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Original Article

Induction of resistance to *Penicillium digitatum* in tangerine fruit cv. Sai Num Phung flavedo by hot water treatment

Sirisopha Inkha[1]* and Danai Boonyakiat[2]

[1] *Postharvest Technology Research Institute,*

[2] *Department of Plant Science and Natural Resources, Faculty of Agriculture, Chiang Mai University, Muaeng, Chiang Mai, 50200 Thailand.*

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Abstract

The effects of hot water treatment (HWT) were investigated for enhancing host resistance to green mold rot caused by *Penicillium digitatum*. Tangerine fruits cv. Sai Num Phung were dipped in hot water at 50±2°C for 3 minutes and 55±2°C for 2 and 3 minutes after inoculation with *P. digitatum* and then stored at 4±2C with 90±5% relative humidity for 30 days. The results showed that the HWT remarkably delayed the onset of disease infection, reduced the number of infected fruits and lowered the severity of infection (lesion diameter). The chitinase and β-1,3-glucanase activities in flavedo tissues of treated fruits increased after storage for 15 days, but activity of peroxidase increased after storage for 25 days, compared with untreated and uninoculated fruits. The protein patterns of tangerine fruit peels treated with HWT appeared to have 112.20 and 100.00 kDa proteins only on the fifth day of storage which indicated that HWT led to heat stress circumstances in the fruit peel tissue and induced biochemical changes. The protein patterns of HWT treated fruit at 22.39 kDa exhibited thicker band compared to untreated and uninoculated fruit peels. The findings indicated that HWT reduced disease incidence partly by inducing defence mechanism in the fruit peel tissue.

Keywords: tangerine fruit, citrus fruit, *Penicillium digitatum*, hot water treatment, inducing resistance

1. Introduction

Tangerine (*Citrus reticulata* Blanco) cv. 'Sai Num Phung' is the main citrus crop in Northern Thailand. Postharvest losses due to *Penicillium digitatum* is the major constraint during handling process of tangerine fruit. The primary infection cause of this pathogen is a wound on fruit where nutrients are available to stimulate spore germination. Wounds can be inflicted during harvest and subsequent handling, and the resulting infections must be eradicated to achieve acceptable levels of control (Smilanick *et al*., 2005). Currently, Penicillium rot in Northern Thailand is controlled by application of Imazalil and Thiabendazole. However, alternative methods are needed due to the widespread use of these agrochemicals in commercial packinghouses has led to proliferation of resistant strains of the pathogens (Palou *et al*., 2007). Furthermore, concerns about human health risks and protection of the environment, associated with fungicide residues have been increased the need for the development of safe and effective alternatives.

Heat treatment technology is a safe and environmentally-friendly procedure with increasing acceptability in commercial operations. It is used successfully to control the incidence of postharvest disease in several commodities (Fallik, 2004). Pre-storage hot-water-dips of fruit at temperatures above 40°C are effective in controlling storage decay, not only by reducing the pathogen inoculum but also by enhancing the resistance of the fruit tissue and influencing

* Corresponding author.
Email address: sirisophai@yahoo.com

host metabolism (Barkai-Golan and Philips, 1991). Postharvest dips are applied for a few minutes at high temperatures, because fungal spores and latent infections are either on the surface or in the first few cell layers under the peel of the fruit (Lurie, 1998). Hot water treatment (HWT) may eliminate incipient infections, by removing spore from wounds and acting directly on their viability and induce fruit defence mechanisms in the outer layers of the epicarp which inhibit pathogen growth (Schirra *et al*., 2000). The benefits of HWT for control of decay caused by *Penicillium* spp. on citrus have been reported (Schirra and D'hallewin, 1997; Porat *et al*., 2000; Palou *et al*., 2001; Ben-Yehoshua, 2003). For citrus, HW dips at 50-53°C for 2-3 minutes were shown to be as effective as curing at 36°C for 72 hours in controlling postharvest decay and chilling injury in various citrus fruits and are much less expensive, mainly because of shorter treatment duration.

Two major groups of proteins may be activated by the HWT that induce fruit resistance: heat shock proteins (HSPs) and pathogenesis-related (PR) proteins. HSPs comprise a diverse group of proteins, ranging in molecular weight from 15 to 115 kDa, that are expressed in all organisms in response to elevated temperatures and are believed to play a major role in thermotolerance (Sabehat *et al*., 1998).

