CHAPTER 4
RESULTS AND DISCUSSION

4.1 Effect of hot water treatment (HWT) on green mold infection in tangerine fruit cv. Sai Num Pung

4.1.1 Effect of HWT on spore germination of *Penicillium digitatum in vitro*

Spore suspension of *P. digitatum* after dipping in hot water at 45±2, 50±2 and 55±2°C for 0.5, 1, 2 and 3 minutes and incubated at 25±2°C for 48 hours in darkness. Results showed that hot water dips at 45±2°C for 0.5, 1, 2 and 3 minutes and 50±2°C for 0.5, 1 minute could not inhibit fungal spore germination (100% germination) (Figure 4.1-4.5). Increasing temperature to 55±2°C with dipping time 0.5 minute could reduce spore germination only 19.00% (81.00% germination) when dipping time was increased to 2 and 3 minutes at 50±2°C could inhibit spore germination to 53.33% and 18.50% after 24 hours, respectively. When the incubation time was increased to 48 hours, all treatments showed 100% spore germination (Figure 4.1, 4.4-4.7). Dipping spore suspension for 1, 2 and 3 minutes at 55±2°C could delay spore germination more than heating at 45±2 and 50±2°C. At 55±2°C of dipping times 1, 2 and 3 minutes, percentage of spore germination were 7.00% (24 hours) 15.00% (48 hours); 1.00% (24 hours) 8.67% (48 hours) and 0.00% (24 hours), 1.67% (48 hours) respectively (Figure 4.1, 4.6-4.7).

The inhibitory effect of HWT on *P. digitatum in vitro* spore germination was found to depend on temperature and duration of treatment (Figure 4.1-4.7). The higher temperature with longer duration could bring effective inhibitory effect. This is in agreement with the findings of other studies showing that spore germination of *P. digitatum* is only partially inhibited by exposure to 56°C for up to 20 seconds, but is

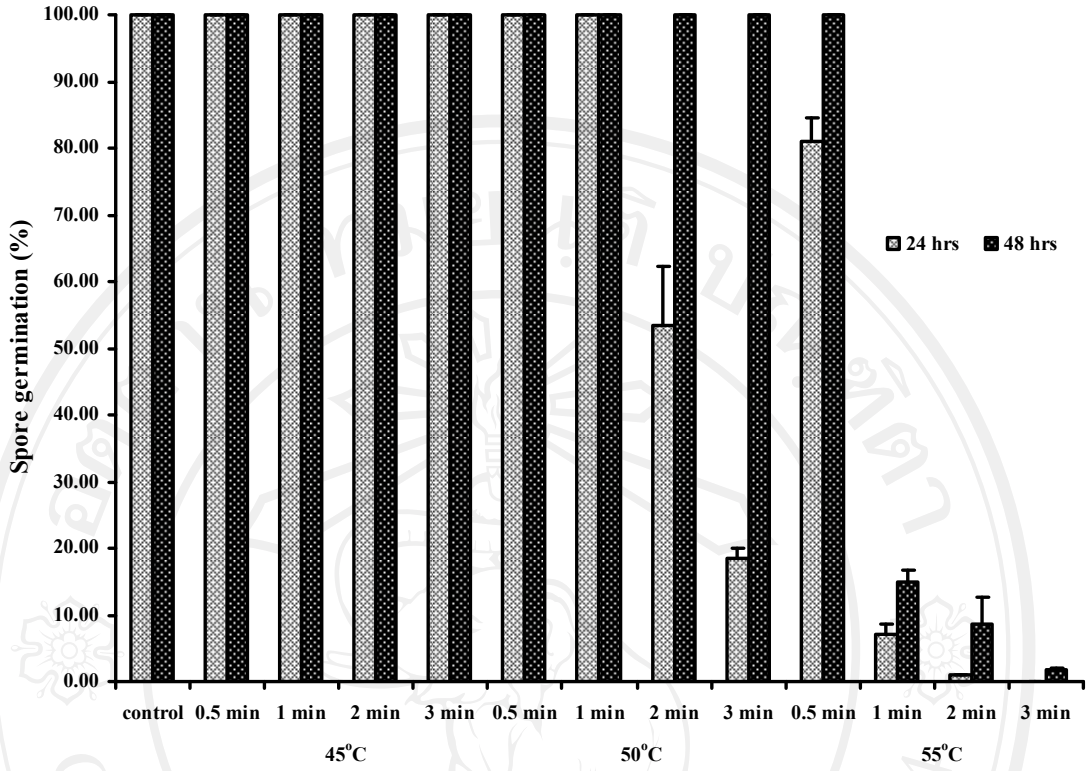

Figure 4.1 Effect of hot water treatments on spore germination of *Penicillium digitatum* incubated at 25±2°C in darkness for 24 and 48 hours at 45±2, 50±2 and 55±2°C for 0.5, 1, 2 and 3 minutes

At 55±2°C for 3 minutes (48 hours) showed best result (1.67% germination), for 2 and 1 minutes gave good inhibition (8.67%, 15% germination). While at 50±2°C for 3 minutes (24 hours) gave rather good effect (18.50% germination). At 45±2°C for 0.5, 1, 2 and 3 minutes and 50±2°C for 0.5 and 1 minutes (48 hours) showed no effect (100% germination).

Note: Vertical bars represent standard deviations, (n=3).

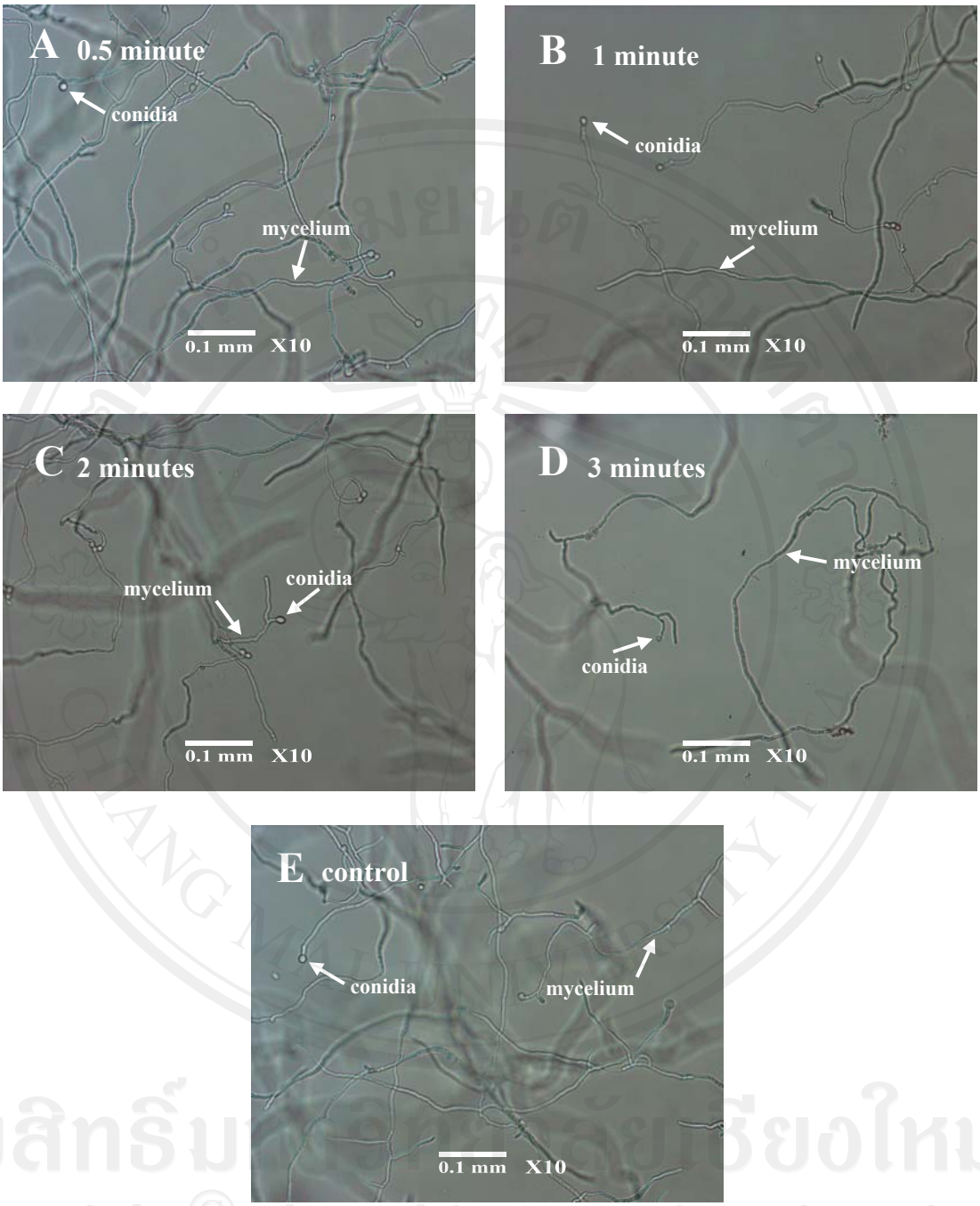

Figure 4.2 Hot water treatments on spore germination of *Penicillium digitatum* incubated at 25±2°C in darkness for 24 hours, at 45±2°C for 0.5, 1, 2 and 3 minutes (A-D) compared with control (E) showed no effect (100% germination)

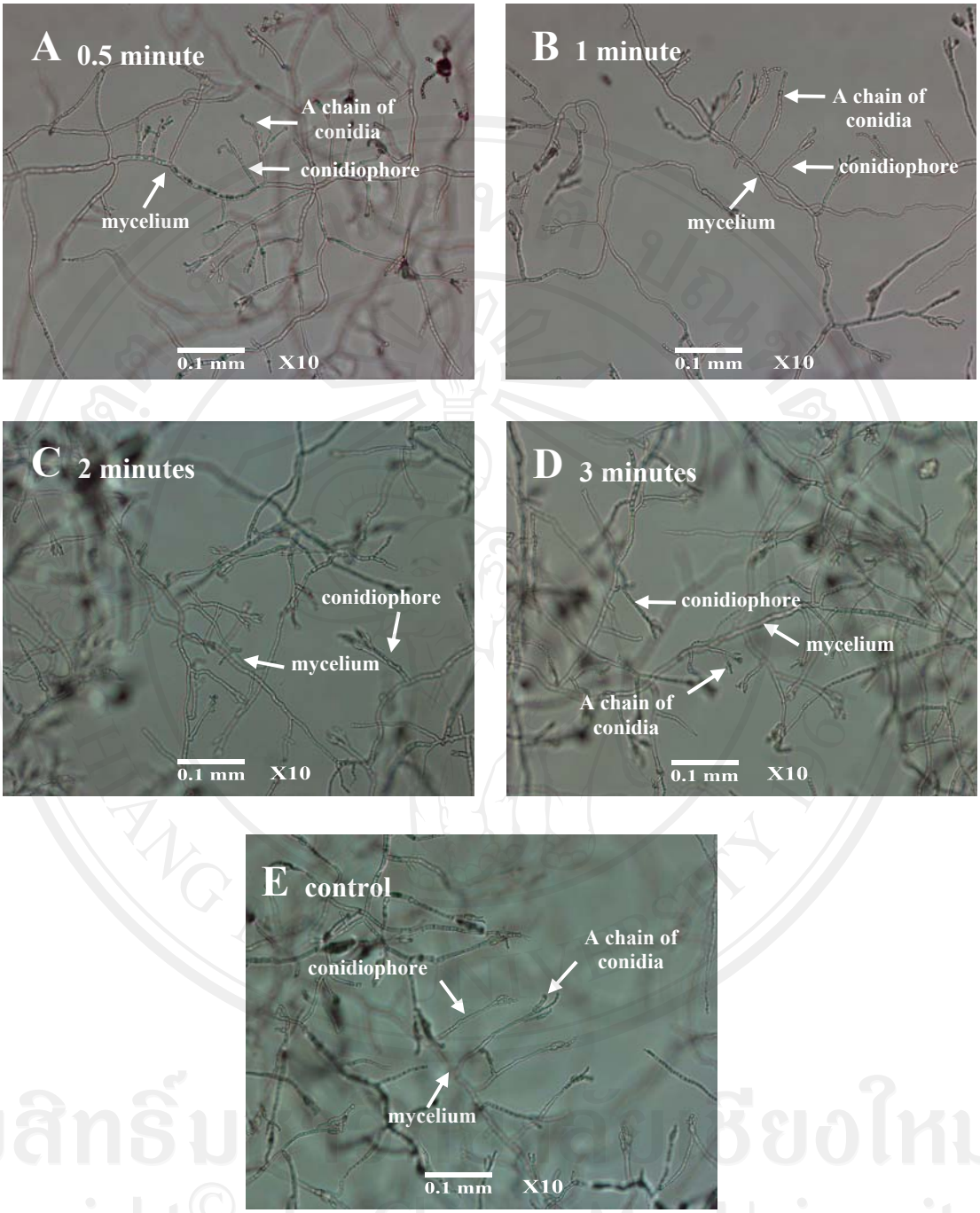

Figure 4.3 Hot water treatments on spore germination of *Penicillium digitatum* incubated at 25±2°C in darkness for 48 hours, at 45±2°C for 0.5, 1, 2 and 3 minutes (A-D) compared with control (E) showed no effect (100% germination)

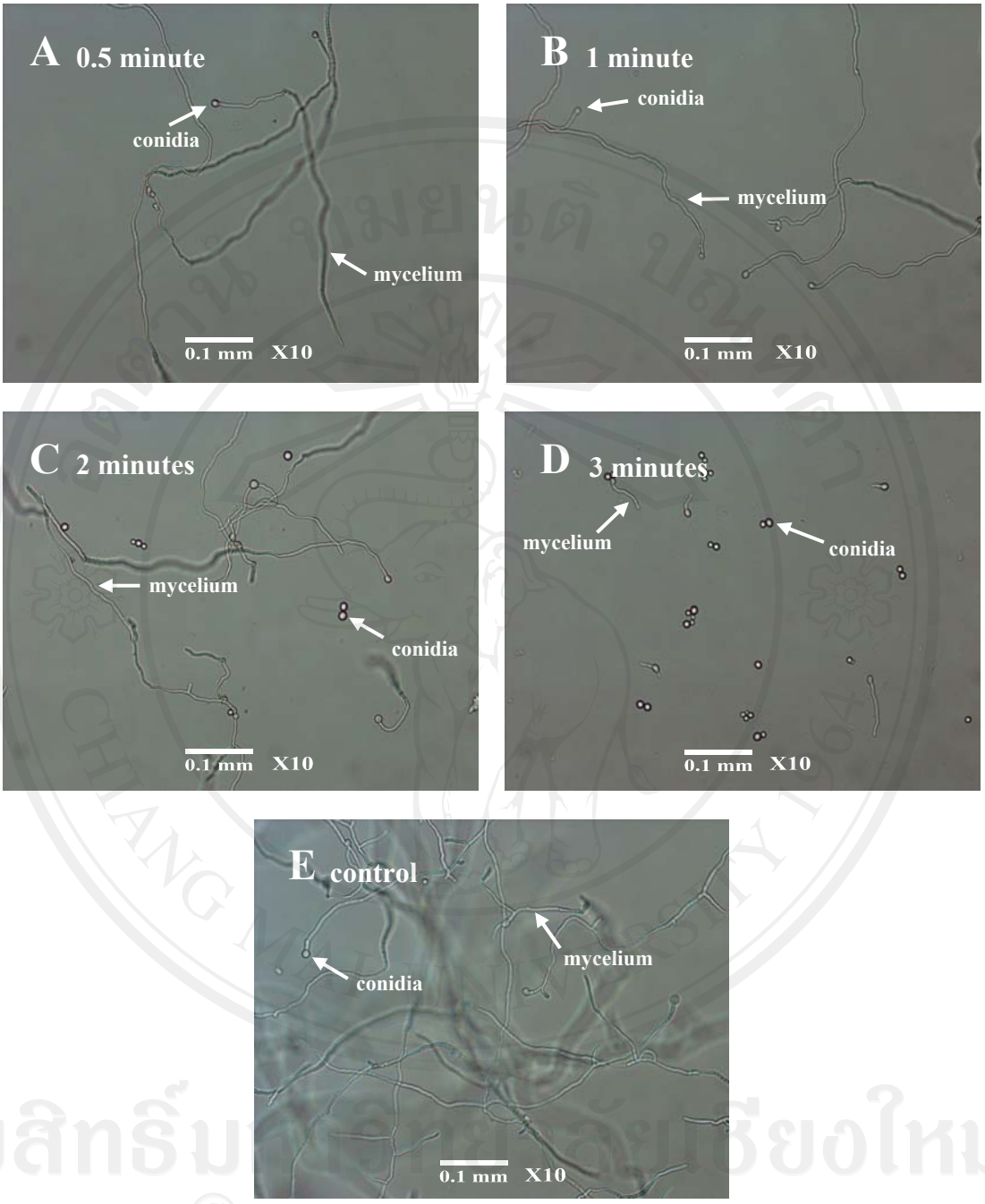

Figure 4.4 **Hot water treatments on spore germination of *Penicillium digitatum* incubated at 25±2°C in darkness for 24 hours, at 50±2°C for 0.5, 1, 2 and 3 minutes**

A = 100% germination: B = 100% germination: C = 53.33% germination: D = 18.50% germination: E = 100% germination

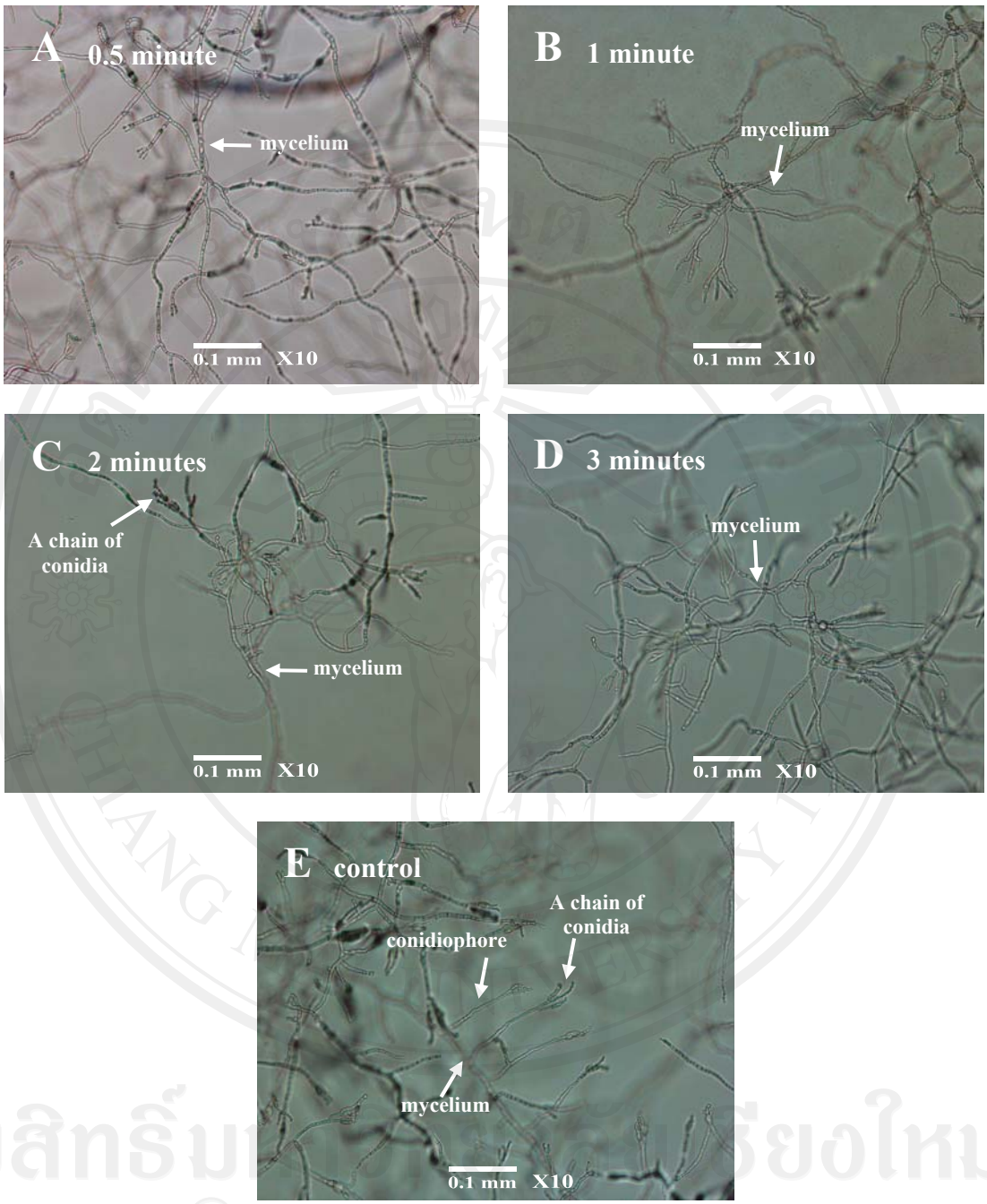

Figure 4.5 Hot water treatments on spore germination of *Penicillium digitatum* incubated at 25±2°C in darkness for 48 hours, at 50±2°C for 0.5, 1, 2 and 3 minutes (A-D) compared with control (E) showed no effect (100% germination)

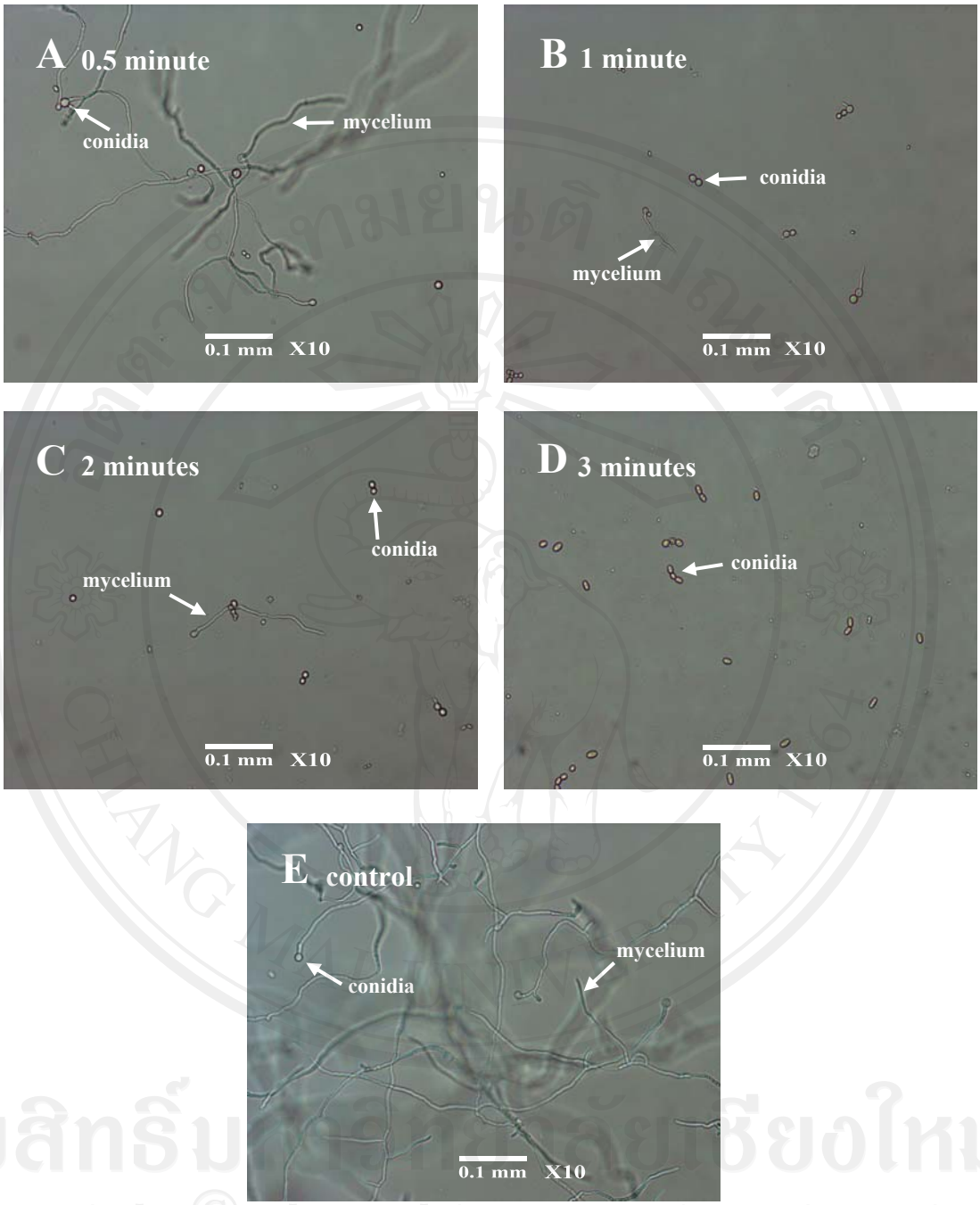

Figure 4.6 Hot water treatments on spore germination of *Penicillium digitatum* incubated at 25±2°C in darkness for 24 hours, at 55±2°C for 0.5, 1, 2 and 3 minutes

A = 81% germination: B = 7% germination: C = 1% germination: D = 0% germination: E = 100% germination

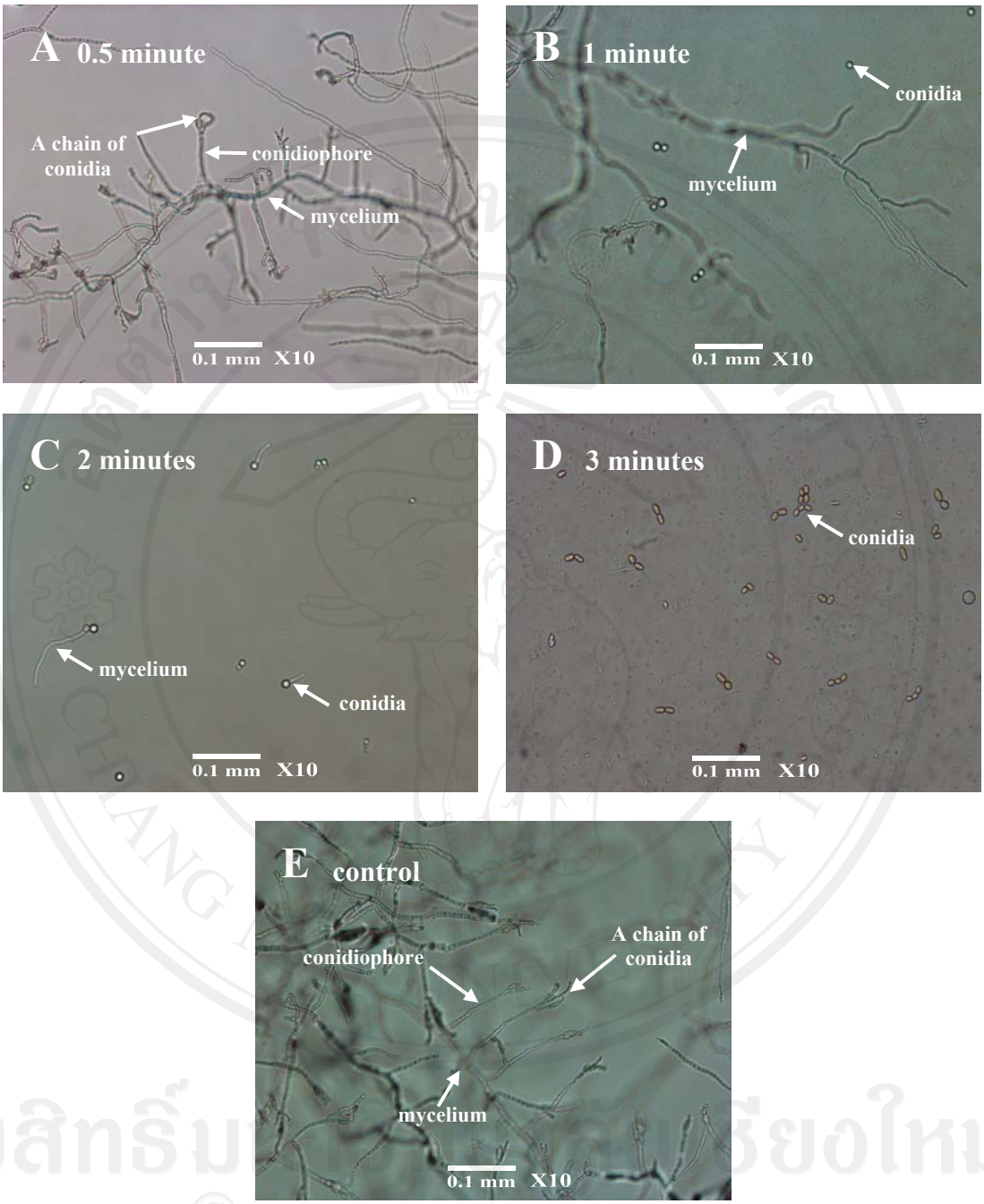

Figure 4.7 **Hot water treatments on spore germination of *Penicillium digitatum* incubated at 25±2°C in darkness for 48 hours, at 55±2°C for 0.5, 1, 2 and 3 minutes**

A = 100% germination: B = 15% germination: C = 8.67% germination: D = 1.67% germination: E = 100% germination

completely inhibited by exposure to 59 or 62°C for 10 or 15 seconds (Porat *et al*., 2000). Schirra *et al*. (2000) reported that heat treatments have a direct effect on fungal pathogens by slowing germ tube elongation or by inactivating or outright killing the germinating spores.

