CHAPTER 1

LITERATURE REVIEW

Wherever plants grow they will be subject to a great variety of stresses, which will restrict their chances of development and survival. Organisms will, naturally, respond differently to a particular stress and the nature and intensity of the response of individual plants may vary considerably, depending upon age, degree of adaptation, and on seasonal and even diurnal activity. Harvested products should, therefore, also not be expected to respond uniformly. Specific mechanisms of adaptation involve all functional levels; in many cases they are elicited by differential gene activation. Also characteristic for a state of stress are non-specific manifestations, which are primarily an expression of the degree of severity of the disturbance. Examples are alterations in membrane properties, increased respiration, inhibition of photosynthesis, reduced dry matter production, growth disturbances and premature senescence. Further non-specific effects of stress are changes in enzyme activities; *de novo* synthesis and accumulation of antioxidants, or stress metabolites, as well as numerous secondary plant substances (Larcher, 1995).

Mesocarp discolouration and external chilling injury will therefore, likely be the result of some kind of stress(es) inflicted on the fruit, which have a cumulative effect of tissue damage.

1.1. FRUIT RIPENING

The avocado is unlike most other fruit in that ripening does not normally take place on the tree and only commences after harvest (Schroeder, 1953). The reason for this phenomenon is not fully understood, but Tingwa and Young (1975) postulated that a ripening inhibitor, possibly an anion, moves either to or from the fruit pedicel once detached from the tree thus preventing on-tree ripening. More, recently Liu *et al*. (2002) suspected that seven-carbon (C7) sugars could control the ripening process.

Ripening may be considered as the first stage in the senescence of fruits in which the characteristic changes in structure and composition occur that make the fruit acceptable to eat (Rhodes, 1980). Ripening, thus, imparts value to fruit as agricultural commodities. It has been suggested that deterioration of fruits, vegetables and other plant materials, either by

natural senescence or by aging due to physiological damage, share a common mechanism (McKersie *et al.*, 1988; Palta, 1990; Stanley, 1991). It will thus be highly advantageous to gain an understanding of the ripening process to aid our manipulation of the process for extending storage and shelf life.

1.1.1 Structural changes

Ripening of avocados is not simply a degradative process and involves many catabolic and anabolic changes (Seymour and Tucker, 1993), requiring large amounts of energy as well as prolonged integrity of membranes (Bruinsma, 1981). The cell membrane system, and in particular the plasma membrane, make up an important aspect of fruit ripening (Bower and Cutting, 1988). In avocados, Golgi bodies and plasma membranes were reported to show increased buoyant density during ripening, while thylakoids and mitochondrial membranes showed no change (Dallman *et al*., 1988). Generally, during ripening, anabolic changes would include the production of new flavour volatiles or new pigments while catabolic changes would include the breakdown of chloroplast thylakoids or in the breakdown of cell wall constituents during tissue softening. In avocados lipid peroxidation may be regarded as one of the earliest detectable processes in fruit ripening (Meir *et al*., 1991). Ultrastructural changes observed in avocados during ripening by Platt-Aloia and Thomson (1981) included a loosening and eventual breakdown of the cell wall, and swelling and vesiculation of the rough endoplasmic reticulum. Montoya *et al*., (1994) also noticed some changes in electrical conductivity. Ultimately in senescence, catabolic processes become dominant (Rhodes, 1980).

There have been numerous reports of changes in activity of various metabolic pathways during the ripening process, with these being expressed as the activation, inhibition, synthesis, or release of rate-limiting enzymes of these pathways (Rhodes, 1980). Blackman and Parija (1928) thought that the activation of enzymes was as a result of changes in cell compartmentation occurring during ripening. In fact many authors have found that a common feature accompanying ripening and senescence, is increased membrane permeability, expressed as increasing leakage of ions (Bain and Mercer, 1964; Thompson, 1988; Stanley, 1991); with similar results in avocado fruit (Ben-Yehoshua, 1964). In apples this leakage correlated with increased membrane viscosity and decreased degree of fatty acid unsaturation (Lurie and Ben-Arie, 1983; Lurie *et al*., 1987). The same occurred in potatoes

(Knowles and Knowles, 1989). Increased phase transition temperatures of membrane lipids and a decline in fluidity have been described in the senescence of flowers; these events preceded enhanced ethylene production and ion leakage (Faragher *et al*., 1986). Together these findings suggest that compositional changes that determine the decreased fluidity of membranes are translated into leakage of ions, and therefore reduced functionality of membranes.

However, Palma *et al*. (1995) reported that the increases in ion leakage observed in senescing tomato fruit were significantly correlated to losses in microsomal membrane K^+-stimulated H^+-ATPase activity and not to the saturation index of membrane lipids. Therefore, in that study at least, degradation of membrane lipids was not the mechanism by which increased ion leakage occurred. However, ion leakage and ATPase activity could be correlated to linolenic acid, a fatty acid particularly prone to oxidation, and which is found in avocado fruit (Seymour and Tucker, 1993). Peroxidation of fatty acids with resulting free radical formation has been described as one of the major deteriorative processes of membranes (McKersie *et al*., 1988; Thompson, 1988; Stanley, 1991; Voisine *et al*., 1991; Voisine *et al*., 1993) and increased free radical production has been observed in a variety of senescent tissues. It is a common belief that changes in membrane lipids resulting in decreased fluidity will affect the functionality of the associated proteins as well. There is some evidence that elevated membrane viscosity is associated with lowered ATPase-specific activity. Furthermore, Vickery and Bruinsma (1973) studied changes in the passive efflux of K^+ under isotonic conditions from slices of tomato pericarp taken from fruit at different stages in ripening and they concluded that the permeability of neither the plasmalemma nor the tonoplast changed during ripening. They suggested that active transport remains under metabolic control throughout the period of ripening. Both Burg (1968) and Vickery and Bruinsma (1973) were of the opinion that changes in cell leakage often reflect changes in the total concentration of the solute available for leakage rather than changes in membrane permeability. There are thus conflicting views on the occurrence or importance of changes in cell permeability during ripening.

1.1.2 Respiration

The avocado is a climacteric fruit and displays a characteristic peak of respiratory activity during ripening, termed the respiratory climacteric. Fruit with high respiratory rates, such as

banana and avocado, tend to ripen very rapidly and hence are more perishable. This has led to the regulation of respiration as a possible mechanism for the biochemical manipulation of shelf life (Tucker, 1993). The substrate for respiration in avocados is not well defined, but the respiratory quotient (RQ) (the ratio of carbon dioxide (CO_2) produced to oxygen (O_2) consumed) remains at around 1 during the climacteric, indicating that the substrate during this period is carbohydrate rather than lipid (Blanke, 1991). Liu *et al.* (2002) reported that the increase in respiration, associated with the onset of ripening, was not initiated until a drop in C7 sugar levels occurred. However, there are also indications that some degradation of the lipid reserve does occur during ripening (Kikuta and Erickson, 1968).

The respiratory pathways utilized by fruit for the oxidation of sugars are those common to all plant tissues, namely glycolysis, pentose phosphate pathway and the tricarboxylic acid pathway (TCA) (Tucker, 1993). The increased respiration of sugars in climacteric fruit seems to be mediated largely by the increased flux through glycolysis (Solomos and Laties, 1974). Studies with inhibitors and isolated mitochondria, in avocados, suggested that the TCA cycle was operating during the climacteric to bring about oxidation of the respiratory substrate, and that this oxidation was coupled to the production of adenosine triphosphate (ATP), probably by electron transport through a cytochrome-mediated pathway (Biale and Young, 1971).

The respiratory climacteric is common to a wide range of fruit, yet its role in ripening is still unclear (Tucker, 1993). In avocados, it has been proposed that the respiratory climacteric represents “maintenance metabolism” of mitochondria in senescent fruit cells (Seymour and Tucker, 1993). Studies with avocado and banana tissue found an increase in leakage of solutes attended the onset of the climacteric, and that the rate of leakage increased exponentially during the respiratory rise (Sacher, 1962). The increase in membrane permeability during ripening would expose mitochondria to harmful substances, and there is evidence to suggest that to retain respiratory control, the mitochondria respond by increasing ATP synthesis (Huang and Romani, 1991). The large increase in ATP synthesis (Solomos and Laties, 1976; Bennett *et al*., 1987) is accompanied by a decrease in the ADP/ATP ratio with the transition from the preclimacteric to the climacteric, indicating a high-energy charge, but low ATP demand. The climacteric may, thus, not be the result of an increased energy demand, but could be a response to changes in the cytosol (availability of substrates, cofactors, activators and inhibitors) or the mitochondria (Blanke, 1991).

Furthermore, excess glucose is believed to cause modification in the structure and function of proteins, some of which may be critical to cellular function (Sharon, 1980). Release of compartmentalised amino or organic acids may lead to pH changes in tissues, with detrimental consequences to a host of metabolic paths (Davies, 1973). Analogous damaging effects could also be elicited by the release into the cytosolic environment of other endogenous compounds, notably phenolics (Frenkel, 1987).

1.1.3 Plant growth regulators

Plant growth regulators (PGRs) have been found to play a large role in avocado fruit ripening (Bower and Cutting, 1988). As the purpose of this research was not to evaluate the role of the PGRs the following information will serve only to acknowledge that there are many components involved in the ripening process. In avocados the onset of ripening is marked by a large increase in ethylene production. The precise role of ethylene is still uncertain, but it appears to be involved in the initiation and coordination of ripening in fruits (Seymour and Tucker, 1993). Increased ethylene biosynthesis is, however, not unique to the ripening of climacteric fruit. Most plant tissues will respond to wounding with an increase in ethylene production. Preharvest water stress has been found to alter the ethylene evolution pattern resulting in uneven ripening and poor fruit quality (Cutting *et al*., 1986). Like ethylene, abscisic acid (ABA) also appears to be a ripening promoter, while auxins, cytokinins and gibberellins, are inhibitors of fruit ripening (Rhodes, 1981). Exogenously applied ABA has been shown to stimulate ethylene biosynthesis and fruit ripening (Vendrell and Palomer, 1997). Similarly, ethylene induces a rise in ABA levels in immature melons (Guillen *et al*., 1998).

Stress is a major factor affecting ABA levels. Hiron and Wright (1973), for example, found a strong relationship between water stress and ABA synthesis. Adato and Gazit (1974) found that the greater the daily water loss from harvested avocado fruits the more rapid the ripening. Infusion of water delayed ripening, and it was thus concluded that moisture stress could be an important factor in ripening. Furthermore, temperature can also affect both ABA levels and the rate of ripening. Wang *et al*. (1972) found that pear fruits subjected to low orchard temperatures ripened faster and also had higher ABA levels. High levels of ABA were also found to significantly increase PPO activity in avocados, reducing the internal fruit quality and time to ripeness (Cutting *et al.*, 1988).