PR proteins coded by host plant genes are induced by pathogen infection or related situations, and are thought to play an important role in plant defence responses against a wide range of pathogens. Among the PR proteins, the most characterized enzymes are those of group 2 that have β-1,3-glucanase activity and group 3 that have chitinase activity (Van Loon and Van Strien, 1999). The substrate of chitinase is chitin which is a common component of fungal cell walls and insect exoskeletons. The major natural role for chitinase is defence, primarily against chitin-containing fungal pathogens by inhibiting spore germination and germ-tube elongation and degrading hyphal tips. β-1,3-Glucanase catalyzes hydrolytic cleavage of the 1,3-β-D glucosidic linkages in β-1,3-glucan, which is a component of mycelial cell walls, thus, directly inhibiting fungal growth (Porat *et al*., 2002; Wang *et al*., 2003).

In the present paper, we reported that a HWT at 50±2°C for 3 minutes and 55±2°C for 2 and 3 minutes induced resistance in 'Sai Num Phung' tangerine fruits against the green mold pathogen *P. digitatum*, which is the main cause for most of the postharvest losses in tangerine fruits. Furthermore, we showed that the HWT induced the accumulation of HSPs, chitinase, β-1,3-glucanase and peroxidase activities in the fruit peel tissue.

2. Materials and Methods

2.1 Plant material

Tangerine fruits cv. 'Sai Num Phung' were obtained from a commercial orchard in Amphoe Fang, Chiang Mai province, Thailand, and harvested at the age of 9 months after full bloom. Fruits were harvested in January, 2007, when commercially mature. After being harvested, the fruits were manually selected by hand from field bins before any commercial postharvest treatment was imposed. Samples of blemish-free fruits of uniform size and appearance were washed with water at room temperature (26±2°C), air-dried and placed at random in plastic baskets.

2.2 Postharvest HWT and storage conditions

The HWT at 50±2°C for 3 minutes and 55±2°C for 2 and 3 minutes for each treatment were applied for tangerine fruit dipping. After treatments, the fruits were incubated at 4±2°C and 90±5% relative humidity (RH) for 30 days. Each treatment comprised three replicated boxes, each containing 10 fruits.

2.3 Fungal cultures

The isolate of *P. digitatum* was obtained from an infected tangerine fruit and cultured on potato dextrose agar (PDA). Spore suspensions were prepared by removing the spores from the sporulating edges of a 1-2-week-old culture with a bacteriological loop, suspending them in sterile distilled water, and filtered through four layers of sterile cheesecloth. The spore concentration was determined with a haemacytometer and adjusted to 10^5 spores ml^{-1}.

2.4 Effects of HWT on fruit resistance against *P. digitatum*

Tangerine fruits were wound-inoculated with a dissecting needle (2 mm long and 1 mm wide) that had been dipped into a spore suspension (10^5 spores ml^{-1}) of *P. digitatum*. A dissecting needle was penetrated at two sites midway between the stem and stylar end position for 3 seconds. Fruits were kept for 3 hours after inoculation at room temperature (26±2C) to simulate actual conditions between harvest and application of control treatments. After 3 hours, fruits were dipped in a water bath at 50±2°C for 3 minutes and 55±2C for 2 and 3 minutes before removing them to rinse with tap water (26±2C), air-dried and placed in plastic pod trays before loading in boxes. The control treatment was divided into 2 groups. The first was inoculated by fungus without hot-water-dipped fruits (untreated fruits) and the second was inoculated by sterile water without hot-water-dipped fruits (uninoculated fruits). Disease incidence, severity (lesion diameter) and sporulation index describing the percentage of the fruit surface covered with green mold spores where 0 = no sporulation on the surface of the fruit; 1 = 1-20%; 2 = 21-40%; 3 = 41-60%; 4 = 61-80% and 5 $\geq$ 80%, as well as external disease appearance, were recorded every 5 days after inoculation at 4±2°C and 90±5%RH for 35 days. The experiment was conducted in a completely randomized design (CRD) with 5 treatments. Each treatment comprised

three replicated boxes, each containing 10 fruits (total of 60 wounds), and the experiment was repeated twice with similar procedures.

