# VAPOUR HEAT TREATMENT OF 'HASS' AND 'FUERTE' AVOCADO (*Persea americana* Mill.) FRUIT FOR EXTENDING STORAGE LIFE

By

Philip Lawrence Weller

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# ABSTRACT

In an attempt to extend storage life of 'Hass' and 'Fuerte' avocado (*Persea americana* Mill.) fruit postharvest vapour heat treatments were used. Vapour heat treatment temperature regimes were 36, 38, 40 and 42°C for either 1, 2, 4 or 8 hours. After vapour heat treatment, fruit were cold stored at 3.5°C, for 5 or 6 weeks each. On removal from cold storage fruit were evaluated for firmness, and ripened at room temperature. Once ripe fruit were evaluated for heat/cold damage, days to ripening, weight loss and physiological disorders. These experiments were conducted during the 1996 and 1997 'Fuerte' and 'Hass' avocado seasons.

Electron microscope analysis of vapour heat treated fruit revealed heat damage to the epidermis and fruit organelles of certain treatments. The effect of vapour heat treatment on protein synthesis on 'Fuerte' and 'Hass' avocado was investigated using gel electrophoresis, to determine if presumed heat shock proteins were synthesized under the treatment conditions. The activity of pectin methyl esterase was also investigated on the heat treated 'Fuerte' and 'Hass' avocados, to investigate if heat alters the activity of this enzyme.

All experiments showed that vapour heat treatment extended 'Fuerte' and 'Hass' avocado storage and shelf life. Evaluation of the time/temperature combination for vapour heat treatment for each cultivar indicated that extension of shelf life was, in some cases, limited by rind heat damage. For the 1996 season, the best time/temperature combination for 'Fuerte' was at 38°C between 4 to 8 hours, 40°C between 4 to 8 hours and 42°C between 2 to 4 hours. For 'Hass', best results were achieved at 38°C between 4 to 8 hours. For the 1997 season, 'Fuerte's best time/temperature combination were at 36°C for 8 hours, 42°C between 1 to 2 hours and marginal results at 38°C between 4 to 8 hours. For 'Hass' the best time/temperature combination were achieved at 38°C and 40°C for 8 hours. Analysis of fruit flesh protein indicated changes associated with vapour heat treatment, and even induction of new proteins, perhaps heat shock proteins, giving 'Fuerte' and 'Hass' avocados beneficial characteristics such as an extension of shelf life. There was a decline in activity of pectin methyl esterase after vapour heat treatment, which was associated with a longer shelf life.

# DECLARATION

I hereby declare that the research work reported in this thesis is as a result of my own investigations, except where acknowledged.

Signed *P. L. Weller*

Philip Lawrence Weller

# ACKNOWLEDGEMENTS

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# INTRODUCTION

The South African avocado industry has a long history of relatively successful exports, mainly to Europe. However, increasing competition and below expected cropping, especially in the 1990's following several years of severe drought, have increased the vulnerability of the industry. In fact the South African share of the avocado European market has declined over the last three years. Data for France (Fig. 1), Germany (Fig. 2) and The Netherlands (Fig. 3) illustrate this trend over three seasons of admittedly low cropping.
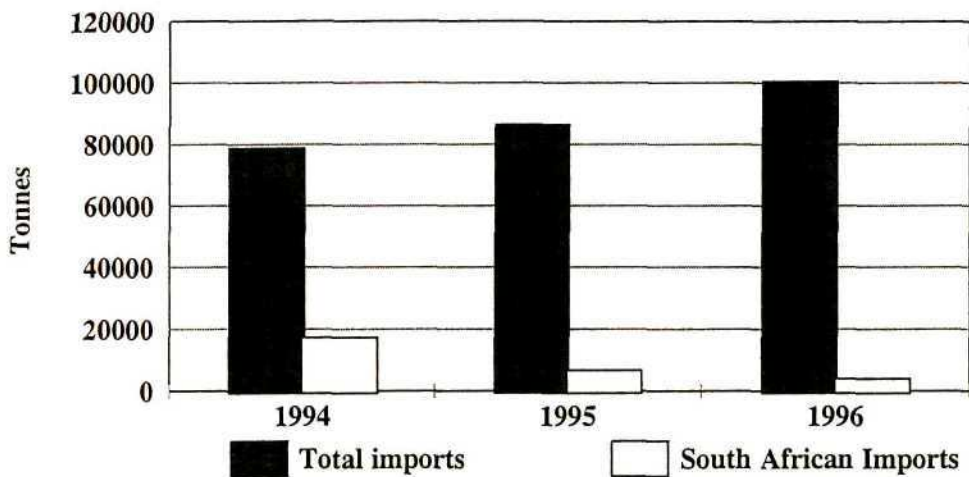


**Figure 1**. South African market share in France (Bard, 1997, pers.comm.)

Bard, Z. J. 1997. Pers. Comm., S. Afr. Avocado Grow. Assoc., Tzaneen.
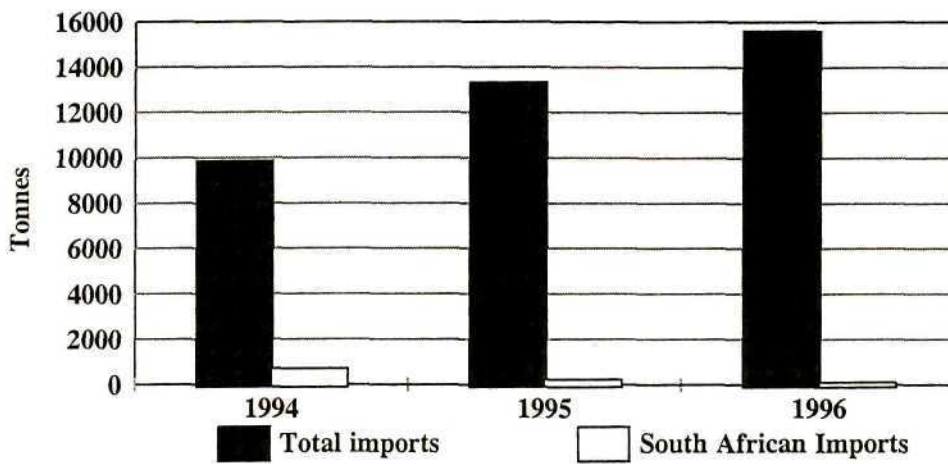
**Figure 2.** South African market share in Germany (Bard, 1997, pers.comm.)
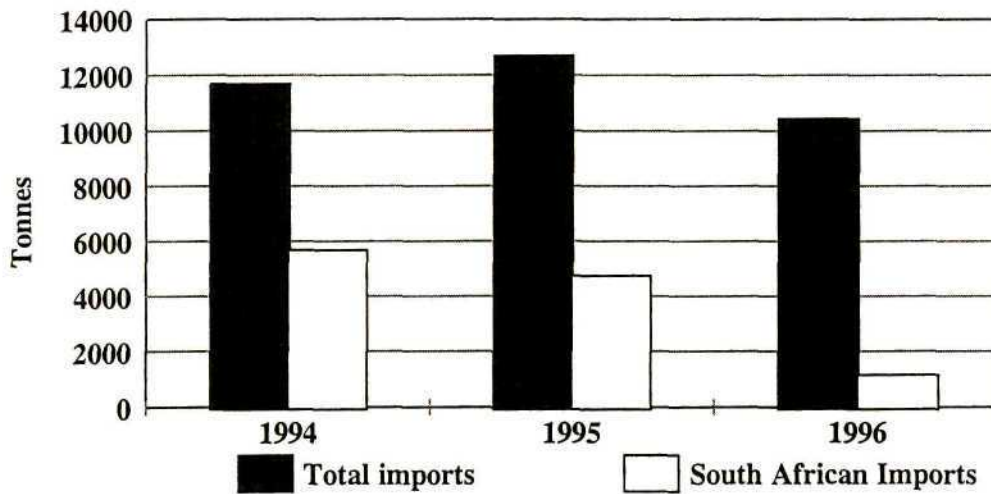


**Figure 3.** South African avocado market share in the Netherlands (Bard, 1997, pers.comm.)

Bard, Z. J. 1997. Pers. Comm., S. Afr. Avocado Grow. Assoc., Tzaneen.

Competition on the European market is fierce and the "window" for South African is getting shrinking due to Spain coming in at the beginning of the season and Israel coming in at the end, while Mexico can supply large volumes year round. This increased competition has led to a tougher market and price is affected accordingly. Excellent quality is essential to ensure high prices and maintain market share. Basically, the European market as currently structured has reached saturation, and one must consider other lucrative markets for greater returns back on the farm. For the future survival of the South African industry new markets must be found and developed. Several potentially lucrative markets such Hong Kong, China, Singapore and Japan could be explored. However, a major problem with distant markets such as Japan is an extension of shipping time. For present shipping routes it takes 27 days to reach Yokohama, Japan, as opposed to 19 days from Durban to France (PPECB shipping timetable, 1997). Another problem to overcome with these new markets is fruit fly disinfestation for avocado, and other phytosanitary regulations.

Due to consumer attitude, a number of synthetic chemicals previously used to control insects, diseases and physiological disorders of harvested fruits have been abandoned in favour of other non-chemical methods (Klein and Lurie, 1991). Heat is an ideal disease control treatment, as it is environmentally friendly and non-chemical. More importantly it has several potential benefits for subtropical fruits such as avocados (Chapter 1). Vapour heat would appear to be the most successful form for heat treatment of fruit, and is already extensively used in the exportation of papaya to Japan from Hawaii (Allan, 1997, pers.comm.). Vapour heat has also successfully been used in the exportation of mangoes to Japan and depending on land of origin, dictates the treatment administered (Table 1; Johnson *et al.*, 1997).

**Table 1.** Fruit fly disinfestation treatments for mangoes for Japan (Johnson *et al.*, 1997).

| Importing country | Exporting country | Required treatment (pest) |
|---|---|---|
| Japan | Australia | VHT (46.5°C, 15 min) |
| | Philippines | VHT (46°C, 10 min) |
| | Taiwan | VHT (46.5°C, 30 min) |
| | Thailand | VHT (46.5°C, 10 min) |

VHT= Vapour heat treatment

Although both dry heat (Woolf *et al.*, 1995) and hot water treatment (Nishijima *et al.*, 1995; Woolf and Lay-Yee, 1997) have been attempted on 'Hass' and 'Sharwil' avocado, it would appear that vapour heat treatment is the most suitable form of heat with which to treat 'Fuerte' and 'Hass' avocado postharvest. Vapour heat is less damaging to the rind of the avocado as opposed to dry heat or hot water (Donkin and Wolstenholme, 1995), as well as easier practical applications as found in Hawaii with vapour heat treatment of papaya (Allan, 1997, pers.comm.).

It is thought that vapour heat treatment of 'Fuerte' and 'Hass' avocado alters protein synthesis which changes cellular metabolism. An example of this is the prevention of the transcription/translation of the catalytic enzyme, cellulase, which results in delayed ripening (Klein and Lurie, 1991). In response to vapour heat treatments, it is thought that heat shock proteins (Hsp) are synthesized which induce beneficial characteristics like longer storage life as well as thermotolerance to otherwise non-permissive temperatures. Subjecting fruit to non-permissive temperatures also delays ripening. This phenomenon is known as the heat-shock response (HSR) (Harrington *et al.*, 1994).

The main objective of the work carried out for the purpose of this thesis was to increase storage life of harvested 'Fuerte' and 'Hass' avocado fruit grown in the KwaZulu-Natal midlands, by means of vapour heat treatment prior to cold storage. By storage life this thesis refers to an extended cold storage period as well as ripening time once removed from cold storage.

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Allan, P. 1997. Pers. Comm., Department of Horticultural Science, University of Natal, Pietermartizburg.

# CHAPTER 1

## LITERATURE REVIEW : POSTHARVEST TREATMENTS OF PLANT PRODUCTS AND THE EVALUATION AND ROLE OF HEAT SHOCK PROTEINS

Temperature is important in determining the rate of metabolism of fruits and vegetables. Decreasing temperature lowers metabolism, hence prolonging storage and shelf life. Refrigeration enables one to maintain fruit and vegetable quality. However, chilling injury is associated with low temperature storage of some fruits and vegetables (Marangoni *et al.*, 1996), especially those of tropical and subtropical origin.

"Chilling injury is the permanent or irreversible physiological damage to plant tissues, cells or organs, which results from the exposure of plants to temperatures below some critical threshold temperature (but above freezing) that causes injury" (Lyons and Breidenbach, 1987). Avocado, a subtropical to tropical fruit, becomes susceptible to chilling injury when exposed to temperatures of less than 7°C and this is a major factor affecting quality of stored fruit (Eaks, 1976; Zauberman *et al.*, 1977). Chilling injury is displayed by avocado by the presence of black spots on the rind and grey or dark brown discolouration of the mesocarp (Chaplin and Scott, 1980; Zauberman *et al.*, 1985).

This literature review considers various postharvest treatments to reduce chilling injury of a variety of horticultural commodities. Little work has been done on trying to extend shelf life of 'Hass' and 'Fuerte' avocado. It appears that the majority of work on avocado has been aimed at reducing chilling injury, ultimately prolonging storage life due to lower storage temepratures. Associated with postharvest treatment of horticultural commodities are physiological changes within the fruit giving it these beneficial properties. In particular, associated with stressful conditions in postharvest

treatments is the production of heat shock proteins. It is thought that the production of heat shock proteins is part of the reason for the beneficial properties like a reduction in chilling injury and an extension of shelf life. Hence, heat shock proteins are reviewed in detail to outline the type of heat shock proteins, their role and function. This will help when analysing protein structures in vapour heat treated avocados.

This review will also consider other tropical/subtropical fruit and vegetables apart from avocados due to the scarcity of literature in this relatively new field.

## 1.1 REDUCING CHILLING INJURY OF FRUITS

The main aim of horticultural research on chilling injury is to find effective methods of reducing this disorder, making it possible for low temperature sensitive horticultural products to have the advantage of low temperature storage (Wang,1993) and, therefore, prolonged storage life.

### 1.1.1. Temperature Conditioning

Many plants and their products, by postharvest treatments of high or low temperature conditioning were found to be more resistant to subsequent chilling injury (Wheaton and Morris, 1967). Table 1.1 illustrates this by displaying temperature and duration of preconditioning treatment to reduce chilling injury in a few horticultural products (Wang, 1993).

**Table 1.1** Precondititioning of horticultural commodities to reduce chilling injury (Wang, 1993)

| Commodity | Precond, Temp (°C) | Duration of Precond. | Storage Temp (°C) |
|---|---|---|---|
| Cucumbers | 18 | 1 - 9 days | 5 |
| | 15 | 1 day | 6.5 |
| | 37 | 1 day | 5 |
| Eggplants | 10 | 5 to 15 days | 1 |
| | 15 and 10 | 1 ady each | 6.5 |
| Grapefruit | 10 or 15 | 7 days | 0 or 1 |
| | 17 | 6 days | 0 |
| | 38 | 17 to 22hrs | 2 or 4.5 |
| | 34.5 | 3 days | 10 |
| Lemons | 5 or 15 | 7 days | 0 - 2 |
| | 21 | 3 days | 1 |
| Mangos | 20 and 15 | 1 and 2 days | 10 |
| | 20 | 12 days | 5 or 10 |
| Papayas | 12.5 | 4 days | 2 |
| Sweet peppers | 10 | 5 or 10 days | 0 |
| | 10 | 5 days | 1, 4 or 7 |
| Sweet potatoes | 32 | 10 days | 7 |
| Tomatoes | 12 and 8 | 4 days each | 5 |
| | 36 to 40 | 3 days | 2 |
| Watermelons | 26 | 4 days | 0 or 7 |
| Zucchini squash | 10 or 15 | 2 days | 2.5 or 5 |

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### 1.1.1.1 Low temperature conditioning

Tomato seedlings conditioned at 12.5°C for 3 hours were more resistant to chilling injury than non-conditioned plants at 1°C (Wheaton and Morris, 1967). Sweet pepper exposed to 10°C for 5 or 10 days had reduced chilling injury at 0°C (Chang, 1982). Grapefruit exposed for 7 days at 10 or 15 °C had reduced chilling injury at 0°C or 1°C (Hutton and Cubbedge, 1980).

With low temperature conditioning, high levels of phospholipids in membrane fatty acids have been maintained, with an increasing unsaturation of membrane fatty acids, the sterol:phospholipid ratio being kept constant, an increase in polyamine, squalene and long-chain aldehyde concentrations occurs. All these factors could lead to a reduction in chilling injury (Wang, 1994), as found with the above-mentioned crops.

### 1.1.1.2 Step-wise temperature conditioning

A two step temperature conditioning in some cases is more effective than a single step temperature conditioning (Wang, 1993). Tomatoes can be exposed for 4 days at 12°C followed by 4 days at 8°C and 7 days at 5°C to reduce chilling injury (Marangoni *et al.*, 1990). Preclimacteric mangoes exposed to 20°C and 15°C for 1 and 2 days respectively, have longer cold storage periods at 10°C as well as better flesh colour and organoleptic qualities on ripening than those treated with a single temperature (Thomas and Oke, 1983). The lowest incidence of chilling injury in bananas was noted when temperature was gradually lowered at 3°C intervals from 21°C to 5°C (Pantastico *et al.*, 1967).

### 1.1.1.3 Postharvest heat treatment of fruit

### 1.1.1.3.1 Mango

McCollum *et al.* (1993) treated 'Keitt' mango (*Mangifera indica* L.) at 38°C for 0, 24 or 48 hours before storage at 5°C for 11 days. On control fruit severe chilling injury symptoms were present. Chilling injury symptoms decreased as the duration of treatment at 38°C increased. This experiment indicates that mango fruit tolerance to chilling temperatures increases after prestorage heat treatment.

### 1.1.1.3.2 Papaya

Paul and Chen (1990) showed that the rate of flesh softening of 'Sunset' papaya (*Carica papaya* L.) slowed when treated with hot water. Ripening was slowed by heat treatment at 42°C for 30 minutes followed by 49°C for 70 minutes. Temperature conditioning at 42°C for 4 hours or 38 to 42°C for 1 hour followed by 3 hours at 22°C induced thermotolerance to otherwise injurious hot water treatment of 49°C for 70 minutes.

### 1.1.1.3.3 Avocado

A variety of different heat treatments has been used on avocados including hot water, vapour heat and dry heat (Donkin and Wolstenholme, 1995).

Woolf *et al.* (1995) found that dry air treatment of 'Hass' avocado at 38°C for 3, 6 or 10 hours and 40°C for 0.5 hours further reduced external chilling injury induced by storage at 2°C. This storage temperature is well below the normal range of cold storage for avocados, viz. 5 to 7°C, hence indicating thermtolerance being induced.

Nishijima *et al.* (1995) discovered that a shortened heat treatment from their original 18 hours to 8 or 12 hours at 38°C was effective in a reduction in chilling injury in 'Sharwil' avocado when pulp temperature was less than 2.2°C during 16 days of cold storage. These fruit were hot air treated. 'Sharwil' is an Australian cultivar which has adapted well to the semi-tropical conditions of Hawaii.

Florissen *et al.* (1996) reported that the minimum time of heat treatment necessary for the production of heat shock proteins (hsp) in 'Hass' avocado was 38°C for 4 hours. Furthermore, it was discovered that heating for 6 - 12 hours induced protection from chilling injury, hence possibly extending storage life, suggesting that hsp may confer resistance to chilling injury.

Results from a preliminary trial by Bard and Kaiser (1996) using vapour heat treated 'Fuerte' avocados, indicated a reduction in chilling injury, extension of shelf life and reduction in postharvest disorders.

Lurie *et al.* (1996) treated 'Hass' and 'Fuerte' avocados with temperatures between 37 and 46°C, then placed the fruit in cold storage at 2°C for 6 weeks. These workers found that a prestorage heat treatment decreased chilling injury in avocados stored at 2°C, reduced the rate of fruit softening and inhibited fungal rot development. Heat damage, however, seemed to be dominant and suggested fine tuning of treatments.

Woolf and Lay-Yee (1997) pretreated 'Hass' avocado in a 38°C water bath for up to 120 minutes before treating fruit in 50°C water for up to 10 minutes. Conditioning fruit at 38°C reduced levels of external browning, skin hardening and internal disorders associated with longer treatments. The most effective conditioning time was 60 minutes.

Woolf (1997) hot water treated 'Hass' avocado at 38°C for up to 120 min. These fruit were placed in cold storage at 0.5°C for up to 28 days. The fruit were allowed to

ripen at room temperature and quality was evaluated. It was found that chilling injury developed in the untreated fruit. The longer durations of hot water treatments tended to reduce chilling injury, with an optimum of 60 min. Woolf also considered electrolyte leakage from heated skin tissue, which increased by 70% during storage as opposed to 480% of non heated skin tissue for the same period. The lower electrolyte leakage highlighting protection conferred by hot water treatments against lower temperatures.

### 1.1.1.3.4 Citrus

Kaiser *et al.* (1996) showed that vapour heat treatment of 'Star Ruby' grapefruit reduced chilling injury in fruit stored at 0°C compared to the control. The most promising treatment was 48°C for 120 minutes. The 0°C (or lower) storage temperature is necessary for insect disinfestation for export to Japan.

Postharvest treatment with hot water dips of benomyl or thiabendazole were found to greatly reduce chilling injury of 'Valencia' oranges (Wild and Hood, 1989). Hot thiabendazole dip treatment was significantly better than other hot dips for reducing chilling injury.

### 1.1.1.4 Intermittent warming

By interrupting cold storage with one or more warm air treatments it is possible to extend storage life of some horticultural produce susceptible to chilling injury. Warm air treatments have to be timed correctly because application too early in the cold storage period would induce softening and too late is of little effect as irreversible chilling injury has already taken place. Correct warm air treatment time varies according to type of horticultural produce (Table 1.2; Wang, 1993).

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**Table 1.2** Warm air periods to to reduce chilling injury in various horticultural products (Wang, 1993)

| Commodity | Storage Temp(°C) | Treatment Frequency | Warm Temp(°C) | Warm Period |
|---|---|---|---|---|
| Apples | 0 | once after 6 or 8 weeks | 15 | 5 days |
| Cranberries | 0.5 | every 4 weeks | 21 | 1 day |
| Cucumbers | 2.5 | every 3 days every 3 days | 20 | 1 day |
|  | 2.5 |  | 12.5 | 18h |
| Grapefruit | 4 | once a week | 21 | 1 day |
|  | 2 | once a week | 21 | 1 day |
|  | 1 | once a week | 21 | 1 day |
| Lemons | 2 | every 21 days | 13 | 7 days |
| Nectarines | 0 | every 3 or 4 weeks |  |  |
| Peaches | 0 | every 4 weeks | 18 | 2 days |
|  | 0 | every 2 weeks | 23-25 | 2 days |
|  | 1 | every 2 weeks | 20 | 1 or 2 days |
|  | 1 | every 2 weeks | 20 | 3 days |
| Plums | -1 | every 15-20 days | 18 | 2 days |
| Potatoes | 0 | every 3 weeks | 15.5 | 1 week |
| Sweet peppers | 1 | every 3 days | 21 | 1 day |
|  | 2.5 | every 3 days | 20 | 1 day |
| Tomatoes | 4 or 8 | once a week | 20 | 3 days |
| Zucchini squash | 2.5 | Every 3 days | 20 | 1 day |

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### 1.1.2 Controlled Atmosphere Storage

Controlled atmosphere (CA) is the removal or addition of gases altering atmospheric conditions surrounding the commodity (air is 78.08% nitrogen, 20.95% oxygen and 0.03% carbon dioxide). Usually this involves a reduction in $O_2$ and/or elevation of $CO_2$ concentration (Kader, 1985). CA is generally beneficial in reducing chilling injury. A general trend of a reduction in oxygen concentration in combination with an increase in carbon dioxide concentration appears to reduce chilling injury on a variety of fruits and vegetables (Table 1.3). In some cases, CA has no effect and can even be detrimental on specific horticultural products (Wang, 1993).

**Table 1.3** Effect of CA on different horticultural produce (Wang, 1993)

| Produce | $O_2\%$ | $CO_2\%$ | Storage Temp (oC) | Effect |
|---|---|---|---|---|
| Asparagus | N | 20 - 30 | 3 | ✘ |
| Avocados | 2 | 10 | 4.4 or 7.2 | ✔ |
| | 21 | 10 | 4.5 | ✔ |
| Cucumbers | 20 | 3 or 5 | 5 | ✘ |
| Grapefruit | N | 20 - 45 | 0 | ✔ |
| | N | 20 | 4.5 | ✔ |
| | 21 | 10 | 4.5 | ✔ |
| Japanese apricots | N | 5 | 0 | ✔ |
| Lemons | N | 40 | 1 | O |
| Limes | N | 30 or 40 | 1.5 | ✘ |
| Nectarines | 1 | 5 | 0 | ✔ |
| Okra | N | 4 -15 | 5 | ✔ |
| Papayas | 1.5 -5 | 2 | 2 | O |
| Peaches | 1 | 5 | O | ✔ |
| Peaches | 0.25 | 0 | 5 | ✔ |
| | N | 20 | 1 | ✔ |
| Pears | 1 | 1.5 | -1 | ✔ |
| Pineapples | 3 | 0 | 8 | ✔ |
| Potatoes | 2.5 | 0 | 1 | ✔ |
| Sweet peppers | 21 | 5 - 20 | 2 | ✘ |
| Tomatoes | 3 - 50 | 20 - 30 | 0 | O |
| Zucchini squash | 21 | 5 | 5 | ✔ |
| | 1 | N | 2.5 | ✔ |

✘ : Detrimental effect

✔ : Beneficial effect

O : No effect

### 1.1.2.1 CA storage of avocados

CA research on avocados has indicated that an atmosphere of 10% $O_2$ and 2% $CO_2$ has beneficial effects on the fruit when in cold storage. Preliminary research Australia, however, found 5% $CO_2$ to be sufficient (Smith *et al.*, 1993). Spalding and Reeder (1975) found a combination of 10% $CO_2$ and 2% Q to be effective in reducing chilling injury in 'Fuchs' and 'Waldin' avocados, which are more tropical in origin and important in Florida, USA. These avocados were stored at 7.2°C for 3 - 4 weeks and anthracnose and chilling injury were significantly reduced. Storage performance is cultivar and area dependent so recommendations for specific areas as well as cultivar must be developed.

### 1.1.3 Packaging

Film packaging of fruit and vegetables has two main benefits, which are a reduction in moisture loss and an alteration of in-package atmosphere (Hardenburg, 1971). Development of chilling injury in fruits can be delayed by reducing the vapour pressure deficit, which decreases water loss (Pantastico *et al.*, 1968). Packaging of fruit maintains a high relative humidity as well as modifying concentrations of $O_2$ and $CO_2$ (Wang, 1993). Increases in $CO_2$ and decreases in $O_2$ levels result in a reduction in chilling injury of avocados, grapefruit, peaches, nectarines, okra, pineapples, potatoes, zucchini squash and cucumber (Wang, 1993; Wang and Qi, 1997).

### 1.1.4 Chemical Treatments

Various chemical applications to fruit have been shown to reduce chilling injury. Chemicals reported to have an effect include calcium ions, ethoxyquin, sodium benzoate,1-(2-(2,4-dichlorophenyl)-2-(2-propenyloxylethyl)-1H-imidazole) (imazalil), 2-(4-thiazolyl) benzimidazole (thiabendazole), mineral oil, safflower oil, vegetable oil and squalene (Wang, 1994).

Experiments have shown that individual avocado fruit with low calcium content were the most susceptible to chilling injury. Chilling injury was also reduced where avocado fruit were vacuum infiltrated with $CaCl_2$ (7.5% m/v) and held at 5°C for 3 weeks (Morris, 1982; Melvin Couey, 1982).

Ethoxyquin and sodium benzoate, which are free radical scavengers, reduce peroxidation of unsaturated fatty acids in membrane lipids hence maintaining membrane integrity and reducing chilling injury (Wang, 1994). Imazalil (McDonald *et al.*, 1990) and thiabendazole (Schiffmann-Nadel *et al.*, 1994) are fungicides which prevent infection and reduce chilling injury in grapefruit when incorporated into the wax coating.

A vegetable oil coating of grapefruit was found to reduce chilling injury (Aljuburi and Haff, 1984). Natural oils are thought to have antitranspirant and antioxidant properties which reduce moisture loss and oxidative damage, hence reducing chilling injury (Wang, 1994).

## 1.2 HEAT SHOCK PROTEINS

Gene expression in plants is altered by environmental conditions such as changes in temperature, light environment, water status or hormone balance. The response of plant parts to elevated temperatures, or 'heat shock', at a molecular level is one of the better characterized environmental responses (Vierling, 1991). For example, exposure of seedlings to temperatures five degrees above optimal growing conditions causes repression of normal proteins and mRNA followed by transcription and translation of a small group of 'Heat Shock Proteins' (Hsps) were initiated. This heat shock response is found in a variety of organisms such as *Drosophila melanogaster, Escherichia coli, Saccharomyces cerevisiae* and humans (Lindquist, 1986).The response of various organisms highlighted two common characteristics:

a) gene induction by heat displays many similarities among diverse eukaryotes in molecular mechanism;

b) major Hsps are highly homologous between eukaryotes. One could strongly suggest, due to the evolutionary conservation of heat shock responses, that the production of Hsps is a fundamental and essential process of an organism's life (Vierling, 1991).

Plants develop tolerance to normally lethal temperatures only if they were first subjected to high but not lethal temperatures. It is thought that short term development of thermotolerance is achieved through the production of Hsps (Lindquist, 1986). In response to elevated temperatures most plant species produce Hsps and the synthesis of Hsps tends to increase with increasing temperature (Collins *et al.*, 1995). The maximum temperature for Hsp synthesis is positively correlated with each species' optimum temperature for growth. Hsp production response depends very much on the physiology of the organism and highlights its biological relevance (Vierling, 1991).