1.2 FACTORS AFFECTING FRUIT QUALITY

Successful marketing of horticultural products requires predictability and consistency of quality and subsequently an understanding of the basis of quality at the cellular and molecular level. It is important to consider that harvested fruit are 'living' structures, which continue to perform the metabolic reactions and maintain the physiological systems, which were present while the fruit were attached to the tree. Respiration and transpiration continue after harvest and, since the fruit is now removed from its normal source of water, photosynthates and minerals, the produce is dependent entirely on its own reserves and moisture content. As the losses of respirable substrates and moisture are not made up after harvest, fruit deterioration starts to occur, making fruit highly perishable commodities (Tucker, 1993). It is this perishability, and inherent short shelf life, that presents the greatest problem to the successful transportation and marketing of fresh fruit. Solutions to the postharvest problems of fruit, as well as improvements in the handling procedures, may therefore come from a better understanding of factors affecting fruit quality. A number of factors can affect fruit quality, but in terms of this study, perhaps the most important, is that of fruit browning.

1.2.1 Fruit browning

The browning potential of various fruits has been directly related to the phenol level, the polyphenol oxidase (PPO) activity, or a combination of these factors (Mapson *et al*., 1963; Ranadive and Haard, 1971; Golan *et al*., 1977). These two components will thus play an important role in discolouration development and this section will give a brief overview of their function and location in the fruit.

1.2.1.1 *Total phenolics*

Plant phenolics include a variety of compounds such as simple phenols, phenolic acids, coumarins, flavonoids, tannins, and lignins. Phenolic acids have a benzene ring, a carboxylic acid, and one or more phenyl hydroxyl groups that may become methylated to produce methoxy groups (Torres *et al*., 1987). Phenolic acids commonly occur as esters and/or ethers in combination with various sugars and aliphatic or aromatic acids and hydroxy acids. Phenolic acids make up significant components of taste and odour, are involved in the browning reaction, and are thought to be involved in growth regulation and in disease and

herbivore resistance. Phenolic acids are generally found in the cell vacuole or in special tissues, and are precursors of many other compounds. When membrane integrity is lost the phenols are released and become oxidized, atmospherically and enzymatically, to quinones (Torres *et al*., 1987). The quinones are irreversibly oxidised to melanin pigments that are brown in colour (Bower and Cutting, 1988).

Total phenolic content can generally vary in different species of the same genus (Gartlan *et al*., 1980), in the same species at different times of year, and in the same tissue at different stages of growth (Lowman and Box, 1983). This was confirmed in avocados where certain cultivars were found to have significantly higher total phenolics than others and where a difference in phenol content was found between the proximal and distal ends of the mesocarp of the same fruit (Golan *et al*., 1977). In the same study a positive correlation was found between total phenolic concentration and fruit browning. Cutting *et al*. (1992) also found that total phenolics in avocado increased with increasing maturity, as did mesocarp discolouration after cold storage. No difference in phenolic concentration was, however, found between cold- and non-stored fruit. Conversely, Graham and Patterson (1982) found that the concentration of fruit phenolics increased at low temperatures. This was attributed to the increased production of two enzymes concerned with the synthesis of phenolic compounds, phenylalanine ammonia lyase (PAL) and hydroxycinnamoyl CoA quinate hydroxycinnamoyl transferase (CQT), increasing at low temperatures.

1.2.1.2 *Polyphenol oxidase*

Polyphenol oxidase (PPO) is a copper-containing enzyme complex which catalyses the conversion of monophenols and *o*-dihydroxyphenols to *o*-quinones in higher plants. The *o*-quinones produced by this reaction can undergo polymerisation and bind covalently to nucleophilic amino acids to form black or brown pigments which cause the characteristic postharvest browning of fruit and vegetables (Mayer and Harel, 1979; 1991; Stewart *et al*., 2001). PPO is a nuclear-encoded protein (Lax *et al*., 1984) that is transported to the plastids, where it is associated with the internal thylakoid membranes (Vaughn *et al*., 1988). As mentioned previously the phenolic substrates for PPO are located in the vacuole, and therefore the enzymic browning reaction only occurs when subcellular compartmentation is lost following tissue damage. PPO gene expression is usually highest in developing tissues and meristematic regions, and decreases during tissue maturation (Hunt *et al*., 1993; Dry and

Robinson, 1994; Boss *et al*., 1995). Generally, PPO can exist in a soluble and an insoluble form (Kahn, 1977a). It is possible to solubilise and release additional PPO activity from the insoluble fraction, and in some cases, soluble PPO can exist in a latent form that is activated by storage, temperature, detergents or denaturing agents (Kahn, 1977a). In avocados an anionic detergent, sodium dodecylsulphate (SDS), has been successfully used to activate latent PPO (Kahn, 1977a). Low temperature storage (Sharon and Kahn, 1979), for increasing lengths of time (Golan and Sadovski, 1977), has also been reported to result in an increase in PPO activity in avocados.

Although the physiological function of PPO in higher plants has not been "unequivocally determined" to date there is evidence to suggest that the enzyme plays a role in plant defence (Vaughn *et al.*, 1988). This is to be expected as cellular damage can result in the loss of PPO latency, decompartmentalization of the phenols and thus interaction of PPO with these phenolic substrates, with the production of bactericidal and fungicidal hydroxyphenolics and quinines which may polymerise to seal off infected tissues. There are, however, studies in avocados in which no correlations or negative correlations have been obtained between PPO activity and disease resistance (Brune and Van Lelyveld, 1982).

The relationship between PPO activity and postharvest fruit quality has also received much attention. In pineapple fruit a correlation was found between PPO activity and the development of the internal browning disorder known as blackheart (Van Lelyveld and De Bruyn, 1977), which is thought to be a chilling disorder. Furthermore, cultivars of grapes with low PPO levels were found to be superior because the wine from these grapes did not turn brown as easily as from varieties having higher PPO levels (Vaughn *et al*., 1988). The possible involvement of PPO in the development of mesocarp discolouration in avocado fruit was also suggested by Engelbrecht (1987). Similarly, Kahn (1975) found that avocado cultivars, which were more susceptible to mesocarp discolouration, had higher PPO activity than those less susceptible. PPO activity has also been linked to preharvest water stress, storage container ventilation (Bower and Van Lelyveld, 1985) and moisture loss during cold storage (Bower and Cutting, 1987).

Senescence is also thought to result in decompartmentalization of PPO and its substrates. This, together with the fact that total phenolics and PPO have been found to increase as the season progresses, may imply browning potential would be higher during this time. However,

a strict correlation between PPO activity and browning capacity does not necessarily exist (Sharon-Raber and Kahn, 1983) as the phenomenon is also both qualitatively and quantitatively substrate dependent.

Some studies have found correlations between the presence of high levels of phenolic compounds and PPO (Golan *et al*., 1977) or low levels of phenolics and no PPO in some tissue types (Vaughn *et al*., 1988). However, these correlations do not always exist. Kahn (1977b) found that in some cases a relatively high substrate concentration inhibited avocado PPO activity. Mueller and Bechman (1978) noted that, although some cell types with vacuolar phenolic depositions had plastids with PPO, other layers of tissue had plastids that lacked PPO activity but had phenolic depositions in the vacuole. While studies found that PPO is not involved in the synthesis of phenolic compounds in healthy, intact cells (Vaughn *et al*., 1988; Strack *et al*., 1986), it is not thought to be totally unrelated to phenolic metabolism.

The fact that PPO is located on the thylakoids of mature chloroplasts, and that it is a redox enzyme, has also led to investigations of a possible role of PPO in energy transduction of the chloroplast. Vaughn and Duke (1984) suggested that PPO might act in mediating photoreduction of molecular oxygen (the Mehler reaction) by photosystem I (PSI). PPO was found to have a low affinity for molecular oxygen and be to a large degree latent on the thylakoid membrane, increasing in its activity during membrane damage. It was thought, therefore, that the regulation of the Mehler reaction by PPO might be modulated by oxygen levels and any factor that might alter PPO latency.

1.2.2 Temperature

1.2.2.1 Factors affecting chilling injury

Several factors influence the incidence of chilling injury. The great diversity in shape, size, and physiology of various fruits and vegetables contributes to the variations in chilling tolerance. Substantial differences in the degree of chilling sensitivity also exist among cultivars and species. In addition, environmental conditions under which the crops are grown have great influence on the susceptibility to chilling injury. Considering all of these factors, it is not hard to understand why we still do not have a universal method, which could be effective in reducing chilling injury in all crops under any circumstances. The degree of

chilling injury incurred by a plant or plant part depends on the temperature to which it is exposed, the duration of exposure, and the species sensitivity to chilling temperatures. The lower the temperature to which a product is exposed below its threshold chilling temperature, the greater the severity of the eventual injury. The rate of development of injury symptoms in storage is also generally decreased with temperature; however, upon removal to non-chilling conditions the full manifestation of the stress becomes apparent (Kays, 1991). Chilling stress and injury does not just occur during storage. Chilling temperatures may be encountered in the field, during handling or transit, during wholesale distribution, in the retail store, and in the home.

1.2.2.2 Symptoms of low temperature disorders

The onset of symptom expression due to chilling injury in plants varies visually and temporally among plant species. In general, greater injury occurs in plants exposed to lower chilling temperatures for longer periods of time. In some plants, injury may be expressed during the chilling period or after the plant tissue has been re-warmed and ripening commences. Symptoms of chilling injury include cellular damage (changes in membrane structure and composition, decreased protoplasmic streaming, electrolyte leakage, and plasmolysis), altered metabolism (increased or reduced respiration, production of abnormal metabolites due to anaerobic conditions), surface lesions on fruits, water soaking of tissues, internal discolouration (including vascular browning), increased susceptibility to decay, and failure to ripen normally (Saltveit and Morris, 1990). Some of the more common symptoms of chilling stress are rapid wilting followed by water-soaked patches which develop into sunken pits that reflect cell and tissue collapse. Following warming the sunken pits usually dry, leaving necrotic patches of tissue.

1.2.2.3 Primary versus secondary chilling injury lesions

It has long been debated whether there is some primary event that leads to chilling injury in plants, and if so what the event is. Evidence has accumulated that chilling stress affects several functions in plants, including biochemical and biophysical structure of membranes, nucleic acid synthesis, changes in protein synthesis, enzyme conformation, affinities and activation energies, water and nutrient (particularly calcium) balances, cellular cytoskeletal structure, and photosynthetic and respiratory function. Which of these represent the primary

event and which are secondary events leading to symptom expression is the basis for the debate. Raison and Orr (1990) propose a single primary event based on temperature transition in the molecular ordering of membrane lipids, which was initially expressed by Lyons and Raison in 1970. This model suggests that chilling injury can be divided into a single primary event and several secondary events (Figure 1). The primary event (probably, membrane phase transition) is initiated when temperature drops below a certain critical temperature. This temperature will vary with species and conditions under which the plant is grown. The primary event is then responsible for initiating numerous secondary events, but the order of initiation, if any, is not clear. If the level of chilling is not too great or too long and the plant is returned to warmer temperatures, the process can be reversed and the plant does not sustain injury. However, if the stress is maintained at too low a temperature for too long, and then returned to warmer temperatures, injury and cellular degradation are accelerated.