López-Cabrera and Marrero-Domínguez (1998) found that the development of *Colletrotrichum musae* and *Fusarium proliferatum* involved in banana crown rot disease were effectively decreased mycelial growth and conidial germination by HWT at 45 to 47.5°C for 15 to 30 minutes. Apparently delayed of spore germination seemed to be the principal mode of action of hot water dips. Possible mechanisms of pathogen control by heating include pectic enzyme inactivation or denaturation of other proteins, lipid liberation, destruction of hormones, and depletion of food reserves or metabolic injury, with or without accumulation of toxic intermediates. More than one of these mechanisms may act simultaneously (Barkai-Golan and Phillips, 1991). Margosan and Phillips (1990) also observed that ultrastructural changes in heat-treated non-germinating spores of *Monilinia fructicula*. Mitochondria and the vacuolar membranes were progressively destroyed, and gaps formed in the conidial cytoplasm. According to their data, the inner membrane of the mitochondria is probably the site that is most sensitive to heat damage in dormant conidia. In addition, Baker and Smith (1970) detected structural changes in the nuclei and in the cell walls of germinating heat-treated conidia of *Rhizopus stolonifer* and *Monilinia fructicola*. Moreover, the efficacy of heat for controlling the growth of *P. digitatum* is known to be influenced by various factors such as the moisture content of the spores, their metabolic activity, age of the the inoculum and inoculum concentration. In general, germinated or moist conidia are more sensitive to heat than dry spores (Barkai-Golan and Phillips, 1991) and mycelial growth (Fallik *et al*., 1993).

4.1.2 Effect of HWT on infection of *Penicillium digitatum* in tangerine fruit cv. Sai Num Pung

To evaluate the effects of HWT on eradication of established infections, 'Sai Num Pung' tangerine fruit were wound-inoculated with *P. digitatum* spore suspension. Dipping tangerine fruit in hot water at 45±2, 50±2 and 55±2°C for 0.5, 1, 2 and 3 minutes before and after inoculation compared with the control fruit which were inoculated with the pathogen another were inoculated with sterile water, both without hot-water treatment (untreated and uninoculated fruit). Acquired results indicated that with increasing of incubation period, green mold rot disease index, severity and sporulation of green mold disease increased in all treatments, except for uninoculated fruit (Figure 4.8-4.10). Fungus developed normal disease symptomatology which early symptoms of green mold rot appeared after 2 to 3 days of incubation in each treatments, except for hot water dips at 55±2°C for 2 and 3 minutes after inoculation appeared disease symptoms after 4 days of incubation were characterized by a soft peel and water soaked lesions of about 0.03 to 1 cm diameter. After 2 to 3 days, the disappearance of oil glands on the surface of the fruit (maceration zone) originated from the point of inoculation, resulted in blister rot. The disease progressed with the development of white mycelia on the surface of the macerated tissue (white mycelia zone) in 3 to 4 days, followed by the appearance of green spores (green spore zone), as time of incubation progressed. There was no infection of the uninoculated fruit by green mold.

The results showed that hot water dips at 50±2°C for 3 minutes and 55±2°C for 2 and 3 minutes after inoculation were more effective in reducing the development of green mold rot, and were significantly different from control untreated fruit. By the end of the 5 day incubation period, disease index on the control treatment untreated fruit reached 96.67% which was higher than the disease index of green molds on fruit treated with hot water dips at 50±2°C for 3 minutes and 55±2°C for 2 and 3 minutes after inoculation which were 41.67, 6.67 and 17.00%, respectively. Moreover, dipping at the temperature and time mentioned above after inoculation reduced disease severity from 9.68 cm diameter of control treatment untreated fruit to 2.61, 0.32 and 1.62 cm diameter of hot water dips at 50±2°C for 3 minutes and 55±2°C for 2 and 3

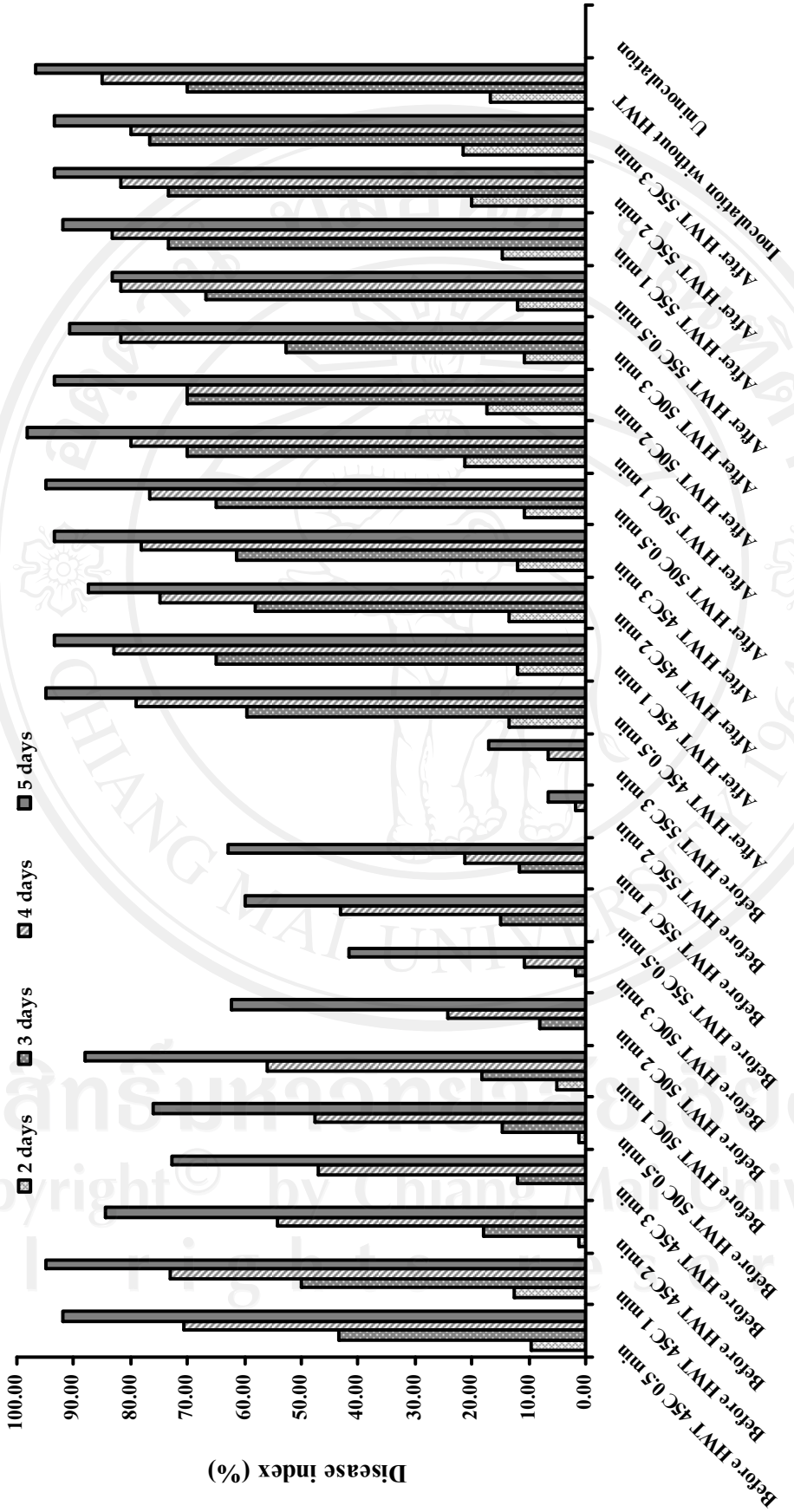

Figure 4.8 Disease index of green mold rot of artificially-inoculated tangerine fruit treated with hot water (HWT) before/after inoculation at 45±2, 50±2 and 55±2°C for 0.5, 1, 2 and 3 minutes during 5 days incubation at 24±2°C and 90±5% RH

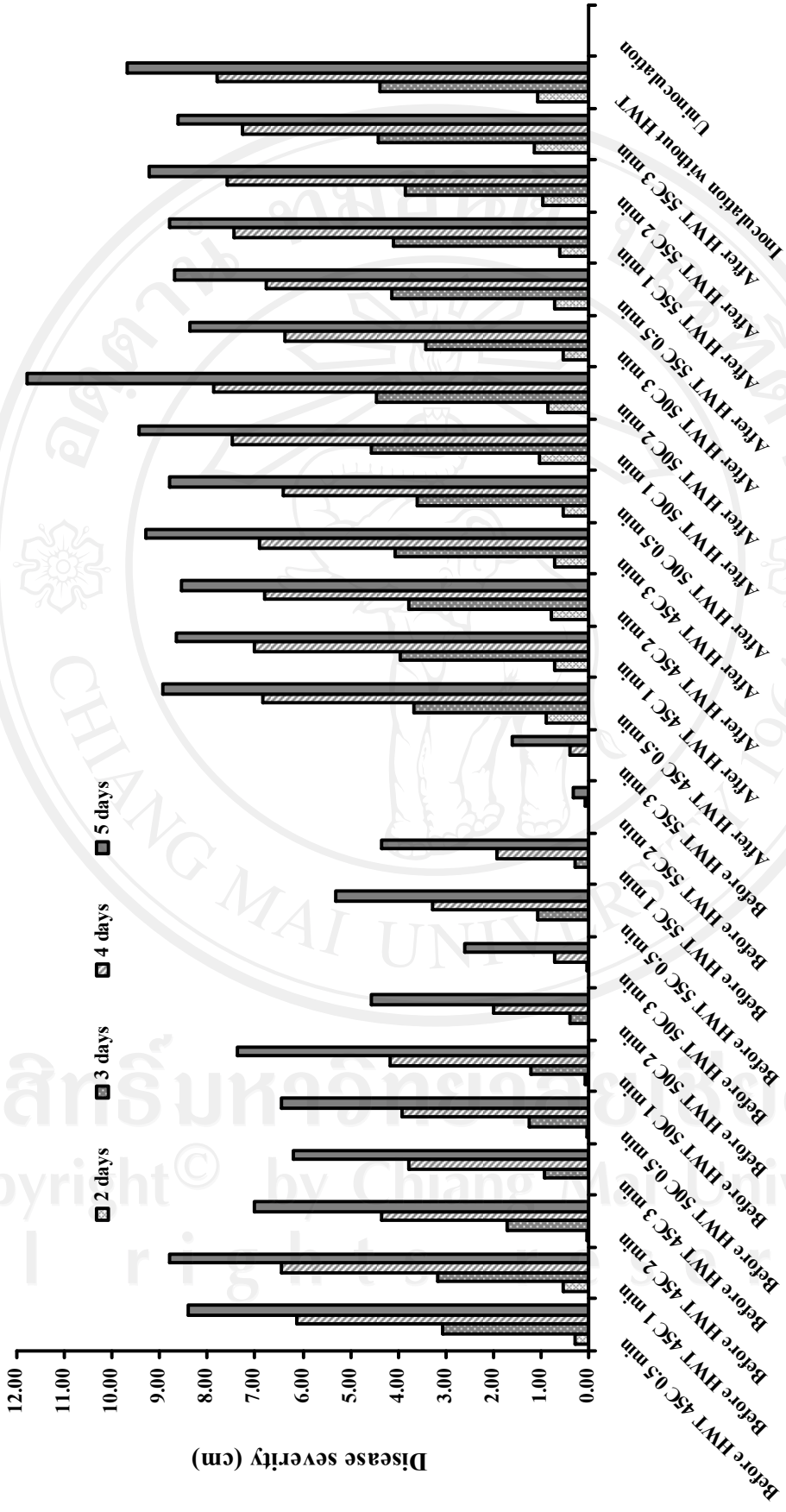

Figure 4.9 Disease severity of green mold rot of artificially-inoculated tangerine fruit treated with hot water (HWT) before/after inoculation at 45±2, 50±2 and 55±2°C for 0.5, 1, 2 and 3 minutes during 5 days incubation at 24±2°C and 90±5% RH

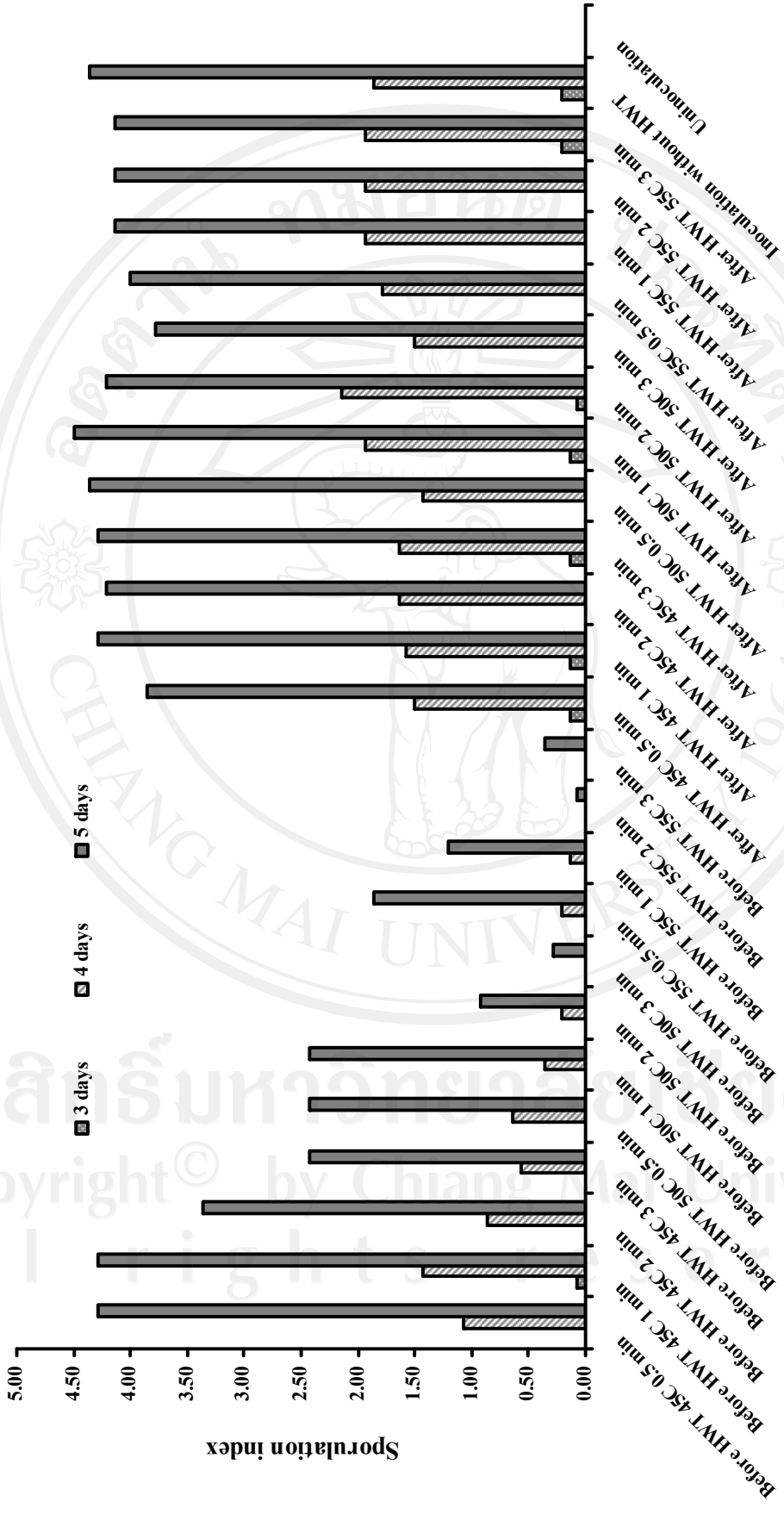

Figure 4.10 Sporulation index of green mold rot of artificially-inoculated tangerine fruit treated with hot water (HWT) before/after inoculation at 45±2, 50±2 and 55±2°C for 0.5, 1, 2 and 3 minutes during 5 days incubation at 24±2°C and 90±5% RH

minutes, respectively, and reduced sporulation index level from 4.36 of control treatment untreated fruit to 0.28, 0.07 and 0.36 of hot water dips at 50±2°C for 3 minutes and 55±2°C for 2 and 3 minutes, respectively, when incubated at 24±2°C and 90±5% RH (Table 4.1-4.3).

Postharvest heat treatments have been used for many years to control fungal disease in fruit and vegetable (Barkai-Golan and Phillips, 1991; Lurie, 1998; Schirra *et al*., 2000). During the last few years, heat treatments have attracted increasing interest as a result of growing demand to reduce the postharvest use of chemical fungicides. Hot water treatment has a number of advantages which include relative ease of use, short treatment time and the killing of skin-borne decay-causing agents (Lurie, 1998). Another important economic advantage of hot water immersion technology is cost of a typical commercial system is about 10% of that of a commercial vapor heat treatment system (Jordan, 1993). In the present study, we found that hot water dips at 50±2°C for 3 minutes and 55±2°C for 2 and 3 minutes after inoculation markedly reduced the development of green mold rot on 'Sai Num Pung' tangerine fruit (Table 4.1-4.3, Figure 4.8-4.13). This result was consistent with Schirra and D'hallewin (1997) found that pre-storage dipping of 'Fortune' mandarins in water at 50, 52 or 54°C for 3 minutes reduced decay both during cold storage at 6°C and simulated shelf-life at 20°C without causing adverse effect to the rind surface. However, temperatures of 56-58°C induced heat damage in form of rind browning, dull-coloration and resulted in enhanced decay development. Similarly, hot water dip at 52°C for 2 minutes reduced decay dramatically of 'Oroblanco' grapefruit (Ben-Yehoshua, 2003). Smoot and Melvin (1963) also reported that immersion of oranges in water at 53°C controlled green molds of artificially-inoculated oranges. In Houck's tests (Houck, 1967), the temperature required to control green mold on 'Eureka' lemons was 52°C.

The complex structure of a given host may greatly influence the rate of heat transfer. Also, heat transfer from tissue to tissue can vary greatly within the leaf, stem, root or fruit. Furthermore, heat transfer may differ among different tissues of the fruit itself. The colored outer layer (flavedo) of the citrus rind, which may have few and small intercellular spaces can transfer heat faster than the underlying white spongy

Table 4.1 Effect of hot water treatments (HWT) on green mold rot disease index of artificially-inoculated tangerine fruit during 5 days incubation at 24±2°C and 90±5% RH

Treatments	Disease index (%)* Day of incubation			
	2	3	4	5
Inoculation before HWT				
45°C 0.5 min	9.67± 8.96^{abc}	43.33± 5.77^{bc}	70.67±27.23^{abcd}	92.00±10.58^{a}
45°C 1 min	12.67± 3.05^{abc}	50.00±17.32^{ab}	73.00±15.72^{abcd}	95.00± 8.66^{a}
45°C 2 min	1.33± 2.31^{c}	18.00± 7.21^{cd}	54.33±23.16^{cde}	84.33± 4.04^{abc}
45°C 3 min	0.00± 0.00^{c}	12.00±10.58^{d}	47.00± 8.18^{def}	72.67±20.03^{abc}
50°C 0.5 min	1.33± 2.31^{c}	14.67±12.86^{d}	47.67±32.50^{def}	76.00±26.23^{abc}
50°C 1 min	5.00± 8.66^{bc}	18.33± 2.89^{cd}	56.00±21.16^{bcde}	88.00±10.58^{ab}
50°C 2 min	0.00± 0.00^{c}	8.00±10.58^{d}	24.33±17.78^{fgh}	62.33±19.40^{bcd}
50°C 3 min	0.00± 0.00^{c}	1.67± 2.89^{d}	10.67±10.07^{h}	41.67± 2.89^{d}
55°C 0.5 min	0.00± 0.00^{c}	15.00± 8.66^{d}	43.00±19.67^{efg}	60.00±34.64^{cd}
55°C 1 min	0.00± 0.00^{c}	11.67±10.41^{d}	21.33±20.13^{gh}	63.00±22.52^{bcd}
55°C 2 min	0.00± 0.00^{c}	0.00± 0.00^{d}	1.67± 2.88^{h}	6.67±11.55^{e}
55°C 3 min	0.00± 0.00^{c}	0.00± 0.00^{d}	6.67±11.55^{h}	17.00±17.52^{e}
Inoculation after HWT				
45°C 0.5 min	13.33±11.55^{abc}	59.67±22.81^{ab}	79.00± 6.56^{abc}	95.00± 8.66^{a}
45°C 1 min	12.00±10.58^{abc}	65.00±25.98^{ab}	83.00± 2.64^{ab}	93.33±11.55^{a}
45°C 2 min	13.33±11.55^{abc}	58.00±25.53^{ab}	75.00± 8.66^{abc}	87.33±15.53^{ab}
45°C 3 min	12.00±10.58^{abc}	61.33±20.13^{ab}	78.00± 7.21^{abc}	93.33±11.55^{a}
50°C 0.5 min	10.67±10.07^{abc}	65.00±25.98^{ab}	76.67±15.27^{abc}	95.00± 8.66^{a}
50°C 1 min	21.33± 2.31^{a}	70.00±17.32^{ab}	80.00± 0.00^{abc}	98.33± 2.89^{a}
50°C 2 min	17.33±16.16^{ab}	70.00±17.32^{ab}	70.00±17.32^{abcd}	93.33±11.55^{a}
50°C 3 min	10.67±10.07^{abc}	52.67±25.32^{ab}	81.67± 2.89^{abc}	90.67±10.07^{a}
55°C 0.5 min	12.00±10.58^{abc}	66.67±23.09^{ab}	81.67± 2.89^{abc}	83.33± 5.77^{abc}
55°C 1 min	14.67±12.86^{abc}	73.33±11.55^{ab}	83.33± 5.77^{ab}	92.00±10.58^{a}
55°C 2 min	20.00± 0.00^{a}	73.33±11.55^{ab}	81.67± 2.89^{abc}	93.33±11.55^{a}
55°C 3 min	21.67± 2.88^{a}	76.67± 5.77^{a}	80.00± 0.00^{abc}	93.33±11.55^{a}
Inoculation without HWT	16.67± 5.77^{ab}	70.00±17.32^{ab}	85.00± 8.66^{a}	96.67± 5.77^{a}
Uninoculation	0.00± 0.00^{c}	0.00± 0.00^{d}	0.00± 0.00^{h}	0.00± 0.00^{e}
C.V. (%)	86.17	38.32	24.87	18.87

*See materials and methods in 3.1.2.4.

$^{a-h}$Means within the same column followed by different letters are significantly different at 95% ($P \leq 0.05$) level by Duncan's Multiple Range Test. Data are mean values ±SD, (n=30).

Table 4.2 Effect of hot water treatments (HWT) on green mold rot disease severity (lesion diameter) of artificially-inoculated tangerine fruit during 5 days incubation at 24±2°C and 90±5% RH

Treatments	Disease severity (cm) Day of incubation			
	2	3	4	5
Inoculation before HWT				
45°C 0.5 min	0.29±0.54^{ef}	3.07±1.75^{e}	6.13±2.84^{d}	8.41±3.68^{bcde}
45°C 1 min	0.55±0.73^{de}	3.17±1.85^{de}	6.43±2.71^{bcd}	8.80±3.34^{bcde}
45°C 2 min	0.03±0.19^{f}	1.70±1.57^{f}	4.36±3.09^{e}	7.01±3.89^{cdef}
45°C 3 min	0.00±0.00^{f}	0.93±1.27^{fg}	3.76±3.08^{e}	6.21±4.34^{efg}
50°C 0.5 min	0.04±0.17^{f}	1.25±1.57^{f}	3.92±3.27^{e}	6.46±3.88^{defg}
50°C 1 min	0.08±0.23^{f}	1.20±1.30^{f}	4.17±2.51^{e}	7.36±3.41^{bcdef}
50°C 2 min	0.00±0.00^{f}	0.38±0.90^{gh}	1.99±2.30^{f}	4.55±3.13^{gh}
50°C 3 min	0.00±0.00^{f}	0.04±0.21^{h}	0.70±1.26^{g}	2.61±2.72^{hi}
55°C 0.5 min	0.00±0.00^{f}	1.06±1.22^{fg}	3.28±2.77^{e}	5.30±4.05^{fg}
55°C 1 min	0.00±0.00^{f}	0.28±0.83^{gh}	1.91±2.31^{f}	4.36±3.68^{gh}
55°C 2 min	0.00±0.00^{f}	0.00±0.00^{h}	0.07±0.38^{g}	0.32±1.19^{j}
55°C 3 min	0.00±0.00^{f}	0.00±0.00^{h}	0.38±1.17^{g}	1.62±3.50^{ij}
Inoculation after HWT				
45°C 0.5 min	0.88±0.99^{abcd}	3.66±2.12^{abcde}	6.82±2.48^{abcd}	8.95±2.85^{bcd}
45°C 1 min	0.71±0.83^{bcd}	3.97±1.53^{abcd}	7.00±1.96^{abcd}	8.66±2.24^{bcde}
45°C 2 min	0.78±0.92^{abcd}	3.78±2.04^{abcde}	6.81±2.41^{abcd}	8.56±2.69^{bcde}
45°C 3 min	0.70±0.83^{bcd}	4.07±1.69^{abc}	6.90±2.47^{abcd}	9.28±2.51^{bc}
50°C 0.5 min	0.52±0.74^{de}	3.58±1.59^{bcde}	6.41±2.21^{cd}	8.78±2.18^{bcde}
50°C 1 min	1.03±0.90^{ab}	4.56±1.37^{a}	7.48±1.94^{abcd}	9.45±3.58^{bc}
50°C 2 min	0.85±0.99^{abcd}	4.44±1.79^{ab}	7.88±1.46^{a}	11.78±4.52^{a}
50°C 3 min	0.53±0.80^{de}	3.43±2.05^{cde}	6.36±2.74^{cd}	8.36±3.98^{bcde}
55°C 0.5 min	0.72±0.82^{bcd}	4.13±1.28^{abc}	6.78±1.76^{abcd}	8.70±2.88^{bcde}
55°C 1 min	0.62±0.76^{cde}	4.11±1.24^{abc}	7.43±1.17^{abcd}	8.81±1.96^{bcde}
55°C 2 min	0.96±0.83^{abc}	3.86±1.95^{abcde}	7.57±1.90^{abc}	9.22±2.31^{bc}
55°C 3 min	1.13±0.83^{a}	4.43±1.28^{ab}	7.25±1.78^{abcd}	8.62±1.89^{bcde}
Inoculation without HWT	1.07±0.88^{ab}	4.37±1.33^{ab}	7.81±1.27^{ab}	9.68±1.26^{ab}
Uninoculation	0.00±0.00^{f}	0.00±0.00^{h}	0.00±0.00^{g}	0.00±0.00^{j}
C.V. (%)	43.68	57.15	44.16	58.79

$^{a-j}$Means within the same column followed by different letters are significantly different at 95% ($P \leq 0.05$) level by Duncan's Multiple Range Test. Data are mean values ±SD**,** (n=30).