A variety of Hsps have been classified in plants. They are distinguished by their approximate molecular weights (kDa) as Hsp110, Hsp90, Hsp70, Hsp60, and low

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molecular weight (LMW) Hsps (15-30 kDa). A small protein involved in ATP-dependent, intracellular proteolysis, ubiquitin, is also classified as a Hsp (Lindquist and Craig, 1988). These proteins are all heat induced in the majority of cell types in a variety of organisms (Nerland *et al.*, 1988).

The value of Hsp extends beyond protecting the plant from high-temperature stress. Initially Hsp were isolated due to their expression when placed in high-temperature stress conditions. Some Hsp though, are found in non-stressful conditions at significant levels, or during the cell cycle or at some stage of development of the plant. Some normal cellular proteins are homologous to Hsps and expression does not increase in response to high temperature. Certain Hsps (i.e. Hsp70, Hsp60 and LMW Hsp) are present in certain cellular compartments, including organelles such as mitochondria and chloroplasts (Craig *et al.*, 1989; Engman *et al.*, 1989; Leustek *et al.*, 1989; Mizzen *et al.*, 1989; Amir-Sharpira *et al.*, 1990; Marshall *et al.*, 1990). The cross reactivity of specific antibodies illutrates the similarity between these proteins and in cases from direct sequence analysis of cloned genes. Hence Hsps are products of a group of multi-gene superfamilies of which not all Hsps are induced by high-temperature stress conditions (Vierling, 1991).

High-temperature stress is not the only condition which leads to the expression of Hsps. A variety of treatments such as ethanol, arsenite, heavy metals, amino acid analogues, glucose starvation and calcium ionophores induce the synthesis of a few or all Hsps in a variety of organisms (Czarnecka *et al.*, 1984; Czarnecka *et al.*, 1988; Edelman *et al.*, 1988; Nover *et al.*, 1989; Winter *et al.*, 1988). Hence Hsps have also been referred to as stress proteins. Hsps though, are not produced in response to all situations where the cell is under stress. Generally, Hsps are not induced in plants placed under water stress (Kimpel and Key, 1985), anaerobic stress (Russell and Sachs, 1989), cold stress (Yacob and Filion, 1986 ; Ougham, 1987; Guy, 1990; Hajela *et al.*, 1990) or salt stress (Czarnecka *et al.*, 1984; Harrington and Alm, 1988). Production of a full complement of Hsp is induced in response to specific

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changes not common to all stresses. The term Hsp cognate (HSC) has been applied to those Hsp family members that are expressed in the absence of heat stress.

It is not yet known how Hsps enable an organism to endure high temperatures. Before one can determine how Hsp enable a plant to withstand heat stress one would need to determine the biochemical activity of individual Hsps. The main line of thought is that Hsps cause stabilization of proteins in a particular state of folding (Ellis, 1987; Lindquist and Craig, 1988; Rothman, 1989; Schlesinger, 1990). Through this concept, evidence tends to suggest that Hsp90, Hsp70, and Hsp60 facilitate a variety of important processes such as protein folding, transport of proteins across membranes, assembly of oligomeric proteins, and modulation of receptor activities (Gatenby, 1992; Gething and Sambrook, 1992). For these functions to be carried out, the alteration or maintenance of specific polypeptide conformations is required. Because Hsp90, Hsp70, and Hsp60 facilitate these activities they have been termed 'molecular chaperones' or 'polypeptide chain binding proteins' (Ellis, 1987; Rothman, 1989).

### 1.2.1  Characteristics and Functions of Hsp70

#### 1.2.1.1 Hsp70 diversity

Hsp70 was one of the initial eukaryotic genes to be cloned. The Hsp70 gene is diverse and partly accounted for by the presence of distinct Hsp70 homologs in the cytoplasm, lumen of endoplasmic reticulum (ER) (Munro and Pelham, 1986; Normington *et al.*, 1989; Rose *et al.*, 1989) and matrix of mitochondria (Amir-Sharpira *et al.*, 1990; Craig *et al.*, 1989; Engman *et al.*, 1989; Mizzen *et al.*, 1989). In the ER the Hsp70 homolog is known as 'binding protein' (BiP) or 'glucose regulated protein' (GRP). Hsp70s are found in chloroplasts in plants (Amir-Sharpira *et al.*, 1990; Marshall *et al.*, 1990). This abundant family of Hsp suggest that Hsp70s perform critical, fundamental cellular functions (DeRocher and Vierling, 1995).

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## 1.2.1.2 Cytoplasmic Hsp70 genes and proteins

HSC70 in plants is essentially found in the cytoplasm of all tissues, and under periods of high temperature stress additional Hsp70 is induced (Chen *et al.*, 1990; Linquist, 1986; Neumann *et al.*, 1987; Neumann *et al.*, 1989). Several of the genes which encode Hsp70 homologs have been isolated from a variety of crops. For eight of these genes the DNA and derived amino acid sequences have been determined. Seven of these genes bear a strong similarity to yeast and human cytoplasmic Hsp70. With this evidence as well as no correct amino-terminal signal sequences necessary for localization to the ER, mitochondria, or chloroplasts, suggested these genes encode the cytoplasmic form of Hsp70.

A comparison between the seven cytoplasmic Hsp70s showed that they are 75% identical and 91.0% similar at amino acid level. If tomato BiP is included in the comparison, identity falls to 54.9% and similarity to 82.5%. Hence, a greater similarity is seen between eukaryotic cytoplasmic Hsp70 homologs from different species than between Hsp70 homologs in different cellular compartments of a single species. Hsp70 of eukaryotes have 50% identity and greater than 65% similarity with *E. coli* DNAk protein, hence Hsp70 are amongst the highest evolutionarily conserved protein biomass.

Hsp70 homologs are also induced by stresses which in general do not induce most Hsp. HSC70 on RNA increased two to four times and a corresponding increase in HSC70 was noted during cold acclimation in several plant species (Berry and Borkman, 1980; Neven *et al.*, 1990). Although the increase in Hsp70 due to low temperature conditions did not induce cold tolerance, a similar response was observed in both cold-tolerant and inter-tolerant species (Vierling, 1991). Due to large numbers and homology of Hsp70 genes, efforts to examine the regulation of Hsp70 expression have been

complicated. In most normal cells the presence of HSC70 RNA and protein adds further complication of the assessment of stress induced changes.

### 1.2.1.3 Cytoplasmic Hsp70 functions

Hsp70 homologs bind ATP and display a weak ATPase activity (Welch and Fermasico, 1985; Flynn *et al.*, 1989; Flaherty *et al.*, 1990). The HSC70 are involved in a variety of functions causing a change in protein conformation. During stress, the function of Hsp70 is thought to be similar to constitutive homologs. Protein sequence characteristics have not yet been isolated as heat-induced or constitutive Hsp70s (Linquist and Craig, 1988). Hence, there are no structural differences in heat-induced Hsp70 homologs to highlight heat-stress-related functions. During heat stress, the total amount of Hsp70 produced is generally less than the amount present in the cell (Bond and Schlesinger, 1987). It is thought that Hsp70 binds to denatured proteins produced when placed under conditions of heat stress. In turn this helps protein reassembly accompanied by hydrolysis of ATP (Pelham, 1986).

The Hsp70 produced when under high-temperature stress conditions is taken up mainly by the nucleolus and is then redistributed into the cytoplasm during recovery. An alteration is binding of Hsp70 to other cellular components or alteration of Hsp70 conformation is involved in controlling intracellular localization (Dang and Lee, 1989; Milarski and Morimoto, 1989). It is suggested that the protein protects preribosomes against denaturation in periods of stress (Pelham, 1986). During stress the HSC70 already present localises to the nucleus, hence it is difficult to distinguish between Hsp70 and HSC70 with regard to their function.

### 1.2.1.4 Hsp70 in the endoplasmic reticulum

During glucose starvation in mammalian cells an ER protein that displayed increased expression was detected as an Hsp70 homolog identical to BiP (Munro and Pelham,

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1986).  Cytoplasmic Hsp70 shares extensive sequence homology with BiP, as well as being able to bind BiP and short peptides (Flynn *et al.*, 1989). This Hsp70 homolog is hardly induced by heat treatment and is found at significant levels in many cells. In mammalian cells, BiP has a much wider function than originally thought. In all plant cells BiP is a large component and ER-bound ribosomes are actively involved in protein synthesis.

### 1.2.1.5 Mitochondrial and chloroplast Hsp70s

Mitochondrial Hsp are found in all species not subjected to heat stress, the same as an essential protein. Two additional Hsp70 homologs have been localized to the chloroplast using antibodies that recognize eukaryotic Hsp70s.  One of these proteins is found between the inner and outer envelope membrane, while the other is located in the stroma.

DNAk homologs have been located in the chloroplast stroma of a variety of plants including pea (Marshall *et al.*, 1990) and spinach (Amir-Sharpira *et al.*, 1990).  These proteins are present in the absence of heat stress and show little change in protein level after heat treatment.

The HSC70s found inside and outside the mitochondria are involved in protein transport, and from this one could assume that HSC70 is also involved in protein transportation into chloroplasts.

### 1.2.2 Hsp 60

These were the first Hsps to be characterized as 'molecular chaperones', and genetic and biochemical studies confirmed this (Ellis, 1987; Hemmingsen *et al.*, 1988). The eukaryotic Hsp60 homologs are nuclear encoded proteins located in the mitochondria and chloroplasts (Hemmingsen *et al.*, 1988). These Hsp are abundant in mitochondria

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and chloroplasts even if heat stress conditions do not prevail. The homolog identified in the chloroplast was ribulose biphosphate carboxylase (Rubisco) binding protein (Roy *et al.*, 1982; Roy, 1989). No other homologous proteins have been isolated in any other eukaryotic cellular compartments.

### 1.2.2.1 Mitochondrial Hsp60

It is thought that Hsp60 interacts with newly imported, unassembled proteins to form a complex in a partially unfolded state (Ostermann *et al.*, 1989; Hartl and Neupert, 1990). Folding is ATP dependent and thought to occur on the surface of the Hsp60 complex.

This Hsp has been found in a variety of plant species, and of these, maize Hsp60 has been extensively characterized (Prasad *et al.*, 1989; Prasad *et al.*, 1990). It has been found with yeast Hsp60 and Hsp60 in maize seedlings, that protein levels increase two to three times during a four hour heat treatment (Prasad *et al.*, 1990). The total protein at imbibition and early seedling growth is made up of a larger percentage of Hsp60 than compared to 4 day old or mature seedlings. This could be due to more Hsp60 being required during germination or at other periods of active mitochondrial division and development (Vierling, 1991).

### 1.2.2.2 Chloroplast Chaperonin 60

This Hsp60 homolog in chloroplasts (the Rubisco subunit binding protein) is also known as chloroplast chaperonin 60. It is thought that chloroplast chaperonin 60 plays a part in the assembly of the rubisco holoenzyme (Roy, 1989).

Chloroplast chaperonin, a member of the Hsp60 family, has got no information displaying its expression during heat stress. In the absence of stress the protein is an abundant chloroplast component, and would be unlikely to increase two to three times if placed in stress conditions (Vierling, 1991).

### 1.2.3 LMW Heat Shock Proteins

A variety of LMW Hsps are synthesized ranging in size from 17 to 28 kDa. At optimal temperatures LMW Hsps are not expressed at detectable levels in leaves, but in many plant species they are the most abundant proteins formed during heat stress (Chen *et al.*, 1990; Mansfield and Key, 1987; Wollgiehn *et al.*, 1994). Plants synthesize up to 30 LMW Hsps that are localized in the cytoplasm, plastids and endoplasmic reticulum (Helm *et al.*, 1995). Studies on genes from various flowers and fruits indicate that most of LMW Hsps belong to one of four multi-gene families. Of these four genes, two encode cytoplasmic proteins, one a chloroplast-localized protein and one an endomembrane protein (Helm and Vierling, 1990). The genes ,therefore, of the LMW Hsps, encode specific proteins destined for different cellular compartments. No homologs for LMW Hsp have been identified. The evidence that most plant LMW Hsps belong to four gene families was obtained considering the amino acid sequence relationship between LMW Hsps from plants. Comparisons display greater identity among certain genes from different species than different genes of the same species (Vierling, 1991).

The various classes of LMW Hsps are displayed in Table 1.4. In class I, the gene similarity ranges from 80.1 to 92.9 % (identity 68.2 - 85.1%). There seems to be a higher value from plants in the same taxonomic family, than compared to genes found within the same species. The values for the comparisons between corresponding nucleic acid sequences of LMW Hsps are even lower. Thus, genes from different classes show little signs of cross hybridization (Vierling, 1991).

Class II cytoplasmic Hsp display similar trends in the sequence of amino acids. The class I and II genes are generally encoded by multigene families (Schoffl and Key, 1983; Nagao *et al.*, 1986).

Several sequences of nuclear genes are available for the third group of LMW Hsps that encode chloroplast-localized Hsps. As with the other classes, interspecies similarity is high within the class although their similarity to other LMW Hsp classes is lower (Vierling, 1991).

**Table 1.4** Amino acid sequence relationship between LMW HSPs from plants (Vierling, 1991)

| Protein | Mol Wt (kd) | pI | Cytoplasmic class I | | | | Cytoplasmic class II | | | | Nuclear-encoded chloroplast - localized | | | | Endomembrane localized | | Unclassified | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | P.sativum HSP18.1 | G.Max HSP17.5-c | A.thaliana HSP17.6 | T.aestivum C3-8 | P.sativum HSP17.7 | G.max HSP17.9-d | Z.mays HSP18.3 | L.longiflorum pEMPR-C6 | P.sativum HSP21 | P.hybrido HSP22 | A.thaliana HSP21 | Z.mays HSP2 | P.sativum HSP22.7 | G.max HSP22 | C.rainho rdu HSP22 | G.max HSP26 |
| **Class I:** | | | | | | | | | | | | | | | | | | |
| P.sativum HSP18.1 | 18.1 | 5.96 | · | | | | | | | | | | | | | | | |
| G.max HSP17.5-E | 17.5 | 6.31 | 85.1 | · | | | | | | | | | | | | | | |
| A.thaliana HSP17.6 | 17.6 | 5.24 | 72 | 71.4 | · | | | | | | | | | | | | | |
| T.aestivum C3-8 | 16.9 | 5.95 | 71.6 | 72.3 | 68.2 | · | | | | | | | | | | | | |
| **Class II:** | | | | | | | | | | | | | | | | | | |
| P.sativum HSP17.7 | 17.7 | 6.82 | 38.3 | 37.5 | 38.4 | 40.5 | · | | | | | | | | | | | |
| G.max HSP17.9-D | 17.9 | 6.29 | 37.1 | 38.4 | 34 | 34.7 | 78.3 | · | | | | | | | | | | |
| Z.mays HSP18.3 | 17.8 | 5.19 | 36.5 | 37.9 | 34 | 39.6 | 62.4 | 64.8 | · | | | | | | | | | |
| L.longiflorum pEMPR-C6 | 17.6 | 8.98 | 40.9 | 37.7 | 35.5 | 36.2 | 53.3 | 58.4 | 54.7 | · | | | | | | | | |
| **Chloroplast-localized:** | | | | | | | | | | | | | | | | | | |
| P.sativum HSP21 | 26.2 | 6.77 | 30.3 | 29.7 | 27.9 | 31 | 28.3 | 32.5 | 25.9 | 23.9 | · | | | | | | | |
| P.hybrida HSP22 | 26.8 | 8.33 | 32.9 | 36.4 | 38 | 34.5 | 30.8 | 30.6 | 33.8 | 31.1 | 67.6 | · | | | | | | |
| A.thaliana HSP21 | 25.6 | 10.2 | 35.1 | 37.4 | 32.9 | 34 | 30.6 | 31 | 36.1 | 34.6 | 61.2 | 65.2 | · | | | | | |
| Z.mays HSP26 | 26.4 | 8.41 | 32.9 | 36.4 | 29.5 | 33.3 | 31.5 | 30.7 | 33.6 | 34.1 | 55.9 | 63.9 | 57.1 | · | | | | |
| **Endomembrane:** | | | | | | | | | | | | | | | | | | |
| P.sativum HSP22.7 | 22.7 | 7.26 | 48.3 | 47.1 | 42.6 | 44.7 | 35.3 | 32.7 | 33.7 | 30.7 | 31.1 | 27.8 | 30.6 | 23.4 | · | | | |
| G.max HSP22 | 22 | 6.69 | 53 | 50.3 | 45.2 | 52.3 | 36.9 | 34 | 36.6 | 35.5 | 31.2 | 31.5 | 28.7 | 24 | 76.2 | · | | |
| **Unclassified:** | | | | | | | | | | | | | | | | | | |
| C.rainhardu HSP22 | 16.7 | 6.53 | 34.6 | 33.3 | 40.1 | 35.3 | 33.5 | 32.9 | 35.1 | 30.2 | 23.8 | 25.7 | 30.6 | 27.5 | 32.3 | 30.5 | · | |
| G.max HSP26 | 26 | 5.31 | 21.7 | 25.2 | 23 | 15.2 | 19.1 | 18.9 | 14.6 | 22 | 17.7 | 12.3 | 14 | 11.7 | 22 | 14.1 | 12.5 | · |

**NOTE :**

1. Percentage identity and similarity were calculated using the GAP program of the Wisconsin GCG DNA sequence anlysis software;

2. Molecular weight and pI were determined from the derived amino acid sequence;

3. Percentage similarity and identity calculated without the transit peptide;

4. Comparisons between proteins in the same class have ben indicated in boldface.

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## 1.2.3.1 LMW Hsp structure

There is a higher sequence of conservation in the carboxyl - terminal portion of the proteins in defined LMW Hsp classes. Amino acid sequence alignment of class I cytoplasmic Hsps highlights this (Fig. 1.1; Vierling, 1991).

```
P.sativum Hsp18.1    : MSLIPSFFSGRRSNVFDPFSLDVWDPLKDFPFSNSSPSASFPRENPAFVSTRV
G.max Hsp17.5-E      : MSLIPGFFGGRRSNVFDPFSLDMWDPFKDFHVPTSSVSA----ENSAFVSTRV
A.thaliana Hsp17.6   : MSLIPSIFGGRRTNVFDPFSLDVFDPFEGFLTP-SGLANAPAMDVAAFTNAKV
T.aestivum C5-8      : MSIV------RRSNVFDPFADLWADPFDTFRSIVPAISGGSS-ETAAFANARV
                       **..        ** ******     **. *               . **    .*


Hsp18.1      DWKETPEAHVFKADLPGLKKEEVKVEVEDDRVLQISGERSVEKEDKNDEWHRVERSSGKFLRRFRLP
Hsp17.5-E    DWKETPEAHVFKADIPGLKKEEVKVQIEDDRVLQISGERNVEKEDKNDTWHRVERSSGKFTRRFRLP
Hsp17.6      DWRETPEAHVFKADLPGLRKEEVKVEVEDGNILQISGERSNENEEKNDKWHRVERSSGKFTRRFRLP
C5-8         DWKETPEAHVFKVDLPGVKKEEVKVEVEDGNVLVVSGERSREKEDKNDKWHRVERSSGKFVRRFRLP
             **.********* *.**..******..**    .* .**** * *.*** *********** ******


Hsp18.1      ENAKMDKVKASMENGVLTVTVPKEEIKKAEVKSIEISG
Hsp17.5-E    ENAKVNEVKASMENGVLTVTVPKEEVKKPDVKAIEISG
Hsp17.6      ENAKMEEIKASMENGVLSVTVPKVPEKKPEVKSIDISG
C5-8         EDAKVEEVKAGLENGVLTVTVPKAEVKKPEVKAIEISG
             *.**.  .** .***** *****   ** .** *.***
```

**Figure 1.1** Amino acid sequence comparison among Class I cytoplasmic LMW Hsps from several plant species. Asterisks highlight residues conserved in all four sequences, periods indicate positions of conservative replacements. Identical residues are indicated in bold, dashes indicate gaps (Vierling, 1991).

Two thirds of the protein in the carboxyl-terminal are 73 identical of 106 amino acids. Although the amino-terminal shows one third less identity, that is only 15 identical of 52 amino acids. Similar multi-species comparisons of class II cytoplasmic are displayed. The sequence divergence between LMW Hsp classes is highlighted by a comparison between class I, II, endomembrane, and chloroplast LMW Hsps from pea (Fig. 1.2; Vierling, 1991).

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```
Hsp18.1 (Class I)
Hsp17.7 (Class II)
Hsp22.7 (endomemb)                                                              MSLK
Hsp21   (Chlplst MAQSVSLSTIASPILSQKPGSSVKSTPPCMASFPLRRQLRPLGLRNVRAQAGGDGDNKDN


Hsp18.1                              MSLIPSFFSGRRSNVFDPFSLDVWDPLKDFPFSNSSPSASFPRENP
Hsp17.7                              MDFRLMDLDSPLFNTLHHIMDLTDDTTEKNLNAPTRTYVRDA
Hsp22.7 PLNMLLVPFLLLILAADFPLKAKASLLP-FIDSPNTLLSDLWSDRFPDP---FRVLEQIPGRNIGGGEI
Hsp21   SVEVHRVNKDDQGTAVERKPRRSSIDISPFGLLDPWSPMRSMRQMLDTMDRIFEDAITIPYGVEKHEPS
                                        *


Hsp18.1 AFVST-RVDWKETPEAHVFKA--DLPGLKKEEVKVEVEDDRVLQISGERSVEKEDKNDEWHRVERSSGK
Hsp17.7 KAMAATPADVKEHPNSYVFMV--DMPGVKSGDIKVQVEDENVLLISGERKREEEKEGVKYLKMERRIGK
Hsp22.7 ITLSHARVDWKETPEGHVIMV--DVPGLKKDDIKIEVEENRVLRVSGERKKEEDKKGDHWHRVERSYGK
Hsp21   ------RVPWEIKDEEHEIRMRFDMPGVSKEDVKVSVEDDVLVIKSDHR--EENGGEDCWSRK--SYSC
                          *.**. ..*.**. .. *  * *
                               CONSENSUS II


Hsp18.1 FLRRFRLPENAKMDKVKASMENGVLTVTVPK---EEIKKAEVKSIEISG
Hsp17.7 LMRKFVLPENANIEAISAISQDGVLTVTVNKLPPPEPKKPKTIQVKVA
Hsp22.7 FWRQFKLPQNVDLDSVKAKMENGVLTLTLHKLSHDKIKGPRMVSIVEEDDKPSKIVNDELK
Hsp 21  YDTRLKLPDNCEKEKVKAELKDGVLYITIPKT------KIERTVIDVQIQ
              . **.*   . .  *  .*** .*. *
                    CONSENSUS I
```

**Figure 1.2** Comparison of amino acid sequences among LMW Hsps of *P.sativum* from different LMW Hsp classes (Vierling, 1991).

Limited homology is displayed between these LMW Hsps compared to the similarity seen with class I LMW Hsps from different species. When comparing different Hsp classes, consensus region I is 27 amino acids long (aa 116 - 143 of Hsp18.1) with 9 identical and 7 conservative replacements. The sequence Pro and Gly - Val - Leu in this region are found in all LMW Hsps of other eukaryotes (Lindquist and Craig, 1988). Consensus region II is 29 amino acids long (aa 66 - 95 of Hsp 18.1) with 9 identical and 8 conservative replacements.

### 1.2.3.2 Plastid LMW Hsps

In a variety of plant species LMW Hsp are found in the chloroplasts (Suss and Yordanov, 1986; Vierling *et al.*, 1988; Glaczinskl and Kloppstech, 1988; Vierling *et al.*, 1989; Chen *et al.*, 1990; Nieto-Sotelo *et al.*, 1990; Chen *et al.*, 1991). From four divergent plant species, LMW chloroplast Hsps are compared in Fig. 1.3 (Vierling, 1991).
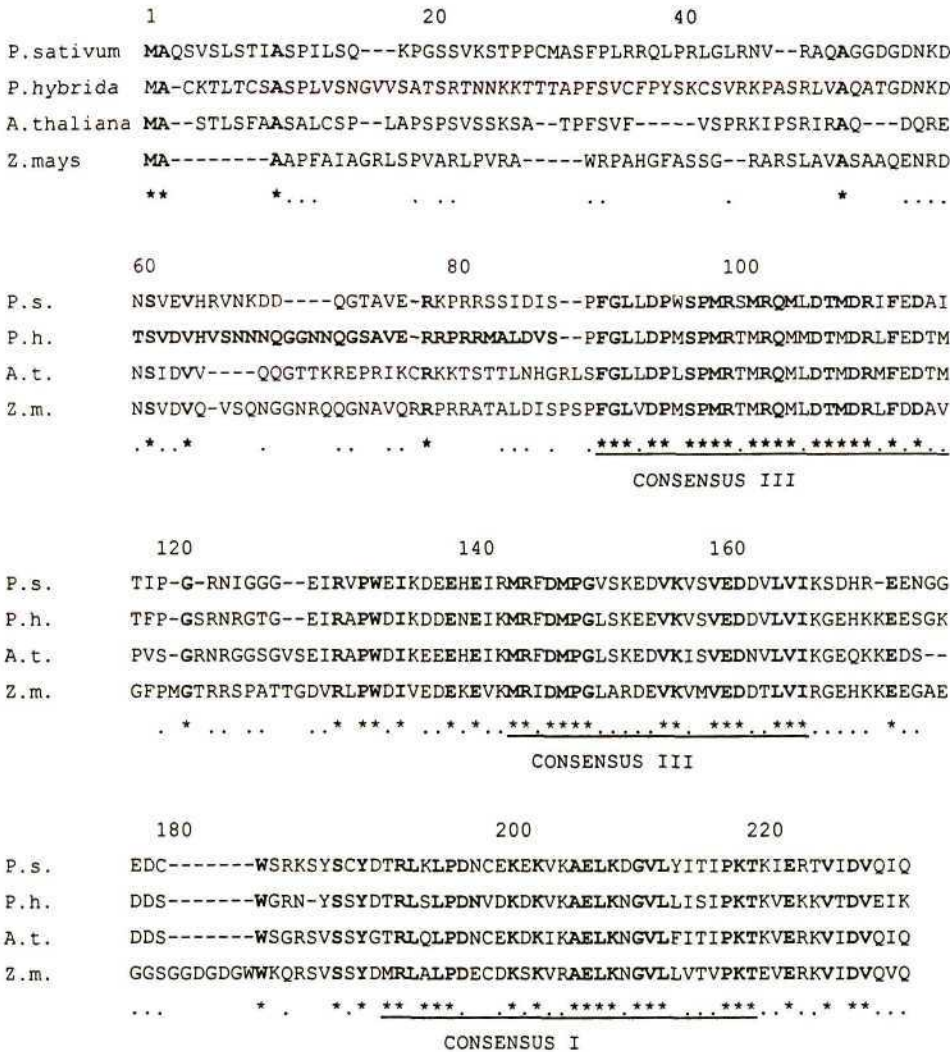
```
            1                    20                    40
P.sativum   MAQSVSLSTIASPILSQ---KPGSSVKSTPPCMASFPLRRQLPRLGLRNV--RAQAGGDGDNKD
P.hybrida   MA-CKTLTCSASPLVSNGVVSATSRTNNKKTTTAPFSVCFPYSKCSVRKPASRLVAQATGDNKD
A.thaliana  MA--STLSFAASALCSP--LAPSPSVSSKSA--TPFSVF-----VSPRKIPSRIRAQ---DQRE
Z.mays      MA--------AAPFAIAGRLSPVARLPVRA-----WRPAHGFASSG--RARSLAVASAAQENRD
            **         *...        .  ..         ..        .       *    ....