2.5 Effects of HWT on activities of the defensive enzymes and protein patterns in tangerine fruit peel

2.5.1 Measurement of enzyme activity

Chitinase, β-1,3-glucanase and peroxidase activities were assayed from flavedo tissues of tangerine fruits treated with HWT and compared with activities in the untreated and uninoculated fruits. Fruit samples in each treatment were obtained from 2.4 and repeated likewise.

Chitinase was extracted according to Cao and Jiang (2006) and assayed using dye-labeled carboxymethylchitin according to the method of Ippolito *et al*., (2000), with slight modifications. Chitinase activity was expressed in international units mg^{-1} protein. 1 unit was defined as the amount of enzyme required to catalyze the formation of 10 mmol min^{-1} of product.

β-1,3-Glucanase was extracted and assayed by measuring the amount of reducing sugar released from the substrate by the dinitrosalicitate method according to Cao and Jiang (2006). The enzyme activity was expressed as unit mg^{-1} protein, where 1 unit was defined as the reducing sugar equivalent to 10 mmol of glucose produced per minute.

Peroxidase was extracted and assayed using guaiacol as substrate according to Flurkey and Jen (1978). Specific activity was expressed as the change in absorbance at 470 nm unit mg^{-1} protein. 1 unit was defined as an increase in A470 of 10 mmol min^{-1} of product.

Soluble protein content was determined according to the method of Bradford (1976), using bovine serum albumin (BSA) as a standard.

2.5.2 Protein pattern analysis

Ten micrograms of proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electroproresis (SDS-PAGE), using a Hoefer™ mini VE vertical electrophoresis system (Amersham Biosciences Limited, Buckinghamshire, England). Gels were stained with Coomassie brilliant blue R250 to visualize the proteins.

2.6 Statistical analysis

All statistical analyses were performed with SPSS. The data were analyzed by one-way analysis of variance (ANOVA). Mean separation within each inspection time was calculated where applicable, and the least-significant difference (LSD) test applied. Differences at P=0.05 were considered as significant.

3. Results and Discussion

3.1 Effects of HWT on fruit resistance against *P. digitatum*

All of the HWT examined in this study significantly reduced disease incidence and severity as compared with untreated fruits (Figures 1 and 2). HWT at 50±2°C for 3 minutes and 55±2°C for 2 and 3 minutes effectively reduced the development of green mold, but were not significantly differ-

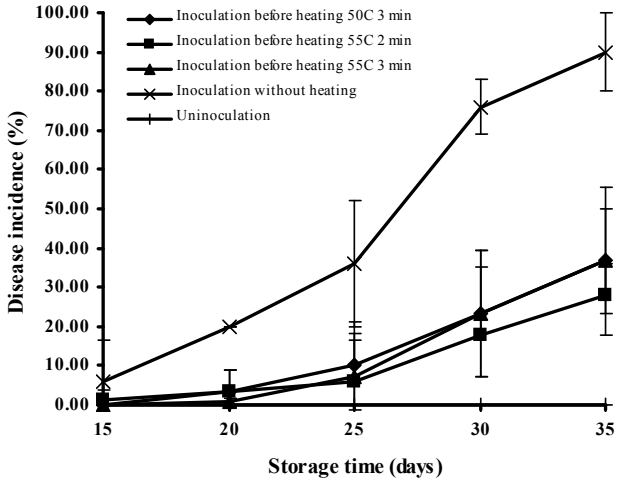

Figure 1. Effects of hot water treatments on green mold disease incidence on artificially-inoculated tangerine fruits stored at 4±2°C, 90±5% RH. Vertical bars represent standard deviations, (n=30).