Table 4.3 Effect of hot water treatments (HWT) on sporulation of green mold rot disease of artificially-inoculated tangerine fruit during 5 days incubation at 24±2°C and 90±5% RH

Treatments	Sporulation index* Day of incubation		
	3	4	5
Inoculation before HWT			
45°C 0.5 min	0.00±0.00^{b}	1.07±0.73^{cd}	4.28±0.47^{a}
45°C 1 min	0.07±0.27^{ab}	1.43±0.65^{bc}	4.28±0.47^{a}
45°C 2 min	0.00±0.00^{b}	0.86±0.66^{de}	3.36±1.08^{b}
45°C 3 min	0.00±0.00^{b}	0.57±0.75^{efg}	2.43±1.34^{c}
50°C 0.5 min	0.00±0.00^{b}	0.64±0.74^{def}	2.43±1.60^{c}
50°C 1 min	0.00±0.00^{b}	0.36±0.50^{fgh}	2.43±0.85^{c}
50°C 2 min	0.00±0.00^{b}	0.21±0.42^{fgh}	0.93±0.83^{de}
50°C 3 min	0.00±0.00^{b}	0.00±0.00^{h}	0.28±0.61^{f}
55°C 0.5 min	0.00±0.00^{b}	0.21±0.42^{fgh}	1.86±1.17^{c}
55°C 1 min	0.00±0.00^{b}	0.14±0.36^{gh}	1.21±1.19^{d}
55°C 2 min	0.00±0.00^{b}	0.00±0.00^{h}	0.07±0.27^{f}
55°C 3 min	0.00±0.00^{b}	0.00±0.00^{h}	0.36±0.63^{ef}
Inoculation after HWT			
45°C 0.5 min	0.14±0.36^{ab}	1.50±0.85^{bc}	3.86±0.86^{ab}
45°C 1 min	0.14±0.36^{ab}	1.57±0.94^{b}	4.28±0.82^{a}
45°C 2 min	0.00±0.00^{b}	1.64±0.74^{ab}	4.21±1.05^{a}
45°C 3 min	0.14±0.36^{ab}	1.64±1.08^{ab}	4.28±0.82^{a}
50°C 0.5 min	0.00±0.00^{b}	1.43±0.75^{bc}	4.36±0.84^{a}
50°C 1 min	0.14±0.36^{ab}	1.93±0.47^{ab}	4.50±0.52^{a}
50°C 2 min	0.07±0.27^{ab}	2.14±0.53^{a}	4.21±0.42^{a}
50°C 3 min	0.00±0.00^{b}	1.50±0.76^{bc}	3.78±0.80^{ab}
55°C 0.5 min	0.00±0.00^{b}	1.78±0.42^{ab}	4.00±0.68^{ab}
55°C 1 min	0.00±0.00^{b}	1.93±0.47^{ab}	4.14±0.36^{a}
55°C 2 min	0.00±0.00^{b}	1.93±0.27^{ab}	4.14±0.36^{a}
55°C 3 min	0.21±0.42^{a}	1.93±0.27^{ab}	4.14±0.36^{a}
Inoculation without HWT	0.21±0.42^{a}	1.86±0.53^{ab}	4.36±0.50^{a}
Uninoculation	0.00±0.00^{b}	0.00±0.00^{h}	0.00±0.00^{f}
C.V. (%)	45.28	54.34	26.99

*See materials and methods in 3.1.2.4.

$^{a-h}$Means within the same column followed by different letters are significantly different at 95% ($P \leq 0.05$) level by Duncan's Multiple Range Test. Data are mean values ±SD, (n=30).

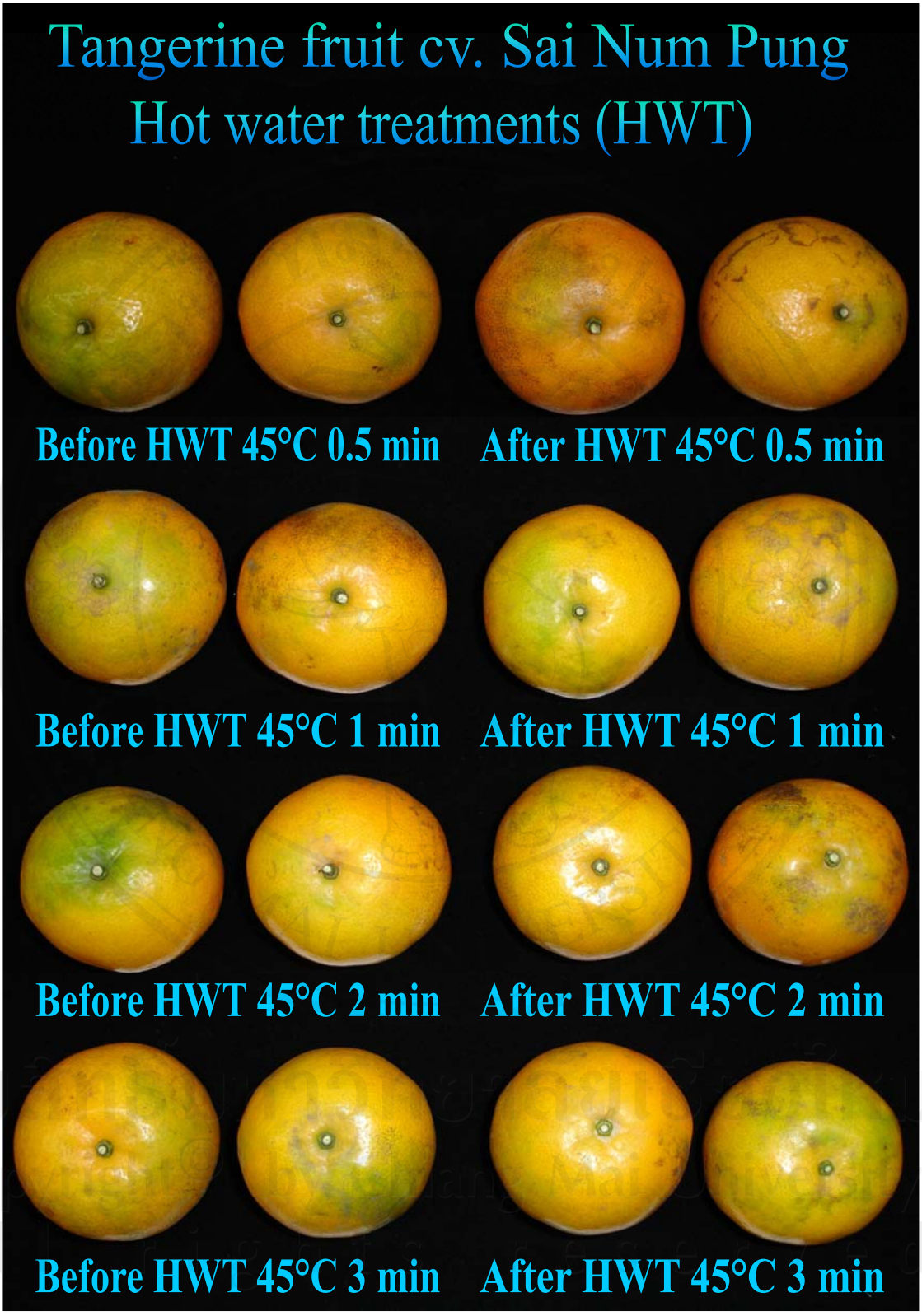

Figure 4.11A Tangerine fruit after inoculated 0 day with *Penicillium digitatum* and treated with hot water (HWT) at 45±2°C for 0.5, 1, 2 and 3 minutes

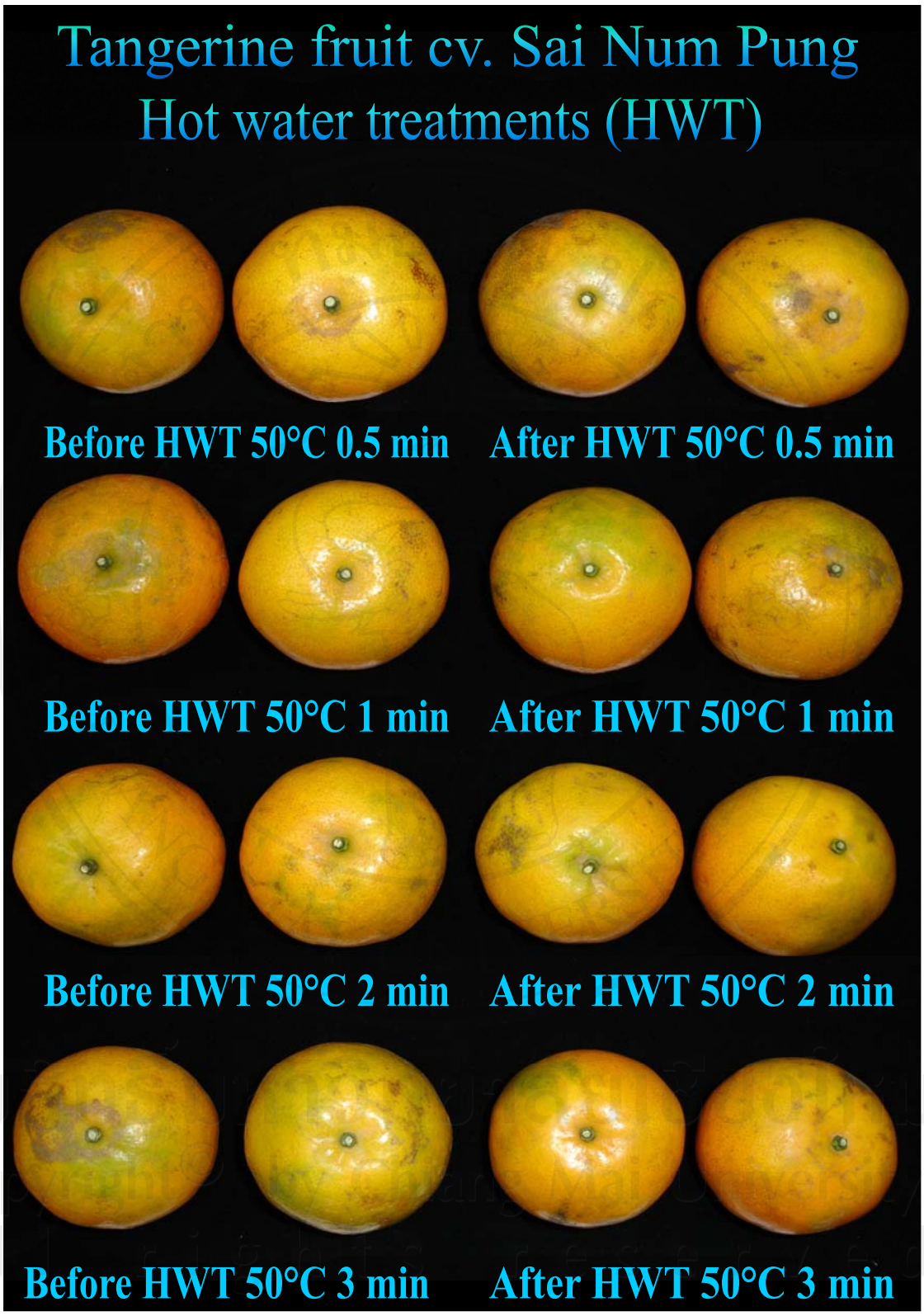

Figure 4.11B **Tangerine fruit after inoculated 0 day with *Penicillium digitatum* and treated with hot water (HWT) at 50±2°C for 0.5, 1, 2 and 3 minutes**

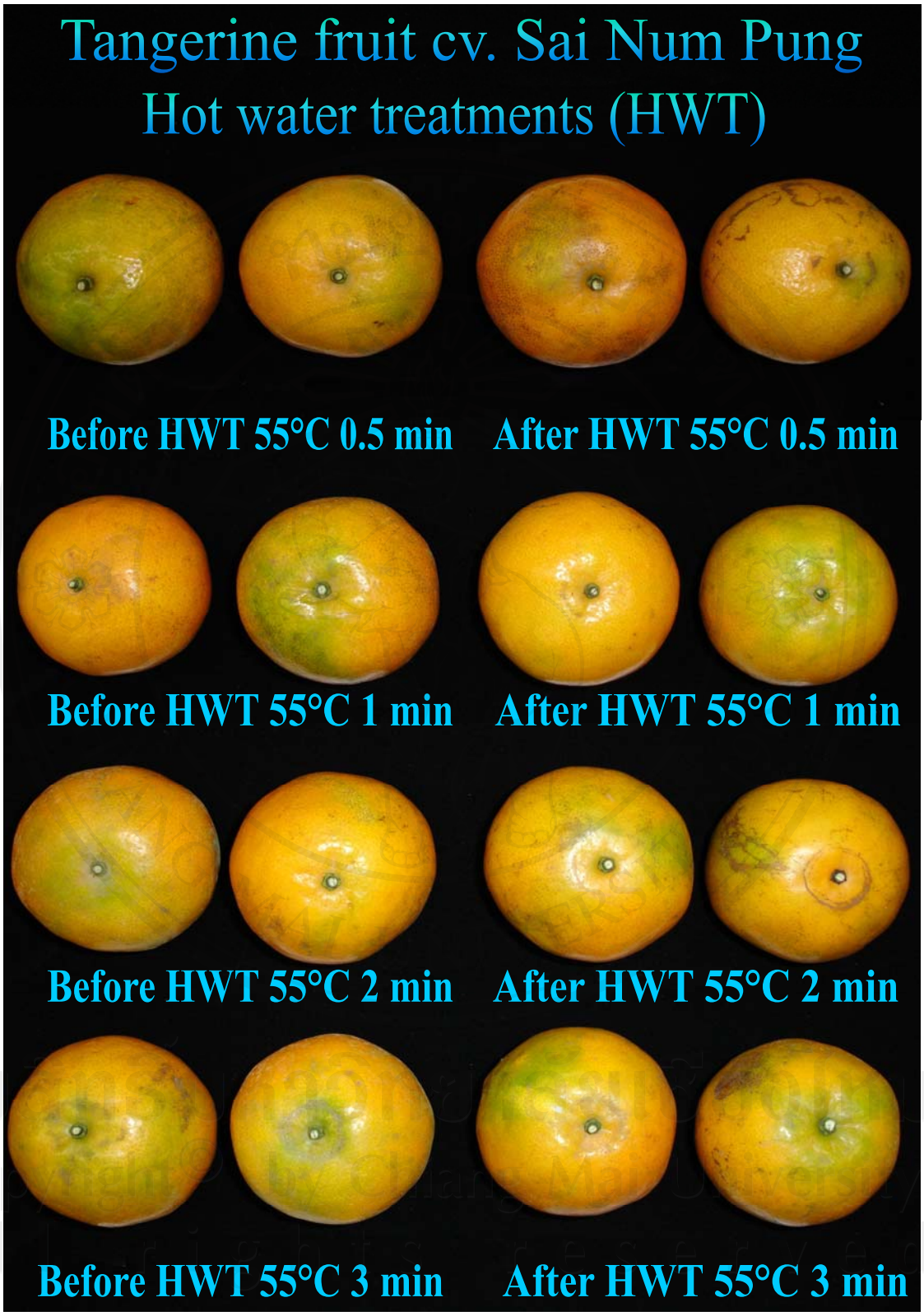

Figure 4.11C Tangerine fruit after inoculated 0 day with *Penicillium digitatum* and treated with hot water (HWT) at 55±2°C for 0.5, 1, 2 and 3 minutes

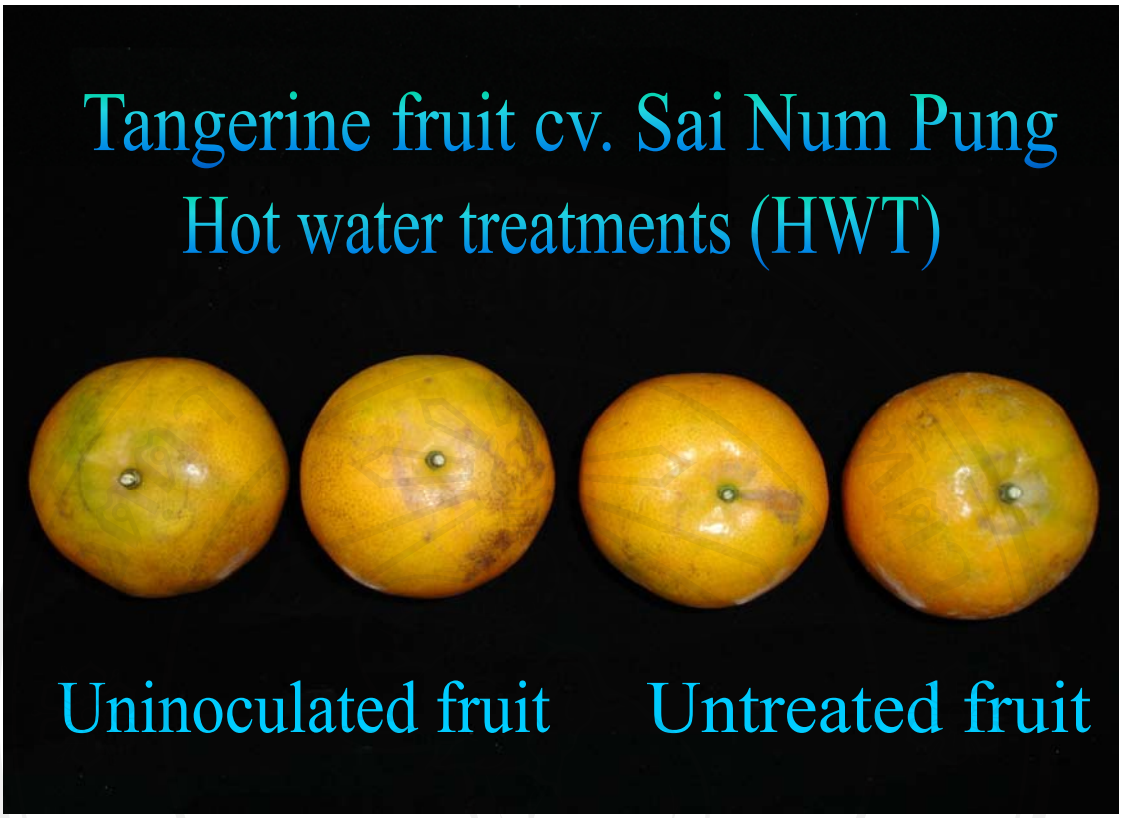

Figure 4.11D Two sets of control uninoculated and untreated fruit 0 day

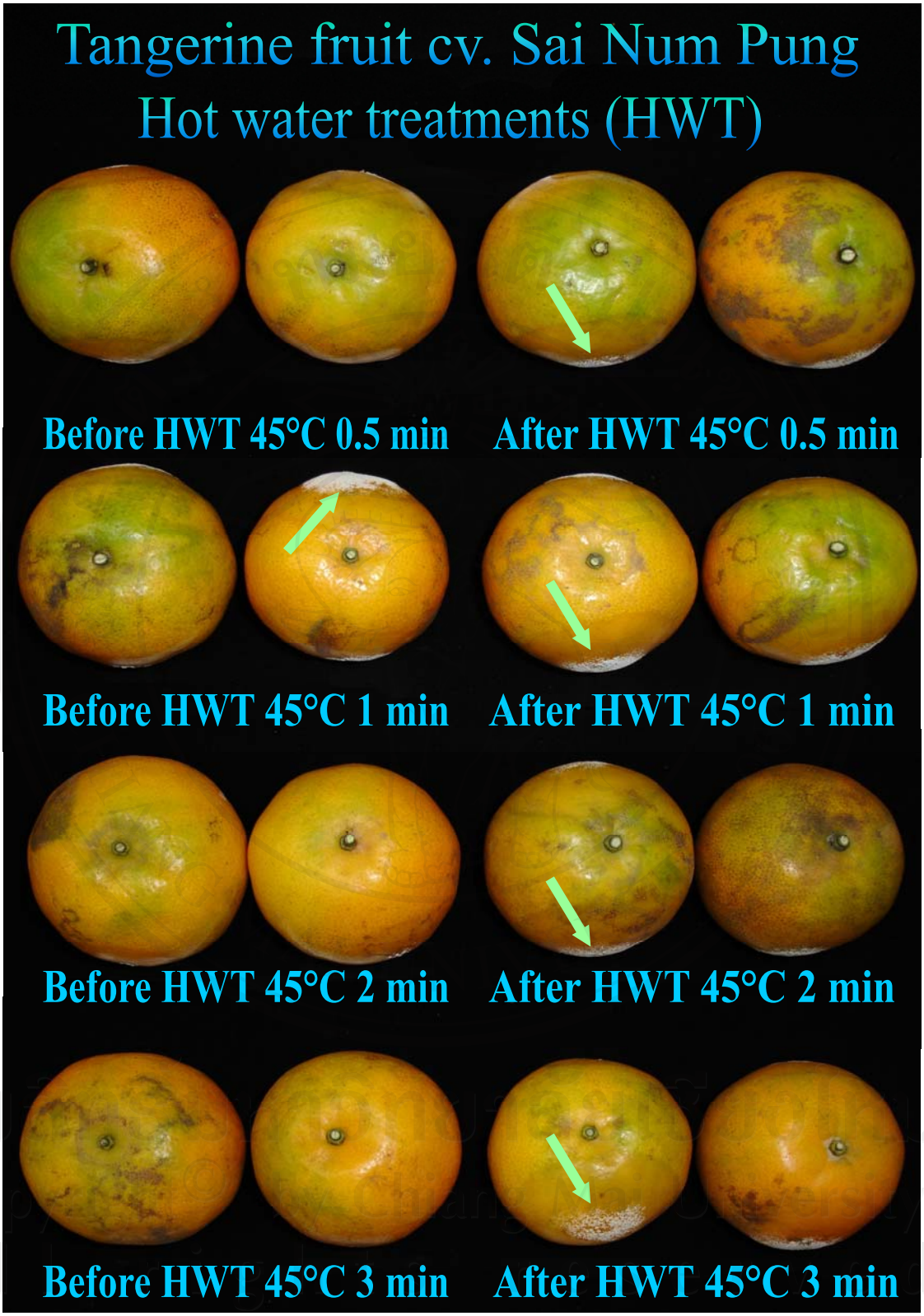

Figure 4.12A **Tangerine fruit 3 days incubation at 24±2°C and 90±5% RH after inoculated with *Penicillium digitatum* and treated with hot water (HWT) at 45±2°C for 0.5, 1, 2 and 3 minutes; arrows indicate where the symptoms begin to show**

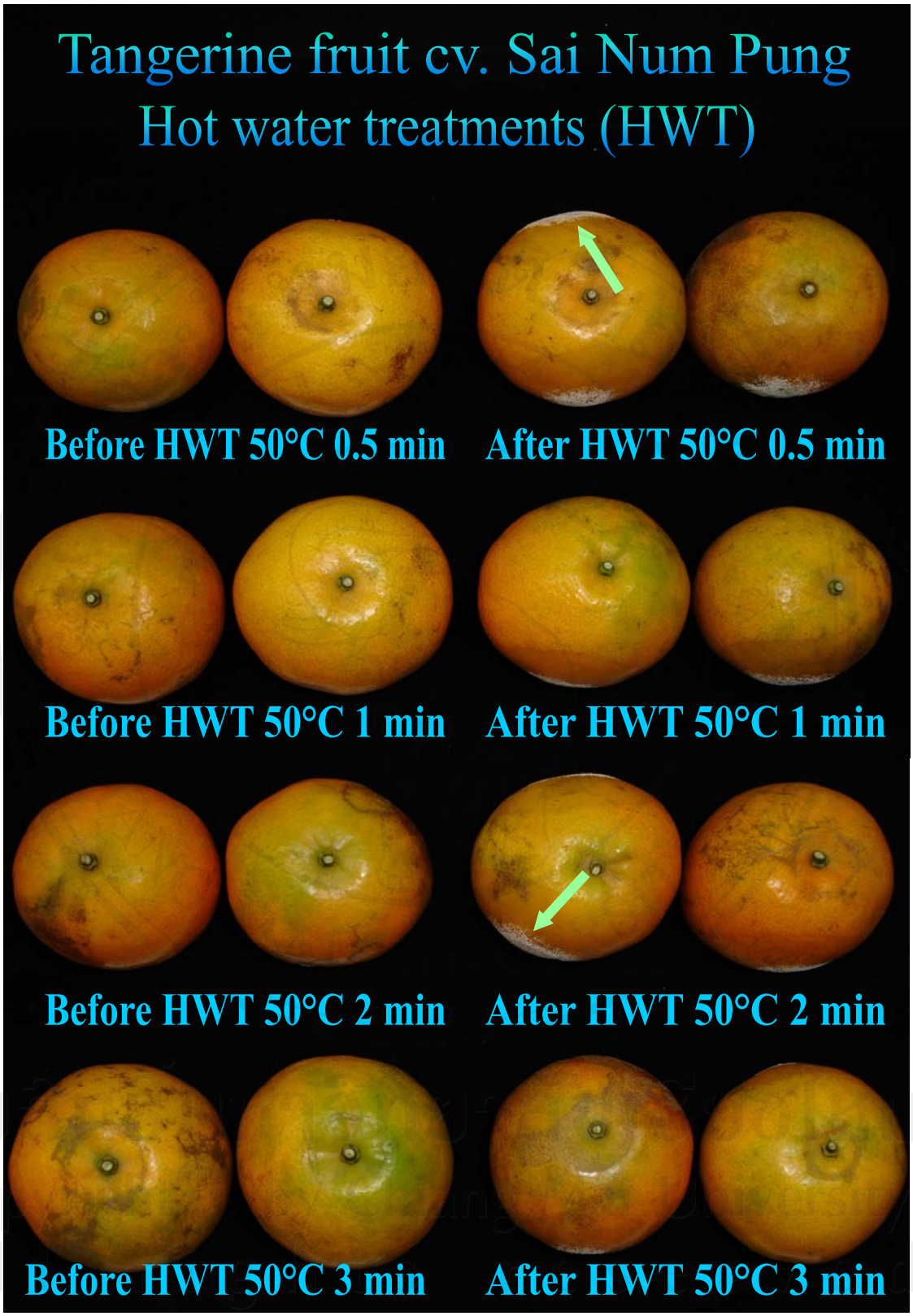

Figure 4.12B **Tangerine fruit 3 days incubation at 24±2°C and 90±5% RH after inoculated with *Penicillium digitatum* and treated with hot water (HWT) at 50±2°C for 0.5, 1, 2 and 3 minutes; arrows indicate where the symptoms begin to show**