            60                    80                    100
P.s.        NSVEVHRVNKDD----QGTAVE-RKPRRSSIDIS--PFGLLDPWSPMRSMRQMLDTMDRIFEDAI
P.h.        TSVDVHVSNNNQGGNNQGSAVE-RRPRRMALDVS--PFGLLDPMSPMRTMRQMMDTMDRLFEDTM
A.t.        NSIDVV----QQGTTKREPRIKCRKKTSTTLNHGRLSFGLLDPLSPMRTMRQMLDTMDRMFEDTM
Z.m.        NSVDVQ-VSQNGGNRQQGNAVQRRPRRATALDISPSPFGLVDPMSPMRTMRQMLDTMDRLFDDAV
            .*..*      .          ..  .. *      ... .  .***.** ****.****.*****.*.*..
                                                    CONSENSUS III


            120                   140                   160
P.s.        TIP-G-RNIGGG--EIRVPWEIKDEEHEIRMRFDMPGVSKEDVKVSVEDDVLVIKSDHR-EENGG
P.h.        TFP-GSRNRGTG--EIRAPWDIKDDENEIKMRFDMPGLSKEEVKVSVEDDVLVIKGEHKKEESGK
A.t.        PVS-GRNRGGSGVSEIRAPWDIKEEEHEIKMRFDMPGLSKEDVKISVEDNVLVIKGEQKKEDS--
Z.m.        GFPMGTRRSPATTGDVRLPWDIVEDEKEVKMRIDMPGLARDEVKVMVEDDTLVIRGEHKKEEGAE
            . *  .. ..      ..* **.* ..*.*..**.****....,**. ***..*** ..... *..
                                                    CONSENSUS III


