Chapter 5: Paper 1

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

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

P. Maxin[1], R.W.S. Weber[2], H. Lindhard Pedersen[1] and M. Williams[1]
([1])Department of Food Science, Aarhus University, Denmark and [2])Esteburg Fruit Research and Advisory Centre, Jork, Germany)

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 preharvest 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, λ_{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 x 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 'Elstar') 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 % O_2, 2.6 % CO_2, 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 (10^6 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_{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_{50} = 39.3 °C). Both *N. galligena* (ET_{50} = 42.9 °C) and *B. cinerea* (ET_{50} = 44.4 °C) were slightly more heat-tolerant, *P. expansum* (ET_{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 HWDat 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).

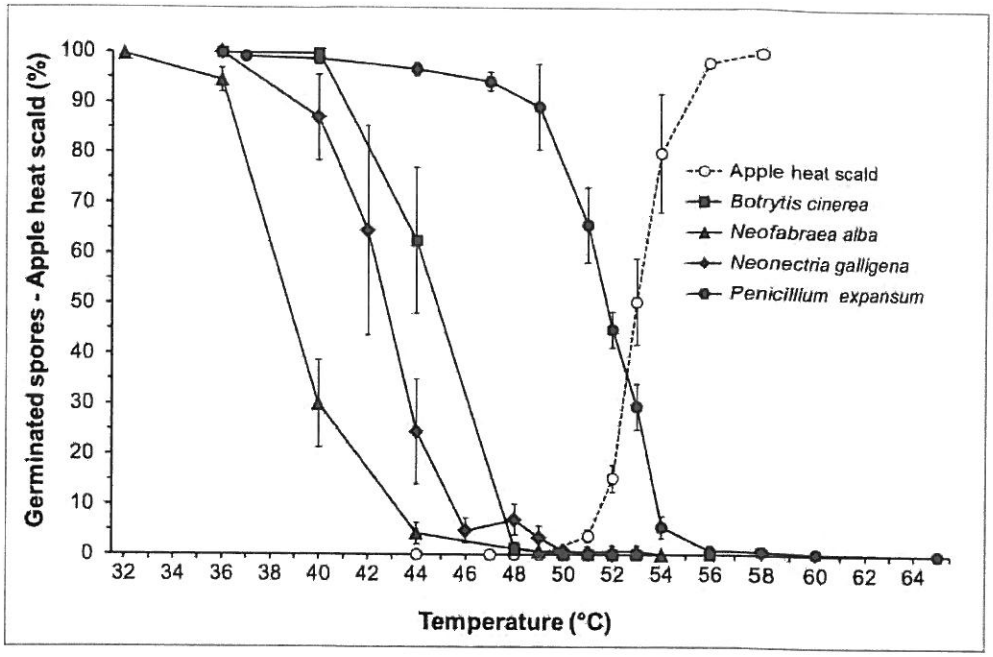

Fig. 5.1. Effect of hot-water dipping (HWD) for 3 min on skin damage of 'Elstar' fruit due to heat scald (percent of fruit with lesions >5 x 5 mm after 15 wk cold storage), and on *in vitro* viability of spore suspensions of four storage-rot fungi (percent germinated spores after 24 h). Standard deviations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