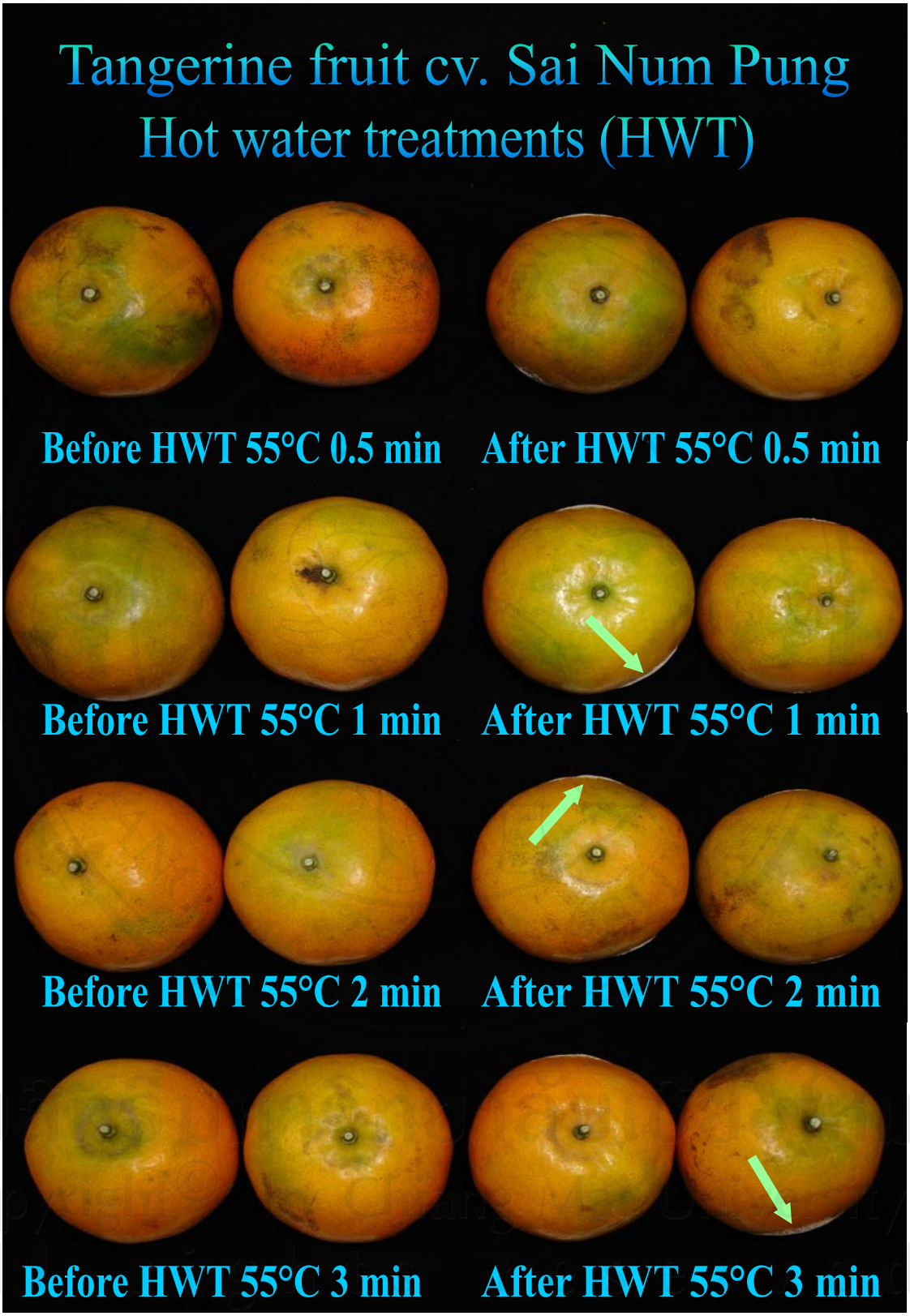

Figure 4.12C **Tangerine fruit 3 days incubation at 24±2°C and 90±5% RH after inoculated with *Penicillium digitatum* and treated with hot water (HWT) at 55±2°C for 0.5, 1, 2 and 3 minutes; arrows indicate where the symptoms begin to show**

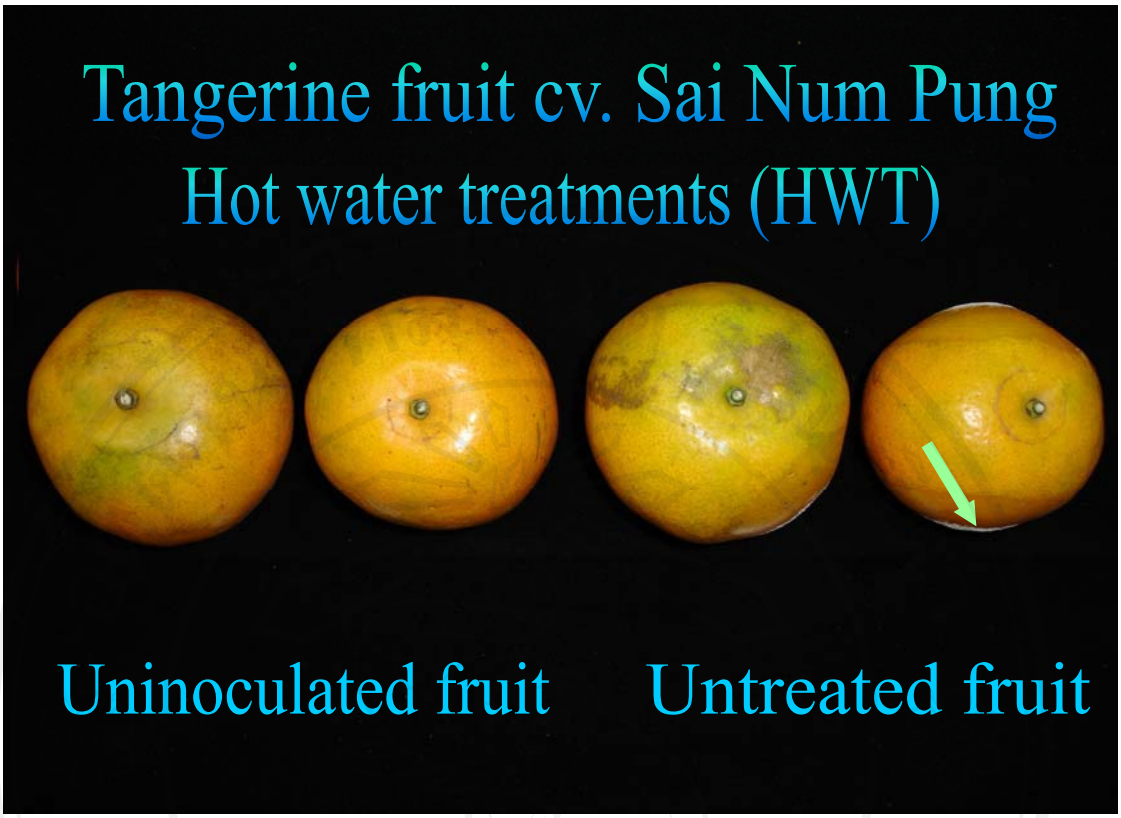

Figure 4.12D Two sets of control uninoculated fruit showed no symptom while inoculated and untreated fruit showed symptoms (arrow) 3 days incubation at 24±2°C and 90±5% RH

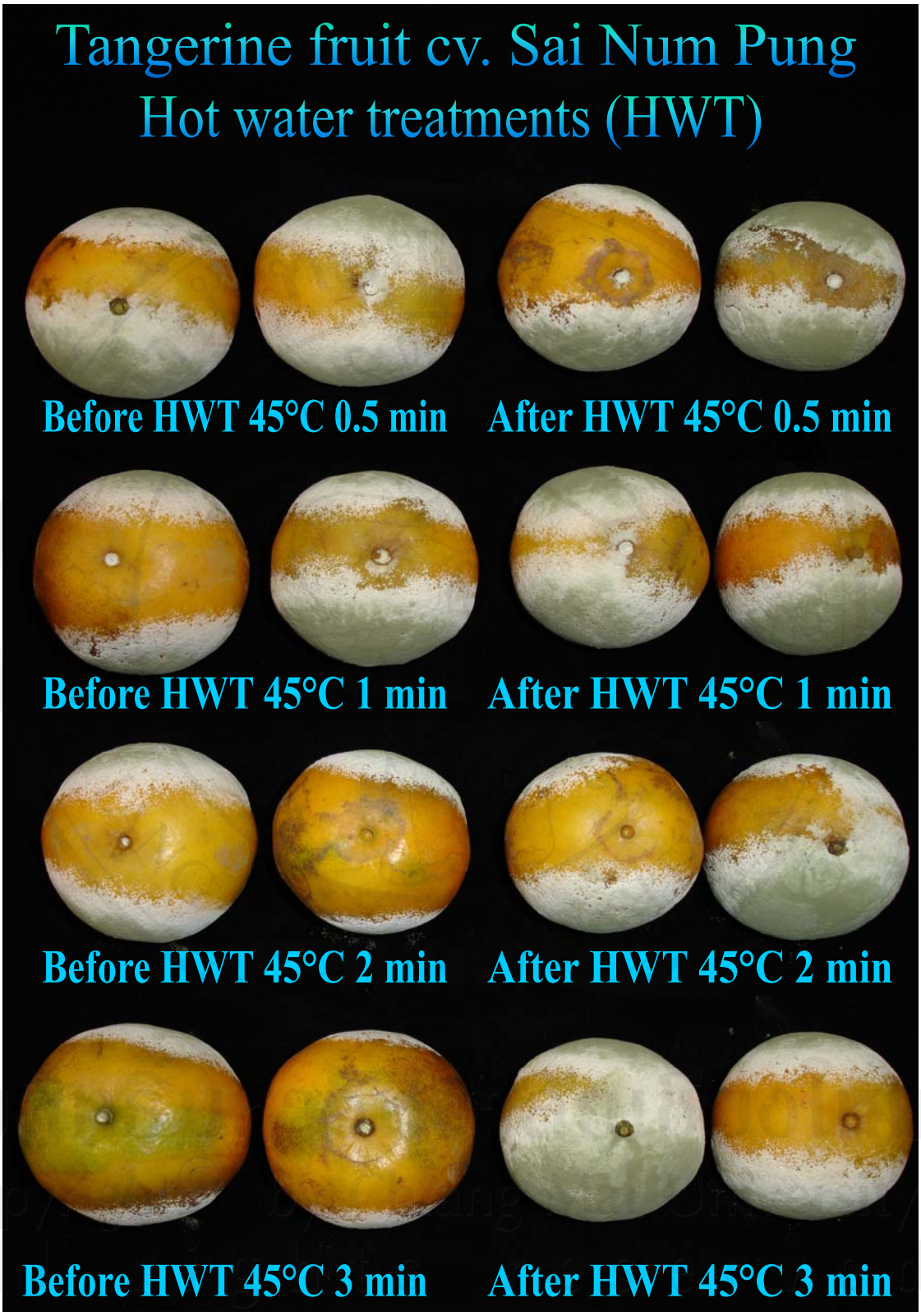

Figure 4.13A **Tangerine fruit 5 days incubation at 24±2°C and 90±5% RH after inoculated with *Penicillium digitatum* and treated with hot water (HWT) at 45±2°C for 0.5, 1, 2 and 3 minutes; all treatments showed symptoms**

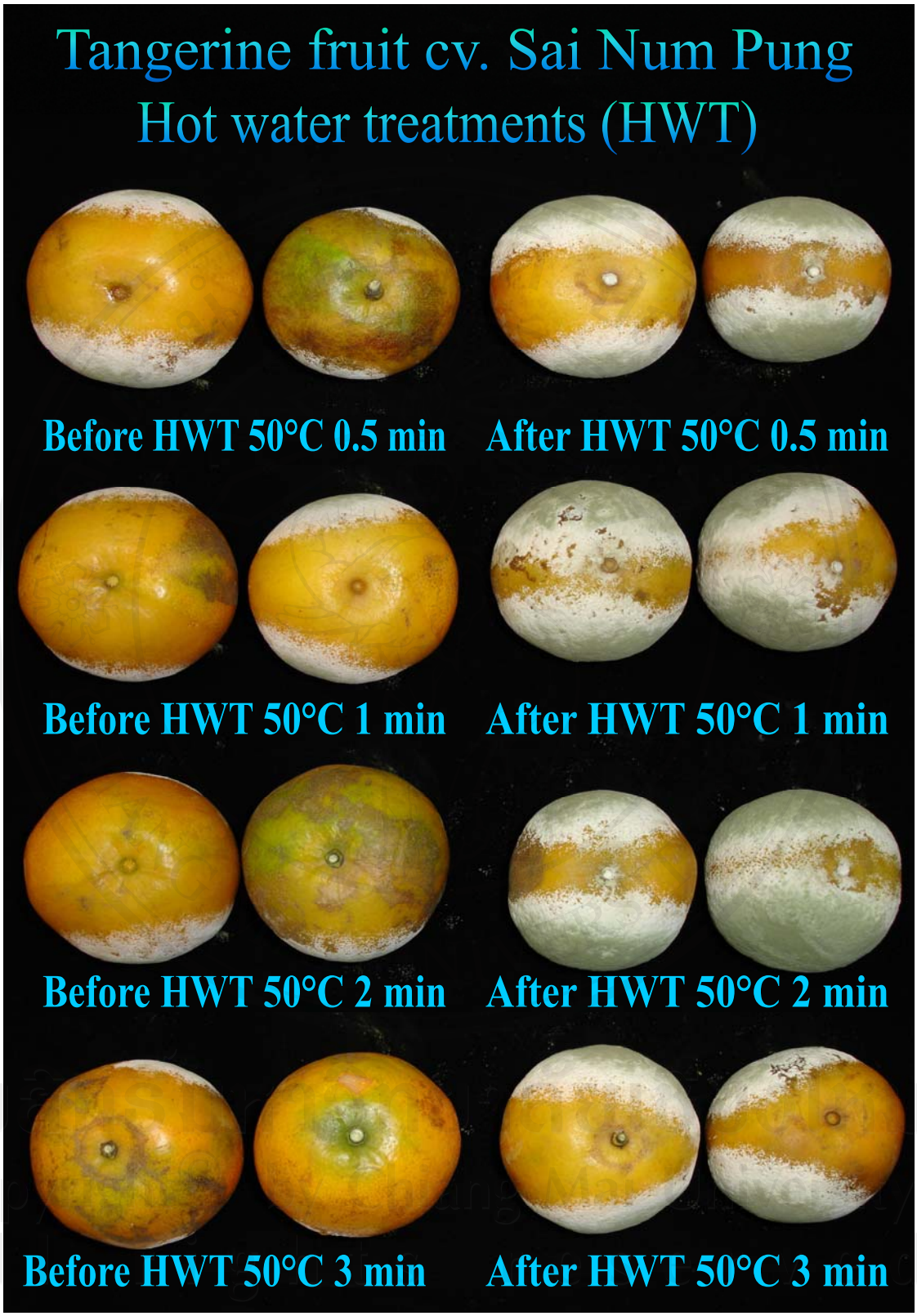

Figure 4.13B **Tangerine fruit 5 days incubation at 24±2°C and 90±5% RH after inoculated with *Penicillium digitatum* and treated with hot water (HWT) at 50±2°C for 0.5, 1, 2 and 3 minutes; inoculation before HWT at 50±2°C for 3 minutes showed best result**

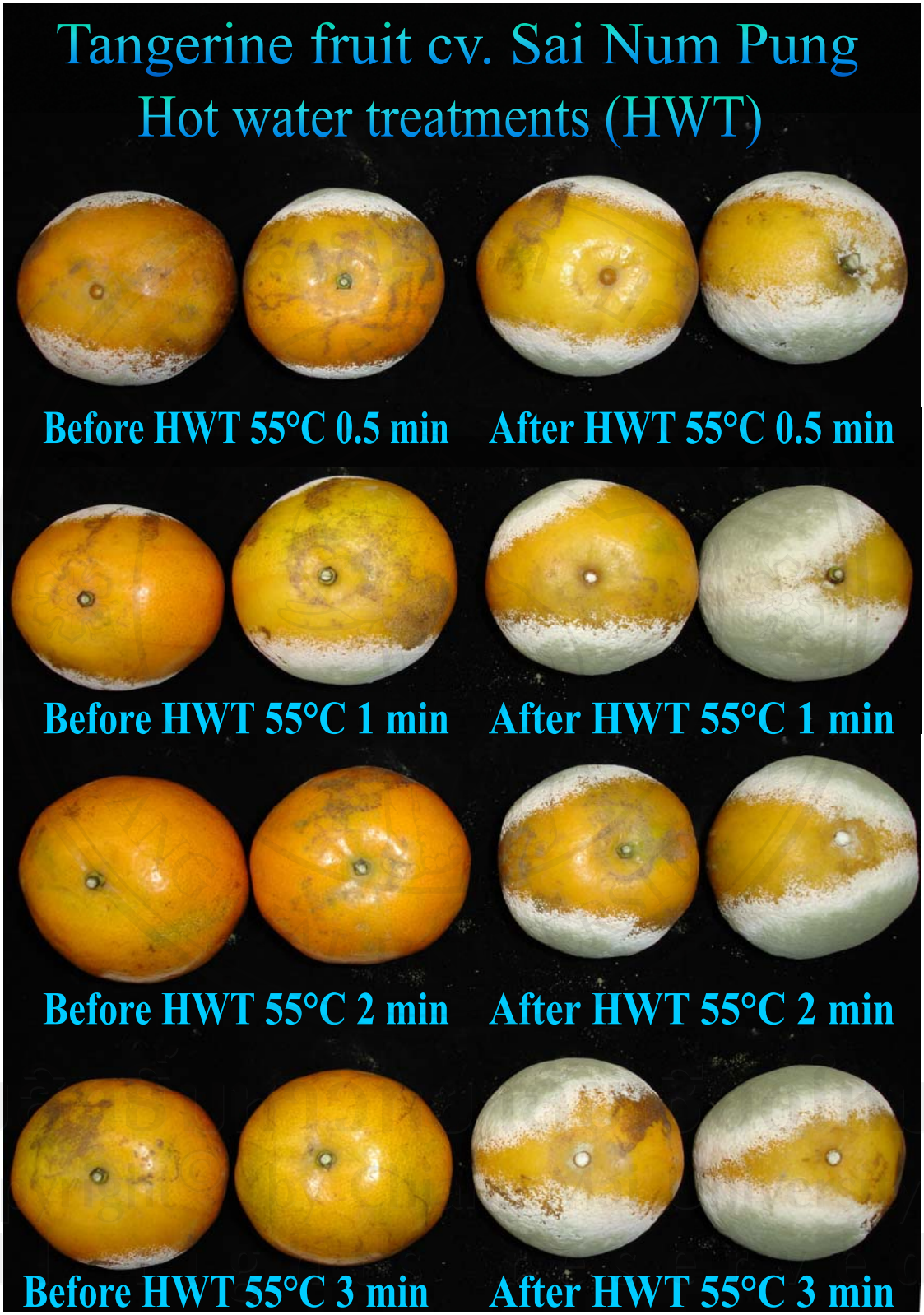

Figure 4.13C **Tangerine fruit 5 days incubation at 24±2°C and 90±5% RH after inoculated with *Penicillium digitatum* and treated with hot water (HWT) at 55±2°C for 0.5, 1, 2 and 3 minutes; inoculation before HWT at 55±2°C for 2 and 3 minutes showed best result**

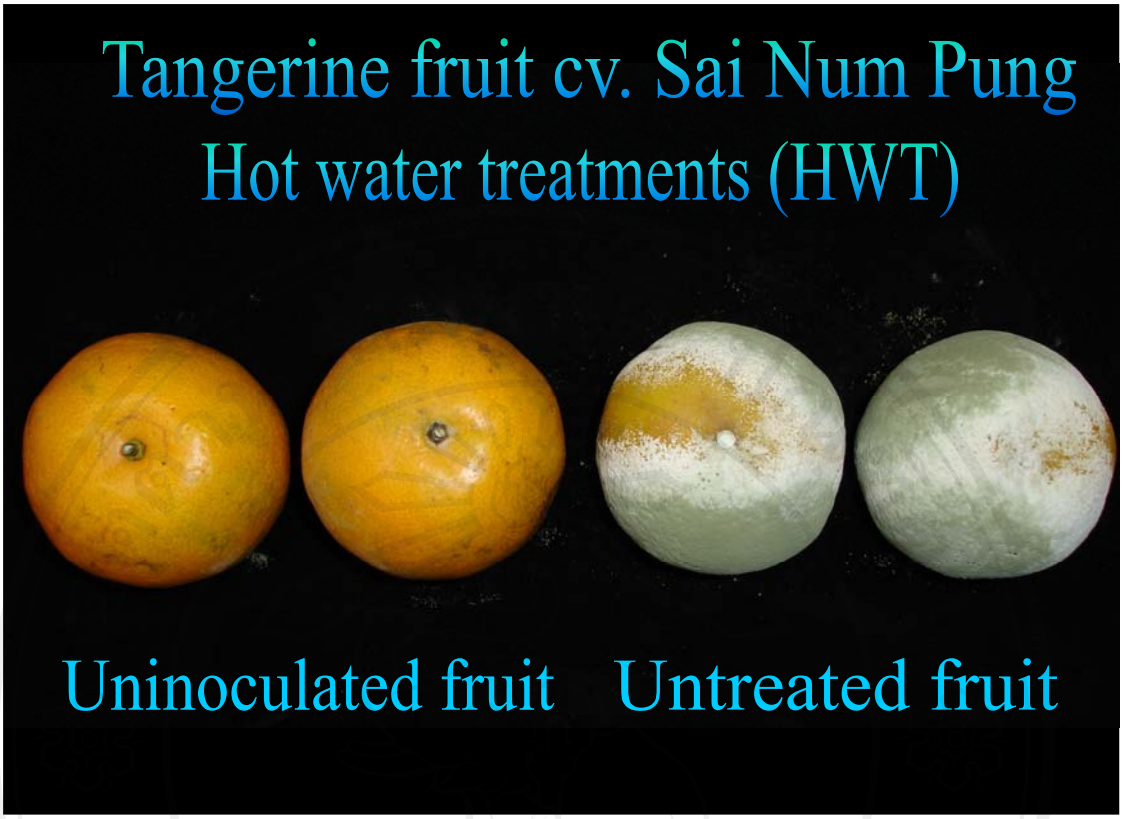

Figure 4.13D Two sets of control uninoculated fruit showed no symptom while inoculated and untreated fruit showed serious symptoms 5 days incubation at 24±2°C and 90±5% RH

tissue (albedo) (Barkai-Golan and Phillips, 1991). Since heat treatments may act directly on the spore population infesting the host surface, a short exposure to heat may sometimes be sufficient to reduce decay incidence markedly. However, heat treatment is also based on the gradual penetration of heat into the host tissues thus the extent of pathogen progress within the tissues may determine the success or failure of the treatment (Barkai-Golan, 2001). The above results obtained by *in vitro* experiment could explain the direct effect of heat treatment *in vivo*. A certain threshold of inoculum level is needed to initiate decay development (Yao and Tuite, 1989; Trapero-Casas and Kaiser, 1992). As a result of heat treatments, which reduce fungal viability, the effective inoculum concentration which causes decay development is reduced, thus reducing rot development.

4.2 Effect of HWT on anatomy and biochemical changes in tangerine fruit during infection of *Penicillium digitatum* under low-temperature storage

4.2.1 Effect of HWT on infection of *Penicillium digitatum* in tangerine fruit under low-temperature storage

From the experiment in section 4.1.2 the suitable temperature and period of HWT were obtained. So the results were used for the following experiments. Dipping tangerine fruit in hot water at 50±2°C for 3 minutes and 55±2°C for 2 and 3 minutes after inoculation showed no visible rind injury on tangerine fruit and significantly reduced both disease index and severity of green mold rot in storage at 24±2°C and 90±5% RH compared to control untreated fruit (Table 4.1-4.3, Figure 4.8-4.13). In this experiment, tangerine fruit were dipped in hot water at 50±2°C for 3 minutes and 55±2°C for 2 and 3 minutes after inoculation compared with the control untreated fruit and uninoculated fruit and stored at 4±2°C and 90±5% RH for 35 days. The results of the experiment indicated that with longer time for storage, green mold rot disease index and severity increased in all treatments, except for uninoculated fruit (Figure 4.14). Tangerine fruit treated with hot water dips at 50±2°C and 55±2°C for 3 minutes after inoculation showed symptoms of infection after 20 days of storage, while fruit treated with hot water dip at 55±2°C for 2 minutes and control untreated fruit, appeared disease symptoms after 15 days of storage. Rot first becomes apparent by a soft peel and water soaked lesions originating from the point of inoculation of about 0.03 to 0.21 cm diameter. After 25 days, the mold begins to appear by the development of white mycelia on the surface of the decaying areas. Nevertheless, sporulation of green mold rot was not found in any treatment and was no infection of the uninoculated fruit by green mold.

