the maturing apples (Stensvand and Ren 2010). During storage, the development of \textit{C. acutatum} decay is similar to \textit{Neofabraea} spp. in that mycelium appears to grow from fungal spores deposited in the lenticels, colonizing the apple
within some weeks. The resulting lesion appears sunken and is often covered by dark grey mycelium extruding yellowish-pink spore drops (Figure 2.3I). Conidia are readily distinguished from *N. perennans* and *N. alba* in being straight, symmetrical and somewhat pointed at one or both ends (Figure 2.3J). However, a reliable distinction between *C. acutatum* and *C. gloeosporioides* is only possible by PCR analysis. Therefore it is important to determine by PCR if reports of infections of Swedish apples with *G. cingulata* (Tahir et al. 2009), teleomorph of *C. gloeosporioides*, can be confirmed.

2.3.5 Brown rot caused by *Monilinia fructigena*

Brown rot caused by *Monilinia fructigena* is an important disease in apple orchards in Europe (Jijakli and Lepoivre 2004). High fruit losses due to *M. fructigena* have been reported in Hungary (Holb 2008), where up to 40% of apples were infected during the production phase in organically managed orchards. In comparison, orchards under IP management suffered losses up to 10% (Holb 2008). In the humid climate of NW Europe, the importance of *M. fructigena* is lower, 5% fruit loss from this fungus being regarded as high (Palm and Kruse 2005).

*M. fructigena* is a wound pathogen on apple fruit (Xu and Robinson 2000). Preharvest infections commonly occur in fruit that have been attacked by biting insects such as larvae of codling moth (*Cydia pomonella*) and leaf rollers (e.g. *Adoxophyes orana*). Mechanical damage caused by wind, birds, hailstorms or passing machinery in the alley ways may also result in *M. fructigena* infections (Holb 2008). The ability of *M. fructigena* to infect a wound depends on the developmental stage of the fruit, wound age and the presence of free water or relative humidity above 97%. The highest rates of infection from *M. fructigena* have been observed in freshly (<24 h) wounded mature fruit at about 20-25°C (Xu and Robinson 2000; Xu et al. 2001).

In the field, fruit may become infected with *M. fructigena* during two time periods; in May when overwintering fruit mummies both in and under trees sporulate to release airborne conidia, and in June when infected fruit drop from the trees and then provide a source of spore inoculum for subsequent storage infections (Holb and Scherm 2007). The infection cycle may be interrupted by the early removal of fruit that have been mechanically or naturally thinned, from the orchard floor. The production of *M. fructigena* spores on infected fruit is induced by light (van Leeuwen
and van Kesteren 1998). During storage in darkness, therefore, infections from one infected fruit to another wounded fruit are interrupted due to the absence of sporulation. In storage, infected fruit become entirely colonised by *M. fructigena*. While the fruit remains firm and retains its original shape, it turns pitch-black in colour (Figure 2.3k). At this late stage, brown rot may be confused with rubbery rot (Figure 2.3g) especially if an investigator is unfamiliar with the latter (Weber 2012). Conidia of *M. fructigena*, if present, are produced from grey cushions of aerial mycelium. These are formed at the tips of apically elongating chains which break up upon contact with water (Figure 2.3l).

Brown rot is an imported disease on peaches and nectarines as well, and a number of studies have shown high efficacies of hot water treatments (HWT) on this disease (Mari et al. 2007; Casals et al. 2010). HWD controls brown rot on nectarines at dipping times of 2 min at temperatures of 40°C. A brief rinse of 20 s at 60°C has also been effective (Mari et al. 2007). HWD of apples has reduced brown rot by 50% at 3 min dipping at 49-53°C (Maxin et al. 2005).

### 2.3.6 Penicillium rot

In Europe, Penicillium rot is mostly caused by three *Penicillium* species, i.e. *P. expansum*, *P. digitatum* and *P. solitum*. Other species such as *P. commune*, *P. verrucosum*, *P. chrysogenum* or *P. rugulosum* only add a very low share to the complex of Penicillium rots (Amiri and Bompeix 2005a). Penicillium rot is easily recognized by a relatively pale, soft lesion on which blue or green conidial pustules are produced (Figure 2.3m). Conidia are globose to subglobose and very small, typically <5 µm (Figure 2.3n). The use of PCR-based methods is helpful for a safe discrimination of the various *Penicillium* spp. which may be involved in blue mould.

Penicillium rot is one of the few postharvest diseases that can arise from infections of fruit during the storage phase (Amiri and Bompeix 2005a). Airborne spores of *Penicillium* spp. are normally unable to penetrate the intact fruit skin, except for lenticels of overripe fruit. Therefore, in most situations infections depend on the fruit being wounded. Suitable wounds for infections by *Penicillium* spp. are pre-harvest insect damage, harvest wounds caused by fingernails, and severe bruising during handling (Amiri and Bompeix 2005c). *P. expansum* may become hyperparasitic.
causing infections on fruit pre-infected by other pathogens and parasitizing their mycelium.

*P. expansum* spores are abundant in soil (Jijakli and Lepoivre 2004); therefore this pathogen can contaminate apples during the preharvest phase. During wet conditions at harvest, contamination of apple boxes with soil in the field may also provide a source of inoculum for the subsequent storage phase (Morales et al. 2010). Spores of *Penicillium* spp. are present almost everywhere and they have a long survival period (Amiri and Bompeix 2005a). Spores may survive from season to season on picking boxes, contaminated bins and on the walls of the storage rooms. Disinfection treatments may help reduce the buildup of inoculum in the storage environment (Amiri and Bompeix 2005a). Spores have also been reported to accumulate in postharvest drench solutions, in flotation tanks, and on packing lines (Morales et al. 2010).

Despite its prevalence, losses in NW Europe attributed to this fungus are generally lower than 1% (Palm and Kruse 2005). In warmer climates, such as Israel and southern France, *P. expansum* is reported as being the most severe storage pathogen, where losses may exceed 10% (Amiri and Bompeix 2010; Fallik et al. 1996). Processing apples infected with *P. expansum* may be contaminated with the mycotoxin patulin (Morales et al. 2010).

The potential of HWT to control *P. expansum* has been critically examined because control efficacies have varied widely in different experimental designs (Spotts and Chen 1987; Fallik et al. 2001; Spadaro et al. 2004; Amiri and Bompeix 2011). Even on artificially inoculated fruit, contrasting results have been obtained, e.g. high efficacies of HWR (Fallik et al. 2001) but low and temporary effects of HWD (Amiri and Bompeix 2011).

### 2.3.7 Grey mould caused by *Botrytis cinerea*

*Botrytis cinerea* is a ubiquitous fungus that infects a wide range of horticultural and agricultural crops. Conidia are abundant and colonize organic matter on the ground. They are readily produced on dead infected plant organs and are dispersed by water splash and air currents. In countries with more continental and arid climates such as France, Belgium or Washington State (USA) and possibly as a result of
different postharvest fungicide treatments, *B. cinerea* may contribute up to 28% to the total postharvest losses (Kader 2002; Jijakli and Lepoivre 2004; Kim and Xiao 2008). In NW Europe grey mould is a minor disease (Berrie 2007; Weber 2009b).

Apples are infected by *B. cinerea* through wounds caused by handling processes and bruising during harvest. The resulting rot is pale brown and of intermediate firmness in texture. Aerial mycelium is produced in coarse bunches and may develop grey clusters of conidiophores in cold storage (Figure 2.3o). Macroconidia are produced at the branched tips of conidiophores in a polyblastic manner (Figure 2.3p). To avoid contamination with *Botrytis* during storage, apples that have fallen to the orchard ground should not be placed in long-term storage. *Botrytis* rot is known as a nesting fungus because infections can spread within storage bins from apple to apple. Therefore, even low numbers of infected fruit may give rise to significant losses because fruit may rot in less than 3 weeks following initial contact (Jijakli and Lepoivre 2004). *Botrytis* infections may give rise to an immense within-sample variation in apple storage trials if fruit are not examined regularly, especially under conditions of low natural infection rates in NW Europe. In this respect grey mould is similar to rubbery rot.

HWD has been carried out to control Botrytis rot of apples (Spadaro et al. 2004), but acceptable control levels were achievable only in combination with different additives or with yeasts. However, HWR was effective in controlling grey mould on pepper, tomatoes and melons (Fallik 2004).

### 2.3.8 Mucor soft rot

Mucor soft rot is an infrequently observed storage disease in NW Europe. However, *Mucor* spp. can spread in storage even at low temperatures (<4°C) and therefore, if present, they have potential to infect several adjacent apples. The resulting fruit rot (Figure 2.3q) is exceptionally soft, infected apples often bursting to release their liquefied contents. A coarse whitish aerial mycelium develops sporangia which are visible to the naked eye as small greyish-black objects. Sporangiospores are large and irregularly globose in shape (Figure 2.3r). The most important fungus associated with Mucor soft rot is *Mucor piriformis*. Its sporangiospores are present in the soil and in organic matter (Guo and Michailides 1997). Infection of apples is possible if spores
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are dislodged by rain or if fruit come into contact with soil. If soil on harvest bins is transferred into storage or into dump tanks, contamination of wounded fruit during storage is possible. In the western USA, high losses in apple and pear production have been reported from Mucor soft rot (Kader 2002).

High-pressure HWTs were able to reduce decay by Mucor rot on pears with a very low efficacy of 29% (Spotts et al. 2006)

2.3.9 Rhizopus soft rot

*Rhizopus stolonifer* has a range of hosts and it is the causal agent of Rhizopus soft rot. Some susceptible genera include *Allium, Ananas, Brassica, Cucumis, Cucurbita, Fragaria, Lycopersicon, Phaseolus, Pisum* and *Solanum* (Lunn 1977). This fungus has a high temperature optimum (27°C), but because of its inability to grow or germinate below 4°C (Pierson 1966) *Rhizopus* is a minor storage-rot pathogen in apples. Due to its temperature requirements, *Rhizopus* can only infect fruit through wounds as a result of pre-harvest damage. *Rhizopus* spores are common in orchards on debris and in the soil. Airborne spores are sometimes found in pack-houses. Infected fruit are often covered by a coarse, grey, hairy mycelium that form a mass of black sporangia at their tips. Infected fruit emit a sour odor. Spread of the fungus between adjacent fruit is possible where temperatures are >4°C, e.g. during transport and retail phases (Kwon et al. 2011)

Short HWD periods for 30 s at 55 and 60°C were able to reduce *Rhizopus stolonifer* decay on strawberries (Zhand et al. 2007). No results seem to have been published for apples as yet.

2.3.10 Wet-core rot caused by *Fusarium* spp.

*Fusarium* spp. have a wide host range but are a minor storage rot in apples. The disease incidence is generally below 1%, although in some cases more than 5% have been reported (Weber 2009b). *Fusarium* infections often begin as wet apple core rot, later spreading outwards to colonize the entire fruit. Whereas abundant white, yellow or pink aerial mycelium may develop on infected fruit (Figure 2.3s), sporulation is frequently absent. Wet growing conditions in the early season, when apple fruit point their blossom end upwards on the tree, enable spore-contaminated water to enter the flower, thereby initiating infections. Depending on the variety, this early infection
of the apple core leads to an increased incidence of Fusarium rot in storage (Sørensen et al. 2009).

Several *Fusarium* spp. have been controlled by the use of hot water in a broad range of commodities. For example, infections of potatoes by *Fusarium solani* were reduced following a 20-30 min dip at 57.5°C (Ranganna et al. 1998), and in melons Fusarium and anthracnose rot were controlled by HWR of 20 s at 59°C (Fallik et al. 2000). To date, control of Fusarium rot of apples by HWT has not yet been reported.

2.3.11 Diseases caused by *Phoma* spp.

Fruit spots caused by *Phoma* spp. on apple fruit have not been reported to cause any economic losses, but this genus is ubiquitous and has a broad range of hosts and substrates to grow on. *Phoma exigua, P. macrostoma* and *P. glomerata* are less frequently reported as a cause of apple leaf and fruit spots than *P. pomorum* (Jones and Aldwinckle 1990). *Phoma* spp. are considered to be weak wound pathogen of apple fruit, and lenticel spots are formed by these fungi at a late stage of saprophytic growth (Jones and Aldwinckle 1990). It is conceivable that such limited infections may develop into a storage rot under favourable conditions, as observed in this Thesis (Figure 2.3.1).

Many *Phoma* spp. are seed-borne, and HWT protocols have been established to control seed-borne diseases in some vegetables, especially for seeds to be used in organic farming (Nega et al. 2003). No specific results of HWT against *Phoma* spp. on apples have been published.
2.4 Invasions of new fungi (neomycota)

Reports on storage losses and the incidence of storage-rot fungi do not reflect the dynamic changes in the composition of fungal pathogens present in any given region over time. Such changes may be due to the occurrence of new species such as *Diplodia seriata* (Quast and Weber 2008) or *P. washingtonensis* (Kim et al. 2005; Weber 2011). From 1961 until 2000, climate change in NW Europe has contributed to an increase in the average temperature by about 0.37 K per decade (Chmielewski et al. 2004). The respective temperature increase for Aarslev, Denmark was estimated at 0.32 K per decade, based on data available from the Danish Meteorological
Institute (DMI) (Figure 2.4). From 1900 to 2005, global warming has been estimated at 0.74 K (Solomon et al. 2007), although this estimate is markedly less than that experienced in NW Europe, where such a temperature increase has been recorded over the last three decades.

Observations of *Diplodia seriata* as a new pathogen in apple production in the Lower Elbe region are probably connected with higher temperatures during the vegetation period (Quast and Weber 2008; Trapman et al. 2008). This fungus is the cause of black rot, a pre-harvest disease of apples. Higher temperatures during the growing season should also favour insect pests (Huang et al. 2011). If global warming continues, most likely the spectrum of climatically limited pests and diseases will change within any given region, and this will have a bearing on the cultivars that can be grown. Management practices may have to be adapted accordingly. So the status of today’s knowledge of fruit cultivation and management of pests and diseases in Northern Germany may become extremely relevant to fruit producers in Denmark (200-300 km further north) within the next few decades.

Figure 2.4: Climate change in Denmark, based on mean annual air temperature at Aarslev (55°18' N, 10°26' E, altitude 47 m). Data obtained with permission from the Danish Meteorological Institute.
A change in the incidence of fungi may also result from the application of new fungicides with a high efficacy against particular species. Such was the case with the introduction of Benomyl in 1968 which gave effective control of *Neofabraea* spp. but permitted a rise in importance of *N. galligena* rot in the Lower Elbe region in the 1970s (Weber and Palm 2010).

Trade activities represent a third important factor favouring new pests and diseases (Anderson et al. 2004). There are several historic examples of global trade being responsible for the introduction of neobiota with catastrophic consequences, as in the case of potato blight caused by *Phytophthora infestans* in Europe in 1845-1848 (Large 1940; Bourke 1991). An important current example threatening fruit production in Europe is the vinegar fruit fly, *Drosophila suzukii*, which originates from South East Asia (Calabria et al. 2011).

### 2.5 Chemical control of storage rots

In Europe, fungicides are applied from bud break until the end of July in most apple orchards to prevent apple scab (*Venturia inaequalis*) and powdery mildew (e.g. *Podosphaera leucotricha*) infections. Infection conditions favouring these two fungi are well known, and knowledge of effective fungicide applications is established in most growing regions. Apple scab infections are suppressed with surface protectant (contact) fungicides such as phthalimides (Captan), dithiocarbamates (Mancozeb) or dithianon (Delan) (Table 2.3). In contrast, curative or eradicant fungicides such as strobilurins (trifloxistrobin), which are absorbed and translocated within plant tissues, are used to control powdery mildew during the growing season. Curative fungicides are expected to make a more substantial contribution to the control of storage rots than contact fungicides (Minar 2006; Granado et al. 2008). In August and September, fungicides are specifically applied to prevent infection of the fruit by storage diseases (Palm and Kruse 2007, Weber and Palm 2010). These preharvest fungicide applications are traditionally used to control apple storage rots in both IP and conventional production systems in Denmark and Germany, but in general the efficacy of these treatments is limited to 50% (Palm and Kruse 2007). Overviews of the registered compounds against scab and powdery mildew (Table 2.3), and for the control of storage rots (Table 2.4), are provided.
Table 2.3: Fungicides registered for scab and powdery mildew control in apple production in Germany and Denmark in 2011.

<table>
<thead>
<tr>
<th>Registered trade name</th>
<th>Active ingredient</th>
<th>Registered use in Germany (G) and Denmark (DK)</th>
<th>Minimum days before harvest G/DK</th>
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</thead>
<tbody>
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<td>Kresoxim-methyl</td>
<td>G; DK</td>
<td>35/70</td>
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<tr>
<td>Delan WG</td>
<td>Dithianon</td>
<td>G; DK</td>
<td>21/21</td>
</tr>
<tr>
<td>Topas</td>
<td>Penconazol</td>
<td>G</td>
<td>14</td>
</tr>
<tr>
<td>Flint</td>
<td>Trifloxystrobin</td>
<td>G</td>
<td>7</td>
</tr>
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<td>Systhane 20 EW</td>
<td>Myclobutanil</td>
<td>G</td>
<td>-</td>
</tr>
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<td>Copper oxychloride</td>
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<td>Score</td>
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<td>Pyraclostrobin</td>
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<tr>
<td></td>
<td>Dithianon</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Consist Plus</td>
<td>Captan</td>
<td>G</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Trifloxystrobin</td>
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<td></td>
</tr>
<tr>
<td>Malvin, Merpan 80 WDG</td>
<td>Captan</td>
<td>G</td>
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<tr>
<td>Dithane NeoTec</td>
<td>Mancozeb</td>
<td>DK</td>
<td>28</td>
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<td>Kumulus/Sulphur</td>
<td>Sulphur</td>
<td>G; DK</td>
<td>7/7</td>
</tr>
<tr>
<td>Scala</td>
<td>Pyrimethanil</td>
<td>DK</td>
<td>28</td>
</tr>
</tbody>
</table>

No postharvest chemical applications are allowed in Germany and Denmark, with the exception of 1-MCP during storage. Imazalil is registered in Spain and Portugal for postharvest dipping of apples. In Belgium, Netherlands, Spain and Italy, the use of Philabuster (Imazalil and Pyrimethanil) as a postharvest dipping treatment is permitted for pears (Vorstermans et al. 2005).
Table 2.4: Fungicides registered as preharvest applications for the control of storage rots in apple production in Germany and Denmark in 2011 and 2012.

<table>
<thead>
<tr>
<th>Registered trade name</th>
<th>Active ingredient</th>
<th>Registered use in Germany (G) and Denmark (DK)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bellis</td>
<td>Pyraclostrobin Boscalid</td>
<td>G</td>
</tr>
<tr>
<td>Signum WG</td>
<td>Pyraclostrobin Boscalid</td>
<td>DK</td>
</tr>
<tr>
<td>Switch</td>
<td>Cypprodinil Fludioxonil</td>
<td>G</td>
</tr>
<tr>
<td>Cercobin FL</td>
<td>Thiophanate-methyl</td>
<td>G</td>
</tr>
<tr>
<td>Consist Plus</td>
<td>Captan Trifloxystrobin</td>
<td>G</td>
</tr>
<tr>
<td>Flint</td>
<td>Trifloxystrobin</td>
<td>G</td>
</tr>
<tr>
<td>Merpan80 WDG</td>
<td>Captan</td>
<td>G</td>
</tr>
</tbody>
</table>

2.6 Fungicides under pressure

The future of fungicide applications as a major strategy to control postharvest disease looks uncertain. Three major trends which may negatively influence fungicide-based crop protection are discussed below.

2.6.1 Public awareness

A survey of the European Food Safety Authority (EFSA) asked 26,900 people in all member states on their view of risks linked to food intake. It was found that 72% of EU citizens were “totally worried” about “pesticide residues in fruit, vegetables or cereals”, this was a 2% increase compared to the 2005 survey (EFSA 2010). Non-governmental organisations, such as Greenpeace and PAN (Pesticide Action Network) described possible risks and published their own surveys of residues in fresh fruit and vegetables in different media in several European countries (Poulsen et al. 2009). Media campaigns were not targeted at the producers or the chemical industries, but focused on retailers even if their products did not exceed governmental Maximum Residues Levels (MRL) and Acute Reference Doses (ARfD). In response to the public focus and in an attempt to avoid future negative press, retailers in Germany
established individual guidelines that limited residues on fruit and vegetables to levels well below the governmental MRLs (Poulsen et al. 2009), or even restricted the maximum number of detectable residues on products, thereby avoiding any concerns with ARfDs. The retailers then challenged the producers and the importers to meet these guidelines (Palm 2009). In 2010, Danish retailers also established standards comparable to the German standards. In Denmark and Germany most organically produced fruit and vegetables are not treated with any synthetic or inorganic chemicals that leave detectable residues. This offers consumers the choice of selecting a residue-free product.

2.6.2 Political framework

In the EU, long-term political programs are focused on reducing the general use of pesticides. The relevant 1993 EU directive (91/414/EEC) has involved reevaluating established pesticides and has led to a reduction of registered compounds by nearly 75%.

"As laid down in Directive 91/414/EEC, in 1993 the European Commission launched the work program on the Community-wide review for all active substances used in plant protection products within the European Union. In this review process, each substance had to be evaluated as to whether it could be used safely with respect to human health (consumers, farmers, local residents and passers-by) and the environment, in particular groundwater and non-target organisms, such as birds, mammals, earthworms, bees.”…"The review of existing pesticides has led to the removal from the market of pesticides which cannot be used safely. Of some 1 000 active substances on the market in at least one Member State before 1993, 26%, corresponding to about 250 substances, have passed the harmonised EU safety assessment. The majority of substances (67%) have been eliminated because dossiers were either not submitted, incomplete or withdrawn by industry. About 70 substances failed the review and have been removed from the market, because the evaluation carried out did not show safe use with respect to human health and the environment” (GD Health & Consumers 2009).

A new directive (2009/128/EC) is currently being introduced into all member states and the overall goal is to “establish a framework for Community action to achieve the sustainable use of pesticides” (GD Health & Consumers 2009). This new directive now
provides the framework for future pesticide use in the EU through higher and harmonised standards in the registration procedure of new chemical compounds to be used in crop protection. The directive also enables registrations that exist in one country to be more easily or automatically accepted in another country. The EU is now divided in three climatic areas; Denmark is part of the Scandinavian group, whereas Germany is part of the Continental group, and a third group includes the Mediterranean countries. Once a compound is registered in one country, accreditation of this registration by other countries within the same climatic area is facilitated. By creating harmonised standards in the registration process and at the same time encouraging accreditation systems, the availability of compounds for orchardists could well change between Denmark and Germany in the future.

2.6.3 Resistance of fungi to fungicides

Spraying chemicals may fail the purpose of protecting the crop if target organisms change their susceptibility to the mode of action. First reports of resistance of storage-rot fungi *Botrytis cinerea* (Bollen and Scholten 1971) and *Penicillium* spp. (Bollen 1971) against benzimidazoles were published shortly after introduction of the compound Benomyl by DuPont in 1968. Subsequently, several benzimidazoles have been introduced into the market and the fungi have adapted to these chemicals. *N. perennans* and *N. album* have developed resistance to all benzimidazoles (Weber and Palm 2010). Risk management of resistance is addressed through a limited use of compounds per season or by releasing formulations with two active ingredients such as “Bellis” containing pyraclostrobin and boscalid. However, the double-compound strategy leaves multiple residues on the fruit and may challenge ARfD limits. In this respect, a multi-site (protectant) fungicide would be preferable. However, at this time captan is the only surface protectant fungicide with a multi-site action registered in Germany and Denmark (Table 2.3).

2.7 Control with heat, GRAS yeasts and hygiene

The use of antagonistic microorganisms to prevent storage decay in apples has been reported (Leibinger et al. 1997). Initial products on the market include “Boni protect” based on *Aureobasidium pullulans*, which is available in Germany and Denmark (Weiss et al. 2006). Generally regarded as safe (GRAS) compounds have recently been reported to have efficacy in controlling storage rots. Mycosin (aluminium sulfate
Acidified clay minerals, yeast and *Equisetum* extracts) and Ulmasud (aluminium oxide acidified clay minerals, silica oxide, sulphur) are used in organic farming in Europe to prevent storage decay caused by *Neofabraea* spp. (Trapman and Jansonius 2008; Zimmer et al. 2011), even though results are inconsistent. Therefore, numerous applications (6-8) of Mycosin are recommended per season (Zimmer et al. 2011).

Sanitation in the orchard environment is becoming more and more important in organic fruit production (Trapman and Jansonius 2008). In several organically managed orchards in the Lower Elbe region of Northern Germany, mummified fruit are routinely removed during winter, and benefits of such an approach have been reported in the literature (Holb et al. 2011).