Naylor (1983) argues against a single primary lesion as being responsible for initiating the cascade of events that lead to injury and symptom development. Instead it is envisioned that chilling stress affects the physical concepts of coordination of metabolic pathways (rate effects) and the stability of complex biological molecules (weak bond effects), either or both of which may be affected at different levels of organization and expression. In the case of rate effects, sensitivity of any one metabolic process, or enzyme within that process, to chilling temperatures could have a significant impact on the functioning of other enzymes in that process or substantially alter the balance among multiple metabolic processes. Naylor (1983) further points out that biochemical structure, including protein configuration, membrane structure, nucleic acid structure, and most biochemical interactions, require a high degree of specificity, which, if not totally, is highly dependent on weak chemical bonds. Thus when considering the importance of just hydrogen bonding (with bonding energies 10 to 12 times weaker than covalent bonds) in protein configuration, the structure and stability of membranes, and protein-membrane and protein-substrate interactions, it becomes clear that there may be numerous opportunities where biochemical functions could be affected simultaneously by chilling stress, making the identification of a single primary lesion difficult if not impossible. The ability of organisms to cope with chilling temperatures and other environmental stresses then lies with the development of physical and/or biochemical strategies that protect the organism against breaking of weak bonds.

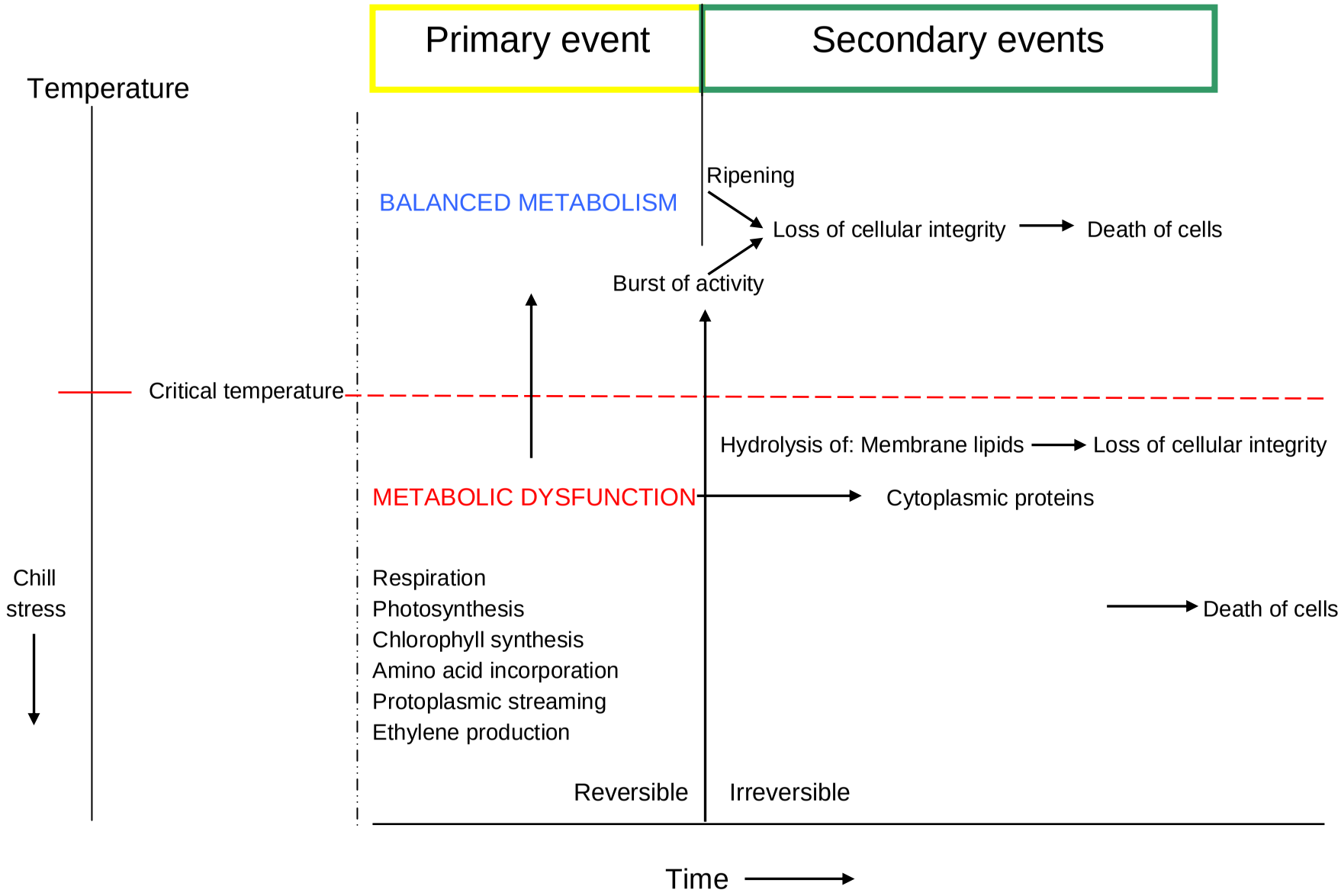

Figure 1. Relationship between “primary” and “secondary” events during chilling injury. (Modified from Raison and Orr, 1990).

1.2.2.4 *Physiological and biochemical effects of chilling stress*

A number of mechanisms have been proposed to accommodate the physiological and biochemical changes associated with acclimation and adaptation of plant cell membranes to different environmental temperatures. A review of these changes indicates that chilling changes the physical stability of the membrane and its ability to function under different conditions (Lyons, 1973; Pantastico e*t al.*, 1975; Quinn, 1988; Nilsen and Orcutt, 1996). Plants of tropical and subtropical origin are generally assumed to contain more highly saturated fatty acids than species growing in cold regions (Pantastico *et al*., 1975). In these plants, phase transitions in membrane lipids have been proposed as the primary event in physiological disorders such as "chilling injury" (Lyons, 1973; Raison, 1973). While this hypothesis received initial support, further studies found that only a small proportion (<10%) of the membrane lipids underwent phase transition at physiological temperatures (Wills *et al*, 1989). A refinement to the lipid-phase transition theory has been based on the presence of heterogenous lipid domains in biological membranes undergoing liquid-crystalline to gel-phase transitions (Stanley, 1991). The membranes of chilling-injured avocado fruit were found by Platt-Aloia and Thomson (1987) to contain particle-depleted regions in the plasmalemma. These particle-depleted microdomains were suspected to be due to lateral phase separations of the membrane components, possibly due to an increase in the viscosity of some membrane lipids, leading to the formation of microdomains of gel phase lipid in the plane of the membrane. This has the potential of forming inverted micelles in the membrane, which could facilitate nutrient or ion leakage. In addition, such restructuring could influence the configuration or positioning of proteins (enzymes) within the membrane altering their functions (Nilsen and Orcutt, 1996). Thus, membranes may not respond to environmental perturbations in a general way as previously thought, but rather, may influence specific organelle membrane lipids and/or affect isolated domains in those membranes, resulting in localized changes in fluidity or perhaps inducing pores as a result of lateral phase transitions resulting from homologous lipid aggregations. Chilling sensitivity was recently correlated with the degree of unsaturation of fatty acids in phosphatidylglycerol of chloroplast membranes (Murata *et al*., 1992).

Thus plants have a mechanism by which they can maintain membrane fluidity by changing the saturation and unsaturation of fatty acyl groups of glycerolipids. It is reported that as temperature increases the degree of unsaturation is reduced, while as temperature

decreases, unsaturation increases. Insertion and removal of sterol from membranes may also be part of the mechanism for membrane fluidity adjustment as temperatures change (Nilsen and Orcutt, 1996). As mentioned in the previous section the physical change in the membrane lipids, with the lowering of temperature, is thought to cause changes in the properties of the membranes (Wills *et al*., 1989). Generally, chilling stress conditions weaken hydrophobic interactions, expose sulfhydryl groups, and can alter the lipid environment surrounding membrane-associated proteins which can ultimately lead to changes in the configuration of proteins and possibly enzyme kinetics (Nilsen and Orcutt, 1996). Similarly, early studies found that an increase in membrane permeability would lead to an upset in ion balance and also to increases in the activation energy (E_a) of membrane-bound enzyme systems, leading to a suppressed reaction rate and establishing an imbalance with non-membrane-bound enzyme systems (Lyons, 1973). For example, below a critical temperature, at which phase transition occurs, the E_a for membrane-bound mitochondrial respiration increases while the E_a for soluble enzyme systems, such as glycolysis, decreases causing an imbalance in the two systems. Enzymes of photosynthetic metabolism are similarly affected. The consequences of such changes in enzyme activities are thought to be imbalances in metabolism, which eventually lead to cell death. Plank (1938) assumed that, under chilling stress conditions, two main types of reactions were involved in the cells – one leading to the accumulation of toxins and the other to their removal. By selecting values for the temperature coefficient used in his equations, he was able to show the critical temperature at which the production and removal of toxins are in equilibrium and below which cell toxins would accumulate, causing chilling injury. Other workers have also accepted the toxin hypothesis of chilling injury, in which toxic products of metabolism accumulate (Eaks and Morris, 1957; Hulme *et al*., 1964; Wills *et al*., 1989). Apparent differences among species or cultivars in chilling sensitivity are suspected to be related to different tolerances in withstanding or metabolising the resulting toxic compounds (Lyons, 1973).

Furthermore, a progressive decline in the capacity of the fruit for oxidative phosphorylation occurs with exposure to low temperature. This could lead to a shortage of high energy, typically ATP, needed for the maintenance of cell organization in the presence of enzymatic processes, constantly tending to disrupt the system. A net breakdown of complex cellular components follows because of the resultant shortage of energy (Pantastico *et al*., 1975).

The temperature-induced phase change in the lipid portion of the membranes is completely reversible, though the effect on the whole organism is reversible only until the system incurs some degenerative injury. Thus, with a short chilling treatment followed by a warmer temperature, respiration increases sharply but only transiently, with the normal metabolism soon re-established. If chilling temperatures continue long enough for degenerative changes to occur, however, the respiration rate remains elevated, reflecting a disrupted metabolism (Lyons, 1973). The effect of temperature on respiration rates (Gonzalez-Meler *et al*., 1999) could, in the short term, be due to the kinetics of most metabolic reactions being highly temperature dependent (Raison, 1980). For most plants respiratory activity at low temperatures remains in balance with glycolysis and other closely related reactions. In contrast, the respiratory activity of many tropical and sub-tropical plants decreases more than other reactions when the temperature is lowered, and this leads to imbalances in metabolism (Raison, 1980). Furthermore, increasing respiratory rates during chilling, accelerated respiration following chilling, and altered respiratory quotients have all been used as indices of chilling sensitivity (Pantastico *et al*., 1975).