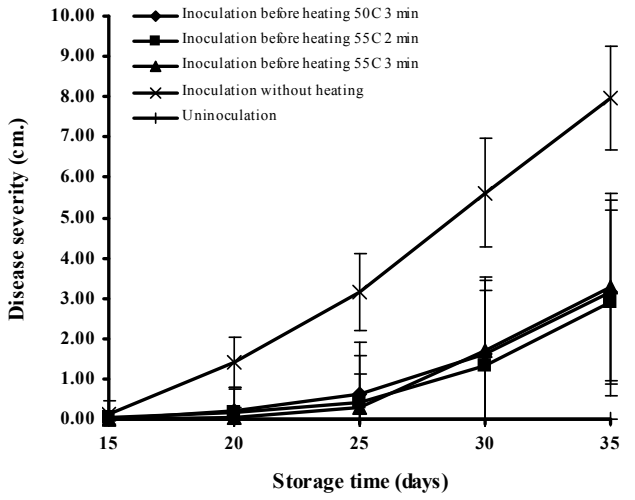

Figure 2. Effects of hot water treatments on green mold disease severity on artificially-inoculated tangerine fruits stored at 4±2°C, 90±5% RH. Vertical bars represent standard deviations, (n=30).

ent from each other. Disease incidence on untreated fruits reached 90%, which was higher than the incidence of green molds on fruit treated with HWT at 50±2°C for 3 minutes and HWT at 55±2°C for 2 and 3 minutes, which were 36.7, 28.0 and 36.7%, respectively. Moreover, HWT conditions that were mentioned above reduced disease severity from 7.7cm of untreated fruits to 3.2, 2.9 and 3.3cm of HWT at 50±2°C for 3 minutes and HWT at 55±2°C for 2 and 3 minutes, respectively, when stored at 4±2°C and 90±5% RH for 35 days. Nevertheless, sporulation of green mold rot was not found in any treatment.

The results of this study indicated that HWT at 50±2°C for 3 minutes and 55±2°C for 2 and 3 minutes after inoculation reduced green mold rot development in 'Sai Num Phung' tangerine fruits about 31.1-40.7% compared with untreated fruits. This result was consistent with those of previous studies on a wide variety of citrus fruit (Rodov *et al*., 1995; Gonzalez-Aguilar *et al*., 1997; Schirra and D'hallewin, 1997; Schirra *et al*., 2004). The significant reduction in decay development of postharvest citrus fruit treated with HWT is considered to be mainly due to the host-pathogen interactions modulated by the treatments and partly to the reduction in the epiphytic microorganism population, compared with untreated fruit (Porat *et al*., 2000; Schirra *et al*., 2000). Hot-water-dipping reportedly had a transient inhibitory effect on *P. digitatum*, arresting its growth for 24-48 hours. During this lag period when the pathogen was arrested, the combined effects of the pathogen and the hot-water-dip induced the build up of resistance in the peel (Ben-Yehoshua, 2003). The effect of HWT on citrus fruit may be associated with melting and redistributing of natural epicuticular wax on the fruit surface, plugging numerous microscopic cuticular cracks and stomata to adapt physical barriers to pathogen penetration (e.g., *Botrytis cinerea* whose spores can germinate and penetrate the surface of fruit) (Porat *et al*., 2000). In fact, natural openings and barely-visible cracks in the epidermis of treated fruit were partially or entirely sealed with rearranged natural wax components present on the cuticle, thus limiting sites of fungal penetration into the fruit (Rodov *et al*., 1995; Schirra and D'hallewin, 1997).

3.2 Effects of HWT on activities of the defensive enzymes and protein patterns in tangerine fruit peel

The activity of chitinase in fruit treated with HWT was markedly increased after 15 days of storage, and increased constantly with higher activity than that of untreated and uninoculated fruits till 30 days of storage. During 20-30 days of storage, the rate of chitinase activity in tangerine fruits which were dipped in HWT at 55±2°C for 2 minutes increased rapidly compared with HWT at 55±2°C for 3 minutes, HWT at 50±2°C for 3 minutes, untreated and uninoculated fruits, respectively. Chitinase activity in tangerine fruits treated with HWT at 55±2°C for 2 minutes and HWT at 55±2°C for 3 minutes were respectively 5.4 and 2.6 times higher than in the untreated fruits at 30 days. However, chitinase activity of uninoculated fruits remained constant for the entire storage period (Figure 3).