### 2.8 Heat treatments

In the past 30 years four ways of heat treatments have been developed for a range of freshly harvested commodities i.e. HWD, vapor heat, hot dry air and HWR with brushing (Schirra et al. 2000). Beneficial effects of these heat treatments are linked to three areas of postharvest quality improvement. Improvements have been reported through changes in physiological processes such as a reduction of chilling injury and delay of ripening processes (Lurie 1998, Ben Yehoshua et al. 1987), killing of critical insect contaminations (Yokoyama et al. 1991), and control of fungal decay in a range of commodities (Schirra et al. 2000). Heat treatments may be divided into short-term (up to 1 h duration) and long-term (14 h to 4 d). Curing is a synonym for long-term heat treatments (Schirra et al. 2000). Water vapour heat (saturated water vapour) or hot dry air in long-term treatments have been used depending on crop and desired effects.

Citrus fruit have played an important role in characterising fundamental aspects of curing. Curing of grapefruit may reduce chilling injury in subsequent storage (Lurie 1998), and it may suppress decay caused by *Penicillium digitatum* and *P. italicum* (Ben Yehoshua et al. 1987). Curing treatments have been introduced in fruit industry for a broad range of subtropical crops such as avocado (Woolf et al. 1995), mango (McCullun 1993), sweet persimmon (Woolf et al. 1997) or Cucurbitaceae (Wang 1994) to prevent cold-temperature injury in subsequent storage. For suitable crops,
curing treatment may be brought about by heating greenhouses prior to harvest (S. Lurie pers. communication). Curing of apples (37°C for 4 d) may improve their shelf life (Conway et al. 2004) and reduce decay by *P. expansum* (Fallik et al. 1996). A three-day treatment at 38°C has been shown to improve apple fruit firmness (Fallik et al. 2001; Tahir et al. 2009), but to the best of our knowledge hot air treatment has not been implemented into the apple fruit industry, perhaps because of high energy costs.

Curing and HWD of fruit is also used to overcome import restrictions aimed against critical insect pests (Sharp and Spalding 1984). A practical example is the decontamination of walnuts from codling moth (*Cydia pomonella*) by heating (Yokoyama et al. 1991). Heating has subsequently been combined with controlled-atmosphere (CA) storage as a standard treatment for critical quarantine trades (Neven and Mitcham 1996).

Hot water is a better vector of energy than air and has provided comparable reductions in fungal decay. Blue mould of grapefruit caused by *P. italicum* or *P. digitatum* has been controlled by dipping fruit for 2 min at 50°C (Schirra et al. 2000). HWR with brushing has been introduced in Israel to control decay and improve quality of a range of commodities. Improvements in the quality of bell pepper, apples, melons, sweetcorn, kumquat, and grapefruit have been reported following postharvest transportation of the products on rotating brushes and treatment with a cold water cleaning and a short hot water rinse (15-25 s at 55-65°C) (Fallik 2004). This combination of HWR with brushing has been patented (Fallik et al. 1999, 2007).

### 2.8.1 Modes of action of HWT

Several modes of action including increasing cold stress tolerance, improved shelf life, and control of postharvest pathogens have been discussed to explain the benefits of heat treatments on a broad range of commodities.

A clear mode of action of any water treatment is to wash-off the spores from the fruit surface (Schirra et al. 2000; Spotts et al. 2006).

Another mode of action is the heat-inactivation of fungal spores and mycelia by HWT (Fallik et al. 1993). This effect has been described for many fungi, e.g. *Botrytis cinerea*, *Alternaria alternata*, *Colletotrichum gloeosporioides*, *Dothiorella daminicana* or
Fusarium solani (Rappel et al. 1991; Fallik et al. 1996, 2000). Mycelial discs of Neofabraea alba were also inactivated by comparable HWT of 3-10 min at 45-52°C (Schirmer et al. 2004; Amiri et al. 2009). Certain combinations of dipping time and temperature were ineffective at killing spores outright, but caused a beneficial delay in germination e.g. in P. expansum (Fallik et al. 1996). Spore germination as well as mycelial growth on grapefruit peel discs inoculated with P. digitatum were decreased and delayed by HWD at 52°C for 2 min (Schirra et al. 2000). The delay of germination of some fungi may enable the plant tissue to develop an effective defence response mechanism (Schirra et al. 2000).

An additional mode of action is that preharvest heat has been reported as a stimulus of heat shock proteins (HSPs) in fruit (Woolf and Ferguson 2000). This stress-induced transcription of proteins involved not only HSPs but also pathogenesis-related proteins (PRP) (Schirra et al. 2000; Pavoncello et al. 2001). HSPs in apples were induced by heating cell suspensions at 38°C for 1 h (Wang et al. 2001). Lurie and Klein (1990) demonstrated that curing (4 d at 38°C) of apples also induced HSPs. Following heat treatment, HSPs and PRPs remain up-regulated for a few days at room temperature or several weeks in cold store (Sabehat et al. 1996; Pavoncello et al. 2001; Wang et al. 2001). Potential PRPs involved in the defence against fungal attack following heat treatment in a range of commodities are chitinase or β-1,3-glucanase (Schirra et al. 2000). HSPs have also been reported to be upregulated in sun versus shade positioned fruit and this *in situ* preharvest treatment effectively mimicked the mode of action reported by postharvest heat treatment (Woolf and Ferguson 2000).

HWTs also influence the structure and composition of epiticular waxes. The covering of cracks and wounds (Tahir et al. 2009) and the formation of anti-fungal substances in the wax after heating (Fallik et al. 1996) are thought to be possible modes of action.

If fruit ripening is delayed by heat inactivation of degradative enzymes, the onset of fruit decay may also be retarded. Many curing treatments have shown this delayed-ripening effect which is regarded as a further possible mode of action (Lurie 1998).
2.8.2 Development of HWD for apples

Whereas Burchill (1964) used a 7 min HWD treatment at 42°C to control Gloeosporium rot, more recent studies have been based on shorter dipping times at higher temperatures (Trierweiler et al. 2003; Maxin et al. 2005; Bompeix and Coureau 2007). HWD of apples has been effective at controlling Neofabraea spp. and Monilia fructigena on apples with increasing efficacy from 1 min to 3 min at 49-53°C, whereas the incidence of rot caused by Neonectria galligena increased by a 1 min dip at these temperatures compared to the untreated control and longer dipping times (Maxin et al. 2005).

In 2010, HWD was used by the apple fruit industry in Germany, the Netherlands, France and Austria (Warlop and Bompeix 2005; Maxin et al. 2006; Trapman and Jansonius 2008). In 2004, HWD was also introduced into the peach industry in France to control brown rot (Warlop and Bompeix 2005). Following HWD of apples, a suspected “microbiological vacuum” (Jijakli and Lepoivre 2004) led to a series of verification experiments (Karabulut et al. 2002; Vorstermans et al. 2007; Zhang et al. 2010). Other experiments showed that the efficacy of HWD could be enhanced if GRAS compounds such as eugenol (Amiri et al. 2009) or calcium salts (Spadaro et al. 2004) were added to the dipping water. None of these combinations may be used in Denmark and Germany, where chemical postharvest treatment of fruit and vegetables is not permitted.

HWD is costly at approximately €0.05 kg\(^{-1}\) depending on the amount of treated fruit per year (calculation based on 200 t per year per orchard; Maxin and Klopp 2004). The use of 3 l diesel fuel per t fruit (Maxin and Klopp 2004) is a key factor when calculating the carbon footprint of apples. At this time only highly priced organic apples are able to offset the costs of HWD.

2.9 Apple Quality

2.9.1 Postharvest quality assessment, state of the art

Once the fruit is placed in storage, all subsequent processes and treatments must be optimised in order to retain apple quality. Preharvest factors that have already influenced fruit quality cannot be changed during storage (Woolf and Ferguson 2000). The most important factor for retaining apple quality in long-term storage is
the maturity of the fruit at harvest (Frasnelli and Casera 1996; Wilcke 1996). After harvest, apples follow a climacteric ripening process, where ethylene is produced and self-regulates the ripening process in which the fruit respires sugar to CO₂ while its flesh softens and loses water. All postharvest treatments are aimed at reducing the rate of respiration and water loss, thereby retaining the fruit in an acceptable stage of maturity for consumption.

For some apple varieties, cold storage alone can delay ripening by approximately three months (Thompson 2010). If fruit are stored in CA conditions, the rate of respiration is lower than in cold store alone, therefore fruit can be maintained for up to 8 months and still meet consumer expectations (Streif et al. 2010; Thompson 2010). Through the development of airtight storage rooms and detection of anoxia by electronic sensors, DCA storage has been established in many fruit growing areas (Veltman et al. 2003). Some commodities that are harvested at the optimal maturity may now be stored for over 12 months, ensuring a year-round supply of regional fruit for consumers (Streif et al. 2010).

Apple fruit quality is based on three principal parameters; firmness of the fruit flesh is measured by a penetrometer (kg cm⁻²) after removal of the skin; soluble solids (sugars) are detected in the fruit juice by a refractometer (°Brix); and the starch content index of the flesh is assessed on a scale ranging from 1 to 10 using an iodine solution on equatorially cut apple slices (Streif 1983; DeLong et al. 1999).

The content of titratable acidity (malic acid and citric acid, (g l⁻¹)) is also routinely determined as a quality parameter based on the fact that acidity has a significant influence on the taste of the fruit (DeLong et al. 1999). Other parameters that indicate the climacteric ripening process of apples are CO₂ and ethylene emissions. CO₂ provides an indication of the respiration rate, and is determined by incubating fruit in sealed jars of a known volume for a known time period, sampling the headspace and injecting the gas sample into a GC (Tahir et al. 2009). A similar methodology can be used to determine ethylene concentrations to indicate stress levels or the ripening stage of the fruit (Fallik et al. 2001).

Once fruit are harvested, their starch and sugar contents are essentially fixed. Therefore any change in quality is attributable to postharvest treatments and the
ability to delay the ripening process. In general, firmness is used to monitor the effects of delayed ripening (Lafer 2010).

2.9.2 Process quality of apple production

During production all management practices including the use of chemicals or fertilizers are documented by the orchardist. These documents are then audited by private companies. Documentation of the critical steps of the production process are necessary in trading activities to show that management practices meet the private food safety standards of retailers. In Germany and Denmark, documentation of standards is guaranteed by fulfilling quality assurance packages such as “Global GAP”, “QS” or “EUREP GAP” (Blanke and Burdick 2005). In addition, product quality is assessed when the fruit and vegetables enter the wholesale trade, but here acceptability can be influenced by seasonal effects and product availability.

Organic products are labeled and guaranteed by governmental standards (EU directive 834/2007) and by private standards such as “Demeter” (Koreleska and Etkowska 2010). In Denmark, orchards are subjected to a governmental audit on an annual basis. In other European countries accredited companies carry out these controls, ensuring high standards in organic production and labeling. Misuse of organic labels and breaches of the rules are penalised with high fines in all European countries.
Chapter 3: General methodology

3.1 Introduction
In this Thesis, apples used for hot water treatment (HWT) experiments had experienced different pre- and postharvest treatments (Figure 3.1). Within the field, apples were exposed to pathogen pressure which varied between trees, or they were subjected to conditions imposed by preharvest treatments. Following harvest, apples were subjected to a brief chilling period prior to postharvest treatments. Fruit were then passed through HWT facilities. Finally, apples ripened during an extended storage period where they either developed heat scald and/or possible decay caused by storage rot pathogens. Chapter 3 provides an outline of experimental methodologies used from the time before harvest until evaluation of results (Section 3.2). In addition, a detailed description of fungal identification methods adopted in this Thesis is provided (Section 3.5) because only a brief summary of this methodology was included in the published manuscript (Chapter 7). Other experimental details specific to particular aspects of research are given in the relevant Chapters 4-7.

HWTs can be established if standard laboratory heating units are available. Up to five apples can be submerged at any one time in a 20 l water bath heated to temperatures of 25 to 80°C. Within 30 s of submerging cold apples, the water temperature will return to the initial set-point temperature. However, the capacity of laboratory heating units is insufficient for large-scale experiments where up to 1.2 t of fruit (approx. 7,500 apples) need to be treated. Therefore, three different methods of HWT were developed or adopted for the experiments performed in this Thesis (Sections 3.3, 3.4.1, 3.4.2).

3.2 Example of the sequence of procedures of an entire experiment
Preharvest treatments
Several experiments were carried out to examine the influence of pre-harvest factors on the postharvest behaviour of apples. Factors of particular interest in the context of this Thesis were the influence of rootstock vigour or of effects of tree positioning on infection levels by postharvest pathogens (Section 4.1.2); a comparison of preharvest fungicide applications with HWD (Section 4.3.1); or differences between fruit from
integrated production (IP) systems and organic orchards (Section 4.2.2). During harvest and transport, great care was taken to avoid bruising or other injury to the fruit skin.

Chilling Period

In all experiments, all fruit were subjected to a short postharvest chilling period (up to 7 d). During this period, there was a lowering of turgor pressure, and to a limited extent apples may have recovered from handling and harvesting bruises inflicted.
during harvest (Tahir et al. 2009). Apples not subjected to a chilling period may have an enhanced susceptibility to postharvest infections by wound pathogens which potentially increase decay results and variability in results.

**Fruit quality analyses**
Depending on the experimental design, fruit quality was measured at critical stages during the experiment. Initial analyses were carried out directly after harvest. In most experiments classical parameters such as firmness, starch content, sugar concentrations and acidity were recorded. When fruit were stored in ambient atmosphere, changes in fruit quality parameters typically became apparent after about 8-10 wk storage (Section 4.2.1). Therefore a second destructive fruit quality analysis should be carried out at about this time.

**Randomisation**
For all experiments the apples were carefully randomised prior to postharvest treatment. For this purpose, apples from a given harvesting container were randomised equally among all experimental replicates by adding one fruit at a time to each successive treatment box (Figure 3.2). If field treatments were to be examined, randomisations were also carried out within each replicate (Sections 4.2.1 and 4.3.1).

**HWT**
Following randomisation, different HWTs were carried out. During all three experimental years, the well-established method of hot water dipping (HWD) was carried out based on a standard protocol (Section 3.3). In contrast, for hot water rinsing (HWR) separate and quite different approaches were developed and implemented during the second and third year of this Ph.D. project (Section 3.4).

**Incubation period**
Following HWT, all fruit (both treated and untreated controls) were stored at 2°C in ambient atmosphere for 15-20 wk. During the storage period, the development of fruit rot was recorded at regular intervals. In all three experimental years a warm storage period of 14 d
Figure 3.2: ‘Ingrid Marie’ fruit randomised into experimental boxes. Each box contains apples added individually from several harvesting boxes. 60 experimental boxes (14 treatments with hot water and a control, each comprising 4 replicates) were used in this experiment. Each box contained 100-110 apples (Chapter 7).

at 15-18°C was added to the end of the cold storage as a final incubation step. At Aarslev cold rooms but no controlled-atmosphere (CA) storage facilities were available. Therefore, an additional experiment was carried out in collaboration with the Esteburg Centre (Jork, Germany), where the effect of CA and dynamic CA (DCA) storage conditions were evaluated on the quality of apples treated with HWD and HWR (Section 4.2.2).

Survey of storage rots
Four weeks after HWT, fruit were first examined for storage rots and for areas of weakness in the surface tissue. Affected apples were separated, labelled and stored in such a way that surface contact to other fruit was avoided (see Figure 2.2). Examination of all apples was repeated at 2- to 3-week intervals.

Fungal growth on infected fruit
Following a major contamination by secondary *Penicillium expansum* infections during prolonged storage of experimental apples in the first year of the Ph.D. project,
all pallets of experimental fruit in years 2 and 3 were wrapped in plastic film to minimise the spread of *P. expansum* spores by air currents. Pallets were stored in cold rooms at 2-4°C. The bottom of each pallet was not sealed in order to permit gas exchange and avoid condensation. Wrapping of pallets in this way provided a constant and near-saturated humidity which facilitated sporulation of fungi at the surface of infected fruit (Figure 2.2).

**Identification of fungi**

The improved sporulation conditions of storage-rot fungi during years 2 and 3 provided good specimens for the identification of fungi, details of which are described in Section 3.5.

**Statistical analyses**

All HWT experiments in this Thesis were set up based on the same system. Each experimental treatment, as well as the untreated control, included four replicates of 45-56 fruit each. In the `Ingrid Marie´ experiment, the number of fruit was increased to 100-110 apples per replicate because this experiment was specifically aimed at characterising HWT responses in as wide a range of pathogens as possible (Chapter 7). In all experiments, fungal infections were recorded as percentage of affected fruit. If most of the means were outside a range of 30-70%, they were square root arc-sin transformed in order to achieve a better fit to a normal distribution (Köhler et al. 2007). An ANOVA analysis was then carried out with the angular transferred percentages of the four replicates of each treatment and the control. To identify significant differences between various treatments or the control, Tukey´s test was performed. All statistical analyses were carried out using R! software (R! Project).

The algorithm of Tukey´s test is based on the formula of the t-test, but the difference is that in the pairwise comparison of the t-test a 95% probability is used every time to accept or reject a hypothesis. Thus, when carrying out 20 pairwise comparisons the probability of occurrence of a type I error would rise to 1. In contrast, Tukey´s test keeps the experimental error to a chosen level of 5% for all of the multiple comparisons. For that Tukey´ s test is also known colloquially as Tukey’s HSD (honestly significant difference) test. In comparison with other ANOVA tests (e.g. Newman-Keul’s test, Duncan’s multiple range test), Tukey’s test gave fewer significant differences in the comparisons but the test gave narrower confidence intervals.
3.3 Hot water dipping method

Following a short-term chilling period, apples were carefully transported to the machine cleaning station at Aarslev. Water in the 350 l dipping tank was heated to the lowest desired temperature (Figure 3.3A), temperatures being monitored with an analogue mercury thermometer scaled to 0.1°C (Carl Roth, Karlsruhe, Germany). A pile of two plastic boxes was added to the dipping tank (Figure 3.3B), and a timer was activated. The box containing the fruit was covered with a similar but weighted box (Figure 3.4) to prevent the fruit from floating to the water surface. Once fruit were submerged, hot water (95°C) was added to the water tank by a commercial stem-jet blower (Figure 3.4); this procedure maintained the temperature within 0.5 K of the desired temperature, as monitored with an electronic four-channel thermometer (Voltcraft K 204; Conrad Electronic SE, Hirschau, Germany) for practical reasons. The electronic thermometer readings were controlled at each temperature step using the analogue mercury thermometer. A beneficial side-effect of the high pressure jet-stream blower was the generation of a strong water current within the tank which equalized the temperature in the dipping tank and in the fruit-box within 30 s of submerging the apples (Figure 3.4). Water was kept at a constant level by an overflow system (Figure 3.4), so that the continuous water flow in the dipping tank was able to eliminate floating items such as insects, leaves or bud-scales. After the designated dipping time, each box was lifted manually and permitted to drip-dry on a pallet for 10-20 min before being placed on the final storage pallet. When all boxes of a given

Figure 3.3: Hot Water Dipping (HWD) performed in Aarslev. (A) The HWD process was performed outside the machine cleaning facility. (B) Apples were submerged within their box in the dipping tank by placing a weighted box on top of the fruit box.
temperature step had been treated, the water temperature was raised to the next level.

![Schematic drawing of HWD process.](image)

**Figure 3.4**: Schematic drawing of HWD process. Fruit in a perforated box were submerged in hot water by a weighted box (WB) in a 350 l water tank (WT). Hot water (95°C) was added by a commercial steam jet blower (SJB) to buffer heat losses and cooling effects. Temperature was read with a four-channel thermometer (Th) and water level was kept constant by an overflow system (Ov).

### 3.4 Hot water rinsing methods

Two radically different HWR methods were developed and used in the two experimental seasons; 2009 and 2010. These are described and illustrated in this section.

#### 3.4.1 HWR method used in 2009

In the 2009 HWR experiments (Figure 3.5), apples were placed on a conveyor belt capable of running at different speed settings. Four spraying units each comprising three flat fan nozzles (Type DG 005 VS TeeJet; Spraying Systems Co.; Wheaton USA) were sequentially arranged along the conveyor belt so that in each spraying unit fruit were treated from above and both sides. The spraying distance between the nozzles and the fruit surface was approx. 10 cm. Apples were transported in single file along the conveyor belt while rotating around their own axis. The duration of the HWR treatment was determined by the speed of the conveyor belt. Fruit emerging from the fourth spraying unit were manually removed and packed into a storage box. During
processing, water temperature was recorded at two positions, i.e. in the water reservoir by its own electronic control device, and upstream of the nozzles with an electronic four-channel thermometer (Voltcraft K 204; Conrad Electronic SE, Hirschau, Germany). The actual treatment temperature was adjusted on the basis of a reading of the temperature of water caught from the processing unit in an insulated beaker using an analogue mercury thermometer scaled to 0.1°C (Carl Roth, Karlsruhe, Germany). A temperature loss of approx. 10 K occurred between the water supply (Voltcraft K 204) upstream of the nozzles and water caught in the beaker. This temperature loss was attributed to the 10 cm spraying distance through ambient air.

Figure 3.5 Schematic drawing of the HWR unit used in 2009. Apples were added to a conveyor belt (CB) at the entry point (En). Rotating fruit (RF) were rinsed by hot water sprayed from flat fan nozzles (No), and manually removed at the exit point (Ex). The HWR treatment time was determined by setting the speed of movement of the conveyor belt. The temperature of water in the reservoir ($T_2$) was approx. 10 K above the water temperature that contacted the fruit surface ($T_1$).

A mobile apple juice production unit was used to provide heated water for this HWR processing line. The maximum capacity of this unit was 30 l water at 75°C per minute at a hot water consumption of 2 l nozzle$^{-1}$ min$^{-1}$. On average each apple was treated with 2 l hot water during a 20 s exposure. During use, several disadvantages of this method became apparent, e.g. high energy losses in an open system (Figure 3.6A), high steam development at HWR temperatures above 40°C (Figure 3.6B), a setup
time of 4 hours to pre-heat the buffering tank of 800 l, and a dependence on third parties to provide equipment. This led to a modification of the system for the 2010 experimental season.

Figure 3.6 The hot water rinsing process in an open system used in 2009. (A) General view, showing development of steam. (B) Close-up of apples leaving the HWR unit in single file.

3.4.2 HWR method used in 2010

High efficacies of HWR obtained in the 2009 season as well as obvious technical shortcomings of the equipment led to the development of a new experimental set-up for the 2010 season (Figure 3.7). In view of possible commercial implementation by orchardists and fruit packers, several key requirements needed to be addressed during the design and development of the experimental protocols. These requirements included the need to develop a closed system to reuse hot water and limit steam emission; protection of all elements requiring lubrication from hot water contact; and the option to increase the output by arranging several lines in parallel. In collaboration with Carsten Sørensen (Innotheque APS, Middelfart, Denmark) and close consultations with regional Danish manufacturers a new HWR set-up was developed as illustrated in Figure 3.7.

In a 400 l insulated stainless steel tank, water was heated by two 4 kW immersion heaters automatically regulated by a digital control unit (ELK 38, ELCO. S.r.l., Pievebelvicino, Italy) to reach the designated treatment temperature; in order to limit energy losses, the pump was stopped while heating water. During HWT of a series of 500 fruit, temperature variations were within ±1 K of the set temperature. Apples entered a HWR processing unit in the shape of a hollow stainless steel tube by rolling
off from a regulated conveyor belt that had already been used during the 2009 set-up. Upon entering the processing unit, each fruit was moved forward by a series of water jets (4 mm nozzles; not shown in Figure 3.7) generated by a second pump until

![Figure 3.7](image.png)

Figure 3.7 Schematic drawing of the hot water rinsing (HWR) equipment used in 2010. Apples were transported by a conveyor belt (CB) towards a ramp (Ra) leading to the entry point of a hollow tube (Tu) immersed in a water bath (WB). Apples were moved forward in a single file by jets of water (not shown) until the first spinning position (SP). The insert shows a transverse section of the tube at a spinning position where a water jet (WJ) forced the apple to spin around its own axis. Apples were maintained in single file by a passing barrier (PB) and brushes (Br). Apples were moved forward by new fruit entering the tube, and were removed manually at the exit point (EP). The water bath was heated by immersion heaters (IH) connected to a digital control unit (CU) and kept under circulation by a pump (Pu).

The first of 12-16 sequential spinning positions was reached. At this point, each fruit was arrested and forced to tumble around its own axis by a water jet injected by a 6 mm nozzle. Fruit were kept in a single file by two brushes positioned at the bottom and at one side, and a firm barrier on the other side. The water jets initiated a left-turning water current in the entire processing tube.

The first apple remained at the first spinning position until it was pushed forward by the fruit following it. By inserting a new fruit every 2 s, a forward-moving row of apples
was created in the processing tube, insertion of an apple at the entry point of the water tube leading to the exit of a fruit from the end of the HWT line. Following treatment each fruit was removed from the end of the treatment line manually. The processing tube had a length of 1.6 m and contained 12-16 (modified according to fruit shape and size) spinning positions alternating with 10-14 non-spinning positions. The HWR processing time was determined by the rate at which fruit were added to the system, and this could be set empirically by adjusting the speed of the conveyor belt. Experimental replicates were separated by the insertion of some green ‘dummy’ apples.