In addition, structural proteins of the cell's cytoskeleton, such as tubulin, are cold-labile and undergo dissociation at low temperatures. This is thought to account for the effect of low temperatures on protoplasmic streaming which is especially sensitive in chilling sensitive plants (Wills *et al*., 1989). Degradation of cell walls plays an obvious role in the development of the visible symptoms of stress response. Localized softening associated with fruit bruising is the most obvious example of enzymatic breakdown of cell walls. Examples of cell-wall breakdown during chilling injury include the development of pitting (Shewfelt, 1993).

The mechanism of low temperature disorders thus encompasses several elements operating independently or simultaneously: imbalances in metabolism, accumulation of toxic compounds, dis-equilibrium of reactions and increased permeability. Levitt (1972), however, proposed that all types of chilling injury can be the result of a change in cell permeability and many studies have in fact concentrated on the effect of membrane permeability, as determined by increased electrolyte leakage, on low temperature disorders (Lyons, 1973; Pantastico *et al*., 1975; Levitt, 1980; Bramlage, 1982; Morris, 1982) and also specifically on avocados (Platt-Aloia and Thomson, 1992; Woolf *et al*., 2000).

1.2.2.5 *Effect on other fruit*

In mango studies, where fruit have a critical storage temperature of about 10°C, a marked increase in sucrose degradation was found when fruit were exposed to temperatures of 2-5°C, which was thought to be indicative of an increase in glycolysis (Chattpar *et al*., 1971). In bananas the respiratory enzymes were found to decrease or lose their activity after exposure to low temperatures (Murata, 1969), with the polyphenol substances, in severely chilled fruits, being oxidised instead of the regular respiratory substrates. Jones (1942) observed that CO_2 production by papaya fruits was higher than expected at chilling temperatures. Miller and Heilman (1952) proposed that in pineapples, the destruction of ascorbic acid constituted the first phase in the development of chilling injury. They proposed that interference in some specific steps in the respiratory process caused quinines to accumulate because of their failure to be converted back to phenols by ascorbic acid, and that this accumulation of quinines resulted in the discolouration noted in many kinds of chilled fruits. In grapefruit, intermediate temperatures were found in some instances to cause greater chilling injury than either higher or lower temperatures (Pantastico *et al*., 1975). It was thought that the greater injuries noted at the intermediate temperatures were possibly restricted to a specific time period (Ulrich, 1958). Preharvest environment is also thought to influence chilling susceptibility (Lyons, 1973). Palmer (1971) cited studies indicating that banana fruits maturing at higher field temperatures were more susceptible than those maturing in a cooler climate.

1.2.2.6 *Effect on avocado fruit quality*

The critical temperature for cold storage of unripe avocados has been reported to be 8°C (Lyons, 1973). Storage at below optimal temperatures has been found to affect fruit ripening (Biale, 1941; Eaks, 1976; 1983; Cutting and Wolstenholme, 1992). Unripe 'Hass' exposed to temperatures of 0°C and 5°C for 4-6 weeks displayed chilling injury symptoms, abnormal ripening, atypical respiratory rate patterns, and greatly reduced ethylene peaks when ripened at 20°C (Eaks, 1983).

Studies have, however, found that the optimum temperature and storage period of avocados depends on a number of factors. For example, not all cultivars are equally sensitive to low temperatures (Vakis, 1982; Bezuidenhout, 1983; Vorster *et al.*, 1987; Eksteen *et al*., 1998).

The stage of fruit development can also be important as sensitivity in 'Hass' was found to be relatively high in the preclimacteric stage, increasing to a maximum at the climacteric peak, and decreasing rapidly as the fruit ripens, and finally reaching a minimum about two days past the climacteric (Kosiyachinda and Young, 1976). Similarly, Bezuidenhout (1983) found that excessive cold prior to the climacteric, in 'Fuerte' fruit, was favourable for chilling injury and pulp spot development. The changes in chilling sensitivity were suspected to be related to changes in the activity of regulatory enzymes such that intermediates accumulated to levels that were toxic to the cells (Kosiyachinda and Young, 1976). These levels of intermediates were found to be lower after the climacteric peak had been reached and thus the storage life of avocados could be extended by transferring the fruit to 2^{o}C after the climacteric peak (Kosiyachinda & Young, 1976). Zauberman and Jobin-Décor (1995) found that unripened 'Hass' could also be stored successfully for four to five weeks at 2^{o}C without developing injury or abnormal ripening. These fruit were still hard after removal from storage and were thus still suspected to be in the preclimacteric stage.

The potential for physiological disorders and cold damage varies throughout the season (Swarts, 1980; Bower *et al.*, 1986; Vorster *et al*., 1987). Early season avocado fruit have been found to be more susceptible to chilling injury than fruit picked later in the season (Swarts, 1980; Smith and Lunt, 1984; Kritzinger and Kruger, 1997). Swarts (1980), however, proved that this was not the effect of picking maturity, but rather the result of a drop in the preharvest ambient orchard temperature to below 17^{o}C. Based on this evidence it was suggested that storage temperature during the early parts of the season should be higher than later in the season. Application of this concept resulted in Vorster *et al*. (1987) finding that 'Fuerte' avocados picked later in the season could be stored at 3.5^{o}C without danger of chilling injury. Zauberman and Schiffman-Nadel (1977) also found that preharvest temperature could have an adverse effect on fruit ripening, *viz.* temperatures above 30^{o}C. Bezuidenhout (1983) attributed up to 50% of the probable causes of internal disorders to "unknown orchard factors". This was confirmed by Rowell (1988) who found that there was a marked variation in the way in which fruit from different areas reacted to temperatures.

1.2.3 Water content

1.2.3.1 Maturity

In South Africa the moisture and oil content of avocado fruit are used as maturity parameters (Swarts, 1982; Kruger *et al*., 1999). Oil content is known to increase and water content to decrease with increasing maturity (Pearson, 1975). In recent years maturity has been considered to be the main causative factor of mesocarp discolouration development in South Africa (Snijder *et al*., 2002; Snijder *et al*., 2003; Kruger *et al*., 2004). In South Africa the moisture content of 'Pinkerton' avocados, being considered for export, should be between 80% (Thompson, 1996) and 73% (Snijder *et al*., 2003). Postharvest time to ripening is thought to be a function of fruit maturity, less time being required with increasing maturity (Zauberman and Schiffman-Nadel, 1972; Adato and Gazit, 1974). Similarly Cutting and Wolstenholme (1992) found that increasing maturity (on-tree storage) decreased the time taken for avocado fruit to ripen after harvest. This is to be expected, as there is a decreasing water percentage with increasing maturity at harvest.

Stage of fruit ripeness, at the time of low-temperature storage, has also been reported to play a role in determining the sensitivity of 'Hass' and 'Fuerte' avocados to chilling injury (Kosiyachinda and Young, 1976). In bananas ripe fruit, regardless of variety, are reportedly less sensitive than green fruit. Among the various varieties of citrus, grapefruit harvested early in the season are found to be more susceptible to pitting with the susceptibility decreasing as fruit became more mature. However, differences in grapefruit susceptibility are also related to season and variety. Tomato fruit are also particularly susceptible to chilling injury at the mature-green stage when they are normally harvested and shipped (Saltveit, 1991).

1.2.3.2 Preharvest orchard conditions

Preharvest water relations also affect the rate of fruit ripening. Bower (1984) found that more negative avocado fruit water potentials at harvest, caused faster ripening after storage. Long term preharvest stress (particularly during the first 3 months after fruit set) also caused an altered ethylene evolution pattern (Cutting *et al.*, 1986). Furthermore, ripening was uneven, and fruit quality poor. Excessive irrigation was also found to reduce calcium uptake as well

as increase levels of ABA in avocado fruit (Bower *et al*., 1986; Bower, 1987). In 'Pinkerton' it was also found that storage potential was drastically reduced during a wet season if there was a very low crop load (Kruger *et al*., 2004).

1.2.3.3 Postharvest handling

The potential for water stress does not stop at harvest; rather in most postharvest products the stress potential increases sharply. When individual plant parts are severed from the parent plant at harvest, their ability to replace water lost through transpiration is eliminated, making them much more susceptible to water stress. Fruit firmness and membrane integrity, during storage, are thought to be affected by relative humidity (Ben-Yehoshua, 1985). When relative humidity is too low, transpiration is enhanced, resulting in loss of moisture. The primary factor controlling the rate of moisture loss is actually the water vapour pressure deficit, which reflects the difference between the humidity in the tissue and the humidity of the air in the storage room or container (Kays, 1991). As water evaporates from the tissue, turgor pressure decreases and the cells begin to shrink and collapse. In studies on avocados Bower and Cutting (1987) also found that the rate of fruit moisture loss during storage was associated with fruit quality. Furthermore, the infusion of avocado fruit with water during ripening (Cutting and Wolstenholme, 1992), as well as storage of fruit under high relative humidity (Bower, 1988), was found to reduce the incidence of physiological postharvest disorders. Water stress caused by prolonged storage can result in increased and early ethylene production, which in turn may enhance ripening processes (Adato and Gazit, 1974). Water status thus plays a very important role in maintaining membrane integrity and slowing down ripening. Storage at very high relative humidity (close to 100%) also can result in adverse effects. Free water on the plant tissue provides an excellent environment for decay microorganisms. Although healthy plant tissue is resistant to decay, cuts, bruises, or additional stress can weaken the endogenous resistance of the tissue.

High relative humidity storage combined with low temperatures has been suggested as a means of maintaining quality of bulk products for extended periods of time. Humidity close to 100% has been found to ameliorate chilling injury in some instances, with low humidity aggravating symptoms. Morris and Platenius (1938) showed that, although the severity of pitting was directly correlated with the rate of transpiration, very rapid rate of water loss did not result in pitting if the fruit had not been exposed to low relative humidity at the same time.

The reduction of chilling injury in citrus by seal-packaging was not attributed to the inhibition of transpiration by the sealing, since the cooling was thought to inhibit transpiration, and chilling injury could be reduced even more effectively by curing fruit for a week at 21^{o}C before exposing it to the sensitive low temperatures. During this curing period, the fruit transpired rapidly (Ben-Yehoshua, 1985).

1.2.3.4 Effect of hydration on membranes

Degree of hydration is very important in affecting the fluidity of plant membranes. In general, if cell water percentage falls to a level of 20% or less of the cell dry weight, this is considered to be a critical level relative to maintaining homeostatic viscosity of the membrane and may even affect the thermodynamic stability of the membrane (Nilsen and Orcutt, 1996). As with temperature stress, lateral phase transitions may occur in which homologous lipids aggregate into different regions or domains of a membrane, resulting in ion leakage. Varying lipid components in membranes exhibit differing degrees of hydration or affinity for water. Glycolipids apparently have a very high affinity for water, which reflects the carbohydrate component of these lipids. Carbohydrates, particularly non-reducing disaccharides such as trehalose and sucrose, appear to be important in organisms capable of survival after complete dehydration. It appears that many organisms produce high levels of these carbohydrates when exposed to dehydration stress, and it is hypothesised that the carbohydrates interact with cellular membranes to increase the stability of the lipid bilayers. The protective mechanism is uncertain, but it has been suggested that under stress, water molecules normally associated with the phospholipid head-groups are replaced with sugars, which prevent lateral phase transition and the formation of lipid domains (Nilsen and Orcutt, 1996).