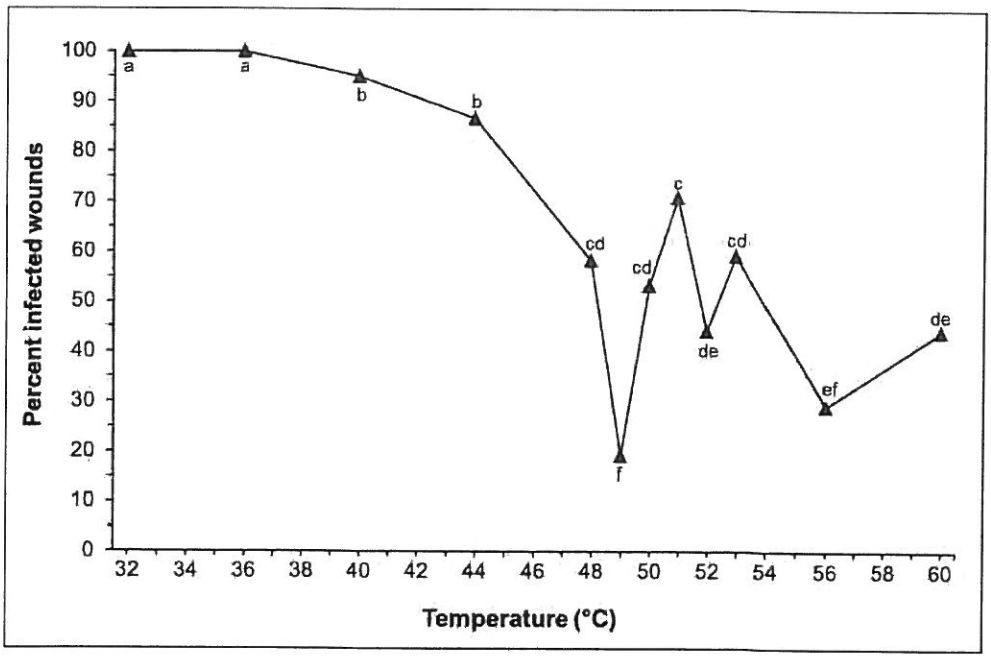

Fig. 5.2. Effect of hot-water dipping (HWD) for 3 min on *Botrytis cinerea* infections (pre-HWD inoculation) after 4 wk storage at 2 °C. Different letters indicate significant differences ($P<0.05$) between treatments. Inoculated control fruit not subjected to HWD showed 100 % infected wounds (significance 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 erspecially 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.

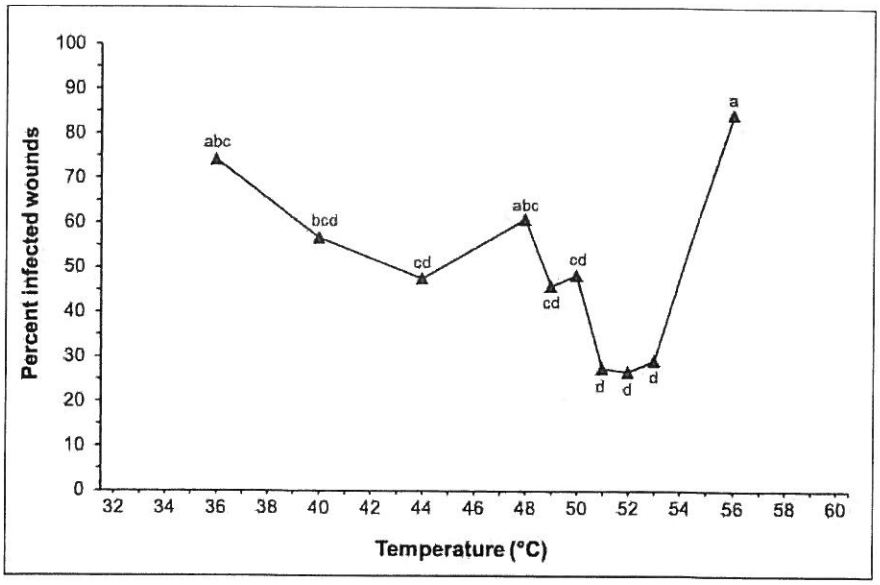

Fig. 5.3 Effect of hot-water dipping (HWD) for 3 min on *Neonectria galligena* infections (pre-HWD inoculation) after 10 wk storage at 2 °C. Different letters indicate significant differences ($P<0.05$) between treatments. Inoculated control fruit not subjected to HWD showed 80.3 % infected wounds (significance range ab).

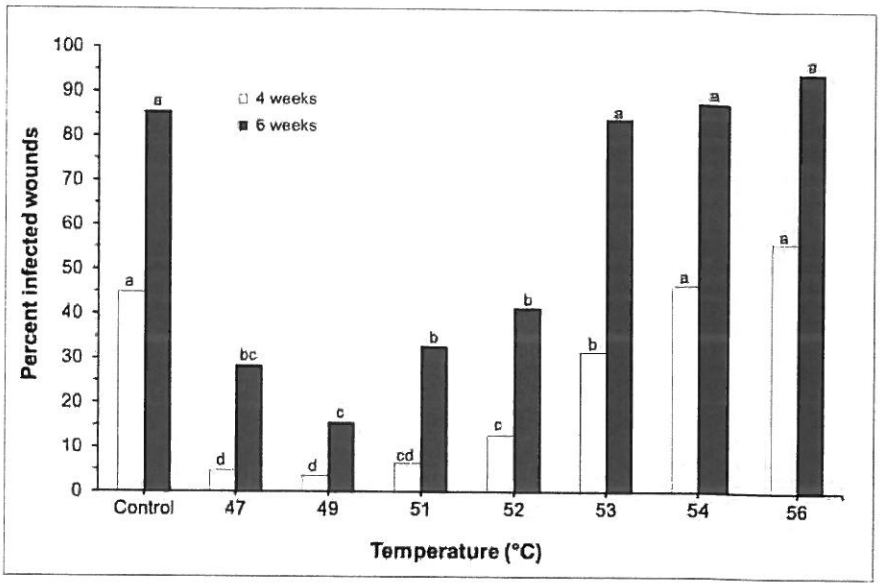

Fig.5.4. Preliminary trial of the effect of hot-water dipping (HWD) on *Penicillium expansum* infections (pre-HWD inoculation) as percentage infected wounds after 4 and 6 wk storage at 2 °C. Different letters above the bars indicate significant differences (P<0.05) between treatments including the positive control (inoculated fruit not subjected to HWD).

