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Pretreatments at 38 °C of 'Hass' Avocado Confer Thermotolerance to 50 °C Hot Water Treatments

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Abstract. **'Hass' avocados [*Persea americana* Mill.] were pretreated in water (38 °C for up to 120 min) immediately before 50 °C hot water treatments of up to 10 min. Fruit were stored for 1 week at 6 °C and ripened at 20 °C. External browning was evaluated immediately upon removal from cold storage, and fruit quality evaluated when fruit were ripe. Pretreatments at 38 °C tended to reduce the levels of external browning, skin hardening, and internal disorders, such as tissue breakdown and body rots, that were associated, and increased, with longer hot water treatments. A pretreatment of 60 min was the most effective for eliminating external browning, and reducing hardening of the skin when fruit were ripe following hot water treatment. Examination of heat shock protein (hsp) gene expression in avocado skin tissue, showed that levels of hsp17 and hsp70 homologous mRNA increased with increasing pretreatment duration. The results demonstrate that 38 °C pretreatments increase the tolerance of avocado fruit to subsequent hot water treatments.**

Some leafroller species, including *Ctenopseusitis obliquana* (Walker), *C. herana* (Felder and Rogenhofer), and *Epiphyas postvittana* (Walker), restrict market access to New Zealand's expanding avocado crop (P. Stevens and D. Steven, pers. comm.). At present, methyl bromide fumigation is used to disinfest avocado fruit. Methyl bromide has deleterious effects on avocado fruit quality (Ito and Hamilton, 1980) and this, along with international initiatives to limit its use due to health and environmental issues (UNEP, 1995), means there is a need to develop alternative disinfestation techniques for this crop.

Heat treatments (HTs) have potential for killing various pest species. Hot air HT is being used commercially in Hawaii to disinfest crops such as papaya (*Carica papaya* L.; Paull, 1990). New Zealand avocado fruit ('Hass') tolerance to air HTs (0.5 h at 40 °C), are well short of the conditions required for effective disinfestation of many of the pests found on this crop in New Zealand (Woolf et al., 1995). For example, >19 h at 40 °C is required for control of *E. postvittana* (Birtles et al., 1991). Sanxter et al. (1994) reported that 38 °C hot air treatments followed by storage at 1.1 °C for 14 days showed potential as a fruit fly disinfestation treatment for 'Sharwil' avocados, but some deleterious effects on fruit quality were observed.

An alternative to hot air HT is hot water treatment (HWT) and fruit response has been examined on a range of crops including mango (*Mangifera indica* L.; Joyce and Shorter, 1994), citrus (*Citrus paradisi* Macf.; McGuire, 1991) and a range of peach, plum and nectarines (*Prunus* sps; Sharp, 1990). For avocado, immersion in 50 °C water for 3 min with 0.05% benomyl (Methy 1-(butylcarbamoyl)-2-benzimidazolecarbamate), followed by 20 days storage at 1 °C, controlled *Bactrocera tryoni* (Froggatt) (Jessup, 1991). However, Jessup (1994) reported in a later study that to maintain fruit quality, the HWT temperature had to be reduced to 46 °C. The most heat tolerance pest of New Zealand avocados is *Epiphyas postvittana* (Jones et al., 1995). The predicted time for 99% mortality of the most heat tolerant stage of *E. postvittana* (fifth instar) when infesting the surface of fruit is 7.2 min in 50 °C water (Lester et al., 1995). However, Hopkirk et al. (1991) reported that a HWT of 50 °C for 5 min results in high levels of skin browning and rots. Thus, for HWTs to become practical for disinfestation of at least *E. postvittana* on New Zealand avocados, the tolerance of the fruit to such treatments needs to be enhanced.