1.2.4 Mineral nutrition

Mineral nutrition plays a very important role in the growth and development of any fruit, and subsequently determines fruit quality and storage life (Ginsberg, 1985). A number of nutrient elements are of importance and also the balances of these elements in relation to each other. Divalent and monovalent cations are known to have a considerable influence on the fluid nature of biomembranes (Nilsen and Orcutt, 1996). How these ions interact with membranes depends on the type and abundance of membrane components present, as well as how they

are arranged in the bilayer. The phospholipids appear to be the most drastically affected components of the membrane; however, interactions with proteins can also occur. Many disorders are prevented by the addition of a specific mineral either during growth or postharvest, although for most disorders the actual role of the mineral in preventing the disorder has not been established (Wills *et al*., 1989). Generally, all plants require a balanced mineral uptake for proper development. However, while leaf and soil norms have been established for optimum avocado production (Koen and Du Plessis, 1991; Köhne *et al*., 1990), there are very few studies that have been conducted to evaluate and establish fruit norms with respect to fruit quality (Cutting *et al*., 1992; Thorp *et al*., 1997; Hofman *et al*., 2002; Snijder *et al*., 2002). Some studies have related fruit quality to soil mineral concentrations (Kremer-Köhne *et al*, 1993), however there are studies that have found no correlation between soil, leaf and fruit concentrations (Thorp *et al*., 1997). Furthermore, there appear to be very few studies that evaluate a wide range of both macro- and micro-elements.

1.2.4.1 Calcium

Calcium has been associated with more physiological disorders than any other mineral (Bangerth, 1979; Wills *et al*., 1989). It is thought to play a significant role in the rate of fruit softening with high endogenous levels of calcium (Tingwa and Young, 1974; Cutting *et al*., 1992) or postharvest calcium applications being found to delay the overall softening process during ripening (Wills and Tirmazi, 1982; Ferguson, 1984). Furthermore, the infiltration of calcium into avocados has been shown to greatly reduce the ethylene peak and respiration rate, resulting in an extended shelf life (Tingwa and Young, 1974; Eaks, 1985; Yuen *et al*., 1994).

Fruit quality is also thought to be affected by fruit calcium concentrations, with higher concentrations resulting in fewer disorders in many avocado cultivars (Chaplin and Scott, 1980; Eaks, 1985; Cutting *et al*., 1992; Hofman *et al*., 2002). The fact that mesocarp discolouration appears first in the distal end of fruit has also been correlated with lower fruit mesocarp calcium concentrations in this part of the fruit (Chaplin and Scott, 1980). The development of mesocarp discolouration, resulting from increased PPO activity, is also thought to be the end result of a calcium deficiency (Kirkby and Pilbeam, 1984). Calcium applications were found to reduce PPO activity and total phenol content in avocado fruit (Van Rensburg and Engelbrecht, 1985), which are related to browning. However, not all studies

regarding postharvest calcium applications have proved to be successful in reducing internal disorder severity (Penter *et al*., 2001). Snijder *et al*. (2002) evaluated the effect of ensuring sufficient calcium concentrations during specific periods of fruit growth and found that a calcium content of between 1000 and 1200 ppm during November (in South Africa) reduced the potential for mesocarp discolouration development.

1.2.4.1.1 Mode of action

Calcium plays an important role in cell division and cell development, especially in root tips, leaves, and fruit (Marschner, 1995). Calcium is thought to play a significant role in membrane stabilisation (Battey, 1990) and in cell wall structure (Ferguson, 1984). It appears that calcium affects membrane permeability by virtue of its intermolecular bridging of phosphate head groups in membranes (Ferguson and Drobak, 1988; Nilsen and Orcutt, 1996). Calcium-deficient tissues are, therefore, less resistant to electrolyte leakage (Simon, 1978) resulting in the leakage of phenols from the vacuole into other cellular compartments where they undergo oxidation (Van Rensburg and Engelbrecht, 1985). Calcium also plays an important role in cell walls, and is a normal constituent of the middle lamella (Conway *et al*., 1992). Calcium ions bind to the pectins in the middle lamella of the cell wall forming cross linkages that appear to provide stability and mechanical strength (Bangerth, 1979; DeMarty *et al*., 1984; Burns and Pressey, 1987). The extensive cross-linking not only facilitates packing of pectic polymers in the middle lamella, but also reduces the accessibility of enzymes that contribute to the breakdown of cell walls and softening (Glenn and Poovaiah, 1990; Conway *et al.*, 1992).

Calcium deficiency disorders are believed to be due to the inefficient distribution of calcium rather than poor calcium uptake (Thorp *et al*., 1997), and are restricted to organs and tissues that have low transpiration rates. Most of the calcium taken up by the roots is transported by mass flow in the xylem. Polar transport of indoleacetic acid (IAA) from an organ is also thought to affect calcium transport (Bangerth, 1979). Physiologically active organs such as developing shoots and fruits show greater IAA export and therefore increased calcium accumulation. However, because of their greater physiological activity and transpiration rate, leaves and shoots are stronger sinks for calcium than fruit (Witney *et al*., 1990). Fruit borne on vigorously growing trees will, therefore, have a lower calcium content, especially during the early stages of fruit growth.

1.2.4.2 Nitrogen

Typically, crops that contain high levels of nitrogen have poorer keeping qualities than the same variety of crop with lower levels (Thompson, 1996). High nitrogen applications have been found to result in faster ripening and more internal disorders in 'Hass' avocado fruit (Arpaia *et al*., 1996), and in some pome and stone fruits (Link, 1980). Similarly Kremer-Köhne *et al*. (1993) found that the percentage of avocado fruit free of physiological disorders was considerably decreased by nitrogen applications. High fruit nitrogen concentrations were also found to increase the severity of mesocarp discolouration in 'Fuerte' fruit after cold storage (Koen *et al*., 1990). In fact in South Africa it was suggested that fruit nitrogen content, especially for 'Pinkerton', be less that 1% by March to reduce mesocarp discolouration development (Snijder *et al*., 2002); and less that 1 % by January to reduce external chilling/black cold injury. Excessive and deficient nitrogen concentrations have also been found to affect chilling injury (Bramlage, 1982). Thus, nitrogen management is important as the type, rate and timing of fertilizer applications have been found to significantly affect fruit yield and quality (Lovatt, 2001).

1.2.4.2.1 Mode of action

Nitrogen is a key component of chlorophyll and is required for the synthesis of plant hormones, which control plant growth. Nitrogen is considered to be very mobile within the plant (Marschner, 1995), and its status often reflects the vigour of the plant. Nitrogen absorption during early fruit development tends to stimulate shoot growth, which acts as a preferential sink for calcium (as well as water and other metabolites) and further reduces calcium transport into fruit (Witney *et al*., 1990). Adding nitrogen to trees also tends to increase fruit nitrogen significantly (Arpaia *et al*., 1996) and creates a high N/Ca ratio, which has been associated with the development of certain internal disorders in apple (Ferguson and Watkins, 1989) and pear (Curtis *et al*., 1990).

Superficial scald in apples (Emonger *et al*., 1994) and translucence in pineapple has been related to high nitrogen (Soler, 1994; Paull and Reyes, 1996). It is also more common in large fruit, which suggests that the disorder is related to fruit growth rates, and water and carbohydrate supply (Ferguson *et al*., 1999). Higher nitrogen concentrations in plant tissues may result in increased ABA production (Nilsen and Orcutt, 1996), weaker cell walls and the

activity of key enzymes involved in phenol metabolism may be reduced, thus decreasing host resistance to fungal attack (Matsuyama and Dimond, 1973).

1.2.4.3 *Boron*

In South Africa boron has been found to be the most common nutrient deficiency in avocado trees (Whiley *et al*., 1996), with soil applications being reported to improve fruit quality (Smith *et al*., 1997). Furthermore, boron has a major effect on avocado fruit shape, as its deficiency causes uneven cell division within the first six weeks of fruit growth (Whiley *et al*., 1996). The exact function of boron is, however, still uncertain and renders it one of the least understood of all plant nutrients (Hu and Brown, 1994; Marschner, 1995). It has, however, been found to be an essential micronutrient in the normal development of root and shoot tips, flowers and fruit, being directly involved in cell division and cell growth (Whiley *et al*., 1996). It has also been proposed that boron plays key roles in cell wall structure and metabolism, plasma membrane integrity, and in phenol metabolism and lignin biosynthesis, which are important processes in plant defence mechanisms against physiological disorders and pathogenic infections (Marschner, 1995; Matoh, 1997).

Boron deficient plants have been found to contain higher calcium, nitrogen, magnesium and iron than boron fed plants (Lal and Subba Rao, 1954). In fact, boron has been closely linked to calcium in plant nutrition (Shear, 1975), probably due to its effect on diffusible auxin (IAA) (Marschner, 1995). In apples boron can enhance calcium transport in apple trees, and help maintain more plant calcium in soluble forms, which may enhance calcium absorption (Faust and Shear, 1968). It is considered to play some part in the regulation of water relations of plasma colloids and helps carbohydrate transformation and utilization (Lal and Subba Rao, 1954).

Boron deficiency initially tends to increase respiration, however a decline is noted as deficiency symptoms become evident (Shelp, 1993). Phenol accumulation, resulting in browning reactions, has also been observed in plants growing in conditions of long-term boron deficiency (Shkolnik, 1984). Marschner (1995) states that under boron deficiency phenols accumulate and polyphenol oxidase activity is increased which leads to highly reactive intermediates such as caffeic quinone in the cell walls. These quinines, as well as light activated phenols, are very effective in producing superoxide radicals potentially capable

of damaging membranes by lipid peroxidation. Where boron is not limiting, it forms complexes with many phenolic compounds thereby reducing phenolic concentration (Cakmak *et al*., 1995).

1.2.4.4 Magnesium

Magnesium is an essential component of chlorophyll, regulates the absorption of other plant nutrients, and is essential for many cellular biochemical functions (Marschner, 1995). Excessive magnesium concentrations in the soil have been found to suppress calcium absorption by roots (Himelrick and McDuffie, 1983). However, an adequate supply of magnesium is important, as the mobility of calcium in the xylem is improved by the presence of divalent cations, such as magnesium, which are also adsorbed onto exchange sites (Himelrick and McDuffie, 1983). A study done in avocados showed that the magnesium concentration decreases as fruit increase in relative maturity (Cutting *et al*., 1992).