3.5 Identification of fungi

Upon transfer of fruit from bulk storage to display crates (containing inlets with 33 separate positions), fruit with disease symptoms were numbered. These numbers were incorporated in a database for subsequent data handling. Pallets of display crates were wrapped with cling film and stored in a cold room at 2°C until April (for 8-20 wk) to permit the development of sporulating structures (see Figure 2.3). In this way it was possible to examine most infected fruit in a concerted effort during 3-4 days. Observations of sporulating colonies with a hand-lens (x10 magnification) were appropriate for species identification of *Monilinia fructigena*, *Botrytis cinerea* or *Phacidiopycnis washingtonensis*, or for identification of *Penicillium* spp., *Phoma* sp. and *Fusarium* spp. to genus level. Microscopic examinations of spores (x100 or x400 magnification) were required in order to unambiguously distinguish between *Neofabraea alba*, *N. perenans*, *Neonectria galligena* and *Colletrotrichum acutatum*. For this purpose, material was removed from a sporulating colony with a pair of watchmaker’s forceps previously cleaned by wiping in a paper towel moistened with 70% (v/v) ethanol, and the material was mounted in a drop of water. Spore mounts were viewed directly without a coverslip.

If no spores were produced by the time of fruit assessment, if fruit-rots were unknown at the time of fruit assessment, or in the case of typical representatives of known fruit rots, specimen apples were kept for the isolation of fungal pathogens in the microbiology laboratory of the Esteburg Fruit Research and Advisory Centre (Jork, Germany) in collaboration with Dr. Roland W.S. Weber. Spores or mycelial fragments were streaked out onto potato dextrose agar (PDA) augmented with 200 mg
penicillin G and streptomycin sulphate 1\(^{-1}\) agar in order to obtain pure-culture isolates. These isolates were incorporated into the culture collection of the Esteburg Centre.

A molecular biological approach was chosen in order to verify identification of fungi. DNA was extracted from 50-100 mg growing mycelium scraped from the surface of a PDA plate, using the FastDNA Spin Kit and the FastPrep 24 Cell Homogenizer (MP Biomedicals, Solon, USA). Extracted DNA was diluted 10- to 100-fold to serve as a template for the PCR reaction. Each reaction vial contained 0.5 µM of both primers, approx. 1-10 ng DNA, and 25 µl DreamTaq PCR Master Mix (Fermentas, Vilnius, Lithuania) in a total volume of 50 µl. Primers chosen to amplify the internal transcribed spacer (ITS) region of the ribosomal DNA gene cluster were ITS4 (5\(^{-}\)TCCTCCGCTTATGATATGC) and ITS5 (5\(^{-}\)GGAAGTAAAAGTCGTAACAAGG) according to White et al. (1990). The PCR conditions were as follows: 2 min pre-heat at 94°C; 35 cycles of 94°C (30 s), 50°C (30 s) and 72°C (60 s); and a final polymerisation at 72°C (10 min). The PCR reaction was performed in a GeneAmp 9700 thermocycler (Applied Biosystems, Foster City, USA).

PCR products (1 µl subsamples) were separated by electrophoresis at 60 V in a 1% agarose gel in TBE buffer (Fermentas), and viewed with a Dark Reader (Clare Chemical Research, Dolores, USA) after staining of the gel for 90 min with SYBR Green I dye (Lonza, Rockland, USA) at 5 µl freshly added stock solution in 100 ml TBE buffer. If DNA bands of the expected size (500-1000 bp) were visible, the remainder of the PCR product was purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany). Following a second gel electrophoresis step to estimate the DNA concentration with reference to a GeneRuler 100-1000 bp DNA ladder (Fermentas), DNA samples were prepared for sequencing by Eurofins MWG Operon (Ebersberg, Germany) according to the company’s instructions.

Sequences were read and edited by eye using Chromas Lite 2.01 (Technelysium, Brisbane, Australia). Sequence searches were performed in GenBank using the BLASTn function (Zhang et al. 2000). The most closely matching GenBank sequences were recorded (see Chapter 7)
Chapter 4: Preliminary results

The results summarised in Chapter 4 represent a series of research activities within the overall theme of this Thesis. These activities were carried out either as pre-trials in order to develop methodologies, or as follow-up work to promising results or to related research questions. The activities presented here could not be pursued in depth due to limitations in time and human resources or as a result of inadequate fruit availability due to the seasonal nature of apple production. The seven studies presented here have been aligned to three themes: pre-harvest effect on the developing storage rots, effect of hot-water treatments (HWT) on fruit quality and physiology, and effect of HWT on pathology. However, because all studies were carried out independently, each is presented as a discrete section with its individual methods, results and discussion.

4.1 Pre-harvest effects on the development of storage rots

4.1.1 Distribution of fruit rots on ‘Elstar’ trees within an orchard

Introduction

Apples are exposed to a number of pests and diseases in the orchard during the growing season. Infection of fruit with Neofabraea spp. causing Gloeosporium rot can take place from shortly after blossom until harvest (Schulte 1997). Diplodia seriata is a newly discovered pathogen of organic apple orchards and causes black rot as a preharvest disease in NW Europe (Trapman et al. 2008). Severe black-rot symptoms become visible on apple fruit during the 3-4 weeks preceding harvest, therefore tree-by-tree quantification of infection levels is possible. Such surveys have demonstrated that infections are spread in concentric circles around individual heavily infected trees serving as foci of infection (Weber and Quast 2009). Neofabraea spp., N. galligena and P. washingtonensis share with D. seriata a passive dispersal mechanism based on rainsplash of conidia. The aim of this study was to determine if inoculum sources of these storage-rot pathogens were distributed non-homogenously in the orchard. An obvious way to address this question was to examine harvested fruit from single trees for the incidence of storage rots.
Materials and Methods

In order to examine the distribution of storage-rot inoculum between individual trees, single-tree harvests were conducted for two production seasons (2008/09 and 2009/10) in an organically managed experimental orchard at the Esteburg research station (53°30’ N, 9°45’ E, altitude -2 m) in Northern Germany.

Four rows of ‘Elstar’ trees were selected. Twenty fruit from every tenth tree were harvested from the inner tree canopy in order to maximize the development of storage rots (Schulte 1997); in total, 24 or 25 trees from each row were examined in this way. The apples were stored in ambient air at 2°C for 6 months and regularly observed for symptoms of decay (described in Section 3.5).

Results

Trials to determine the distribution of fungal inoculum within an orchard showed that stored apples were infected by all the common storage rots present in NW Germany (Figure 4.1). There were considerable variations in the relative importance of different storage rots in both years of the trial. *N. alba* making the largest single contribution in 2009 whereas *N. perennans* was dominant in 2010 (Figure 4.1). Over both years, 31.4% of stored apples rotted due to different fungi including *N. perennans* (17.4%), *N. alba* (4.6%), *N. galligena* (2.9%), *M. fructigena* (1.0%), *C. acutatum* (1.4%), *Penicillium* spp. (2.3%), *Fusarium* spp. (0.7%) and minor storage-rot pathogens (1.1%).

In 2009, *N. perennans* infected on average 1.1 (range 0 to 6) of 20 harvested apples per tree, whereby infections were highly variable along the length of individual rows (Figure 4.2, l.h. column). In 2010, the average number of infected fruit was 5.6 (range 0-17) of 20 harvested apples (Figure 4.2, r.h. column). Infections by *N. alba* were higher and more evenly distributed than by *N. perennans* in 2009, whereas only traces of this fungus were detected in 2010. *N. galligena* was similar to *N. perennans* in its frequency in 2009 (average 1.0, range 0-4 infected apples per 20 harvested fruit), and altogether 57 of 99 harvested trees bore infected fruit. In 2010, *N. galligena* was rare (average 0.2 of 20 fruit), and was present on only 12 trees.
Figure 4.1 Relative contributions (%) of different fungi to the total fungal storage decay in ‘Elstar’ in 2009 and 2010.

Discussion

The experiment described here was carried out as preliminary trial to evaluate the distribution of inoculum within harvested apples as a prerequisite for further detailed studies examining the efficacy of HWT in the course of this Thesis (Section 3.2). A highly heterogeneous occurrence of storage-rots, notably of *N. perennans*, in individual trees was determined in this trial (Figure 4.2). Such a clustered incidence of *N. perennans* has also been reported recently from Brazilian apple orchards (Spolti et al. 2012). The differences in infection rates observed between two successive years (2009 and 2010) in the same trees of the same orchard demonstrated the necessity for multiple year studies of storage-rot fungi. In 2010, high infection rates of *N. alba* were also observed in ‘Pinova’ fruit grown at a 500 m distance to the ‘Elstar’ orchard (Chapter 7). This specific ‘Pinova’ plot was chosen based on the high infection level of *N. perennans* (>50%) in 2009. In conclusion, storage experiments should preferably be carried out on apples from the same trees for a minimum of two seasons to exclude weather and inoculum effects.
Figure 4.2 Decay of ‘Elstar’ apples by *N. perennans* (black), *N. alba* (white) and *N. galligena* (hatched) from four rows of apple trees harvested in autumn 2009 (l.h. column) and autumn 2010 (r.h. column).
4.1.2 Survey of fruit rots on ‘Ingrid Marie’ apples grown on different rootstocks with and without root-pruning

**Introduction**

The rootstock of an apple tree is known to influence tree growth, the age of the tree when it sets its first fruit, and the balance between vegetative growth and fruiting (Saure 2007). Root pruning is an orchard management practice to restrict tree growth and thereby to influence fruit set (Byers et al. 2004). Root pruning also affects the density of foliage (Saure 2007) which in turn may have effects on other parameters such as leaf wetness, fruit russetting and cracking, and infection of storage rots.

This experiment was first set up in 2008 (results not shown) as a control to clarify possibilities of mixing fruit of the different rootstocks for later experiments, following an observation that apples grown on different rootstock were strikingly different in their shape and general appearance. When root pruning was introduced in 2009, the setup of the experiment described here was adopted. The aim of this study was to determine if root pruning or the type of rootstock had an effect on the incidence of infections by different fungal pathogens, as assessed by the extent and identity of fruit rots developing during storage.

**Materials and Methods**

‘Ingrid Marie’ trees were grafted onto four different rootstocks (M9, J9, M26, MM106) that were either root-pruned or left unpruned. This study was carried out on 11-year-old trees located at Aarslev, Denmark (Aarhus University; 55°18’ N, 10°26’ E, altitude 47 m). In the first year (2009/2010), every second row was root-pruned on one side by a tractor-pulled blade reaching 50 cm into the soil at a distance of 15 cm to the apple trunk. This treatment, which was conducted in March 2009, cut off approx. 30% of the root zone. At harvest, plots were chosen from 4 experimental replicates. In the second year (2010/2011), the untreated rows from the first year were root-pruned as described above and the treated trees from the first year were left untreated. As a result, all trees in the 2010/2011 trial had been root-pruned once either in 2009 or 2010.
Fruit from trees not root pruned in the season of harvest were separated into a control batch and a hot water dipping (HWD)-treated batch (52°C for 3 min) which was treated as described in Section 3.3. Fruit (4 replicates x 100) were sampled from each of the J9, M26 and MM106 plots in the field. Samples of 100 fruit were also harvested from adjacent trees grafted on M9, in order to examine any effect of local inoculum. Fruit were homogenized within all four replicates of the same rootstock/pruning combination (Section 3.2). Fruit were stored for 4 months at 2°C followed by 14 d at 15°C. Storage and identification of fruit rots were performed in 2010 and 2011 as described in Section 3.5.

Results

Fruit assessment of ‘Ingrid Marie’ apples stored for 22 wk gave rise to 32.9% storage rot in 2009 and 11.6% in 2010 (average 22.3%). On average (over 2009 and 2010) the following fungi were recorded: *N. alba* (14.4%), *N. perennans* (0.3%), *N. galligena* (3.9%), *M. fructigena* (1.2%), *Penicillium* spp. (0.8%), *C. acutatum* (0.6%), *Fusarium* spp. (0.6%), and other species (0.9%). The relative incidence of each storage rot in each year of the trial is shown in Figure 4.3. In 2009, statistical evaluations using ANOVA were hampered by high variations between the four replicates of each treatment. Therefore, no significant effect of root pruning on the incidence of *N. alba* or any other pathogen (Figure 4.4A) was observed. In 2010, a significant reduction in the infection rates of *N. alba* in non-pruned rootstocks was found on M9, M26 and J9 (Figure 4.4B). For all other fungi, there were no significant effects of the rootstock or of the root pruning treatments. HWD (3 min at 52°C) reduced the incidence of *N. alba* by 76-92% in apples harvested from all treatments, without causing any heat damage (results not shown).

Discussion

In 2009, the effects of the rootstock and of the root-pruning treatments on the incidence of storage rots were non-significant. Differences between the 2009 and 2010 results may be attributed to a reduced level of inoculum in 2009 following the initial root pruning, due to fewer fruit mummies, earlier leaf drop, a less dense canopy permitting a faster evaporation of moisture due to improved ventilation, and internal parameters of the apple trees that inhibited the development of fungal inoculum (Holb and Scherm 2007). It is possible that a reduced inoculum in one year may lead
to a reduced infection pressure in the following year. Future studies should focus on the relationships between the incidence of fruit russetting and the development of storage rots, and between root pruning and the incidence of fruit rot and inoculum development.

It is clear from this preliminary study that single trees should not be used as replicates for storage trials. If single trees have to be used as replicates, then an extremely high number of replicates must be provided. An alternative approach was taken in all subsequent trials in this Ph.D. study, in which large numbers of fruit were harvested from individual trees, these fruit were carefully distributed across all trial boxes (replicates). Boxes containing fruit from numerous different trees were then used as the experimental replicates, thereby eliminating the between-tree variability as a source of statistical error in HWT trials.

![Figure 4.3 Relative incidence (%) of different fungi that caused storage decay in an ‘Ingrid Marie’ rootstock trial carried out in 2009 and 2010.](image)
Figure 4.4 Decay caused by *N. alba* in ‘Ingrid Marie’ apples grafted on different rootstocks with and without root pruning in the growing seasons 2009 (A) and 2010 (B). The untreated rootstocks in 2010 had been subjected to root pruning in the 2009 growing season. Data are presented as means (n=4), small letters on bars indicate significant differences. ANOVA was evaluated using Tukey’s test (P< 0.05) in 2010.
4.2 Effects of HWT on fruit quality and physiology

4.2.1 Effect of pre-harvest conditions on the occurrence of fruit damage caused by HWT

Introduction

HWD can be employed to control postharvest pests and diseases in crops from organic as well as integrated production (IP). In NW Europe, organic farmers have been particularly keen to introduce HWD (Maxin et al. 2006; Trapman and Jansonius 2008). HWD treatment is so effective in controlling Neofabraea spp. rot in apples that the method is economically profitable to orchardists (Maxin and Klopp 2004). In addition the treatment ensures a consistently high quality product both in the sales chain and in private households. Nevertheless, fruit losses as a direct result of heat damage have been reported, and studies have been carried out to minimise these losses by lowering HWD temperature or by adding clove oil to promote HWD efficacy at reduced temperatures (Amiri et al. 2009). Previous studies on single apples indicated that some fruit may be more susceptible to heat treatment than others (Maxin, unpublished data). Indeed, there are many factors in the orchard which may affect the susceptibility of apples to heat damage by HWD (Woolf and Ferguson 2000). In previous studies, slightly immature ‘Elstar’ fruit appeared to be more susceptible to heat damage than mature fruit (Maxin, unpublished data). In one out of four years, the climate during the growing season influenced the susceptibility of ‘Boskoop’ apples to heat damage as a result of HWD (Maxin, unpublished data). Preliminary studies on management practices have shown that fruit grown under integrated IP management were more susceptible to heat damage as a result of HWD than organic grown fruit (J. Zimmer, personal communication). Different apple varieties also differed in their susceptibility to heat damage (Schirmer et al. 2004). This trial was carried out to determine if individual apples, differing in their nutrient status and harvested from trees with different crop loads, varied in their susceptibility to heat scald as a result of HWD treatment.

Materials and Methods

‘Elstar’ apples were harvested from a fertiliser trial in Aarslev, Denmark (55°18’ N, 10°26’ E, altitude 47 m). Trees were 5 years old and were grown under IP management. Trees were grown under three different nitrogen (N) regimes: (1) no
fertilization during the growing season of harvest; (2) moderate N fertilization (50 kg N ha\(^{-1}\) supplied in March as calcium ammonium nitrate); (3) excessive N fertilization (100 kg N ha\(^{-1}\) supplied as two calcium ammonium nitrate applications in February and May).

For each treatment, fruit were harvested from two trees of each of four replicate plots in a randomized trial. Fruit were harvested at an early maturity stage suitable for long-term storage (Starch index 3-4). For each HWT, 42 apples (fruit size 150–180 g) were selected from the harvest of each field replicate, and placed in a plastic dipping box (30 x 40 x 20 cm; 24 l volume; half the size of those used in Chapter 7). HWD was carried out as described in Section 3.3, following a chilling period of 7 d in cold store at 2°C in ambient atmosphere.

In the same orchard, trees with different crop loads were separated into four categories; those with a high load (>150 fruit per tree), medium (100-120 fruit per tree), low (60-80 fruit per tree) and very low (<50 fruit per tree). Trees were harvested individually. Due to the different number of trees required to obtain the desired number of apples for the trial, fruit from trees within each category were pooled and then separated into the boxes containing 46 fruit per replicate. All treatments were replicated four times.

The nutrition and crop load trials were carried out in two successive years (2008 and 2009). In 2008, apples were subjected to HWD for 2 min (50 or 52°C) or 3 min (48, 50 or 52°C). In 2009, fruit were dipped for 3 min at 50, 52, 54 or 56°C. Post-HWD storage conditions were as described in Section 3.2. Apples were assessed for skin damage and colour change after 11 weeks’ storage at 2°C. Skin damage was classified into four categories; (1) no damage; (2) minimal damage of small brown lesions <5 x 5 mm; (3) heat-damaged areas >5 x 5 mm but covering <50% of the total fruit surface; and (4) heat-damaged areas covering >50% of the fruit surface (see Chapters 6 and 7). Colour changes were also grouped into four classes; (1) no change as shown in Figure 4.5A; (2) possible colour change visible as a slight darkening; (3) readily visible colour change restricted to an area <50% of the fruit surface; and (4) severe colour change covering >50% of the fruit surface, as shown in Figure 4.5B.
Results of colour change and skin damage were enumerated as an index per replicate (n total = 42), as follows:

\[
\text{Index} = \frac{1 \times n1 + 2 \times n2 + 3 \times n3 + 4 \times n4}{n \text{ total}}
\]

Figure 4.5 ‘Elstar’ apples after 12 wk storage. (A) Control fruit without surface defects. (B) Severe colour change caused by HWD for 3 min at 56°C. Areas not showing darkening are indicated by arrows.

Physiological parameters including total soluble sugars, firmness, starch and colour were determined on 4 x 10 fruit from each field replicate at the time of HWD, and on 10 fruit per field replicate and HWD treatment after 12 wk storage. Fruit colour readings were taken at the border of the blush of each fruit, using a colorimeter (Chroma Meter, CR-400, Konica Minolta Sensing Inc., Japan) to measure \( L \) (lightness/luminosity), \( a \) (trend towards red or green) and \( b \) (trend towards yellow or blue) according to the manufacturer’s instruction manual. On the same apples, flesh firmness was determined after removal of the fruit skin, using a penetrometer (GS20 Fruit Texture Analyser, Güss Ltd., South Africa) fitted with an 8 mm probe. The force needed to break the resistance of the fruit flesh was expressed in kg cm\(^{-2}\). Soluble
solids content was determined using a temperature-compensated digital refractometer (RFM712, Bellingham Stanley Ltd., UK). Flesh samples were removed from the cortical regions of the fruit (approx. 1 g per analysis). The flesh was squeezed with a garlic press to provide juice. The value was expressed in °Brix. Finally, fruit were sliced open transversely to determine the starch content using a standard starch-iodine test (Streif 1983). Starch content was ranked on a visual scale of 1 (black, starch) to 10 (clear fruit surface without dark staining).

Results
In 2008, HWT treatments did not cause any detectable superficial heat damage or colour change on fruit harvested from either the nutrition or the crop load trials. No significant statistical differences were observed with respect to any of the physiological fruit quality parameters (results not shown).

In 2009, using excessive HWD conditions, adverse responses by apples were observed. Heat damage was observed as skin browning in fruit which had been treated at 54 or 56 °C. Following HWD at 56°C, apples from trees with a high crop load were less severely affected than fruit from trees with lower crop loads (Figure 4.6A).

Incipient colour changes were observed in fruit treated at temperatures ≥52°C. At 54°C, the discoloration index increased from the high to the poor crop load levels (Figure 4.6B), meaning that fruit grown on trees with a high crop load were least affected by the HWT. Following treatment at 56°C, all fruit showed a marked colour change. However, this change could not directly be correlated with any $L \times a \times b$ colour space measurements.

In general, the $b$ value, where positive numbers indicate fruit yellowing on a green-to-yellow scale, increased during storage of apples subjected to HWD at 54°C and 56°C. The $a$ value also decreased following treatment at 56°C, indicating that the intensity of the red colour was reduced. No trend in relation to the HWT was observed with respect to the $L$ value, representing lightness. However, due to high variations within the fruit batches, none of the $L \times a \times b$ colour space numbers (Table 4.1) showed clear correlations with any significant differences between the four crop load treatments and fruit colouration after storage. Destructive fruit analysis for firmness
and sugar content showed significant differences between the different crop load levels (Tukey test; data not shown), but not in response to the HWD treatments (Figures 4.6C,D).

Table 4.1 Colour ($L \times a \times b$ colour space) of ‘Elstar’ fruit treated with hot water dipping for 3 min at 52, 54 and 56°C. Fruit were harvested from trees at four different crop load schedules, i.e. very low (20-50 fruit), low (60-80 fruit), medium (100-120 fruit), and high (160-180 fruit). Data are presented as means (n=4), SD indicates standard deviation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>L</th>
<th>SD (L)</th>
<th>a</th>
<th>SD (a)</th>
<th>b</th>
<th>SD (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-HWD high</td>
<td>68.45</td>
<td>3.04</td>
<td>12.40</td>
<td>6.11</td>
<td>43.44</td>
<td>2.92</td>
</tr>
<tr>
<td>52°C high</td>
<td>69.54</td>
<td>1.85</td>
<td>10.53</td>
<td>5.02</td>
<td>43.73</td>
<td>2.47</td>
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<tr>
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<td>67.30</td>
<td>1.52</td>
<td>12.74</td>
<td>2.58</td>
<td>41.74</td>
<td>1.79</td>
</tr>
<tr>
<td>56°C high</td>
<td>66.89</td>
<td>4.49</td>
<td>6.37</td>
<td>3.34</td>
<td>40.89</td>
<td>3.71</td>
</tr>
<tr>
<td>Non-HWD medium</td>
<td>70.78</td>
<td>0.95</td>
<td>11.82</td>
<td>1.62</td>
<td>45.00</td>
<td>0.50</td>
</tr>
<tr>
<td>52°C medium</td>
<td>68.71</td>
<td>2.92</td>
<td>13.57</td>
<td>5.18</td>
<td>43.10</td>
<td>2.41</td>
</tr>
<tr>
<td>54°C medium</td>
<td>69.59</td>
<td>2.18</td>
<td>11.96</td>
<td>2.90</td>
<td>43.29</td>
<td>1.25</td>
</tr>
<tr>
<td>56°C medium</td>
<td>61.97</td>
<td>1.21</td>
<td>10.72</td>
<td>3.34</td>
<td>37.55</td>
<td>1.54</td>
</tr>
<tr>
<td>Non-HWD low</td>
<td>69.21</td>
<td>2.87</td>
<td>14.60</td>
<td>4.90</td>
<td>44.83</td>
<td>2.46</td>
</tr>
<tr>
<td>52°C low</td>
<td>71.27</td>
<td>1.98</td>
<td>10.54</td>
<td>3.69</td>
<td>47.20</td>
<td>2.57</td>
</tr>
<tr>
<td>54°C low</td>
<td>71.94</td>
<td>2.41</td>
<td>7.59</td>
<td>3.37</td>
<td>47.68</td>
<td>1.89</td>
</tr>
<tr>
<td>56°C low</td>
<td>66.33</td>
<td>2.40</td>
<td>9.53</td>
<td>1.73</td>
<td>42.35</td>
<td>1.51</td>
</tr>
<tr>
<td>Non-HWD very low</td>
<td>70.66</td>
<td>0.98</td>
<td>12.56</td>
<td>1.76</td>
<td>47.36</td>
<td>0.86</td>
</tr>
<tr>
<td>52°C very low</td>
<td>70.74</td>
<td>0.95</td>
<td>12.98</td>
<td>1.20</td>
<td>48.30</td>
<td>0.15</td>
</tr>
<tr>
<td>54°C very low</td>
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<td>1.09</td>
<td>9.94</td>
<td>1.48</td>
<td>46.50</td>
<td>1.09</td>
</tr>
<tr>
<td>56°C very low</td>
<td>66.53</td>
<td>2.23</td>
<td>11.49</td>
<td>1.02</td>
<td>43.41</td>
<td>2.22</td>
</tr>
</tbody>
</table>

Start values
- High crop load: 75.01, -3.96, 40.25
- Medium crop load: 69.36, 9.32, 34.79
- Low crop load: 71.91, 7.29, 37.49
- Very low crop load: 71.06, 13.37, 36.72
Following HWT, destructive analyses of firmness and sugar content were carried out for fruit from plots with three different nutrition levels. The results revealed no significant difference in the $L \times a \times b$ colour space measurements, and no difference in firmness and sugar content (results not shown). The colour change index also showed no significant difference between the different nutrition levels, although a colour change in all treatments was obvious following HWD at 54°C and 56°C (Figure 4.7A).