Pretreatments (or "preconditioning") using hot air or water have been found to increase tolerance to subsequent HTs in papaya (Couey and Hayes, 1986; Paull and Chen, 1990), cucumber (*Cucumis sativus* L.; Chan and Linse, 1989), and mango (Joyce and Shorter, 1994). Paull and Chen (1990) reported that thermotolerance of papaya was increased by a 1-h 38 °C water pretreatment followed by 3 h in air at 22 °C prior to a 49 °C HWT. They also reported that a 38 °C water treatment for 2 h induced the production of heat shock proteins (hsps). Hsps have also been found in hot air treated apples (*Malus domestica* Borkh.; Lurie and Klein, 1990) and tomato (*Lycopersicon esculentum*, Mill.; Lurie and Klein, 1991). Expression of hsp genes, for example hsp17 and hsp70 (members of gene families associated with the heat response in a range of organisms; Vierling, 1991), is induced in heat-treated tomato (Lurie et al., 1993) and avocado fruit flesh (Woolf et al., 1995). Hsps have been reported to be associated with induced thermotolerance in various organisms (Parsell and Lindquist, 1993).

The objective of this work was to determine whether pretreating fruit at 38 °C can reduce damage to avocado fruit associated with 50 °C hot water treatments, and, because of a possible role for hsps in acquired thermotolerance, we examined hsp gene expression in response to pretreatments.

Materials and Methods

Fruit. 'Hass' avocado fruit (export grade, 190 to 220 g) were obtained from a commercial orchard in Whangarei, New Zealand, held overnight at ambient packhouse temperatures (13 to 17 °C) then graded and packed. Fruit were transported from the packhouse 1 day after packing and arrived at the laboratory early on the morning of treatment (2 days after harvest). Before treatment, fruit were further graded for freedom from blemishes and damage. All fruit handling, HWTs and fruit ripening were carried out in a controlled environment room (20 ± 2 °C; relative humidity 65% ± 10%).

Water baths. Hot water treatments were carried out in water baths consisting of fiberglass tubs (volume 82 L) with Grant temperature controller units (±0.1 °C, 1.4 kW heater; model GRAVF, U.K.) with the stirrer blade removed. Uniform water distribution, and thus temperature within the baths, was achieved by pumping water (model UPS 20-60B 150; Grundfos pumps, Denmark) recirculated past the heater, through perforated PVC tubing (22-mm ID) arranged in a grid pattern in the base of the baths. Additional heating at the time of fruit immersion was provided using manually operated Eutron (Auckland, New Zealand) water heaters (2.3 kW) switched on 0.5 min before, and off 1 min after, fruit immersion. This system resulted in water temperatures varying by no more than ±0.2 °C spatially throughout the baths, or over time.

Hot water treatment. Fruit were pretreated at 38 °C for 0, 5, 15, 30, 60, or 120 min in water and then immediately subjected to HWTs of 50 °C for 0, 0.5, 1, 3, 5, 7.5, or 10 min. Each treatment combination consisted of seven fruit placed in plastic netting baskets that were weighted to submerge fruit during hot water treatments. The entire experiment was carried out twice on the same day with the same fruit lot.

After treatment, fruit were manually dried with cotton towels, placed in open commercial trays and into storage at 6 ± 0.5 °C within 5 min

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of treatment along with control fruit. The following day, trays were closed and fruit stored at 6 °C for a further week to simulate fruit transport. Upon removal from storage, external browning was rated on a relative scale of 0 to 3 (0 = none, 0.5 = <10%, 1.0 = 10% to 20%, 1.5 = 21% to 50%, 2 = 51% to 75%, 2.5 = 76% to 90%, 3.0 = >90% of the fruit surface). Fruit were then held in a 20 ± 2 °C controlled environment room and allowed to ripen. Fruit ripeness was measured by flesh firmness, as assessed daily by gentle hand-squeezing by one trained assessor. When each fruit became "ripe to eat" [equivalent to a "firmometer" (Swarts, 1981) reading of ≈99], the number of days to become ripe was recorded (shelf life). The fruit was cut longitudinally into quarters, and the following factors evaluated: hard skin (hardness or brittleness of the skin determined when cutting and peeling back the skin), tissue breakdown (breakdown of emerald green flesh adjacent to the skin such that tissue adhered to the skin when peeled), body rots (rots entering through the skin), stem-end rots (rots entering only through the fruit peduncle), uneven ripening (uneven flesh softening such that flesh tissue adhered to the seed when fruit was cut in half), vascular browning (browning of the vascular strands running vertically through the fruit tissue), and flesh browning (browning of the fruit tissue not due to disease or vascular browning). Each factor was rated on a scale of 0 to 3 (Hopkirk et al., 1994) where 0 = no occurrence; 1 = slight; 2 = a level at which the consumer would notice, and possibly reject the fruit; 3 = severe. An overall fruit quality rating (% sound) was calculated as the percentage of fruit with no damage that would be detectable by the consumer (i.e., no factor with a severity rating >1).