1.2.4.5 Potassium

Potassium has several important roles in plants, such as the regulation of water balance through controlling the opening and closing of stomata on leaves and the synthesis and movements of starches, sugars, and oils, which may have a direct affect on fruit quality. Potassium also has a prominent effect on the conformation of enzymes and thus regulates the activities of a large number of enzymes (Marschner, 1995). Both high and low levels of potassium have been associated with abnormal metabolism (Wills *et al*., 1989). It is a relatively mobile mineral in the plant (Terblanche, 1972) and its concentration has been shown to have a distinct fluctuating trend with increasing maturity in avocados (Cutting *et al*., 1992). Like magnesium, very high soil levels of potassium may suppress calcium absorption by roots (Himelrick and McDuffie, 1983). In plants potassium may increase phloem transport, which can further depress the calcium status of fruit because the ratio of xylem to phloem transport into fruit is reduced (Bangerth, 1979).

In avocado, the balance of fruit magnesium, potassium and calcium concentrations has proven to be important in internal disorder severity. Strong negative correlations were found between mesocarp discolouration severity in ‘Hass’ fruit, and mesocarp calcium, magnesium and (Ca+Mg)/K ratio (Hofman *et al*., 2002). Cutting and Bower (1990) also indicated that

high magnesium levels and a high (Ca+Mg)/K ratio were positively related to a high PPO activity, which is involved in the browning reaction. Marschner (1995) also stated that potassium deficient plants exhibit a much higher activity of oxidases such as polyphenol oxidase than do tissues of normal plants. Koen *et al*. (1990) found a positive relationship between proximal fruit potassium concentrations and mesocarp discolouration, but a strong negative correlation between leaf and soil (Ca+Mg)/K and grey pulp (mesocarp discolouration). Du Plessis and Koen (1992) also found that the incidence of mesocarp discolouration was strongly correlated with the subsoil (Ca+Mg)/K ratio. They also reported a significant reduction in the incidence of pulp spot with high levels of subsoil potassium, which, conversely, was found to aggravate mesocarp discolouration. In New Zealand, however, no relationship was found between leaf, soil and fruit mineral concentrations (Thorp *et al*., 1997).

1.2.4.6 *Zinc*

Zinc is taken up predominantly as a divalent cation. High concentrations of other divalent cations, such as calcium, inhibit zinc uptake to a certain extent (Marschner, 1995). Zinc acts either as a metal component of enzymes or as a functional, structural, or regulatory cofactor of a large number of enzymes. Zinc also plays an essential role in the production of certain plant hormones, such as auxins, although the mode of action is still obscure. Zinc-deficient plants have been found to exhibit lower levels of indoleacetic acid and ABA (Nilsen and Orcutt, 1996). Zinc also has a regulatory role in the absorption of water, and is necessary for normal chlorophyll production (Marschner, 1995). Zinc plays a part in cellular oxidation and the fundamental processes involved in cellular metabolism and respiration. Zinc is also considered to act as a catalytic agent on some essential reactions bringing about inhibition of carbohydrate transformation. It is also a component of a catalytic system necessary for the phosphorylation of glucose (Lal and Subba Rao, 1954). Deficient plants contain more reducing sugar but less sucrose and starch than healthy plants. It is not very mobile either in the soil or in the tree and it tends to accumulate in roots (Marschner, 1995).

Zinc is thought to have a synergistic effect on the entry and accumulation of calcium in plant tissues (Testoni and Pizzocaro, 1980). Zinc applications to apples, were found to increase fruit calcium concentrations and reduced the severity of the internal disorder bitter pit (Shear, 1980). The higher zinc concentrations were thought to result in more bound calcium being

released from various chelating and complexing agents (such as lignin, organic acids, and proteins) for transport to the fruit.

Zinc and copper are both important components of superoxide dismutase (SOD). Under zinc deficiency the level of toxic oxygen species is high because of both depressed SOD activity and lower export rates of carbohydrates as a result of low sink activity (Marschner, 1995). This results in the peroxidation of membrane lipids and an increase in membrane permeability. Chvapil (1973) reported that zinc has the greatest affinity for membranes followed by copper, iron and calcium and found considerable evidence that zinc may increase the stability of membranes. Shear (1980) speculated that increases in zinc status in fruit may increase fruit quality.

Vorster and Bezuidenhout (1988) found that 'Fuerte' fruit with poor internal quality had significantly lower fruit zinc and calcium concentrations than unaffected fruit. Furthermore, high leaf zinc concentrations were associated with less pulp spot in fruit after cold storage (Bezuidenhout and Vorster, 1991). Zinc has been found to have a direct role on avocado fruit size and shape, with fruit from zinc deficient trees tending to be smaller and rounder than fruit from trees of the same cultivar with adequate zinc concentrations (Kadman and Cohen, 1977; Crowley *et al.*, 1996).

1.2.4.7 *Phosphorus*

Phosphorus is important for energy metabolism in maintenance and overall fruit growth, and can also be involved in membrane stability and carbon partitioning (Marschner, 1995). Phosphorus also controls some key enzyme reactions. It is incorporated in membrane lipids and nucleic acids and affects the photosynthetic efficiency of chlorophyll molecules. Studies have found that high concentrations of phosphorus in cells, specifically chloroplasts, can lead to the inhibition of starch synthesis. Low phosphorus concentrations have also been associated with chilling injury in certain fruit (Bramlage, 1982).

1.2.4.8 *Copper*

Copper is known to activate a group of oxidising enzymes – polyphenol oxidase, monophenol oxidase, laccase and systems of oxidising ascorbic acid. It acts as a catalyst for the enzymic

systems which lead to enzymic browning, the browning of cut or damaged tissues that are exposed to air. In copper deficient leaves the activity of polyphenol oxidase has been found to be almost absent (Marschner, 1995). Copper is also an important constituent of tyrosinase. Tyrosinase plays a part as a terminal oxidase in respiration of plants. Absence of copper also reduces apparent photosynthesis (Lal and Subba Rao, 1954).

1.2.4.9 *Iron*

All known enzymatic systems depending on iron involve porphyrin molecules. Enzymes like polyphenol oxidase and succinic dehydrogenase have also been known to be interrelated in effect with iron (Lal and Subba Rao, 1954). Injections of iron have been found to induce symptoms similar to low temperature breakdown and superficial scald in apple, but whether it has a direct role is still uncertain (Wills *et al*., 1989). Certain respiratory enzymes required for salt accumulation are mediated by iron respiratory enzymes. The role of iron in chlorophyll production and its relation to other metals is of great significance (Lal and Subba Rao, 1954). It is suggested that potassium may displace iron from the enzymes involved in chlorophyll formation.

1.2.4.10 *Manganese*

Bezuidenhout and Vorster (1991) previously suspected that manganese could play a role in fruit quality, although the exact mechanism by which it did was uncertain. Manganese plays an important role in chlorophyll formation (Marschner, 1995). Generally zinc, copper, iron and manganese are all important components of detoxifying enzymes, being directly involved in the photosynthetic electron transport chain. The photolysis of water is mediated by a manganese-containing enzyme attached to photosystem II (Marschner, 1995). In this water-splitting system manganese clusters act as a device for storing energy prior to the oxidation of the two water molecules. Manganese also presumably acts as the binding site for the water molecules that are oxidised. Furthermore, manganese is thought to play an important role in sugar formation and sugar metabolism (Lal and Subba Rao, 1954). It is involved in respiration in that small doses increase oxygen intake. It is also suggested to catalyse aerobic respiration (Lal and Subba Rao, 1954).

1.2.5 Plant growth regulators

Ismail and Grierson (1977) reported that growth regulators applied preharvest, as well as postharvest, can alter the susceptibility of grapefruit to chilling injury. All the classical plant growth regulators (auxin, gibberellins, cytokinins, abscisic acid (ABA), and ethylene) have been shown to influence biological responses through changes in membrane characteristics (Nilsen and Orcutt, 1996), generally by increasing membrane permeability.

1.2.5.1 Abscisic acid

Abscisic acid (ABA) has been found to help in the prevention of chilling injury in cucumber seedlings and cotton plants (Rikin and Richmond, 1976; Rikin *et al*., 1979), grapefruit (Kawada *et al*., 1979) and zucchini squash (Wang, 1991). ABA may protect plants against chilling injury through its action as an anti-transpirant agent (Christiansen and Ashworth, 1978) and membrane stabilizer (Markhart, 1986). ABA was also found to induce protein synthesis, which may be associated with increased chilling tolerance (Xin and Li, 1991). Cold, drought and salinity stress can all cause a reduction in tissue water content. Many studies have shown an endogenous increase in ABA during cold stress. Furthermore, a cause-and-effect relationship between cold tolerance and endogenous ABA has been established by Heino *et al*., (1990). Therefore, it is likely that desiccation stress caused by cold shock or water stress influences the activity of ABA, which regulates genes that code for stress-induced proteins. Terpenoid analogues of ABA have been shown to retard chilling-induced electrolyte leakage and phospholipid loss and to reduce chilling injury in cucumber seedlings (Flores *et al*., 1988). Stressful temperatures can alter the internal water status and thereby ABA production (Walton, 1980), or they can directly affect ABA levels. It is, thus, sometimes difficult to separate the direct effects of temperature on ABA levels from the effects of temperature on plant water relations and subsequent ABA levels. In avocados, Cutting *et al*. (1988) showed that high levels of ABA significantly increased PPO activity, reduced internal fruit quality and reduced time to ripeness.

1.2.5.2 Ethylene

Ethylene had been described as a ripening hormone. It can thus be envisaged that fruits that increase their resistance to chilling injury with ripening would benefit from prestorage ethylene

treatment, for example tomatoes and papayas. On the contrary, chilling injury in some fruit becomes more severe after exposure to ethylene. Advancing the ripeness of avocados with ethylene increases anthracnose infection and chilling injury, and reduces shelf life (Chaplin *et al*., 1983). Avocado fruit stored in an atmosphere containing high ethylene levels also showed more chilling injury than those kept in air at the same temperatures (Chaplin *et al*., 1983; Lee and Young, 1984). Similarly "wild-type" melons develop more chilling injury when exposed to ethylene during cold storage than do "antisense" 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase melons in which ethylene production is greatly suppressed (Jones *et al*., 2001). Ethylene has been shown to increase the rate of respiration, alter the cellular compartmentalization, and alter auxin transport and/or metabolism (Pratt and Goeschl, 1969).

Biale (1941) was unable to detect a climacteric rise in 'Fuerte' fruit stored at 4-5^{o}C for 5 weeks. In 'Pinkerton' fruit, no ethylene was detected during a cold storage period of 16 days (Fuchs *et al*., 1986). In 'Hass' and 'Fuerte' fruit, chilling sensitivity was found to be dependant on the stage of the ethylene climacteric, with fruit at the climacteric rise being less sensitive than those at the climacteric peak. It was thought that the periods of sensitivity were correlated with periods of high metabolic activity. Kosiyachinda and Young (1976) proposed that a change in the activity of regulatory enzymes during low temperature storage could lead to the accumulation of intermediates to levels that become toxic to cells.