Superficial browning appeared after HWD at 52, 54 and 56°C. Following HWD at 54 and 56°C, fruit from trees supplied with 50 kg or 2 x 100 kg N ha$^{-1}$ showed significantly less browning (Figure 4.7B) than apples from non-fertilised trees.

**Discussion**

HWD at excessive temperatures (54 and 56°C) resulted in heat damage to the fruit, as observed by a colour change that would be unacceptable for retailers or consumers. However, the 2008 results had already indicated that 50°C and 52°C were suitable temperatures for HWD of ‘Elstar’ fruit. None of the results reported here demonstrated any negative effect of HWD on fruit quality. Fallik (1996) and Tahir (2009) reported that fruit treated with hot air had a higher firmness following cold storage as compared to control fruit, but this improvement in fruit quality was not confirmed here for HWD with ‘Elstar’ or with ‘Ingrid Marie’ (P. Maxin, previous unpublished data). Wide variations in $L \times a \times b$ colour space readings prevented the characterization of any significant differences in colour change between HWD-treated and untreated fruit following storage. The red blush of the ‘Elstar’ variety Elshof, used in this study was not ideal for such assays. To describe an obvious yellowing of HWD treated fruit (Figure 4.5), an uncoloured variety such as ‘Golden Delicious’ or a poorly pigmented ‘Elstar’ type would have been preferable. From this study, 54°C appeared to be the critical temperature at which susceptibility to colour
Figure 4.6 (A) discolouration index, (B) browning index, (C) firmness (kg cm$^{-2}$), and (D) sugar content (°Brix) of ‘Elstar’ fruit, treated with HWD for 3 min at 52, 54 and 56°C. Fruit were harvested from trees carrying 4 different crop loads, i.e. very low (20-50 fruit), low (60-80 fruit), medium (100-120 fruit), and high (160-180 fruit). Data are presented as means (n=4), error bars indicating standard deviation.
change and surface browning became apparent. In order to determine any predisposing influence of pre-harvest factors to HWD damage, further studies need to be carried out with different varieties and other factors (e.g. colouration, tree age, sun exposure).

Although HWD did not improve physiological fruit quality to any measurable extent, the observed benefit of suppressing postharvest fruit rots remains important. Further, the possibility that HWR could improve apple quality should be further investigated.

Figure 4.7 Discolouration index (A) and browning index (B) of ‘Elstar’ fruit treated by HWD for 3 min at 52, 54 and 56°C. Fruit were harvested from trees at 3 nitrogen (N) fertilisation levels, i.e. 0 kg N, 50 kg N and 2 x 100 kg N ha⁻¹. Data are presented as means (n=4), error bars indicate standard deviation.
4.2.2 Development of skin spots in 'Elstar' apples in response to postharvest treatments

Introduction
Technological progress has enabled growers to store apples for longer periods at high fruit quality. Cooling in a controlled atmosphere (CA) has led to ultra-low oxygen (ULO) conditions to be introduced. The most recent step along this road is the concept of dynamically controlled atmosphere (DCA) (Veltmann 2003) which is being introduced into commercial practice (Streif et al. 2010). DCA is characterised by a stepwise reduction and dynamic adjustment of oxygen during the storage phase. Development of anaerobic compounds in the fruit is prevented by use of the non-destructive “Harvest Watch” system or by the destructive HPLC analysis of fruit samples at different time-points.

‘Elstar’ trees contribute 22% of the apples grown in the Northern German fruit production area Altes Land (Schwartau and Görgens 2011). In most orchards up to three ‘Elstar’ pickings are conducted to enable the development of pigmentation on apples in the inner canopy regions. First-picked apples are the most suitable ones for long-term CA/DCA storage. Fruit from the second harvest are typically sold within three months, and those from the third harvest are sold directly, mostly for processing purposes. During long-term CA storage, ‘Elstar’ fruit may develop a physiological storage disorder in which there is a skin browning during and after storage. This has been described as Elstar Skin Spots (ESS) by Roelofs (1996) and Poldervaart (2002). ESS development is highly variable between different growing seasons. In recent years high economic losses due to ESS have occurred on many fruit farms in Northern Germany where second-picked fruit had to be stored for extended periods in order to avoid market saturation. No information on the underlying physiological causes of this disorder is available. DCA has been shown to decrease the incidence of ESS, whereas 1-methylcyclopropene (1-MCP) treatments may promote it (Hennecke et al. 2008). This study was therefore carried out in order to examine whether HWD and HWR have any promoting or retarding effect on ESS.

Materials and Methods
‘Elstar’ fruit were harvested at the second picking from two orchards, one under organic and the other under IP management, both belonging to the ‘Esteburg’ site
Chapter 4: Preliminary results

(Jork, Northern Germany, 53°30’ N, 9°45’ E, altitude -2 m). Apples were separated on a colour grader, and only fruit with low skin pigmentation (5-20%) were selected and randomised into 40 kg plastic boxes. Pre-storage hot water treatments (HWD, 3 min at 50°C; HWR, 20 s at 55°C) and 1-MCP treatments were carried out as described in Chapters 3 and 5. All treatments were repeated four times and all combinations were stored in two different regimes. In CA storage, conditions were fixed at 1.4% O₂ and 2.6% CO₂ at 2°C, whereas in DCA the O₂ concentration was reduced stepwise to 0.7%, and a Harvest Watch system (Satlantic Inc., Halifax, Nova Scotia, Canada) system was used to monitor the fruit to prevent anoxia.

Following 6 months’ storage in CA or DCA and again after a further 20 d at 2°C in ambient atmosphere, fruit were assessed for the development of ESS using the following index; 0 (no symptoms), 1 (traces of ESS), 2 (total ESS area <1 cm²), 3 (ESS = 1-5 cm²), and 4 (ESS >5 cm²). The index was calculated per replicate (n total = 60) using the following equation:

\[
\text{Index} = \frac{0 \times n0 + 1 \times n1 + 2 \times n2 + 3 \times n3 + 4 \times n4}{n \text{ total}}
\]

Samples of 30 fruit per replicate per measurement were destructively analyzed for sugar, firmness and starch using a semi-automatic Pimprenelle fruit analyser (Setop Giraud Technologie, Cavaillon, France) as described by Lafer (2010). Fruit were analysed at four times; at the beginning of the experiment (at harvest), following 6 months’ storage at CA or DCA, after 20 d cold storage at 2°C, and after a further 7 d at 15°C. Decay was recorded only directly after removing fruit from CA/DCA storage.

Results

There were no differences in sugar, starch and firmness between apples grown under IP or organic management. Fruit quality was not influenced by any pre-storage treatment (HWD, HWR or 1-MCP) with respect to sugar or starch content (results not shown). However, 1-MCP influenced fruit firmness significantly in the final analysis following 7 d at 15°C in all treatments of IP and organic fruit (Tables 4.2-4.5). ESS was observed in all treatments at different levels. Initially, DCA reduced the incidence of ESS in all pre-storage treatments and in control fruit. A significant increase in the ESS incidence was observed in 1-MCP treated IP fruit after 6 months in CA storage plus 20 d storage in ambient atmosphere (Figure 4.8A), whereas for organically grown fruit no
significant differences were observed (Figure 4.8b). No increase in ESS incidence was apparent in IP fruit subjected to 6 months’ DCA storage with or without 1-MCP treatment (Figure 4.9a), but a significant increase was observed in organic apples treated with 1-MCP, stored in DCA and 20 d at 2°C (Figure 4.9b). No HWT had any effect on ESS in any treatment combination.

Table 4.2: Firmness (kg cm$^{-2}$) of ‘Elstar’ fruit grown in an integrated production system and stored in controlled-atmosphere conditions. Pre-storage batches were treated with 1-methylcyclopropene (1-MCP), hot water rinsing (HWR) for 20 s at 55°C and hot water dipping (HWD) for 3 min at 50°C. Significant differences of treatments relative to the control at the final time-point of measurements (P<0.05; Tukey test) are indicated by different letters.

<table>
<thead>
<tr>
<th>Timeline</th>
<th>Control</th>
<th>HWR</th>
<th>HWD</th>
<th>1-MCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Start</td>
<td>6.2</td>
<td>6.2</td>
<td>6.2</td>
<td>6.2</td>
</tr>
<tr>
<td>6 months</td>
<td>5.8</td>
<td>6.1</td>
<td>5.9</td>
<td>5.8</td>
</tr>
<tr>
<td>+ 20 d at 2°C</td>
<td>6.0</td>
<td>5.9</td>
<td>5.9</td>
<td>5.9</td>
</tr>
<tr>
<td>+ 7 d at 15°C</td>
<td>5.3 a</td>
<td>5.0 a</td>
<td>5.2 a</td>
<td>6.1 b</td>
</tr>
</tbody>
</table>

Table 4.3: Firmness (kg cm$^{-2}$) of organically grown ‘Elstar’ fruit, stored in controlled atmosphere (CA) conditions. Pre-storage batches were treated with 1-methylcyclopropene (1-MCP), hot water rinsing (HWR) for 20 s at 55°C and hot water dipping (HWD) for 3 min at 50°C. Significant differences of treatments relative to the control at the final time-point of measurements (P<0.05; Tukey test) are indicated by different letters.

<table>
<thead>
<tr>
<th>Timeline</th>
<th>Control</th>
<th>HWR</th>
<th>HWD</th>
<th>1-MCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Start</td>
<td>6.1</td>
<td>6.1</td>
<td>6.1</td>
<td>6.1</td>
</tr>
<tr>
<td>6 months</td>
<td>6.1</td>
<td>5.6</td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td>+ 20 d at 2°C</td>
<td>6.1</td>
<td>6.0</td>
<td>6.0</td>
<td>6.3</td>
</tr>
<tr>
<td>+ 7 d at 15°C</td>
<td>5.1 a</td>
<td>5.3 a</td>
<td>5.0 a</td>
<td>6.2 b</td>
</tr>
</tbody>
</table>
Figure 4.8: Incidence of ‘Elstar’ skin spots as ESS index on (A) IP and (B) organically grown apples after 6 months’ controlled atmosphere (CA) storage (white bars) or after a further storage in ambient atmosphere for 20 d at 2°C (black bars). Pre-storage batches were treated with 1-methylcyclopropene (1-MCP), hot water rinsing (HWR) 20s 55°C and hot water dipping (HWD) 3 min 50°C. Data are presented as means (n=4), significant differences of treatments relative to the control at the same time-point (P<0.05; Tukey test) are indicated by different letters.

Table 4.4: Firmness (kg cm\(^{-2}\)) of organically grown ‘Elstar’ fruit, stored in dynamically-controlled atmosphere conditions. Pre-storage batches were treated with 1-methylcyclopropene (1-MCP), hot water rinsing (HWR) for 20 s at 55°C and hot water dipping (HWD) for 3 min at 50°C. Significant differences of treatments relative to the control at the final time-point of measurements (P<0.05; Tukey test) are indicated by different letters.

<table>
<thead>
<tr>
<th>Timeline</th>
<th>Control</th>
<th>HWR</th>
<th>HWD</th>
<th>1-MCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Start</td>
<td>6.2</td>
<td>6.2</td>
<td>6.2</td>
<td>6.2</td>
</tr>
<tr>
<td>6 months</td>
<td>5.9</td>
<td>6.1</td>
<td>5.9</td>
<td>5.8</td>
</tr>
<tr>
<td>+ 20 d at 2°C</td>
<td>6.1</td>
<td>6.1</td>
<td>6.1</td>
<td>6.1</td>
</tr>
<tr>
<td>+ 7 d at 15°C</td>
<td>5.5 a</td>
<td>5.3 a</td>
<td>5.4 a</td>
<td>6.1 b</td>
</tr>
</tbody>
</table>
Table 4.5 Firmness of organically grown ‘Elstar’ stored in a dynamically controlled atmosphere (DCA) conditions. Pre-storage batches were treated with 1-methylcyclopropene (1-MCP), hot water rinsing (HWR) 20 s 55°C and hot water dipping (HWD) 3 min 50°C. Significant differences of treatments relative to the control at the final time-point of measurements (P<0.05; Tukey test) are indicated by different letters; these were observed only at the final time-point.

<table>
<thead>
<tr>
<th>Timeline</th>
<th>Control</th>
<th>HWR</th>
<th>HWD</th>
<th>1-MCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Start</td>
<td>6.1</td>
<td>6.1</td>
<td>6.1</td>
<td>6.1</td>
</tr>
<tr>
<td>6 months</td>
<td>6.1</td>
<td>6.0</td>
<td>6.2</td>
<td>6.1</td>
</tr>
<tr>
<td>+ 20 d at 2°C</td>
<td>6.0</td>
<td>6.2</td>
<td>6.3</td>
<td>6.2</td>
</tr>
<tr>
<td>+ 7 d at 15°C</td>
<td>5.1 a</td>
<td>5.2 a</td>
<td>5.1 a</td>
<td>6.1 b</td>
</tr>
</tbody>
</table>

Discussion

This study presents the first report on the occurrence of ESS in organically grown fruit. ESS has not yet resulted in any fruit losses in organic farming because the second picking of ‘Elstar’ fruit, which would typically be susceptible to ESS (Hennecke et al. 2008), are sold early in the marketing season. This high demand for fresh regionally produced organic apples is not comparable to fruit harvested from the IP sector.

No differences in the incidence of ESS were observed between fruit from the different production systems, therefore it is unlikely that pre-harvest application of compounds that are capable of irritating the fruit skin, such as copper or wettable sulphur, contributed to the post-storage incidence of ESS. DCA reduced the incidence of ESS, although no additional reduction could be achieved by any other pre-storage treatment. In this trial the negative influence of 1-MCP in combination with CA and DCA (Hennecke et al. 2008) was confirmed. HWD and HWR showed no influence on any parameter of fruit quality. There was no correlation with storage environment or production system.
Figure 4.9: Incidence of Elstar skin spots as ESS index on (A) IP and (B) organically grown apples after six months’ dynamically controlled atmosphere (DCA) storage (white bars) or storage in ambient atmosphere for a further 20 d at 2°C (black bars). Pre-storage batches were treated with 1-methylcyclopropene (1-MCP), hot water rinsing (HWR) for 20 s at 55°C and hot water dipping (HWD) for 3 min at 50°C. Data are presented as means (n=4), significant differences of treatments relative to the control at the same time-point (P<0.05; Tukey test) are indicated by different letters.

4.2.3 Effect of HWD and HWR on water loss of apples during storage

Introduction

In 2009 and 2010, the surface of ‘Ingrid Marie’ apples subjected to HWR (20 s at 55 or 58°C) showed appreciably different surface properties from HWD-treated and control fruit in that they lacked the greasy surface texture characteristic of ‘Ingrid Marie’ after long-term storage. Temperatures above 54.8°C have been shown to cause a melting and rearrangement of apple surface waxes (Aggarwal 2001). Further, several ultrastructural studies have demonstrated that the morphology of the
wax layer may change in response to heat treatment such as hot air at 38°C for >72 h (Fallik 2004; Tahir et al. 2009). If surface waxes change their properties by melting and rearrangement, they may cover cracks or other openings in the fruit skin. As a consequence, transmission of water vapour might be expected to differ between HWR-treated fruit as compared to control fruit. A study was therefore conducted in order to determine the rates of water loss of apples with or without preceding HWR treatment.

**Materials and Methods**

‘Ingrid Marie’ fruit (65-75 mm diameter) were harvested at Aarslev (Denmark, 55°18’ N, 10°26’ E, altitude 47 m), and the blossom end of each fruit was sealed with a circular piece of glue tape sticker (20 mm diam.). Ten fruit per replicate were weighed to an accuracy of 1 mg using a fine balance (FZ-300i-EC; A&D Co. Ltd., Tokio, Japan), sealed in a 40 x 60 cm zip-lock plastic bag together with 2 x 150 g silica gel, and stored at 16°C. Fruit were weighed initially and thereafter hourly for 8 h; at each time point fresh silica gel packs were placed in the bag. During handling, the apples were held for <60 s in ambient atmosphere. After 8 h, one set of fruit was subjected to HWD at 50°C for 3 min (based on the methodology described in Section 3.3); one set to HWR at 58°C for 20 s (based on the methodology described in Section 3.4.2); and a control set was subjected to cold-water dipping (CWD) at 9°C. Following the different treatments, the fruit continued to be incubated in zip-lock bags, being weighed at hourly intervals for 12 h. The first 5 h period of this second weighing period was excluded from the results because the HWR treatment temporarily increased the weight of the single fruit. This period is presented by the gap between 8 and 10 h at the x axis in Figure 4.10. The measurement at 5 h after HWD, HWR or CWD was used as a new zero point for calculations of hourly weight losses for a period of 7 h. A final measurement was performed 12 h later. A fourth batch of fruit was incubated throughout this experiment in an atmosphere of saturated humidity created by placing a damp tissue paper in the plastic bags. Weight loss per fruit was calculated in mg cm⁻² of fruit surface. Assuming that apples were spherical, the fruit surface (a) was calculated by $a = \pi \times d^2$, where d was the fruit diameter.
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Results
No weight loss was recorded in control fruit stored in a water-saturated atmosphere at 16°C throughout this experiment (not shown). Fruit incubated in the presence of a desiccant (silica gel) had an average water loss of 1.46 mg cm\(^{-2}\) during the first 8 h, varying between 0.86 and 2.44 mg cm\(^{-2}\) (Figures 4.10A-L). Slightly more water was lost during the 7 h period of hourly measurements post-HWD, CWD or HWR incubation, averaging 1.94, 1.86 and 1.96 mg cm\(^{-1}\), respectively. The results were highly variable and no significant differences were found. Average water loss for the 10 apples treated by CWD (Figure 4.11A) as well as those subjected to HWD and HWR (not shown) was strictly linear. The final weight of apples 24 h after HWD, HWR or CWD showed a significant reduction of cumulative water loss for fruit treated with HWR (Student’s T test; p< 0.05) as compared to HWD and CWD (Figure 4.11B).

Discussion
Water loss varies both between and within individual apples because cracked areas on a fruit surface resulted in a 12-fold increased water loss compared to intact areas (Maguire et al. 1999). In this short-term study respiration could be excluded as a factor influencing weight loss because fruit incubated in a water-saturated atmosphere showed no weight loss during the measurement period. Because the blossom-end of each apple was sealed, we can conclude that water vapour transmission was the main factor responsible for the recorded weight losses. The strictly linear relationship between water loss and time (Figure 3.19A) showed that no upper limit for water loss was reached during the experimental time period. The significant result in this trial after 24 h incubation post-HWR suggests the potential use of HWT to reduce water loss and to increase the uniformity of fruit during long-term storage. Reduced weight loss, and increased crispness and juiciness are important quality attributes that indicate the fruit has been optimally stored. These are also important quality criteria from a consumer perspective (Jaeger et al. 1998). A planned modified repetition of this trial was unable to be conducted because a continuous frost period from mid-November 2010 to March 2011 in Denmark made it impossible to carry out any HWR activities. It is recommended that this trial is repeated to focus further on hot-water treatments in relation to water loss and fruit quality. The experimental design should be modified in an attempt to reduce some of the variability observed here, and longer-term trials should be conducted.
Figure 4.10 Cumulative hourly water loss (mg cm\(^{-2}\)) in four replicates of apples (10 fruit per replicate) subjected to hot-water dipping (HWD; images A-D), cold-water dipping (CWD; images E-H) and hot-water rinsing (HWR; images I-L). Measurements were recorded for 8 h prior to the treatment (1-8), and for 7 h (10-16) after the treatment following a 5-h equilibration period.
Figure 4.11: (A) Linear regression of average cumulative water loss (mg cm\(^{-2}\) fruit surface) during 8 h before (1-8 h) and 19 h after cold water dipping. (B) Average water loss (mg cm\(^{-2}\) fruit surface) after 24 h exposure to dry atmosphere following hot water rinsing (HWR) for 20 s at 58°C, cold water dipping (CWD) for 3 min at 9°C, and hot water dipping (HWD) for 3 min at 50°C. Data are means of four replicates each comprising 10 fruit; error bars indicate standard deviation. * Significant difference (Student’s T test, P < 0.05).
4.3 Effects of HWT on pathology

4.3.1 Comparing pre-harvest spraying strategies with postharvest hot water dipping

Introduction
During the growing season, apple orchards are sprayed with fungicides to prevent apple scab and powdery mildew infections. Depending on the seasonal weather patterns, in integrated production (IP) of fruit in Northern Germany 8-12 apple scab and 3-4 powdery mildew sprays are applied during the first half of the growing season to reduce infections to an acceptable level (Palm 2009). IP in Denmark includes a similar number of applications of a similar range of compounds including Kresoxim-metyl (Candit), Dithianon (Delan), Mancozeb (Merpan), and Sulphur (Kumulus) (Table 2.3). As a curative treatment Pyrimethanil (Scala) may be used against these two diseases (Nielsen 2011). The efficacies of pre-harvest application of chemicals on the incidence of storage rots have been shown in numerous trials (Palm and Kruse 2005; Palm and Kruse 2007). Fungicide applications during the first half of the season may also have an effect against postharvest decay (Minar 2006). In addition, pre-harvest sprays of Pyraclostrobin and Boscalid (co-formulated in Signum WG) are applied to control Neofabraea spp. and other storage diseases (Nielsen 2011). The substantial number of compounds available in IP present is in a striking contrast to the strictly limited selection of registered fungicides for use in organic production systems in Denmark. At present, this is confined to wettable sulphur (Kumulus) (Nielsen 2011).

As described in Section 2.6, the use of fungicides shortly before harvest is under scrutiny, and HWD has been shown to be a viable alternative in NW Europe (Maxin et al. 2005). The experiment summarized in this Section was aimed at evaluating summer spraying schedules aimed at apple scab control in an ‘Ingrid Marie’ orchard, where the fungicide applications were terminated at different time-points prior to harvest (i.e. fully sprayed, stopped in July, stopped in May, comparison with a full schedule based on wettable sulphur). A comparable trial was carried out in an ‘Elstar’ orchard, where a full spraying schedule was compared with a reduced spraying schedule (stopped in June at the end of the primary scab season).
Efficacies of the different spraying schedules in controlling the storage pathogens were compared to the efficacies of HWD.

Materials and Methods

In 2008 and 2009, ‘Elstar’ apples were harvested from two plots of an apple scab fungicide testing field at Aarslev, Denmark (Aarhus University, 55°18’ N, 10°26’ E, altitude 47 m). The spray schedules for both years are summarized in Tables 4.6 and 4.7. In 2008, ‘Ingrid Marie’ apples were harvested from four different spraying schedules (Table 4.8) in a field at the same location. Each spray schedule was available in four replications.

Table 4.6 Fungicide applications in the ‘Elstar’ orchard in the 2008 season (all application rates are given per ha).

