Chapter 3

OPTIMISATION OF WARM WATER TREATMENT IN CITRUS PACKHOUSES, AND INTEGRATION THEREOF WITH *BACILLUS SUBTILIS* *

ABSTRACT

Postharvest pathogens account for serious losses of export citrus fruit from South Africa. Warm water and warm water supplemented with antagonist treatments were evaluated for control of citrus postharvest decay. Inoculated fruit were dipped in a warm water bath at different temperatures and time periods. All warm water treatments effectively controlled postharvest decay. Effect of different warm water treatments on anti-fungal activity was determined by means of TLC bioassay using *Penicillium digitatum* as test organism. Levels of fungal inhibitors causing clear inhibition were detected at 36 and 40 °C (1, 2.5 and 5 min) treatments, which correlated with levels of disease control. Only slight inhibition of *P. digitatum* could be detected from control treatments. Warm water enhanced the efficacy of *Bacillus subtilis* treatment and this integrated treatment was as effective as the chemical treatment in reducing decay although not as consistent.

* DE VILLIERS, E.E., VAN DYK, K., SWART, S.H., SMITH, J.H. & KORSTEN, L. 1997. Potential alternative decay control strategies for South African citrus packhouses. *Proceedings of the 8th Congress of the International Society of Citriculture* 1: 410-414.

INTRODUCTION

Citrus (*Citrus sinensis* (L.) Osbeck) is affected by various postharvest diseases, including *Alternaria* rot (*Alternaria citri* Ellis & N. Pierce), *Aspergillus* rot (*Aspergillus niger* Tiegh.), anthracnose (*Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc.), stem-end rot (*Lasiodiplodia theobromae* (Pat.) Griffon & Maubl.), sour rot (*Geotrichum citri-aurantii* (Ferraris) E.E. Butler), green mould (*Penicillium digitatum* (Pers.: Fr. Sacc.), blue mould (*Penicillium italicum* Wehmer), *Rhizopus* rot (*Rhizopus stolonifer* (Ehrenb.: Fr.) Vuill), *Trichoderma* rot (*Trichoderma viride* Pers.: Fr.) (Brown & Eckert, 1989; Eckert & Brown, 1989) and whisker mould (*Penicillium ulaiense* Hsieh, Su & Tzean) (Holmes *et al.*, 1994). In the past, heat treatment was widely used in citrus packhouses to control postharvest diseases (Fawcett, 1922; Smoot & Melvin, 1963), but has since been replaced by chemical treatment of fruit with fungicide such as guazatine, imazalil and thiabendazole (Shachnai & Barash, 1982; Eckert & Ogawa, 1985; Pelser, 1988). However, the development of resistance by pathogens to fungicides (Bancroft *et al.*, 1984; Bus *et al.*, 1991; Eckert *et al.*, 1994) and the increased demand for fruit free of hazardous chemicals (Brown & Baraka, 1996), have revived interest in heat treatment (Rodov *et al.*, 1996).

Heat is usually applied to a fruit commodity via air or water (Barkai-Golan & Phillips, 1991). However, water is more efficient than air by virtue of its higher heat transfer coefficient (Jacobi *et al.*, 1993; Shellie & Mangan, 1993). Numerous investigations with heated water treatments have shown improved citrus postharvest disease control in many countries, including Israel (Rodov *et al.*, 1996), Italy (Dettori *et al.*, 1996), and the USA (Brown & Baraka, 1996). Hot water treatment of other fruit commodities such as nectarine, peach, avocado and mango substantially reduced postharvest decay (Spalding & Reeder, 1986; Plumbley *et al.*, 1993; Margosan *et al.*, 1997). In the papaya industry, immersion of fruit in hot water has been the principal postharvest treatment for decay control since 1964 (Akamine, 1967). Disinfectants (Smilanick *et al.*, 1995) and fungicides such as imazalil (Smilanick *et al.*, 1997) are often integrated with hot water treatments to enhance the efficacy of postharvest disease control.

Hot water dips reported in the literature for citrus fruit involve temperatures of 50-54 °C and exposure periods of 2-3 min (Dettori *et al.*, 1996), compared to the 40-45 °C of warm water baths in citrus packhouses. This chapter describes the optimisation of warm water treatment in

citrus packhouses for postharvest disease control, and integration of the heat treatment with application of *Bacillus subtilis*.