            180                   200                   220
P.s.        EDC-------WSRKSYSCYDTRLKLPDNCEKEKVKAELKDGVLYITIPKTKIERTVIDVQIQ
P.h.        DDS-------WGRN-YSSYDTRLSLPDNVDKDKVKAELKNGVLLISIPKTKVEKKVTDVEIK
A.t.        DDS-------WSGRSVSSYGTRLQLPDNCEKDKIKAELKNGVLFITIPKTKVERKVIDVQIQ
Z.m.        GGSGGDGDGWWKQRSVSSYDMRLALPDECDKSKVRAELKNGVLLVTVPKTEVERKVIDVQVQ
            ...         * .     *.* ** ***.   .*.*..****.***  ...***..*..* **...
                                                    CONSENSUS I
```

**Figure 1.3** Comparison of amino acid sequences among LMW chloroplast Hsps from four plant species. Sequence numbers refer to *P.sativum* sequence (Vierling, 1991).

The 45-50 residues display no sequence conservation between species. The conserved regions of the mature protein are referred to as consensus regions I, II and III. Consensus regions I and II are homologous with other LMW Hsps (Figure 1.2). A highly conserved domain is found in region III of chloroplast Hsps corresponding to amino acids 90 - 117 of the complete pea sequence and thought to form a Met-rich, amphipathic alpha helix (Chen and Vierling, 1991).

### 1.2.3.3 Endomembrane LMW Hsps

Heat-shocked plant material has displayed LMW Hsps located in the ER through cell-fractional studies (Hooper and Ho, 1987; Sticher *et al.*, 1990). Due to conclusive evidence, soyabean Hsp22 and pea Hsp22.7 have been classified as endomembrane localized proteins.

### 1.2.3.4 LMW Hsp function

Little is known about the function of LMW Hsps (Vierling, 1991). The genetic approach was instrumental in identifying the function of Hsp70, but did not help in locating a role for LMW Hsp. Biochemical experiments also failed to locate a function for LMW Hsp. To determine the function of these proteins one will have to consider LMW Hsp complexes. The composition of the 10 - 20S particles and heat shock granules appear to contain LMW cytoplasmic Hsps, Hsp 70, other unidentified proteins and an RNA component (Nover *et al.*, 1989). It is suggested the heat shock granules function to protect cellular mRNA during stress ( Arrigo and Welch, 1987; Arrigo *et al.*, 1988).

As heat shock granules only accumulate in cells when concentrations are high, which is brought about by very severe heat shock, LMW Hsps are not found as heat shock granules under most conditions. This in turn suggests the active form of the LMW Hsps could be at the 10 - 20s particle. Alternatively the 10 - 20s particle could be

non- functional, and would become functional heat shock granules only under severe stress (Vierling, 1991).

Collier *et al.* (1988) have proposed that 10 - 20s LMW Hsp particles have an enzymatic function and more substantial aggregates are an inactive, "stored" form of enzyme. Speculation based on homology of LMW Hsps to α-crystallin, structural protein of the eye lens suggest LMW Hsps maintain structural integrity of heat stressed cells (Lindquist and Craig, 1988).

It is suggested that the chloroplast Hsp functions to protect or repair photosystem II during stress. Photosystem II is a heat sensitive component of the chloroplast (Berry and Bjorkman, 1980). Below 38°C this protein showed no localization in the thylakoid membrane.

Chen *et al.* (1990) proposed that if Hsps in chloroplasts protect photosynthetic mechanisms, they do so as a result of general alteration of enzymatic properties instead of direct interaction with photosystem II. Chen *et al.* (1990) found that the pea chloroplast Hsp was also produced at significant levels in nonphotosynthetic plastids. This and its homology to cytoplasmic and endomembrane LMW Hsps support the hypothesis of that chloroplast Hsps perform functions in addition to protecting photosynthesis.

### 1.2.4 Other Hsps

#### 1.2.4.1 Hsp 90

Hsp 90 class of proteins ranges from 80 - 94 kDa in size. Proteins encoding genes with an homology to Hsp 90 have only recently been located in plants. During heat stress, Hsp 90 mRNA level is strongly induced, although at extremely high temperatures the appearance of a high molecular weight transcript would indicate the

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mRNA is not being completely sliced (Vierling, 1991). Krishna *et al.* (1995) found that a significant increase in Hsp90 mRNA level in *Brassica napus* seedlings subjected to 5°C. The cold-induced accumulation of Hsp90 mRNA correlates closely with the expression of two previously identified cold-regulated genes of *B. napus*. Cold regulation of Hsp90 mRNA in spinach (*Spinacea oleracea*) was also confirmed. From these results a role for Hsp90 was suggested in adaptation to cold temperature stress.

### 1.2.4.2 Hsp 110

Most plants synthesize a high molecular weight Hsp of approximately 110 kDa, which appears to be a soluble cellular component (Cooper and Ho, 1987, Lindquist and Craig, 1988). In plants the synthesis of Hsp 110 is more transient than other Hsps, synthesis being limited to the first hour of heat stress.

### 1.2.4.3 Ubiquitin

In many eukaryotes during heat stress, ubiquitin transcription is increased (Burke *et al.*, 1985; Lindquist and Craig, 1988; Christensen and Quail, 1989). Ubquitin is a 76 amino acid protein which covalently attaches to other proteins, labelling them for degradation. This ubiquitin-protein complex is reversible (Hershko, 1988). The production of ubiquitin increases during heat stress, indicating a possible increase in the removal of proteins damaged by stress.

### 1.2.4.4 Additional heat-regulated proteins

The synthesis of several proteins displayed an increase during heat stress have been identified in addition to the ones above. Due to elevated cell temperatures of 5-15°C above normal, this is expected. Generally, other heat stress induced proteins are species or tissue type specific, and the level of induction is significantly lower than Hsp70 or LMW Hsp (Vierling, 1991).

## 1.2.5 Relevance of Hsps to Plant Growth and Development

### 1.2.5.1 Expression of Hsps in the natural environment

In the laboratory, Hsp expression has been studied, normally under nonphysiological abrupt changes in temperature. Hsp gene transcripts start to accumulate after being placed in heat stress conditions for five minutes (Roberts and Key, 1990). Work has indicated that Hsp synthesis does not require abrupt stress. A gradual increase in temperature induces Hsp expression, which is what a plant would be exposed to under natural conditions (Altschuler and Mascarenhas, 1985; Kimpel and Key, 1985; Burke *et al.*, 1988; Vierling *et al.*, 1989; Chen *et al.*, 1990). Although little work has been done on Hsp expression in the field, Hsp expression does occur in the natural environment (Vierling, 1991).

Kimpel and Key (1991) showed that LMW Hsp mRNAs accumulate in field grown soyabeans on hot days. Hsp mRNA expression was higher in water stressed plants than well watered plants, and this could be due to differences in leaf temperature.

Hsp expression has been studied in a simulated field environment in which plants are placed under natural stress (Vierling *et al.*, 1989; Chen *et al.*, 1991). In the simulated field environment, young pea plants were subjected to morning, midday and decreasing afternoon temperatures with a set level of humidity to limit transpirational cooling. The number of LMW Hsps was directly proportional to midday leaf temperatures (Chen *et al.*, 1991). Class 1 cytoplasmic Hsps were detectable when maximum leaf temperatures were as low as 32°C, indicating that Hsps are frequently expressed even under low stress conditions. Plants were analysed during and after stress, and the half life of Hsps was identified as being 52hr and 37hr for chloroplast and cytoplasmic Hsp, respectively. Due to the persistence of these Hsps they could play an important role in the recovery process (Vierling, 1991).

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In plant structures which are not effectively cooled by transpiration, induction of Hsps may occur frequently. Reproductive structures which are too large for rapid heat exchange or which have a limited number of stomates will be at higher temperatures than the leaves, for example (Vierling, 1991).

At particular stages of plant development Hsp expression is more dominant (for example during seedling emergence where cooling mechanisms are limited). During seed maturation continual high temperatures are detrimental (Keigand Mullen, 1986). Seed temperatures high enough to induce Hsp have been found to be at optimal growing temperatures. These seeds have high levels of Hsp mRNA in axis and cotyledon tissue and exhibit maximum germinating potential.

In the natural environment more investigation into Hsp expression might display that the production of Hsps is a vital part of a plant's life, including species growing in optimum conditions (Vierling, 1991).

### 1.2.5.2 Hsp Expression during plant development

The induction of Hsp synthesis due to high-temperature stress tends to occur in transcriptionally active tissue. The transcription of Hsp genes and Hsp protein synthesis have been found to occur in all vegetative tissue, mid-maturation seeds (Altschuler and Mascarenhas, 1985; Chrispeels and Greenwood, 1987), aleurone of imbibed seeds (Brodl et al., 1990; Sticher et al., 1990) and in germinating embryos (Helm et al., 1989; Howarth, 1989; Helm and Albernathy, 1990). The stages of plant development where heat shock response is not activated occur at very early embryo development (Pitto et al., 1983; Zimmerman et al., 1989) and pollen germination (Xiao and Mascarenhas, 1985; Van Herpen et al., 1989).

### 1.2.5.3 Role of Hsps in thermotolerance

Pretreatment of plants at nonlethal high temperatures induces acquired thermotolerance, enabling the plant to withstand otherwise lethal temperatures. Experiments have shown that condititons under which thermotolerance is acquired are also those under which Hsps are induced. Lin *et al.* (1984), using etiolated soybean seedlings, showed that the rate of synthesis of LMW Hsps was proportional to the development of thermotolerance. This would suggest that Hsps are valuable against heat stress.

### 1.2.6 Expression of avocado Hsps

Woolf and Lay-Yee (1997) conditioned 'Hass' avocados at 38°C which gave positive results of thermotolerance to 50°C hot water treatments. Due to the possible role of heat shock proteins in acquired thermotolerance, heat shock protein gene expression in response to pretreatments was examined. Levels of Hsp17 and Hsp70 homologous mRNA increased with increasing pretreatment duration, perhaps indicating thermotolerance increases with an increase in pretreatment duration.

## 1.3 CONCLUSIONS

Postharvest heat treatment of horticultural commodities such as avocados would appear to be beneficial. Initially one needs to consider which type of heat to use. Dry heat, although it has been used, would almost certainly be detrimental to the fruit. A lot of work has been done on hot water and results were positive, as described earlier. Hot water has disadvantages on a larger scale, such as how to treat a shipment of avocados in a packshed. Initially the problem of putting a hot water bath in a packshed to accomodate enough fruit and maintain a stable temperature will be difficult and expensive. Vapour heat would appear to be a better option. Vapour heat firstly has 95% relative humidity or greater, hence not being detrimental to the fruit and extracting moisture. Secondly, vapour heat is a simpler form of heat to treat fruit on a commercial basis as it is easy to maintain temperature and treat large amounts of fruit simultanously. In Hawaii this has been illustrated by the vapour heat treating of containers of papaya for fruit fly disinfestation before being exported to Japan.

With vapour heat treatment of avocados it is thought that there will be physiological alterations such as formation of Hsp. In this literature review one can see how Hsps could be beneficial to avocados if they are heat induced. An analysis of the protein structure of 'Hass' and 'Fuerte' avocado which have been vapour heat treated is important to highlight any alteration in protein structure and if there is a correlation between the literature, beneficial properties displayed by heat treated avocados and any proteins produced under these conditions. If there is a correlation it could mean these beneficial properties of avocado are due to the presence of Hsp.

# CHAPTER 2

# POSTHARVEST VAPOUR HEAT TREATMENT OF 'HASS' AND 'FUERTE' AVOCADO

## 2.1 INTRODUCTION

Due to the traditional European avocado markets reaching saturation, alternative markets should be considered to avoid oversupply and lower prices. Markets such as Japan and Canada are an option if one can increase the storage life of fruit and meet the specific phytosanitary requirements. A major concern with the export of avocados is that fruit must arrive with acceptable firmness. The South African avocado industry is investigating various options for remunerative marketing of the expected larger crops, especially once the effects of prolonged drought and other climatic upsets have been overcome. The export crop of 6.5 - 7 million cartons in 1996 and 1997 will inevitably be followed by a crop far exceeding the record of over 10 million cartons. New markets and the adoption of CA storage are inevitable. The industry has also noted the promising reports of post-harvest heat treatments in Israel and New Zealand, and is supporting local research in this field.

Klein and Lurie (1991) stated that vapour heat treatment of avocados has the following benefits:

1) It reduces rate of ripening in climacteric fruit and hence extends shelf life;

2) increases tolerance of harvested products to low temperatures, thereby reducing chilling injury;

3) reduces incidence of postharvest diseases;

4) controls pests (disinfestation) such as with fruit fly found in papaya(*Carica papaya* L.) in Hawaii.

A variety of heat treatments have been tested such as dry heat, vapour heat and hot water in subtropical fruit such as avocado and papaya (Donkin and Wolstenholme, 1995). Heat treatment temperatures differ according to type of fruit. The oil storing avocado appears to be more heat sensitive than sugar storing fruit, hence lower temperatures are used for heat treatment in avocados (Bard and Kaiser, 1996).

## 2.2 MATERIALS AND METHODS

In this experiment two cultivars of avocado were used, viz. 'Fuerte' donated by Everdon Estate near Howick and 'Hass' donated by Cooling farm in the Bruyns Hill area. Both are large, well-managed farms in which the objective is to maximise the quantity and quality of export fruit. Both cultivars were exposed to the same treatment. The experimental design for the vapour heat treatments used on both cultivars is illustrated in Fig. 2.1. Both cultivars were exposed to temperatures of 36, 38, 40 and 42°C for a duration of 1, 2, 4 and 8 hrs. Control fruit were not heat treated and placed in cold storage. The statistical design was completely randomised, with 17 treatments and 32 ('Fuerte') or 36 ('Hass') single-fruit replicates.



**Figure 2.1** Experimental design for vapour heat experiment

For each treatment and storage period there were 32 replications for 'Fuerte' and 36 for 'Hass'. For example, 32 'Fuerte' avocados were treated for 38°C for 4 hrs, and stored for 5 weeks. The count size used (per 4 kg export carton) for 'Fuerte' was 16 (236-265g) and for 'Hass' was 18(211-235g). Fruit were treated in a vapour heat chamber, a Paxton Electrotherm heater and humidification unit contructed by Agrelek, Cedara College, and attached to a modified shipping container. For each treatment, air and fruit pulp temperatures were recorded with the use of a Campbell Scientific® data logger (Appendix 1) attached to several thermocouples. Fruit pulp temperatures were measured by inserting thermocouples (1cm) into fruit mesocarp. From information obtained by the data logger, graphs were obtained to ensure conditions were correct (Fig. 2.2) for every treatment. Once fruit had been treated, they were placed in cold storage at approximately 3.5°C, which is lower than the traditional 5.5°C used in South Africa. Every treatment had a storage period of 5 and 6 weeks.

The temperature of cold storage was recorded for the entire trial for the 1996 (Fig. 2.3) and 1997 (Fig. 2.4) avocado season to ensure condition were correct. For the 1997 season fruit pulp temperatures were also recorded (Fig. 2.4) and it can be seen fruit pulp temperatures lagged behind the air temperature. Following cold storage, fruit were removed and allowed to ripen at room temperature. The overall experiment was geared to both the 1996 and 1997 'Hass' and 'Fuerte' avocado export seasons for KwaZulu-Natal midlands. This is necessary as it is well known in the industry that fruit quality varys significantly from season to season.

Once fruit was removed from cold storage it was evaluated with regard to firmness, days to ripening, heat/cold damage, physiological disorders and weight loss. Firmness was measured, with a firmometer in the 1996 season and with a densimeter in the 1997 season, as soon as fruits were removed from cold storage. The values obtained using the firmometer were recorded in hundreds as opposed to tens in order to illustrate a clearer trends. With firmometer values the lower the value the harder the fruit. Firmness in the 1997 season was recorded with a densimeter. The reason for

changing from a firmometer to a densimeter was to keep up to date with instrumentation as used by the South African Avocado Growers Association to evaluate exported fruit arriving at the various ports of destination. Unlike the firmometer values, the higher the densimeter value the harder the fruit. Days to ripening was time taken from when fruits were removed from cold storage to eating ripe at room temperature (ca 20°C). Heat/cold damage was evaluated on removal from cold storage as well as at time of ripening. Physiological disorders were evaluated when fruit were ripe. Weight loss of fruit was determined after vapour heat treatments as well as after 5 and 6 week storage periods. The overwhelming bulk of this loss was believed to be due to water loss from the fruit.
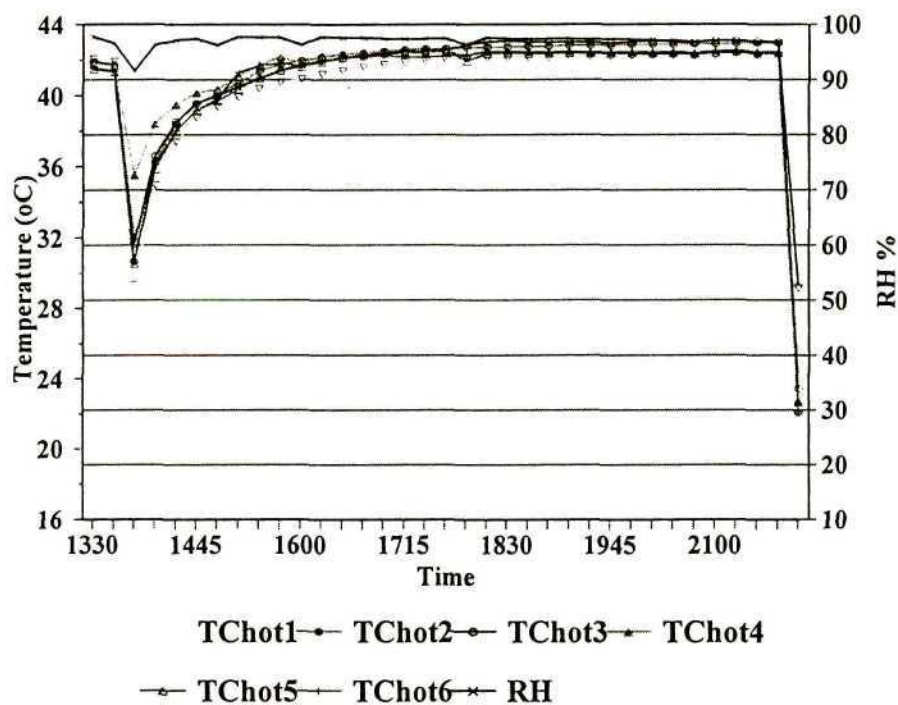
**Figure 2.2** A typical graph displaying fruit pulp temperatures of vapour heat treatment at 42°C (TC=thermocouple, RH=relative humidity)
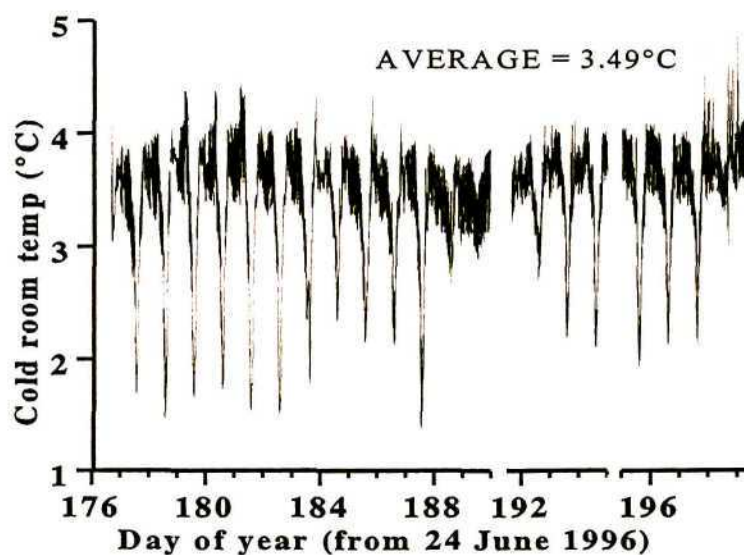


**Figure 2.3** The cold storage air temperatures for the1996 season

41
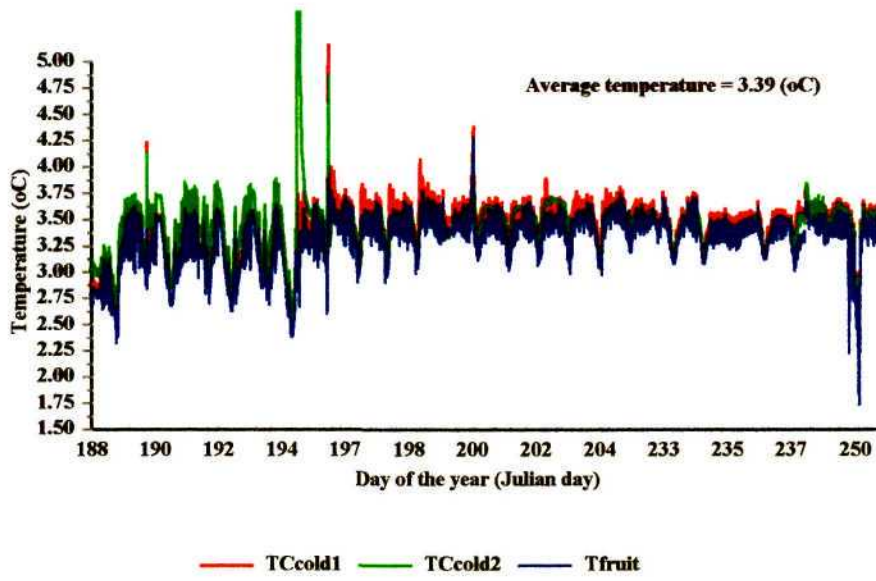
**Figure 2.4** Cold storage air and fruit pulp temperatures for the entire 1997 season

# 2.3 RESULTS AND DISCUSSION FOR 1996 AVOCADO SEASON

## 2.3.1 Firmness

### 2.3.1.1 'Fuerte'

Firmometer readings for 'Fuerte' immediately after 5 weeks of cold storage displayed a trend of fruit being firmer as duration and temperature of vapour heat treatment increased (Fig. 2.5). There was no significant difference in firmometer readings between the control and fruit receiving different treatments. The differences between firmometer readings for different temperatures and times were both highly significant ($P < 0.001**$) while the temperature and time interaction was not significant. After 6 weeks of cold storage (Fig. 2.6) the same trends for 'Fuerte' were similar, although the control was significantly different ($P < 0.05*$) from the rest of the treatments.

**Figure 2.5** Fruit firmness (firmometer readings) for 'Fuerte' after 5 weeks of cold storage at 3.5°C
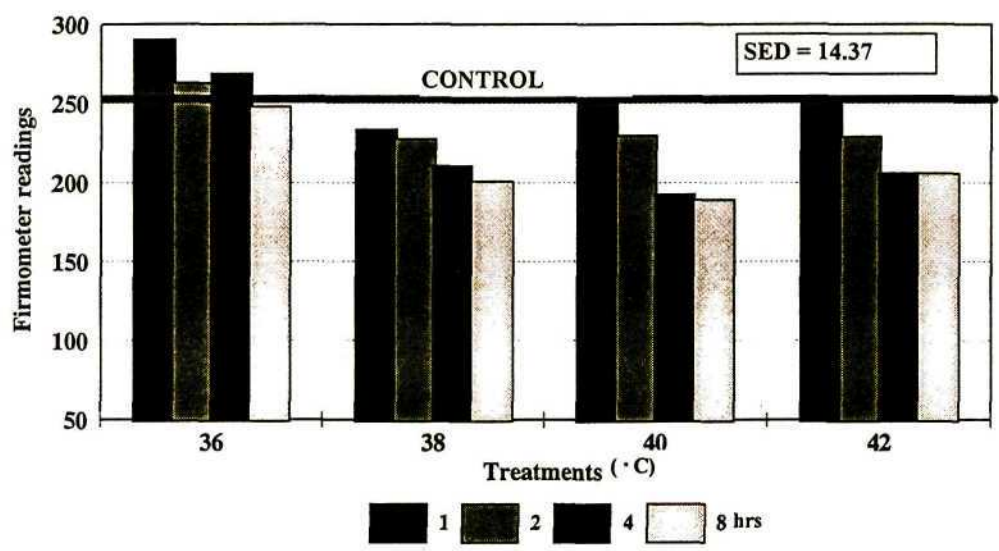