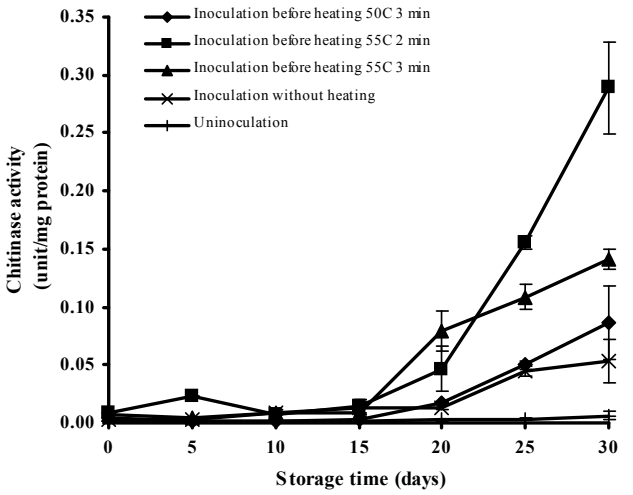

Figure 3. Effects of hot water treatments on change of chitinase activity in flavedo tissue of tangerine fruits inoculated with *P. digitatum* stored at 4±2°C, 90±5% RH. Vertical bars represent standard deviations, (n=5).

Levels of β-1,3-glucanase activity in all treatments were slightly increased during the early 15 days of storage. However, on day 25 of storage, it was found that β-1,3-glucanase activity of tangerine fruits dipped in HWT at 55±2°C for 2 minutes and 50±2°C for 3 minutes were markedly increased and diminished on day 30, while β-1,3-glucanase activity of tangerine fruits which were dipped in HWT at 55±2°C for 3 minutes increased continuously during the storage period. In case of untreated fruits, β-1,3-glucanase activity increased on day 20 and remained constant until the last day of storage. On day 30, β-1,3-glucanase activity from all of the heat treatments was significantly higher than that of the untreated and uninoculated fruits, which was 1.9 and 9.9 times, respectively (Figure 4).

Peroxidase activities in all treatments remained relatively constant until 25 days of storage, then surprisingly increased on day 30, especially tangerine fruits dipped in HWT at 55±2°C for 3 minutes, which had the highest peroxidase activity, followed by HWT at 55±2°C for 2 minutes, HWT at 50±2°C for 3 minutes, untreated and uninoculated fruits, respectively, with significant differences (Figure 5).

The present data show that HWT was effective in controlling green mold rot of tangerine fruits. A reduction in disease may indicate the expression of resistance induction. The results imply that HWT reduced disease incidence partly by inducing the accumulation of chitinase, β-1,3-glucanase and peroxidase in tangerine fruits over and above the stimulation of these enzymes in untreated and uninoculated fruits.

The protection of fruit from invasion of fungal pathogens is largely due to activation of a highly-coordinated biochemical and structural defence system that helps ward off the spread of pathogens (Lawton *et al*., 1996). Chitinase and

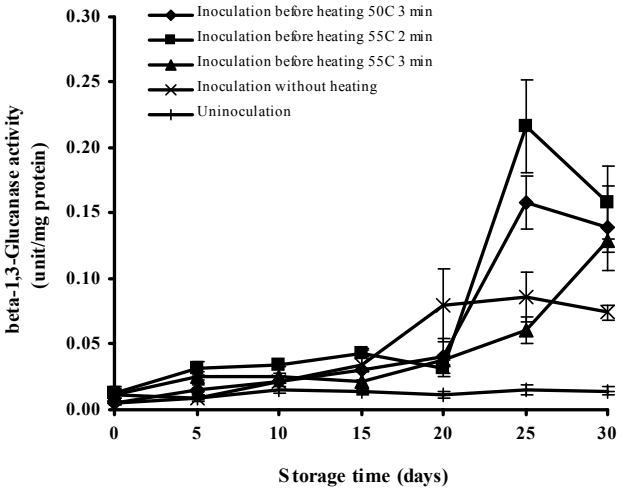

Figure 4. Effects of hot water treatments on change of β-1,3-glucanase activity in flavedo tissue of tangerine fruits inoculated with *P. digitatum* stored at 4±2°C, 90±5% RH. Vertical bars represent standard deviations, (n=5).