MATERIALS AND METHODS

Warm water supplemented with antagonist treatments

Preparation of inoculum: Citrus fruit naturally infected with *A. citri, C. gloeosporioides*, *D. natalensis*, *G. citri-aurantii, P. digitatum*, *P. italicum* and *T. viride*, were macerated, filtered through four layers of cheesecloth. The suspension was diluted to obtain a concentration of 10^5 *Penicillium* conidia ml^{-1} in experiment 1 and 10^6 conidia ml^{-1} in experiment 2.

Inoculation procedure: Valencia orange fruit provided by Letaba Estates (Tzaneen, Northern Province, SA) were pretreated, injured and inoculated as described in Chapter 2.

Preparation of antagonist: A mass culture of *B. subtilis* (B246) (obtained from L. Korsten, Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, SA), known to inhibit growth of citrus postharvest pathogens (De Villiers *et al.,* 1993), was prepared according to Korsten *et al.* (1995). The antagonist concentration was determined using a Petroff Hauser counting chamber and ajusted to 10^7 cells ml^{-1}.

Treatment of fruit: Each treatment consisted of 800 fruit. The following treatments were applied 24 h after artificial inoculation: 1) untreated control; 2) 10 min dip in water at 40 °C; 3) 3 min dip in water containing the following chemicals: Deccotine (guazatine - 1000 ppm) (Rhône-Poulenc Agrichem SA (Pty) Ltd., Onderstepoort, SA), Fungazil (imazalil - 500 ppm) (Janssens Pharmaceutica, Olifantsfontein, SA) and Tecto (thiabendazole - 1000 ppm) (Logos Pharmaceuticals (Pty) Ltd., Midrand, SA); 4) 10 min dip in 40 °C water supplemented with B246 (10^7 cells ml^{-1}). After treatment fruit were air-dried, packed in cartons (80 fruit/box) and stored at 25 °C for 14 days. Percentage sound fruit was recorded for each box. Data were statistically analysed using ANOVA and Student's t-least significant differences calculated to determine differences between treatments ($P = 0.05$). The experiment was repeated and treatments included tap water dip and tap water supplemented with B246 (10^7 cells ml^{-1}).

Different temperature regimes

Valencia orange fruit obtained from Letaba Estates were artificially inoculated with a postharvest pathogen suspension containing 10^5 ml^{-1} *Penicillium* conidia as described above. Each treatment consisted of 480 fruit and included water dips at 23, 39, 43, 47, 51, 55 and 59 °C for 3, 6 or 9 min. An inoculated control not dipped in water was also included. After treatment fruit were air-dried, packed in boxes (80 fruit/box) and stored at 25 °C for 14 days. Percentage sound fruit was recorded per box for each treatment. Data were analysed using CSS Non-linear Estimations to calculate the relationship between treatments (temperature and time) and response (percentage sound fruit).

***In vivo* determination of optimal warm water temperature**

Pathogen preparation: *Penicillium digitatum* was isolated from decayed citrus fruit and cultured on potato-dextrose agar (PDA) (Biolab, Midrand) at 25 °C until sporulation occurred. A conidial suspension was prepared by washing the cultures with sterile distilled water and adding it to 25 l tap water to a final concentration of 10^5 conidia ml^{-1}.

Inoculation procedure: Valencia orange fruit were rinsed in chlorine (100 ppm) and air-dried. Wounds were made at five random sites on each fruit with an inoculation needle (0.5 mm diameter) to a depth of 2 mm. Fruit were subsequently dipped for 3 min in the conidial suspension of *P. digitatum*, air-dried and incubated overnight at 25 °C.

Warm water treatment: Treatments consisted of three replicates, each comprising five fruit. Inoculated fruit were dipped in a water bath at different temperatures (36, 40, 44, 48, 52 °C) for 1, 2.5, 5, 7.5 or 10 min at each temperature. Uninoculated and inoculated fruit not subjected to warm water treatment were included as controls. After treatment fruit were air-dried, packed in polystyrene containers, and stored at 25 °C for 21 days. The experiment was repeated twice.