<table>
<thead>
<tr>
<th>Date</th>
<th>Fully Sprayed</th>
<th>Stopped in June</th>
</tr>
</thead>
<tbody>
<tr>
<td>31.03</td>
<td>Scala 1.1 l</td>
<td>Scala 1.1 l</td>
</tr>
<tr>
<td>12.04</td>
<td>Scala 1.1 l + Kumulus 4 kg</td>
<td>Scala 1.1 l + Kumulus 4 kg</td>
</tr>
<tr>
<td>25.04</td>
<td>Scala 1.1 l + Kumulus 4 kg</td>
<td>Scala 1.1 l + Kumulus 4 kg</td>
</tr>
<tr>
<td>07.05</td>
<td>Candit 0.2 kg</td>
<td>Candit 0.2 kg</td>
</tr>
<tr>
<td>16.05</td>
<td>Candit 0.2 kg</td>
<td>Candit 0.2 kg</td>
</tr>
<tr>
<td>19.05</td>
<td>Delan 1.0 kg</td>
<td>Delan 1.0 kg</td>
</tr>
<tr>
<td>27.05</td>
<td>Candit 0.2 kg + Baycor 1.3 kg</td>
<td>Candit 0.2 kg + Baycor 1.3 kg</td>
</tr>
<tr>
<td>17.06</td>
<td>Delan 1.0 kg</td>
<td>Delan 1.0 kg</td>
</tr>
<tr>
<td>10.07</td>
<td>Baycor 1.3 kg</td>
<td></td>
</tr>
<tr>
<td>06.08</td>
<td>Delan 1.0 kg</td>
<td></td>
</tr>
<tr>
<td>19.08</td>
<td>Delan 1.0 kg</td>
<td></td>
</tr>
</tbody>
</table>

Fruit picked from plots subjected to different spraying schedules were carefully randomized within each treatment. For each fungicide treatment each of four replicates comprising 40-42 fruit was placed in a perforated plastic box (30 x 40 x 20 cm; 24 l volume). HWD was performed as described in Section 3.3. In 2008, ‘Ingrid Marie’ and ‘Elstar’ fruit of all different spraying schedules listed in Tables 4.6 and 4.8 were dipped for either 2 or 3 min. The 2 min treatments were at either 50 or 52°C, whereas the 3 min dips were carried out at 48, 50 or 52°C. These five HWD
treatments were compared to an undipped control. In 2009, only ‘Elstar’ fruit from two spraying schedules (Table 4.7) were harvested and treated with HWD. Fruit were dipped for 3 min either at 50, 52 or 54°C. These HWD treatments were compared

Table 4.7 Fungicide applications in the ‘Elstar’ orchard in the 2009 season (all application rates are given per ha).

<table>
<thead>
<tr>
<th>Date</th>
<th>Fully Sprayed</th>
<th>Stopped in June</th>
</tr>
</thead>
<tbody>
<tr>
<td>22.04.</td>
<td>Scala 1.1 l + Kumulus 4 kg</td>
<td>Scala 1.1 l + Kumulus 4 kg</td>
</tr>
<tr>
<td>03.05.</td>
<td>Scala 1.1 l + Kumulus 4 kg</td>
<td>Scala 1.1 l + Kumulus 4 kg</td>
</tr>
<tr>
<td>16.05.</td>
<td>Delan 1.0 kg + Kumulus 4 kg</td>
<td>Delan 1.0 kg + Kumulus 4 kg</td>
</tr>
<tr>
<td>27.05.</td>
<td>Delan 1.0 kg</td>
<td>Delan 1.0 kg</td>
</tr>
<tr>
<td>04.06.</td>
<td>Merpan 2.25 kg</td>
<td>Merpan 2.25 kg</td>
</tr>
<tr>
<td>10.06.</td>
<td>Baycor 1.3 kg</td>
<td>Baycor 1.3 kg</td>
</tr>
<tr>
<td>08.07.</td>
<td>Delan 1.0 kg</td>
<td></td>
</tr>
<tr>
<td>24.07.</td>
<td>Delan 1.0 kg</td>
<td></td>
</tr>
<tr>
<td>21.08.</td>
<td>Delan 1.0 kg</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.8 Fungicide applications in the ‘Ingrid Marie’ orchard in 2009 (all application rates are given per ha).

<table>
<thead>
<tr>
<th>Date</th>
<th>Fully sprayed</th>
<th>Stopped in July</th>
<th>Stopped in May</th>
<th>Sulphur applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>31.03.</td>
<td>Scala 1.1 l</td>
<td>Scala 1.1 l</td>
<td>Scala 1.1 l</td>
<td>Kumulus 4 kg</td>
</tr>
<tr>
<td>12.04.</td>
<td>Scala 1.1 l</td>
<td>Scala 1.1 l</td>
<td>Scala 1.1 l</td>
<td>Kumulus 4 kg</td>
</tr>
<tr>
<td>25.04.</td>
<td>Scala 1.1 l</td>
<td>Scala 1.1 l</td>
<td>Scala 1.1 l</td>
<td>Kumulus 4 kg</td>
</tr>
<tr>
<td>07.05.</td>
<td>Candit 0.2 kg</td>
<td>Candit 0.2 kg</td>
<td>Candit 0.2 kg</td>
<td>Kumulus 4 kg</td>
</tr>
<tr>
<td>16.05.</td>
<td>Candit 0.2 kg</td>
<td>Candit 0.2 kg</td>
<td>Candit 0.2 kg</td>
<td>Kumulus 4 kg</td>
</tr>
<tr>
<td>27.05.</td>
<td>Candit 0.2 kg + Baycor 1.3 kg</td>
<td>Candit 0.2 kg + Baycor 1.3 kg</td>
<td>Candit 0.2 kg + Baycor 1.3 kg</td>
<td>Kumulus 4 kg</td>
</tr>
<tr>
<td>17.06.</td>
<td>Delan 1.0 kg/ha</td>
<td>Delan 1.0 kg</td>
<td></td>
<td>Kumulus 4 kg</td>
</tr>
<tr>
<td>10.07.</td>
<td>Baycor 1.3 kg/ha</td>
<td>Baycor 1.3 kg</td>
<td></td>
<td>Kumulus 4 kg</td>
</tr>
<tr>
<td>06.08.</td>
<td>Delan 1.0 kg/ha</td>
<td></td>
<td></td>
<td>Kumulus 4 kg</td>
</tr>
<tr>
<td>19.08.</td>
<td>Delan 1.0 kg/ha</td>
<td></td>
<td></td>
<td>Kumulus 4 kg</td>
</tr>
</tbody>
</table>
Chapter 4: Preliminary results

with an undipped control. All HWD experiments were replicated four times. After dipping, all fruit were stored in ambient atmosphere at 2°C for 100 d followed by a warm storage of 14 d at 16°C. At 14 d intervals, decayed fruit were removed, labeled and stored separately. Results of different rot types were obtained, but are not shown here.

Results

In ‘Ingrid Marie’ apples harvested in 2008, all replicates not treated with HWD produced similar levels of postharvest rots irrespective of pre-harvest fungicide spray regime (Table 4.9). HWD significantly (Tukey’s Test, P<0.05) reduced storage rot decay in the fungicide treatments “fully sprayed”, “stopped in May” and “sulphur applications” as compared to undipped control fruit (Table 4.9). The HWD treatments most effective in controlling storage rots were 2 min at 52°C (73.7% efficacy) and 3 min 50°C (75.1% efficacy).

Table 4.9 Incidence of storage rot (% affected fruit) on ‘Ingrid Marie’ apples harvested in 2009 from plots with different pre-harvest spraying schedules, and treated with hot water dipping (HWD). The average efficacy (%) of each HWD treatment in controlling storage rot is also indicated. Significant differences of HWD treatments relative to the HWD-untreated control (P<0.05; Tukey test) are indicated by different letters, where ns represents no significant difference.

<table>
<thead>
<tr>
<th></th>
<th>Fully sprayed</th>
<th>Stopped in July</th>
<th>Stopped in May</th>
<th>Sulphur applications</th>
<th>Average efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23.2 a</td>
<td>22.9 ns</td>
<td>25.3 a</td>
<td>29.9 a</td>
<td></td>
</tr>
<tr>
<td>2 min, 50°C</td>
<td>4.6 b</td>
<td>11.4 ns</td>
<td>12.8 ab</td>
<td>15.1 ab</td>
<td>57.4</td>
</tr>
<tr>
<td>2 min, 52°C</td>
<td>4.4 b</td>
<td>8.0 ns</td>
<td>5.6 b</td>
<td>8.7 b</td>
<td>73.7</td>
</tr>
<tr>
<td>3 min, 48°C</td>
<td>22.3 a</td>
<td>17.9 ns</td>
<td>15.9 ab</td>
<td>16.8 ab</td>
<td>26.6</td>
</tr>
<tr>
<td>3 min, 50°C</td>
<td>8.3 ab</td>
<td>6.3 ns</td>
<td>6.3 b</td>
<td>3.4 b</td>
<td>75.1</td>
</tr>
<tr>
<td>3 min, 52°C</td>
<td>9.9 ab</td>
<td>10.3 ns</td>
<td>12.5 ab</td>
<td>5.9 b</td>
<td>60.8</td>
</tr>
</tbody>
</table>

When comparing 2008 and 2009 harvests of the ‘Elstar’ trial, strongly differing decay rates were observed. In 2008, an average of 10.7% of the fruit suffered from decay in storage, and no difference in the incidence of decay was observed in fruit harvested from the two spraying schedules. The highest HWD efficacy 84.2%, was observed in fruit that had been treated for 3 min dipping at 50°C (Table 4.10). In 2009, only 1.4%
of the apples in the control were infected by any storage rot. No significant differences in efficacies were observed between the treatments at such low levels of infection (Table 4.10).

Table 4.10 Incidence of storage rot (% affected fruit) on ‘Elstar’ apples harvested in 2008 from plots with differing pre-harvest fungicide treatments, and subjected to hot water dipping (HWD). The average efficacy (%) of each HWD treatment in controlling storage rot is also indicated. Significant differences of HWD treatments relative to the HWD-untreated control (P<0.05; Tukey test) are indicated by different letters.

<table>
<thead>
<tr>
<th></th>
<th>Fully sprayed</th>
<th>Stopped in June</th>
<th>Average efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.3 a</td>
<td>9.0 a</td>
<td></td>
</tr>
<tr>
<td>2 min, 50°C</td>
<td>3.7 b</td>
<td>3.2 b</td>
<td>65.8</td>
</tr>
<tr>
<td>2 min, 52°C</td>
<td>2.6 b</td>
<td>3.0 b</td>
<td>71.3</td>
</tr>
<tr>
<td>3 min, 48°C</td>
<td>1.2 b</td>
<td>2.8 b</td>
<td>78.9</td>
</tr>
<tr>
<td>3 min, 50°C</td>
<td>1.2 b</td>
<td>1.8 b</td>
<td>84.2</td>
</tr>
<tr>
<td>3 min, 52°C</td>
<td>2.0 b</td>
<td>1.9 b</td>
<td>80.4</td>
</tr>
</tbody>
</table>

Table 4.11 Incidence of storage rot (% affected fruit) on ‘Elstar’ apples harvested in 2009 from plots with differing pre-harvest fungicide treatments, and subjected to hot water dipping (HWD). The average efficacy (%) of each HWD treatment in controlling storage rot is also indicated. Significant differences of HWD treatments relative to the HWD-untreated control (P<0.05; Tukey test) are indicated by different letters, where ns represents no significant difference.

<table>
<thead>
<tr>
<th></th>
<th>Fully sprayed</th>
<th>Stopped in June</th>
<th>Average efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.4 ns</td>
<td>1.4 ns</td>
<td></td>
</tr>
<tr>
<td>3 min, 50°C</td>
<td>0.5 ns</td>
<td>0.9 ns</td>
<td>50.0</td>
</tr>
<tr>
<td>3 min, 52°C</td>
<td>1.0 ns</td>
<td>0.9 ns</td>
<td>33.3</td>
</tr>
<tr>
<td>3 min, 54°C</td>
<td>1.4 ns</td>
<td>1.8 ns</td>
<td>-16.1</td>
</tr>
</tbody>
</table>

**Discussion**

In 2008, a beneficial effect of HWD against storage-rot development could be demonstrated for both ‘Ingrid Marie’ and ‘Elstar’ fruit. The efficacies of HWD (75%-84%) following 3 min 50°C were comparable to published results of pre-harvest applications of Signum WG (Vorstemans and Creemers 2007). However, by using HWT the two chemical residues of Signum, boscalid and pyraclostrobin, were
avoided (Palm 2009). In 2009, the infection rate of ‘Elstar’ apples with storage rot pathogens was unusually low so that no statistically reproducible effect of HWD was observable. There were no clear-cut effects of different pre-harvest spray applications in either 2008 or 2009. However, before jumping to any conclusions concerning the non-efficacy of fungicides tested, it should be considered that (i) no fungicide-untreated control was available in this experiment, and that (ii) fungicides with high efficacies against storage-rot fungi were not applied during the period of 4-6 weeks preceding harvest, which is considered to be the most relevant window of time for infections by postharvest pathogens (Schulte 1997; Palm and Kruse 2005, Palm and Kruse 2007). A further issue to be considered is that regular fungicide applications during the growing season collaterally control non-target fungi such as ‘sooty blotch’ or ‘fly speck’ diseases. Therefore, removing these sprays could potentially increase the incidence of these fungi. These fungi often are observed in organic or unsprayed orchards and cause high fruit losses (Section 4.3.2, Figure 4.12).
4.3.2 Efficacy of hot water dipping against sooty blotch

Introduction

Sooty Blotch is a colloquial term to describe the colonization of fruit surfaces by dark-pigmented (melanised) fungi. Sooty blotch is largely a cosmetic problem leaving the fruit physiologically intact. However, sooty blotch causes significant losses in organic orchards all over Europe (Trapman 2004) because affected apples cannot be sold as top-grade dessert apples. In Germany, sooty blotch is a common disease in organic fruit production and is caused by several different fungi (Gleason et al. 2011). No severe incidence has been observed in German IP orchards, presumably because sooty blotch is suppressed by fungicide applications aimed at scab, powdery mildew or storage-rot control. Because HWD is known to be effective against storage scab, i.e. *Venturia inaequalis* infections originating prior to harvest but developing visible symptoms only during prolonged storage (Palm & Kruse 2007; P. Maxin, unpublished), HWD might also have the potential to prevent sooty blotch symptoms visible at harvest from developing further during storage. This preliminary trial was established to test this assumption.

Materials and Methods

Apples (cultivar ‘Elastar’) were harvested on 12 Sept. 2008 from an organically managed orchard (Alexander Maxin, Jork, Germany; 53°53’ N, 9°76’ E altitude +1 m). Fruit showing 30-50% surface colonization by sooty blotch were selected for this experiment. The sooty-blotch colonies on the fruit surface were outlined with three different waterproof marker pens. In order to exclude any effect of the marker pens on sooty-blotch development, 100 fruit with and without outlined colonies were photographed at harvest and at the end of cold storage. For the HWD trial, samples of 40 apples were randomly distributed into 16 plastic boxes. Apples were then dipped in hot water for 3 min at either 48, 50 or 52°C, or left untreated. After 5 months’ cold storage at 2°C, treated and untreated fruit were examined by comparing the original outlined borders of the colonies, with the colony size following storage. Apples were categorized into three groups according to changes in sooty-blotch area, as follows: (1) No change in shape and size of sooty-blotch colonies during storage; (2) expansion (>1 cm²) of sooty-blotch area during storage; and (3) reduction of sooty-blotch area (>1 cm²).
Chapter 4: Preliminary results

Results

The three HWD temperature steps did not cause any reduction or expansion of the sooty-blotch area relative to the initial sooty-blotch coverage (Table 4.12). Further, no increase of sooty blotch was observed in control fruit not subjected to HWD. In fact, in all treatments on >97% of the apples the areas of sooty blotch remained in the original shape and incidence (Figure 4.12).

Table 4.12 Changes in fruit surface coverage by sooty blotch (SB). Percentages of fruit showing reductions, expansions or no changes in the covered area on 18 March 2009 compared to the starting point (3 October 2008) are indicated (n = 4 replicates, each comprising 40 apples per treatment).

<table>
<thead>
<tr>
<th></th>
<th>Reduced sooty-blotch area (%)</th>
<th>Constant sooty-blotch area (%)</th>
<th>Expanded sooty-blotch area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.3</td>
<td>99.7</td>
<td>0</td>
</tr>
<tr>
<td>3 min 48°C</td>
<td>1.3</td>
<td>97.7</td>
<td>1</td>
</tr>
<tr>
<td>3 min 50°C</td>
<td>0.5</td>
<td>99.5</td>
<td>0</td>
</tr>
<tr>
<td>3 min 52°C</td>
<td>0.8</td>
<td>98.5</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Discussion

Although HWD has been shown to be effective in controlling a range of storage-rot pathogens of apples (see Chapters 5, 6 and 7), in the present trial no reduction of the incidence of sooty blotch was observed in response to HWD (Table 4.10). In fact, no growth of sooty blotch was recorded even on control fruit not subjected to HWD. Sooty blotch is caused by >70 different species of fungi, of which only some are capable of growing at the very low storage temperatures (Batzer et al. 2010). Because HWD was unable to remove existing sooty blotch colonization, its use against this disease must be considered marginal. In contrast, chlorine bleaching has been demonstrated to be effective (Batzer et al. 2002), and in German pack-houses for organic apples a postharvest mechanical cleaning by scrubbing is frequently applied in an attempt to control sooty blotch. The use of post-storage HWR may improve the efficacies of such mechanical cleaning. Considering that a group of apple waxes undergoes melting at temperatures above 54.8°C (Aggarwal 2001), a combination of HWR with brushing should be tested for efficacy against sooty blotch.
This combination of treatments could also be effective against a wide range of storage-rot fungi (Schirra et al. 2000; Fallik 2004).

Figure 4.12 ‘Elstar’ apples superficially colonized by sooty blotch. (A) Control fruit on 3 Oct. 2008 and (B) the same fruit after storage on 18 March 2009. (C) Fruit on 3 Oct. 2008 prior to HWD for 3 min at 50°C, and (D) the same fruit after storage on 18 March 2009.
Chapter 5: Paper 1

Hot-Water Dipping of Apples to Control *Penicillium expansum*, *Neonectria galligena* and *Botrytis cinerea*: Effects of Temperature on Spore Germination and Fruit Rots

Summary

The efficacy of hot-water dipping against apple storage rots caused by *Neonectria galligena*, *Botrytis cinerea* and *Penicillium expansum* was examined. Pure spore suspensions as well as artificially inoculated ‘Elstar’ apples were incubated for 3 min in a water bath heated to specific temperatures in the range of 32 °C to 70 °C, followed by incubation at 2 °C (fruit) or 20 °C (spores). Whereas there were striking interspecific differences in the dipping temperatures survived by spores, storage rots caused by all three species were significantly reduced by dipping temperatures around 50 °C. Temperatures above 52 °C caused serious heat scald on the fruit surface and gave rise to increasing levels of fruit rot in the case of *N. galligena* and *P. expansum*. Very similar temperature-response curves of blue mould development were observed in apples inoculated with *P. expansum* before or after hot-water dipping, except for the highest temperature tested (70 °C). It is concluded that the major effect of hot-water dipping against these fruit rots is mediated by heat-induced acquired resistance of fruit rather than heat-mediated spore mortality. These results suggest possible applications for hot-water dipping of apples at harvest, after short-
term cold storage or after the opening of controlled-atmosphere storage rooms in order to improve fruit quality during subsequent storage periods.

**Keywords.** apple – *Botrytis* grey mould – *Malus domestica* – *Nectria* storage rot – *Penicillium* blue mould – post-harvest disease – storage rot

**Introduction**

Hot-water dipping (HWD) of freshly harvested apple fruit prior to long-term storage is an important strategy for the control of post-harvest diseases especially in the organic production sector (SCHIRMER et al. 2000; MAXIN et al. 2006). In the mild and humid current climate of Western Europe, >50 % of storage rots can be attributed to *Neofabraea perennans* (Syn. *Pezicula perennans*) which is commonly called ‘*Gloeosporium perennans*’, and to *N. alba* (Syn. *Pezicula alba*) commonly called ‘*Gloeosporium album*’, the latter species being dominant in Southern Europe (WEBER 2009). Numerous trials have documented the efficacy of HWD against *Neofabraea* spp. (BURCHILL 1964; TRIERWEILER et al. 2003; MAXIN et al. 2005; NERI et al. 2009).

For several reasons, the effects of HWD on other important storage rots of apples are less well understood. In the case of grey mould caused by *Botrytis cinerea*, HWD trials are difficult to evaluate because of the ability of this fungus to spread from fruit to fruit during storage, thereby producing infection clusters. Further, *B. cinerea* may cause both pre-harvest infections (e.g. blossom-end rot) and post-harvest infections of wounded as well as intact fruit (TRONSMO and RAA 1977; DE KOCK and HOLZ 1992). The blue mould pathogen *Penicillium expansum* is another fungus for which erratic results have been produced following hot water treatments (SPOTTS and CHEN 1987; FALLIK et al. 2001; SPADARO et al. 2004; AMIRI and BOMPEIX 2011). Possible explanations are that *P. expansum* typically infects apple fruit during storage (AMIRI and BOMPEIX 2005), i.e. after apples have been dipped, or that it has a high heat tolerance (PIERSON 1968; BALDY et al. 1970) which may enable its spores to survive HWD. The occurrence of *P. expansum* storage rot is highly variable, depending on inoculum availability, storage conditions and mechanical damage to the fruit surface (AMIRI and BOMPEIX 2005). A third important Western European post-harvest pathogen is the apple canker fungus *Neonectria galligena*. This fungus may cause blossom-end rot as well as quiescent infections of fruit before harvest in a manner similar to *Neofabraea* spp. (XU and
ROBINSON 2010; BERESFORD and KIM 2011). Despite the considerable economic importance of *N. galligena* as a storage rot (PALM 1986; WEBER 2009), no critical HWD trials seem to have been reported as yet.

The current experiments were therefore carried out in order to determine the efficacy of HWD against *B. cinerea*, *P. expansum* and *N. galligena*, using wound-inoculated fruit for maximum reproducibility. Heat treatments of apples were compared to equivalent treatments of spore suspensions *in vitro* in order to characterise host and pathogen responses to heat. Large-scale HWD experiments with naturally infected fruit were also evaluated and will be published separately (P. Maxin et al., in preparation).

**Materials and Methods**

**Fungal inoculum**
Representative isolates of *B. cinerea*, *P. expansum* and *N. galligena* were obtained from infected stored fruit and have been deposited as lyophilised spore suspensions in the Culture Collection of the Esteburg Fruit Research and Advisory Centre (accession numbers OVB10-017, -018 and -019, respectively). For spore production, these fungi were cultivated on potato dextrose agar (PDA; Carl Roth, Karlsruhe, Germany) for 7-14 d at 20 °C. Cultures of *B. cinerea* were exposed to a 10-min burst of near-UV light (BLB-20W, $\lambda_{\text{max}} = 365$ nm) once every day to induce sporulation. Conidia were harvested in 10 ml sterile dist. water by scraping the surface of a sporulating PDA culture with a microscope slide. Spores of *Neofabraea alba* were harvested directly from infected fruit in order to compare *in vitro* spore mortality with the above three species. All spore suspensions were kept at 4 °C and were used within 24 h of being harvested. No spore germination was observed under these conditions.

**In vitro tests**
To characterise the effect of heat treatments on spore germination, 0.25 ml aliquots of conidial suspensions ($2 \times 10^6$ conidia ml$^{-1}$) in standard 1.5 ml Eppendorf vials were suspended by vortexing, and incubated for 3 min in a water bath heated to the appropriate temperature. For each HWD temperature, a 20 µl drop was plated out onto PDA and incubated at 20 °C for up to 5 d. After 24 h incubation, 2 x 100 spores
were scored for germination which was defined as the stage at which the length of the germ-tube exceeded the spore diameter. Three separate replicate runs were performed for this experiment. Microphotographs of representative results were taken with a digital camera ICc 3 fitted to an Axio Scope A1 equipped with differential interference contrast (DIC) optics, using a x40 Plan-Neofluar objective (Carl Zeiss, Göttingen, Germany).

Hot water treatment of fruit

Organically grown apples (cultivar ‘Estar’) harvested in Sept. 2009 from the Esteburg Fruit Research and Advisory Centre (Jork, Germany) were obtained from commercial controlled-atmosphere (CA) facilities (2 °C, 1.4 % O₂, 2.6 % CO₂, 95% humidity) after 3-5 months’ storage. All experimental steps except the HWD process were performed in a cold room with air circulation (2 °C, ambient atmosphere, 95 % humidity). On each apple, three wounds (2 mm diam, 1 mm deep) arranged in an even-sided triangle (3 cm side length) were created by gently pressing the fruit onto a flat wooden surface with three protruding nails. For ease of detection of fungal infections, the nonpigmented side was wounded. Conidial suspensions (10⁶ spores ml⁻¹) of *B. cinerea, N. galligena* and *P. expansum* were applied to the wounds with a fine paintbrush, ensuring that a drop of suspension (approx. 20 µl) covered the entire wound surface. Apples were inoculated before HWD (all three fungi) or after HWD (*P. expansum* only). Aliquots of 10 apples were packed in 24 l perforated plastic boxes, stored for 24 h at 2 °C, and dipped for three minutes in a 200 l plastic tank with overflow containing hot water. The lids of these boxes were covered with a 5 kg weight, ensuring that all fruit were submerged throughout the 3-min HWD. Fruit dipping temperatures were 40, 44, 47, 49, 51, 52, 53, 54, 56, 58, 60 and 70 °C for *P. expansum; 32, 36, 40, 44, 48, 49, 50, 51, 52, 53, 56, and 60 °C for *B. cinerea*; and the same for *N. galligena* except that 32 °C and 60 °C were omitted. These temperatures were chosen according to the results of in vitro spore germination trials. For each replicate run, HWD was carried out at descending temperatures, adding hot water (90 °C) to the dipping tank in order to maintain the chosen temperature at a constant level (± 0.5 K). After HWD, apples were stored at 2 °C in a commercial store-room, the residual HWD water evaporating within 24 h due to the ventilation system. Fruit were visually examined twice weekly for the growth of storage rots and their lesion
diameter. Apples naturally contaminated with other fungi were eliminated. After 6 wk storage, representative apples were photographed.