All of the HWT examined in this study significantly reduced disease index and severity as compared with control untreated fruit (Table 4.4 and 4.5, Figure 4.14-4.19). HWT at 50±2°C for 3 minutes and 55±2°C for 2 and 3 minutes effectively reduced the development of green mold rot, but were not significantly different from

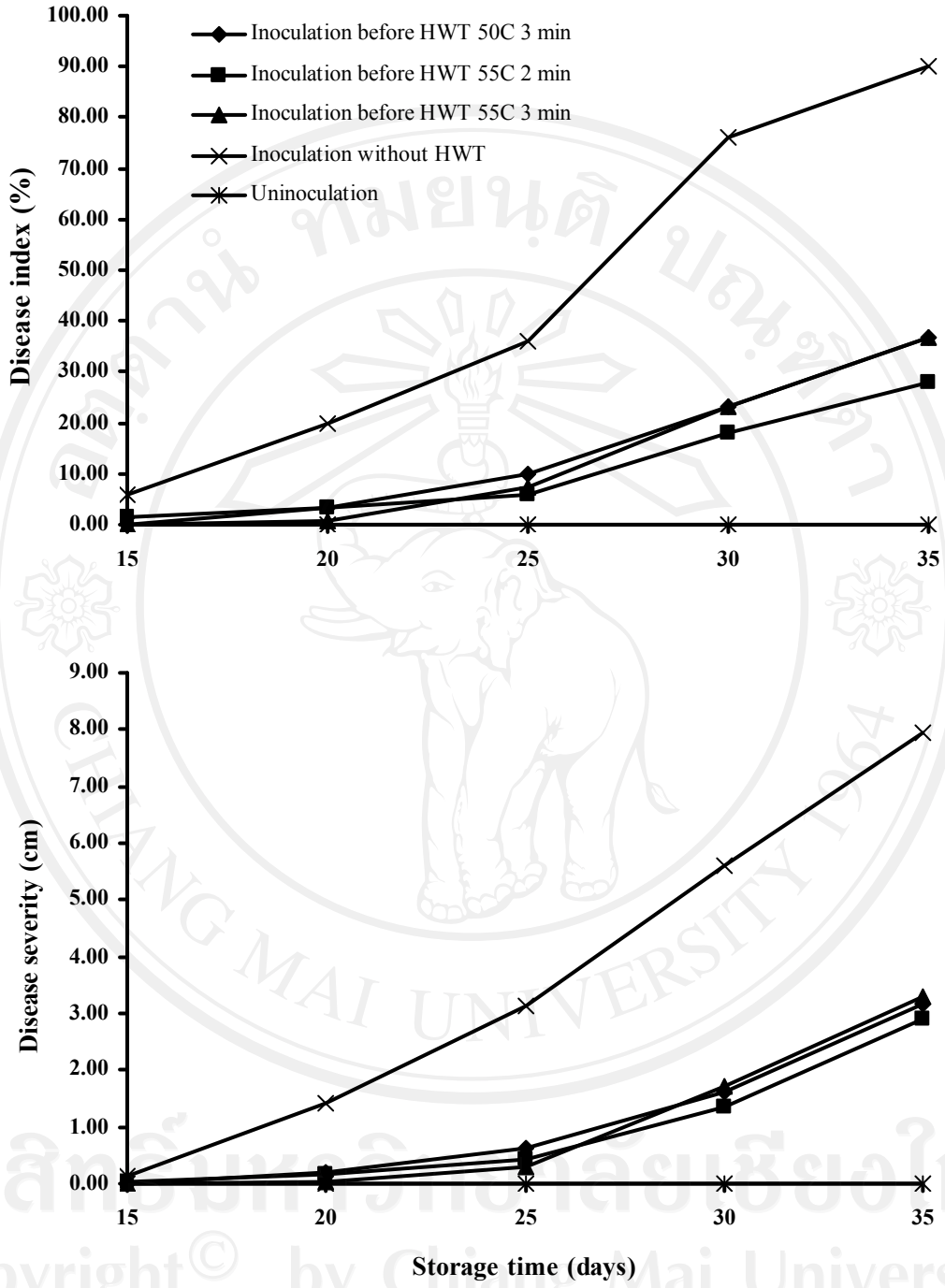

Figure 4.14 Effect of hot water treatments (HWT) on green mold rot disease index and severity (lesion diameter) of artificially-inoculated tangerine fruit during 35 days storage at 4±2°C and 90±5% RH

Table 4.4 Effect of hot water treatments (HWT) on green mold rot disease index of artificially-inoculated tangerine fruit during 35 days storage at 4±2°C and 90±5% RH

Treatments	Disease index (%)* Day of storage				
	15	20	25	30	35
Inoculation before HWT					
50°C 3 min	0.00± 0.00	3.33±5.77[b]	10.00±11.13[b]	23.33±16.04[b]	36.67±18.85[b]
55°C 2 min	1.33± 2.31	3.33±5.77[b]	6.00±10.39[b]	18.00±17.09[b]	28.00± 8.00[b]
55°C 3 min	0.00± 0.00	0.67±1.15[b]	7.33±11.01[b]	23.33±16.04[b]	36.67±13.32[b]
Inoculation without HWT	6.00±10.39	20.00±0.00[a]	36.00±16.00[a]	76.00± 6.93[a]	90.00±10.00[a]
Uninoculation	0.00± 0.00	0.00±0.00[b]	0.00± 0.00[b]	0.00± 0.00[b]	0.00± 0.00[c]
$LSD_{0.05}$	8.66	6.71	20.08	23.78	21.06
C.V. (%)	32.46	67.45	33.02	46.47	30.26

*See materials and methods in 3.1.2.4.

[abc]Means within the same column followed by different letters are significantly different at 95% ($P \leq 0.05$) level by Least Significant Difference Test. Data are mean values ±SD, (n=30).

Table 4.5 Effect of hot water treatments (HWT) on green mold rot disease severity (lesion diameter) of artificially-inoculated tangerine fruit during 35 days storage at 4±2°C and 90±5% RH

Treatments	Disease severity (cm) Day of storage				
	15	20	25	30	35
Inoculation before HWT					
50°C 3 min	0.00±0.00[b]	0.21±0.59[b]	0.63±1.28[b]	1.61±1.90[b]	3.15±2.28[b]
55°C 2 min	0.03±0.16[b]	0.16±0.57[bc]	0.43±1.15[b]	1.34±1.87[b]	2.89±2.30[b]
55°C 3 min	0.00±0.00[b]	0.03±0.26[bc]	0.31±0.81[bc]	1.72±1.73[b]	3.29±2.33[b]
Inoculation without HWT	0.14±0.30[a]	1.41±0.63[a]	3.14±0.95[a]	5.61±1.35[a]	7.96±1.30[a]
Uninoculation	0.00±0.00[b]	0.00±0.00[c]	0.00±0.00[c]	0.00±0.00[c]	0.00±0.00[c]
C.V. (%)	46.55	13.15	10.52	75.17	54.25

[abc]Means within the same column followed by different letters are significantly different at 95% ($P \leq 0.05$) level by Duncan's Multiple Range Test. Data are mean values ±SD, (n=30).

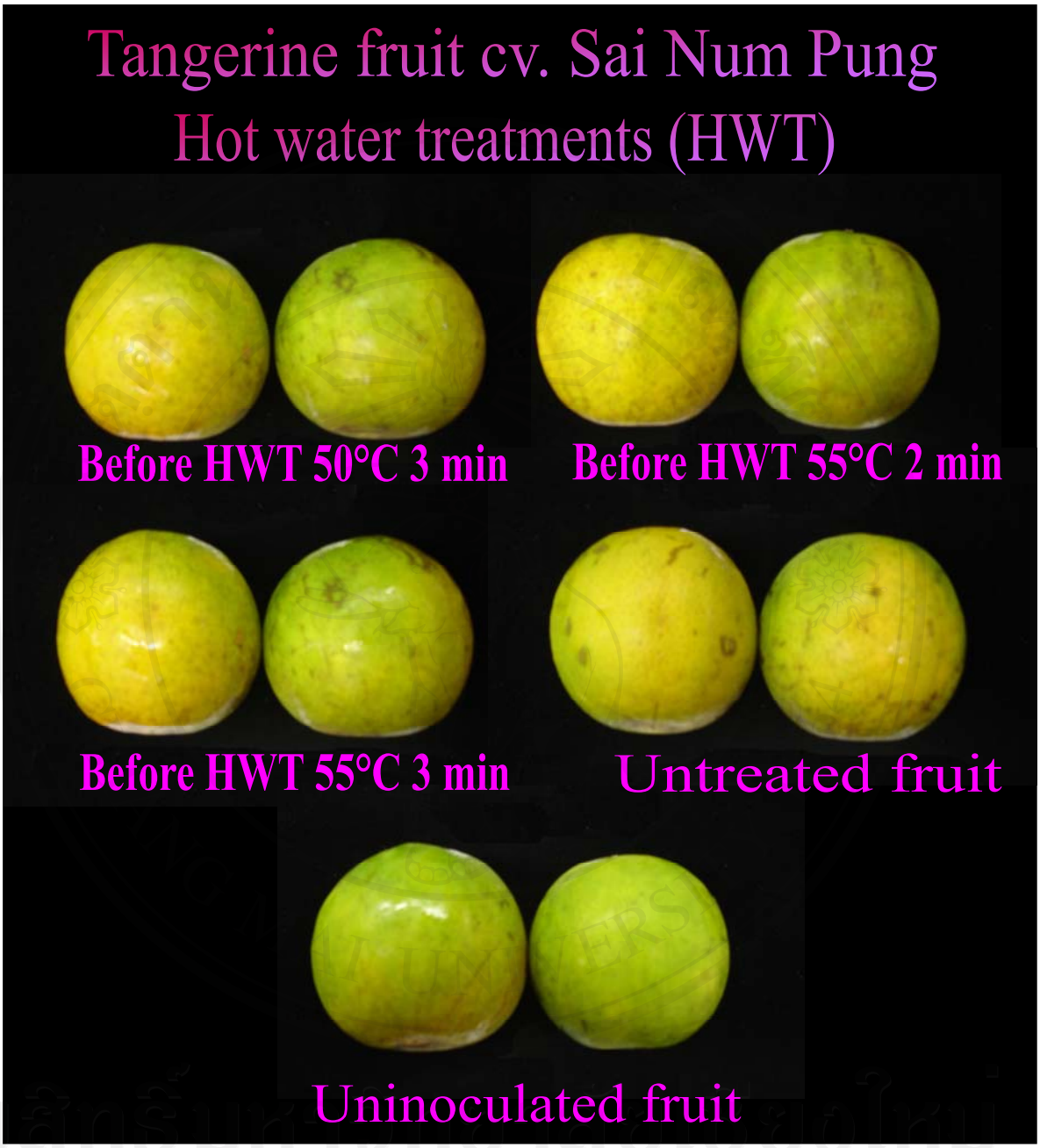

Figure 4.15 Effect of hot water treatments (HWT) at various temperature and time on green mold rot disease development of artificially-inoculated tangerine fruit 10 days storage at 4±2°C and 90±5% RH

No infection was found.

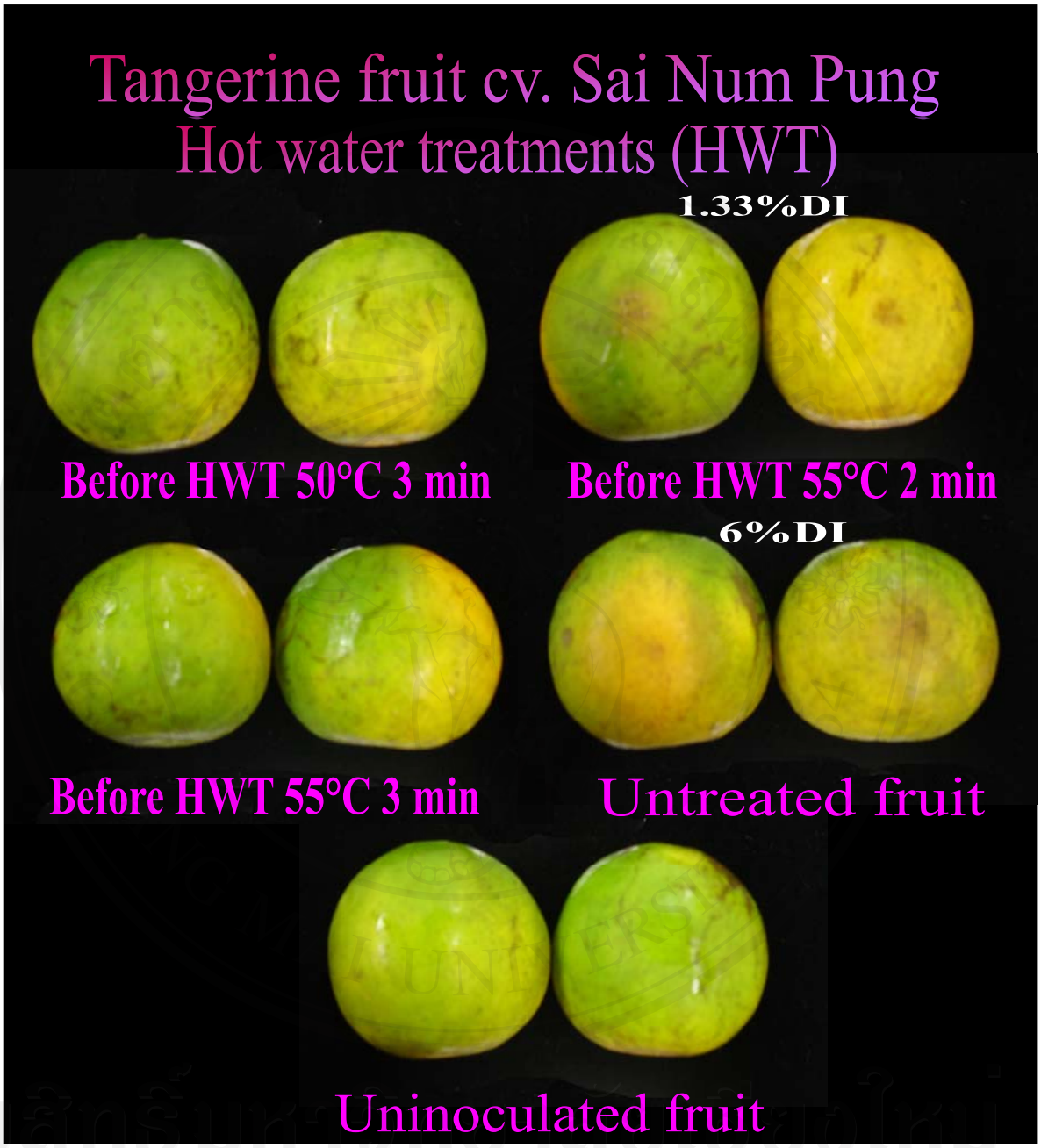

Figure 4.16 Effect of hot water treatments (HWT) at various temperature and time on green mold rot disease development (percentage of disease index: DI) of artificially-inoculated tangerine fruit 15 days storage at 4±2°C and 90±5% RH

Symptoms appeared on the inoculated fruit treated with HWT at 55±2°C for 2 minutes and control (untreated fruit) only.

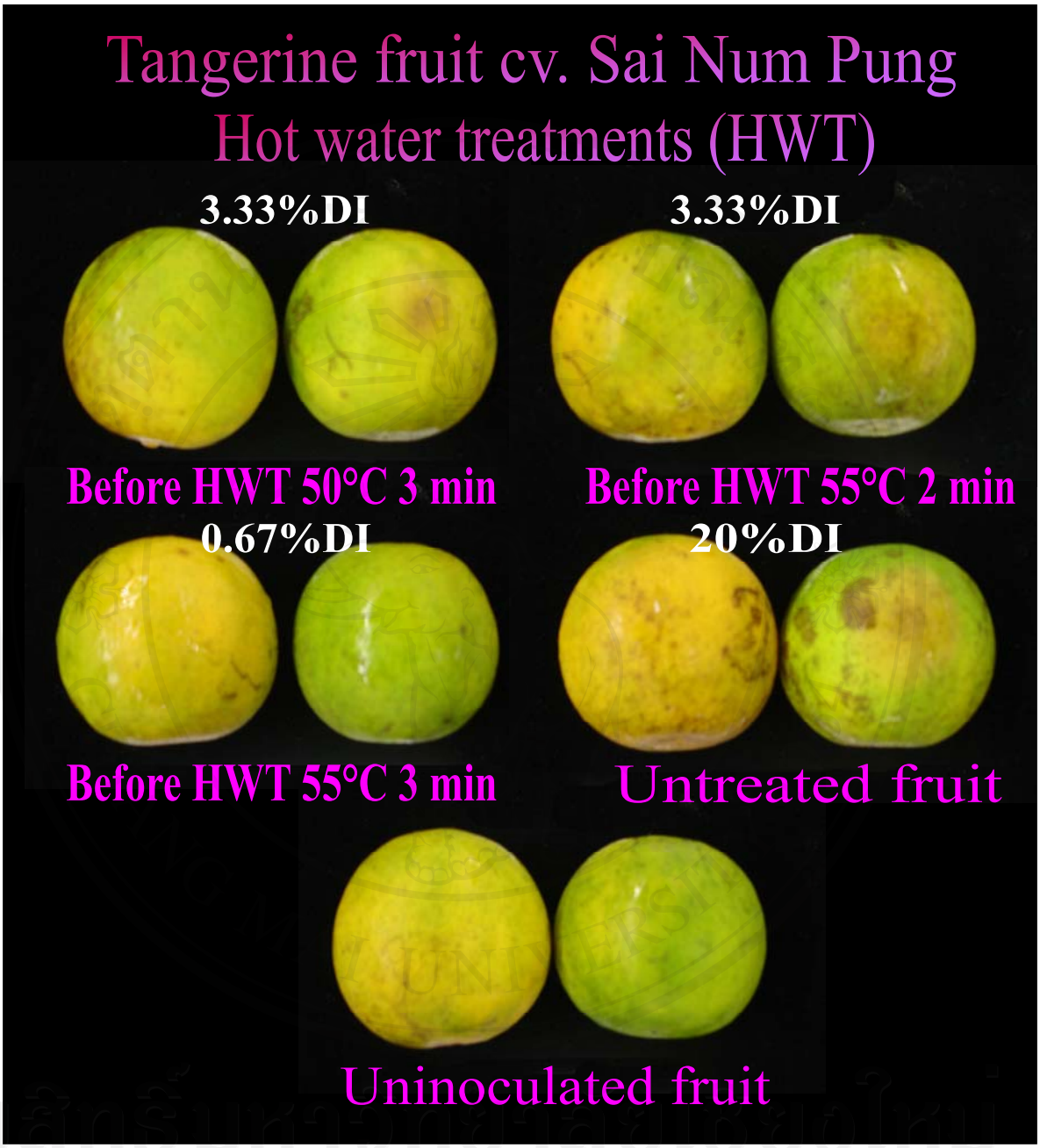

Figure 4.17 Effect of hot water treatments (HWT) at various temperature and time on green mold rot disease development (percentage of disease index: DI) of artificially-inoculated tangerine fruit 20 days storage at 4±2°C and 90±5% RH

Symptoms appeared on the inoculated fruit treated with HWT and control (untreated fruit).

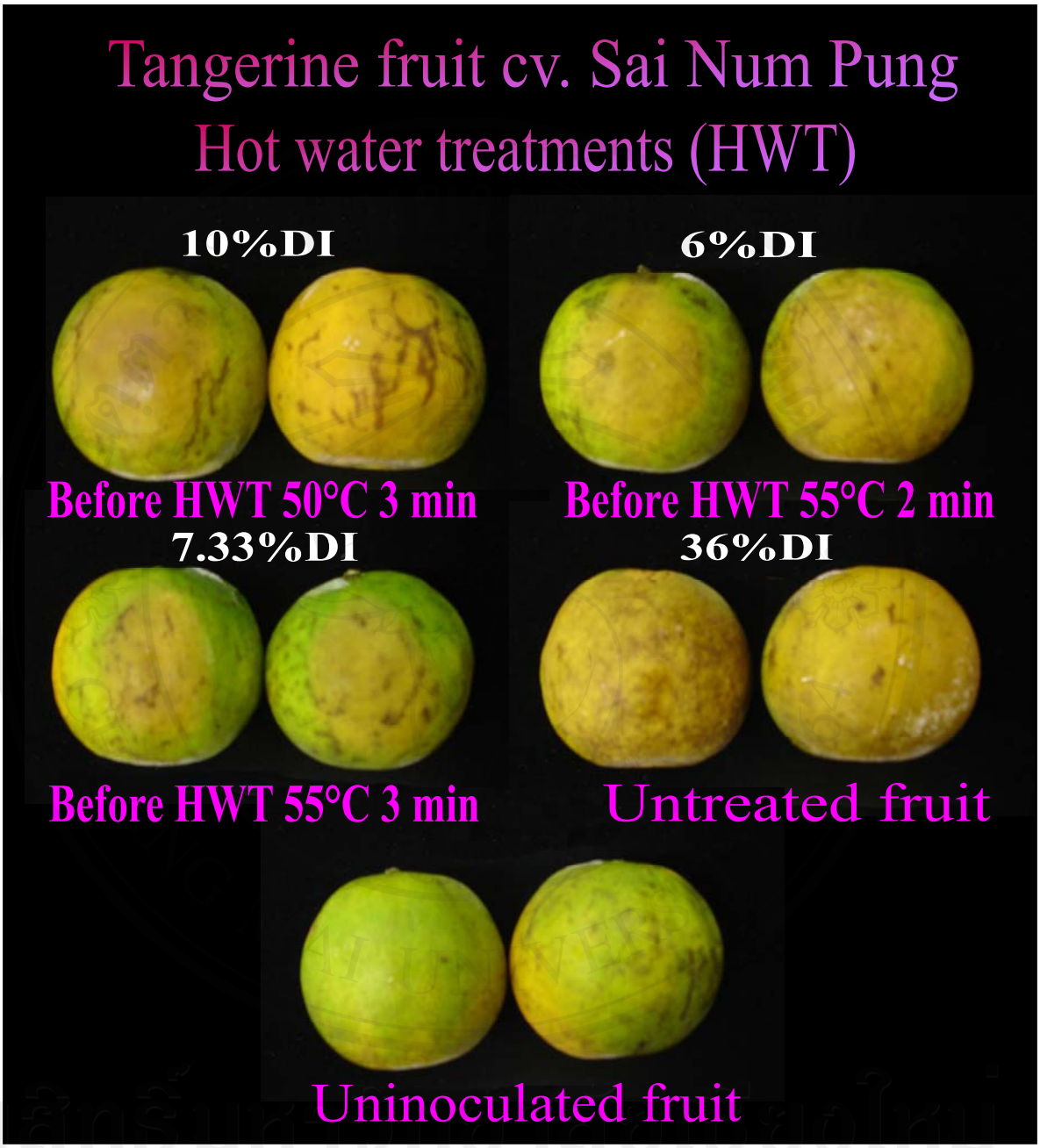

Figure 4.18 Effect of hot water treatments (HWT) at various temperature and time on green mold rot disease development (percentage of disease index: DI) of artificially-inoculated tangerine fruit 25 days storage at 4±2°C and 90±5% RH

Symptoms appeared on the inoculated fruit treated with HWT and control (untreated fruit).

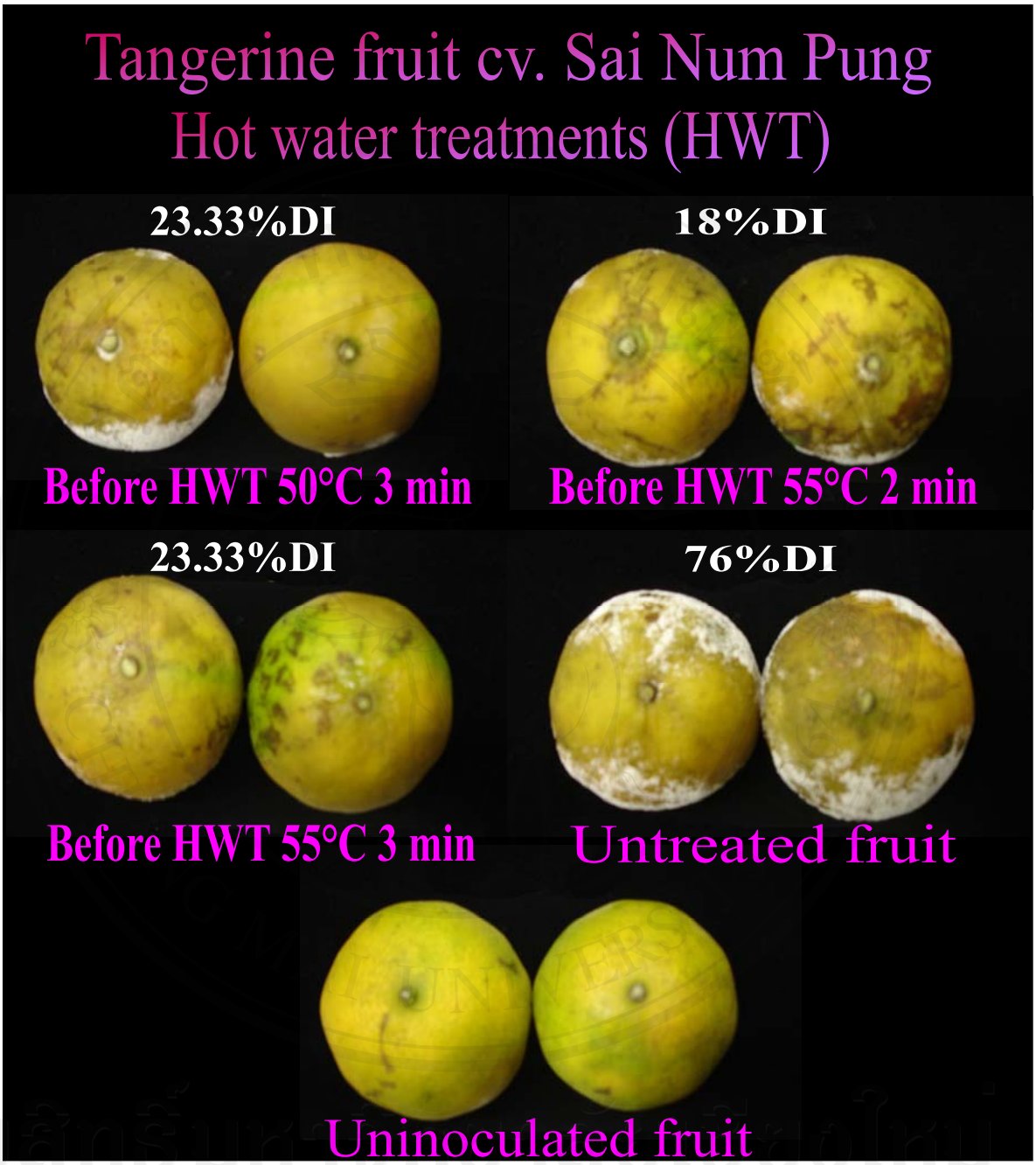

Figure 4.19 Effect of hot water treatments (HWT) at various temperature and time on green mold rot disease development (percentage of disease index: DI) of artificially-inoculated tangerine fruit 30 days storage at 4±2°C and 90±5% RH

Symptoms appeared on the inoculated fruit treated with HWT and control (untreated fruit).

each other. By day 25, the control untreated fruit had 36.00% disease index, while fruit treated with hot water dips at 50±2°C for 3 minutes and 55±2°C for 2 and 3 minutes had 10.00, 6.00 and 7.33% disease index, respectively. On day 30, the control untreated fruit had 76.00% disease index, while fruit treated with hot water dips at 50±2°C for 3 minutes and 55±2°C for 2 and 3 minutes had 23.33, 18.00 and 23.33% disease index, respectively. By the end of the 35 day storage period, disease index on control untreated fruit reached 90.00% which was higher than the disease index of green molds on fruit treated with hot water dips at 50±2°C for 3 minutes and 55±2°C for 2 and 3 minutes which were 36.67, 28.00 and 36.67%, respectively. Moreover, HWT conditions that were mentioned above reduced disease severity from 7.69 cm diameter of control untreated fruit to 3.15, 2.89 and 3.29 cm diameter of hot water dips at 50±2°C for 3 minutes and 55±2°C for 2 and 3 minutes, respectively, when stored at 4±2°C and 90±5% RH (Table 4.4 and 4.5). This result was similar to the hot water dip treatments at 52 to 53°C for 2 to 3 minutes effectively reduced decay development in lemon, mandarin, orange, oroblanco and kumquat citrus fruit (Ben-Yehoshua *et al*., 2000; Rodov *et al*., 1995; Schirra and Mulas, 1995; Schirra and D'hallewin, 1997). Similarly, hot water dip reduced crown rot in banana (Reyes *et al*., 1998) and botrytis rot in kiwi, pepper and tomato (Cheah *et al*., 1992; Fallik *et al*., 1993, 1996).