Preparation of crude extracts: A crude extract was prepared from fruit of each treatment according to the procedure described by Ben-Yehoshua *et al.* (1987) and Kim *et al.* (1991). Flavedo tissue from mechanically-injured areas was excised with a scalpel. Samples (20 g in total) were extracted for 24 h with petroleum ether (1:4 v/v) (Biolab) at 25°C. The tissue was homogenised in a Waring-blender and the homogenate filtered *in vacuo* through Whatman No 1 filter paper. Residues were rehomogenised with the same volume of

petroleum ether and filtered again. Filtrates were concentrated *in vacuo* in a Buchi Rotovaporator-RE 120, and the extract collected with 6 ml dichloromethane (Biolab). All samples were stored in vials at 7 °C until further use.

Chromatography of crude extracts: Samples of the crude extracts (20 µl in total) were spotted on TLC plates (Kieselgel 60 F_{245}) (Sigma, Johannesburg, SA) and developed with toluene:ethyl acetate (1:1 v/v) as solvent. Scoparone (6,7-dimethoxycoumarin) (Department of Biochemistry, Rand Afrikaans University, Johannesburg) at a concentration of 1 000 ppm was used as standard. Developed plates were air-dried overnight at 25 °C and exposed to UV light. Fluorescent bands were compared to that of scoparone.

Detection of antifungal activity: Antifungal activity in TLC bands was bio-assayed directly on the plates (Kim *et al.*, 1991). TLC plates were sprayed Czapeck-Dox medium (Homans & Fuchs, 1970) containing a conidial suspension of *P. digitatum* (10^6 conidia ml^{-1}) harvested from 14-day-old cultures. Plates were placed in a moist tray and incubated at 25 °C for three days. Antifungal activity was indicated by the presence of inhibition zones around bands.

Optimisation of warm water treatments in the packhouse

Valencia orange fruit provided by Letaba Estates were artificially inoculated as described in Chapter 2 with a postharvest pathogen suspension containing 10^6 *Penicillium* conidia ml^{-1}. Each treatment consisted of 480 fruit and included warm water dips at 36, 38, 40 and 42 °C for 1, 2 or 3 min. A control consisting of mechanically injured fruit artificially inoculated but not subjected to warm water treatement, was also included. The chemical treatment consisted of a 3 min dip in tap water containing the following chemicals: Deccotine (guazatine - 1000 ppm), Fungazil (imazalil - 500 ppm) and Tecto (thiabendazole - 1000 ppm). After treatment, fruit were air-dried, packed (80 fruit/box) and stored at 25 °C. Percentage sound fruit per box was recorded for each treatment after 14 days. Data were analysed statistically using ANOVA and Student's t-least significant differences were calculated to determine differences between treatments (P = 0.05).

RESULTS

Antagonist integrated with warm water treatment

In both experiments, warm water treatment reduced decay significantly in comparison with the control (Fig. 1 & 2). Supplementation with B246 enhanced efficacy of the warm water treatment although not significantly. In the first experiment (Fig. 1) warm water, whether supplemented with antagonist or not, was as effective as the chemical treatment. However, in the second experiment (Fig. 2) the chemical dip yielded significantly more sound fruit than any of the other treatments.

Different temperature regimes

Exposure for 3, 6 or 9 min to temperatures of 39 °C or above increased the percentage sound fruit to more than 75 % compared to the 60 % sound fruit of the non-heat-treated control (Fig. 3).

***In vivo* determination of optimum warm water temperature**

Effect of heat treatment on decay development: Green mould development was delayed for longer than 14 days in fruit exposed for 1, 2.5 or 5 min to 36 and 40 °C in comparison to control fruit not heat-treated. These warm water treatments had no deleterious physiological effects on the fruit. Fruit treated at 52 °C for 2.5 min or longer showed no decay, but became hard and developed off-odours during storage. Fruit of the remaining treatments rotted within 7-14 days of storage.

Presence of induced compounds: Exposure of developed TLC plates to UV light verified the presence of scoparone when bands were compared to that of the scoparone standard (Rf 0.18). Scoparone was detected in the crude extracts of all treatments, including the controls. Crude extracts of the 36 and 40 °C treatments showed more distinct bands (Rf 0.20) than those of other treatments. Another band (Rf 0.13), not corresponding with that of scoparone, was observed on plates prepared from crude extracts of 36 °C for 1, 2.5 and 5 min treatments.

Antifungal activity of induced compounds: Antifungal activity of scoparone varied between treatments (Table 1). Clear inhibition of *P. digitatum* was evident with extracts of fruit treated at 36 or 40 °C for 1, 2.5 and 5 min (Fig. 4). Extracts from fruit of all other heat treatments showed either slight or no inhibition of the pathogen. *Penicillium digitatum* was inhibited only slightly by flavedo extracts of fruit from both control treatments (Fig. 3). The unknown compound from extracts of fruit heat-treated at 36 °C for 1, 2.5 and 5 min also

caused inhibition of the fungus.