1.3 PREVENTION OF CHILLING INJURY

One of the main goals of research on chilling injury in horticultural commodities is to find effective methods to reduce the injury induced by chilling. Chilling stress is usually limited to plants native to or growing in tropical or subtropical regions of the world. The temperature range for chilling stress in such plants ranges from just above freezing to 15 to 20^{o}C in some chilling-sensitive plants. Generally, plants are more sensitive to chilling under non-dormant conditions (high metabolic activity), during younger stages of development, under drought stress, and when nutrients are limiting. Thus, fresh produce sensitive to chilling cannot receive the full advantage of cold storage but deteriorates rapidly if not refrigerated. If the tolerance to chilling in these sensitive tissues can be increased, or if the development of chilling injury can be delayed, then it would be feasible to store these commodities at lower temperatures to reduce the rate of deterioration. Furthermore, cold treatment is an approved quarantine treatment. During the past 60 years numerous techniques have been used to

lessen injury during the postharvest period, with results varying between different crops and different times in the season. Thus the search for new techniques continues.

1.3.1 Methods used to prevent external chilling injury

To a horticulturist, perhaps the most important aspect of studying chilling injury is to develop a means of alleviating it during both the preharvest and postharvest periods. Postharvest techniques which have been shown to alleviate low temperature injury include temperature preconditioning, heat treatment, intermittent warming, controlled atmosphere storage, treatments with calcium or other chemicals, waxing, film packaging, genetic modification, and applications with ethylene, abscisic acid, methyl jasmonate, polyamines, or other natural compounds

1.3.1.1 Temperature pre-conditioning

Prestorage temperature significantly affects the susceptibility of commodities to chilling injury (Saltveit, 1991). Low temperature conditioning involves holding cold-sensitive tissue at temperatures just above those at which injury occurs before placing them in storage to induce tolerance to these normally damaging low temperatures and delay the development of injury symptoms (Wheaton and Morris, 1967; Hatton, 1990; Woolf *et al*., 2003). This form of conditioning has been very successful in a wide range of fruits and vegetables (Wang, 1993). In zucchini squash temperature preconditioning treatment at 15^{o}C for 2 days before storage at 5^{o}C was effective in reducing chilling injury, not only in delaying the onset of chilling injury but also in reducing the rate of the development of injury symptoms (Wang *et al*., 1992). Exposure of papaya fruit for 4 days at 12.5^{o}C prior to storage at 2^{o}C reduced chilling damage (Chen and Paull, 1986). Hatton and Cubbedge (1982) found that preconditioning of grapefruit at 10^{o}C or 15^{o}C for 7 days reduced chilling injury at 0^{o}C or 1^{o}C. Temperature conditioning is also effective in alleviating chilling injury in other citrus fruits (Houck *et al*., 1990; Spalding and Reeder, 1983). In nectarines preconditioning fruit for 2 days at 20^{o}C, before storage at 0^{o}C, also substantially reduced chilling injury (Zhou *et al*., 2000). The most effective conditioning period for mature green bell peppers was 5 days at 10^{o}C before storage at 1^{o}C (Risse and Chun, 1987).

1.3.1.1.1 Physiological changes induced by temperature conditioning

Temperature-conditioning treatments are thought to induce an adaptive response in fruits and vegetables to chilling stress. This adaptation to lower temperatures is the result of various physiological modifications induced by the conditioning treatment. Many biochemical and physiological alterations in chilling-sensitive plants have been associated with temperature conditioning or hardening treatments. For example, in cotton plants increases in sugar and starch, and decreases in RNA, protein, and lipid-soluble phosphate occur during exposure to 15^{o}C for 2 days, which also reduces leakage of metabolites and prevents subsequent chilling injury at 5^{o}C (Guinn, 1971). In citrus cold hardening was initially characterized by light-enhanced and cool temperature-induced accumulation of carbohydrates (Yelenosky, 1978). Chilling degrades lipids in cucumber fruit and tomato pericarp and temperature conditioning reduces the loss of lipids. A transition in the molecular ordering and fluidity of membrane lipids is thought to be the primary event causing chilling injury (Raison and Orr, 1990). The fluidity of the lipid bilayer is determined, to a large extent, by the fatty acid composition of the phospholipids. The flexibility of the membranes is associated with the relative proportion of saturated and unsaturated fatty acids in membrane glycerollipids. Temperature conditioning has been reported to increase the degree of unsaturation of fatty acids in phospholipids and to prevent chilling injury. The temperature conditioning also suppresses the increase of sterol/phospholipid ratio during chilling. This ratio is closely associated with membrane viscosity and permeability. It also affects the fluidity of membranes and, in turn, influences the capacity of tissue to withstand chilling stress. The decline in the ratio of unsaturated to saturated fatty acids may indicate an increase in lipid peroxidation during chilling (Wang *et al*., 1992), which is thought to contribute to the development of chilling injury.

Sugar content has been correlated with changes in chilling sensitivity (Purvis, 1990). It has also been shown that low temperature acclimation increases the levels of proline and reducing sugars in grapefruit (Purvis and Yelenosky, 1983a). The high concentration of proline in the peel of grapefruit was reported to enhance its resistance to chilling temperatures in storage (Purvis, 1981). Other changes that take place inside the plant tissues in response to low temperature hardening include reduced leakage of electrolytes, a decrease in ATP during subsequent chilling, increases in the phosphatidylcholine and phosphatidylethanolamine, increases in unsaturation of fatty acids and increases in the degree of unsaturation of fatty acids.

In nectarines the delayed storage treatment (2 days at 20^{o}C) was thought to prevent the occurrence of the physiological disorder 'wooliness' by allowing softening to continue during the 0^{o}C storage (Zhou *et al*., 2000). The 2 days delayed-storage fruit softened more during storage than control fruit. This difference in firmness was reflected by the differences in cell wall components of delayed-storage fruit compared to control fruit, and was thought to be reminiscent of changes found in fruit from intermittent warming.

1.3.1.1.2 Quarantine

A quarantine treatment must satisfy two conflicting goals: kill all quarantined insects present and prevent significant damage to the commodity, which, in the case of fresh produce, is also alive. The success of preconditioning, in alleviating chilling injury, has become very important to countries who need to meet certain quarantine regulations in order to export their fruit and who therefore have to subject their fruit to cold treatment. Cold treatment is an approved quarantine treatment for citrus grown in areas infested with a number or tropical fruit flies and involves storage of fruit below 2.2^{o}C for specified periods. Preconditioning grapefruit, which are susceptible to chilling injury during extended cold storage, has helped to maintain fruit quality while meeting quarantine requirements (Hatton and Cubbedge, 1983). In studies on 'Hass' avocado fruit low temperature conditioning, at 6^{o}C for 3 days, before cold disinfestation was found to effectively eliminate external chilling injury as well as improve internal mesocarp quality (Hofman *et al*., 2003).

1.3.1.2 Packaging and waxing

The use of semi-permeable films, to package fruit and vegetables, which are sensitive to chilling temperatures, is a simple and inexpensive method to increase CO_2 and lower O_2 concentrations and to maintain a high humidity in the atmosphere. Under these modified atmosphere conditions the respiration rate of the fruit is decreased and the ethylene climacteric rise is delayed (Meir *et al*., 1995). The reduction of water loss from the tissues under high humidity apparently inhibits the collapse of epidermal and underlying cells. These factors are believed to be responsible for the reduction of chilling injury by packaging films (Forney and Lipton, 1990; Wang, 1993). Studies using perforated bags have found that the O_2 and CO_2 concentrations changed very little from the ambient atmosphere (Wang and Qi,

1997). The value of high humidity in suppressing chilling injury was recognized as early as the 1930's (Morris and Platenius, 1938), with the development of chilling injury also being delayed by decreasing the vapour pressure deficit and thus reducing moisture loss from commodities (Wardowski *et al*., 1973). Film packaging has been reported to be successful in preventing or reducing chilling injury in many crops including avocados and various citrus cultivars (Wang, 1993). In grapefruit and lemons, stored at 5^{o}C and 2^{o}C respectively, high-density polyethylene film seal-packaging was found to create a water-saturated atmosphere around fruit and inhibit chilling injury (Ben-Yehoshua *et al*., 1981). In some commodities, the reduction of chilling injury by film packaging is attributed largely to the modification of the microenvironment within the package and to the alleviation of water stress (Ben-Yehoshua *et al*., 1983a). The modified atmosphere was found to extend ripening time, improve firmness, and maintain fruit quality (Ben-Yehoshua *et al*., 1983a; Wang, 1993). Studies of 'Fuerte' avocados packed in polyethylene bags, and stored at 5.5^{o}C for 33 days before ripening at 20^{o}C, showed that these fruit exhibited no chilling injury and had an extended shelf life, compared to control fruit, although this treatment resulted in an increase in anthracnose rot (Eksteen and Truter, 1985). This was attributed to the higher relative humidity within the bag and the longer period taken to ripen once removed from storage. Storage of 'Hass' avocados was also extended by packing individual fruit in sealed polyethylene bags, especially when an ethylene absorbent was included in the bag (Oudit and Scott, 1973). The use of micro-perforated polypropylene bags, with anti mist coatings, have been successful in reducing moisture stress in 'Pinkerton' avocados while modifying respiration rates. This, in combination with low temperature storage (2^{o}C), was found to significantly reduce external chilling injury, preserve internal quality and decrease the rate of softening, after storage (Bower and Jackson, 2003).

Waxing of fruits and vegetables restricts gas exchange and transpiration of the fresh produce, and thus has effects similar to film packaging. Waxing grapefruit was found to significantly decrease the incidence of chilling injury (Chalutz *et al*., 1985). Waxing has also been reported to reduce moisture loss, delay softening and improve appearance of 'Fuerte' avocados (Lunt *et al*., 1981). Unfortunately, in some cases waxing has been reported to lead to the increased incidence of mesocarp discolouration in avocados (Cutting *et al*., 1989). Ben-Yehoshua *et al*. (1987) found that film wrapping drastically reduced chilling injury (pitting) in citrus, compared to waxed fruit subjected to the same conditions. Similarly, Bower and Magwaza (2004) found that polypropylene packaging in 'Fuerte' avocados was more effective in reducing external

chilling injury than waxing. In 'Fuerte' avocados waxing was found to cause a slight build-up of CO_2 and a possible reduction in internal O_2 concentrations during the climacteric. Furthermore, waxing caused a one day delay in fruit softening under extended cold storage (Durand *et al.*, 1984). The variation in the effectiveness of waxing has been ascribed to differences in the composition of the actual wax formulation that in turn affect the properties of the coating (Amerante and Banks, 2001). In studies on mango and avocado it was found that coating type significantly affected the external damage of fruit (Bower *et al*., 2003).

1.3.1.2.1 Effects of individual seal-packaging on gas exchange of fruit

Ben-Yehoshua *et al*. (1983b) calculated that waxing (1 µm thickness) increases only slightly the resistance of fruit-to water vapour, but raises the resistance to CO_2, O_2 and ethylene by 140%, 250%, and 100%, respectively. Conversely, seal-packaging (10-20 µm) was found to raise the resistance to fruit-to water vapour by 1375%. However, resistance to CO_2, O_2 and ethylene was raised by only 72%, 233%, and 25%, respectively. Studies using a scanning electron microscope found that waxing plugged the stomatal pores of citrus either partially or completely, and this was suspected to restrict CO_2, O_2 and ethylene transport. Furthermore, a wax coating was thought to have a low resistance to water, because the new surface layer, which is formed, has many pits and breaks. The plastic film of high-density polyethylene was not selectively impermeable to water, but the film reduced water loss by 14-times without substantially inhibiting gas exchange, because, initially, the fruit has far less resistance to water than to CO_2, O_2 and ethylene.