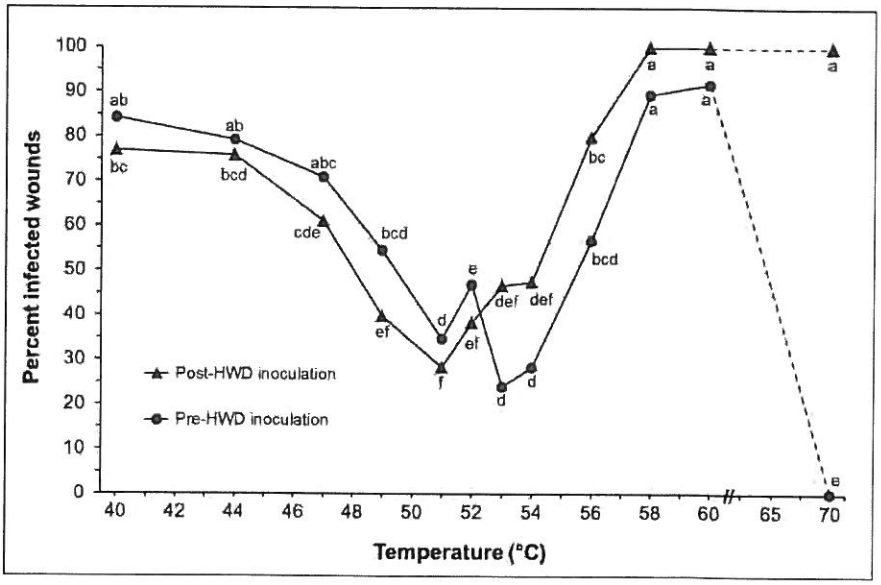

Fig. 5.5. Effect of hot-water dipping (HWD) on *Penicillium expansum* wound infections (pre- and post-HWD inoculations) after 6 wk storage at 2 °C. Different letters indicate significant differences (P<0.05) between treatments including non-HWD control fruit inoculated at the pre-HWD (87.5 % infected wounds; significance range ab) and post-HWD time points (92.1 % infected wounds; significance range ab).

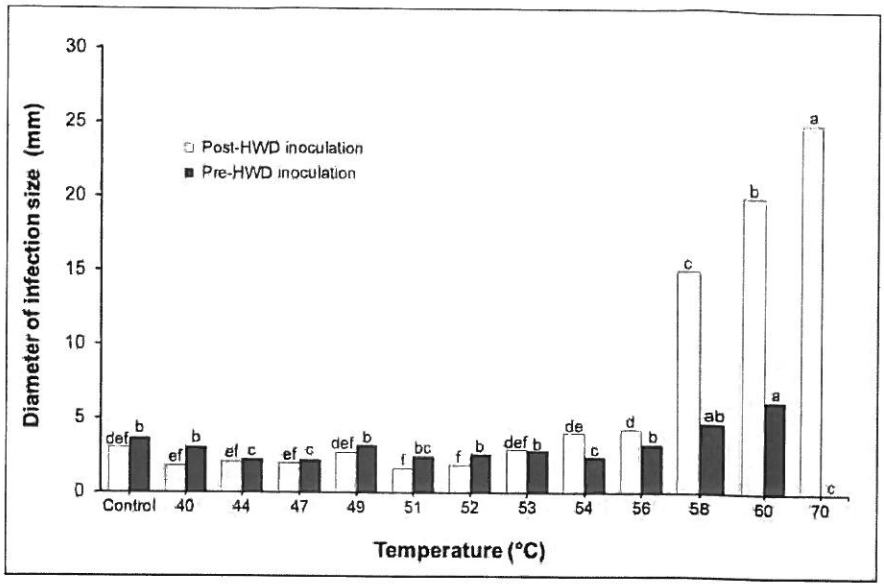

Fig. 5.6. Diameter of *Penicillium expansum* lesions on fruit inoculated before and after hot-water dipping (pre- and post-HWD, respectively) and in the positive controls (inoculated fruit not subjected to HWD) after 6 wk incubation at 2 °C. Different letters indicate significant differences between 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