Optimisation of warm water treatments in the packhouse

All treatments significantly reduced decay in comparison with the control (Fig. 5). Chemical treatment nevertheless was significantly more effective than the warm water treatments.

DISCUSSION

Amongst the technologies available to extend shelf-life of fruit, pre-storage heat treatment appears to be one of the most promising for postharvest decay control (Couey, 1989). This study demonstrated that warm water treatment of Valencia oranges at 36 or 40 °C for 1, 2.5 or 5 min, can provide effective control of *P. digitatum in vivo*. These temperatures are not high enough to kill conidia of *P. digitatum* and fall in the range supporting growth of the organism (Domsch *et al.*, 1980). From the results obtained it can be deducted that conidia were probably inactivated at high temperatures. Barkai-Golan *et al.* (1969) observed 30% conidial survival after heat treatment for 5 min at 50 °C, while Dettori *et al.* (1996) observed that dip treatment at 50-52 °C for 2 min caused only a delay in conidial germination for a period of up to 48 h. Since neither scoparone nor the unidentified fungistatic compound was produced at temperatures higher than 44 °C, the effect of heat treatment at temperatures of 44 °C and lower therefore seems to be due to respons of the host. It is known that wounding and pathogen infection of the citrus peel can induce resistance (De Lange *et al.*, 1976; Brown & Barmore, 1983). In addition, heat treatment enhances this effect, causing even low pathogen infestation to successfully elicit resistance (Kim *et al.*, 1991). This resistance comprises two mechanisms. Firstly, alterations to the host cell wall or other structures could provide a more efficient barrier against pathogen penetration e.g. lignification of wounded areas (Brown & Barmore, 1983). Secondly, antifungal compounds such as scoparone are produced in the host tissue (Kim *et al.*, 1991; Ben-Yehoshua *et al.*, 1992).

In this study, mechanically injured-inoculated fruit showed an increase in decay and insignificant production of scorparone compared to mechanically injured-inoculated fruit heat-treated at 36 or 40 °C. Ben-Yehoshua *et al.* (1987) and Kim *et al.* (1991) also reported that low concentrations of scoparone accumulated as a result of mechanical injury and inoculation, but substancially increased (20-fold) following heat treatment (36 °C for 3 days). However,

antifungal activity did not increase following heat treatment alone.

Commercially, heat treatment may be most useful when applied in tandem with an additional postharvest decay control measures such as biocontrol (Brown & Baraka, 1996; Shellie & Skaria, 1998). Huang *et al.* (1995) demonstrated that biocontrol of green mould with *Pseudomonas glathei* can be enhanced when heat is applied to retard conidial germination of *P. digitatum* while simultaneously stimulating multiplication of the bacterium. Although heat treatment enhanced the efficacy of *B. subtilis* (B246) in the present study, the enhancement was not significant and furthermore not comparable to decay control achieved with chemical treatment. This is probably due to the high inoculum level (10^5 and 10^6 conidia ml^{-1}) and low antagonist concentration (10^7 cells ml^{-1}) (Chapter 2). Another possibility is that effective biocontrol may dependent on successful establishment of the antagonist at the wound site prior to challenge by the pathogen (Huang *et al.*, 1995). Arras (1996) observed scoparone accumulation 19 times higher than in untreated inoculated fruit when the antagonist *Candida famata* (FC Harrison) SA Meyer & Yarrow was inoculated 24 h prior to *P. digitatum*, and only four times higher if inoculated 24 h after the pathogen.

In conclusion, there is renewed interest in heat treatment for disease control. Advances in understanding the metabolic responses of citrus fruit to heat could lead to more effective and rational use of heat treatment. This study demonstrated that packhouse dipping of fruit in water for 1-5 min at the relatively low temperatures of 36 and 40 °C reduced postharvest decay whilst maintaining fruit quality. Heat treatment has the further advantage of ease of implementation in South African citrus packhouses and the absence of chemical residues.