**Figure 2.6** Fruit firmness (firmometer readings) for 'Fuerte' after 6 weeks of cold storage at 3.5°C

### 2.3.1.2 'Hass'

After 5 weeks cold storage 'Hass' did not display such clear trends such as those obtained for 'Fuerte' and tended to be equal to control (Fig. 2.7). The difference between firmometer readings for different temperatures was highly significant ($P < 0.001$**) while differences between times were significant ($P < 0.05$*). Time/temperature interaction was highly significant ($P < 0.001$**). At 42°C fruits were less firm after cold storage. Similar trends after 6 weeks cold storage were also illustrated (Fig. 2.8). The differences between different temperatures and times and temperature/time interaction were highly significant ($P < 0.001$**). It is interesting to note all fruit of both cultivars, including controls, had acceptable firmness of less than 300, which is suitable for export markets.
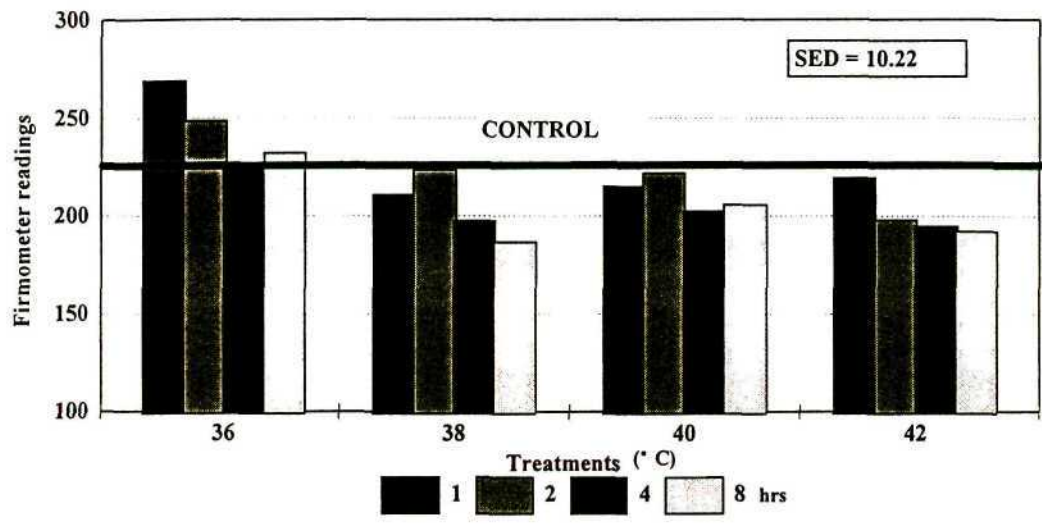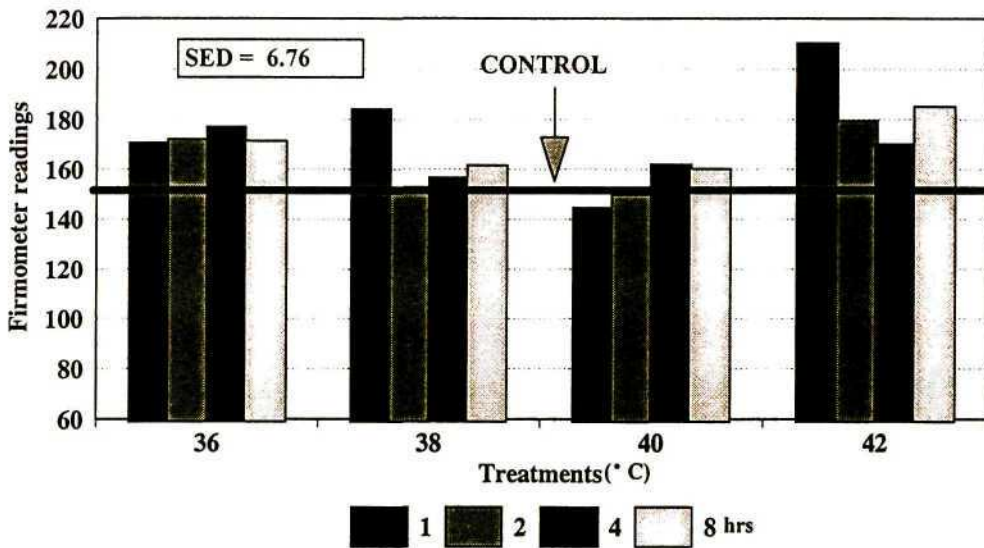


**Figure 2.7** Fruit firmness (firmometer readings) for 'Hass' after 5 weeks of cold storage at 3.5°C
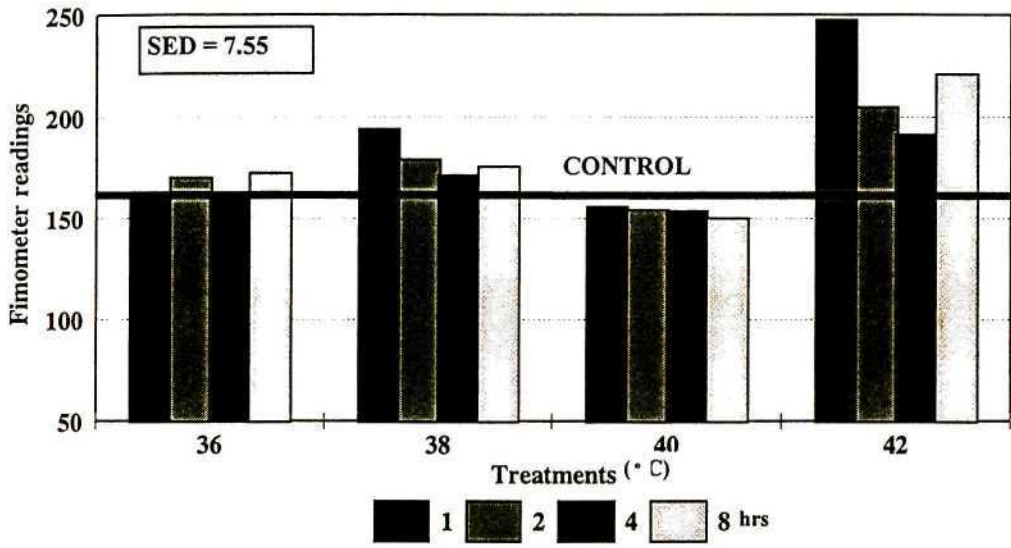
**Figure 2.8** Fruit firmness (firmometer readings) for 'Hass' after 6 weeks of cold storage at 3.5°C

## 2.3.2 Days to Ripening

### 2.3.2.1 'Fuerte'

For 'Fuerte' which was cold stored for 5 weeks, as duration of heat treatments increased shelf life also increased (Fig. 2.9). No significant differences were found between the control and the heat treatments, but as many as 2 extra days of shelf life were achieved by 36°C for 8 hrs. Highly significant differences ($P < 0.001$**) were found between the different times and temperature as well as time and temperature interaction, emphasizing the importance in choosing the correct treatment to obtain maximum shelf life. After 6 weeks (Fig. 2.10) similar trends were observed although in the 36°C heat treatment shelf life was virtually identical to the control. The difference between the control and the heat treatments, different temperatures and times and time/temperature interaction were all highly significant ($P < 0.001$**). An extra 4 days of shelf life was achieved by heat treatment at 40°C for 8 hrs. Although statistically this is no different to the 8 days in the control.



**Figure 2.9** Days to ripening for 'Fuerte' after 5 weeks of cold storage at 3.5°C

47
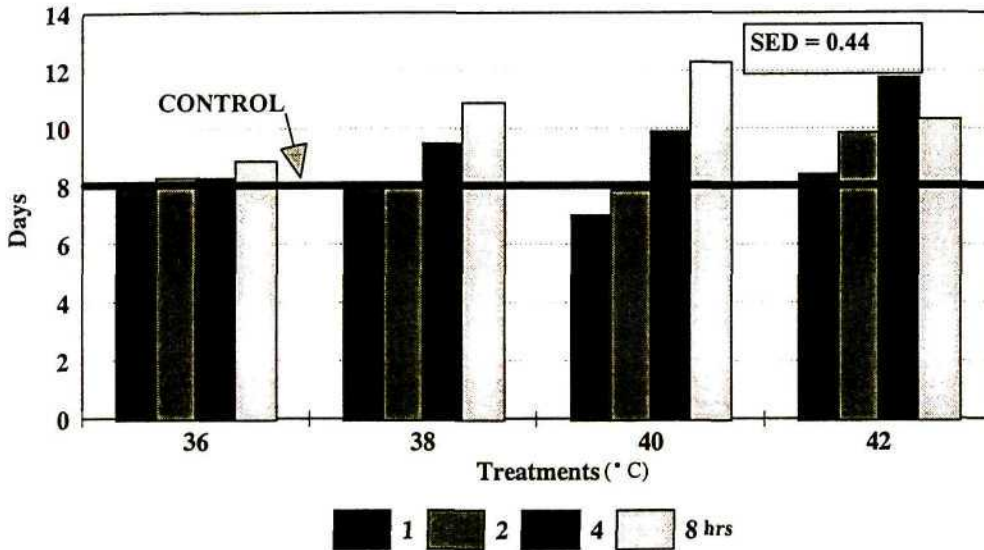
**Figure 2.10** Days to ripening for 'Fuerte' after 6 weeks of cold storage at 3.5°C

### 2.3.2.2 'Hass'

After 5 weeks of cold storage for 'Hass' only the longer treatments seem to have increased shelf life (Fig. 2.11). The control was not significantly different in ripening time from the rest of the treatments, although 40°C for 8 hrs induced an extra four days of shelf life above that of the control. Differences between different times and temperatures as well as time/temperature relationships were highly significant ($P < 0.001**$). This significant difference illustrates different effects the different treatments have on extending ripening time. After 6 weeks of cold storage (Fig. 2.12) similar trends were seen but the control was found to be significantly different from the treatments ($P < 0.001**$).
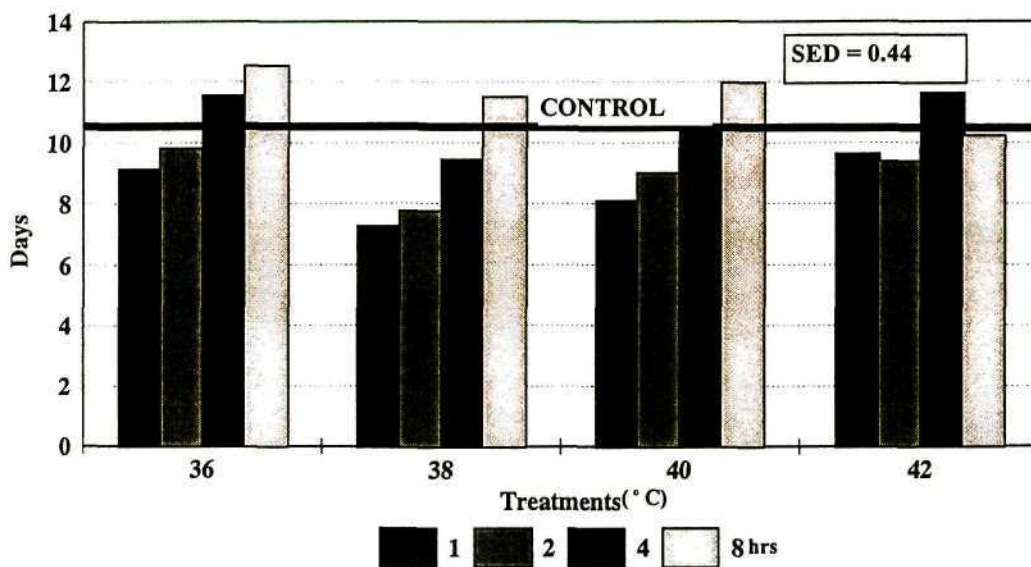
48
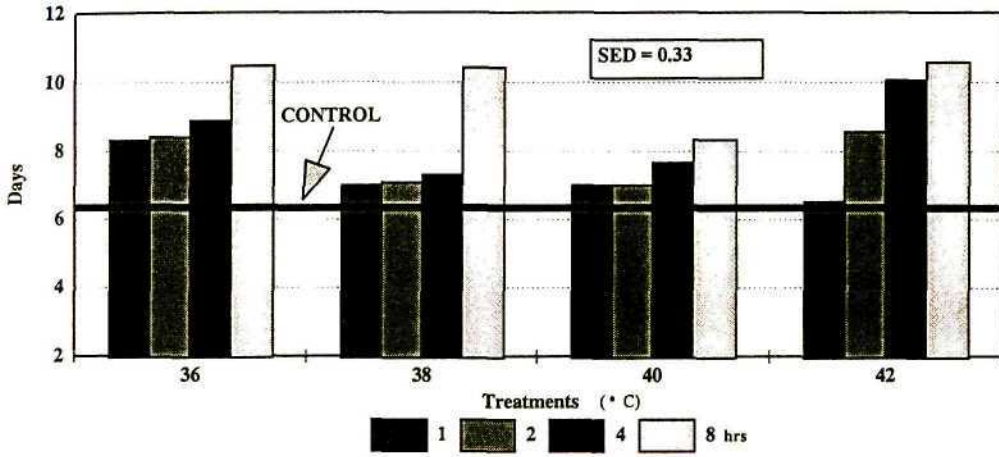
**Figure 2.11** Days to ripening for 'Hass' after 5 weeks of cold storage at 3.5°C
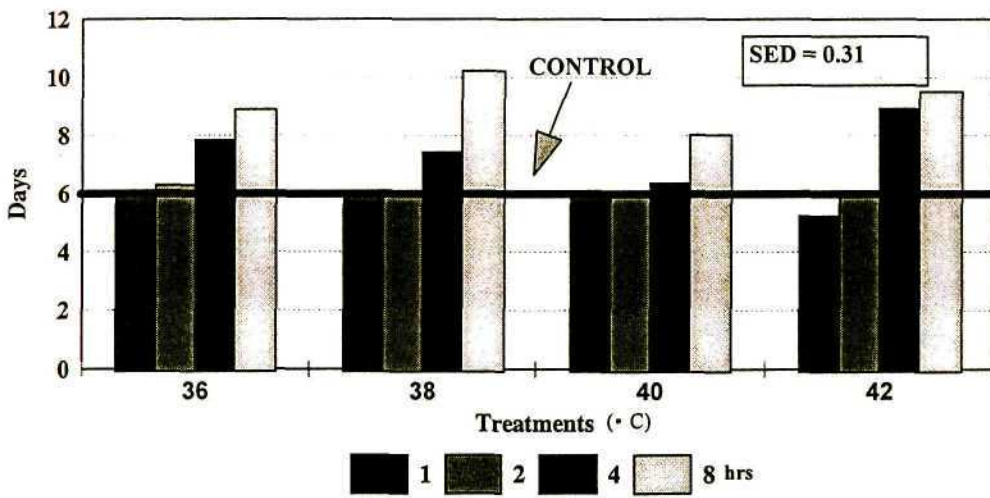


**Figure 2.12** Days to ripening for 'Hass' after 6 weeks of cold storage at 3.5°C

49

Interestingly the 38°C heat treatment of 'Hass' delayed ripening after 5 weeks cold storage. Three days after fruit from this treatment were removed from cold storage rind colour was evaluated and the results are shown in Table 2.1.

**Table 2.1 'Hass' rind colour at different durations of 38°C, after 5 weeks cold storage at 3.5°C**

| Duration at 38°C | Rind colour score of 18 fruit |
|---|---|
| 1 hour | 18 black |
| 2 hour | 10 black, 8 green |
| 4 hour | 4 black, 14 green |
| 8 hour | 18 green |

From Table 2.1, the delay in ripening and the increase in shelf life due to the different treatments can be observed, with the 8 hour treatment being the most effective. This is represented by a series of photographs shown in figures 2.13 - 2.16.
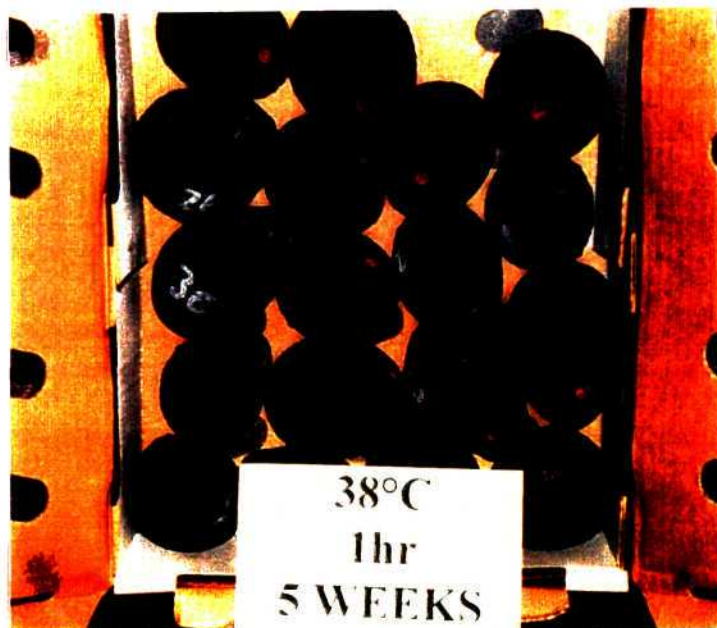


**Figure 2.13** 'Hass' treated at 38°C for 1 hour, and 3 days out of cold storage at 3.5°C for 5 weeks
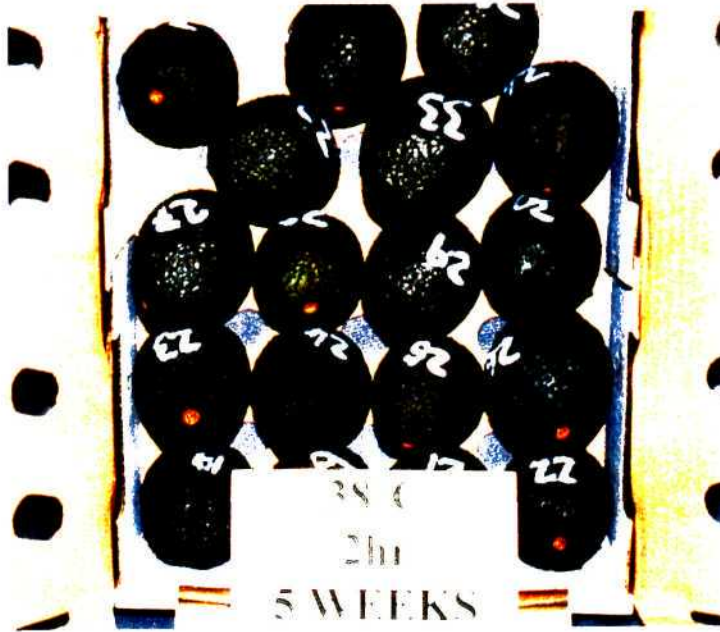
**Figure 2.14** 'Hass' treated at 38°C for 2 hrs, and 3 days out of cold storage at 3.5°C for 5 weeks



**Figure 2.15** 'Hass' treated at 38°C for 4 hrs, and 3 days out of cold storage at 3.5°C for 5 weeks
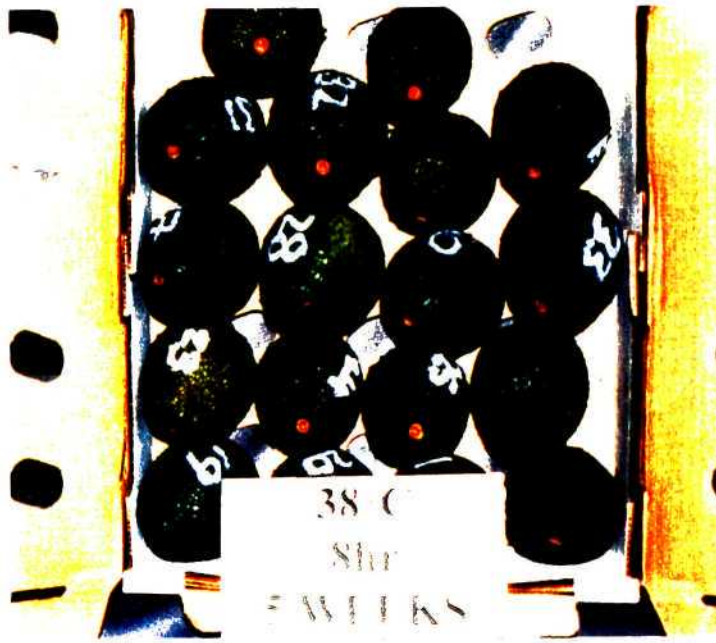
**Figure 2.16** 'Hass' treated at 38°C for 8 hrs, and 3 days out of cold storage at 3.5°C for 5 weeks

### 2.3.3 Heat/Cold Damage

Heat/cold damage was determined by visual evaluation of the external appearance of the avocado fruit. Heat/cold damage being any external damage to the rind usually in the form of blackening. A subjective rating of 0 - 10 was used, where 0 indicated a fruit free of any heat/cold damage and 10 indicated a fruit with severe damage and of no commercial value.

#### 2.3.3.1 'Fuerte'

After 5 weeks of cold storage for 'Fuerte' no chilling injury was observed (Fig. 2.17; see control), and any damage can be assumed to be heat damage. This finding was confirmed by electron microscopy work done in Chapter 3. As treatment increased in temperature and duration so did rind heat damage. The time/temperature interaction was highly significant (P<0.001**). Fuerte after 6 weeks displayed similar trends (Fig. 2.18), with the time/temperature interaction again being highly significant (P<0.001**).

To highlight the heat damage to 'Fuerte' rinds induced by vapour heat treatment, the 42°C treatments were analysed after being in cold storage for 1 week. The control fruit (Fig. 2.19) had no external damage; 42°C for 4 hrs (Fig. 2.20) also had no heat damage; whereas 42°C for 8 hrs (Fig. 2.21) had extensive damage to the rind, illustrating how detrimental vapour heat can be if the "damage threshold" is passed.
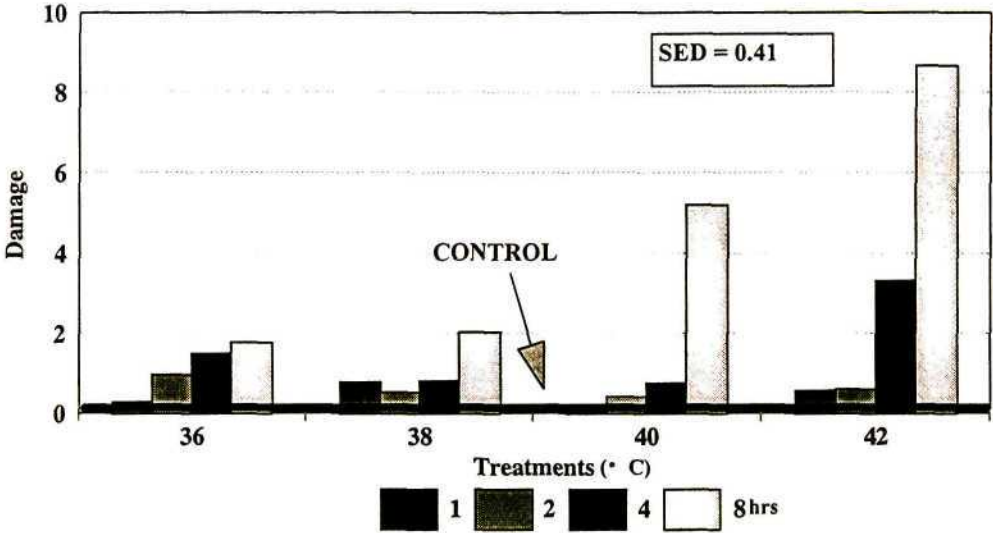


**Figure 2.17** Heat/cold damage for 'Fuerte' after 5 weeks of cold storage at 3.5°C (damage to the rind was rated on a scale of 0 - 10)

53
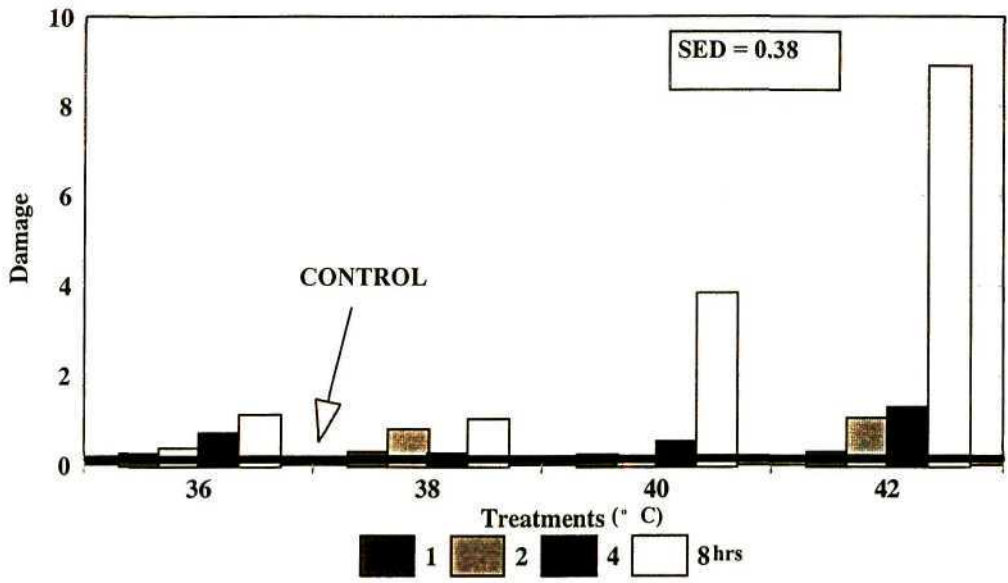
**Figure 2.18** Heat/cold damage for 'Fuerte' after 6 weeks of cold storage at 3.5°C (damage to the rind was rated on a scale of 0 - 10)



**Figure 2.19** Control fruit after 1 week in cold storage at 3.5°C, no heat/cold damage present
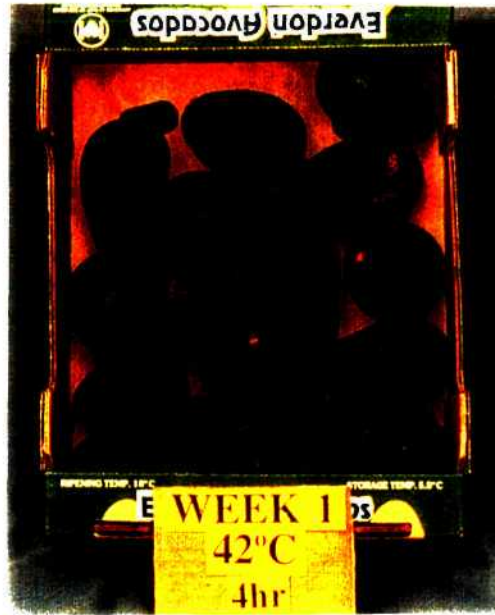
**Figure 2.20** 'Fuerte' treated at 42°C for 4 hrs after 1 week in cold storage at 3.5°C, with little heat/cold damage present



**Figure 2.21** 'Fuerte' treated at 42°C for 8 hrs after 1 week in cold storage at 3.5°C, illustrating severe heat damage

55

### 2.3.3.2 'Hass'

'Hass' after 5 weeks storage at 3.5°C showed little sign of chilling injury and the graph displaying heat/cold damage had to be magnified because with a rating out of 10 it was difficult to obtain a visible presentation (Fig. 2.22). Longer treatments at 42°C resulted in a slightly higher level of heat damage. The time/temperature relationship was highly significant (P<0.004**), showing that longer treatments were more detrimental to fruit quality. After 6 weeks, similar trends were found where longer heat treatments tended to increase heat damage (Fig. 2.23). Again time/temperature relationships were significantly different (P<0.001**).
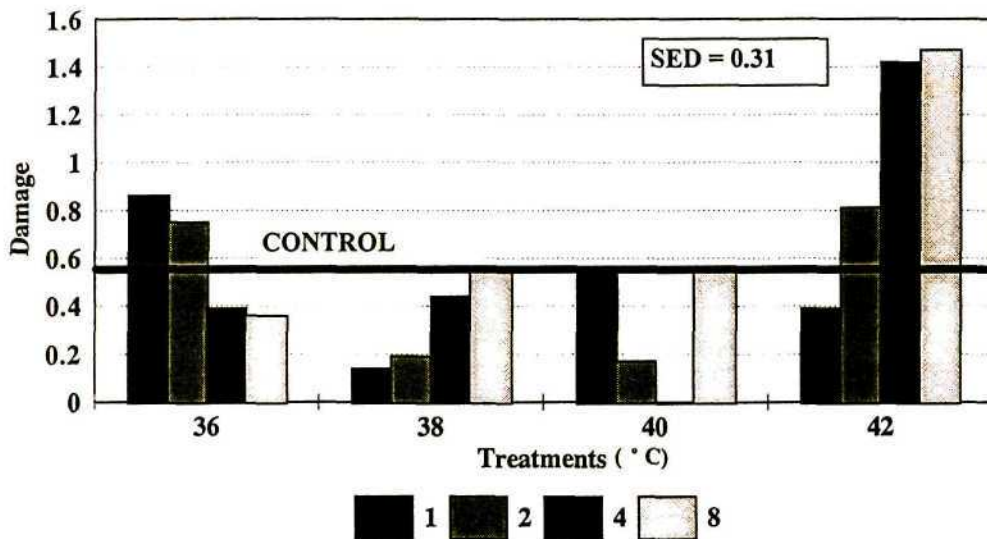


**Figure 2.22** Heat/cold damage for 'Hass' after 5 weeks of cold storage at 3.5°C (damage to the rind was rated on a scale of 0 - 10)

56
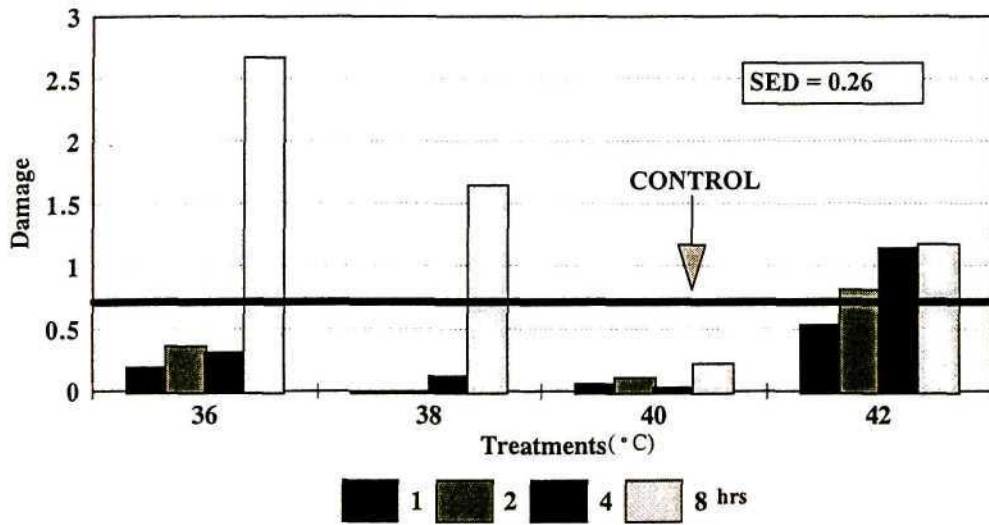
**Figure 2.23** Heat/cold damage for 'Hass' after 6 weeks of cold storage at 3.5°C (damage to the rind was rated on a scale of 0 - 10)

### 2.3.4 Physiological disorders

In both cultivars, the control fruit displayed no physiological problems for both storage periods. The problems are generally associated with longer and hotter treatments, hence it is important to determine where beneficial vapour heat treatment becomes detrimental.

'Fuerte' showed few signs of physiological disorders. Fruit treated at 42°C for 8 hrs displayed physiological problems with respect to pulp spot, vascular browning and mesocarp discolouration. A rating of 0 - 10 was used for physiological disorders, with 10 being the most severe. At 42°C for 8 hrs the heat appeared to have totally destroyed the tissue resulting in an average rating of 8 for physiological problems.

Mesocarp discolouration at 36°C treatments was greater than in control fruit. 'Hass' showed very few physiological disorders for both storage periods and highlighted that, in 'Hass', physiological disorders were not induced by this temperature regime.

### 2.3.5 Weight loss

Weight loss by fruit of both cultivars immediately after vapour heat treatments was around 1 gram (+/- 0.3% weight loss), indicating that vapour heat treatments did not cause substantial moisture loss in treated fruits. There were no significant differences between weight before and after vapour heat treatment. This would also indicate that the vapour heat used in these trials was close to the design target of 95% or higher relative humidity.

### 2.3.5.1 'Fuerte'

After 5 weeks cold storage 'Fuerte' fruit had lost 9 grams in the control, while 38, 40, and 42°C for 4 hrs caused an average reduction in weight loss of only 7 grams (2% weight loss). The 38°C for 8 hr treatment had the largest weight loss of 14 grams (4.6% weight loss), (Fig. 2.24). There were no significant differences present in weight loss between all treatments when compared to the control after 5 weeks of cold storage. 'Fuerte' displayed similar trends after 6 weeks although control fruit increased in moisture loss to 11 grams (3.6%), which is a significant difference ($P < 0.05*$) from the treated fruit.
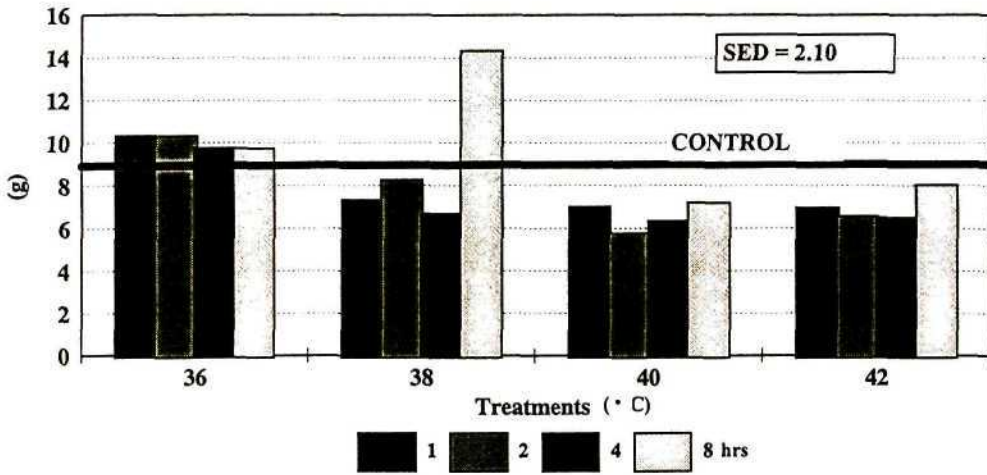
**Figure 2.24** Fresh weight loss (g) for 'Fuerte' after 5 weeks of cold storage at 3.5°C

### 2.3.5.2 'Hass'

There were no significant differences in weight loss between control and treated fruit after 5 weeks of cold storage at 3.5°C. The trend, in fact, was for heat treated fruit to lose less weight than control fruit. After 6 weeks of cold storage at 3.5°C, treated fruit on average lost significantly less (P < 0.001**) weight than control fruit. Fruit treated at 42°C, the highest temperature used, lost significantly (P < 0.001**) more weight than control irrespective of duration of heat. Most of this weight loss can be attributed to moisture loss.

With both cultivars vapour heat treated fruit tended to have a reduced weight loss when compared to the control after its specific storage period. This perhaps suggests a "conditioning" of the fruit due to heat treatment, resulting in longer storage and shelf life.

## 2.4 CONCLUSIONS

Initially for the 1996 Kwazulu-Natal avocado season, vapour heat treatment of 'Hass' and 'Fuerte' avocado showed potential to extend storage life. Two major factors in considering which vapour heat treatments to use for 'Hass' and 'Fuerte' avocado, are the trade-off in an extension of storage life versus heat damage. Heat damage is a limiting factor, and this is demonstrated by 'Fuerte' fruit treated at 42°C for 8 hrs. This treatment extended shelf life by 4 days, although not sinificantly, but also had a heat damage rating of 5 out of 10. For 'Fuerte' and 'Hass', treatments have been evaluated and are clearly represented in Figure 2.25 and 2.26 respectively. For 'Fuerte' possibilities for treatments exist at 38°C between 4 and 8 hrs, 40°C between 4 and 8 hrs and 42°C between 2 and 4 hrs. For 'Hass' a possibility exists for 38°C between 4 and 8 hrs.

| | 36°C | 38°C | 40°C | 42°C |
|---|---|---|---|---|
| 1hr | X | X | X | X |
| 2hr | X | X | X | ☺ |
| 4hr | X | ☺ | ☺ | ☹ |
| 8hr | X | ☹ | ☹ | X |

**Figure 2.25** Vapour heat treatment ratings for 'Fuerte' for the 1996 season (cross=no good, happy face=good but could but the heat treatment could be longer due to lack of heat damage, sad face=good, but induces heat damage)

**Figure 2.26** Vapour heat treatment ratings for 'Hass' for the 1996 season. Notation as for Figure 2.25

# 2.5 RESULTS AND DISCUSSION FOR 1997 AVOCADO SEASON

Promising results were obtained from the 1996 avocado season, but many environmental factors can change from year to year. For this reason it was necessary to repeat the experiment for the 1997 avocado season to evalute if vapour heat treament of avocados is a postharvest technique that can be considered to extend shelf life from year to year, and not dependent on the varying fruit quality from year to year.

## 2.5.1 Firmness

### 2.5.1.1 'Fuerte'

Denismeter readings for 'Fuerte' after 5 weeks of cold storage (Fig. 2.27) for treatments at 36°C and 38°C displayed no trend, while at 40°C increasing duration of treatment resulted in firmer fruit.At 42 °C similar trends were noted except for the 8 hr treatment which resulted in softer fruit, perhaps indicating that this treatment is the cut off point between beneficial and detrimental treatments. The densimeter readings for the control vs effects of treatment temperature, effect of time and temperature:time interaction were not significantly different. Densimeter readings for 'Fuerte' after 6 weeks storage (Fig. 2.28) showed similar trends to the 5 week storage period, but slightly poorer results at 38°C. The differences in temperature were significant ($P<0.01$**) for the 6 week storage period.

**Figure 2.27** Fruit firmness (densimeter readings) for 'Fuerte' after 5 weeks of cold storage at 3.5°C
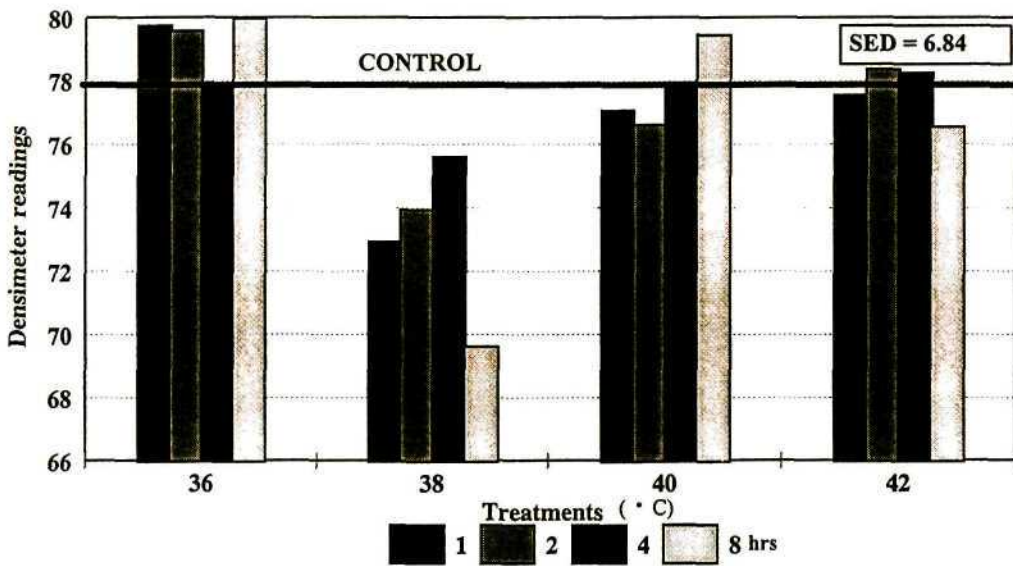


**Figure 2.28** Fruit firmness (densimeter readings) for 'Fuerte' after 6 weeks of cold storage at 3.5°C.

### 2.5.1.2 'Hass'

Densimeter readings for 'Hass' after 5 weeks of cold storage for all treatments were below the control, i.e. softer fruit, and no trends were observed (Fig. 2.29). Although, the densimeter readings for the treatments were below the control, the difference between the control and the lowest densimeter reading is only 7 units. The differences in duration of temperatures and the time/temperature interaction between different temperatures were not significant. Untreated fruit ($P<0.01**$) and different temperatures ($P<0.05**$) were significantly different when comparing densimeter readings. For 'Hass' after 6 weeks of cold storage (Fig. 2 .30) no obvious trends were seen, and again all values for heat treated fruit were less than the control. The difference between the control and lowest value was 10 units. The control vs treatment effects of temperature and temperature/time interaction were significantly different ($P<0.001**$). The differences in treatment time were not significant.

In 1997 all heat treatments failed to keep fruits firmer than untreated fruits, unlike 1996. Although when the rest of the seasons data were analysed, a positive result similar to 1996 was found with regard to extending shelf life. The results obtained from 1997 illustrating the fruit are softer that the control could be due to the use of the densimeter. This could be due to the densimeter having a small ball bearing testing the pressure of the mesocarp as opposed to a firmometer which tests a far greater area and to a greater depth, hence it would suffice to say the readings from the firmometer could be more accurate.
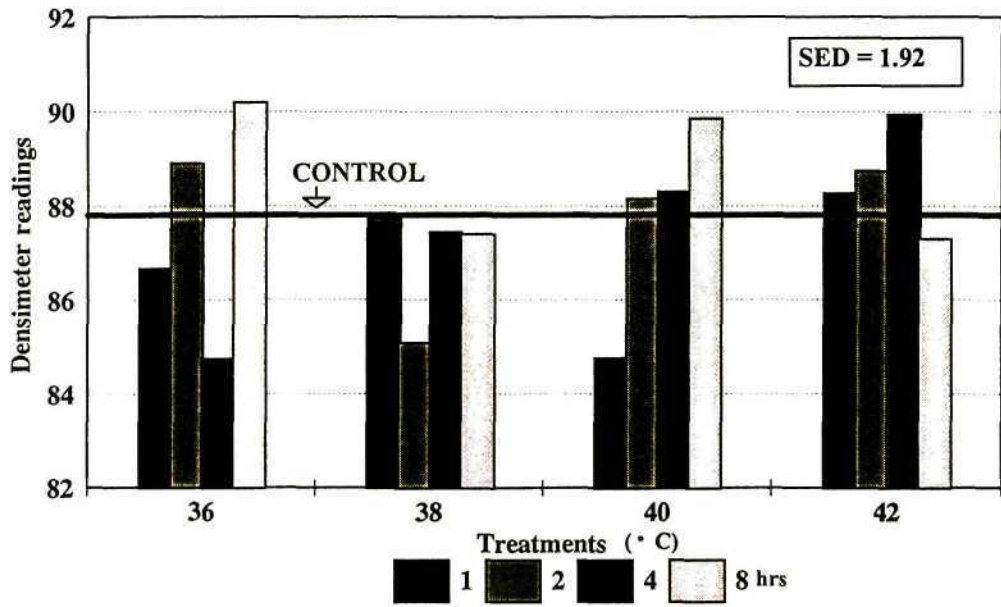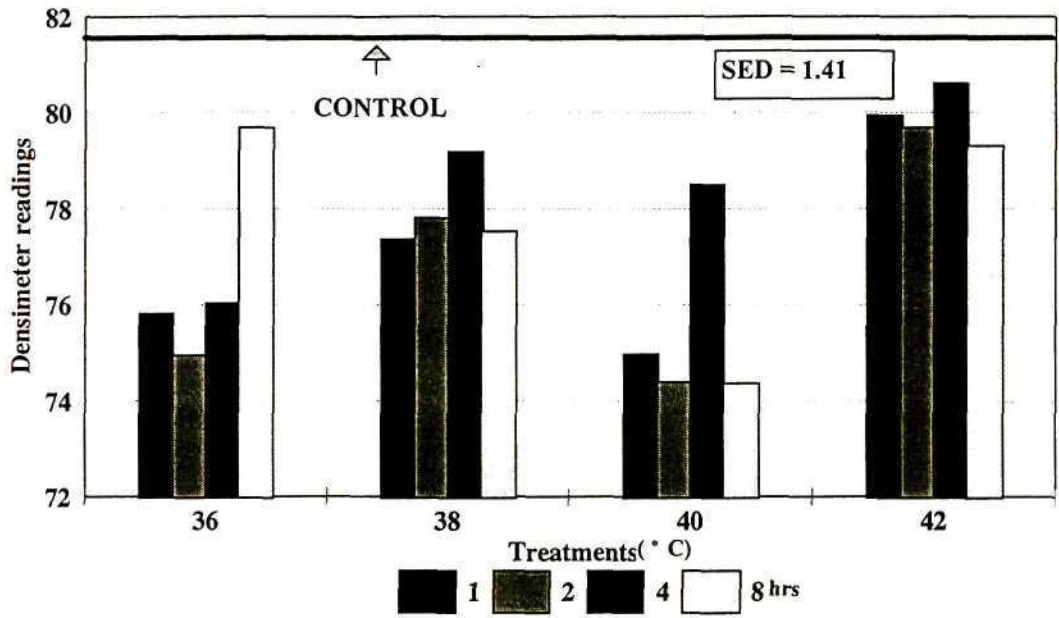
**Figure 2.29** Fruit firmness (densimeter readings) for 'Hass' after 5 weeks of cold storage at 3.5°C
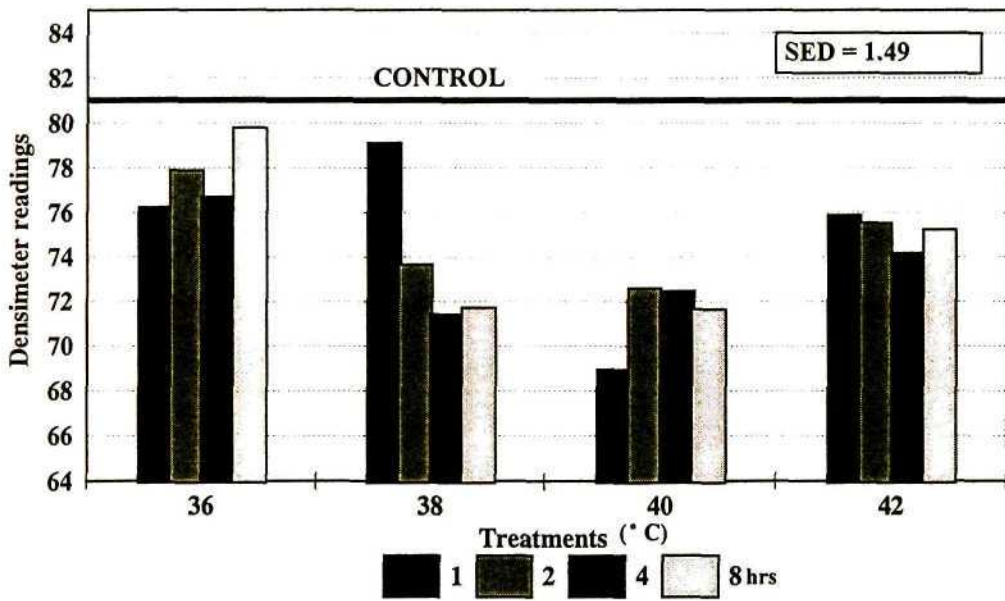


**Figure 2.30** Fruit firmness (densimeter readings) for 'Hass' after 6 weeks of cold storage at 3.5°C

**2.5.2 Days to Ripening**

**2.5.2.1 'Fuerte'**

As each temperature treatment increased in duration so too did days to ripening for 'Fuerte' after 5 weeks of cold storage (Fig. 2.31). The days to ripening for the control vs treatment, differences in temperature, differences in duration and the time/temperature interaction were all significantly different (P<0.001**). The control was eating ripe 4 days after removal from cold storage while heat treatment of 40°C for 8 hrs increased shelf life by 4 days and 38°C for 8 hrs achieves an extra 3 days of shelf life. 'Fuerte' after 6 weeks of cold storage illustrated (Fig. 2.32) similar trends to the 5 week storage period, also with 40°C for 8 hrs achieving a maximum extra storage life of 3 days above that of the control. The control vs treatments, differences in temperature, differences in duration and temperature/time interaction are all significantly different (P<0.001**). The 42°C for 8 hrs displays a decline in days to ripening perhaps illustrating the detrimental effect of this treatment.
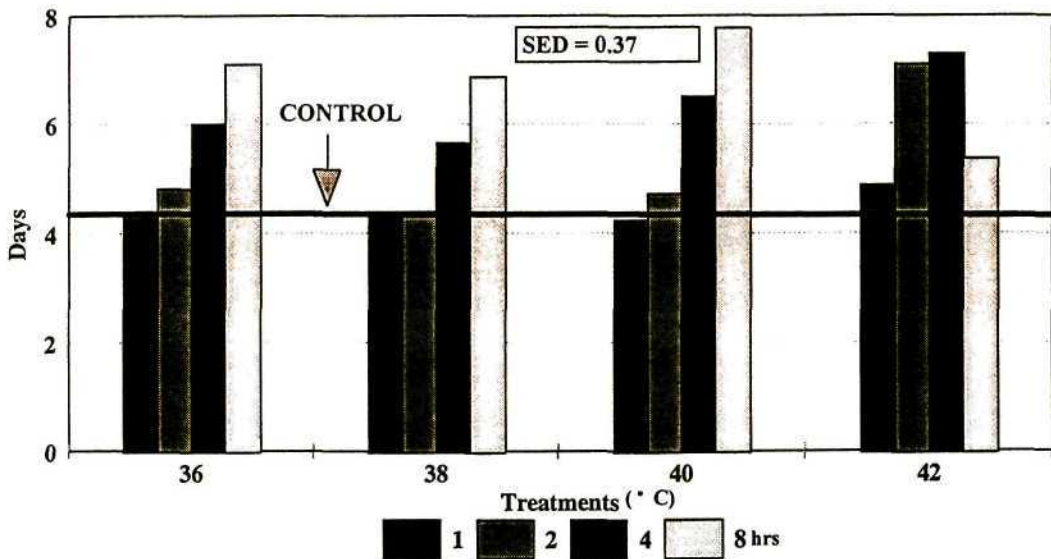


**Figure 2.31** Days to ripening for 'Fuerte' after 5 weeks of cold storage at 3.5°C

66
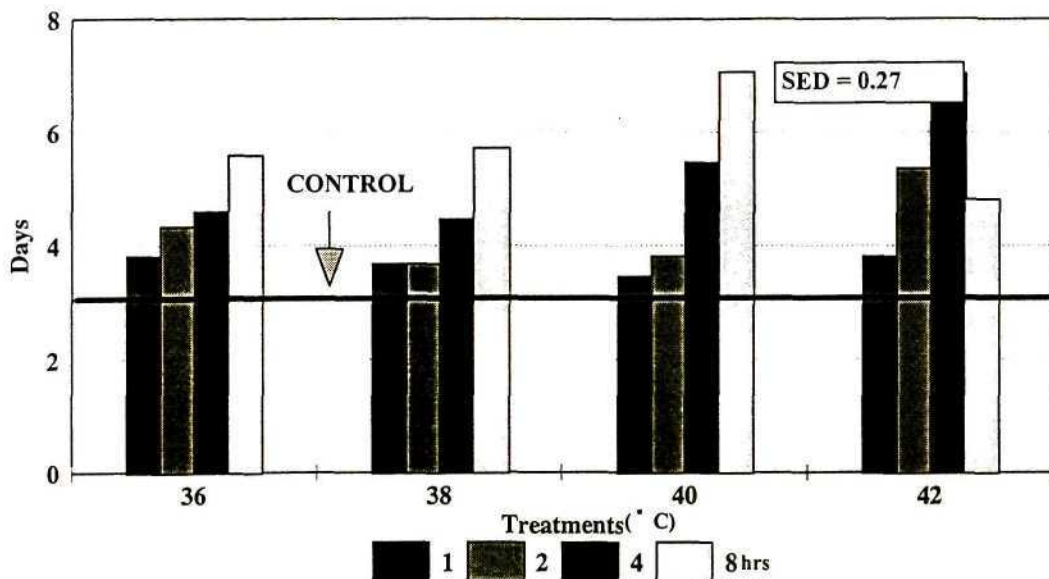
**Figure 2.32** Days to ripening for 'Fuerte' after 6 weeks of cold storage at 3.5°C

### 2.5.2.2 'Hass'

For 'Hass' similar trends were clearly seen as with 'Fuerte'when analysing an extension of shelf life. As the duration of the heat treatment increased so too did the days to ripening for both storage periods except for 42°C for 8 hrs (Figs. 2.33; 2.34). The days to ripening for the control vs treatments, differences in temperature, differences in duration and temperature/time interaction were all significantly different for both storage periods (P<0.008** for 5 weeks; P<0.001** for 6 weeks storage period). Temperatures of 40°C and 38°C for 8 hrs produced roughly an extra 3 days of shelf life.

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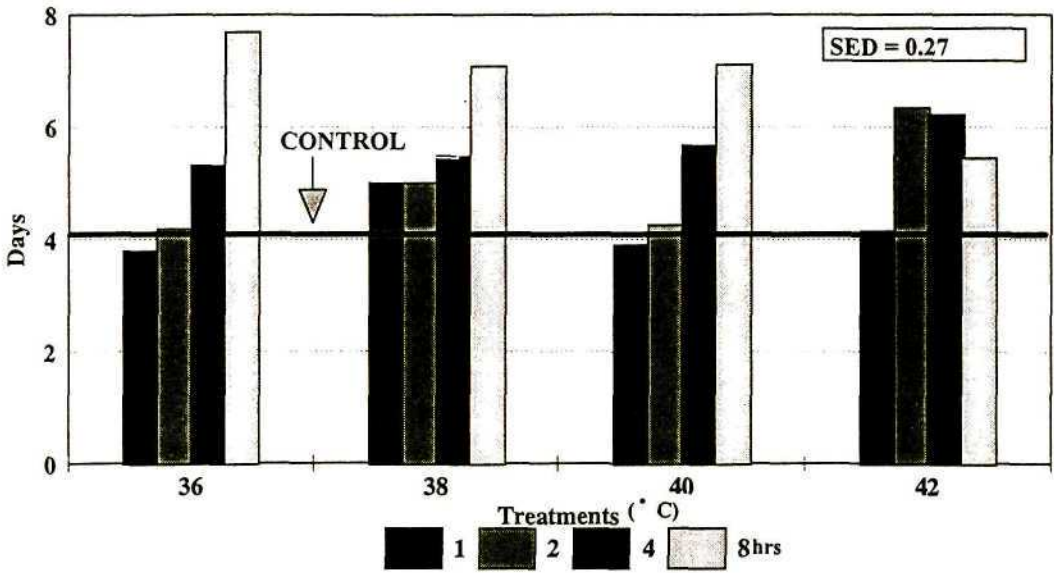
**Figure 2.33** Days to ripening for 'Hass' after 5 weeks of cold storage at 3.5°C
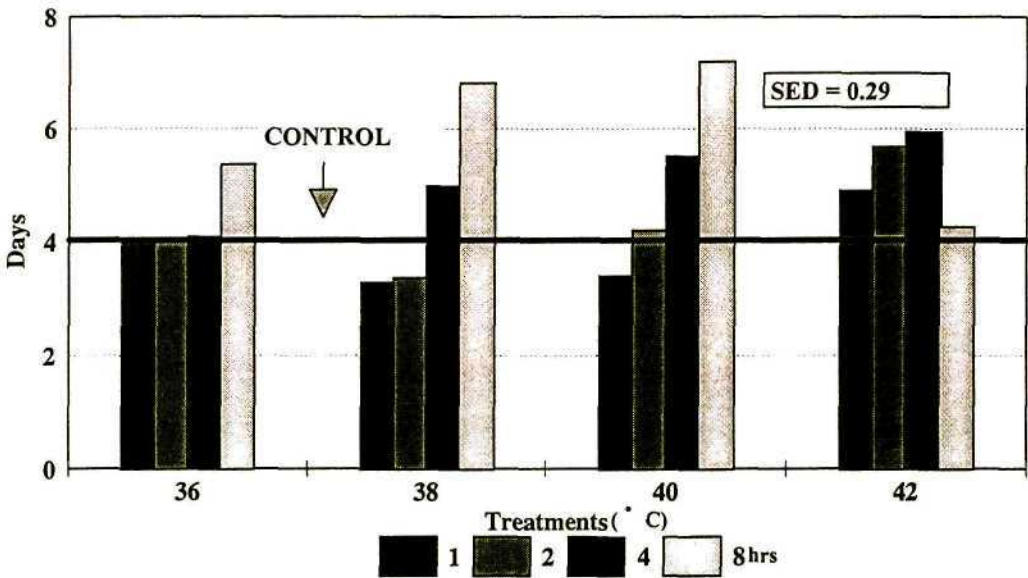


**Figure 2.34** Days to ripening for 'Hass' after 6 weeks of cold storage at 3.5°C

### 2.5.3 Heat/Cold Damage

#### 2.5.3.1 'Fuerte'

For 'Fuerte' after 5 weeks of cold storage, untreated (control) fruit had a rating of below 0.5 (Fig. 2.35), hence any rind damage present is heat damage. This is illustrated by the longer heat treatments having a higher heat damage rating. At 40°C for 8 hrs and 42°C for 8 hrs scores were highly unsatisfactory at 6 and 10, respectively. The heat damage rating for the control, differences in temperature, differences in duration and temperature/time interaction were all significantly different (P<0.001**). After 6 weeks of cold storage untreated 'Fuerte' had a chilling injury rating of 2 (Fig. 2.36). The longer treatments induced severe heat damage; e.g 42°C for 8 hrs scoring 8.5. The heat damage rating for the control, differences in temperature, differences in duration and time/temperature interaction are all significantly different (P<0.001**).
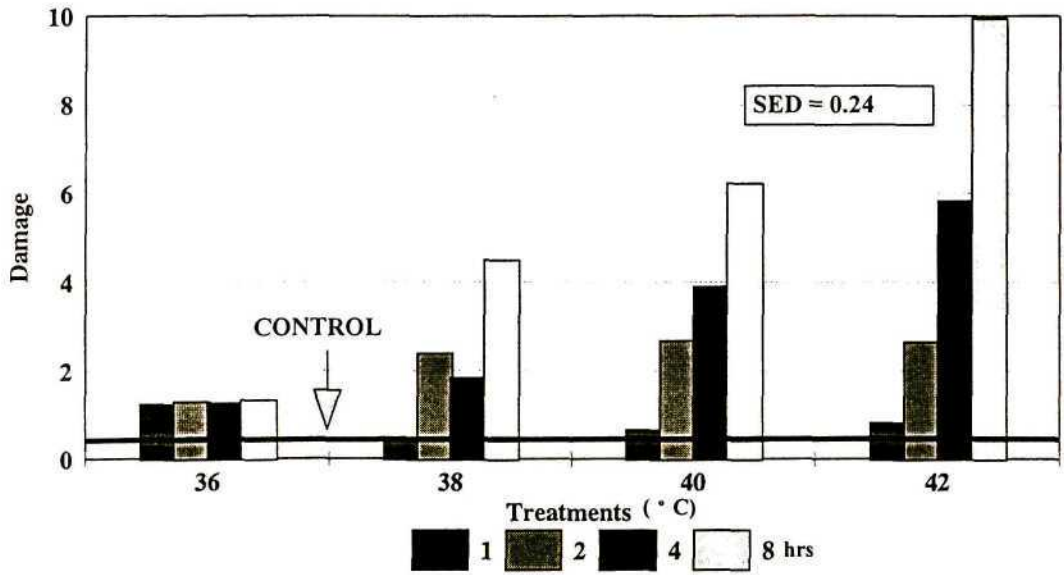
**Figure 2.35** Heat/cold damage for 'Fuerte' after 5 weeks of cold storage at 3.5°C (damage to the rind was rated on a scale of 0 - 10)
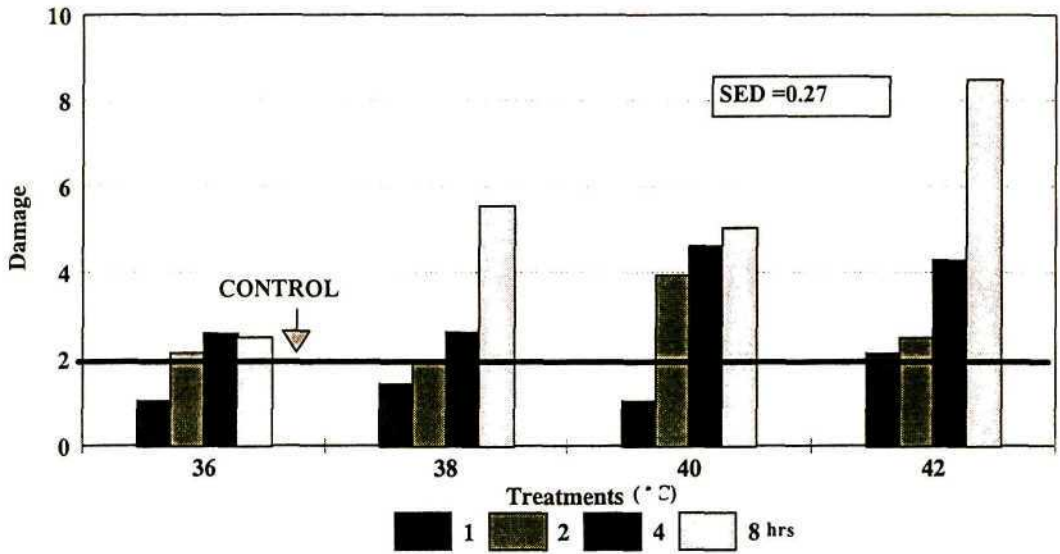


**Figure 2.36** Heat/cold damage for 'Fuerte' after 6 weeks of cold storage at 3.5°C (damage to the rind was rated on a scale of 0 - 10)

### 2.5.3.2 'Hass'

For untreated 'Hass' after 5 weeks of cold storage almost no chilling injury was observed (Fig. 2.37). At 42°C, damage was noted at 1, 2 and 8 hrs, with the 8 hr treatment scoring 7. Similar trends were seen for 'Hass' after 6 weeks (Fig. 2.38) of cold storage, heat damage at 42°C for 8 hrs, with a rating of 7 was present. The heat damage rating for the differences in temperature, differences in duration and time/temperature interaction were all significantly different ($P<0.01**$).