Experiments were carried out as four independent replicates, each comprising 10 inoculated apples per HWD treatment as well as 10 inoculated apples not subjected to HWD (positive control). For each replicate run, 10 wounded but noninoculated apples were also subjected to HWD at each temperature tested.

**Assessment of heat scald**
Heat damage was recorded after 15 wk storage at 2 °C as localised sunken areas of epidermal browning. Apples were graded according to the following key: (1) no heat scald; (2) sporadic brown lesions <5 x 5 mm; (3) heat-scalded areas >5 x 5 mm but covering <50 % of the fruit surface; and (4) heat-scalded areas >5 x 5 mm covering >50 % of the surface. The percentage of apples belonging to categories (3) and (4) was determined for each HWD temperature.

**Statistical analyses**
Data were expressed as percentages of germinated spores, infected wounds or heat-damaged fruit. An analysis of variance (ANOVA) test of arcsin-transformed percentages was performed, and significant differences (P<0.05) were calculated using the Tukey test. For *in vitro* tests, the effective temperature causing a 50 % inhibition of spore germination (ET\textsubscript{50}) was calculated by a linear regression of the percentage of germinated spores against dipping temperature.

**Results**

**Effect of temperature on spore germination in vitro**
The four fungi showed striking differences in their sensitivity to heat treatments recorded as percentage of germinated spores after 24 h incubation following a 3-min exposure to heat (Fig. 5.1). The most heat-sensitive fungus was *N. alba* (ET\textsubscript{50} = 39.3 °C). Both *N. galligena* (ET\textsubscript{50} = 42.9 °C) and *B. cinerea* (ET\textsubscript{50} = 44.4 °C) were slightly more heat-tolerant, *P. expansum* (ET\textsubscript{50} = 51.7 °C) more strongly so. Temperature inactivation curves of all four fungi followed a sigmoidal shape with a slow onset of spore mortality at low temperatures and a long tail of residual viability in the upper temperature range (Fig. 5.1), meaning that a substantial proportion of *P.*)
expansum spores as well as low proportions of conidia of N. galligena and B. cinerea would have survived HWD conditions of 52 °C for 3 min. In the case of P. expansum, <10 % of swollen but non-germinated spores were observed in addition to germinating ones at temperatures of 53-60 °C (see Fig. 5.7f,i). These swollen spores displayed a delayed germination after a further 2-4 d incubation (not shown). No such delayed germination was observed after exposure to higher temperatures (65 and 70 °C) where no swollen spores were apparent after 24 h (see Fig. 5.7l,o). No delayed germination was observed for N. alba, N. galligena or B. cinerea.

Heat scald of apples caused by HWD
Severe heat scald with individual lesions covering areas >5 x 5 mm was observed after HWD for 3 min at 52 °C, its incidence rising sharply to 98 % affected fruit after HWD at 58 °C (Fig. 5.1). Following HWD at 70 °C, the entire apple surface had become necrotic during subsequent cold storage (see Fig. 5.7m,n).

Effect of HWD temperature on B. cinerea and N. galligena in artificially inoculated apples
Due to the rapid progress of grey mould symptoms caused by B. cinerea, apples inoculated with this fungus were examined after 4 wk incubation in cold storage. A significant suppression of grey mould development was observed on inoculated fruit subjected to HWD at or above 48 °C (Fig. 5.2) and was apparent even at HWD temperatures causing severe damage to the fruit surface. In the case of N. galligena, the cold-storage period had to be extended to 10 wk for full symptom development. A significant suppression of Nectria fruit rot was evident following HWD of inoculated fruit at 51-53 °C, whereas after a 56 °C dip the incidence of fruit rot was as high as in the positive control, i.e. fruit inoculated with N. galligena but not subjected to HWD (Fig. 5.3). The highest control efficacies were 80.8 % at 49 °C for B. cinerea, and 67.0 % at 52 °C for N. galligena.

Effect of HWD temperature on P. expansum in artificially inoculated apples
A preliminary trial of apples inoculated with P. expansum prior to HWD revealed a significant suppression of blue mould development by HWD temperatures of 47-52 °C, corresponding to control efficacies of 52-82 %. In striking contrast, higher HWD temperatures gave rise to disease levels equivalent to the inoculated control fruit not subjected to HWD (Fig. 5.4) in a manner similar to N. galligena. This suppression of
blue mould by a narrowly delimited range of HWD temperatures was recorded after 4 and 6 wk cold storage (Fig. 5.4) and was still evident after 10 wk (not shown).

![Graph showing the effect of hot-water dipping (HWD) on skin damage and in vitro viability of spore suspensions of four storage-rot fungi.](image)

**Fig. 5.1.** Effect of hot-water dipping (HWD) for 3 min on skin damage of ‘Estar’ fruit due to heat scald (percent of fruit with lesions >5 × 5 mm after 15 wk cold storage), and on in vitro viability of spore suspensions of four storage-rot fungi (percent germinated spores after 24 h). Standard deviations of 3 (spore viability) or 4 (heat scald) independent experimental replicates are indicated by error bars.

![Graph showing the effect of hot-water dipping (HWD) on Botrytis cinerea infections.](image)

**Fig. 5.2.** Effect of hot-water dipping (HWD) for 3 min on *Botrytis cinerea* infections (pre-HWD inoculation) after 4 wk storage at 2 °C. Different letters indicate significant differences (*P*<0.05) between treatments. Inoculated control fruit not subjected to HWD showed 100 % infected wounds (significance range a).
We wondered whether such a complex temperature response pattern might be explained by differential effects of HWD temperatures on the fruit and on the *P. expansum* inoculum. In order to examine this possibility, a second trial was performed in which apples inoculated with *P. expansum* prior to HWD were compared with others inoculated post-dipping. The results revealed that fruit inoculated before or after HWD showed a similar suppression of blue mould symptoms by critical HWD temperatures of 49-54 °C (Fig. 5.5). During cold-storage following yet higher HWD temperatures (56-60 °C), a strong increase in the incidence of blue mould was observed in fruit inoculated pre-HWD or post-HWD. At these elevated temperatures, a significant increase in the average diameter of fruit-rot lesions caused by *P. expansum* was also observed especially in post-HWD inoculated apples (Fig. 5.6, Fig. 5.7j,k). At 70 °C, disease incidence remained at levels of the positive control in fruit inoculated post-dipping, whereas on pre-HWD inoculated apples there was no development of blue mould despite systemic tissue necrosis due to heat damage (Fig. 5.7m,n). No *P. expansum* infections were observed in wounded but noninoculated apples subjected to the same range of HWD temperatures (not shown), thereby ruling out chance contaminations by *P. expansum* during storage as the source of the observed disease symptoms.

![Fig. 5.3 Effect of hot-water dipping (HWD) for 3 min on *Neonectria galligena* infections (pre-HWD inoculation) after 10 wk storage at 2 °C. Different letters indicate significant differences (*P*<0.05) between treatments. Inoculated control fruit not subjected to HWD showed 80.3 % infected wounds (significance range ab).](image-url)
Fig. 5.4. Preliminary trial of the effect of hot-water dipping (HWD) on *Penicillium expansum* infections (pre-HWD inoculation) as percentage infected wounds after 4 and 6 wk storage at 2 °C. Different letters above the bars indicate significant differences (*P*<0.05) between treatments including the positive control (inoculated fruit not subjected to HWD).

Fig. 5.5. Effect of hot-water dipping (HWD) on *Penicillium expansum* wound infections (pre- and post-HWD inoculations) after 6 wk storage at 2 °C. Different letters indicate significant differences (*P*<0.05) between treatments including non-HWD control fruit inoculated at the pre-HWD (87.5 % infected wounds; significance range ab) and post-HWD time points (92.1 % infected wounds; significance range ab).
Fig. 5.6. Diameter of *Penicillium expansum* lesions on fruit inoculated before and after hot-water dipping (pre- and post-HWD, respectively) and in the positive controls (inoculated fruit not subjected to HWD) after 6 wk incubation at 2 °C. Different letters indicate significant differences between treatments (*P* < 0.05).

**Discussion**

The current work has provided evidence of a substantial efficacy of HWD against *B. cinerea*, *N. galligena* and *P. expansum* in artificially inoculated apples. It has also characterised a significant discrepancy between HWD temperatures lethal to spore suspensions *in vitro* and those causing the inhibition of fruit rot development during subsequent cold storage. A suppression of fruit rots was initiated at HWD temperatures at or above 47 °C in all three pathogen species tested, even though the ET$_{50}$ values of spore viability were lower by 3-4 K in two species (*B. cinerea, N. galligena*) and higher by 4.5 K in a third species (*P. expansum*). Thermal sensitivity of the inoculum of storage-rot fungi was clearly not the sole determinant for HWD control efficacy. Indeed, in the case of *N. galligena* and *P. expansum* an increasing incidence of fruit rot was observed following HWD temperatures at or above 53 °C, at which point physiological damage of the fruit surface due to heat scald became severe. Such a pattern has been reported previously for *P. expansum* on apples (Fallik et al. 2001), and we have also observed it in apples naturally infected with several storage rots and subjected to HWD at harvest (P. Maxin et al. in preparation). A direct inhibitory effect of heat on *P. expansum* in apples was evident only in pre-inoculated fruit exposed to HWD at 70 °C, at which point both fruit tissue and fungal inoculum were killed (Figs. 5.5 and 5.7). There is therefore a temperature range of
approx. 47 to 52 °C in which a 3-min HWD can suppress storage rots caused by *N. galligena, B. cinerea* and *P. expansum*, as well as other fungi (MAXIN et al. 2005; MAXIN

Fig. 5.7. Visual effects of hot-water dipping (HWD) for 3 min on *Penicillium expansum* at representative dipping temperatures. Apples were inoculated with *P. expansum* pre- or post-HWD and stored for 6 wk at 2 °C. Conidia were incubated at 20 °C for 24 h following HWD.
and Weber 2011). The upper limit of this range may vary depending on heat sensitivities of different apple varieties (Schirmer et al. 2004).

These findings suggest an important role of the physiological state of the fruit in the development of storage rot following HWD. Substantial support for this hypothesis is provided by the present work in which apples were resistant to P. expansum rot even if fruit were wound-inoculated with this fungus after HWD. This observation has practical relevance because P. expansum usually infects apples during storage (Amiri and Bompeix 2005). Similar results of a HWD effect on subsequent infections by storage-rot fungi have been obtained for pear fruit inoculated with Mucor piriformis or Phialophora malorum (Spotts and Chen 1987), and for grapefruit inoculated with Penicillium digitatum (Porat et al. 2000a).

Sub-lethal heat treatments are known to have profound effects on the physiology of plant cells and tissues, including ripening fruit. Heat may trigger the transcription not only of heat-shock proteins (HSPs; Woolf and Ferguson 2000), but also of pathogenesis-related proteins (PRPs) with putative or proven roles in suppressing microbial plant pathogens (Schirra et al. 2000; Pavoncello et al. 2001). In apples, HSPs have been induced by incubating fruit cell suspensions at 38 °C for 60 min (Wang et al. 2001), or by hot-air treatments of intact fruit for 4 d at 38 °C (Lurie and Klein 1990). Following heat shock, the transcripts of HSPs and PRPs as well as the proteins themselves may remain active for a few days at room temperature, but for several weeks in cold-storage (Sabehat et al. 1996; Pavoncello et al. 2001; Wang et al. 2001). In apples, a fungistatic antimicrobial effect against P. expansum was induced by HWD at 50 °C for 3 min, and was maintained during subsequent cold-storage for 100 d whilst disappearing within 150 d (Amiri and Bompeix 2011). Furthermore, Fallik et al. (1996) have demonstrated that crude extracts from the peel of heat-treated but not from untreated apple fruit were inhibitory to the growth of P. expansum in vitro. Therefore, the heat-shock induced activation of PRPs such as chitinases or β1,3-glucanases, and possibly phytoalexin-like substances, is a simple explanation as to how the physiological state of a fruit can have an effect on fruit rot development (Schirra et al. 2000; Pavoncello et al. 2001). This effect may be regarded as an act of acquired resistance induced by heat. Temperatures above the effective range may inhibit the heat-shock response by killing plant tissue. A low
proportion of fungal inoculum surviving such temperatures may give rise to a resurgence of storage rots associated with heat scald, as observed in the present study for *P. expansum* or *N. galligena*.

If heat shock is a trigger of resistance, a brief rinse with water, far shorter than that required for killing fungal spores, might be sufficient. This was indeed observed by PORAT et al. (2000a) and PAVONCELLO et al. (2001) who briefly rinsed grapefruit (20 s at 62 °C) and performed post-heat shock inoculations with *Penicillium digitatum*. Similarly, hot-water rinsing has been shown to be effective against *P. expansum* in apples (FALLIK et al. 2001) and numerous other fungal storage rots in a range of fruit (FALLIK 2004). Overall, the effect of HWD and other heat treatments in apples is best explained as a combination of the indirect fungistatic effect of induced resistance of fruit, and the variable, direct and fungicidal effect of heat on inoculum viability. Depending on experimental design, pathogen species and type of inoculum, the relative importance of these two effects in apples may differ (FALLIK et al. 1995; SCHIRRA et al. 2000; NERI et al. 2009). Further relevant effects of heat treatments could be morphological alterations of the fruit surface by rearrangements of epicuticular waxes, or the inhibition of fruit ripening processes (LURIE and KLEIN 1990; SCHIRRA and D’HALLEWIN 1997; PORAT et al. 2000b; FALLIK et al. 2001; TAHIR et al. 2009).

If induced resistance due to heat shock is an important factor, then the physiological state of the fruit at the time of heat treatment may be critical. In our work, high efficacies of HWD treatments were obtained with maturing apples that had already been stored in CA for 3-5 months prior to HWD. The HWD responsiveness of fruit at different physiological states caused by different pre-HWD and post-HWD storage conditions would be an important subject for further investigation. The present study may have benefited from using physiologically homogeneous stored apples, as opposed to freshly harvested apples which may be heterogeneous depending on whether they have experienced heat shock by exposure to solar irradiation before harvest (WOOLF and FERGUSON 2000). In such sun-exposed apples, there may be a renewed synthesis of HSPs on each sunny day (FERGUSON et al. 1998). In this context, orchardists’ observations of a reduced incidence of *Neofabraea* storage rots on highly pigmented apple fruit picked from outer and upper canopy regions (SCHULTE 1997) may be explained in connection with pre-harvest heat shocks. Such an effect
of pre-harvest temperatures on the development of post-harvest rots has been clearly demonstrated for avocado (WOOLF et al. 2000).

As discussed above, HWD can be effective against pre- and post-HWD inoculations with a wider range of fruit-rot fungi than previously realised. Since the introduction of HWD into organic farming practice is hampered by the perceived need to perform it at the peak work period during harvest time, the treatment of fruit after a few days of cold-storage or immediately after the opening of a long-term CA storage room provides new options for prolonging their subsequent storage life.

**Acknowledgements**

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References


**Chapter 5: Paper 1**


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Chapter 6: Paper 2

Control of *Phacidiopycnis washingtonensis* storage rot of apples by hot-water treatments but not by 1-MCP

Abstract

*Phacidiopycnis washingtonensis*, cause of rubbery rot of apples during long-term storage, was first observed in Denmark in April 2010 on fruits of the 2009 harvest. Hot-water treatments were examined as a possible way to control *P. washingtonensis*. The effective temperature causing a 50% mortality of infectious spores (conidia) after a 3-min submersion in water was 40.2°C. A significant reduction of rubbery rot was achieved by dipping artificially infected fruit in a water bath at 47–52°C for 3 min. Using naturally infected apples, *P. washingtonensis* as well as the widespread storage-rot pathogen *Neofabraea perennans* were effectively controlled by a post-harvest dip at 50°C for 3 min or by a rinse at 55°C for 20 s, followed by cold storage in controlled-atmosphere conditions. In contrast, a treatment of freshly harvested fruits with 1-methylcyclopropene instead of hot water failed to control *P. washingtonensis*.

Key words: Controlled-atmosphere storage, Malus domestica, 1-methylcyclopropene, *Neofabraea perennans*, post-harvest disease, rubbery rot
Abbreviations: CA = controlled atmosphere; DCA = dynamically controlled atmosphere; HWD = hot-water dipping; HWR = hot-water rinsing; 1-MCP = 1-methylcyclopropene

1. Introduction

*Phacidiopycnis washingtonensis* has been described as the cause of a new storage rot of apples in North America (Xiao et al. 2005, Kim & Xiao 2006) and, more recently, in Northern Germany where the disease has been named ‘rubbery rot’ (Weber 2011). Although the incidence of rubbery rot is generally low even after long-term storage under controlled atmosphere (CA) or dynamically controlled atmosphere (DCA) conditions, crop losses of 5–10% after storage have been recorded in harvests from some Northern German orchards (Weber 2011). There was no obvious correlation between disease severity and pre-harvest orchard management by integrated or organic approaches (RWS Weber, unpublished).

Irrespective of orchard management, *Neofabraea perennans* and *N. alba* are by far the most important storage-rot fungi of apples in Northern Europe (Palm & Kruse 2005, Weber 2009). Hot-water dipping (HWD) or rinsing (HWR) of freshly harvested apples prior to their placement in long-term storage is an effective way to control *Neofabraea* spp. as well as other post-harvest pathogens such as *Neonectria galligena*, *Botrytis cinerea* and *Penicillium expansum* (Fallik et al. 1995, Trierweiler et al. 2003, Maxin et al. in preparation). Similarly, a pre-storage treatment with 1-methylcyclopropene (1-MCP), an inhibitor of the fruit ripening hormone ethylene, may retard the development of storage rots (Rizzoli & Acler 2009). In view of the growing importance of rubbery rot even in orchards treated with standard fungicide spray programmes (captan and QoI compounds; RWS Weber, unpublished), we used naturally and artificially infected fruits to examine the efficacy of HWD, HWR and 1-MCP against *P. washingtonensis*. In addition, the present paper provides the first report of *P. washingtonensis* from Denmark.

2. Materials and methods

2.1 Artificial inoculation trials

*Phacidiopycnis washingtonensis* was isolated on 20 April 2010 from infected stored apples (cv. Elstar) which had been harvested in Sept. 2009 from an orchard located...
in Aarslev (Aarhus University, Denmark; 55°18’ N, 10°26’ E, altitude 47 m). This orchard (tree density 1 m by 3.5 m, rootstock M9, planted in autumn 2003) had been managed with a low-input fungicide spray schedule after petal fall based solely on applications of wettable sulphur. A representative isolate of *P. washingtonensis* has been incorporated as accession number OVB10-012 into the Culture Collection, Esteburg Fruit Research and Advisory Centre (Jork, Germany). Conidia were harvested from cultures sporulating on potato dextrose agar (PDA), and diluted to 10⁶ ml⁻¹ in distilled water. Apples (cv. Elstar) previously stored in CA conditions for 6 months were wounded (2 mm diam., 1 mm depth) at three positions, and each wound was inoculated with approx. 2 ×10⁴ conidia using a soft paintbrush. Following incubation at 2°C for 24 h, pre-inoculated fruits were subjected to HWD for 3 min at 40, 44, 47, 49, 51, 52, 53, 54, 56, 58 or 60°C. For each dipping temperature, 4 repeats each comprising 10 inoculated apples were examined. Apples were then stored for 6 weeks at 2°C in ambient atmosphere prior to the evaluation of results as percent of inoculated wounds showing rubbery rot.

The heat sensitivity of conidia *in vitro* was examined by dipping 250 µl aliquots of spore suspension of strain OVB10-012 in 1.5 ml Eppendorf vials for 3 min at 37, 40, 44, 47, 49, 51, 52, 53, 54, 56 or 58°C, and observing the germination of spores on PDA after 40 h incubation at 20°C. This experiment was performed three times independently.

### 2.2 Treatments of natural infections

Apples (cv. Elstar) were obtained from an organically managed orchard at the Esteburg site (53°30’ N, 9°45’ E, altitude -2 m). In order to maximise the incidence of natural infections of fruits by storage-rot fungi, the second-picked harvest was used (Palm & Kruse 2005). Pale fruits were selected because of the ease of assessing skin disorders (Hennecke et al. 2008). For each hot-water treatment, 4 replicates each containing 8 kg fruit (55–65 apples) were subjected to HWD for 3 min at 50°C, or to HWR for 20 s at 55°C. Alternatively, apples were treated with 1-MCP (Smartfresh®; Rohm and Haas Inc., Spring House, Pennsylvania, USA) according to the distributor’s recommendations. All apples were initially stored at 2°C for 10 d in ambient atmosphere, followed by storage at 2°C either in CA with 1.4% O₂ and 2.6% CO₂, or in DCA where O₂ was reduced from 1.4% to 0.7% within 4 months (Veltman et al. 2003).
A Harvest Watch® system (Satlantic Inc., Halifax, Nova Scotia, Canada) was used to prevent anoxia of fruit. After 6 months’ CA or DCA storage, apples with fruit-rot symptoms were separated and incubated for a further 2 months at 2°C in ambient atmosphere for full symptom development. Infections by *P. washingtonensis*, *Neofabraea alba* and *N. perennans* were identified unambiguously by the appearance of affected fruits and by spore morphology (Verkley 1999, Weber 2009, 2011).

### 2.3 Statistical analyses

Data were expressed as percentages of germinated spores, wounds with rubbery rot lesions, or rotted fruits. The effective temperature causing a 50% inhibition of spore germination (**ET**\textsubscript{50}) was calculated by a linear regression of critical dipping temperatures *versus* percentage of germinated spores relative to the undipped control. For artificial and natural fruit infections, an analysis of variance of arcsin square-root transformed percentages was performed, and significant differences (**P**<0.05) relative to a control not subjected to heat treatments were calculated using the Tukey test. All statistical analyses were performed using the computing environment ‘R project’ (http://www.r-project.org).

### 3. Results and discussion

Identification of *P. washingtonensis* on stored apples of the Sept. 2009 harvest from the Aarslev site was unambiguous on the basis of disease symptoms, colony morphology on PDA, conidial dimensions, and 100% ITS sequence identity with Northern German and North American isolates (GenBank accessions AY608643-AY608648 and JF732919; Xiao et al. 2005, Weber 2011). In the 2010 harvest from the same orchard, 9.8% of 4000 fruits examined after 8 months’ cold-storage in May 2011 were affected by this fungus. To the best of our knowledge, this is the first report of *P. washingtonensis* as well as the rubbery rot disease of apples in Denmark. As discussed by Kim & Xiao (2006) and Weber (2011), the true distribution and importance of *P. washingtonensis* may well be underestimated, and we anticipate further reports of apple infections from other European countries.
Table 6.1: Effects of pre-storage treatments of naturally infected apples (cv. Elstar) on the incidence of storage rot (percent affected fruits) caused by *Neofabraea perennans* and *Phacidioptynis washingtonensis* after 6 mo controlled atmosphere (CA) or dynamically controlled atmosphere (DCA) storage.

<table>
<thead>
<tr>
<th></th>
<th>Neofabraea perennans</th>
<th>Phacidioptynis washingtonensis</th>
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<tbody>
<tr>
<td></td>
<td>CA</td>
<td>DCA</td>
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<tr>
<td>Control</td>
<td>9.4 a</td>
<td>2.9 bc</td>
</tr>
<tr>
<td>1-MCP</td>
<td>4.2 b</td>
<td>3.5 bc</td>
</tr>
<tr>
<td>HWD</td>
<td>0.2 c</td>
<td>0.6 c</td>
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<tr>
<td>HWR</td>
<td>0.4 c</td>
<td>1.3 bc</td>
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1 Treatment with 1-methylcyclopropene (SmartFresh®)
2 Hot-water dipping for 3 min at 50 °C
3 Hot-water rinsing for 20 s at 55 °C
4 For each fungus, significant differences according to the Tukey test at $P<0.05$ are indicated by different letters

Xiao et al. (2005) reported relatively low growth temperatures for *P. washingtonensis*, and a high sensitivity of conidia to high temperatures was confirmed in our study which revealed an ET$_{50}$ value of 40.2°C in a 3-min dip typical of HWD. This was similar to the ET$_{50}$ of *N. alba* (39.2°C) but lower than values recorded for *N. galligena* (43.2°C), *B. cinerea* (43.8°C) and *P. expansum* (52.2°C) under identical conditions (Maxin et al. in preparation). The incidence of rubbery rot in artificially infected apples showed a nearly linear decline with increasing HWD temperatures (Fig. 6.1). Significant ($P<0.05$) suppression of rubbery rot was achieved by HWD temperatures at or above 47°C. In contrast to storage rots caused by *P. expansum* and *N. galligena* (Maxin et al. in preparation), there was no increase in the incidence of rubbery rot in heat-damaged apples following exposure to HWD temperatures above 53°C.
Fig. 6.1: Effect of a 3-min hot-water dip on Phacidioxyenis washingtonensis in terms of spore viability in vitro (○) and development of rubbery rot in artificially infected apple wounds (▲). Error bars indicate standard deviations (spore viability; \( n = 3 \)) and different letters indicate significant differences according to the Tukey test at \( P<0.05 \) (wound infections; \( n = 4 \)). Control values without heat treatments were 67.8% germinated spores, and 85.5% infected fruit. The linear regression equation for spore viability was \( y = -10.47x + 471.0 \) (\( R^2 = 0.98 \)) for temperatures up to 44°C.