β-1,3-glucanase are considered as key enzymes having direct activity against pathogens in plant disease-resistance systems (Cao and Jiang, 2006). Peroxidase activity produces the oxidative power for cross-linking of proteins and phenylpropanoid radicals, resulting in reinforcement of cell walls against attempted fungal penetration (Kristensen *et al*., 1999). Our results indicate that HWT induces higher activities of chitinase and β-1,3-glucanase in tangerine fruits than the untreated and uninoculated fruits after storage for 15 days (Figures 3 and 4). The increase in chitinase and β-1,3-glucanase activity in fruit treated with HWT seems to correlate with a reduction in lesion diameter of fruit. This is in line with previous findings on responses of plant-fungus systems to heat treatment (Pavoncello *et al*., 2001). In lemon fruits, transient thermal inhibition of pathogen growth was attributed to the build-up of resistance factors, such as increased chitinase and β-1,3-glucanase activities, enabling the degradation of fungal wall components (Arlorio *et al*., 1992). Nevertheless, results obtained here showed that peroxidase did not contribute noticeably to the induced resistance in tangerine fruits, as activity of peroxidase increased only on day 30 as responses to the HWT (Figure 5). This was contrary to mature green tomatoes, in which heat treatment had an effect on peroxidase activity and ability of the fruit tissue to withstand fungal attack (Lurie *et al*., 1997). In citrus, chitinase and β-1,3-glucanase seem to be involved in the enhancement of pathogen resistance. Thus, callus cultures of 'Femminello' lemons that appeared to be tolerant to *Phoma tracheiphila* have an enhanced release of these enzymes into the culture medium, and exogenous application of a callus medium rich in chitinase and β-1,3-glucanase inhibited the growth of *P. digitatum* in Petri dishes (Porat *et al*., 2002). Pavoncello *et al*. (2001) showed that hot-water-brushing (HWB) treatment at 62°C for 20 seconds induced resistance against *P. digitatum* in 'Star Ruby' grapefruit. HWB using heated water induced the accumulation of the 21, 22 and 25 kDa chitinase proteins and of the 38 and 43 kDa β-1,3-glucanase proteins, which were observed 1 and 3 days after the HWB treatment when the fruit appeared to be more resistant to *P. digitatum*. Porat *et al*., showed using RNA gel blot hybridizations that the expression of the genes coding chitinase (2001) and β-1,3-glucanase (2002) were markedly induced both by HWB and by UV illumination.

Protein profiles separated by SDS-PAGE 10% after HWT challenged by *P. digitatum* are shown in Figure 6. The results showed that the protein patterns of tangerine fruit peels treated with HWT revealed 112.20 and 100.00 kDa proteins only on the fifth day of storage, whereas on the other days of storage there were no incidence of these protein patterns. Moreover, there were apparent differences in protein patterns or synthesis of novel proteins compared with untreated and uninoculated fruit peels. The protein patterns of HWT treated fruit at 22.39 kDa exhibited a thicker band compared to that of untreated and uninoculated fruit peels.

The HWT was sufficient to administer a heat shock to the cells of the fruit peel tissue, as indicated by its capability to induce the 112.20 and 100.00 kDa proteins (Figure 6). These proteins mentioned above had molecular mass close to the protein from 'Star Ruby' grapefruit which was 105.00 kDa (Pavoncello *et al*., 2001). Usually, HSPs are induced only after longer incubation periods of at least 2-3 hours but at lower temperatures of 37 to 40°C (Chen *et al*., 1990). Therefore, the combination of a short exposure and a higher temperature is probably equivalent to a longer exposure at a lower temperature for the induction of HSPs. HSPs produced in response to high temperature are believed to prevent irreversible protein denaturation that would be detrimental to the cell. The lag period for induction of heat shock

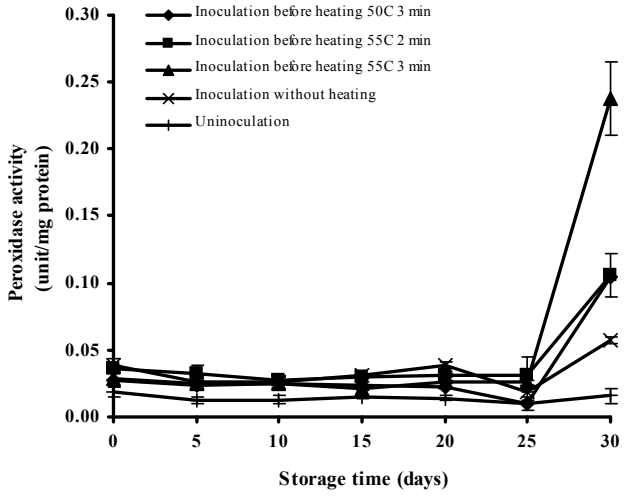

Figure 5. Effects of hot water treatments on change of peroxidase activity in flavedo tissue of tangerine fruits inoculated with *P. digitatum* stored at 4±2°C, 90±5% RH. Vertical bars represent standard deviations, (n=5).