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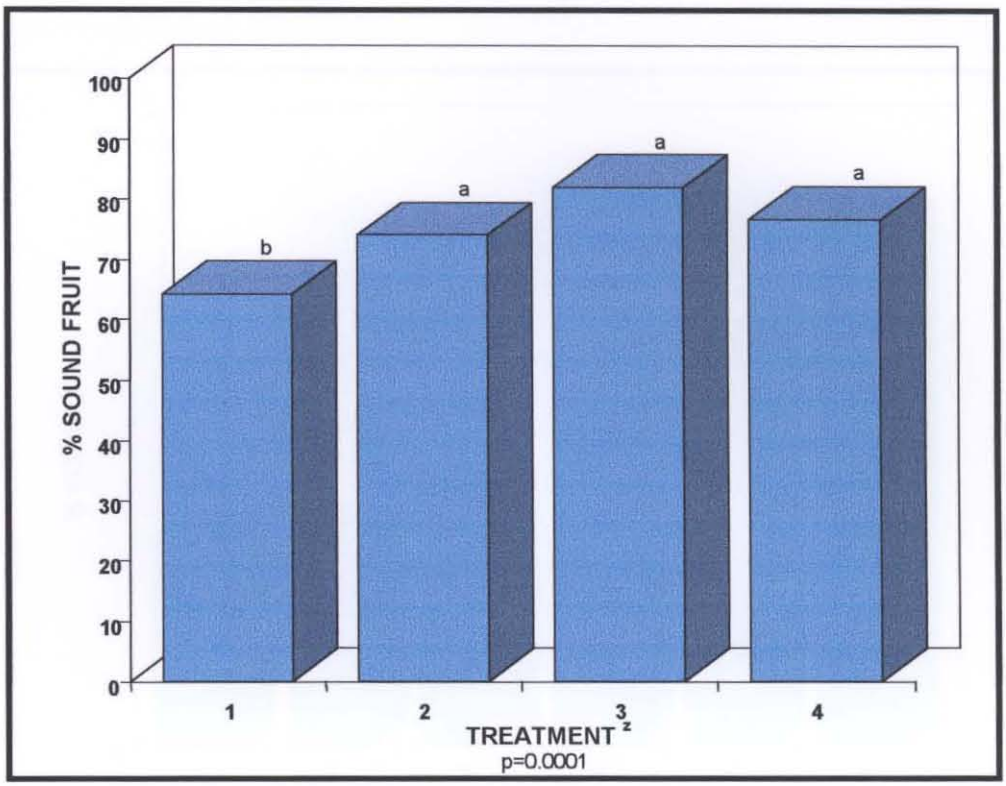
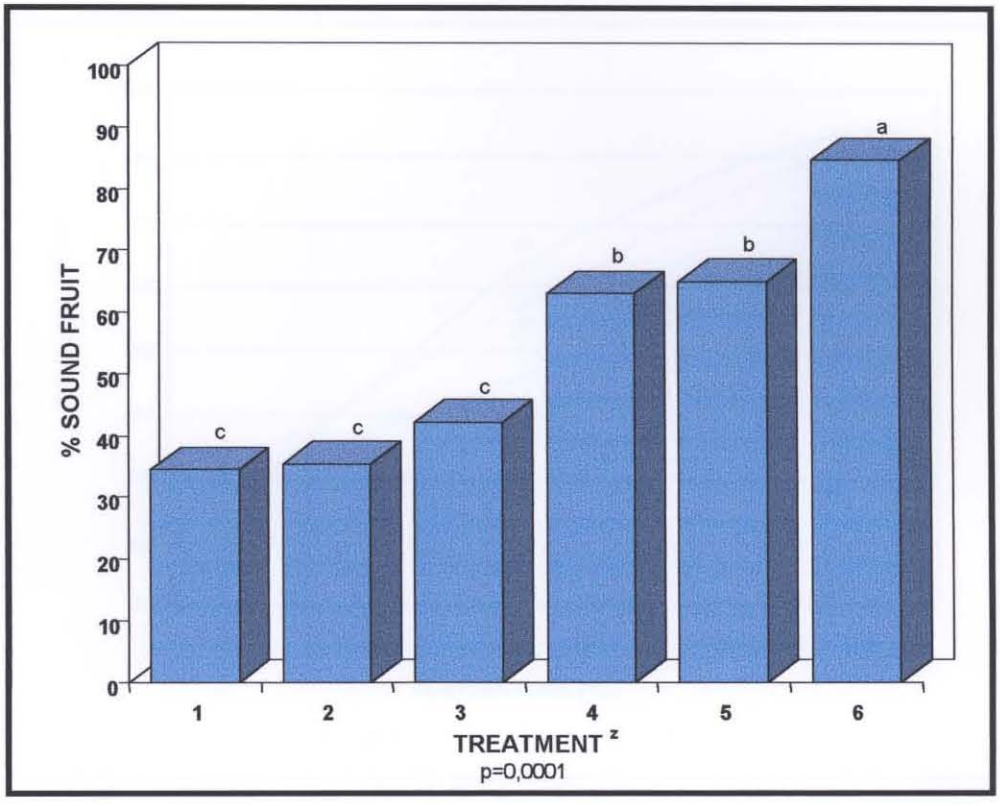