It seems that little cosmetic damage was caused except in the 42°C treated fruit. It is noteworthy that storage temperatures of 3.5°C, well below the industry standard, caused almost no rind chilling injury even for untreated fruit.
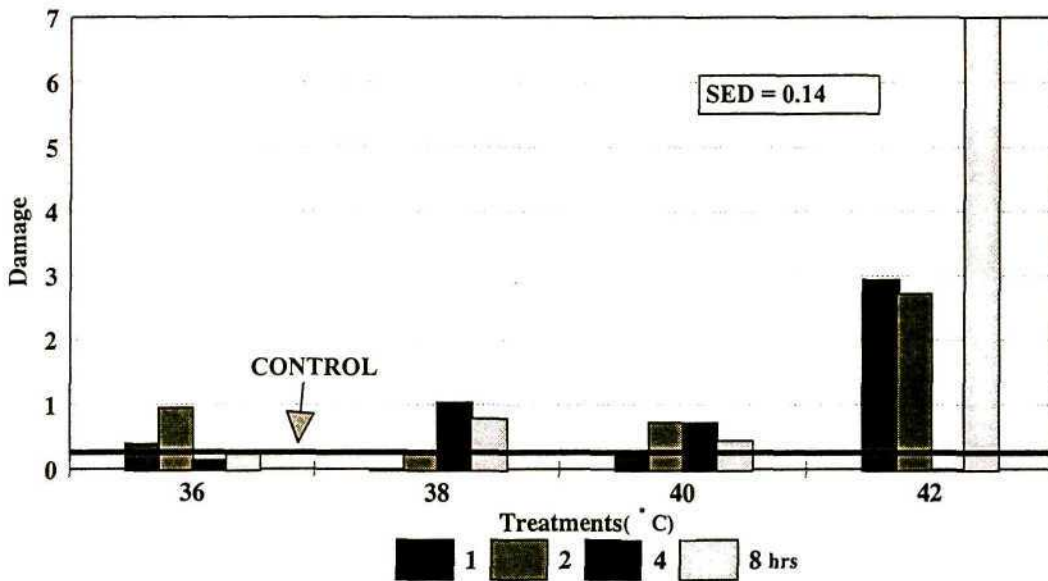


**Figure 2.37** Heat/cold damage for 'Hass' after 5 weeks of cold storage at 3.5°C (damage to the rind was rated on a scale of 0 - 10)
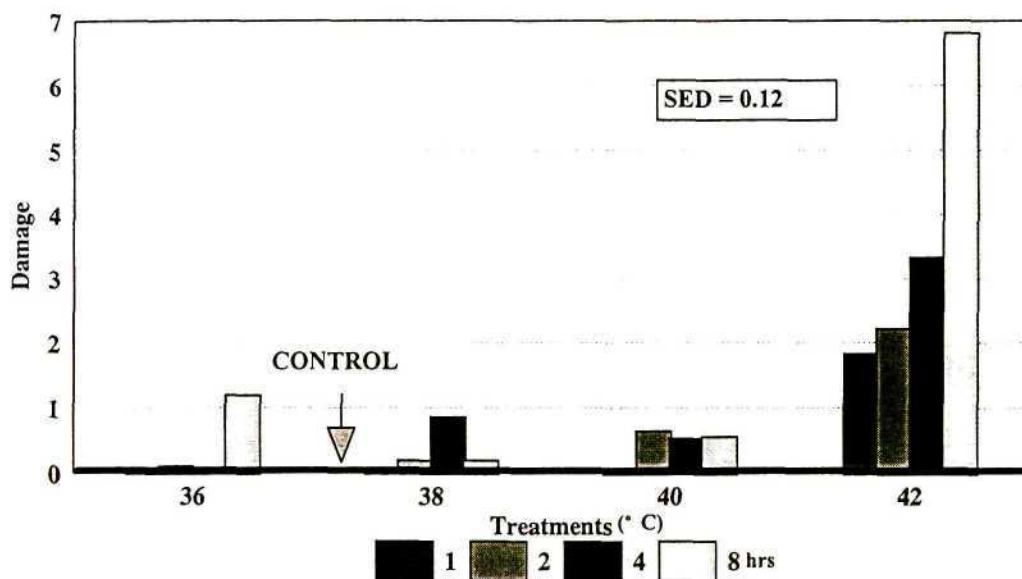
**Figure 2.38** Heat/cold damage for 'Hass' after 6 weeks of cold storage at 3.5°C (damage to the rind was rated on a scale of 0 - 10)

### 2.5.4 Physiological Disorders

Untreated 'Fuerte' fruit displayed few physiological internal/mesocarp disorders on cutting, irrespective of cold storage period. There were however, signs of mesocarp discoloration, expressed more in the 6 week storage period. Untreated 'Hass' fruit displayed no physiological disorders. Physiological disorders were associated only with fruit exposed to high temperatures for long periods. 'Fuerte' fruit was far more prone to internal disorders. At 42°C for 8 hrs though, both cultivars displayed severe pulp spot, vascular browning and mesocarp discolouration. These physiological disorder seemd to be as a result of heat damage.

## 2.6 CONCLUSIONS

Similar conclusions were reached for the 1997 Kwazulu-Natal avocado season as for the previous season, viz. that vapour heat treatment of 'Fuerte' and 'Hass' avocado fruits has shown potential to extend storage life. The two major factors to consider when choosing which vapour heat treatments to use for 'Fuerte' and 'Hass' avocado were an extension of storage life versus heat damage. A summary for both cultivars has been done on which treatments are suitable and are clearly represented in Figure 2.39 and 2.40 respectively. For 'Fuerte' good possibilities exist at 36°C for 8 hrs, 42°C between 4 and 8 hrs and a marginal possibility at 38°C for between 4 and 8 hrs. For 'Hass' good possibilites exist at 38°C and 40°C for 8 hrs.



**Figure 2.39** Vapour heat treatment ratings for 'Fuerte' for the 1997 season (cross=no good, happy face=good but could but the heat treatment could be longer due to lack of heat damage, sad face=good, but induces heat damage)

|      | 36°C | 38°C | 40°C | 42°C |
|------|------|------|------|------|
| 1hr  | X    | X    | X    | X    |
| 2hr  | X    | X    | X    | X    |
| 4hr  | X    | X    | X    | X    |
| 8hr  | X    | ☺    | ☺    | X    |

**Figure 2.40** Vapour heat treatment ratings for 'Hass' for the 1997 season. Notation as for Figure 2.25

## 2.7 CONCLUSIONS FOR 1996 AND 1997 AVOCADO SEASON

In conclusion, vapour heat treatment of both 'Hass' and 'Fuerte' avocado has potential to extend shelf life. This can be achieved if the correct time/temperature regime is utilised for each cultivar. The optimum time/temperature regime varied slightly between the two seasons.

For 'Fuerte' 38°C between 4-8 hrs for both seasons gave good results. In the 1996 season (Fig. 2.25) for 'Fuerte' 40°C between 4-8 hrs was positive whereas in the 1997 season the 40°C treatment was not an option. The 42°C treatment of 'Fuerte' in 1996 showed potential between the 2-4 hr duration, whereas in the 1997 season potential was shown between the 1-2 hr duration. In the 1997 season treating 'Fuerte' at 36°C for 8 hrs also showed potential. The 38 °C treatment for 'Fuerte' between 4-8 hrs, tends to correlate with work done on 'Sharwil'(Nishijima *et al.*, 1995) and 'Hass'(Woolf et al., 1995; Florissen et al., 1996; Lurie *et al.*, 1996; Woolf and Lay-Yee, 1997) avocado. Little work has been done on 'Fuerte' avocado, although a preliminary trial by Bard and Kaiser (1996) who used short treatments with higher temperatures to reduce chilling injury. The majority of the work done though has not been with vapour heat so it is difficult to compare the duration of possible treatments at 38°C if they were treated with hot water or dry heat.

'Hass' in the 1996 season had only one possible combination and that was 38°C between 4-8 hrs. In the 1997 season possibilities existed at 38°C and 40°C for 8 hrs durations. Again, as with 'Fuerte', the 38°C treatment between 4-8 hrs seemed a good possibility if one combines both seasons data. This also would tend to correlate with the literature that 38°C is a suitable temperature to consider.

# CHAPTER 3

# EFFECTS OF VAPOUR HEAT TREATMENT ON 'HASS' AND 'FUERTE' AVOCADO FRUIT ANATOMY

## 3.1 INTRODUCTION

The avocado fruit, a botanical berry, consists of the exocarp (rind), the fleshy mesocarp and endocarp, and the seed surrounded by the seed coat (Fig. 3.1). A thin waxy cuticle is the outermost layer of the exocarp. Below the cuticle is a single layered epidermis, sometimes impregnated with suberin. Below the epidermis there are one to three layers of brick-shaped hypodermal cells, layers of parenchyma cells and a single layer of sclerenchyma or stone cells (Schroeder, 1952; 1953). Collectively these form the rind of 'Fuerte', which is a thin-rinded cultivar, in contrast to 'Hass' which has a thicker and rougher rind.
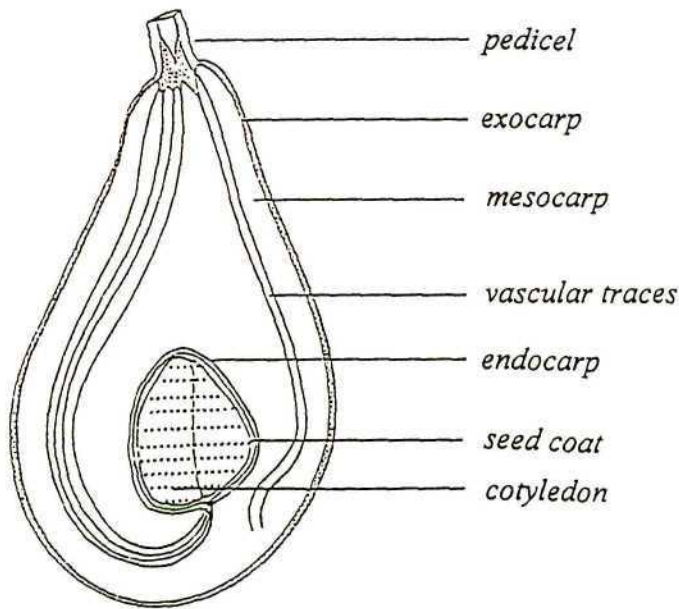


**Figure 3.1** Longitudinal section showing avocado fruit anatomy (x1) (Valmayor, 1967).

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Heat treatment of avocado fruits used to extend storage and shelf life can lead to heat injury of the external layers of the exocarp (Lurie *et al.*, 1996). Heat damage is highlighted by blackening of the epidermis and associated tissue. Any blemish of the external appearance of the exocarp (rind) reduces the marketability of the fruit, and ultimately profit.

The objective of this study was to investigate the effect of vapour heat treatment on the rind tissues of 'Hass' and 'Fuerte' fruit, using transmission electron microscopy.

## 3.2 MATERIALS AND METHODS FOR TRANSMISSION ELECTRON MICROSCOPY

### 3.2.1 Materials

The following apparatus was used for the transmission electron microscope (TEM) study; (1) Haraeus® oven, (2) LKB Ultratome III® ultramicrotome, (3) 200 mesh copper grids, (4) tungsten-coated glass knives, (5) perspex stubs, (6) Genchem® superglue and (7) Jeol 100-CX® TEM. The following reagents were used; (1) 3% glutaraldehyde in 0.05M sodium cacodylate buffer, (2) 0.05M sodium cacodylate buffer, (3) ethanol, (4) 2% osmium tetroxide in 0.05M sodium cacodylate buffer, (5) Spurr's resin (Spurr, 1969), (6) chloroform, (7) lead citrate, (8) uranyl acetate, (9) Ladd's multiple stain.

### 3.2.2 Procedure

In this study 8 samples per vapour heat treatment from 8 different fruit were taken for both 'Hass' and 'Fuerte' avocado. Samples, 1-3mm$^3$ in size were taken from the outermost layer of the exocarp in order to see if the vapour heat treatment in question was having a detrimental effect on the fruit rind. These samples were taken immediately after each specific treatment.

Freshly cut samples were fixed in 3% glutaraldehyde in a 0.05 M sodium cacodylate buffer (pH 7.1) for 8 mins and then washed twice for 30 min in fresh 0.05 M sodium cacodylate buffer. Samples were then postfixed for 2 hours in 2% osmium tetroxide in 0.05M sodium cacodylate buffer, and washed in fresh sodium cacodylate buffer twice for 30 min each.

Samples were then dehydrated in a graded ethanol series (10-100%) with the material being placed into each solution for a duration of 10 min. Samples were dehydrated in 100% alcohol for three 10 min periods.

Specimens were then infiltrated with resin:

    2 hrs : 25% Spurrs:75% ethanol

    2 hrs : 50% Spurrs:50% ethanol

    2 hrs : 75% Spurrs:25% ethanol

After this, specimens were embedded into fresh Spurr's resin, in small labelled aluminium dishes. These dishes were placed into a Haraeus® oven at 70°C for 16 hrs, to polymerize the resin. Once the resin had polymerized and cooled, specimens were cut out of the resin blocks and glued with Genchem® Superglue onto perspex stubs, and then trimmed with glass knives on an LKB Ultratome III® ultramicrotome. Thin sections (2μm) were then cut and placed on a glass slide and stained with Ladd's multiple stain and viewed with a compound light microscope to identify tissues of interest.

Ultrathin gold sections were cut (60 - 70nm thick) using a tungsten-coated glass knife and floated sections onto water in the attached boat. The sections were expanded using chloroform then picked up onto formvar coated grids. These sections were stained using uranyl acetate followed by lead citrate (Reynolds, 1963) and viewed using a Jeol 100CX® TEM at an accelerating voltage of 80kV. Regions of interest within sections were photographed (Plates 1- 5). The technique described above was adapted from that in standard use at the Centre for Electron Microscopy of the University of Natal, Pietermaritzburg (cf Appendix 2).

## 3.3 RESULTS AND DISCUSSION

Vapour heat treatment of 'Fuerte' fruit can be extremely detrimental to the epidermal cell wall if duration of treatment is too long. The longest and hottest treatment at 42°C for 8 hrs (Plate 2.4), destroyed the epidermal cell wall causing a black appearance, and produced fruit of no commercial value. Considering the 'softer' treatments one can see that the detrimental effect on the epidermal cell wall varied according to treatment. The effect of 38°C for 8 hrs (Plate 2.3) was still detrimental to the epidermal cell wall, and it would appear that this treatment was just slightly too long, causing the epidermis to show signs of initial breakup. Although the waxy cuticle was destroyed by a 36°C for 8 hr treatment (Plate 1.2), compared to the control (Plate 1.1) little signs of damage to the cell wall of the epidermis were present.

Mesocarp tissue from 'Fuerte' fruit heated at 42°C for 8 hrs shows how heat damage can be detrimental to structures like the cell wall of internal tissue (Plate 3.7). It would appear that the majority of tissue in this plate has been heated to the extent of irreversible damage, ultimately leading to tissue blackening. Healthy mesocarp tissue of 'Fuerte' fruit (Plate 3.5, Plate 3.6) illustrates the contrast between unheated and heated 'Fuerte' fruit.

Heat treatment of 'Hass' fruit was not as detrimental to the epidermal cell wall as for 'Fuerte' (Plate 4.9). The epidermal cell wall of 'Hass' fruit treated at 42°C for 8 hrs was only damaged in the upper layers of the cell wall, unlike 'Fuerte' epidermal cell wall which showed major disintegration. An example of an undamaged and a healthy epidermal cell wall from control 'Hass' fruit is shown in Plate 4.8.

Considering heat treated fruit mesocarp tissue of 'Hass', irreversible damage is present (Plate 5.11) at 42°C for 8 hrs. Heat damage appeared to also be in the form of disintegration of the cell wall and internal organelles as opposed to control 'Hass' fruit (Plate 5.10).

## 3.4 CONCLUSIONS

It would appear that vapour heat treatment of 'Fuerte' and 'Hass' avocados both have a cut off point before the benefit of vapour heat treatment is offset by irreversible anatomical damage. 'Fuerte' seems to be more susceptible to heat damage than 'Hass' and this can be seen if one compares the two epidermal cell walls at 42°C for 8 hrs. Damage was far greater in 'Fuerte' than in 'Hass'. For both cultivars the 8 hr duration at 42°C was too severe, causing irreversible damage. The lower temperature of 36°C, although causing no damage, did not produce the desired physiological effect of extending storage and shelf life, unlike the slightly higher temperatures of 38 and 40°C depending on treatment duration. Ultimately, a balance between duration and temperature is required for the optimal treatment and this treatment should leave no permanent damage to the exocarp which would ultimately reduce fruit marketability. Finally this electron microscopy work reaffirmed the findings of heat damage, which should not be confused with chilling injury which can also lead to a blackening of the exocarp.

Little electron microscopy has been done on examining heat damaged rind of 'Hass' and 'Fuerte' avocado, thus making it difficult to compare results with other researchers in this field.

Plate 1

1.1 - Transmission electron micrograph of epidermal cell wall of 'Fuerte' fruit exocarp (control fruit, ECW = epidermal cell wall, WC = waxy cuticle).

1.2 - Transmission electron micrograph of epidermal cell wall of 'Fuerte' fruit exocarp (heat treated at 36°C for 8 hrs, ECW = epidermal cell wall, WC = waxy cuticle).

WC

ECW

2.5μm

①

WC

ECW

2.5μm

②
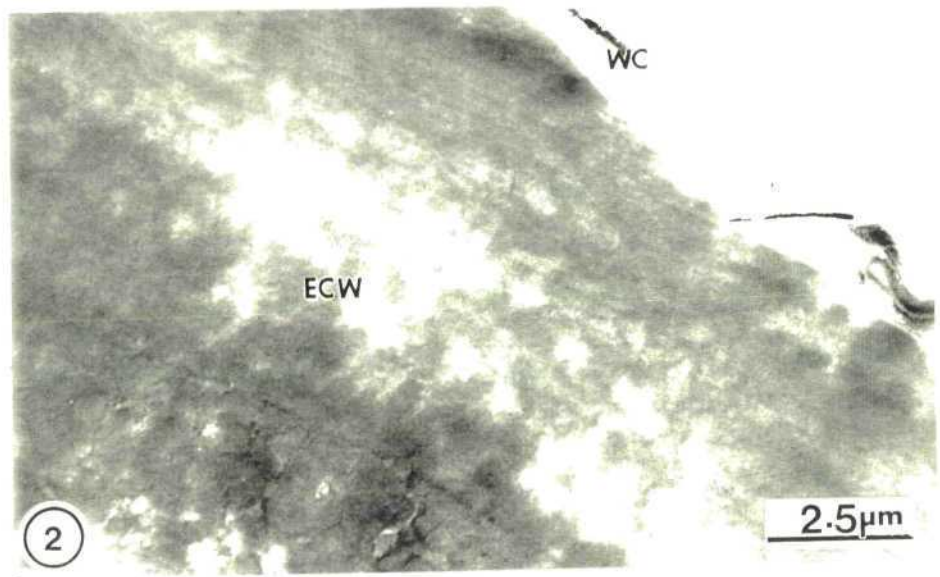
## Plate 2

3 - Transmission electron micrograph of epidermis of 'Fuerte' fruit exocarp (heat treated at 38°C for 8 hrs, ECW = epidermal cell wall, C = cytoplasm, M = mitochondrion).

4 - Transmission electron micrograph of epidermis of 'Fuerte' fruit exocarp (heat treated at 42°C for 8 hrs, ECW = epidermal cell wall, C = cytoplasm).

3  M  C  ECW  0.5μm

4  C  ECW  1μm
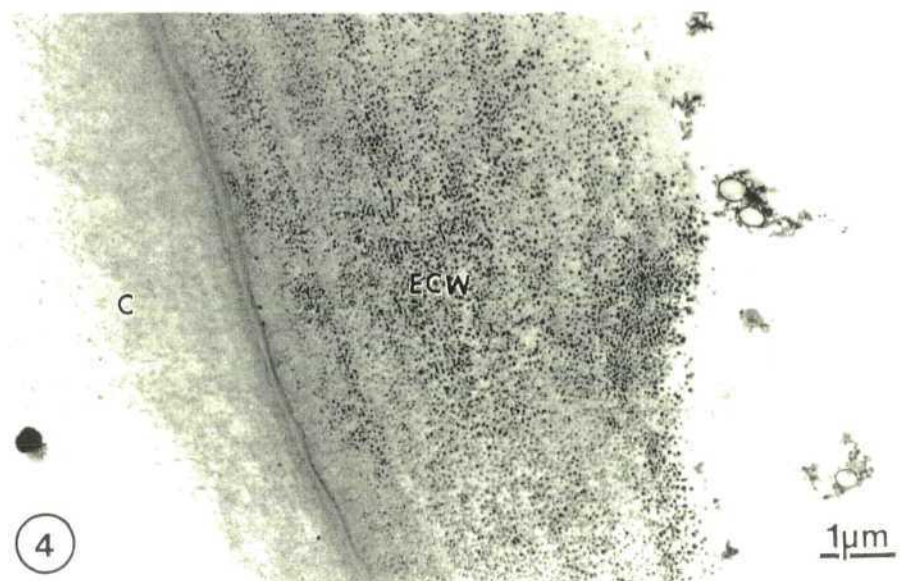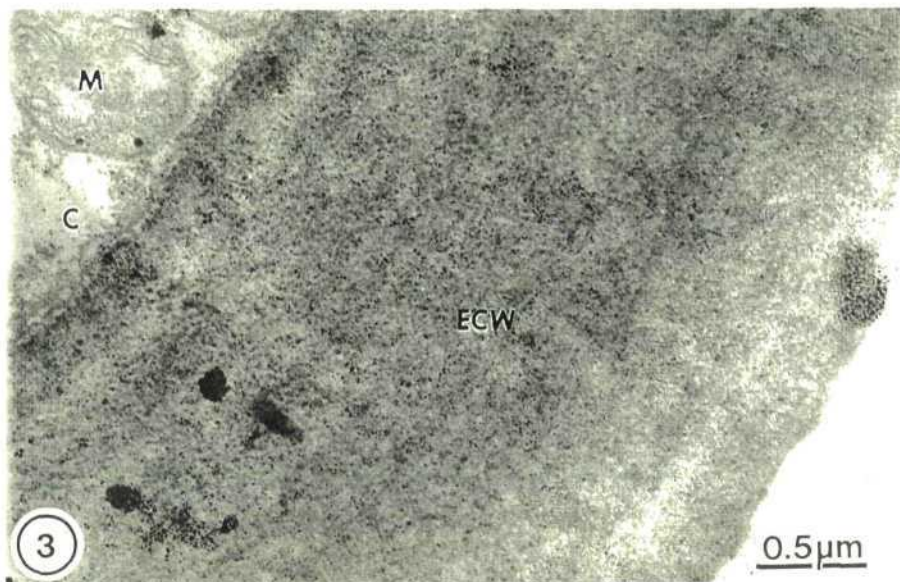
## Plate 3

5 - Transmission electron micrograph of a parenchyma cell of 'Fuerte' fruit mesocarp tissue (control fruit, CW = cell wall, P = Phenolics).

6 - Transmission electron micrograph of a cell wall of 'Fuerte' fruit mesocarp tissue (control fruit, CW = cell wall).

7 - Transmission electron micrograph of a cell wall of 'Fuerte' fruit tissue (heat treated at 42°C for 8 hrs, CW = cell wall, P = plasmodesmata, C = cytoplasm).

5

CW

P

10 µm



6

CW

1 µm



7

C

CW

P

C

1 µm
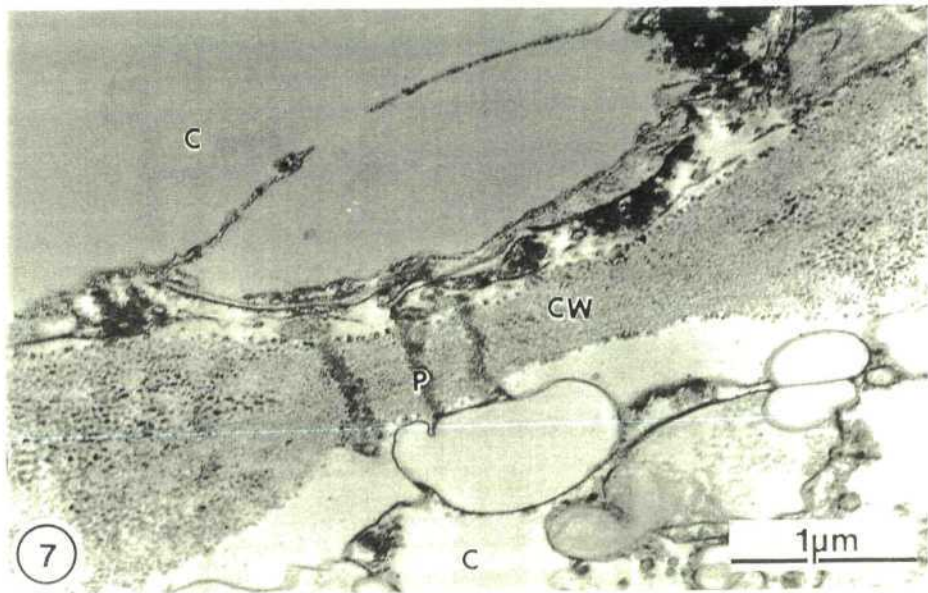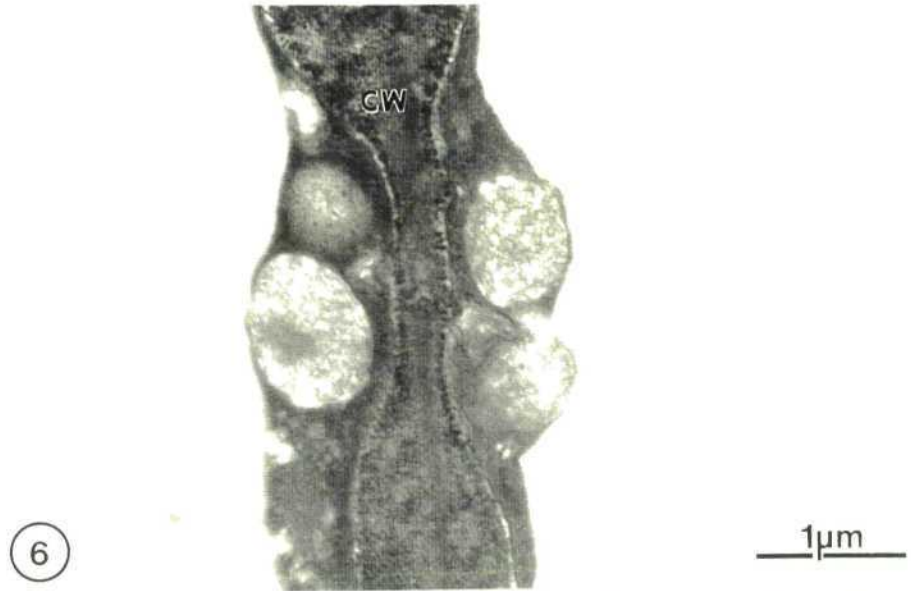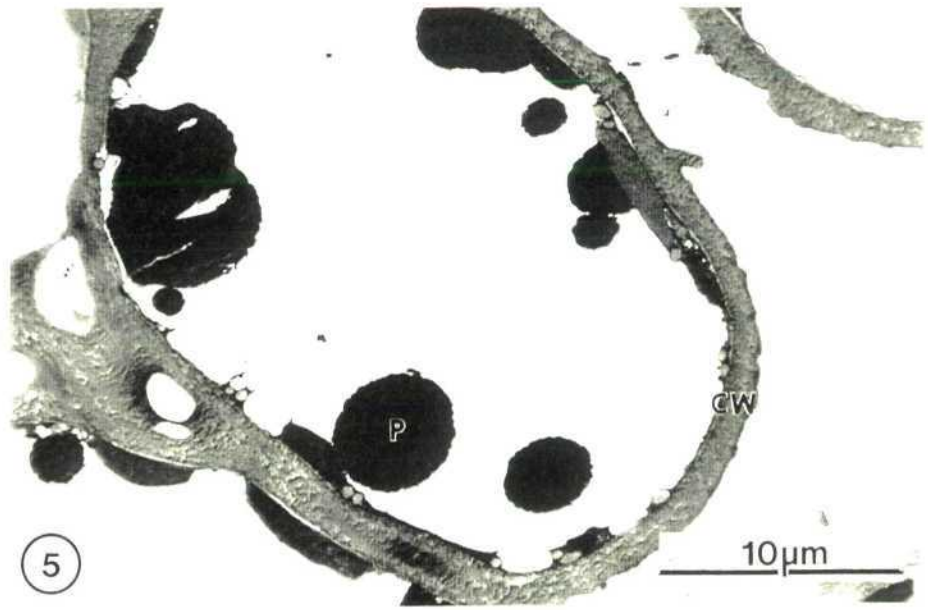
# Plate 4

8 - Transmission electron micrograph of epidermal cell wall of 'Hass' fruit exocarp (control fruit, ECW = epidermal cell wall).

9 - Transmission electron micrograph of epidermis of 'Hass' fruit exocarp (heat treated at 42°C for 8 hrs, ECW = epidermal cell wall, C = cytoplasm).

8

ECW

1μm

9

ECW

C

1μm
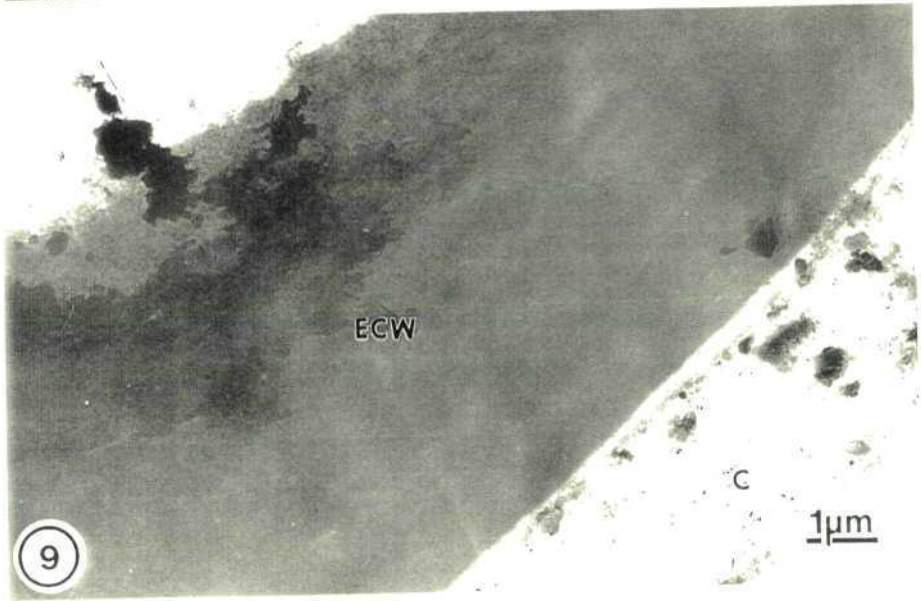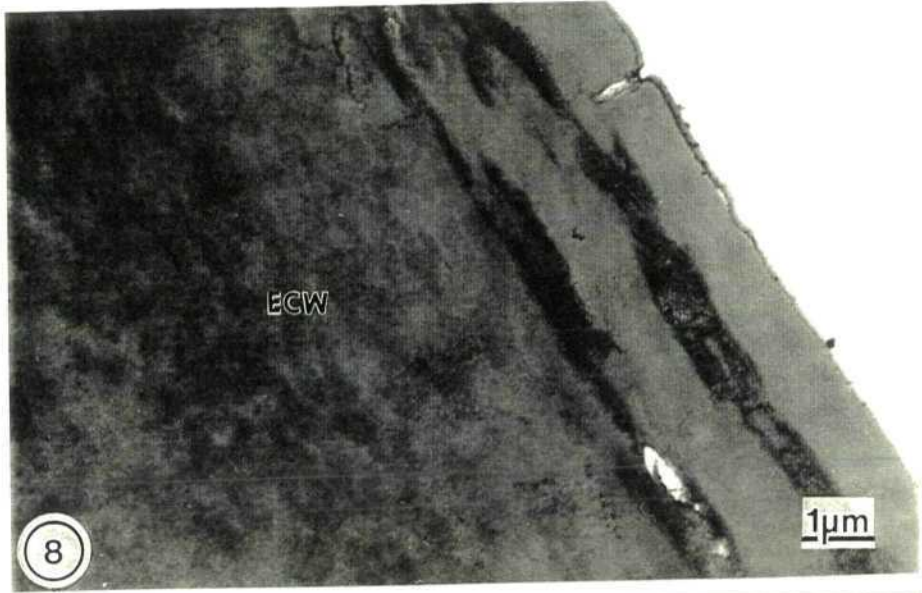
# Plate 5

10 - Transmission electron micrograph of healthy rind tissue of 'Hass' fruit (control fruit, N = nucleus, n = nucleolus, M = mitochondria, CW = cell wall).

11 - Transmission electron micrograph of tissue of 'Hass' fruit (heat treated at 42°C for 8 hrs, CW = cell wall, M = mitochondria).

N
n
M
CW
10
1μm

CW
M
11
1μm
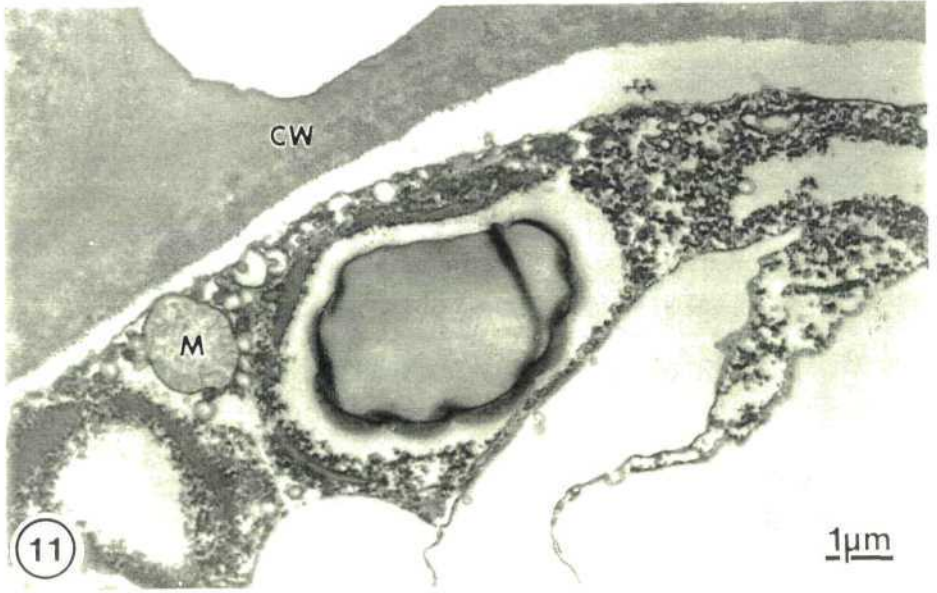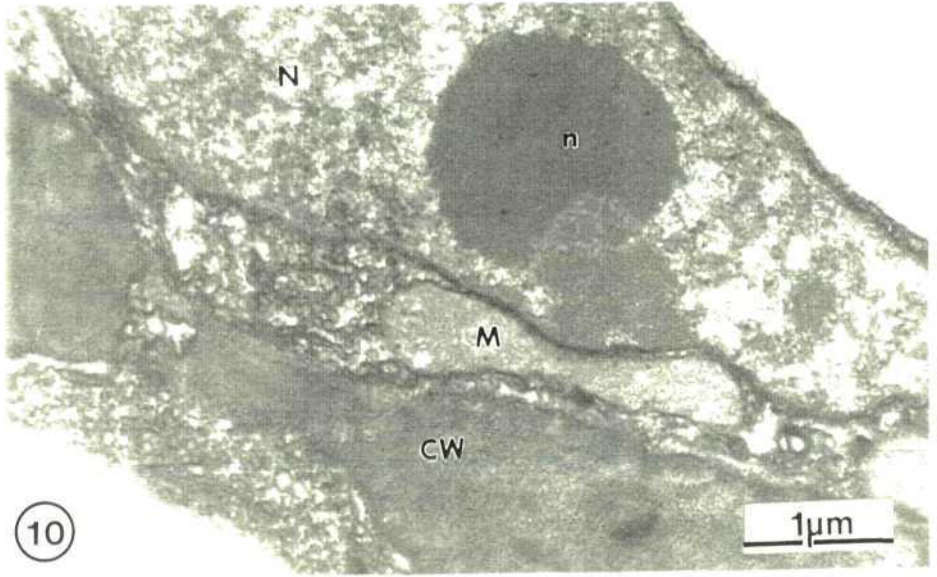
# CHAPTER 4

## PROTEIN ANALYSIS

## OF

## VAPOUR HEAT TREATED 'HASS' AND 'FUERTE' AVOCADO

### 4.1 INTRODUCTION

The extension of storage and shelf life of 'Fuerte' and 'Hass' avocado to certain vapour heat treatments could be attributable *inter alia* to alteration of the protein composition and subsequent hypothetical presence of heat shock protein production. To investigate if vapour heat treatment of 'Hass' and 'Fuerte' avocado alters the protein composition, proteins were isolated from vapour heat treated fruit mesocarp and subjected to polyacrylamide gel electrophoresis (PAGE). The null hypothesis of this particular experiment is that vapour heat treatment has no effect on the protein composition of 'Hass' and 'Fuerte' avocado.

When isolating proteins from fruit tissue there are several conditions which one must consider in order to have an effective isolation. Initially when homogenising the tissue, the acid sap (pH 2-4) could denature proteins present. This can be avoided by macerating tissue with a neutral buffer. Also, on maceration of tissue, phenolases are released which react with phenols and oxygen giving quinones, which react irreversibly with protein to form brown pigments. Detrimental effects of phenolase can be prevented by adding a reducing compound, ascorbic acid or methyl mercaptan, or performing extraction in a nitrogen atmosphere. Natural tannin can also be a problem as it complexes readily with protein. Natural tannin can be extracted by absorbtion to polyvinyl-pyrrolidone (PVP, Polyclar-AT), added to the initial extraction buffer. Lastly, rapid isolation at low temperatures (0-4°C) prevents effects of proteolytic enzymes (Dawson *et al.*, 1995).

The principle of electrophoresis involves moving charged molecules such as proteins and nucleic acids, through application of an electric field. The speed of migration of molecules within an

electric field is dependent on charge, shape and size. In "straightforward" gel electrophoresis, a gel is made as a continuous slab of polyacrylamide with thin sample wells. An electric potential is applied and electrophoresis commences, with the gel acting as a "sieve" and imposing steric hindrance to migration of proteins. The "sieving" effect of the gels is proportional to molecular charge, hence some molecules will be retarded to a greater extent than other molecules, depending on the charge/mass ratio. In gel electrophoresis size of protein molecules and concentration of the gel affect separation (Hoefer Scientific Instruments, San Francisco).

In particular, sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis separates proteins according to their molecular weight. SDS is an ionic detergent that denatures proteins by covering the polypeptide backbone. In doing so SDS confers a net negative charge to the polypeptide chain in proportion to its length. Once treated with SDS and a reducing agent, polypeptides become negatively charged with equal "charge densities". SDS-PAGE can separate complex protein mixtures into a wide range of bands on a gel. The position of a protein along a lane on the gel gives a good indication of its size, also stained band intensity is a rough indicator of amount present in the sample (Hoefer Scientific Instruments, San Francisco).

The extension of shelf life on 'Hass' and 'Fuerte' avocado achieved by using vapour heat treatment is postulated to have some relation with heat shock proteins. The objective of this research was to investigate the possible correlation between production of heat shock proteins and an extension of shelf life.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Protein Extraction

Before one can proceed with gel electrophoresis, proteins need to be extracted from both vapour heat treated 'Hass' and 'Fuerte' avocado fruits and untreated controls. The following protein extraction protocol was used for both cultivars was carried out for all the vapour heat treatments of 36, 38, 40, 42°C for 1, 2, 4 and 8 hrs. One sample was performed per treatment.

Radial sections were taken from several fruit of which 5g of frozen tissue was ground to a fine powder in a mortar and pestle in liquid $N_2$. This powder was transferred to a 50ml tube with 20ml of extraction buffer (50mM Tris pH 7.5, 5% sucrose, 4% SDS, 5% mercaptoethanol, 2 mM PMSF), vortexed well and incubated (10 min., 0°C). Following this, the mixture was filtered through cheesecloth into another graduated tube. Equal volumes of Tris-buffered phenol were added,the tubes shaken for 10 min. and centrifuged. The phenol phase (bottom phase) was recovered and re-extracted with fresh extraction buffer, shaken for 10 min. and centrifuged. Proteins were precipitated in 5 volumes of 0.1M ammonium acetate in methanol and left overnight at -20°C . The following day samples were centrifuged, washed 3 times with ammonium acetate in methanol and once with acetone, and finally dried under nitrogen. Samples were resuspended in sample treatment buffer, transferred to Eppendorf tubes and protein concentration determined (Lurie, 1996, pers.comm). Since these protein samples were all treated the same way, any difference in the results on the gel were postulated to be due to the heat treatment.