Elstar apples harvested from the Esteburg site were naturally infected with \( P. \) washingtonensis as well as \( N. \) perennans. We were therefore able to compare the efficacy of HWD, HWR and 1-MCP treatments against both fungi (Table 6.1). The combination of 1-MCP treatment with CA or DCA storage failed to produce any consistent synergistic effect in either fungus, confirming previous reports for \( Neofabraea \) spp. (Lafer 2009). When naturally infected apples from Aarslev were treated with 1-MCP and then kept in cold-storage at ambient atmosphere for 8 months, the incidence of \( P. \) washingtonensis remained unaffected by 1-MCP whereas infections by \( N. \) alba were significantly reduced by 88% relative to the untreated control (M Bertelsen, P Maxin & RWS Weber in preparation).

In contrast, both HWD and HWR treatments were highly effective against \( P. \) washingtonensis and \( N. \) perennans in naturally infected fruit (Table 6.1). For \( N. \) perennans and its sister species \( N. \) alba, there is a substantial body of literature describing the efficacy of HWD and HWR (e.g. Burchill 1964; Trierweiler et al. 2003). Our results confirm that \( P. \) washingtonensis is similarly susceptible to heat treatments.
either alone or in combination with commercially applied CA or DCA storage methods.

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References


Chapter 7: Paper 3 Control of a wide range of storage rots in naturally infected apples by hot-water dipping and rinsing

Abstract
Hot-water rinsing (15, 20 or 25 s) and dipping (3 or 4 min) at a range of incubation temperatures was applied to apples (cv. ‘Ingrid Marie’ and ‘Pinova’) naturally infected with a range of North West European storage-rot fungi. Significant reductions in the incidence of fruit rot were achieved by incubation periods of 3 min at 50-54 °C (dipping) and 20 or 25 s at 55 °C (rinsing), followed by up to 100 d cold-storage at 2 °C and 14 d at 18 °C. Pathogens controlled in this way were Neofabraea alba, N. perennans, Monilinia fructigena, Colletotrichum acutatum, Phacidiopycnis washingtonensis and Cladosporium spp. Neonectria galligena was reliably controlled by dipping but not rinsing. No effects of either heat treatment on Gibberella avenacea and Botrytis cinerea were apparent. Following rinsing at 65 °C for 20 s, the incidence of P. washingtonensis, Penicillium expansum, Mucor spp. and Phoma exigua was higher than in untreated control fruit or in apples rinsed at lower temperatures, and was associated with heat damage. The relative contributions of heat effects on inoculum viability and activation of defence responses of apple fruit are discussed. Hot-water rinsing has several advantages over hot-water dipping.
related to the efficient processing of fruit either directly after harvest or after long-term storage.

**Keywords**

*Malus domestica*, defence response, heat damage, latent infection, post-harvest disease

1. Introduction

Mild and humid climatic conditions such as those prevalent in North Western Europe favour post-harvest fruit rots caused by fungi. The most important pathogens which infect apples prior to harvest are *Neofabraea* spp. (*N. alba* and *N. perennans*), *Neonectria galligena* and *Monilinia fructigena* in descending order of importance (Palm and Kruse, 2005). *Penicillium* spp. and *Botrytis cinerea* may infect fruit before and also during storage (Jijakli and Lepoivre, 2004). Although modern storage technologies aimed at retarding fruit ripening have an effect on many fruit rots (Spotts et al., 2007; Lafer, 2010), repeated sprays with fungicides (e.g., captan and strobilurin-type compounds) during the two months preceding harvest remain an essential component of the current strategy to control storage-rot fungi (Palm and Kruse, 2005; Minar, 2006).

The use of fungicides shortly before harvest is under scrutiny because of retailers’ demands to reduce pesticide residues well below the legally permissible thresholds, or to restrict the number of detectable residues (Poulsen et al., 2009). Furthermore, resistance development may impair the efficacy of fungicides against key pathogens (Weber and Palm, 2010). Alternative strategies to control fungal post-harvest diseases are therefore required, and this is especially important for organic orchardists who may experience elevated storage losses because of the non-availability of chemical fungicides (Holb and Scherm, 2007; Granado et al., 2008).

Heat treatments of apples have shown promise in reducing the subsequent development of storage rots (Fallik et al., 2001). High efficacies against *Neofabraea* spp. and *Penicillium expansum* have been obtained after incubation in hot air (e.g. 72 h at 40 °C; Fallik et al., 2001; Tahir et al., 2009) or by hot-water dipping (HWD) for up to 3 min (Maxin et al., 2005; Amiri and Bompeix, 2011). Hot-water rinsing (HWR) for
<30 s at temperatures above 50 °C has been developed in Israel to control post-harvest pests and diseases of a range of horticultural products (Fallik, 2004). In Northern Germany, HWD has been introduced into organic apple production (Maxin et al., 2006), although acceptance of this technology by fruit orchardists has been hampered by high energy costs and the need for added labour during the peak work time at harvest (Maxin and Klopp, 2004). Furthermore, there is only limited information on the range of fungi that can be controlled by HWD and especially HWR.

In preliminary studies, Maxin and Weber (2011) and Maxin et al. (2012) had shown that HWD could successfully control various storage rots on artificially inoculated apples. The aim of the present report was to characterise the full range of fungal pathogens susceptible to HWD and HWR as natural infections, and to evaluate the potential of HWR as an alternative to HWD in commercial organic fruit production.

2. Materials and methods

2.1. Apples

On 22 Sept. 2009 and 29 Sept. 2010, apples (cv. ‘Ingrid Marie’) were harvested from an experimental orchard at Aarslev (Aarhus University, Denmark; 55°18’ N, 10°26’ E, altitude 47 m) because previous surveys at this site had shown a high incidence of storage rots (Maxin and Weber, unpublished). Apples were harvested at a starch index of 3.5–4.0 according to Streif (1983) and a fruit flesh firmness of 6.5–7.5 kg cm\(^{-2}\) (measured with a GS20 fruit texture analyser, Güss Ltd., Strand, South Africa). In order to maximise natural infections by storage-rot fungi, this orchard was not exposed to any fungicide treatment after petal fall.

Apples cv. ‘Pinova’ harvested on 4 Oct. 2010 from the Esteburg experimental farm in Northern Germany (53°30’ N, 9°45’ E, altitude -2 m) were also included in the evaluations. The starch index at harvest was 4.0–5.0, and the fruit flesh firmness was 8.0–9.0 kg cm\(^{-2}\). This orchard had been under organic management since 1995, and stored fruit from previous harvests had shown a reliable incidence of bull’s-eye rot caused by Neofabraea spp. (Maxin, unpublished).

In view of the highly localised occurrence of storage-rot inoculum on individual trees (Spolti et al., 2011; Maxin and Weber, unpublished), apples from different trees were
mixed after harvest. Aliquots of 90-110 fruit (cv. ‘Ingrid Marie’) or 40-46 fruit (cv. ‘Pinova’) were packed in perforated plastic boxes (40 l volume; 60 x 40 x 17 cm; 35% perforated area in side walls and bottom), stored for 5 d at 2 °C at ambient atmosphere, and then subjected to HWD or HWR. All treatments were replicated four times, each replicate comprising apples from one box.

2.2. Hot-water dipping (HWD)
Plastic boxes containing apples were dipped in 350 l heated water. The top of each box was covered with another box containing a 5 kg weight, thereby ensuring that all fruit remained entirely submerged throughout the HWD period. Heat loss and cooling effects were buffered by adding 95 °C water from a commercial steam-jet blower that introduced water currents into the dipping unit to ensure that a uniform temperature was maintained around the apples within 30 s of submersion. During dipping, temperatures were monitored between apples in the centre of the dipped box using an electric thermometer (Voltcraft K 204; Conrad Electronic SE, Hirschau, Germany). Prior to each HWD step, the temperature of the water bath was equilibrated and checked with a certified analog mercury thermometer scaled to 0.1 °C (Carl Roth, Karlsruhe, Germany). In 2009, HWD was carried out at four temperatures (48, 50, 52, 54 °C) in combination with two dipping times (3 and 4 min) which were chosen on the basis of previous results with apples showing reduced efficacies after 1 or 2 min HWD (Trierweiler et al., 2003; Maxin et al., 2005). In 2010, HWD was carried out for 3 min at 50, 52 and 54 °C.

2.3. Hot-water rinsing (HWR)
For the 2009 trial, single apples were removed from the plastic boxes, placed on a conveyor belt with rotating elements, and sprayed with hot water from 12 flat fan nozzles (Type DG 005 VS TeeJet; Spraying Systems Co.; Wheaton USA). Hot water consumption was 2 l nozzle⁻¹ min⁻¹, and each apple was treated with 2 l hot water during a 20 s exposure. High losses of energy were observed, a 10 cm spraying distance between the nozzles and the apple surface reducing the temperature by approx. 10 K. The actual treatment temperature (T₁) was measured with an analog mercury thermometer in water samples collected from the processing line. The adjusted water temperature (T₂ = T₁ + 10 K) was controlled with a second thermometer incorporated in the water supply unit upstream of the nozzles. In the
2009 season, apples were rinsed for a standard period (20 s) at different temperatures (55, 58 or 62 °C).

For the 2010 trial, equipment modifications and parameter changes were introduced (Fig. 3.7). To ensure that temperatures were within ±1 K of the required values, a volume of 400 l water was heated in a closed system to the specified treatment temperature by electronic heaters connected to an automatically regulated digital control unit (ELK 38, EL.CO. S.r.l., Pievebelvicino, Italy). During HWR processing, apples were rotated and floated in a row formed by water currents at 16 positions on one side and a border of fixed plastic brushes on the other side. The addition of a new apple at the beginning of the row resulted in a forward movement of the row of apples by one position. The last fruit leaving the row at the end of the line was removed manually. Experimental repeats were separated by inserting green dummy apples. The duration of HWR treatments was determined by the speed of adding apples into the process line which was controlled by using the regulated conveyor belt from the 2009 trial. In the 2010 trial, HWR temperatures were combined with different exposure times, i.e. 55 °C for 15, 20 and 25 s, 60 °C for 7, 15, 20 and 25 s, and 65 °C for 20 s.

2.4. Storage after hot-water treatments
Following HWD or HWR, apples were stored for 100 d at 2 °C and 14 d at 18 °C in ambient atmosphere, and examined at 14-d intervals. Apples showing incipient fruit rot were isolated from healthy fruit, labelled, and kept at 2 °C until the onset of sporulation.

2.5. Identification of fruit rots
Fungi associated with fruit rots were identified for each infected apple by the appearance of macroscopic symptoms, sporulating structures and microscopy of spores produced. Pure-culture isolates were obtained from representative infections by streaking out spores onto potato dextrose agar augmented with 200 mg penicillin G and streptomycin sulphate l⁻¹ agar (supplied by Carl Roth). These isolates were incorporated into the culture collection, Esteburg Fruit Research and Advisory Centre, Germany. DNA extraction from mycelium, PCR amplification and sequencing of the internal transcribed spacer (ITS) region of ribosomal DNA were carried out as
described in detail by Weber (2011). Sequence searches were performed in GenBank using the BLASTN function (Zhang et al., 2000).

2.6. Assessment of heat damage
Physiological damage due to heat was examined after 70 d at 2 °C. Heat damage was identified as slightly sunken regions of brownish discolouration which did not spread during further incubation at 2 °C. Four categories were distinguished, i.e. 1 (no damage), 2 (small occasional spots <5 x 5 mm), 3 (spots >5 x 5 mm covering <50% of the fruit surface), and 4 (severe damage covering >50% of the fruit surface).

2.7. Statistical analyses
Data were expressed as percentages of heat-damaged fruit (categories 3-4) or apples infected by a given fungal pathogen. Efficacies of HWD or HWR treatments against fruit-rot development were calculated according to Abbott (1925). In case of fruit showing multiple infections, each identifiable fungus was recorded as a separate infection event, whereas multiple infections by the same fungus on the same apple were counted as one. An analysis of variance (ANOVA) test of arcsin square root-transformed percentages was performed, and significant differences ($P<0.05$) were calculated using the Tukey test. The computing environment ‘$R$-project’ (http://www.r-project.org) was used for all statistical analyses.

3. Results
3.1. Identification of storage-rot fungi
During the two years of these experiments, approx. 2,000 rotten apples were examined visually and by microscopy for the occurrence and identity of pathogenic fungi. *Botrytis cinerea*, *Monilinia fructigena* and *Neonectria galligena* were unequivocally identifiable by these means (Jones and Aldwinckle, 1990). For other common or unusual species, microscopic identification was confirmed by ITS sequence analysis of representative isolates (Table 7.1). In the case of minor rots occurring as species complexes (*Mucor, Cladosporium*), identification to species level was not attempted.

In both years of the trials, *Neofabraea alba* was the dominant storage-rot fungus on ‘Ingrid Marie’ fruit from the Aarslev site. An exceptionally wide range of additional pathogens was identified in these apples (Table 7.2). Because only traces of *N.*
perennans were discovered in ‘Ingrid Marie’ harvested from Aarslev, we obtained apples cv. ‘Pinova’ from another orchard (Esteburg site) which in previous years had shown infections by \textit{N. perennans}. The 2010 harvest from this orchard was heavily colonised by both \textit{N. alba} and \textit{N. perennans} (Table 7.2).

3.2. Heat damage associated with HWD and HWR

No heat damage was observed on ‘Ingrid Marie’ fruit subjected to HWD at temperatures up to 50 °C, or to HWR up to 58 °C. HWD caused significant (\(P<0.05\)) skin damage (categories 3 and 4) at 52 and 54 °C (Fig. 7.1A). Significant skin damage was also caused by HWR at 60 °C or above (Fig. 7.2A).

Table 7.1: Important or unusual storage-rot fungi on ‘Ingrid Marie’ apples from Aarslev (Denmark) identified by ITS sequence analysis.

<table>
<thead>
<tr>
<th>Species</th>
<th>Esteburg accession number</th>
<th>Reference for microscopic identification</th>
<th>Representative GenBank sequences (% identity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Neofabraea perennans}</td>
<td>OVB11-006</td>
<td>Verkley (1999)</td>
<td>AF281389, AF281390, AF281391, AF281392, AF281393, AF281395, AF281396, AF281397 (all 100%)</td>
</tr>
<tr>
<td>\textit{Neofabraea alba}</td>
<td>OVB11-007</td>
<td>Verkley (1999)</td>
<td>AF141190, AF281366, AF281367, AF281368, AY359235, AY359236, EU098116, EU098124, HQ166293, HQ166318, HQ166337, HQ166387, HQ166390, HQ166503 (all 100%)</td>
</tr>
<tr>
<td>\textit{Penicillium expansum}</td>
<td>OVB11-008</td>
<td>Pitt (1979)</td>
<td>DQ339547, DQ339548, DQ339552, DQ339558, DQ339562 (all 100%)</td>
</tr>
<tr>
<td>\textit{Phacidiopycnis washingtonensis}</td>
<td>OVB10-012</td>
<td>Weber (2011)</td>
<td>see Maxin and Weber (2011)</td>
</tr>
<tr>
<td>\textit{Gibberella avenacea}</td>
<td>OVB11-004 and 11-005</td>
<td>Booth (1971)</td>
<td>AY147282, AY147283, AY147284 (all 100%)</td>
</tr>
<tr>
<td>\textit{Phoma exigua}</td>
<td>OVB11-003</td>
<td>-</td>
<td>EU343139, EU167567, AJ608976, EF136400 (all 100%)</td>
</tr>
</tbody>
</table>
3.3. Effect of HWD and HWR on storage rots

HWD in the range of 48-54 °C controlled *N. alba* infections at efficacies above 80% (Fig. 7.1B). The commercially applied conditions of HWD for 3 min at 50 °C reduced *N. alba* by 86% and 84% in 2009 and 2010, respectively. HWR also significantly reduced fruit rot due to *N. alba* (Fig. 7.2B). The highest efficacy was 82% in ‘Ingrid Marie’ in 2010 (Table 7.3) and 77% in ‘Pinova’ (Table 7.4) following HWR for 25 s at 55 °C. When ‘Pinova’ apples from Northern Germany were treated by HWD for 3 min at 54 °C, *N. perennans* fruit rot was reduced by 96% (Table 7.4). Reduced control of *N. perennans*, at efficacies of 59-73%, was obtained by HWR (Table 7.4). Therefore both Neofabraea spp. responded similarly to hot-water treatments. Storage rots caused by *N. galligena* were present in ‘Ingrid Marie’ in both years. In 2009, all HWD and HWR treatments except 15 s at 55 °C significantly (*P*<0.05) reduced fruit rot caused by this fungus (not shown). In 2010, *N. galligena* rot was significantly reduced by moderate HWD treatments such as 3 min at 50 °C, but not by HWR. The highest incidence of *N. galligena* was associated with the most severe HWR treatment of 20 s at 65 °C which caused major heat damage.

Table 7.2: Incidence of different storage-rot fungi (percent of total fruit examined) in untreated control fruit of cv. ‘Ingrid Marie’ (Aarslev, Denmark) and cv. ‘Pinova’ (Esteburg, Germany) after storage for 100 d at 2°C and 14 d at 18°C (n.d., not determined).

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>‘Ingrid Marie’ 2009 (%)</th>
<th>‘Ingrid Marie’ 2010 (%)</th>
<th>‘Pinova’ 2010 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Neofabraea alba</em></td>
<td>28.6</td>
<td>37.0</td>
<td>62.0</td>
</tr>
<tr>
<td><em>Neofabraea perennans</em></td>
<td>0</td>
<td>0.8</td>
<td>24.5</td>
</tr>
<tr>
<td><em>Neonectria galligena</em></td>
<td>5.3</td>
<td>4.5</td>
<td>0</td>
</tr>
<tr>
<td><em>Monilinia fructigena</em></td>
<td>4.1</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td><em>Cladosporium spp.</em></td>
<td>3.3</td>
<td>1.4</td>
<td>0</td>
</tr>
<tr>
<td><em>Penicillium expansum</em></td>
<td>2.3</td>
<td>1.9</td>
<td>0</td>
</tr>
<tr>
<td><em>Phacidiopycnis washingtonensis</em></td>
<td>n.d.</td>
<td>2.4</td>
<td>0</td>
</tr>
<tr>
<td><em>Colletotrichum acutatum</em></td>
<td>0.3</td>
<td>1.8</td>
<td>0</td>
</tr>
<tr>
<td><em>Gibberella avenacea</em></td>
<td>0.6</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td><em>Botrytis cinerea</em></td>
<td>0</td>
<td>0.5</td>
<td>0</td>
</tr>
</tbody>
</table>

Blue mould caused by *P. expansum* was not controlled by any hot-water treatment, and its incidence significantly (*P*<0.05) increased following exposure of the fruit to
high temperatures, such as HWD at 54 °C for 4 min or HWR at 62 °C for 20 s in 2009 (not shown), or HWR for 20 s at 65 °C in 2010 (Table 7.3). In line with this finding, *P. expansum* infections were positively correlated with increasing severity of physiological heat damage in ‘Ingrid Marie’ (Fig. 7.3).

Due to low infection rates in 2010, no clear-cut effects of hot-water treatments were obtained for *M. fructigena*. However, in 2009 a significant suppression of Monilinia fruit rot was obtained by HWD for 3 min at 54 °C, or HWR for 20 s at 58 °C and 62 °C (Table 7.3).

---

**Fig. 7.1.** Effects of hot-water dipping (HWD) at various temperatures for 3 min (black bars) and 4 min (white bars) in apples cv. ‘Ingrid Marie’. (A) Heat damage presented as percentage of fruit with lesions >5 x 5 mm, and (B) control efficacy (%) of *Neofabraea alba* storage rot on naturally infected fruit. Means of experimental replicates for 2009 are shown (n = 4). Error bars indicate standard deviation.
Fig. 7.2. Effects of hot-water rinsing (HWR) at various temperatures for 15 s (black bars), 20 s (striped bars) and 25 s (empty bars) in apples cv. ‘Ingrid Marie’. (A) Heat damage presented as percentage of fruit with lesions >5 x 5 mm, and (B) control efficacy (%) of Neofabraea alba storage rot on naturally infected fruit. Means of experimental replicates for 2010 are shown (n=4). Error bars indicate standard deviation.
Table 7.3: Incidence of storage-rot fungi on ‘Ingrid Marie’ apples treated by hot-water dipping (HWD) and hot-water rinsing (HWR) relative to the untreated control after 100 d storage at 2°C, followed by 14 d at 18°C (n.d., not determined). For each fungus, data are presented as means (n=4), and significant differences of treatments relative to the control (P<0.05; Tukey test) are indicated by different letters.

<table>
<thead>
<tr>
<th>Storage-rot fungus</th>
<th>Year of treatment</th>
<th>Control</th>
<th>Hot-water dipping (HWD)</th>
<th>Hot-water rinsing (HWR)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>3 min at 50°C</td>
<td>3 min at 52°C</td>
</tr>
<tr>
<td><em>Neofabraea alba</em></td>
<td>2010</td>
<td>37.0 a</td>
<td>5.8 bc</td>
<td>5.5 c</td>
</tr>
<tr>
<td><em>Neonectria galligena</em></td>
<td>2010</td>
<td>4.5 a</td>
<td>2.5 ab</td>
<td>1.0 b</td>
</tr>
<tr>
<td><em>Monilinia fructigena</em></td>
<td>2009</td>
<td>4.1 a</td>
<td>1.4 ab</td>
<td>0.6 c</td>
</tr>
<tr>
<td><em>Phacidiopycnis washingtonensis</em></td>
<td>2010</td>
<td>2.4 b</td>
<td>0.5 c</td>
<td>0.3 c</td>
</tr>
<tr>
<td><em>Penicillium expansum</em></td>
<td>2010</td>
<td>1.9 b</td>
<td>4.8 ab</td>
<td>1.3 b</td>
</tr>
<tr>
<td><em>Colletrotrichum acutatum</em></td>
<td>2010</td>
<td>1.8 a</td>
<td>0.0 b</td>
<td>0.5 b</td>
</tr>
<tr>
<td><em>Cladosporium spp.</em></td>
<td>2010</td>
<td>1.4 a</td>
<td>0.0 b</td>
<td>0.0 b</td>
</tr>
<tr>
<td><em>Gibberella avenacea</em></td>
<td>2009</td>
<td>0.6</td>
<td>1.1</td>
<td>0.6</td>
</tr>
<tr>
<td><em>Botrytis cinerea</em></td>
<td>2009</td>
<td>0.0</td>
<td>0.3</td>
<td>0.0</td>
</tr>
<tr>
<td><em>Mucor spp.</em></td>
<td>2010</td>
<td>0.0 b</td>
<td>0.0 b</td>
<td>0.0 b</td>
</tr>
<tr>
<td><em>Phoma exigua</em></td>
<td>2010</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>
Table 7.4: Incidence of *Neofabraea alba* and *N. perennans* (percent infected fruit) on apples (cv. ‘Pinova’) treated by hot-water dipping (HWD) and hot-water rinsing (HWR) relative to the untreated control after 100 d storage at 2°C and a further 14 d at 18°C. Data are presented as means (n=4), significant differences of treatments relative to the control (P<0.05; Tukey test) are indicated by different letters.

<table>
<thead>
<tr>
<th>Storage-rot fungus</th>
<th>Control</th>
<th>HWD</th>
<th>HWR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3 min at 50°C</td>
<td>3 min at 52°C</td>
</tr>
<tr>
<td><em>Neofabraea alba</em></td>
<td>62.0 a</td>
<td>13.0 cd</td>
<td>7.5 d</td>
</tr>
<tr>
<td><em>Neofabraea perennans</em></td>
<td>24.5 a</td>
<td>8.5 bc</td>
<td>4.0 bc</td>
</tr>
</tbody>
</table>

Fig. 7.3. Correlation of Penicillium expansum fruit rot (percent infected fruit) with heat damage (percent of apples with lesions >5 x 5 mm) on ‘Ingrid Marie’ apples subjected to hot-water dipping (HWR) at temperatures above 51 °C (2009 data). The regression equation was $y = 1.19 e^{0.0234x}$ (R² = 0.76).