Bars not sharing a common letter differ significantly according to Student's t-LSD (P=0.05).

[z] Treatments consisted of mechanically injured fruit which were artificially inoculated by dipping in 25 l inoculum of *Alternaria citri*, *Colletotrichum gloeosporioides*, *Geotrichum citri-aurantii*, *Lasiodiplodia theobromae*, *Penicillium digitatum*, *P. italicum* and *Trichoderma viride*, containing 1.4 x 10^5 *Penicillium* conidia ml^{-1}. Fruit were incubated overnight and treated the following day. Treatments included 1) Untreated control; 2) Warm water (40 °C) dip for 7 min; 3) Warm water (40 °C) supplemented with *Bacillus subtilis* (B246) (1 x 10^7 cells ml^{-1}) dip for 7 min; 4) Chemical dip (1 000 ppm guazatine, 500 ppm imazalil, 1 000 ppm thiabendazole) for 3 min.

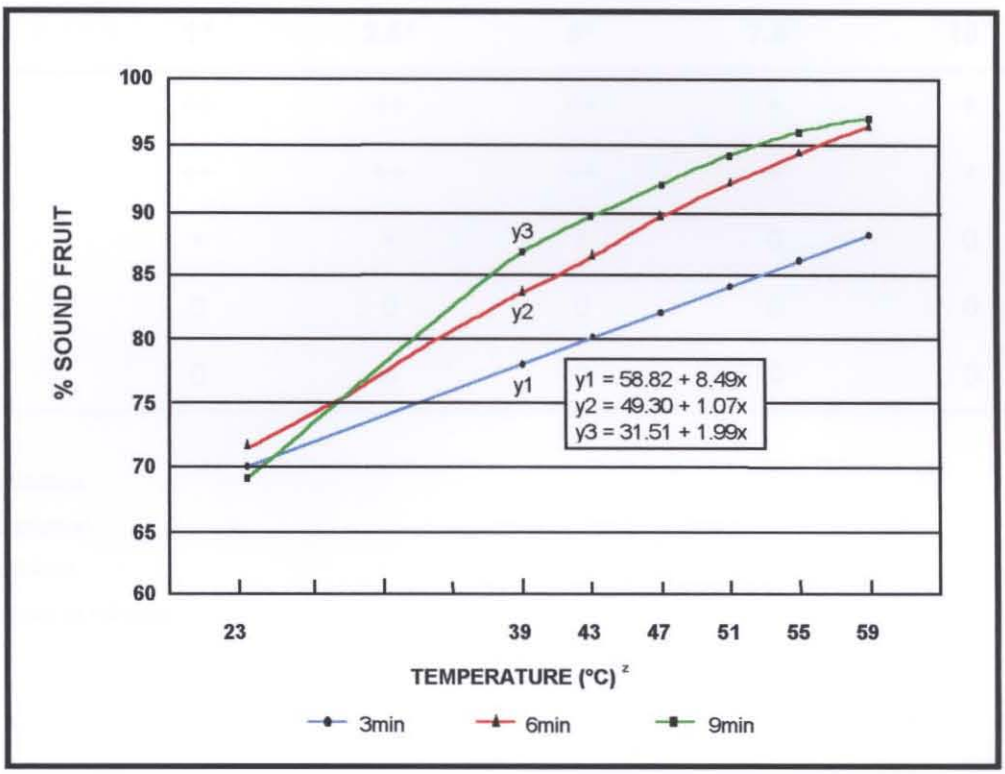
Fig. 1 Effect of chemical, warm water and warm water supplemented with *Bacillus subtilis* dip treatments on postharvest deterioration of Valencia orange fruit (First experiment).



Bars not sharing a common letter differ significantly according to Student's t-LSD (P=0,05).