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. The primary mode of action of hot water is killing or damaging of infection structures of fungi present on the fruit surface or in the first layers under the skin (Porat *et al*., 2000; Schirra *et al*., 2000). *In vitro* studies showed that germination declined when fungi were dipped in hot water at 55±2°C for 1, 2 and 3 minutes and incubated at 25±2°C for 48 hours in darkness. 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 (Nafussi *et al*., 2001; Ben-Yehoshua,

2003). It is possible that tangerine fruit treated with hot water dips at 50±2°C for 3 minutes and 55±2°C for 2 and 3 minutes may be related to a partial removal and/or inhibition of pathogen spores. The pathogen is markedly inhibited by both thermal inhibitions as well as by the enhanced resistance of the fruit against the pathogen.

4.2.2 Effect of HWT on anatomy changes of tangerine fruit peel during infection of *Penicillium digitatum* under low-temperature storage

The effect of HWT at 50±2°C for 3 minutes and HWT at 55±2°C for 2 and 3 minutes compared with the control untreated fruit on anatomy changes of tangerine fruit peel was observed by SEM on day 0 following treatment. The skin of untreated fruit exhibited a number of plate-like structures with deep surface cracks or a rough surface (Figure 4.20 D), whereas the skin of tangerine fruit treated with hot water dips at 50±2°C for 3 minutes and 55±2°C for 2 and 3 minutes did not have similar cracks, but rather a much more homogeneous surface, especially, tangerine fruit dipped in HWT at 55±2°C for 3 minutes which had the highest homogeneous surface (Figure 4.20 C), followed by HWT at 55±2°C for 2 minutes (Figure 4.20 B), HWT at 50±2°C for 3 minutes (Figure 4.20 A) and untreated fruit (Figure 4.20 D), respectively. Acquired results indicated that the heat treatments cleaned the fruit. Moreover, we found that the heat treatments smoothed the fruit epicuticular waxes, so that it covered and sealed the stomata (Figure 4.21 A-C) and microscopic cracks on the fruit surface (Figure 4.22 A-C) as compared with the control untreated fruit (Figure 4.21 D and 4.22 D).

Roy *et al*. (1994) investigated on apples and reported that the epicuticular wax of non-heated fruit displayed a number of deep surface cracks that formed an interconnected network on peel surface. Following a hot air treatment at 38°C for 4 days, the cuticular cracks disappeared, probably as a result of the melting of the wax platelets that had occurred in the cracks. Similar changes in epicuticular wax structure have been observed in various fruit species subjected to heating, such as hot water dip treatments at 50 to 54°C for 2 minutes in 'Oroblanco' grapefruit, 'Fortune' mandarin and cactus pear (Rodov *et al*., 1996; Schirra and D'hallewin, 1997; Schirra *et al*., 1999), as well as after hot water rinsing and brushing treatments at 54°C for 20 seconds in sweet pepper, melon and organically grown citrus (Fallik *et al*., 1999, 2000; Porat *et al*., 2000). Thus, fruit response to various types of heat treatments, in term of changes of ultrastructure of epicuticular wax, appears to be quite similar. The

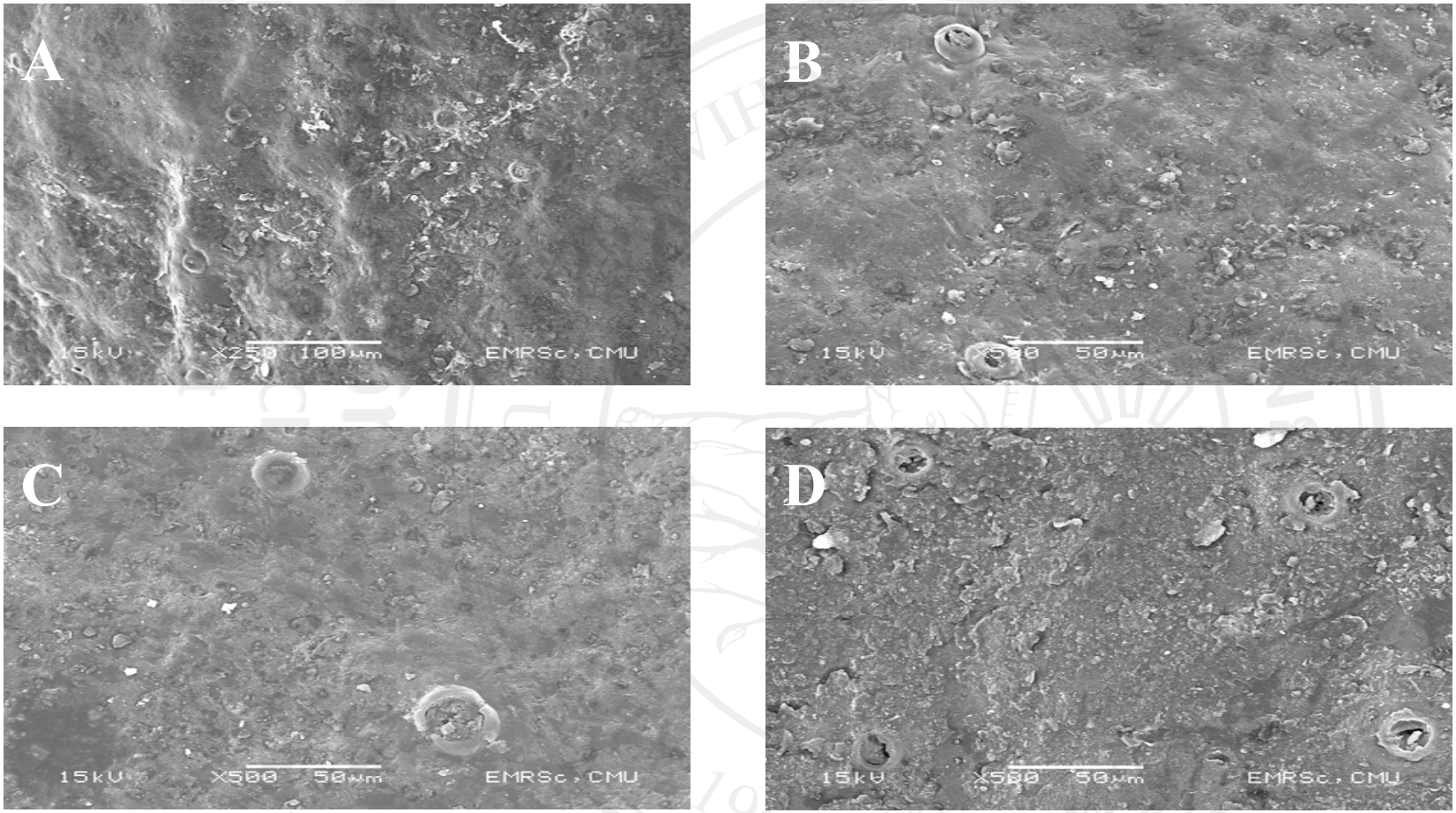

Figure 4.20 SEM showed effect of hot water treatments (HWT) on 'Sai Num Pung' tangerine fruit surfaces after treated with HWT at 50±2°C for 3 minutes (A) HWT at 55±2°C for 2 minutes (B) and 3 minutes (C) (0 day) the peel was smooth homogeneous but control (D) was rough

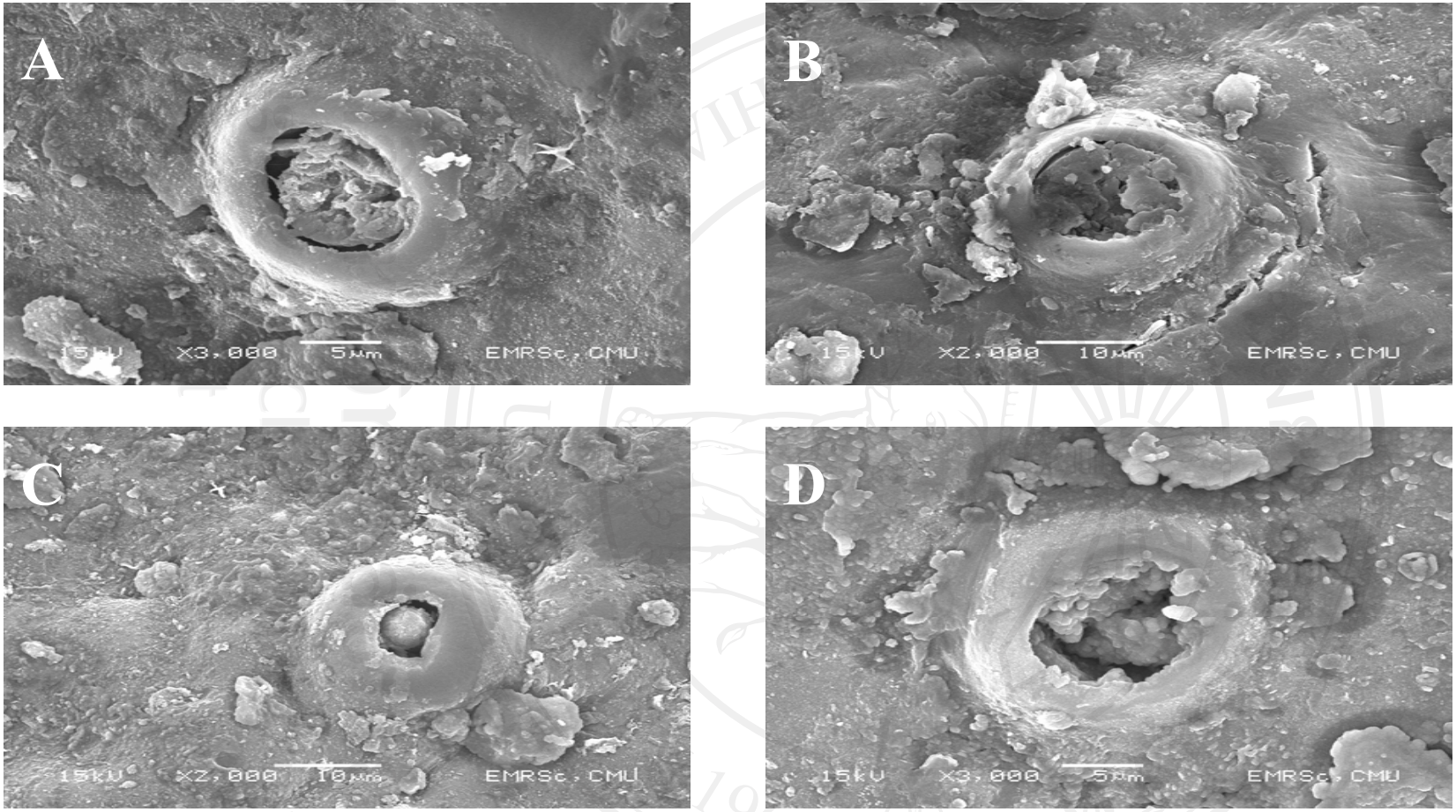

Figure 4.21 SEM showed effect of hot water treatments (HWT) (Day 0) on 'Sai Num Pung' tangerine fruit surfaces after treated with HWT at 55±2°C for 3 minutes having narrow stoma (C) while HWT at 50±2°C for 3 minutes and HWT at 55±2°C for 2 minutes stomata were plugged with melted cuticle (A, B) compared with control stoma was wide opened and unplugged (D)

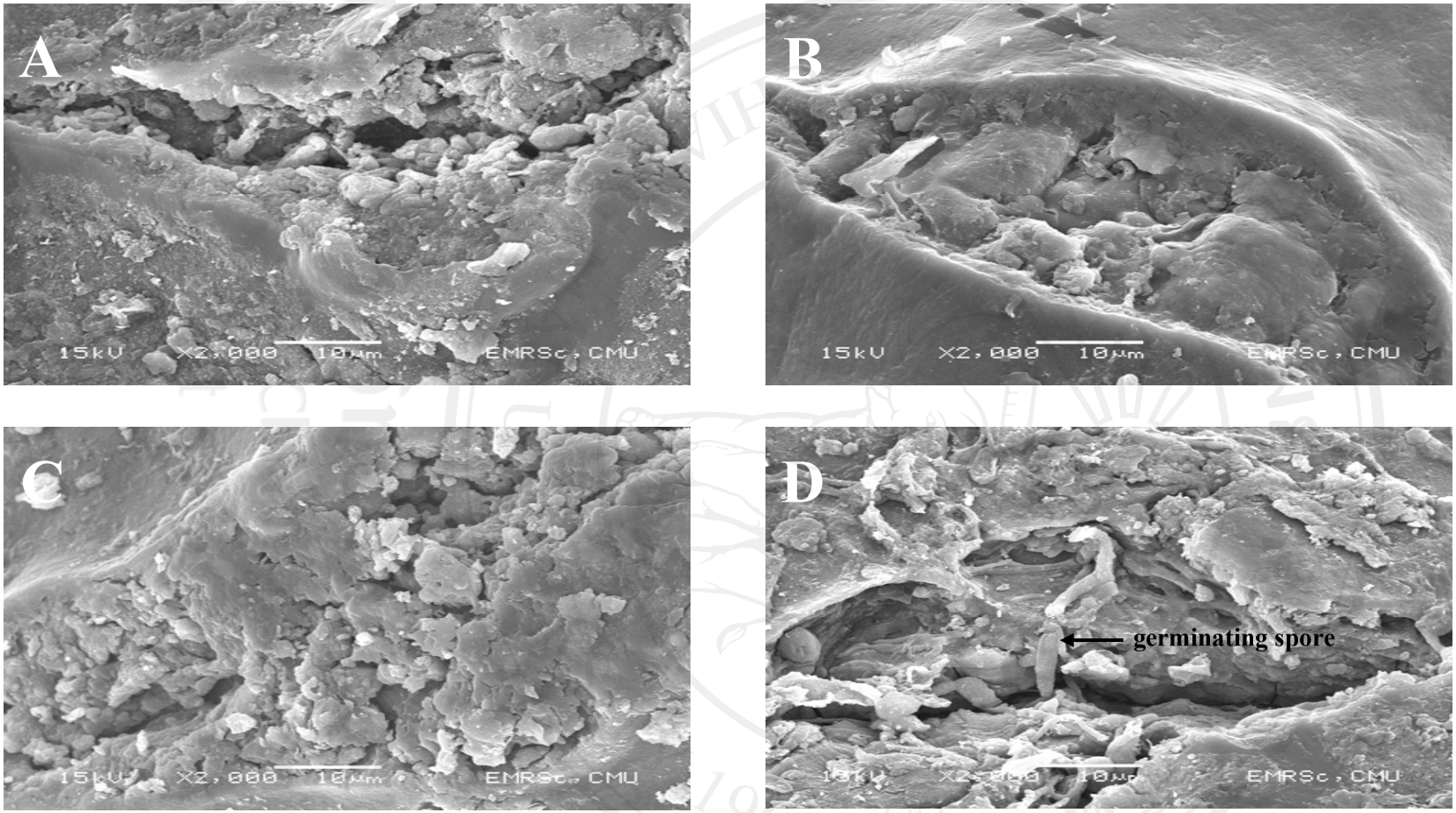

Figure 4.22 **SEM showed effect of hot water treatments (HWT) on crack of the peel light after treated with HWT at 50±2°C for 3 minutes (A) HWT at 55±2°C for 2 minutes (B) and 3 minutes (C) (0 day) showed the cracks were shallow filled with melted cuticle whereas control (D) the crack was wider with a germinating spore (arrow)**

results strengthen the suggestion that the heat may change the physical status of the wax making it more plastic so that it may be stretched to occlude the micro-cracks (Ben-Yehoshua and Porat, 2005). The effect of HWT on tangerine 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).

SEM examination of *P. digitatum*-inoculated samples collected 0 day from tangerine fruit treated with hot water dips at 50±2°C for 3 minutes and 55±2°C for 2 and 3 minutes showed that the heat treatments removed fungus spores and hyphae from its surface, so that the distribution of *P. digitatum* spore was sparse (Figure 4.23 A-C) and collapsed, evidenced by the shrinked appearance of the *P. digitatum* cell surface, especially, tangerine fruit dipped in HWT at 55±2°C for 2 and 3 minutes, respectively (Figure 4.24 B and C), as compared with the control untreated fruit (Figure 4.23 D and 4.24 D). Observation of samples from untreated fruit, collected 15 days of storage at 4±2°C and 90±5% RH, revealed that spores of *P. digitatum* had germinated and the fungus proliferated rapidly at the cell surface (Figure 4.25 A-C), whereas samples from tangerine fruit treated with hot water dips at 50±2°C for 3 minutes (Figure 4.26 A-C) and 55±2°C for 2 and 3 minutes (Figure 4.27 and 4.28 A-C) showed that very few spores were germinated and the mycelial mat was very thin due to markedly reduced branching. Moreover, we found that the heat treatments collapsed spores and mycelia (Figure 4.26-4.28, A-C). In contrast, normal spore and mycelial shapes on control untreated fruit was turgor and lush (Figure 4.25 A-C).

The results imply that mode of action of HWT at 50±2°C for 3 minutes and HWT at 55±2°C for 2 and 3 minutes in reducing the development of green mold rot partly by inhibition of pathogen development. Additionally, spore germination and mycelial growth were inhibited by spores collapsing and mycelia dislodgement from

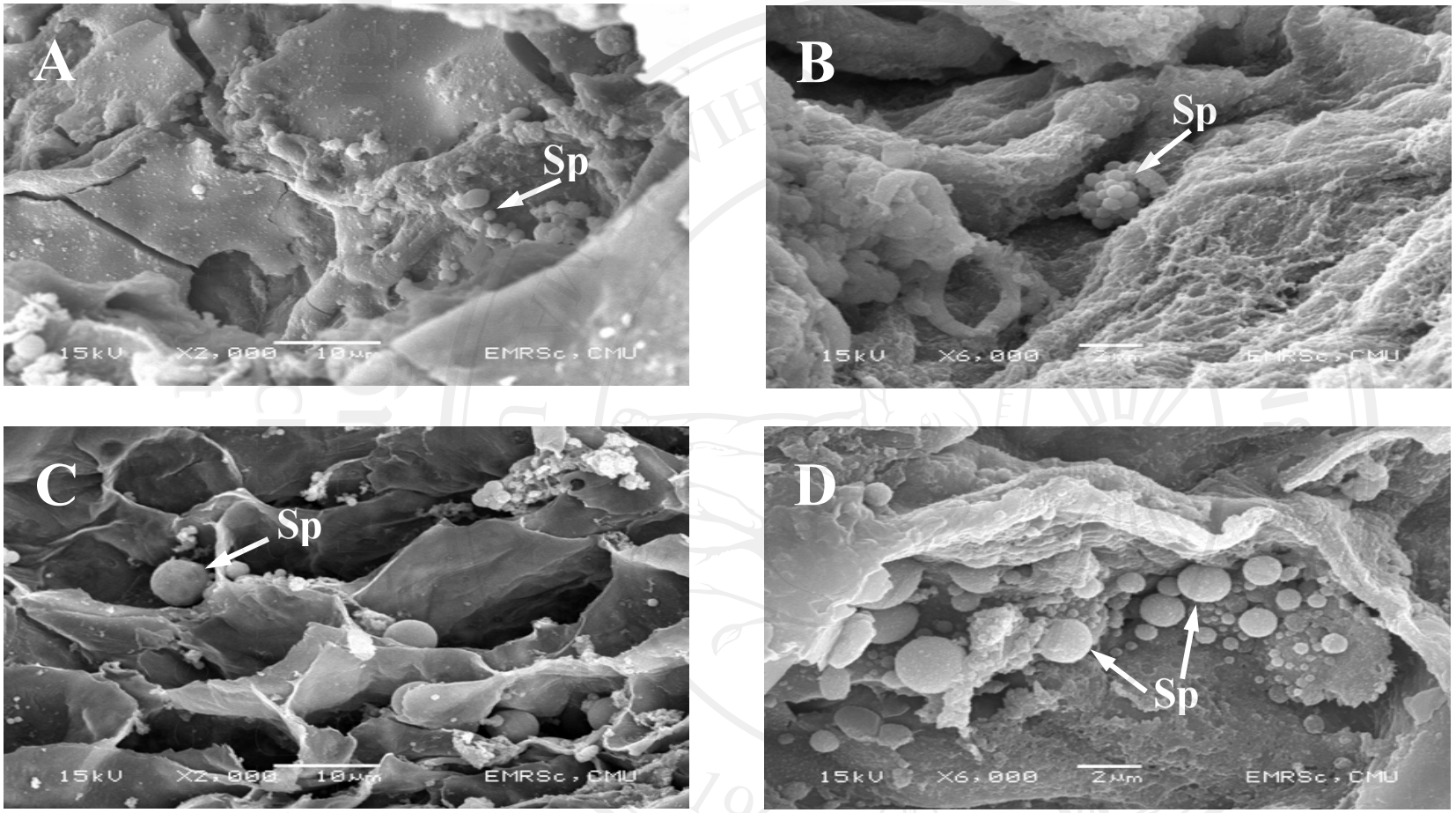

Figure 4.23 **SEM showed effect of hot water treatments (0 day) on *Penicillium digitatum* spore (Sp) number exist on the peel after treatment at various temperature and time, all treatments (A, B, C) showed small number of spore compared to high number in control (D)**

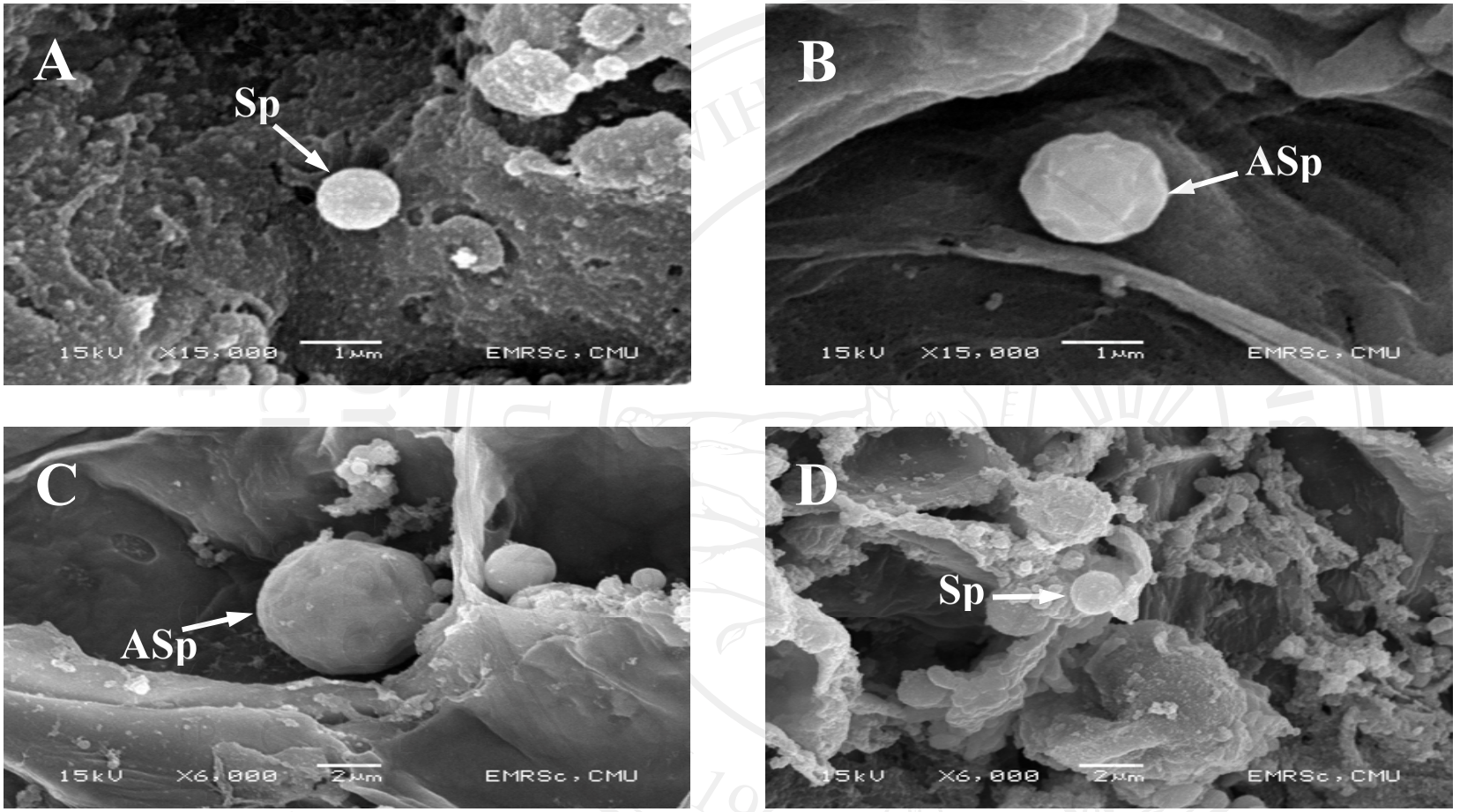

Figure 4.24 **SEM showed higher magnification of spore (Sp) on the fruit peel affected by hot water treatments (0 day) at various temperature and time, all treatments (A, B, C) showed denature of spore (ASp = abnormal spore) while the spore in control (D) was normal**

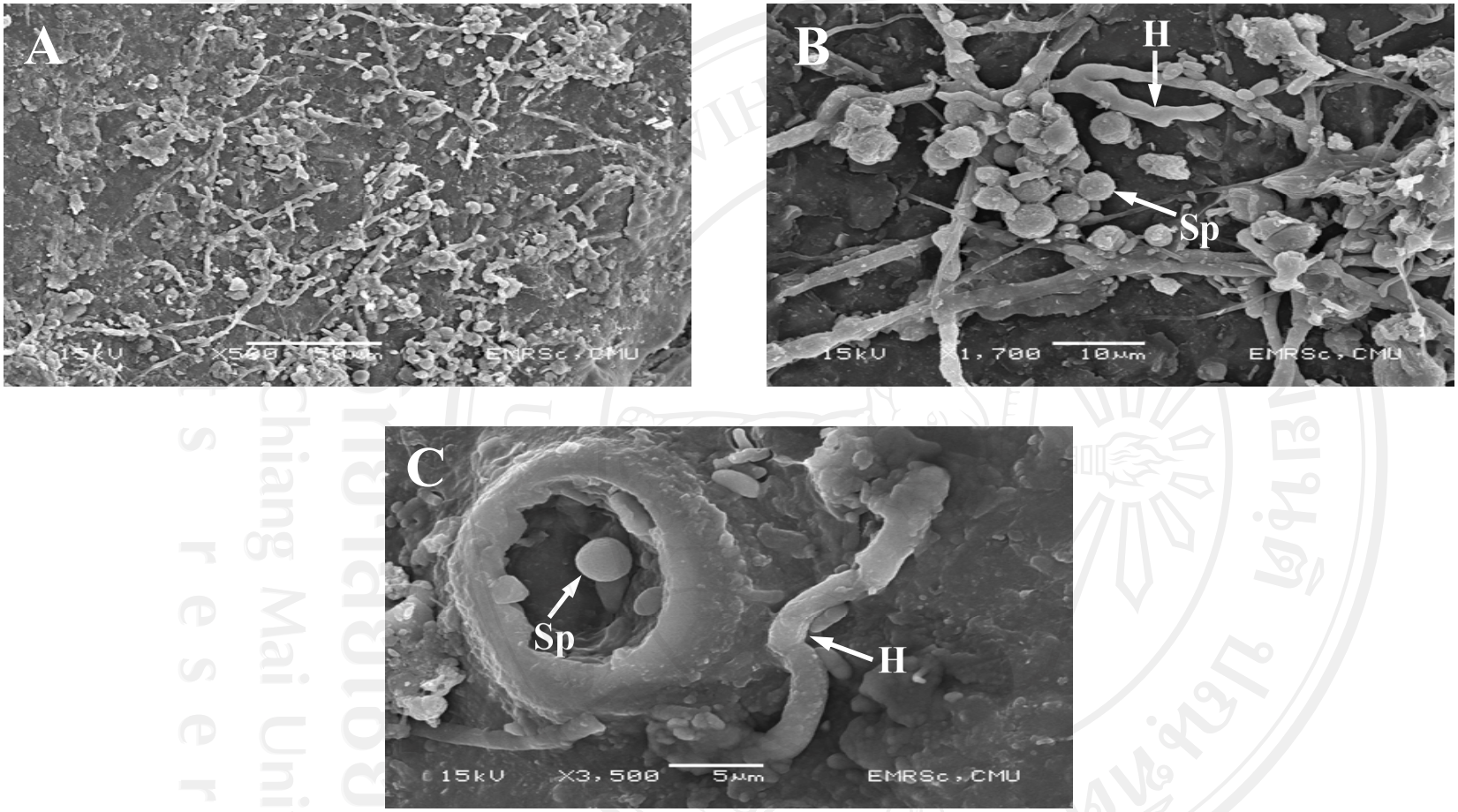

Figure 4.25 SEM showed numerous spores (Sp) and mycelium (H = hyphae) of *Penicillium digitatum* on the peel of tangerine fruit (A, B, C) 15 days after inoculation stored at 4±2°C and 90±5% RH without hot water treatment (control)