RNA extraction and Northern analysis. Skin disks were taken from fruit exposed to the range of 38 °C pretreatments as described above. Tissue plugs were removed using a 10-mm cork borer, and skin disks were cut from the plug with a scalpel just above the line where the skin would normally separate from the flesh tissue when ripe. The skin disks were frozen in liquid N within 10 min after removal of fruit from the water baths, and stored at –80 °C. Total RNA was extracted using the method of Lopez-Gomez and Gomez-Lim (1992), denatured, fractionated (30 µg/lane), and transferred to a Hybond-N+ (Amersham, Buckinghamshire, U.K.) membrane as described by Veierskov et al. (1992). The cDNA inserts from pMON9575 (petunia hsp70 cDNA; Winter et al., 1988) and pFS1968 (soybean hsp17 cDNA; Schoffl et al., 1984) were labeled with ^{32}P using a random primers DNA labeling system (BRL). Hybridization was carried out according to the Amersham Hybond-N+ protocol. Following hybridization, the membrane was washed twice for 10 min in 2 × SSC (3 M NaCl and 0.3 M Na Citrate, pH 7.0), 0.1% details SDS (Na dodecylsulfate) at room temperature, then once in 1 × SSC, 0.1% SDS at 65 °C for 15 min. The membrane was exposed to Kodak XAR film with intensifying screens at –80 °C.

Statistics analysis. The design had two factors as follows: pretreatment time, with six levels (0 to 120 min), and time of 50 °C HWT, with seven levels (0 to 10 min). There were seven fruit per treatment replication, and the experiment was carried out twice with the same batch of fruit. We analyzed the data as a randomized block design, fitting cubic orthogonal polynomial contrasts, and their interactions, in both factors. Data are presented as average severity (average of the attribute rating for replications), and as percent incidence (percentage of treatment replications with attribute rated unacceptable to a consumer; i.e., severity rating >1). Percent incidence data were transformed using an angular transformation [arcsin(sqrt(X))], and transformed means are presented.

Results

Fruit quality. Immediately after cold storage, external damage associated with HWTs was evident as skin browning/blackening that became more distinctly brown or "bronzed" as fruit ripened. In nonpretreated fruit (0 min at 38 °C), 50 °C HWTs resulted in increased severity of skin browning with a peak level at 1 and 3 min 50 °C (Table 1). The severity of skin browning appeared to decrease with longer 50 °C treatments, such that a 10 min 50 °C HWT had a similar severity rating as a 0.5-min treatment. The incidence of external browning (i.e., percent fruit exhibiting a severity rating >1), followed a similar pattern to that of the severity data, with more than 61% and 58% incidence following HWTs of 1 and 3 min, respectively, without pretreatment (Table 1).

In general, water pretreatments at 38 °C reduced both the severity and incidence of external browning of avocado fruit associated with 50 °C HWTs. Increasing pretreatment duration (from 5 to 60 min) progressively reduced external browning in response to HWTs, with a 60-min pretreatment virtually eliminating the incidence of browning in response to all HWT durations (average severity of <0.5; Table 1). External browning following the 120-min pretreatment was similar to the 60-min pretreatment, but following the 5-, 7.5-, and 10-min HWTs, there was a slight increase in severity of browning relative to that found in the 60-min pretreatment.