[z] Treatments consisted of mechanically injured fruit which were artificially inoculated by dipping in 25 l inoculum of *Alternaria citri*, *Colletotrichum gloeosporioides*, *Geotrichum citri-aurantii*, *Lasiodiplodia theobromae*, *Penicillium digitatum*, *P. italicum* and *Trichoderma viride*, containing 1.1 x 10^6 *Penicillium* conidia ml^{-1}. Fruit were incubated overnight and treated the following day. Treatments included 1) Untreated control; 2) Tap water dip for 7 min; 3) Tap water supplemented with *Bacillus subtilis* (B246) (1 x 10^7 cells ml^{-1}) dip for 7 min; 4) Warm water (40 °C) dip for 7 min; 5) Warm water (40 °C) supplemented with B246 (1 x 10^7 cells ml^{-1}) dip for 7 min; 6) Chemical dip (1 000 ppm guazatine, 500 ppm imazalil, 1 000 ppm thiabendazole) for 3 min.

Fig. 2 Effect of chemical, warm water and warm water supplemented with *Bacillus subtilis* (B246) dip treatments on postharvest deterioration of Valencia orange fruit (Second experiment).



[z] Temperature treatments were applied to mechanically injured fruit which were artificially inoculated by dipping in 25 l inoculum of *Alternaria citri*, *Colletotrichum gloeosporioides*, *Geotrichum citri-aurantii*, *Lasiodiplodia theobromae*, *Penicillium digitatum*, *P. italicum* and *Trichoderma viride*, containing 1.2×10^5 *Penicillium* conidia ml^{-1}. Fruit were incubated overnight and treated the following day.

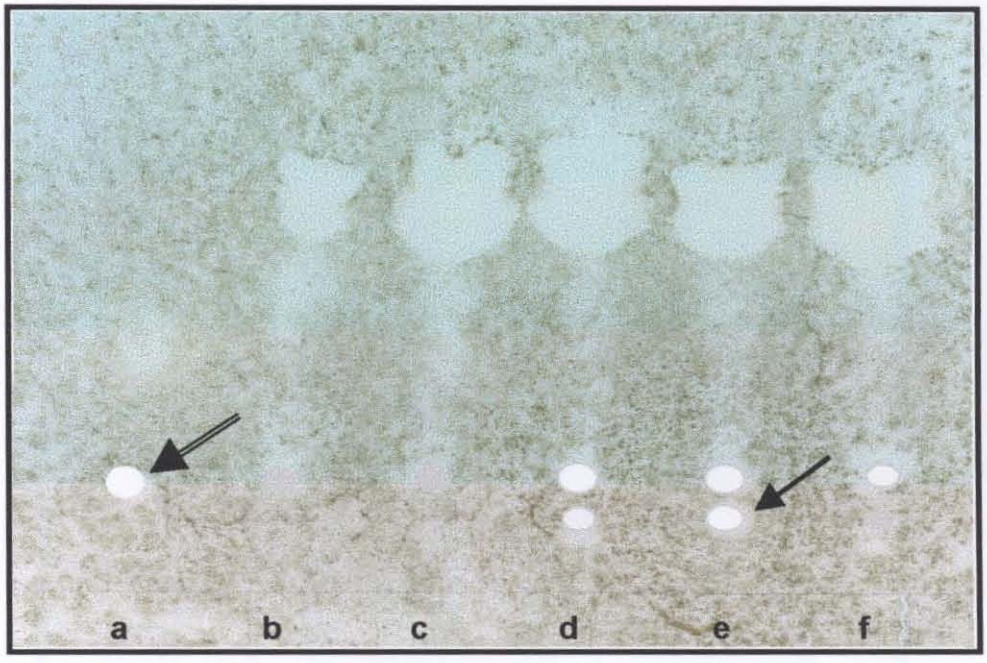
Fig. 3 Effect of temperature and exposure time on postharvest deterioration of Valencia orange fruit.