### 4.2.2 Protein determination

Whatman paper (3mm) was divided with a pencil into 1cm squares. For standards 0, 5, 10, 15, and 20 μg of BSA were put into 5 separate squares and dried with a hair drier. Squares were cut out, placed in a petri dish and Coomassie Blue stain (2g Coomassie blue, 500ml MeOH, 70ml acetic acid, 430ml $H_2O$) was added, and shaken for 10 min.. Squares were washed 4 times with destaining solution (30% methanol, 10% acetic acid), and dried with a hair drier. These squares with spots were placed in individual test tubes, to which 2ml of 1% SDS was added, then shaken for 1.5 hrs. Absorbance was read at 600 nm (Lurie, 1996, pers.comm).

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Lurie, S. 1996. Pers. Comm., Department of Postharvest Science, Israel.

### 4.2.3 Gel Electrophoresis

#### 4.2.3.1 Reagents

This method is a modification of the Laemmli (1970) system. It has the advantage that the same stock solutions are used for both PAGE gels (non-denaturing) and SDS-PAGE gels (denaturing) simply by the omission or addition of SDS. Reagents were made according to the Hoefer manual (Hoefer Scientific Instruments, San Francisco).

**Stock solutions**

**A: Monomer Solution.** Acrylamide (58,4 g) and Bis (N,N' methylene-bisacrylamide) (1,6 g) were dissolved and made up to 200 ml with dist. $H_2O$.

**B: 4 x Running Gel Buffer.** Tris (36,3 g) was dissolved in approximately 100 ml of dist. $H_2O$, titrated to pH 8,9 with 1M HCl and made up to 200 ml.

**C: 4 x Stacking Gel Buffer.** Tris (3,0 g) was dissolved in approximately 30 ml dist. $H_2O$, and titrated with 1M HCl to pH 6,8 and solution was made up to 50 ml.

**D: Stock Tank Buffer (pH 8,3).** Tris (12,0 g) and glycine (57,6 g) were dissolved and made up to 4 litres with dist. $H_2O$. 10 ml of SDS stock (reagent E) was added per litre.

**E: SDS stock.** Sodium dodecyl (lauryl) sulfate (50 g) was dissolved in 500 ml dist. $H_2O$. Gentle heating helped dissolve the solution.

**F: Treatment Buffer.** Buffer C (2,5 ml), SDS solution E (4,0 ml), glycerol (2,0 ml) and β-mercaptoethanol (1 ml) were made up to 10 ml, using the fume hood.

**G: Initiator.** Ammonium persulfate (0,5 g), was made up to 5 ml just <u>before use</u>.

**H: Staining solution**(0.125% (m/v) Coomassie blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid)

**I: Destaining solution I** (50% (v/v) methanol, 10% (v/v) acetic acid). Methanol (500ml) is mixed with acetic acid (100ml) and made up to 1litre with dist. $H_2O$.

**J: Destaining solution II** (7% (v/v) acetic acid, 5% (v/v) methanol). Acetic acid (70ml) is mixed with methanol (50ml), and made up to 1 litre with dist. $H_2O$.

### 4.2.3.2 Preparation of the running gel (10%T, 2,7%C).

The following reagents were mixed together (swirling avoided excess oxygen entering the solution):

| | |
|---|---|
| Monomer solution | 10,0 ml |
| Buffer B | 7,5 ml |
| SDS solution (E) | 0,3 ml |
| Water | 12,2 ml |

150 µl initiator and 10 µl TEMED were added. Swirling gently helped to mix the solution. This preparation is for the "Mighty small" or mini gel. For the larger gel, the Hoefer SE 600 apparatus, exactly double the amounts of reagents were used.

### 4.2.3.3 Preparation of the stacking gel.

The following reagents were mixed together. (As with the running gel consistent swirling avoided excess oxygen entering the solution).

| | |
|---|---|
| Monomer solution | 2,6 ml |
| Buffer B | 5,0 ml |
| SDS solution (E) | 0,2 ml |
| Water | 12,2 ml |
| Initiator | 100 µl |
| TEMED | 10 µl |

This preparation is for the "Mighty small" or mini gel. For the larger gel, the Hoefer SE 600 apparatus, exactly double the amounts of reagents were used (Hoefer Scientific Instruments, San Francisco).

### 4.2.3.4 Procedure

A vertical slab gel unit has two gels running at one time. Two plates are separated by lightly greased plastic spacers onto which glass plates are attached by plastic clamps. The two gel plates are separated by a plastic housing onto which the clamps go, and water runs through to aid cooling of the system. This plastic housing with attached plates is placed on a flat glass plate with hot agarose (1%) solution at the base to prevent the running gel from leaking out of the "sandwich" of plate and glass. This whole apparatus is known as a pod.

Freshly prepared running gel solution was introduced into the "sandwich" to a depth of 80 mm using a Pasteur pipette, and a further 5 mm dist. $H_2O$ was carefully layered on top of this, with care being taken to ensure that a smooth interface was formed between layers. The gel was allowed to polymerise (about 30 min.).

When the running gel had polymerised, the water layer was removed using a twist of tissue paper. Freshly-prepared stacking gel solution was added and a comb was inserted into the stacking gel to form wells, ensuring no stacking gel was open to air (about 20 min.). For the Hoefer SE600, the gel apparatus was assembled according to manufacturers instructions (Hoefer Scientific Instruments, San Francisco).

At this stage the lower electrode compartment was filled with tank buffer (10 ml of reagent E added per litre) and, after polymerisation of the stacking gel the comb was removed and the wells were filled with tank buffer. Care was taken to exclude bubbles.

Using a blunt-ended microlite syringe, each sample (100 μl) was loaded and molecular weight markers (5 μl) onto separate gels, underlying the solutions beneath the buffer. The upper reservoir was filled with electrode buffer, and a potential was applied to give a constant current of 36 mA per pod (16 mA/gel) until the bromophenol blue marker has traversed the gel (Fig. 4.1). Electrophoresis was completed in 1.5-2 hrs. The gels were stained (in stain 4hrs), then placed in destain I overnight and then left in destain II to be analysed. The gels were analysed to determine the effect of vapour heat treatment on the protein composition of 'Fuerte' and 'Hass' avocado fruit .mesocarp

This is the basic procedure for running mini-gels but due to the complex nature of the protein sample resulting in poor resolution of the bands on gels, larger gels were run using the same basic procedure, employing the Hoefer SE 600 apparatus. Final gel dimensions were 11cm x 8cm, affording better separation of protein bands. Electrophoresis was conducted at constant voltage (80mv per gel) for 4.5-5 hrs.

**Figure 4.1** Gel electrophoresis being performed

## 4.3 RESULTS AND DISCUSSION

### 4.3.1 Protein determination

#### 4.3.1.1 'Fuerte'

The results of the protein determination for 'Fuerte' fruit mesocarp are illustrated in Figure 4.2. Note that protein concentrations represented here reflect proteins extracted by the phenol extraction, and as such are not an indication of total protein content.
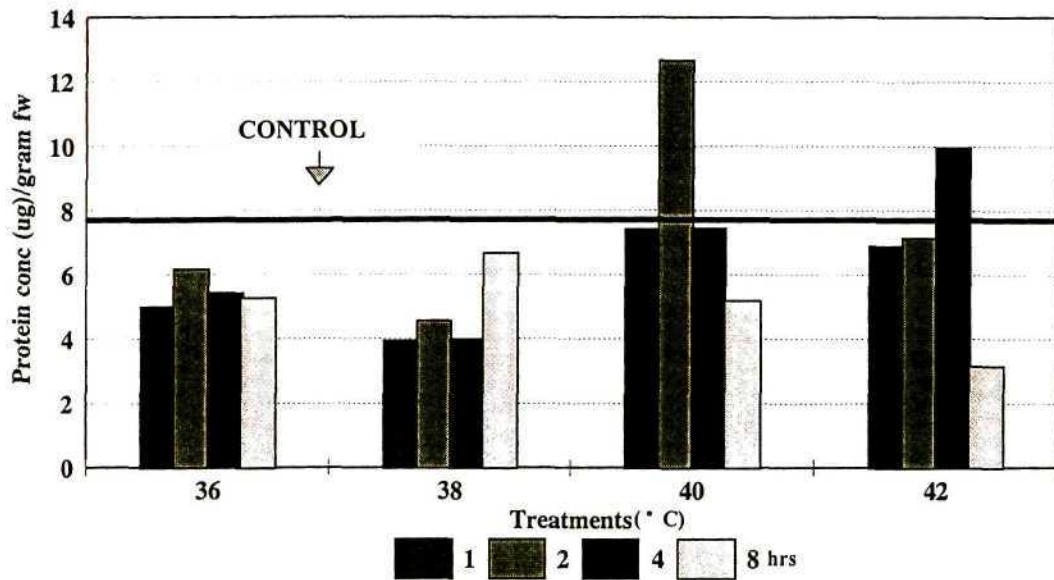
**Figure 4.2** Protein concentration (µg) for 'Fuerte' fruit mesocarp per gram of fresh weight determined for each treatment

Protein concentrations for the 36°C vapour heat treated fruit seemed unaffected by treatment duration, but were all lower than the control (untreated). Protein concentrations for 38°C were also unaffected by treatment duration, although at the 8 hr duration the protein concentration was higher, although still below the control. At 40°C for 2 hr protein concentration increased dramatically, followed by a decreasing trend of protein concentration from the 2 to 8 hr treatment. At 42°C at 8 hrs a low protein concentration was found, suggesting that protein synthesis had been inhibited at this temperature or, just as feasibly, that protein degradation was enhanced.

A reduction in protein concentration at 38°C could possibly be associated with a reduction in metabolic activity associated with longer storage life of fruit, a result which correlates well with the work presented in Chapter 2, showing this to be the optimal temperature for increased shelf life of both cultivars. A decrease in protein concentration suggests a reduced metabolism, coupled to a decrease in production of enzymes, which the latter may contribute to tissue

turnover during ripening. The observation that, at higher temperatures of 40°C and 42°C, slightly higher protein concentrations were obtained (except at 8hrs), is consistent with the results obtained in chapter 2 where these temperatures casued tissue damage. It is therefore thought that these temperatures caused heat denaturation of proteins allowing them to be more easily extracted ; resulting in an overall increase in protein concentration. These results appear annomolous

### 4.3.1.2 'Hass'

For the 36°C heat treatment all durations of treatment seem to have had little effect on altering protein concentrations, although these were slightly higher than the control (Fig. 4.3). No consistent trends were seen at 38°C. At 40°C for 2 hrs there was an increase in protein concentration followed by a decrease. Heat treatment of 42°C also displayed similar trends to 40°C with increasing duration of treatment.
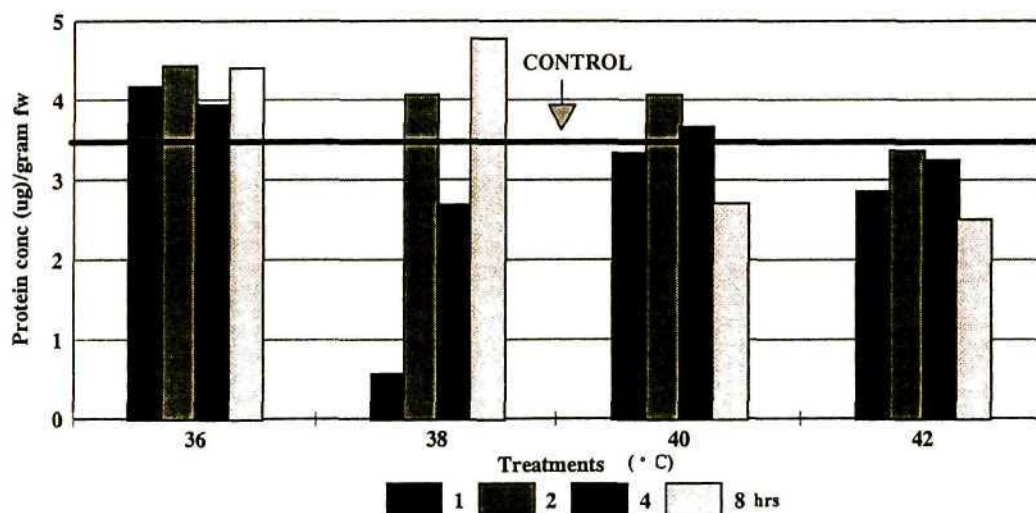


**Figure 4.3** Protein concentration (μg) for 'Hass' fruit mesocarp per gram of fresh weight determined for each treatment.

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If one compares these cultivars, 'Fuerte' had a mesocarp protein concentration of almost double that of 'Hass'. For both cultivars at 42°C for 8 hrs the protein concentration displayed a decline, suggesting that a maximum temperature forthe most suitable protein composition has been surpassed. It is also curious that for 'Hass', at 36°C, the protein concentration was greater than control (untreated samples), while in 'Fuerte', at 36 and 38°C the protein concentration was lower than the control. The control values vary, which suggests an intrinsic difference between the cultivars.

The results presented here suggest that protein concentration and composition are indeed affected by heat treatment. Further, they suggest that certain heat treatments cause an overall decrease in protein concentration which could indicate a prologed shelf-life. The critical finding would be to determine the temperature at which protein synthesis is decreased, but at which little thermal denaturation has occurred. Due to financial constraints, these are preliminary results, and variation in values shows the necessity for a more statistically representative trial to be conducted.

### 4.3.2 Gel Electrophoresis

Initially, several mini gels were run and due to there being a variety of bands on the gels poor resolution was obtained. Larger gels were run to obtain better resolution of the bands. Only the longer heat treatment durations i.e. 4 and 8 hrs were considered on the larger gels, as these treatments seemed to have the greatest effect on extending storage life for both cultivars (Chapter 2).

#### 4.3.2.1 'Fuerte'

A wide variety of proteins were found in the analysis of vapour heat treated and untreated 'Fuerte' avocado mesocarp (Plate 1). Interestingly, there was greater expression of proteins evident in the longer durations for each temperature as shown by darker staining, although these proteins were present in the shorter duration treatments but at lower intensity. The major bands

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on the gel present for the control and heat treated fruit were proteins of +\- 64, 30 and 14 kDa (Table 4.1). The proteins which seem to have increased in expression due to vapour heat treatment were 66, 61, 56, 45, 42, 36, 33, 17 and 16 kDa. Some of these proteins were suppressed at 42°C for 8 hrs, for example 56 kDa, and it would appear that this has exceeded the maximum temperature for protein synthesis.

Considering 38°C for 8 hrs (the optimal treatment for prolonged shelf life, perhaps) resulted in the majority of protein bands being expressed. As an example, 66 kDa is considered. It is expressed as a major band at 38°C for 8 hrs and as the temperature increased, it was repressed in the remainder of the treatments to a minor band. Protein band intensity increased until 42°C for 8 hrs (shown by darker staining) where synthesis appeared to be greatly retarded, hence a reduction in band intensity.

### 4.3.2.2 'Hass'

A wide variety of proteins were found in the analysis of vapour heat treated 'Hass' avocado (Plate 2). The major bands on the gel present for the control and heat treated fruit were proteins of 13, 26, 45 and 61 kDa (Table 4.2). Judging by band intensity, 'Hass' demonstrates more clearly than 'Fuerte' where optimum protein synthesis occurs. Proteins which appeared to have been expressed due to heat treatment are 7, 17 and 90 kDa were exclusively found in 38°C at 8 hrs. Speculatively these proteins may be labelled as Hsps due to the fact that they were only expressed under heat treated conditions. Several proteins were suppressed, i.e. found in the control only namely 19, 25, 52, 62 and 72 kDa. A very clear contrast can be seen between 38°C at 8 hrs and 40°C at 8 hrs. It would appear perhaps that 40°C at 8 hrs has exceeded the maximum temperature for optimum protein synthesis in 'Hass' avocado (again these results corroborate those obtained by protein synthesis Table 4.3)

Comparison of the two cultivars showed that they have different protein complements. 'Fuerte' had a far more constant protein complement than 'Hass' for the different treatments. The untreated 'Hass' fruit had several proteins which were suppressed when fruit were heat treated.

For both cultivars it seems that the longer treatments induce greater protein expression. This is not consistent for 'Fuerte' (Table 4.3) and this could be associated with induction of new Hsps, or expression of Hsps which are usually present in the cell. Proteins such as 70 kDa form the largest group of Hsps, Hsp70 are present, according to the literature (Chapter 1), in all plant tissue including non-treated tissue. Hyper expression of Hsp 70 could contribute to a longer shelf-life as it is thought that expression of Hsp could suppress the synthesis of normal tissue degradative enzymes. This correlates with the findings in Chapter 5 where a reduction of pectin methyl esterase activity was associated with longer storage life. In order to confirm the identity of Hsps, it would be necessary to vapour heat treat fruit in the presence of radiolabelled amino acids. Following isolation of proteins, those proteins which have been synthesised during heat stress would contain the radiolabelled amino acids.

The literature states (Chapter 1 - LMW Hsps) that LMW Hsps (17-28 kDa) could be responsible for induced thermotolerance, for example Hsp17, which is present as a putative Hsp in 'Hass' and has been further expressed in 'Fuerte' (Table 4.1 and 4.2). This protein in 'Hass' has clearly been expressed under the influence of vapour heat. The literature also states that LMW Hsps are the most induced proteins during heat stress (Chapter 1). The expression of these LMW proteins or presumed LMW Hsps could perform a very valuable function of 'Hass' and 'Fuerte' fruit cells to endure the stress under which the fruit is being placed giving it an extended shelf life.

The proteins expressed in 'Hass' at 38°C for 8 hrs are presumed to be Hsps as they have been induced under vapour heat treated conditions. Woolf and Lay-Yee (1997) found that at 38°C 'Hass' avocado fruit mesocarp levels of Hsp17 mRNA increased with increasing duration of heat, and this correlated with an increase in thermotolerance. These findings correlated with the findings of putative Hsp17 in 'Hass' during this investigation.

The main findings of heat treatments (Chapter 2) were that as the temperature and duration of the treatments increased the storage life increased accordingly, but that the time-temperature interaction must be taken into account. If we consider both gels, the trend seems to be that the 8 hr treatments resulted in greater protein intensity until 42°C for 4 hrs for 'Fuerte'and 38°C for

8 hours for 'Hass', where protein synthesis was reduced. These cut off time/temperature relationships for each cultivar is where the benefit of the vapour heat treatment seems to be minimised or even detrimental, particularly in respect of rind damage. These findings correlate with an extension of shelf life and general benefit to both 'Hass' and 'Fuerte' avocado treated as such.

# PLATE 1

**Figure 4.4** 12.5% SDS PAGE Gels stained with comassie blue, illustrating Hsp expression during certain vapour heat treatments of 'Fuerte' avocado. Lane 1, molecular weight markers; lane 2, 36°C for 4 hrs; lane 3, 36°C for 8 hrs; lane 4, 38°C for 4 hrs; lane 5, 38°C for 8 hrs; lane 6, control; lane 7, 40°C for 4 hrs; lane 8, 40°C for 8 hrs; lane 9, 42°C for 4 hrs; lane 10, 42°C for 8 hrs.
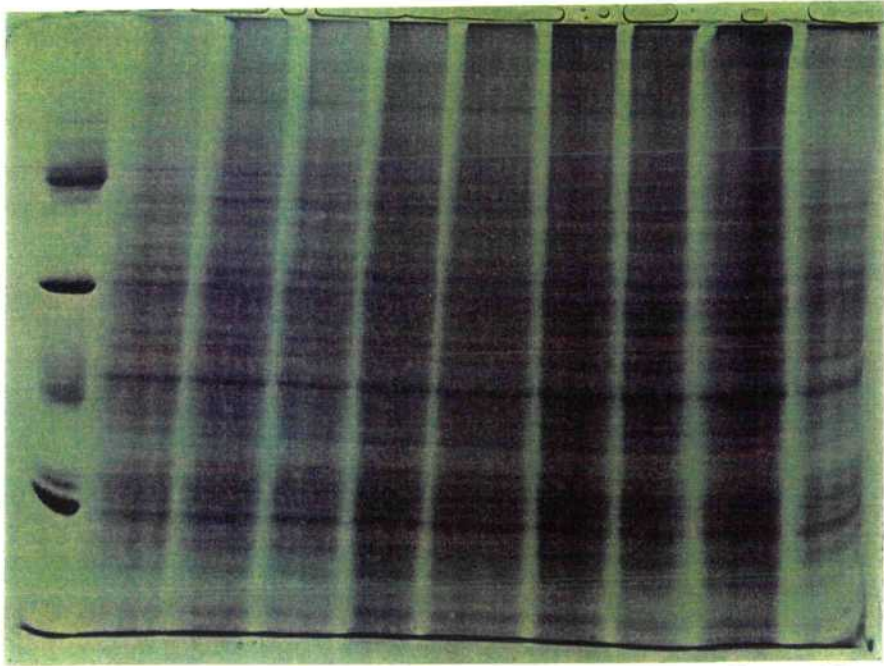
PLATE 2

**Figure 4.5** 12.5% SDS PAGE Gels stained with comassie blue, illustrating Hsp expression during certain vapour heat treatments of 'Hass' avocado. Lane 1, molecular weight markers; lane 2, 36°C for 4 hrs; lane 3, 36°C for 8 hrs; lane 4, 38°C for 4 hrs; lane 5, 38°C for 8 hrs; lane 6, control; lane 7, 40°C for 4 hrs; lane 8, 40°C for 8 hrs; lane 9, 42°C for 4 hrs; lane 10, 42°C for 8 hrs.
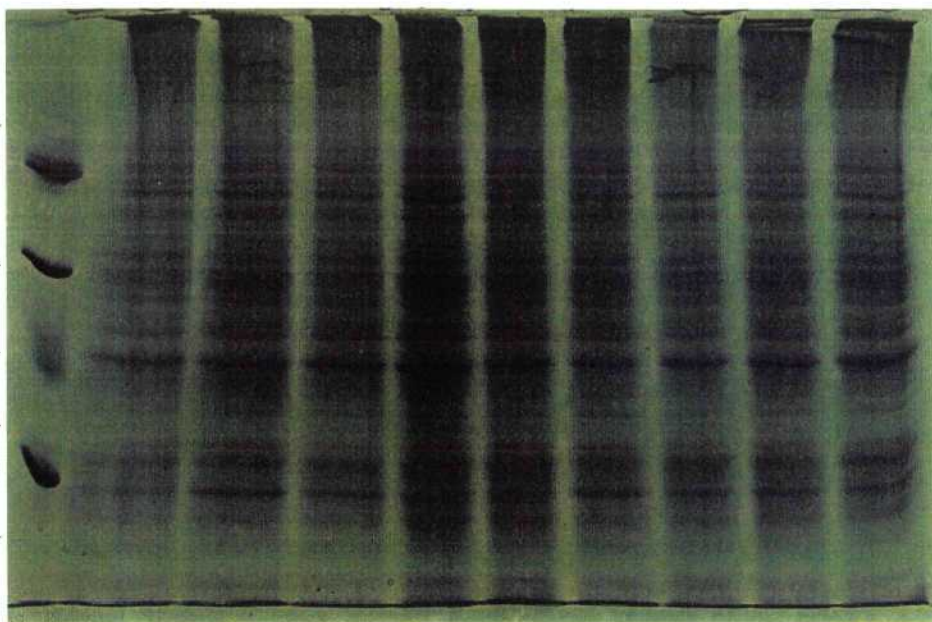
**Table 4.1** Analysis of the different proteins found in Plate 1 for different treatments of 'Fuerte' avocado (Weight in kDa).

| 36°C/4hrs | 36°C/8hrs | 38°C/4hrs | 38°C/8hrs | CONTROL | 40°C/4hrs | 40°C/8hrs | 42°C/4hrs | 42°C/8hrs |
|---|---|---|---|---|---|---|---|---|
| 102 | 102 | 102 | 102 | 102 | 102 | 102 | 102 | |
| 86 | 86 | 86 | 86 | 86 | 86 | 86 | 86 | |
| | | | | | | | | 75 |
| 72 | 72 | 72 | 72 | 72 | 72 | 72 | 72 | 72 |
| | | | | | | 68 | | |
| 66 | 66* | 66* | 66* | 66 | 66 | 66 | 66 | 66 |
| 61 | 61* | 61 | 61* | 61 | 61* | 61* | 61* | |
| | | | | | | | | 59 |
| 56 | 56* | 56 | 56* | 56 | 56* | 56* | 56* | |
| 48 | 48 | 48 | 48 | 48 | 48 | 48 | 48 | 48 |
| | | | | | | | | 47 |
| 45* | 45* | 45* | 45* | 45 | 45* | 45* | 45* | 45* |
| 42 | 42* | 42 | 42* | 42 | 42* | 42* | 42* | 42* |
| 40 | 40* | 40 | 40* | 40 | 40* | 40* | 40* | 40 |
| 36 | 36 | 36 | 36* | 36 | 36 | 36* | 36 | 36 |
| 33 | 33* | 33* | 33* | 33 | 33* | 33* | 33* | |
| 31 | 31* | 31* | 31* | 31* | 31* | 31* | 31* | 31 |
| 26* | 26* | 26* | 26* | 26* | 26* | 26* | 26* | 26 |
| 24 | 24 | 24 | 24 | 24 | 24 | 24 | 24 | 24 |
| 21 | 21 | 21 | 21 | 21 | 21 | 21 | 21 | 21 |
| 18 | 18 | 18 | 18 | 18 | 18 | 18 | 18 | 18 |
| 17 | 17* | 17 | 17* | 17 | 17* | 17 | 17 | 17 |
| 16 | 16* | 16 | 16* | 16 | 16* | 16 | 16 | 16 |
| 14* | 14* | 14* | 14* | 14* | 14* | 14* | 14* | 14* |
| 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 |
| 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 |

* Major band has been expressed while unstarred numbers are minor bands

**Table 4.2** Analysis of the different proteins found in Plate 2 for different treatments of 'Hass' avocado (Weight in kDa).

| 36°C/4hrs | 36°C/8hrs | 38°C/4hrs | 38°C/8hrs | CONTROL | 40°C/4hrs | 40°C/8hrs | 42°C/4hrs | 42°C/8hrs |
|---|---|---|---|---|---|---|---|---|
|  | 98 | 98 | 98 | 98 | 98 | 98 | 98 | 98 |
|  |  |  | 90 |  |  |  |  |  |
|  | 83 |  | 83 | 83 |  |  | 83 |  |
|  |  |  | 79 | 79 | 79 |  |  |  |
| 72 | 72 | 72 | 72 | 72* | 72 | 72 | 72 | 72 |
| 66 | 66 | 66 | 66 | 66 | 66 | 66 | 66 | 66 |
| 62 | 62 | 62 | 62 | 62* | 62 |  | 62 | 62 |
| 56 | 56* | 56 | 56 | 56* | 56 | 56 | 56 | 56 |
|  |  |  |  | 52 |  |  |  |  |
| 49 | 49 | 49 | 49 | 49 | 49 | 49 |  | 49 |
| 45 | 45* | 45* | 45* | 45* | 45* | 45 | 45* | 45* |
| 42 | 42* | 42* |  | 42* | 42 | 42 | 42* | 42* |
| 40 | 40* | 40* | 40** | 40* | 40 | 40 | 40* | 40* |
| 35 | 35* | 35 | 35 | 35 | 35 | 35 | 35* | 35 |
| 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 |