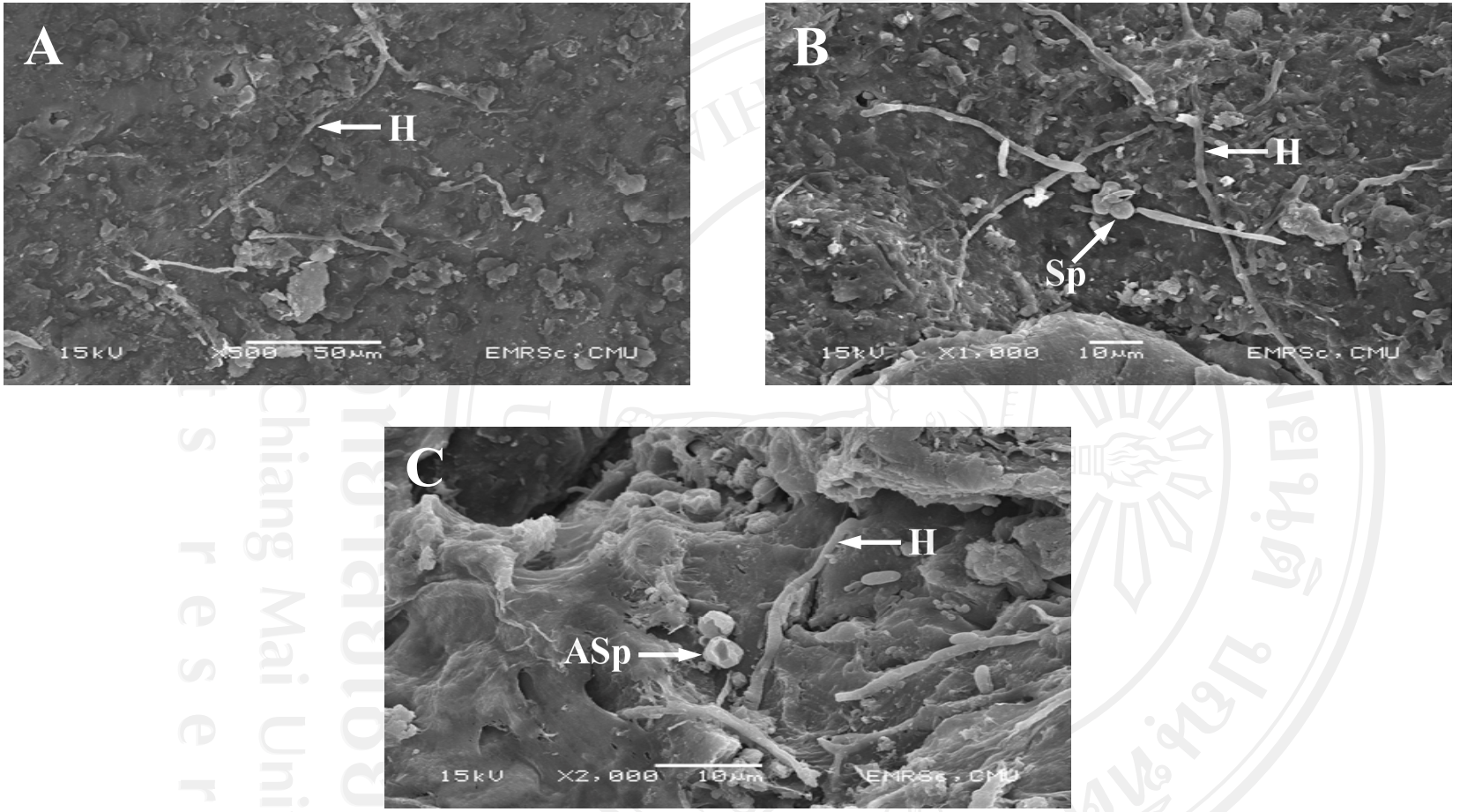

Figure 4.26 SEM showed few hyphae (H), small number and denature of spore (Sp) of *Penicillium digitatum* on the peel of tangerine fruit affected by hot water treatment at 50±2°C for 3 minutes (A, B, C) 15 days after inoculation stored at 4±2°C and 90±5% RH; ASp = abnormal spore

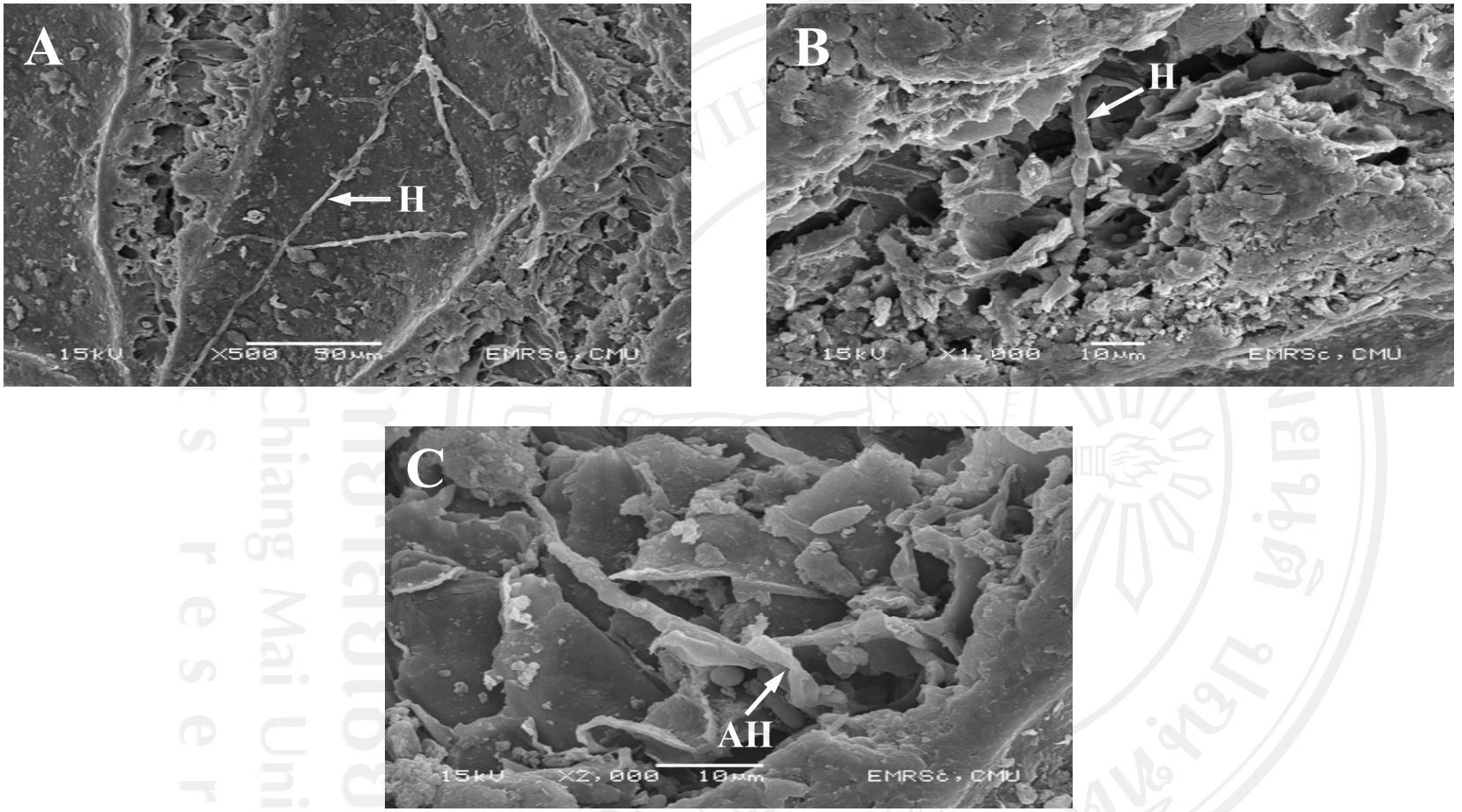

Figure 4.27 SEM showed small number and denature of hyphae (H) of *Penicillium digitatum* on the peel of tangerine fruit affected by hot water treatment at 55±2°C for 2 minutes (A, B, C) 15 days after inoculation stored at 4±2°C and 90±5% RH; AH = abnormal hyphae

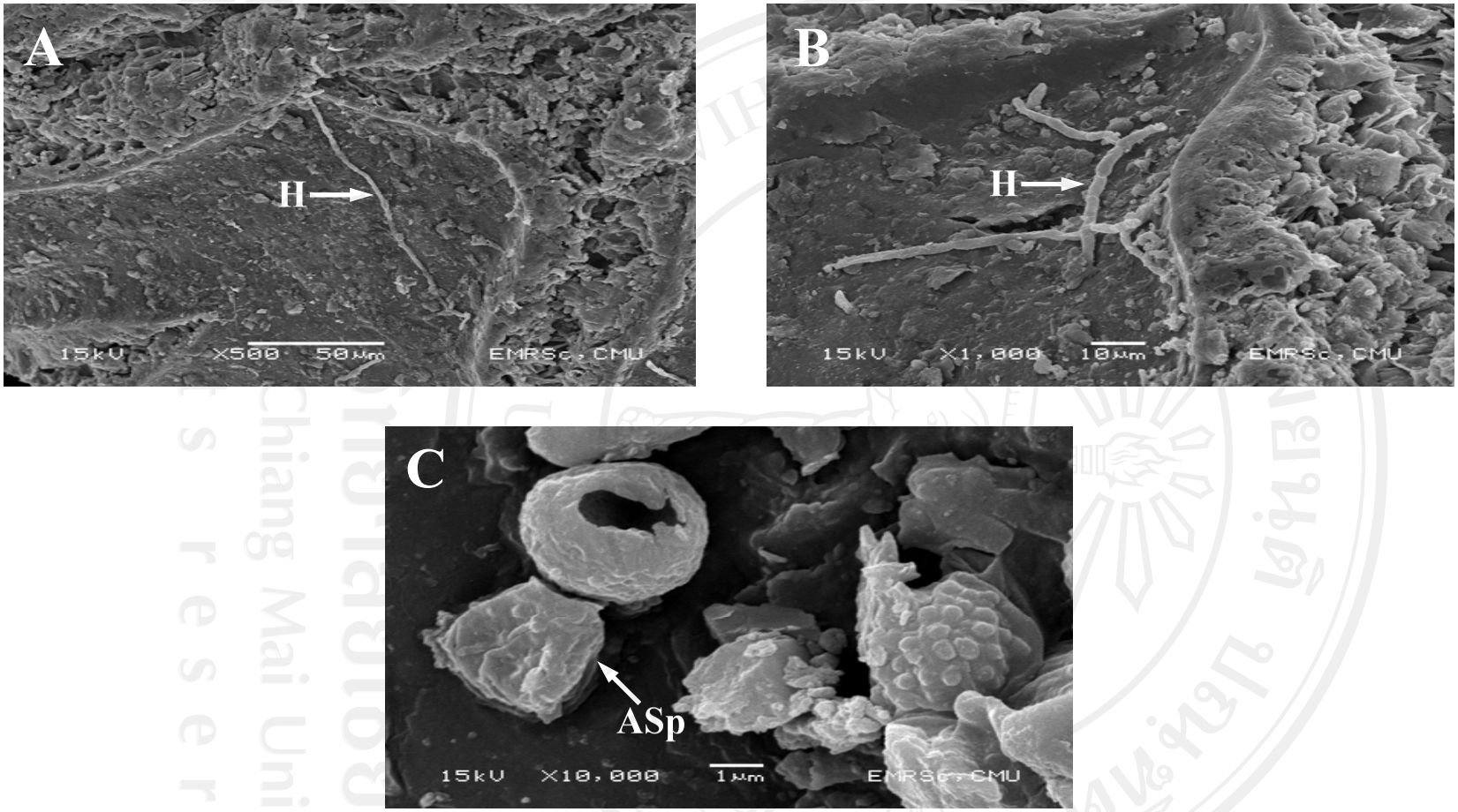

Figure 4.28 SEM showed few hyphae (H) and denature of spore (Sp) of *Penicillium digitatum* on the peel of tangerine fruit affected by hot water treatment at 55±2°C for 3 minutes (A, B, C) 15 days after inoculation stored at 4±2°C and 90±5% RH; ASp = abnormal spore

the fruit surface (Figure 4.26-4.28, A-C). This result was consistent with Eckert and Eaks (1988) who reported that a hot water rinsing and brushing treatment at 56°C applied to 'Minneola' tangerine fruit for 2 seconds cleaned and disinfected the fruit surface from fungal spores and hyphae and also partially melted and smoothed the fruit's natural wax platelets, which then covered and sealed stomata openings on the fruit surface. Without such treatment, these stomata may undergo severe alterations during postharvest storage and become important invasion sites for wound pathogens. Porat *et al*. (2000) also reported that drenching and brushing the 'Star Ruby' grapefruit with tap water alone reduced the population of naturally occurring epiphytic microflora on the fruit surface to only 1.4% of that on control unwashed fruit. Increasing the hot water temperature to 56, 59 and 62°C resulted in further reduction in microbial counts (colony-forming units-CFUs) to 24, 12 and <1%, respectively, of those observed on tap water-washed fruit. It is possible that heat may cause changes in nuclei and cell walls, denature proteins, destroy mitochondria and outer membranes, and disrupt vacuolar membranes and formation of gaps in the spore cytoplasm (Barkai-Golan and Phillips, 1991). Nevertheless, morphological studies performed by Dettori *et al*. (1996) who dipped 'Star Ruby' grapefruit at 50°C for 2 minutes, the morphogenesis of the mycelium of *Penicillium* spp. appeared to be markedly different for growth *in vivo* from that *in vitro*. In fact, *in vivo* morphological studies with *P. italicum* growing on 'Star Ruby' grapefruit showed that when fruit were dipped in hot water 1 hour after wounding and inoculation, the mycelia become thinner, with reduced branching, and were unable to spread into the albedo. In contrast, *in vitro* treatments with the pathogen growing in Petri dishes did not affect mycelium thickness and branching. Thus, in addition to physical effects, and direct effects on the pathogenic organisms, the other major way in which heat may be effective in reducing disease is in inducing defence mechanisms.

4.2.3 Effect of HWT on activities of the defensive enzymes and protein patterns in tangerine fruit peel during infection of *Penicillium digitatum* under low-temperature storage

The analysis of the induction kinetics of chitinase, β-1,3-glucanase and peroxidase in flavedo tissues of tangerine fruit treated with hot water dips at 50±2°C for 3 minutes and 55±2°C for 2 and 3 minutes, then challenge-inoculated with *P. digitatum*, and compared with activities in the control untreated and uninoculated fruit are shown in Figure 4.29.

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 fruit till 30 days of storage. During 20-30 days of storage, the rate of chitinase activity in tangerine fruit 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 fruit, respectively. On day 30, chitinase activity in tangerine fruit treated with hot water dips at 55±2°C for 2 and 3 minutes were 5.42 and 2.64 times, respectively, and higher than in the untreated fruit. While, 45.86, 22.36 and 13.63 times of fruit treated with hot water dips at 55±2°C for 2 and 3 minutes and 50±2°C for 3 minutes, respectively, and higher than in the uninoculated fruit which had the chitinase activity increased slightly after 20 days of storage (Table 4.6, Figure 4.29).

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 fruit 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 fruit which were dipped in HWT at 55±2°C for 3 minutes increased continuously during storage period. In case of untreated fruit, 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 fruit which was 1.91 and 9.94 times, respectively (Table 4.7,Figure 4.29).

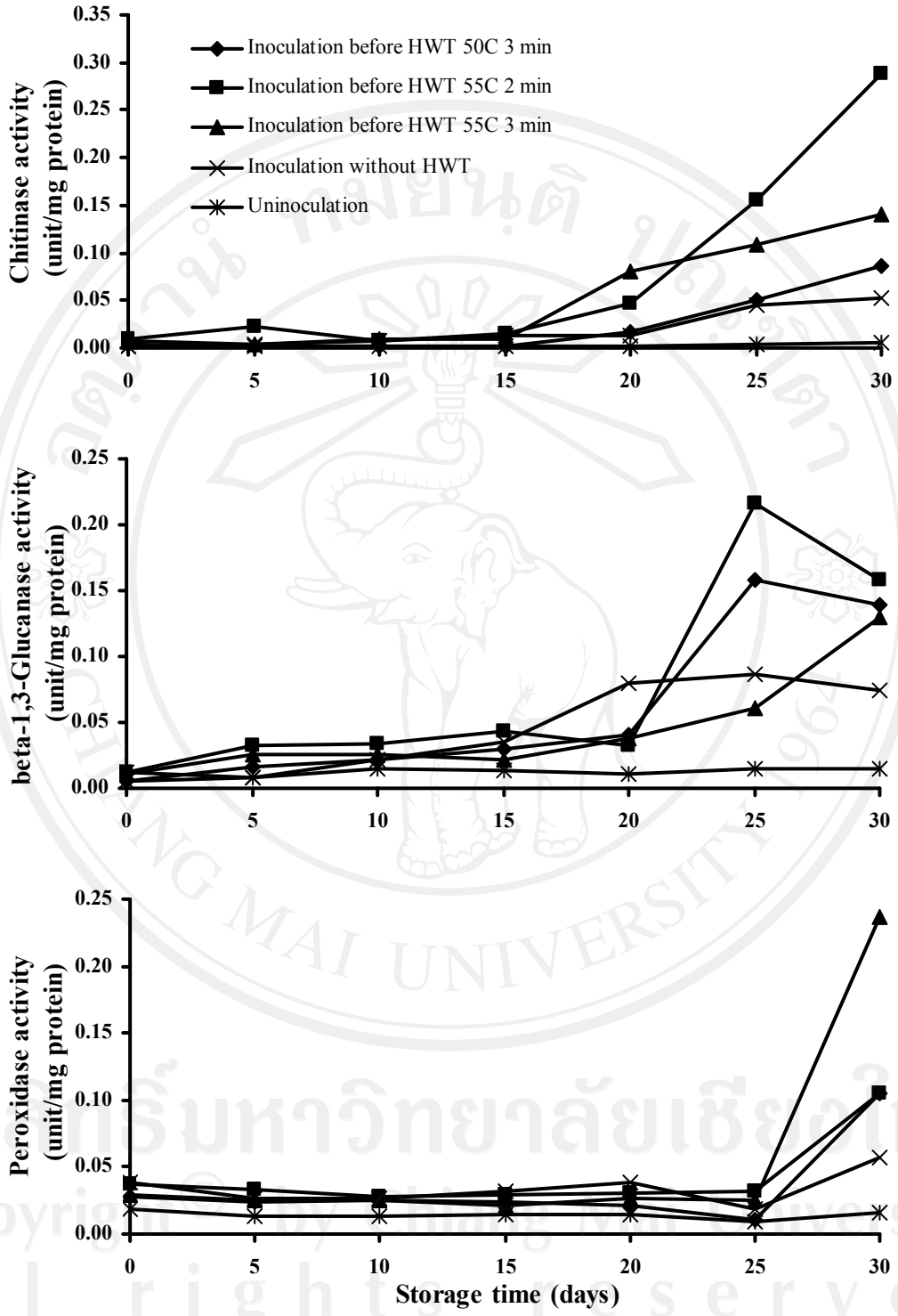

Figure 4.29 Effect of hot water treatments (HWT) on changes of chitinase, β-1,3-glucanase (beta-1,3-Glucanase) and peroxidase activities in flavedo tissue of tangerine fruit inoculated with *Penicillium digitatum* during 30 days storage at 4±2°C and 90±5% RH

Table 4.6 Effect of hot water treatments (HWT) on chitinase activity in flavedo tissues of artificially-inoculated tangerine fruit during 30 days storage at 4±2°C and 90±5% RH

Treatments	Chitinase activity (unit/mg protein) Day of storage						
	0	5	10	15	20	25	30
Inoculation before HWT							
50°C 3 min	0.0039±0.0007^{b}	0.0013±0.0001^{c}	0.0021±0.0009^{b}	0.0026±0.0007^{bc}	0.0170±0.0007^{c}	0.0498±0.0041^{c}	0.0859±0.0315^{c}
55°C 2 min	0.0086±0.0020^{a}	0.0227±0.0001^{a}	0.0074±0.0047^{a}	0.0143±0.0059^{a}	0.0465±0.0197^{b}	0.1550±0.0058^{a}	0.2889±0.0391^{a}
55°C 3 min	0.0079±0.0007^{a}	0.0037±0.0008^{b}	0.0092±0.0014^{a}	0.0090±0.0034^{ab}	0.0796±0.0173^{a}	0.1082±0.0107^{b}	0.1409±0.0086^{b}
Inoculation without HWT	0.0027±0.0006^{bc}	0.0029±0.0005^{b}	0.0081±0.0005^{a}	0.0127±0.0049^{a}	0.0131±0.0021^{c}	0.0450±0.0046^{c}	0.0533±0.0184^{c}
Uninoculation	0.0019±0.0005^{c}	0.0011±0.0000^{c}	0.0014±0.0002^{b}	0.0010±0.0001^{c}	0.0025±0.0005^{c}	0.0032±0.0010^{d}	0.0063±0.0033^{d}
$LSD_{0.05}$	0.0019	0.0009	0.0041	0.0069	0.0214	0.0110	0.0441
C.V. (%)	20.00	15.62	24.81	12.66	31.45	13.85	21.28

$^{a-d}$Means within the same column followed by different letters are significantly different at 95% ($P \leq 0.05$) level by Least Significant Difference Test. Data are mean values ±SD, (n=5).

Table 4.7 Effect of hot water treatments (HWT) on β-1,3-glucanase activity in flavedo tissues of artificially-inoculated tangerine fruit during 30 days storage at 4±2°C and 90±5% RH

Treatments	β-1,3-Glucanase activity (unit/mg protein) Day of storage						
	0	5	10	15	20	25	30
Inoculation before HWT							
50°C 3 min	0.0049±0.0009^{b}	0.0157±0.0038^{b}	0.0211±0.0012^{c}	0.0298±0.0013^{bc}	0.0407±0.0134^{b}	0.1581±0.0204^{b}	0.1391±0.0323^{a}
55°C 2 min	0.0122±0.0061^{a}	0.0322±0.0051^{a}	0.0337±0.0012^{a}	0.0434±0.0028^{a}	0.0319±0.0067^{bc}	0.2162±0.0355^{a}	0.1578±0.0279^{a}
55°C 3 min	0.0112±0.0026^{a}	0.0258±0.0036^{a}	0.0251±0.0032^{b}	0.0210±0.0087^{cd}	0.0378±0.0041^{b}	0.0607±0.0101^{c}	0.1297±0.0093^{a}
Inoculation without HWT	0.0120±0.0028^{a}	0.0087±0.0015^{c}	0.0215±0.0013^{bc}	0.0347±0.0121^{ab}	0.0802±0.0280^{a}	0.0864±0.0188^{c}	0.0745±0.0057^{b}
Uninoculation	0.0051±0.0010^{b}	0.0087±0.0025^{c}	0.0150±0.0025^{d}	0.0137±0.0019^{d}	0.0113±0.0023^{c}	0.0154±0.0035^{d}	0.0143±0.0031^{c}
$LSD_{0.05}$	0.0060	0.0063	0.0038	0.0126	0.0261	0.0375	0.0346
C.V. (%)	10.99	54.94	34.48	35.09	35.00	18.62	19.40

$^{a-d}$Means within the same column followed by different letters are significantly different at 95% ($P \leq 0.05$) level by Least Significant Difference Test. Data are mean values ±SD, (n=5).

Peroxidase activities in all treatments remained relatively constant until 25 days of storage, then surprisingly increased on day 30, especially, tangerine fruit 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 fruit, respectively, with significant differences. Peroxidase activity in tangerine fruit treated with hot water dips at 55±2°C for 3 and 2 minutes and 50±2°C for 3 minutes were 4.14, 1.84 and 1.83 times, respectively, and higher than in the untreated fruit. While, 15.21, 6.76 and 6.71 times of fruit treated with hot water dips at 55±2°C for 3 and 2 minutes and 50±2°C for 3 minutes, respectively, and higher than in the uninoculated fruit at 30 days (Table 4.8, Figure 4.29).

The present data show that HWT at 50±2°C for 3 minutes and HWT at 55±2°C for 2 and 3 minutes were effective in controlling green mold rot of tangerine fruit. A reduction in disease may indicate the expression of resistance induction. The results imply that HWT at 50±2°C for 3 minutes and HWT at 55±2°C for 2 and 3 minutes reduced disease index partly by inducing the accumulation of chitinase, β-1,3-glucanase and peroxidase in tangerine fruit over and above the stimulation of these enzymes in untreated and uninoculated fruit.

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 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). The results in this study indicated that HWT at 50±2°C for 3 minutes and HWT at 55±2°C for 2 and 3 minutes induces higher activities of chitinase and β-1,3-glucanase in tangerine fruit than the untreated and uninoculated fruit after storage for 15 days (Figure 4.29). 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 fruit, transient thermal

Table 4.8 Effect of hot water treatments (HWT) on peroxidase activity in flavedo tissues of artificially-inoculated tangerine fruit during 30 days storage at 4±2°C and 90±5% RH

Treatments	Peroxidase activity (unit/mg protein) Day of storage						
	0	5	10	15	20	25	30
Inoculation before HWT							
50°C 3 min	0.0275±0.0027^{c}	0.0242±0.0040^{b}	0.0251±0.0037^{a}	0.0235±0.0009^{c}	0.0219±0.0017^{d}	0.0100±0.0012^{c}	0.1047±0.0028^{b}
55°C 2 min	0.0366±0.0024^{ab}	0.0329±0.0059^{a}	0.0275±0.0023^{a}	0.0294±0.0009^{b}	0.0308±0.0014^{b}	0.0317±0.0133^{a}	0.1055±0.0162^{b}
55°C 3 min	0.0290±0.0061^{bc}	0.0249±0.0034^{b}	0.0254±0.0039^{a}	0.0207±0.0008^{d}	0.0262±0.0016^{c}	0.0257±0.0020^{ab}	0.2373±0.0273^{a}
Inoculation without HWT	0.0381±0.0053^{a}	0.0267±0.0035^{ab}	0.0267±0.0010^{a}	0.0316±0.0012^{a}	0.0392±0.0014^{a}	0.0182±0.0055^{bc}	0.0573±0.0029^{c}
Uninoculation	0.0187±0.0042^{d}	0.0127±0.0022^{c}	0.0130±0.0035^{b}	0.0148±0.0009^{e}	0.0143±0.0019^{e}	0.0098±0.0048^{c}	0.0156±0.0061^{d}
$LSD_{0.05}$	0.0079	0.0072	0.0031	0.0016	0.0028	0.0126	0.0265
C.V. (%)	33.33	41.15	42.55	41.67	65.36	52.36	13.58

[a-e]Means within the same column followed by different letters are significantly different at 95% ($P \leq 0.05$) level by Least Significant Difference Test. Data are mean values ±SD, (n=5).