Pretreatment of fruit at 38 °C for 60 or 120 min also reduced skin hardening (evident when fruit were ripe) induced by 50 °C HWTs. Without pretreatment, skin hardening increased with longer HWT duration to a maximum for the 7.5-min treatment with severity of 2.4, and incidence of 79% (Table 1). Of the pretreatments tested, 60 min was most effective in reducing skin hardening following HWTs for durations up to 7.5 min. A pretreatment of 120 min was slightly less effective. Shorter pretreatment durations (5 to 30 min) had little to no effect. Pretreatment on its own (38 °C with no subsequent 50 °C HWTs) did not increase the severity or incidence of external browning or skin hardening relative to nontreated controls (Table 1).

When no pretreatment was employed, tissue breakdown increased with longer duration of 50 °C HWT to a maximum at 10 min (Table 1). Pretreatments tended to reduce tissue breakdown, with 60 min being most effective. Pretreatment with no subsequent 50 °C HWTs had very little effect on tissue breakdown relative to nontreated controls.

The level of body rots was high in nontreated controls (Table 1). With HWT, both severity and incidence of body rots increased with longer HWT duration up to 3 min and then remained relatively constant. Pretreatment for 60 min tended to reduce the severity and incidence of body rots relative to nonpretreated hot water treated fruit and resulted in fruit with incidence of body rots similar to that of control fruit (40% to 50%), with the exception of the 7.5 min HWT, which had an incidence of 63%.

In contrast to body rots, the severity and incidence of stem end rots was low in control fruit (0.75% and 27%, respectively, data not shown). For nonpretreated fruit, HWTs increased the severity and incidence of stem end rots to 1.5% and 50%, respectively, after 7.5 min at 50 °C. Pretreatments had little consistent influence on stem-end rots (data not shown). However, a 60-min pretreatment before all 50 °C HWTs maintained stem-end rot incidence at levels lower than for the controls, with the exception of the 7.5- and 10-min HWTs (incidence of 37% and 32%, respectively).

The incidence of uneven ripening and vascular and flesh browning was low and contributed little to the reduction in the number of sound fruit. Days to eating ripeness varied only slightly with HWT and pretreatment duration, ranging from 6.8 to 8.7 d depending on treatment (data not shown).

With nontreated control fruit, i.e., fruit not treated in either 38 or 50 °C water, the percent sound fruit was 34%, with rots being the main contributor to reduction in fruit quality. Hot water treatments of ≥1 min resulted in <16% sound fruit (Table 1). Pretreatments of 15 and 30 min at 38 °C provided some improvement in overall fruit quality following HWTs, particularly at shorter durations (0.5 and 1 min). However, only the 60-min pretreatment consistently maintained a level of sound fruit similar to that found with nontreated control fruit.

The levels of RNA homologous to pFS1968 cDNA (hsp17) were low in nonheated skin tissue and were elevated when sampled directly after 38 °C pretreatments (Fig. 1). Levels increased progressively with durations of 5 min and longer and were highest after a 120-min pretreatment. Levels of RNA hybridizing to pMON9575 (hsp70) exhibited a similar pattern (data not shown).

Discussion

A water pretreatment at 38 °C for 60 min significantly reduced the incidence and severity of external browning and skin hardening of fruit caused by HWTs of 0.5- to 5-min duration. This pretreatment effect is similar to that found in papaya where increased tolerance to

49 °C HWTs is imparted by previous pretreatments at 38 to 42 °C (Paull and Chen, 1990). Similarly, Chan and Linse (1989), working with cucumbers, found that exposure to 32.5 °C air for 24 h enhanced fruit tolerance to 45 °C HWTs from 30 min to at least 60 min. Joyce and Shorter (1994) also noted some beneficial effects of 37 °C air pretreatments on external damage following 47 °C HWTs of mango.

Pretreatments at 38 °C were associated with increased levels of hsp17 and hsp70 homologous mRNA in the skin relative to nonheated controls (Fig. 1). This pattern of increase corresponded to reduced heat damage to the skin (browning and hardening when ripe; Table 1) and to the flesh tissue adjacent to the skin (tissue breakdown; Table 1). Since 50 °C HWTs were applied immediately following 38 °C pretreatments, the levels of hsps present directly following pretreatment are those present at the time of exposure to 50 °C HWTs, and thus, may be acting to protect the tissue from heat damage. Induction of hsp gene expression has also been found to be associated with acquired thermotolerance in other plant tissues (Paull and Chen, 1990; Vierling, 1991).