| 29 | 29* | 29 | 29* | 29 | 29 | 29 | 29 | 29 |
| 26* | 26* | 26* | 26* | 26* | 26* | 26* | 26* | 26* |
|  |  |  |  | 25 |  |  |  |  |
|  | 23 | 23 | 23 | 23 | 23 | 23 | 23 | 23 |
|  |  | 22 | 22 | 22 |  |  |  |  |
| 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 |
|  |  |  |  | 19 |  |  |  |  |
|  |  |  | 17 |  |  |  |  |  |
| 15 | 15* | 15 | 15* | 15* | 15* | 15 | 15 | 15 |
| 13 | 13* | 13* | 13* | 13* | 13* | 13* | 13* | 13* |
|  |  |  | 12 | 12 |  |  |  |  |
| 11 | 11 | 11 | 11 | 11 | 11 | 11 | 11 | 11 |
| 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
|  |  |  | 7 |  |  |  |  |  |
| 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 |

* Major band has been expressed while unstarred numbers are minor bands

## 4.4 CONCLUSIONS

Generally, for both 'Fuerte' and 'Hass', heat treatments resulted in the expression of proteins, with a greater expression of proteins in the longer duration treatments for 'Fuerte' and 'Hass' up till 38°C for 8hrs. Hence we can reject our null hypothesis that vapour heat treatment of 'Hass' and 'Fuerte' avocado has no effect on protein synthesis. For 'Hass' presumed Hsps (Hsp 17 in particular) have been expressed and this agrees with the literature (Chapter 1). Also the optimum expression of proteins tended to be at 38°C for 8 hrs, although 'Fuerte' was not as clearly divided as 'Hass'. This temperature of 38°C has been found to be effective for 'Hass' for various scientists working around the world (Nishijima *et al.*, 1995; Woolf *et al.*, 1995; Florissen *et al.*, 1996; Lurie *et al.*, 1996; Woolf and Lay-Yee, 1997), whereas little work has been done on 'Fuerte'. This work correlates well with previous work (chapter 2) and with pectin methyl esterase assay (Chapter 5) which shows a general decrease in enzyme activity associated with longer treatments. In order to conclusively identify proteins as Hsps, further work would be necessary, labelled amino acids or the use of antibodies would confirm Hsp identity. Such as the use of labelled amino acids to identify induced proteins, or the employment of specific antibodies to Hsps to identify proteins in Western blotting . Finally, the author wishes to reitterate the preliminary nature of these results, acknowledging the statistical shortfalls of this work, and noting the potential for collaberative work with researchers in this field.

# CHAPTER 5

## SPECTROPHOTOMETRIC ASSAY FOR PECTIN METHYL ESTERASE IN VAPOUR HEAT TREATED 'FUERTE' AND 'HASS' AVOCADO

### 5.1 INTRODUCTION

Ripening in fruit is associated with a variety of changes. Fleshy fruits normally have a colour change where chlorophyll is degraded and other pigments are expressed. Simultaneously fruit mesocarp softens as a result of enzymatic digestion of pectin, the principle component of the middle lamella of the cell wall. Chemically, organic acids and starches and avocados oils are metabolised into sugars at the same time (Raven *et al.*, 1992). These fruit are now ready for human consumption and are at their cosmetic and internal quality peak of appeal to the consumer.

The ripening process in avocado, once initiated, cannot be stopped. Avocados are different from most fruit in that they only start to ripen once harvested(Bower and Cutting, 1988). Several enzymatic changes are initiated once the avocado fruit ripening process commences.

Pectin degradation activity is an important event in fruit ripening (Hobson *et al.*, 1984). Cell wall carbohydrates, and especially pectin, are enzymatically digested resulting in softening. Pectin consisting of $\alpha$ 1,4 - linked galacturonic acid and galacturonic acid methyl esters, is broken by various pectinases. Galacturonic acid methyl esters are hydrolysed by pectin methyl esterase (equation 1).

pectin-$COOCH_3$ + $H_2O \rightarrow$ pectin-$COO^-$ + $H^+$ +$CH_3OH$
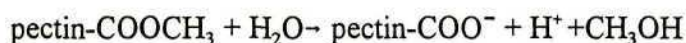
**Equation 1** (Hagerman and Austin, 1986)

The principle of this enzyme assay is a colour change of a pH indicator during the PME - catalyzed reaction. As ester bonds are hydrolysed, acid groups are produced (equation 1) and pH is altered, resulting in changes in colour, and therefore absorbance changes accordingly.

Pectin methyl esterase is one of several enzymes associated with avocado fruit ripening and its activity in stored avocados has been shown to be initially high, declining until the beginning of softening (Zauberman and Schiffman-Nadel, 1972). It follows that decreased PME activity could predict prolonged shelf life, and the purpose of this investigation was to test this, by various heat treatments of avocado fruit. It is thought that heat treatment delays ripening in avocados by altering protein synthesis (Klein and Lurie, 1991). Initial activity of enzymes like pectin methyl esterase could be reduced by vapour heat treatment, hence prolonging shelf life. In this experiment this hypothesis was tested.

## 5.2 MATERIALS AND METHODS

'Hass' and 'Fuerte' avocado fruit were vapour heat treated at 36, 38, 40 and 42°C for 1, 2, 4 and 8 hours for each temperature. For each treatment before cold storage a radial section of avocado fruit mesocarp was excised and frozen. A sample of 5 g of this section of frozen avocado fruit mesocarp was weighed out and 25 ml of 1M NaCl added. The pH of this suspension was adjusted to 7.5 with either acid or base. This was homogenised and left to extract on ice for 1 hour. Samples were centrifuged for 10 minutes. 100μl of supernatant was added to 2.5 ml of solution A at 25°C (Solution A; Bromothymol blue (0.01% w/v) : citrus pectin (0.5% w/v)- 1:10; with pH corrected to 7.5). Absorbance was read over 30 seconds at 660 nm wavelength (Hagerman and Austin, 1986). Values were taken to correlate with activity, ie. increased absorbance showed increased activity. There were 8 replicates per treatment.

## 5.3 RESULTS AND DISCUSSION

For 'Fuerte', activity for PME was significantly less (P<0.001**) for treated fruit compared to control fruit (Fig. 5.1). As the duration for each temperature increased, activity values tended to decrease. Differences between the different temperatures were highly significant (P<0.001**),

as were differences between treatment times (P<0.001**) while interaction between temperature and time was non significant. At 42°C for 8 hour duration the lowest activity was recorded. For 'Hass', activity values for PME in heat treated fruit were significantly less (P<0.001**) than in the control (Fig. 5.2). As with 'Fuerte' as the duration of each treatment increased, initial activity of PME tended to decrease. Differences between temperatures and times were highly significant (P<0.001**) and interaction between temperature and time was also highly significant (P<0.001**).
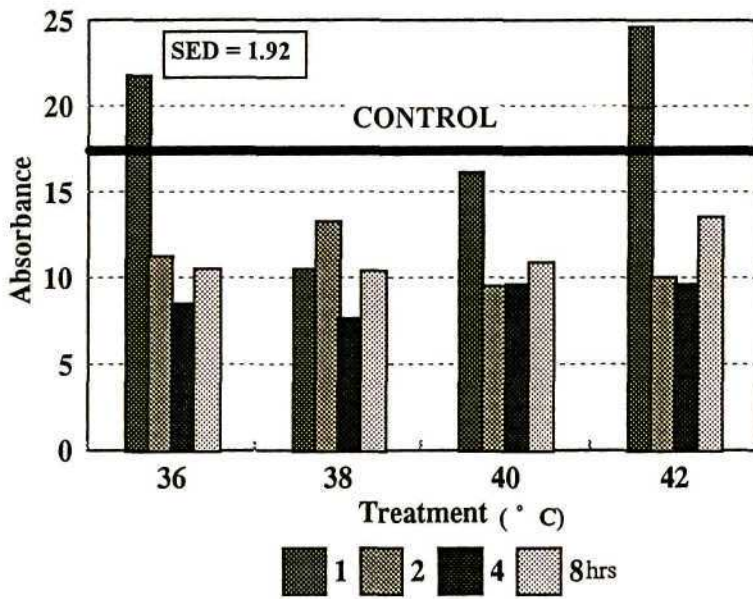


**Figure 5.1** PME activity (absorbance) for 'Fuerte' mesocarp in fruits subjected to four heat treatments for four durations
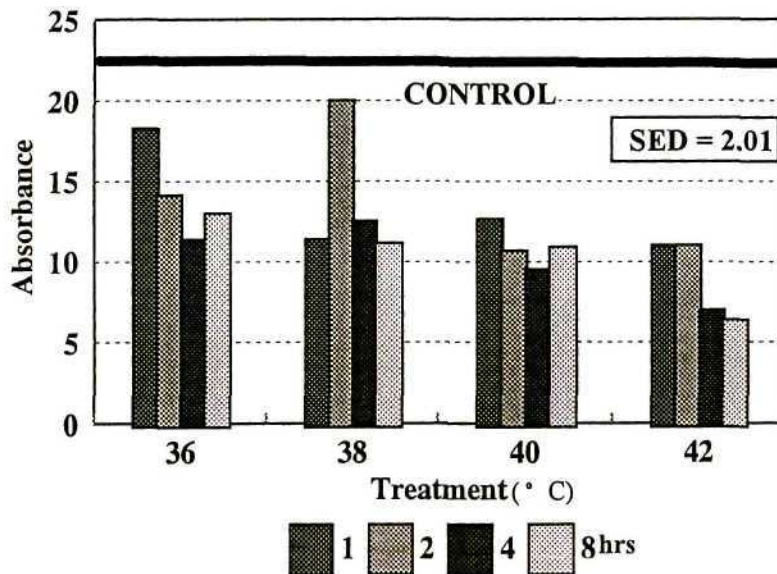
**Figure 5.2** PME activity (absorbance) for 'Hass' mesocarp in fruits subjected to four heat treatments for four durations

## 5.4 CONCLUSIONS

Considering equation 1, the more PME present and/or the higher the PME activity, the greater the pectin-COO⁻ produced and the greater absorbance due to indicator colour change. Vapour heat treated fruit displayed lower absorbance than control fruit, hence less PME activity was present. As duration of vapour heat treatment increased, PME activity decreased and significant differences were noted between treatments. The reduction of PME activity induced by vapour heat treatments correlated with the delay in ripening also induced by these treatments. The same fruit used for PME sampling were also used to evaluate an extension of shelf life. Results

generally showed fruit which had an extension of shelf life had an initial reduction in PME activity (Chapter 2). Future research could involve examining all the enzymes associated with avocado ripening and this would perhaps create a clearer picture.

# GENERAL DISCUSSION AND CONCLUSIONS

It appears that the majority of postharvest treatments of subtropical fruit have aimed to minimize chilling injury. With a reduction in chilling injury one is able to extend fruit storage life due to lower temperature tolerance, but ripening time cannot be extended after removal from cold storage. The main focus of this thesis was to try to extend storage life which includes extending ripening time once being removed from cold storage (shelf life). From the vapour heat trials of 'Fuerte' and 'Hass' avocado in 1996 and 1997, one can conclude that an extension of storage time is achieved by the correct vapour heat treatment for each cultivar. The time/temperature interaction is critical. Rind damage is a major limiting factor when considering the length of the treatment to use, as any external blemishes will reduce its export potential. A general recommendation for both cultivars is at 38°C for between 4 to 8 hrs. This temperature regime is in agreement with several other workers (Nishijima *et al.*,1995; Woolf *et al.*, 1995; Lurie *et al.*, 1996; Woolf and Lay-Yee,1997; Woolf, 1997).

To the best of the authors knowledge no other research trial on a large scale has been done (some 2048 'Fuerte' and 2304 'Hass' avocado fruit were evaluated each season, thus making a total of 8704 avocados involved in this experiment). The large scale of this experiment provides a result base from which further, more in depth, refining experiments can be based. Other researchers such as Woolf (1997) used smaller experiments ( a single temp. treatment with 6 time regimes on small samples with only 2 replicates) which, however, leave much to speculation about further temperature/time interactions.

The other potential benefits of vapour heat treatment include environmentally friendly disinfestation of fruit and a reduction in chilling injury. There is no reason why vapour heat treatment of avocados cannot be used to meet the phytosanitary requirements for fruit fly on avocados, exported to Japan, for example. This appears feasible since vapour heat treatment is being used to treat papaya in Hawaii for fruit fly which are exported into Japan. There is a fairly extensive procedure to get fruit into Japan which is clearly outlined in Figure 6.1. Only once this procedure has been performed will it be decided if this form of treatment is sufficient.

There are practical problems of treating avocado fruit on a large scale. If they are to be treated at the end of the pack line in a full pallet in a container, the airflow within the container will have to be carefully designed. This will be to insure the fruit are all treated equally. Also the use of closed boxes will prevent equal heat treatment of the fruit, thus open boxes will have to be used.

Due to marketing strategies, the 1996 season fruit was harvested 3 weeks earlier than the 1997 fruit. Therefore 1997 fruit was more mature when picked, allowing for seasonal differences in maturity. Consequently 'Fuerte' and 'Hass' avocados from the 1997 season ripened up more quickly. For example, the control fruit for 'Fuerte' after 5 weeks of cold storage took just over 10 days to ripen in 1996, compared with the control 'Fuerte' from 1997 which took just over 4 days to ripen after 5 weeks of cold storage. Both seasons, even with this difference in maturity, illustrated a positive effect on extending ripening time due to selected postharvest vapour heat treatment .

Fruit from both seasons were of good quality, as evidenced by very low levels of physiological disorders in treated and untreated fruit after low temperature storage at 3.5°C (lower than industry norm), and storage periods of 5 and 6 weeks (instead of the normal 3 or 4 weeks).

**QUARANTINE PROTOCOL FOR THE MINISTRY OF
AGRICULTURE, FISHERIES AND FOOD, JAPAN**

THE STEPS NECESSARY FOR OBTAINING APPROVAL FOR ENTRY OF A FRUIT
ARE AS FOLLOWS:

| | |
|---|---|
| TECHNOLOGY FOR PERFECT DISINFESTATION OF THE PESTS AND SUBMITTAL OF NECESSARY DATA TO JAPAN | BY THE GOVERNMENT OF A COUNTRY REQUESTING THE REMOVAL OF THE BAN |
| | WHEN THE DATA SENT ARE NOT SUFFICIENT |
| EVALUATION OF TEST DATA | BY THE JAPANESE PLANT QUARANTINE QUTHORITY |
| WHEN THE DATA SENT ARE SUFFICIENT | |
| INVESTIGATIONS IN THE EXPORTING COUNTRY BY JAPANESE EXPERTS | BY THE PLANT QUARANTINE AUTHORITIES IN JAPAN AND IN THE EXPORTING COUNTRY |
| CONSULTATION BETWEEN THE TWO COUNTRIES | BY THE PLANT QUARANTINE AUTHORITIES IN JAPAN AND IN THE EXPORTING COUNTRY |
| PUBLIC HEARING IN JAPAN | |
| AMENDMENT OF RELATED LAWS AND REGULATIONS IN JAPAN AND THE EXPORTING COUNTRY | |
| IMPORTATION COMMENCES | DESPATCHING OF JAPANESE PLANT INSPECTOR TO EXPORTING COUNTRY |

**Figure 6.1** The steps involved in obtaining clearance for export of 'Fuerte' and 'Hass' avocado into Japan (Johnson *et al.*, 1997)

In an attempt to correlate all the work done in this thesis, a spider diagram (Fig. 6.2) will be presented, followed by a brief discussion . Some of the points have already been discussed above, but it also hopes to incorporate some of the physiological aspects. While the experiments have already been discussed in full and conclusions drawn from the results, this spider diagram will hopefully give an overview of the whole thesis.
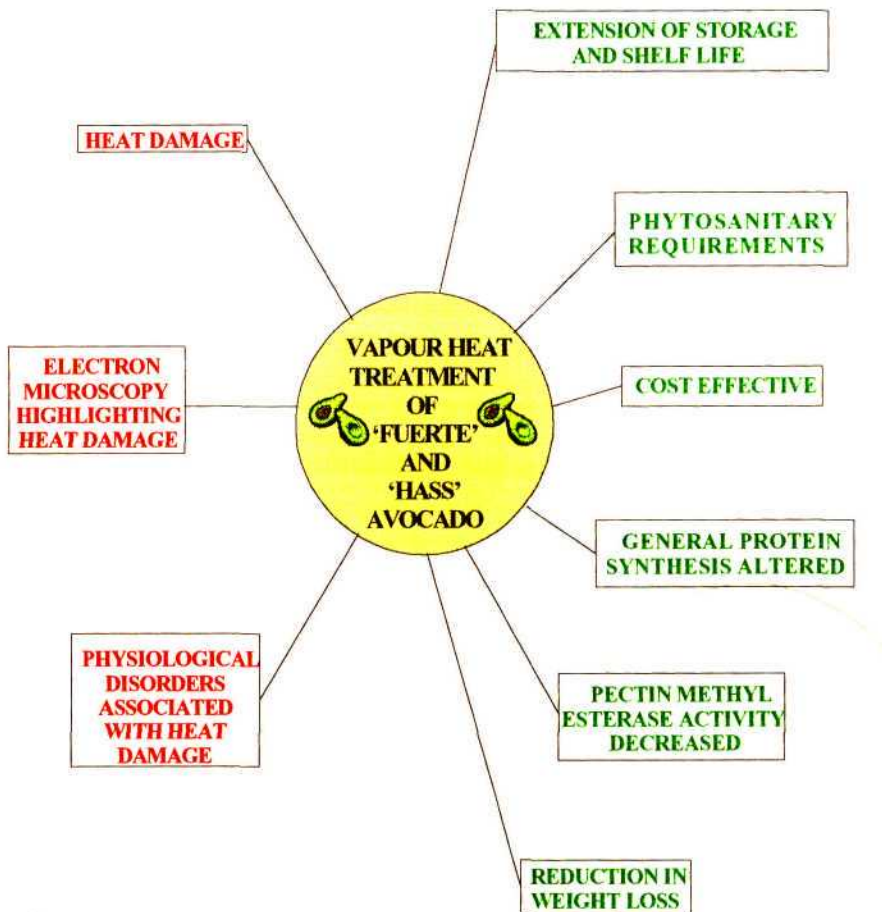


**Figure 6.2** Spider diagram illustrating the beneficial/detrimental effects of vapour heat treatment on 'Fuerte' and 'Hass' avocado in correlation with various techniques used in this thesis (red writing = detrimental, green writing = beneficial)

Heat damage (working anticlockwise around the spider diagram) is the major limiting factor when deciding which treatment to use. It will obviously have a cut off point (*"critical" temperature* for a particular duration) and it has been found that 42°C above about 2 hrs has devastating effects on the fruit. 'Fuerte' were definitely more susceptible to heat damage than 'Hass'. The electron microscopy displayed similar findings and highlighted the irreversible damage if the "critical" temperature:time interaction is surpassed. Physiological disorders were generally not a problem and tended be proportional to heat damage.

A reduction in weight loss over the cold storage periods was noted in correlation with vapour heat treatments, perhaps highlighting a conditioning of 'Fuerte' and 'Hass' avocado. This general conditioning of fruit was emphasized when it was found that pectin methyl esterase activity decreased with heat treatment. Pectin methyl esterase is important in the ripening process, and a reduction in activity of this enzyme was correlated with an extension of ripening time. Gel electrophoresis further highlighted the alteration of protein synthesis associated with vapour heat treatment. However more detailed investigations are needed to test this. It is thought that several of these proteins induced by vapour heat treatment, are heat shock proteins and give the fruit several beneficial properties such as an extension of storage life. It was seen that fruit stored for 5 weeks at 3.5°C had no problems, but a 6 week storage period could be too long, due to a slight increase in physiological disorders and chilling injury. Remarkably, very little chilling injury was observed in either season, in spite of the extended storage period at the lower than normal storage temperature of 3.5°C. This could be due to the good quality of fruit as well as constant cold storage conditions ie. no breaks in the cold chain.

Most importantly to the farmer, is this whole procedure cost effective? The average price in Europe this season was around R28 per carton compared to R48 per carton in Singapore. Unfortunately no prices were obtained from Japan, but it is thought they would be greater than Singapore (Seele, 1997, pers.comm).

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Seele, W. 1997. Pers. Comm., Cooling Estate, Wartburg.

With such good prices, it looks as if vapour heat treatment will be a very viable option and essential for increasing the viability of avocado farming in South Africa. This would , however, depend on successful commercialization of the technique, after further "fine-tuning", to successfully treat large consignments of fruit under packhouse conditions.

This thesis has highlighted future research that would be necessary, specifically fine tuning of the different vapour heat treatments on 'Fuerte' and 'Hass' avocado. The temperatures evaluated so far were accurately applied, but an exact time/temperature timetable for the entire season needs to be produced, for both cultivars. This fine tuning of treatments would also need to be achieved with the view of meeting the specific phytosanitary requirements of lucrative markets, such as Japan.

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# APPENDIX 1

## DATA LOGGER PROGRAMME FOR EVALUATION OF TEMPERATURE OF 'HASS' AND 'FUERTE' AVOCADO DURING VAPOUR HEAT TREATMENT AND COLD STORAGE

Name: Phil Weller
Project: Heatshock of avocados
Year: 1996-1997
;{21X}
*Table 1 Program
 01: 1.0000   Execution Interval (seconds)

1: Batt Voltage (P10)
 1: 1      Loc [ _____ ]

2: Internal Temperature (P17)
 1: 2      Loc [ _____ ]

3: Thermocouple Temp (SE) (P13)
 1: 12     Reps
 2: 1      5 mV Slow Range
 3: 1      SE Channel
 4: 2      Type E (Chromel-Constantan)
 5: 2      Ref Temp Loc [ _____ ]
 6: 3      Loc [ _____ ]
 7: 1      Mult
 8: 0      Offset

4: Volts (SE) (P1)
 1: 1      Reps
 2: 5      5000 mV Slow Range
 3: 13     SE Channel
 4: 15     Loc [ _____ ]
 5: .1     Mult
 6: -40    Offset

5: Saturation Vapor Pressure (P56)
 1: 15     Temperature Loc [ _____ ]
 2: 16     Loc [ _____ ]

6: Volts (SE) (P1)
 1: 1      Reps
 2: 5      5000 mV Slow Range
 3: 14     SE Channel
 4: 17     Loc [ _____ ]
 5: .1     Mult
 6: 0      Offset

7: Z=X*Y (P36)
 1: 16     X Loc [ _____ ]
 2: 17     Y Loc [ _____ ]
 3: 18     Z Loc [ _____ ]

8: Z=X*F (P37)
 1: 18     X Loc [ _____ ]
 2: .01    F
 3: 18     Z Loc [ _____ ]

9: If time is (P92)
 1: 0      Minutes into a
 2: 15     Minute Interval
 3: 10     Set Output Flag High

10: Real Time (P77)
 1: 1220   Year,Day,Hour/Minute (midnight = 2400)

11: Average (P71)
 1: 18     Reps
 2: 1      Loc [ _____ ]

12: Serial Out (P96)
 1: 30     SM192/SM716/CSM1

*Table 2 Program
 01: 0.0000   Execution Interval (seconds)

*Table 3 Subroutines

End Program (Savage, 1996)

# APPENDIX 2

### Acetate buffer

Measure out 5.72ml of 99.8% acetic acid, add 0.2198 g $CaCl_2.H20$, add NaOH ($\pm$10 pellets in 20ml water) until pH = 5 and then make up to 1$\ell$ with distilled water.

Note:

(1) A 0.1 M acetate buffer requires $0.1 \times$ molecular mass of acetic acid $dm^{-3} = (0.1 \times 60.05)$ g $dm^{-3} = 6.005$ g $dm^{-3}$. However, acetic acid is a liquid with a density of 1.05, where density = mass $\div$ volume. Therefore the volume of acetic acid required = mass $\div$ density = $6.005 \div 1.05$ = 5.72 m$\ell$ of 99.8% acetic acid.

(2) A Ca concentration of 60 to 70 mg$\ell^{-1}$ is required in the buffer solution for optimal action of the Termamyl®. The fraction of calcium that is found in $CaCl_2.2H_2O$ is $40.08 \div 147.02 =$ 27.3%. Therefore to achieve a Ca concentration of 60 mg$\ell^{-1}$ Ca, $100 \div 27.3 \times 60 = 219.8$ mg $CaCl_2.2H_2O$ must be added $\ell^{-1}$ of buffer.

### Spurr's Resin Formula

10.0g Vinyl Cyclohexene Dioxide
6.0g Diglycidyl ether of Polypropylene Glycol
26.0g Nonenyl Succinic Anhydride
0.6g Dimethylaminoethanol

### Ladd Multiple Stain

A combined nuclear and cytoplasm stain. Each 50m$\ell$ contains 0.365g Toluidine Blue and 0.135g Basic Fuchsin in 30% ethyl alcohol, intended for *in vitro* use and stored at room temperature.