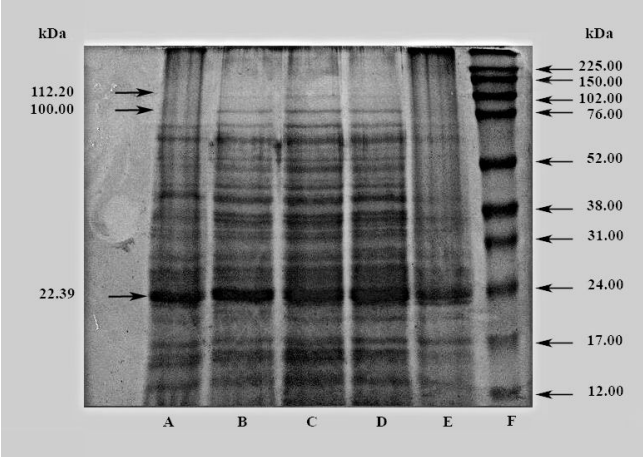

Figure 6. Pattern of proteins from tangerine fruit peel tissue in response to the HWT: (A) non-inoculation, (B) inoculation before heating 50±2°C 3 min., (C) inoculation before heating 55±2°C 2 min., (D) inoculation before heating 55±2°C 3 min., (E) inoculation without heating, (F) molecular mass markers given in kDa stored at 4±2°C, 90±5% RH on day 5 by 10% SDS-PAGE. The arrows and values on the left indicate the approximate molecular mass of tangerine fruit peel tissue cross-reacting molecular mass markers.

response is slower than that of other stress responses. A decay of HSPs occurs, with a corresponding loss in thermotolerance. This phenomenon appears to confer a temporary, acquired heat resistance to sub-lethal temperatures. There is a fundamental role for HSPs in cellular function during high temperature stress (Sabehat *et al*., 1998; Paull and Chen, 2000).

Beyond being an indicator of the heat stress, the accumulation of protein 22.39 kDa following the HWT was probably related to the induction of fruit resistance against *P. digitatum*. In the literature, it was reported that the 22.00 kDa protein is one of the chitinase isoform that is normally abundant in heat-stressed grapefruit (Mccollum *et al*., 1997; Pavoncello *et al*., 2001).

Overall, enhancement of fruit resistance against pathogen infection requires the induction of a wide array of proteins involved in various defence responses, such as lignin formation, phytoalexin production, synthesis of antifungal enzymes, etc. (Porat *et al*., 2002). The induction of chitinase and β-1,3-glucanase activities and some chitinase protein following the HWT may be part of the complex biochemical mechanisms involved in the induction of fruit resistance to *P. digitatum*.

The protein bands at 112.20, 100.00 and 22.39 kDa might have significance in genetic-related areas and lead to more useful information for further studies. Since the objectives of this research were mainly the postharvest process, the study of those three protein bands, which requires extensive knowledge related to molecular biology, should be included in further study.

4. Conclusion

This experiment indicated that a postharvest HWT at temperatures 50±2°C for 3 minutes and 55±2°C for 2 and 3 minutes remarkably reduced green mold rot in 'Sai Num Phung' tangerine fruits due possibly to induction of defence system in the fruit peel tissue, as evidenced by the increased chitinase and β-1,3-glucanase activities and some chitinase protein. There is a narrow range of temperatures and duration that can delay green mold rot development and the suitability of this treatment in Thai citrus packinghouse operations. The advantages of HWT are that it also cleans the fruit and improves its general appearance. In addition, it is simple to apply in the citrus industry since it can be incorporated into the packinghouse sorting line and does not require any special handling. However, up-scaling of the hot dip method from the laboratory to packinghouse scale demands additional technical solutions to maintain a desired treatment regime with large masses of fruit.

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