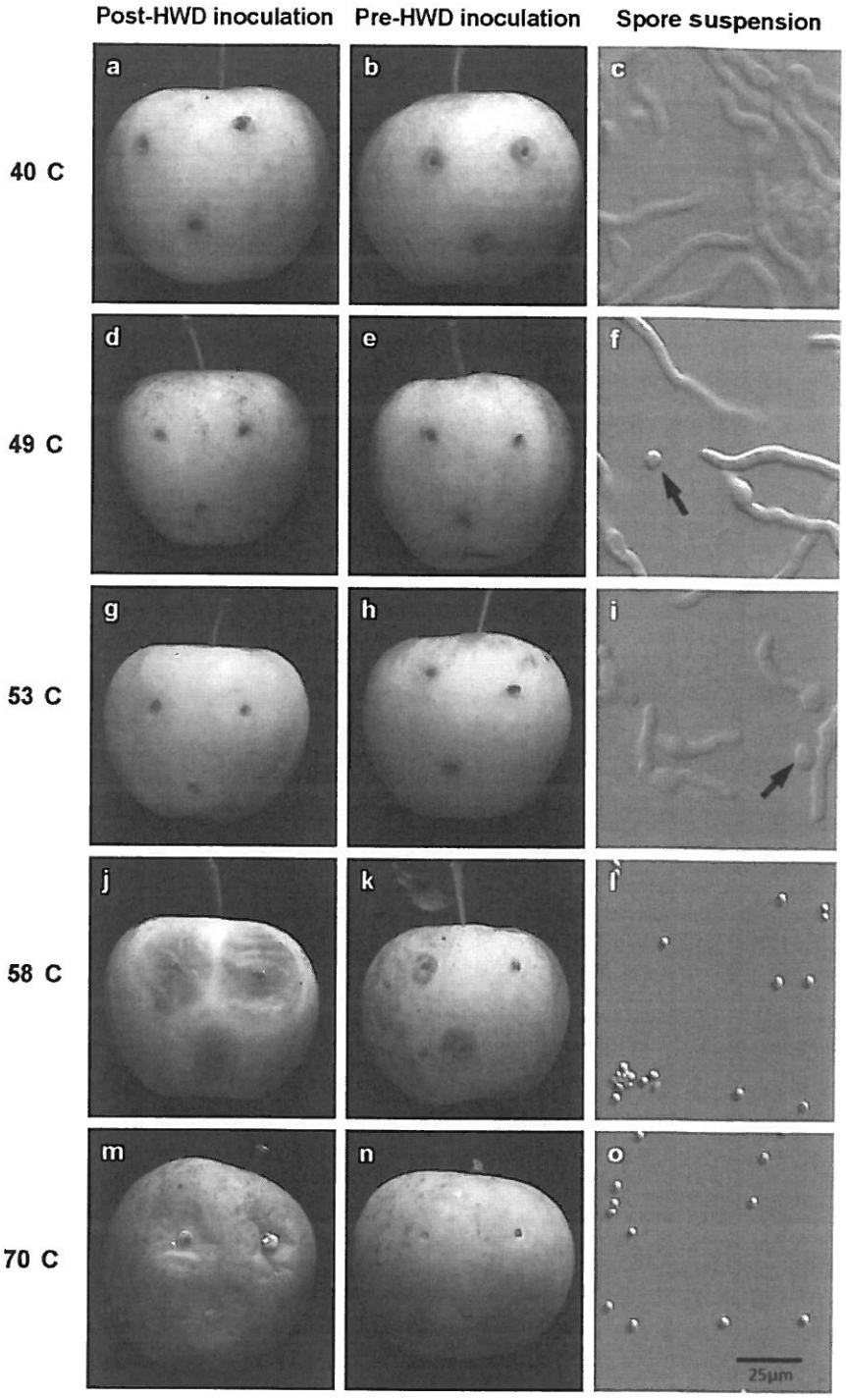

Fig. 5.7. Visual effects of hot-water dipping (HWD) for 3 min on *Penicillium expansum* at representative dipping temperatures. Apples were inoculated with *P. expansum* pre- or post-HWD and stored for 6 wk at 2 °C. Conidia were incubated at 20 °C for 24 h following HWD.

storage rots caused by *N. galligena*, *B. cinerea* and *P. expansum*, as well as other fungi (MAXIN et al. 2005; MAXIN 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 β-1,3-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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Addresses of authors: Peter Maxin, Hanne Lindhard Pedersen and Michelle Williams, Institut for Fødevarekvalitet, Aarhus Universitet, Denmark; Roland W. S. Weber (corresponding author), Esteburg-Obstbauzentrum Jork, Moorende 53, 21635 Jork, Germany, e-mail: roland.weber@lwk-niedersachsen.de