Table 1 Scoparone activity in Valencia orange fruit exposed for different periods to different temperatures, determined by means of a TLC-bioassay with *Penicillium digitatum*

Temperature	**Inhibition of *P. digitatum* [y]**				
(°C)	**1 [z]**	**2.5 [z]**	**5 [z]**	**7.5 [z]**	**10 [z]**
36	++	++	++	+	+
40	++	++	++	+	+
44	+	+	+	0	0
48	0	0	0	0	0
52	0	0	0	0	0

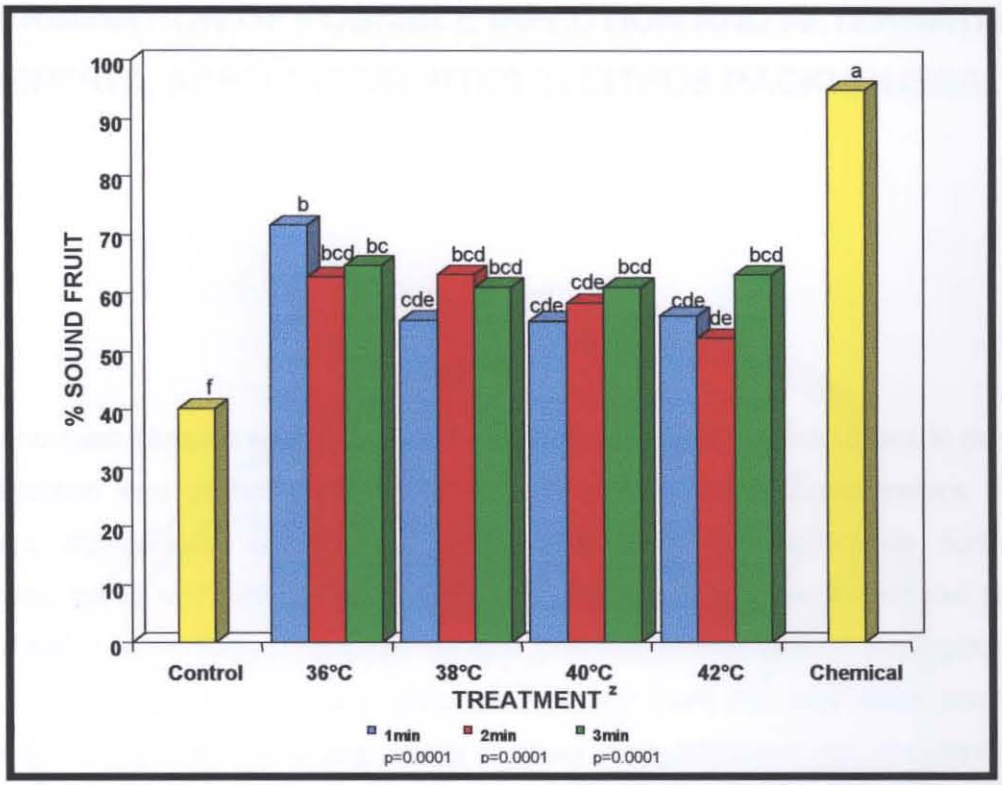
[y] ++ Clear inhibition
\+ Slight inhibition
0 No inhibition

[z] Exposure times in minutes



za	Scoparone
b	Control (mechanically injured)
c	Control (mechanically injured fruit + inoculated with *P. digitatum* (10^6 conidia ml^{-1}))
d	Mechanically injured + inoculated with *P. digitatum* + 36°C warm water dip for 1 min
e	Mechanically injured + inoculated with *P. digitatum* + 36°C warm water dip for 2.5min
f	Mechanically injured + inoculated with *P. digitatum* + 36°C warm water dip for 5 min
←	Scoparone
←	Unknown antifungal compound

Fig. 4 Inhibition of *Penicillium digitatum* on a TLC plate run with petroleum ether extracts of Valencia orange fruit dipped for different time periods in water at 36 °C.



Bars not sharing a common letter differ significantly according to Student's t-LSD (P=0.05).

[y] Control and chemical treatment data are included for comparison.

[z] Treatments consisted of mechanically injured fruit which were artificially inoculated by dipping in 25 l inoculum of *Alternaria citri*, *Colletotrichum gloeosporioides*, *Geotrichum citri-aurantii*, *Lasiodiplodia theobromae*, *Penicillium digitatum*, *P. italicum* and *Trichoderma viride*, containing 2.3 x 10^5 *Penicillium* conidia ml^{-1}. Fruit were incubated overnight and treated the following day. Chemical treatment involved dipping fruit for 3 min in guazatine (1 000 ppm), imazalil (500 ppm) and thiabendazole (1 000 ppm).

Fig. 5 Effect of different warm water regimes on postharvest deterioration of Valencia orange fruit.