Table 1. Response of 'Hass' avocado fruit to 38 °C water pretreatments (0, 5, 15, 30, 60, or 120 min) followed immediately by 50 °C hot water treatment (HWT) for 0, 0.5, 1, 3, 5, 7.5, or 10 min. Following treatment, fruit were stored for 1 week at 6 °C and then ripened at 20 °C. Factors were rated on a scale of 0 to 3 (0 = none to 3 = severe) and data are presented as severity (average severity rating) and incidence (percent fruit with severity ratings >1). Incidence data are presented in the transformed form.

		Fruit characteristics								
HWT	Pretreatment	External damage		Hard skin		Tissue breakdown		Body rots		
duration (min)	duration (min)	Severity (rating)	Incidence (%)	Severity (rating)	Incidence (%)	Severity (rating)	Incidence (%)	Severity (rating)	Incidence (%)	Sound (%)
0	0	0.0	0	0.0	0	0.1	8	1.6	44	34
	5	0.1	0	0.0	0	0.0	0	0.9	27	49
	15	0.0	0	0.0	0	0.3	16	1.4	37	41
	30	0.0	0	0.0	0	0.2	11	1.3	36	36
	60	0.1	0	0.1	0	0.2	11	1.4	41	32
	120	0.1	0	0.0	0	0.4	20	1.7	56	25
0.5	0	0.4	11	0.2	11	0.4	16	1.4	41	41
	5	1.0	22	0.1	11	0.6	27	1.6	45	37
	15	0.1	0	0.1	11	0.6	16	1.7	45	41
	30	0.2	0	0.0	0	0.0	0	1.5	45	45
	60	0.0	0	0.0	0	0.1	0	1.6	45	41
	120	0.1	0	0.4	11	0.6	20	1.9	56	20
1	0	2.0	61	0.7	32	0.8	25	1.9	54	11
	5	1.9	53	0.6	16	0.4	16	1.7	41	27
	15	0.4	0	0.0	0	0.6	22	1.5	41	41
	30	0.1	0	0.1	0	0.4	22	1.2	32	49
	60	0.5	11	0.0	0	0.0	0	1.6	41	32
	120	0.2	0	0.4	16	0.6	27	2.4	63	22
3	0	2.1	58	1.2	45	0.6	27	2.4	74	0
	5	1.4	37	1.2	40	1.4	45	2.9	90	0
	15	1.1	20	1.1	41	0.7	27	2.4	58	27
	30	1.0	25	0.8	20	1.0	29	2.3	65	25
	60	0.3	0	0.4	11	0.1	11	1.6	45	41
	120	0.1	0	0.6	11	0.1	0	1.9	53	20
5	0	1.0	11	1.1	32	1.5	45	2.5	70	16
	5	0.6	11	1.4	45	1.4	45	2.6	79	0
	15	0.5	0	1.9	58	0.8	25	2.4	70	16
	30	0.5	0	1.0	37	1.1	29	2.2	58	16
	60	0.1	0	0.3	11	0.2	11	1.7	49	32
	120	0.7	0	0.6	20	1.1	37	2.9	90	0
7.5	0	0.4	0	2.4	79	1.8	50	2.6	74	0
	5	0.4	0	2.0	54	2.1	53	2.8	79	0
	15	0.2	0	2.0	58	2.1	58	2.6	74	16
	30	0.5	0	1.8	53	1.3	37	2.6	68	11
	60	0.2	0	0.6	16	0.9	36	2.2	63	27
	120	0.5	0	1.2	45	0.9	32	2.3	58	11
10	0	0.3	0	2.0	63	2.1	58	2.5	79	11
	5	0.2	0	1.9	58	2.4	74	2.9	90	0
	15	0.3	0	1.5	50	1.7	50	3.0	90	0
	30	0.4	0	2.1	58	2.0	53	2.8	79	0
	60	0.2	0	1.6	53	0.6	27	1.9	49	27
	120	0.5	0	1.4	49	0.9	32	2.9	79	11
$LSD_{0.05}$[z]		0.6	21	0.7	22	1.2	33	0.9	33	29
Significance										
HWT		*	*	***	***	***	***	***	***	***
L		NS	NS	***	***	***	***	***	***	***
Q		***	NS	***	*	NS	NS	*	*	*
C		***	NS	NS	NS	NS	NS	NS	NS	NS
Pretreatment		***	***	***	***	NS	NS	***	*	*
L		***	***	***	***	*	NS	NS	NS	NS
Q		***	***	***	***	*	NS	***	***	***
C		NS	*	NS	NS	NS	NS	***	NS	NS
HWT × pretreatment		***	***	***	NS	NS	NS	NS	NS	NS
L × L		***	*	NS	NS	NS	NS	NS	NS	NS
Q × L		***	*	NS	NS	NS	NS	NS	NS	NS
L × Q		NS	*	NS	NS	NS	NS	NS	NS	NS