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 fruit, which activity of peroxidase increased on day 30 as responses to the HWT (Figure 4.29). Generally, induction of chitinase, β-1,3-glucanase and peroxidase was found to be the strongest at the point of infection and decreased rapidly as the distance from the infection site increased (Metraux and Boller, 1986; Dore *et al*., 1991). The result of peroxidase enzyme activity in this study was contrary to mature green tomatoes which heat treatment had an effect on peroxidase activity and ability of the fruit tissue to withstand fungal attack (Lurie *et al*., 1997). Furthermore, it was also found that a hot water dip at 50°C for 40-50 seconds increased cucumber resistance to *Cladosporium cucumerinum* and peroxidase enzyme activity (Stermer and Hammerschmidt, 1984). In citrus, chitinase and β-1,3-glucanase seem to be involved in the enhancement of pathogen resistance. As a result, the hot water dip at 53°C for 2 minutes raised the level of chitinase (Rodov *et al*., 1996) and hot-water-rinsing and brushing (HWRB) treatment at 62°C for 20 seconds, induced the accumulation of 21, 22 and 25 kDa proteins that cross-reacted with citrus and tobacco chitinase antibodies and 38, 42 and 43 kDa proteins that cross-reacted with citrus and tobacco β-1,3-glucanase antibodies in grapefruit peel tissue. This suggests that the increases in the accumulation of glucanase and chitinase proteins may be part of the complex of fruit disease resistance mechanisms induced by the HWRB treatment (Pavoncello *et al*., 2001). Porat *et al*, also showed that a RNA gel blot hybridizations that the expression of the genes coding chitinase (2001) and β-1,3-glucanase (2002) were markedly induced by both HWRB as well as by UV illumination. However, these correlations cannot yet be interpreted to mean that they have causative relationship.

The Protein profiles extracted from flavedo tissues of tangerine fruit treated with hot water dips at 50±2°C for 3 minutes and 55±2°C for 2 and 3 minutes, then challenge-inoculated with *P. digitatum*, and compared with the control untreated and uninoculated fruit were separated on 10% SDS-PAGE gel and stained with CBB R-

250. The results showed that the protein patterns of tangerine fruit treated with hot water dips at 50±2°C for 3 minutes and 55±2°C for 2 and 3 minutes appeared the 112.20 and 100.00 kDa proteins only on the fifth day of storage and there were apparent differences in protein patterns or synthesis of novel proteins compared with untreated and uninoculated fruit. Moreover, we found that the protein patterns of tangerine fruit treated with hot water dips at 50±2°C for 3 minutes and 55±2°C for 2 and 3 minutes at 22.39 kDa exhibited thicker band compared to that of untreated and uninoculated fruit (Figure 4.30). Whereas on the other days of storage, there were no incidences in these protein patterns (the 112.20 and 100.00 kDa proteins) and no differences in protein patterns or synthesis of novel proteins for any of the heat treatments compared with untreated and uninoculated fruit.

The HWT was sufficient to administer a heat shock to the cells of the fruit peel tissue; as indicated by its capability to appear the 112.20 and 100.00 kDa proteins (Figure 4.30). 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, heat shock proteins (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 response is slower than other stress responses. The 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 literatures, it was reported that the 22.00 kDa protein is one of the chitinase isoform which is normally abundant in heat-stressed grapefruit (Mccollum *et al*., 1997; Pavoncello *et al*., 2001).

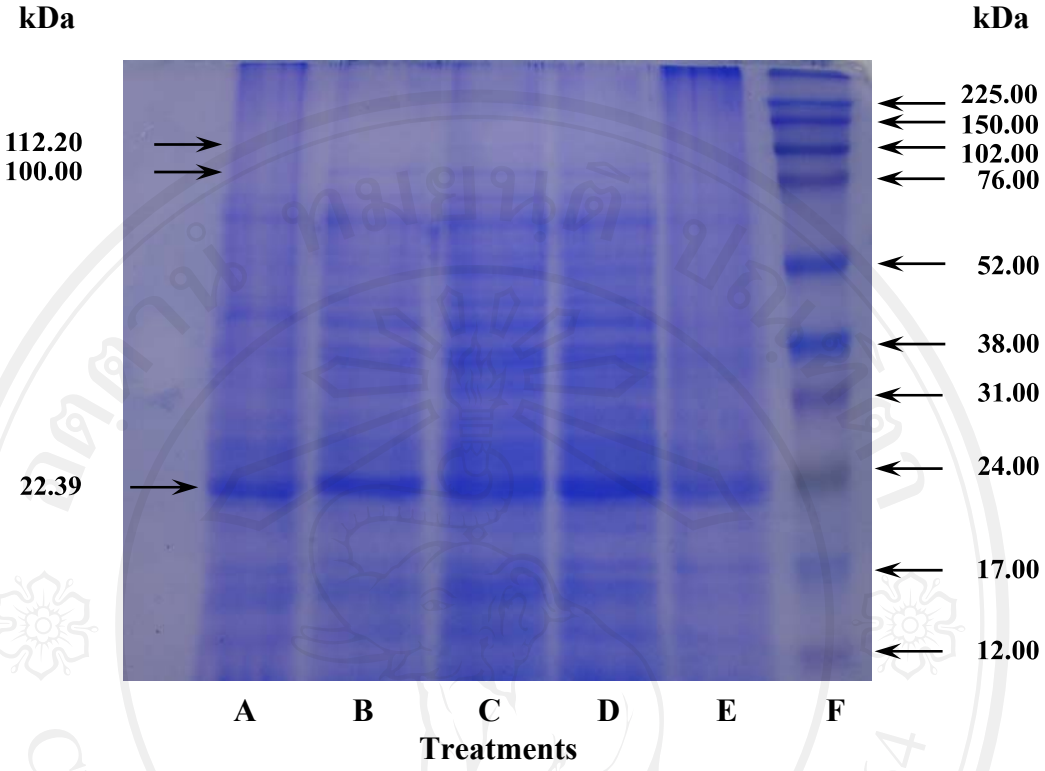

Figure 4.30 Protein bands from tangerine fruit peel tissue in response to hot water treatments (HWT) 5 days storage at 4±2°C and 90±5% RH by 10% SDS-PAGE

(B; HWT at 50±2°C for 3 minutes C; HWT at 55±2°C for 2 minutes D; HWT at 55±2°C for 3 minutes) appeared at 112.20 and 100 kDa and at 22.39 kDa showed thicker band compared with control treatments (A; uninoculated fruit E; untreated fruit) and (F; standard protein)

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*.

4.3 Effect of HWT on chemical component changes and chilling injury in tangerine fruit under low-temperature storage

Tangerine fruit were dipped in hot water at 50±2°C for 3 minutes and 55±2°C for 2 and 3 minutes and then stored at 2±2°C and 90±5% RH for 30 days. No visible symptoms of chilling injury were observed in all treatments for the entire storage period (Table 4.9, Figure 4.31 and 4.32). While, tangerine fruit are subjected to chilling injury when stored at temperatures below 4-7°C and 90-95% RH. The highest freezing temperature reported was -0.8°C (Ladaniya, 2002). In 'Nova' and 'Forture' mandarin fruit chilling injury symptoms appeared after 14 days of storage at 2.5°C (Sala, 1998). Symptoms of chilling injury of fruit associated with the changes in membrane permeability. Membrane permeability is an expression of the freedom that water and solutes can pass through the membrane. Increased permeability of membranes may cause the promotion of an enzyme-substrate interaction, resulting in the occurrence of scald, discoloration and browning. Permeability can be assessed by the measurement of the rate of leakage of solutes, including ions from the tissues (Wang, 1990). In the present study, it was found that electrolyte leakage in all treatments remained constant for the entire storage period, which varied between 35.50-47.54% without significant different (Table 4.10, Figure 4.33). The constant of percentage of electrolyte leakage correlated with no observed chilling injury symptoms of tangerine fruit in all treatments like mentioned above. Nonetheless, the application of HWT at 50±2°C for 3 minutes and 55±2°C for 2 and 3 minutes also had no effects on electrolyte leakage of tangerine fruit. Besides, Schirra *et al*. (1997) reported that no change in electrolyte leakage due to hot water dips of 'Tarocco' oranges at 53°C for 3 minutes.

Maintenance of membrane integrity at low temperature has been considered important in the resistance to low temperature (Saruyama *et al*., 2004). The chemical composition of the membranes, in particular the fatty acid composition, determines the temperature at which the membrane changes from the gel phase to the liquid crystalline phase. This transition is suggested to be the cause of loss of membrane

Table 4.9 Effect of hot water treatments (HWT) at various temperature and time on chilling injury index of tangerine fruit storage at 2±2°C and 90±5% RH for 20, 25 and 30 days

Treatments	Chilling injury index* Day of storage		
	20	**25**	**30**
HWT 50°C 3 min	0.67±0.00[1]	0.69±0.03	0.69±0.03
HWT 55°C 2 min	0.69±0.03	0.69±0.03	0.69±0.03
HWT 55°C 3 min	0.69±0.03	0.71±0.07	0.71±0.07
Control	0.67±0.00	0.69±0.03	0.69±0.03
$LSD_{0.05}$	0.04	0.09	0.09
C.V. (%)	3.60	6.89	6.89

*See materials and methods in 3.2.2.2.

[1]Means within the same column are not significantly different at 95% ($P \leq 0.05$) level by Least Significant Difference Test. Data are mean values ±SD, (n=30).

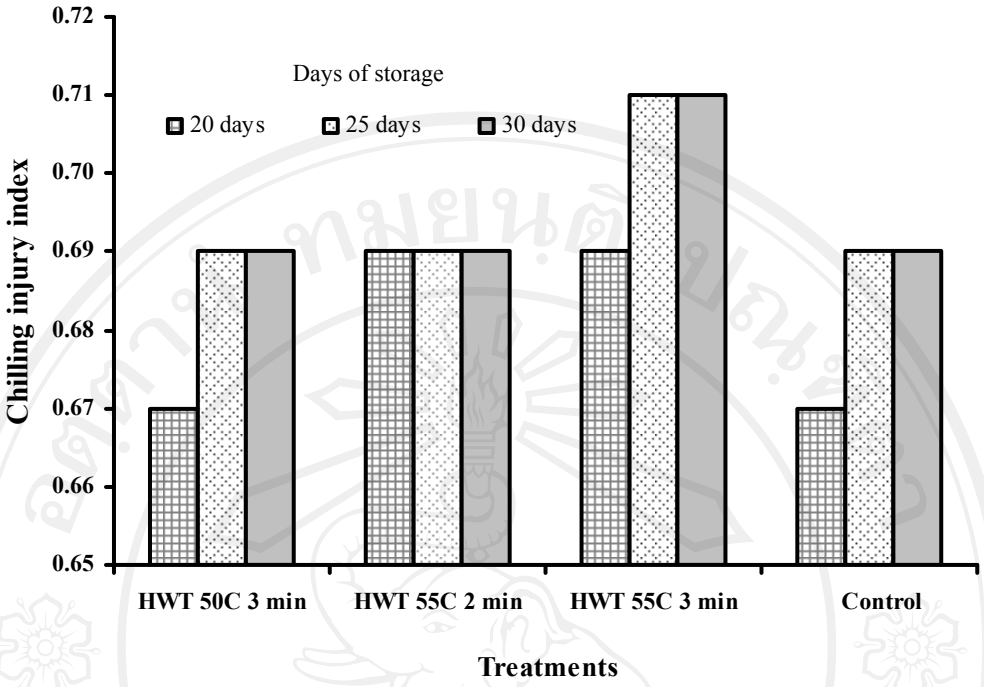

Figure 4.31 Effect of hot water treatments (HWT) at various temperature and time on chilling injury index of tangerine fruit storage at 2±2°C and 90±5% RH for 20, 25 and 30 days

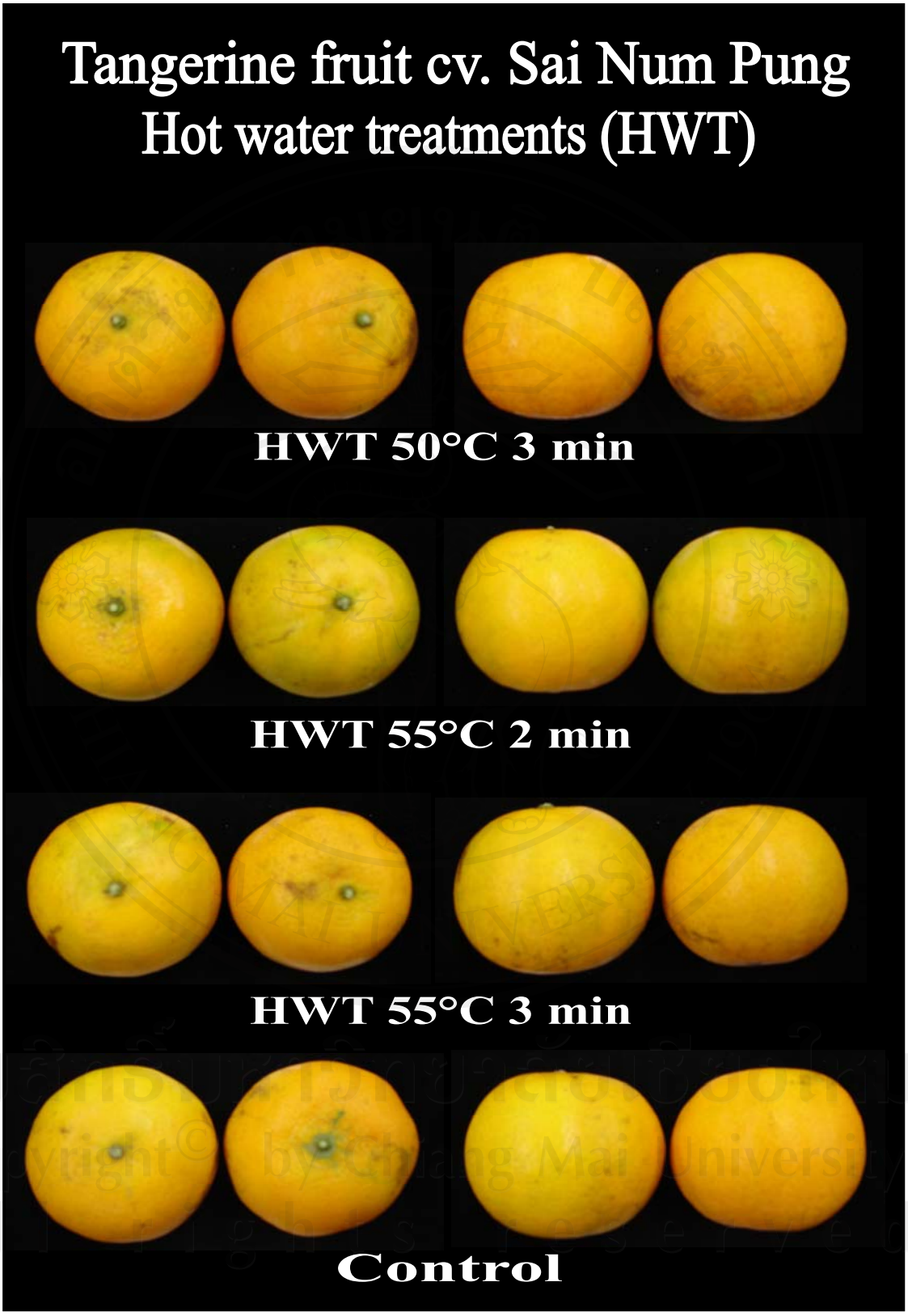

Figure 4.32 Effect of hot water treatments (HWT) at various temperature and time on chilling injury of tangerine fruit 30 days storage at 2±2°C and 90±5% RH, no chilling injury were found

Table 4.10 Effect of hot water treatments (HWT) on electrolyte leakage (EL) in flavedo tissues of tangerine fruit during 30 days storage at 2±2°C and 90±5% RH

Treatments	Electrolyte leakage (%)* Day of storage						
	0	5	10	15	20	25	30
HWT 50°C 3 min	41.17±2.40	40.93±3.88	38.83±4.82	41.52±2.23	45.31±2.86	45.22±4.00	39.20±4.11
HWT 55°C 2 min	42.12±1.73	37.26±8.79	40.81±4.66	44.52±2.57	44.18±6.20	43.19±2.69	38.62±3.14
HWT 55°C 3 min	42.44±4.64	39.64±5.57	41.62±3.26	42.95±1.74	47.54±4.09	45.99±4.09	45.01±7.24
Control	38.71±6.38	35.50±4.67	37.57±3.56	41.12±3.81	44.99±3.65	42.49±5.25	39.33±3.25
$LSD_{0.05}$	5.65	8.08	5.54	3.62	5.87	5.52	6.36
C.V. (%)	10.25	15.71	10.40	18.70	9.62	9.30	11.69

*Means within the same column are not significantly different at 95% ($P \leq 0.05$) level by Least Significant Difference Test. Data are mean values ±SD, (n=5).

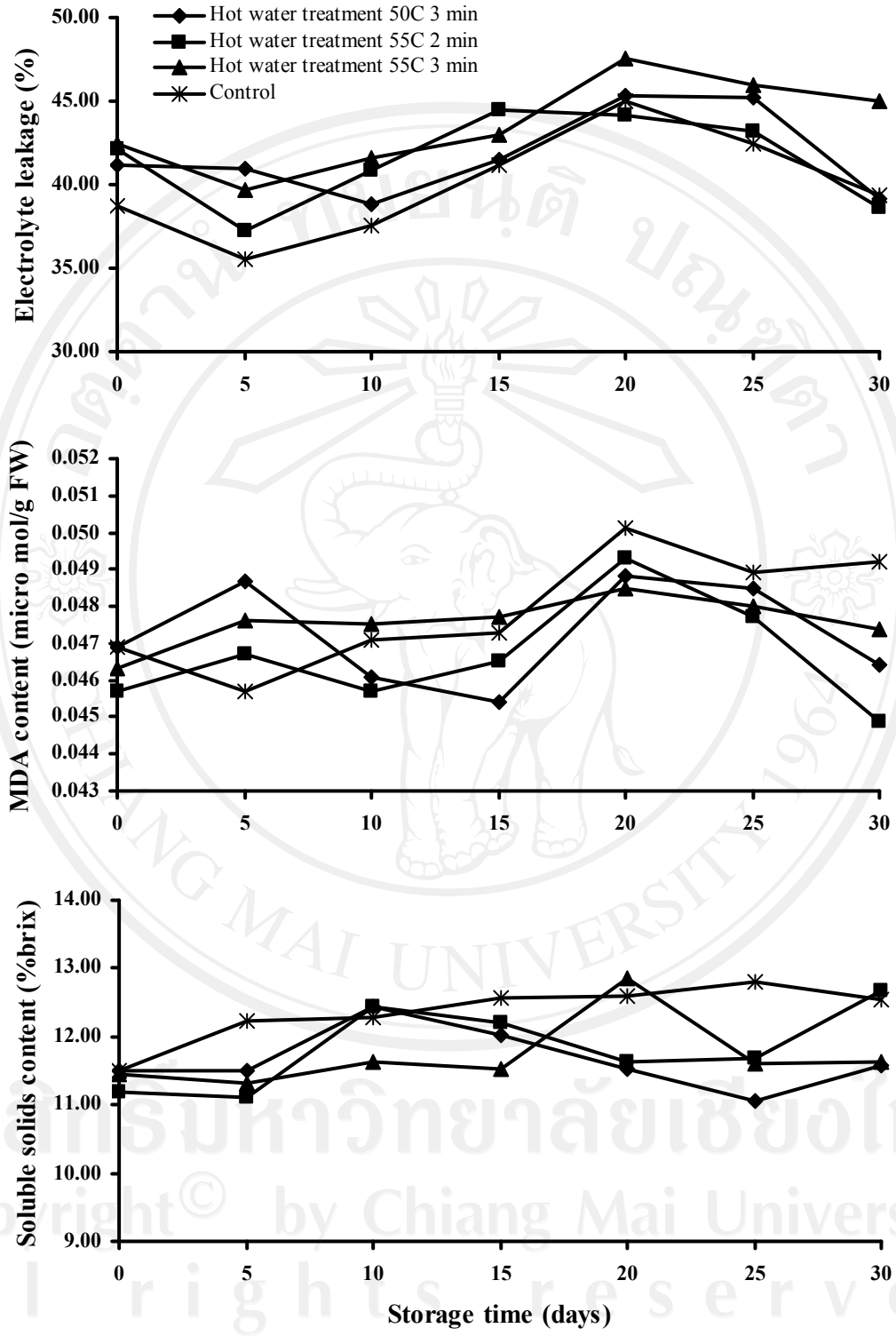

Figure 4.33 Effect of hot water treatments (HWT) on electrolyte leakage, malondialdehyde (MDA) and soluble solids content of tangerine fruit during 30 days storage at 0±2°C and 90±5% RH

semipermeability, and thus to loss of separate cell compartments. Chilling injury is also accompanied by lipid degradation. In coffee plant, for example, varieties with a high susceptibility to chilling injury showed the highest lipid degradation (Campos *et al*., 2003). Part of lipid breakdown can be due to lipoxygenase, which is often activated during chilling injury (Maalekuu *et al*., 2006). The most common lipoxygenase substrates in plants are linoleic acid and linolenic acid (Grechkin, 1998). The degradation of such polyunsaturated fatty acids results in peroxide ions and malondialdehyde. Accumulation of malondialdehyde is often taken as an indicator of chilling injury (Queiroz *et al*., 1998). In this study, the changes in malondialdehyde content correlated to trends with electrolyte leakage, which were remained constant for the entire storage period. Malondialdehyde contents in all treatments were varied between 0.0449-0.0501 μmol g^{-1} FW, without significant different (Table 4.11, Figure 4.33). In addition, it was found that tangerine fruit in all treatments had no lipoxygenase activity incidence, during low-temperature storage. Because there was no lipid degraded in membrane which related to malondialdehyde content. Lipoxygenase might be inactivated by the suppression of lipoxygenase gene expression. Whereas, lipoxygenase activity of chilled lemon fruit stored at 2±2°C and 90±5% RH for 10 days, had 5.18 unit mg^{-1} protein (measured by the same method). The result shown that 'Sai Num Pung' tangerine fruit might tolerate to low-temperature storage (2±2°C) due to no observed evidence related to chilling injury symptoms, electrolyte leakage and malondialdehyde occurrences. Cold stress tolerance is a complex quantitative characteristic (Guy, 1999). Further, it depends on factors such as time of harvesting (Schirra *et al*., 1998; Nordby and McDonald, 1995), the part of canopy from which the fruit was harvested (Nordby and McDonald, 1995), genotype (McDonald *et al*., 1991; Yuen and Tridjaja, 1995) and maturity (Lafuente *et al*., 1997). Among mandarins there are cultivars tolerant to low-temperature storage, such as 'Clemenules' and 'Clementine' (Martínez-Jávega and Cuquerella, 1984; Puppo *et al*., 1988; Martínez-Jávega *et al*., 1991), whereas other cultivar such as 'Nova' and ' Forture' are susceptible to chilling injury (Cuquerella *et al*., 1990; Martínez-Jávega *et al*., 1991, 1992; Sala, 1998).

Table 4.11 Effect of hot water treatments (HWT) on malondialdehyde (MDA) concentration in flavedo tissues of tangerine fruit during 30 days storage at 2±2°C and 90±5% RH

Treatments	Malondialdehyde concentration (μmol g^{-1} FW)* Day of storage						
	0	5	10	15	20	25	30
HWT 50°C 3 min	0.0469±0.0032	0.0487±0.0051	0.0461±0.0052	0.0454±0.0048	0.0488±0.0067	0.0485±0.0087	0.0464±0.0040
HWT 55°C 2 min	0.0457±0.0057	0.0467±0.0043	0.0457±0.0033	0.0465±0.0050	0.0493±0.0070	0.0477±0.0044	0.0449±0.0041
HWT 55°C 3 min	0.0463±0.0074	0.0476±0.0036	0.0475±0.0068	0.0477±0.0058	0.0485±0.0061	0.0480±0.0061	0.0474±0.0053
Control	0.0469±0.0064	0.0457±0.0051	0.0471±0.0036	0.0473±0.0045	0.0501±0.0081	0.0489±0.0049	0.0492±0.0047
$LSD_{0.05}$	0.0042	0.0034	0.0037	0.0037	0.0051	0.0045	0.0034
C.V. (%)	8.60	5.60	10.29	10.49	11.49	8.54	10.20

*Means within the same column are not significantly different at 95% ($P \leq 0.05$) level by Least Significant Difference Test. Data are mean values ±SD, (n=5).

Dipping tangerine fruit in hot water at 50±2°C for 3 minutes and 55±2°C for 2 and 3 minutes had no effects on soluble solids content when stored at 2±2°C and 90±5% RH for 30 days. The soluble solids content in all treatments remained constant for the entire storage period, which varied between 11.06-12.86% with not significant different (Table 4.12, Figure 4.33). This result accorded with Schirra *et al*. (1997), who reported that dipping 'Tarocco' oranges in hot water at 53°C for 3 minutes did not influence the total soluble solids and total acid. Hot water brushing at 56°C for 2 seconds did not affect juice total soluble solids and total acid in 'Minneola' tangerines, 'Shamouti' oranges and 'Star Ruby' grapefruit (Porat *et al*., 2000). Similarly, no measurable effects on fruit quality parameters were reported after hot water dips (Schirra and Mulas, 1995; Schirra *et al*., 1997).

Table 4.12 Effect of hot water treatments (HWT) on soluble solids content (SSC) of tangerine fruit during 30 days storage at 2±2°C and 90±5% RH

Treatments	Soluble solids content (% brix)* Day of storage						
	0	5	10	15	20	25	30
HWT 50°C 3 min	11.50±1.32	11.50±0.49	12.44±0.79	12.02±0.85	11.52±0.92	11.06±1.18	11.58±1.08
HWT 55°C 2 min	11.20±1.35	11.12±0.72	12.44±0.42	12.20±0.55	11.62±0.45	11.68±1.39	12.68±0.61
HWT 55°C 3 min	11.46±1.13	11.32±0.58	11.64±0.99	11.52±1.22	12.86±1.11	11.60±1.11	11.62±0.31
Control	11.50±0.81	12.22±0.89	12.28±0.74	12.58±1.54	12.60±0.90	12.80±0.73	12.54±0.99
$LSD_{0.05}$	1.57	0.92	1.02	1.48	1.18	1.51	1.08
C.V. (%)	10.26	5.94	6.24	9.17	7.25	9.59	6.68

*Means within the same column are not significantly different at 95% ($P \leq 0.05$) level by Least Significant Difference Test. Data are mean values ±SD, (n=5).