In 2009, *Colletotrichum acutatum* occurred as a minor storage rot in ‘Ingrid Marie’ apples, resulting in low degrees of infection that failed to produce any significant result. However, in 2010 HWD for 3 min at 50 °C and HWR for 20 s or 25 s at 55 °C gave significant control (Table 7.3). In 2010, fruit rot associated with *Cladosporium* spp. was observed almost exclusively on untreated fruit, and this was significantly controlled by all hot-water treatments (Table 7.3). In marked contrast, an increase in
the incidence of fruit rots was observed for several fungal pathogens in heat-
damaged apples (Table 7.3). The incidence of *Mucor* spp. was significantly \((P<0.05)\)
confined to fruit with severe heat damage treated by HWR for 20 s at 65 °C, but was
not associated with heat damage caused by HWD. A similar observation was made
for *Phoma exigua* which caused a sporadic fruit rot with black lesions physically
closely associated with heat-damaged areas (not shown). Significant \((P<0.05)\) control
of rubbery rot caused by *Phacidiopycnis washingtonensis* was achieved by all HWD
treatments and by all HWR treatments except at 65 °C where the incidence of
rubbery rot was significantly higher than in the untreated control.

4. Discussion

The present study has shown that HWD of apples is capable of controlling natural
infections by *Colletotrichum acutatum*, *Neonectria galligena* and *Cladosporium* spp.
for which no critical data have been reported previously, in addition to confirming
HWD susceptibility of *Neofabraea* spp. (Burchill, 1964), *Monilinia fructigena* (Maxin et
al., 2005) and *Phacidiopycnis washingtonensis* (Maxin and Weber, 2011). Whereas
excessive HWD conditions (3 min at 54 or 56 °C) had significant deleterious effects
on the fruit surface in terms of heat damage (this study) and pigment bleaching
(Maxin, unpublished), no effects on internal quality parameters such as flesh firmness
or sugar and starch contents were observed (Maxin, unpublished). Lower
temperatures (50 or 52 °C) did not produce such surface defects whilst still affording
high efficacies of storage-rot control. Further, our study is the first report of the
successful use of HWR to control apple storage-rot fungi other than *P. expansum*
(Fallik et al., 2001) and *P. washingtonensis* (Maxin and Weber, 2011). Although
different kinds of equipment were used in 2009 and 2010, both approaches gave rise
to comparable results.

In general terms, HWR was not quite as effective as HWD in our trials, although we
acknowledge that this technique has not yet been fully optimised. From Fig. 7.2 it is
apparent that further experiments should test prolonged exposure times up to 30 s at
55 °C, or explore temperatures between 55 and 60 °C at a rinsing time of 20 s. Such
an approach may be worthwhile because many apple varieties are much less prone
to heat damage than the highly sensitive ‘Ingrid Marie’ (Schirmer et al., 2004).
HWD and HWR were able to control a similar range of fungal pathogens. A few species such as *G. avenacea*, *B. cinerea* and *P. expansum* were relatively indifferent to both kinds of heat treatment (Table 7.3). In the case of the latter two species, this result was unexpected because our previous HWD experiments with artificially infected fruit had given good control (Maxin et al., 2012). Poor or variable efficacies of hot-water treatments in naturally infected fruit may be due to deep-seated centres of natural infection located in the apple core in the case of *G. avenacea* (Weber, unpublished) or in the blossom end of the fruit (*B. cinerea*, *N. galligena*, Jijakli and Lepoivre, 2004). Although *Penicillium expansum* is widely regarded as being tolerant to hot-water treatments (Vorstermans et al., 2008), critical experiments have demonstrated that inoculum present at the time of HWD can be effectively controlled (Amiri and Bompeix, 2011; Maxin et al., 2012). Therefore, problems in *P. expansum* control by hot-water treatments are perhaps best explained by late infections during prolonged storage (Amiri and Bompeix, 2005).

There are at least two components which may contribute to the mode of action of hot-water treatments: (1) a direct and lethal effect of heat on fungal inoculum within or outside the apple, and (2) an indirect effect mediated by a stress-induced physiological response of the fruit. Support for the latter aspect has been provided by studies in which the production of heat-shock or pathogenesis-related proteins was induced by heat treatments in different kinds of fruit (Schirra et al., 2000; Pavoncello et al., 2001; Widiastuti et al., 2011). Further, in inoculation experiments hot-water treatments had a retarding effect on fungal pathogens even when fruit were inoculated shortly after the heat shock (Pavoncello et al., 2001; Widiastuti et al., 2011; Maxin et al., 2012).

It is plausible that both direct and indirect heat effects on fungal inoculum may influence the efficacy of HWD and HWR. In particular, prolonged exposure times above 1 min are required to permit subcuticular regions of the fruit to be exposed to significant increases in temperature (Trierweiler et al., 2003). The heat destruction of superficial apple tissues, without deeper heat penetration, may explain why certain pathogens such as *P. washingtonensis*, *P. exigua* and *Mucor* spp. caused an elevated incidence of storage rot in association with heat damage in HWR- but not HWD-treated fruit (Table 7.3). At least in the slowly-growing *P. exigua*, infections were
clearly co-located with heat-damaged areas on individual apples. Therefore, the association of pathogens with heat damage caused by HWR can be likened to the effect of the herbicide paraquat, which releases fungal endophytes from dormancy by killing plant tissues while sparing fungal inoculum (Biggs, 1995).

Pathogens with a high heat tolerance might be expected to be able to grow on apple tissue killed by HWD, and this was indeed observed for *P. expansum* in artificially inoculated fruit which showed a significantly elevated incidence of infection after HWD at destructive temperatures of 56-60 °C as compared to 50-54 °C (Maxin et al., 2012). For a complete inhibition of *P. expansum* rot, temperatures of 70 °C were required (Maxin et al., 2012). Taken together, therefore, several lines of evidence support induced resistance as the primary factor determining the efficacy of HWD and HWR in apple.

HWR is particularly attractive to fruit producers because the method could be incorporated into fruit grading lines directly after harvest without extensive technical modifications. This would result in substantial economic savings as compared to HWD which requires specialised equipment and expertise (Maxin and Klopp, 2004). There is also the possibility to introduce a HWR step at pack-out because storage-rot fungi retarded by controlled-atmosphere conditions (Lafer, 2010) can break out in cold-storage during the remainder of the marketing chain. Further, HWR of cold fruit may provide a substantial saving of heat energy as compared to HWD. In view of the short duration of the heat-shock response at room temperature (Pavoncello et al., 2001), it is uncertain if HWR is able to prolong shelf-life during the retail phase.

5. Conclusions

A wide range of fungal pathogens of stored apples can be effectively controlled by HWD and HWR. High efficacies of HWR have been demonstrated for the first time on naturally infected apples. HWR has potential to become a sustainable alternative for fruit orchardists and packers because it is less costly than HWD and because its short treatment times enable it to be integrated into existing fruit grading lines. There is scope for further optimisation of HWR parameters.
Acknowledgements

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References


Phytopathological Problems in Organic Fruit-Growing. FÖKO, Weinsberg, Germany. pp. 118–120.


Chapter 8: General Discussion

8.1 Hypotheses underlying this Thesis
The present work was based on eight hypotheses concerning the mode of action of hot water dipping (HWD), its efficacies against different storage-rot fungi, and the influence of hot water treatment (HWT) on apple fruit quality. The eight hypotheses examined in this Thesis were as follows:

Hypothesis 1: HWD is able to improve apple fruit quality. Based on the results elaborated in Sections 4.2.1 and 4.2.2, this hypothesis was rejected because HWD and hot water rinsing (HWR) failed to give rise to any obvious improvement in the interior quality of fruit. However, if HWR or HWD are to be used in replacement of preharvest fungicide applications (Section 3.2.1), an improved processing quality of fruit (Section 2.9.2) might be obtained by a reduction of pesticide residues, thereby improving quality aspects in the process of fruit production and addressing consumers’ and retailers’ demands (see Section 2.6).

Hypothesis 2: HWD is effective in controlling all major current storage rots in Danish apples. This hypothesis was aimed chiefly at funding bodies interested in supporting research with relevance to Danish interests. Major storage rots in Danish apples in the 2008-2010 seasons were identified, i.e. Neofabraea alba, Monilinia fructigena and Neonectria galligena (Chapter 7). All three storage rots were significantly reduced by hot water treatment (HWT) with efficacies of 78-100%. This hypothesis may therefore be accepted (Chapter 7).

Hypothesis 3: HWD is able to control existing minor storage rots on Danish apples. This hypothesis opened up possibilities of examining storage-rots for which no or insufficient national and international literature existed at the start of the project. Fundamental scientific work with critical identification of fungal pathogens was required to address this hypothesis. The work presented in Chapters 5, 6 and 7 showed that HWD was able to control minor storage rots on Danish apples. This hypothesis may therefore be accepted. There are implications of this work beyond the borders of Denmark.
Hypothesis 4: HWR is an alternative to HWD. This hypothesis was developed during the first 6 months of the Ph.D. project. This hypothesis was accepted in two successive experimental seasons, i.e. the 2009 and 2010 harvests (Chapter 7).

Hypothesis 5: The mode of action of HWT is based on killing fungal inoculum. This statement, widely held to be true among organic fruit orchardists as well as their consultants throughout NW Europe, was the starting point for hypotheses 5 to 8. Hypothesis 5 was initiated during experiments addressing hypothesis 2. It was rejected because experimental results, supported by data arising from previously published physiological experiments, suggested that HWD and HWT acted primarily via an induction of the immune response of the fruit (Chapter 5).

Hypothesis 6: HWD is able to reduce storage-rot on artificially inoculated apples. This was the logical progression of experimental results arising from work on hypothesis 5. This hypothesis was rejected during the first year (2008) with the chosen dipping temperatures and dipping times, but it was subsequently accepted using Penicillium expansum as a model system in 2010 (Chapter 5).

Hypothesis 7: Penicillium rot increases on fruit damaged by excessive HWD temperature. This hypothesis was accepted in general terms in Chapter 7, and with artificially inoculated fruit in Chapter 5, intriguingly against a background of effective control of Penicillium rot at lower temperatures (>52°C).

Hypothesis 8: The reduction of Penicillium rot in artificially inoculated apples is due to an effect of HWD on the fruit, not on the fungus. This hypothesis was accepted in Chapter 5, thereby also addressing hypotheses 5 and 6. In Section 8.3 general aspects on the acceptance of this hypothesis are discussed.

8.2 Effects of delayed ripening on the incidence of storage rots

Apple storage methods have been revolutionized by the concepts of controlled atmosphere (CA) and dynamically controlled atmosphere (DCA) which enable fruit to be kept for much longer time periods than in cold storage under ambient atmosphere (Veltman et al. 2003). The main mode of action of CA and DCA is to delay fruit ripening. Treatment with 1-methyl-cyclopropene (1-MCP) achieves the same result through the different means of inhibiting ethylene production and
activity. However, none of these methods directly eliminates fungal inoculum. This explains why there are few, if any, synergies between 1-MCP and CA or DCA (Chapter 5). In terms of efficacy against the development of fungal postharvest diseases, CA or DCA after 8 months may be comparable to cold storage with 1-MCP after 6 months, or simple cold storage after 3 months (Spotts et al. 2007; Lafer 2010; see Chapter 6).

In order to reduce the incidence of fruit rots after the end of a CA or DCA storage period, i.e. the time of fruit distribution, sale and consumption, fungal inoculum must be eliminated. This may be achieved by preharvest fungicide applications which are under public scrutiny, hygiene measures (e.g. removal of fruit mummies) which are labour-intensive and therefore costly, and HWD or HWR which have not (yet) gained full acceptance by fruit producers. The research presented in this Thesis indicates that these HWT hold significant unrealized potential.

8.2.1 Interactions between HWT and ripening inhibition

A summary of existing literature knowledge of the efficacies of the above-mentioned methods to control postharvest diseases (pre-harvest fungicides, removal of mummies, low-temperature storage with or without CA or DCA, application of 1-MCP) is presented in Table 8.1. The incidence of the major storage rots, caused by Neofabraea spp., has been reported to be decreased by a delay in fruit ripening associated with CA, DCA, reduced storage temperatures, and 1-MCP (Spotts et al. 2007; Lafer 2010). The effect of CA on controlling blue mould caused by Penicillium expansum may be due in part to a retarded fruit ripening, and partly to direct effects of the modified atmosphere on the fungus (Yackel et al. 1971). In contrast, other fungal storage rots such as Botrytis cinerea or Neonectria galligena were not affected by CA or DCA (Berrie et al. 2011). Similarly, the recently discovered Phacidiopycnis washingtonensis failed to show any obvious response to CA, DCA and 1-MCP (Chapter 6). This provides a plausible explanation for the frequent discoveries of rubbery rot after prolonged DCA and CA storage (Weber 2011), in contrast to a lowered incidence of Neofabraea spp. under these conditions.

Although, the efficacy of HWD against Neofabraea spp., P. expansum and M. fructigena has been well documented (Burchill 1964; Maxin et al. 2005; Amiri and Bompeix 2010), recent reports have so far indicated a similar potential for HWR only
against *P. expansum* (Fallik et al. 2001). Prior to the publications originating from this Thesis, no literature existed on the efficacy of HWR on any other storage-rot fungus on apples. This Thesis has provided a new insight in to the potential of HWR and HWD in controlling major storage rot fungi (Table 8.1). In the present study high efficacies of both HWD and HWR were characterized for a wide range of storage-rot fungi including *C. acutatum, Cladosporium* spp., *N. galligena, P. washingtonensis*, *N. alba* and *N. perennans* (Chapter 7). Further, *P. expansum* and *B. cinerea* were controlled in artificially infected fruit subjected to HWD (Chapter 5). These findings greatly expand our understanding of the potential offered by the physical HWT of apples. It is apparent from Table 8.1 that the range of fungi susceptible to HWD or HWR is wider than that of species controlled by 1-MCP, CA or DCA. Further, HWD and HWR can be expected to show synergies with ripening delay caused by CA, DCA or 1-MCP treatments because they are based on different modes of action.

### 8.2.2 Preharvest chemical treatments vs. postharvest HWT

Efficacies of HWT in reducing storage rots and their possible positive interactions with any ripening delay procedures are an important basis for evaluating risks and benefits of HWR and HWD in the context of registration of new chemical substances in the EU for the use in plant protection (Section 2.6). The EU 2009/128/EC directive, which is currently being transformed into national legislation by all EU member states, requires that the new pesticide registration system “also establishes a mechanism for the substitution of more toxic pesticides by safer (including non-chemical) alternatives” (GD Health and Consumer, 2010). In providing such an alternative to chemical treatments, HWR and HWD may be expected to gain importance and acceptance as methods to control postharvest fungi in future. A similar focus may also be anticipated for preharvest hygiene measures aimed at reducing inoculum during the growing season or at harvest. These may consist of the removal of fruit mummies or sporulating cankers. However, although such measures may significantly reduce post-harvest decay caused by specific fungi (Holb and Scherm 2007), the efficacy of such measures against a wider range of species under NW European conditions remains to be proven. On biological grounds, synergistic effects between (1) hygiene measures, (2) HWT and (3) ripening delay caused by CA, DCA or 1-MCP may be expected.
8.3 The influence of heat on spores and fruit

‘Elstar’ apples artificially inoculated with *Penicillium expansum* and subjected to 3 min HWD at 55°C or above showed a higher incidence of blue mold than fruit subjected to HWD at 52°C. The choice of a relatively high temperature was based on the assumption that HWD

Table 8.1: Efficacies of various methods for the reduction of post-harvest storage rots of apples, indicated as +++ (>75%), ++ (50-75%), + (<50%), and - (none). Red symbols represent new knowledge arising from results generated in this Thesis, whereas black symbols are based on previous literature (Burchill 1964; Fallik et al. 2001; Jijakli and Lepoivre 2004; Maxin et al. 2005; Palm and Kruse 2005; Kim and Xiao 2006; Holb and Scherm 2007; Tahir et al. 2009; Lafer 2010; Berrie et al. 2011). Question marks indicate that knowledge on the efficacy of a treatment is not yet available.

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Pre-harvest fungicide treatments</th>
<th>Removal of fruit mummie s</th>
<th>Lowering storage temperature</th>
<th>CA or DCA</th>
<th>1-MCP</th>
<th>HWR</th>
<th>HWD</th>
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<tbody>
<tr>
<td><em>Neofabraea spp.</em></td>
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<td>?</td>
<td>+++</td>
<td>+++</td>
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<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td><em>Neonectria galligena</em></td>
<td>?</td>
<td>?</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><em>Penicillium expansum</em></td>
<td>-</td>
<td>?</td>
<td>+</td>
<td>++</td>
<td>?</td>
<td>++</td>
<td>++/</td>
</tr>
<tr>
<td><em>Botrytis cinerea</em></td>
<td>+</td>
<td>?</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><em>Phacidiopycnis washingtonensis</em></td>
<td>?</td>
<td>?</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td><em>Monilinia fructigena</em></td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>?</td>
<td>?</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><em>Colletotrichum acutatum</em></td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>++</td>
<td>?</td>
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</tr>
</tbody>
</table>

had a direct inhibitory effect on fungal inoculum, i.e. on spores and mycelium underneath the cuticle. Initial results did not support this assumption because it was found that *P. expansum* was mainly associated with the heat-damaged regions of the apple surface. A more detailed study was therefore initiated to determine the exact temperatures at which increased levels of decay were observed. Surprisingly, the results showed a decline in the incidence of *P. expansum* infections following HWD from 44 to 52°C, but resurgence after HWD temperatures above 52°C, which coincided with superficial heat damage. This result suggested that the fruit itself must have influenced the efficacy of HWD. As discussed in detail in Chapter 5, the heat-
induced activation of pathogen defence responses within the fruit is a possible physiological explanation of this phenomenon.

To test this theory, fruit were inoculated pre- or post-HWD, thereby permitting a distinction between the effects of temperature on the fruit and on the spores themselves. Results of this temperature effect, described in Chapter 5 as percent wound infections, are here considered as control efficacy (Abbott 1925). These efficacies of HWD against the development of blue mould on artificially infected fruit showed two curves that were essentially superimposable on one another at HWD temperatures up to 52°C (Figure 8.1), thereby confirming that the heat-induced defense response of the apples themselves is indeed the active principle in HWT of apples.

![Figure 8.1: Efficacy of hot water dipping (HWD, %) on Penicillium expansum, modified from Figure 5.5. Schematic summary of temperature responses of a spore suspension (broken line, open triangles), and of fruit inoculated pre-HWD (solid line, open circles) and post-HWD (dotted line, black circles).](image)

Ultimately, the efficacy of HWD and HWR depends on two parameters, i.e. dipping temperature and dipping time. These physical parameters show differential effects on different apple cultivars and different species of storage-rot fungi. As shown
above, incipient heat damage due to HWD can give rise to an increased incidence of heat-tolerant storage-

Figure 8.2: Schematic drawings of biological effects of HWD. (A) Effects on storage-rot fungi which cause infections shortly after HWD (solid line), or after prolonged post-HWD storage (dotted line), or as pre-HWD infections by a heat-sensitive species such as *Neofabraea* spp. (long dashes) or a moderately heat-tolerant fungus such as *Neonectria galligena* with a resurgence of fruit-rot after HWD at 52–56°C (short dashes). (B) Efficacy of HWD on *Neofabraea* storage-rot in apple varieties with a low (e.g. ‘Ingrid Marie’), moderate (e.g. ‘Elstar’) or strong (e.g. ‘Topaz’) heat tolerance.
rots such as *P. expansum* (*ET*$_{50}$ = 51.7°C), whereas such a response was not observed with *N. alba* (*ET*$_{50}$ = 39.3°C) or *P. washingtonensis* (*ET*$_{50}$ = 40.2°C). Intriguingly, incipient heat damage caused by HWR (20 s at 65°C) was associated with a resurgence of heat-sensitive storage-rot fungi such as *P. washingtonensis* (see Table 7.3). This discrepancy may be explained by the depth of heat penetration into subcuticular regions of the fruit, which is expected to be much greater during 3 min HWD than 25 s HWR. Thus, excessive temperatures of both HWD and HWR are likely to kill apple tissue, but only HWD may be capable of killing subcuticular inoculum of heat-sensitive storage-rots which would otherwise be able to colonise dead heat-scaled tissue. Moderately or highly heat-tolerant storage-rot fungi such as *N. galligena* and *P. expansum* (respectively) showed a bimodal response curve to increasing HWD temperatures (Figure 8.2A). The first increase of HWD efficacy up to 50-52°C may be explained by the heat-induced resistance of the fruit, the second phase after HWD at 58°C or 60°C by a direct lethal effect of dipping temperatures on fungal inoculum within the fruit.

A schematic response curve of different apple varieties to HWT is provided in Figure 8.2B. For cv. ‘Elstar’, the highest HWD efficacies were obtained at 50-52°C. For yet more heat-sensitive cultivars such as ‘Ingrid Marie’, temperatures should not exceed 50°C. This corresponds to current advice given to organic fruit growers in Northern Germany where the recommended HWD conditions are 50°C for 4.5 min. This prolonged HWD period was necessary due to technical constraints in treating large numbers of fruit in large boxes at the required temperature (Trierweiler 2004). Varieties with enhanced heat tolerance such as ‘Topaz’ (Trierweiler 2004) may remain free from heat damage at temperatures up to 53°C. Where possible, the upper temperature limit of HWD should be determined and used as a treatment temperature, because at 53°C a higher efficacy of storage-rot control will be obtained than at 50°C (Figure 8.2B).

### 8.4 Perspectives

Results obtained in this study clearly indicate that HWD and HWR have a high efficacy of 80% or more in controlling various storage-rot fungi, including *Neofabraea* spp. which are the most important pathogens in NW Europe. Such a strong effect is equivalent to the highest efficacy obtainable with repeated preharvest applications
of fungicides (Palm and Kruse 2005). These results open new possibilities of non-chemical storage-rot control for fruit industries both in organic and integrated production systems. In modern storage facilities fruit ripening is delayed by CA and DCA at cold temperatures. Such treatments retard the development of a range of fungi during the storage phase (Table 8.1), but their effects on fruit-rot during the remainder of the fruit distribution chain are extremely limited. Hot water treatments reduced the incidence of storage rots by >50% and therefore provide a valuable tool that broadens options available to fruit growers and pack-house managers. Hot water treatments may be incorporated either pre- or post-storage into CA and DCA strategies and have the potential to reduce fruit wastage during the distribution and consumer phases and to enhance consumer satisfaction.

The technology evaluated in this study showed that HWR gave rise to only slightly reduced disease control efficacies as compared to HWD, but that HWR holds several other advantages over HWD. The cost of HWR per unit of treated apples is expected to be much lower than for HWD because no additional labour is required and the amount of energy required to heat the water for a 30 s rinse should be about 20% of the energy used in a 3 min HWD process. A further advantage of HWR is that it can be incorporated into existing fruit grading lines, thus avoiding the need for additional equipment or parallel structures. Traditionally, harvested fruit in storage boxes are emptied by submersion in cold water, followed by floating the fruit to a conveyor belt, where they are dried and passed to the grading unit. Apples are then separated by size and pigmentation. Several exits on the grading unit are opened or closed, depending on the desired parameters. A HWR unit could easily be placed on such an exit, permitting a fraction of the total crop to be treated with hot water. Alternatively, all fruit could be treated by HWR prior to entering the drying unit. A closed HWR unit needs to be developed to minimize the emission of water vapor in the packing hall.

Additional positive effects of HWR on fruit quality can be expected. In particular, it has been observed that fruit were less waxy and more homogenous following HWR and storage than after HWD or without hot-water treatments (see Section 4.2.3). Further, HWR may be associated with enhanced fruit firmness (Fallik et al. 2001). These attributes, which may be due to a partial melting of epicuticular waxes above 55°C (Aggarwal 2001), should be explored in future studies and may further address
Hypothesis 1 which was rejected based on the grounds of the preliminary work reported in this Thesis.

Further research should also be aimed at identifying the proteins and/or low-molecular weight substances induced by heat shock in apples. An understanding of their identity should enable us to optimise parameters and time-points of HWT in apples. An additional area of interest is a detailed characterisation of the responsiveness of fruit to HWR directly after harvest in comparison with fruit subjected to HWR after short- or long-term storage. Finally, the question remains whether apples treated by HWR before storage are capable of mounting an additional heat-shock response if they are exposed to a second HWT during the packing phase, following a pre-storage HWR and, if so, how this might further improve fruit quality during the distribution phase.
Chapter 9: Literature


approaches for the Control of Postharvest Diseases and Disorders, Bologna, Italy, 149-155.


Large EC (1940) *The Advance of the Fungi*. London: Jonathan Cape


Lunn JA (1977) *Rhizopus stolonifer*. CMI Descriptions of Fungi and Bacteria **524**.


R! Project, The R Project www.r-project.org


Yokoyama VY, Miller GT, Dowell RV (1991) Response of codling moth (Lepidoptera: Tortricidae) to high temperature, a potential quarantine treatment for exported commodities. Journal of Economic Entomology 84, 528-531.