[z]LSD = least significant difference for the two-way interaction at $P \leq 0.05$.

NS, *, ***Nonsignificant or significant at the $P \leq 0.05$ or 0.001, respectively; L = linear, Q = quadratic, C = cubic.

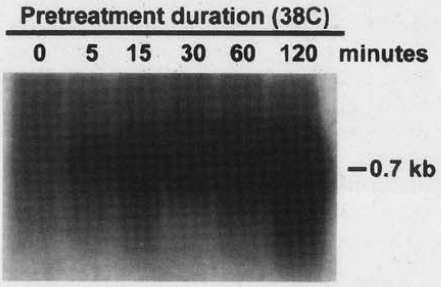

Fig. 1. Northern analysis of total RNA extracted from avocado skin sampled directly (<10 min) after pretreatments at 38 °C for the given durations. An equal amount of RNA (30 µg) was loaded on each lane, and hybridized with 32P-labeled insert of pFS1968 (soybean hsp17 cDNA; Schoffl et al., 1984).

Hot water treatments were associated with a range of damage symptoms in avocado fruit. The main form of heat damage was browning or "bronzing" of the skin, which did not markedly differ from the natural ripening color of 'Hass' (purple-black). However, this browning, which occurs at a stage when fruit are unripe, is commercially unacceptable since consumers use color change as an indicator of the onset of ripening. The nature of the heat-induced browning of avocado skin is, as yet, unknown, although possible mechanisms such as membrane damage and activity of browning enzymes have been suggested (Woolf and Laing, 1996). Interestingly, following storage, severity of skin browning first increased, then decreased as duration of HWT increased. Subsequent work has shown that in fruit not cold stored following 50 °C HWTs for 10 min, damage development is only delayed rather than reduced (Woolf and Laing, 1996). This result suggests that the lack of external browning observed following the 10-min HWT used in the present experiment (where external browning was rated immediately after cold storage), may be due to a delay rather than elimination of browning development.

The incidence of body rots was high in non-hot-water-treated fruit, reflecting the characteristically high levels of postharvest rots that develop in New Zealand avocados (Hopkirk et al., 1994). Hot water treatments of >3 min were associated with an increase in disease incidence, possibly as the result of decay organisms invading through damaged skin tissue (Chan and Linse, 1989). A 60-min 38 °C pretreatment reduced the incidence of rots associated with 50 °C HWTs >3 min to levels similar to those found in nontreated control fruit, possibly through reducing skin damage and thus the opportunity for disease entry. In addition, further research has indicated that application of the fungicide Sportak 45EC (Prochloraz; *N*-propyl-*N*-[2-(2,4,6-trichlorophenoxy) ethyl] imidazole-1-carboxamide; Nufarm Agricultural Chemicals, Auckland, New Zealand) with the HWT significantly reduced the incidence of rots relative to nonfungicide-treated fruit (unpublished data).

This initial examination of the response of avocados to 38 °C pretreatments and 50 °C HWTs has demonstrated that pretreatments can impart significant levels of thermotolerance to avocados subsequently treated at 50 °C. However, although fruit thermotolerance is increased, further research is required to improve fruit quality at durations effective as disinfestation treatments.

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