1.4 PROLINE

1.4.1 Accumulation of proline

An increase in the concentration of the total free amino acids in plant tissues when the plant is exposed to low temperatures has been frequently recorded (Draper, 1972; Srivastava and Fowden, 1972; Chu *et al*., 1978). In plants, L-Proline (henceforth referred to as proline) is synthesized from either glutamate or ornithine (Sánchez *et al*., 2002). However, some studies indicate that most of the proline accumulated in the vegetative tissues of mature plants in response to stress is the result of enhanced synthesis from glutamate (Tayler, 1996; Roosens *et al.*, 1998). Proline accumulation, induced by stress conditions, is thought to be

mediated both by increased synthesis and reduced oxidation of the amino acid (Hare, 1998). Although proline may also be synthesised from ornithine (Roosens *et al*., 1998), a decrease in proline oxidation frequently accompanies prolonged stress (Madan *et al*., 1995), although this in itself is unlikely to account for the levels of proline often accumulated (Chiang and Dandekar, 1995).

In establishing why in comparison with other amino acids, proline metabolism appears to be extremely sensitive to adverse environmental conditions, it is useful to compare its metabolism with that of other amino acids. In comparison with most other amino acids, proline has the metabolic advantage of being the terminal product of a relatively short and highly regulated pathway. Proline accumulation therefore affects fewer metabolic reactions than the buildup of multi-use substrates such as glutamate, which are participants in many equilibrium reactions central to intermediary metabolism. Because of the secondary amines of the nitrogens, proline cannot participate in the transamination or decarboxylation reactions common to other amino acids (Phang, 1985).

Proline represents a unique class of molecule among the amino acids. With its peptide bond within the pyrrolidone ring, proline confers rigidity and three-dimensional stability to proteins (Phang, 1985). A specific system of enzymes with special properties has evolved to mediate the metabolism of proline. Another feature of proline as a result of its unusual metabolic feature is that it easily crosses cellular and organellar barriers (Abrahamson *et al*., 1983).

1.4.2 Role of proline accumulation

Proline has been found to accumulate in plants after they have been exposed to different stresses (Aspinall and Paleg, 1981). Much remains to be understood concerning the mechanisms whereby proline accumulates under stress. Proline accumulation is argued by some researchers to be advantageous to a plant as far as stress tolerance in concerned (Singh *et al*., 1973). Different roles have, however, been proposed for proline accumulation as an adaptive response; it has been suggested that proline may function as an osmoticum (Wyn Jones *et al*., 1977), a sink of energy and reducing power (Blum and Ebercon, 1976), a nitrogen-storage compound (Ahmad and Hellebust, 1988), a hydroxy-radical scavenger (Smirnoff and Cumbes, 1989), and a compatible solute that protects enzymes (Charest and Chon, 1990). It may also play a role in the regulation of cellular redox potentials. Conversely,

some researchers suggest the opposite to be true, that is, that the mere correlation between the accumulation of proline and the development of stress conditions does not provide sufficient evidence for any adaptive advantage with regards to stress (Kramer, 1983) and that proline accumulation is simply an indication of the damage suffered by the plant during stress conditions (Hanson *et al*., 1979). Blum and Ebercon (1976) suggested that the positive effect of proline accumulation under stress conditions is that it augments growth upon relief from stress, rather than serving any direct function during the period of exposure to stress.

The proline biosynthetic pathway from glutamate, although short, involves an extremely high rate of consumption of reductants. Furthermore, proline degradation is capable of high-energy output. The accumulation of proline appears to be an excellent means of storing energy since the oxidation of one molecule of proline can yield 30 ATP equivalents (Atkinson, 1977). These two features may have contributed substantially to a role for proline in plants as a resource of value either in the acclimation to stress or in recovery upon relief from stress. The benefit of possessing a metabolic system displaying extreme sensitivity to stress may derive more from its regulatory effects on apparently unrelated pathways than on accumulation of the end product itself (Hare, 1998).

Under stressful conditions proline synthesis may ameliorate the effects of the concomitant reduction of the pyridine nucleiotide pools, particularly the accumulation of excessive amounts of nicotinamide adenine dinucleotide hydrogen phosphate (NADPH). The oxidation of NADPH accompanying proline synthesis may assist in restoration of the terminal electron acceptor of the photosynthetic electron transport chain (Berry and Björkman, 1980). High levels of proline during stress play a role in the maintenance of the nicotinamide adenine dinucleotide phosphate ($NADP^+$) /NADPH ratio, as even a small increase in the biosynthesis has a large impact on the $NADP^+$ pool (Hare and Cress, 1997). This is the result of stomatal closure, which leads to the intercellular decrease of CO_2 as the leaf water stress increases. As the overall protein synthesis declines during drought stress (Van der Mescht and De Ronde, 1993), proline biosynthesis may substitute for protein synthesis in the turnover of ATP and the oxidation of $NADP^+$ (Hare, 1995).

The extensive accumulation of active oxygen species and their contribution to cell damage induced by stressful conditions such as temperature extremes and water deficit is well known. In order to deal with this effect, plants have evolved a number of protective, scavenging or

antioxidant defensive mechanisms. Apart from an enzymatic defensive system (Bowler *et al*., 1992), the accumulation of free-proline may also contribute to the scavenging of these active oxygen species by enhancing photochemical electron transport activities (Alia *et al*, 1991; Saradhi *et al*., 1995). It has even been suggested that the accumulation of proline might contribute to the detoxification of the active oxygen species (Floyd and Nagy, 1984).

Many plants accumulate organic osmolytes upon exposure to abiotic stresses that cause depletion of cellular water (drought and temperature extremes) (Aspinall and Paleg, 1981). Of the organic osmolytes, proline is the most widely distributed osmolyte (Tayler, 1996) and extensively studied (Hare, 1998). The proposed role of proline as an osmoregulator (Wyn Jones and Storeys, 1978) can be supported by the involvement of proline in the maintenance of membrane integrity as an adaptation to conditions of reduced water availability (Hare, 1995). The molecules accumulated in the cells during an osmotic stress prevent damage from cellular dehydration by balancing the osmotic strength of the cytoplasm with that of the environment. Proline can affect the solubility of various proteins due to its interaction with hydrophobic residues on the protein surface (Schobert and Tschesche, 1978). The increase in the total hydrophilic area of the protein stabilises it by increasing its solubility in an environment with low water availability (De Ronde, 2000).

1.4.3 Examples and conditions of proline accumulation

Proline accumulation in plants subjected to low temperatures and water stress appears to be a universal phenomenon. The free proline content of the leaves of many species increases with a decrease in leaf water potential (Singh *et al*., 1973). The accumulation induced by low temperature, however, is not the result of a concomitant decrease in leaf water potential (Chu *et al*., 1974) and appears to be a more direct response to the decrease in temperature. In citrus the accumulation of free proline is one of the features of water stressed-induced cold hardening (Yelenosky, 1979). A linear relationship was found to exist between free proline concentration and xylem pressure potential in lemon trees (Levy, 1980). Proline accumulation was also found to enhance drought tolerance, and recovery, in sorghum (De Ronde, 2000).

In barley proline accumulation at low temperature was shown to be light-dependent (Chu *et al*., 1978). A critical temperature was found to exist at which proline accumulation did not occur and it was suggested that the accumulation of proline was a consequence of a specific

metabolic event rather than the result of a continuous spectrum of temperature-affected changes in the total amino acid pool. Proline accumulation in response to water stress was not found to be light-dependent.

Accumulation of sugars and proline in citrus tissues is associated not only with cold hardening of trees, but also with the midseason resistance of grapefruit to chilling injury (Purvis, 1981; Purvis and Grierson, 1982). Grapefruit which have accumulated relatively high concentrations of carbohydrates and proline are less likely to be injured by low, non-freezing temperatures (Purvis, 1981; Purvis and Grierson, 1982). Increases in soluble carbohydrates and proline may be more a consequence of low temperature stress conditions rather than a direct factor in tissue hardening; but, sugars and proline levels, nevertheless, do correlate well with the chilling resistance of grapefruit as well as with cold hardiness of other citrus tissues (Purvis, 1981; Purvis and Grierson, 1982). Grapefruit harvested during midseason are generally found to be more resistant to chilling injury than fruit harvested either earlier or later in the season (Purvis *et al*., 1979). In addition, unexposed interior canopy fruit are found to be more resistant to chilling injury than exposed exterior canopy fruit (Purvis, 1980). While midseason chilling injury resistance has been related to a high level of reducing sugars, which accumulate in the peel of grapefruit at low orchard temperatures, no differences were observed between sugar levels in the peels of unexposed interior canopy and exposed exterior canopy fruit (Purvis, 1980). Proline contents were, however, found to be higher in interior canopy fruit peels, than exterior canopy fruit, and this correlated well with chilling injury resistance (Purvis, 1981). The accumulation of proline in citrus fruit tissues during stress is thought to result from a reduction in the utilization or oxidation of proline (Stewart and Hanson, 1980), which is translocated into them. Low temperatures may result in decreased proline oxidase activity in mitochondria of citrus fruit tissues (Purvis and Yelenosky, 1983b).

1.5 CHLOROPHYLL FLUORESCENCE

Chlorophyll fluorescence technology has been used to assess the responses of plants to a diverse range of stresses. More recently the application of chlorophyll fluorescence has been extended to studying the response of fruits and vegetables to postharvest stresses (Tijskens *et al*., 1994; Woolf and Laing, 1996). DeEll *et al*. (1999) also reported that chlorophyll fluorescence might be useful in evaluating ripening and senescence of fruits. Furthermore,

chlorophyll fluorescence is a non-invasive and non-destructive measurement that can be performed fairly quickly and it is reported to detect cellular damage before the development of visible symptoms.

Chlorophyll fluorescence techniques are based on the theory that light energy is absorbed by chlorophyll molecules within plant tissue and is used to drive photosynthesis. Energy surplus to that utilised in photosynthesis is dissipated as fluorescence or heat. The fluorescence of green plants is almost exclusively emitted by chlorophyll *a* (DeEll *et al*., 1999). At ambient temperatures the vast majority of emitted fluorescence is derived from processes occurring in Photosystem II (PSII). When healthy plant tissue is suddenly illuminated after a period in darkness, a time-dependent fluorescence induction (Kautsky effect) is observed, the amplitude of which is proportional to the incident light level.

It has been found that a parameter derived from chlorophyll fluorescence, the ratio of variable/maximum fluorescence (Fv/Fm), can be used as a quantitative measure for the basic functioning of photosynthetic electron transport. When Fv/Fm is measured in dark-adapted tissue it is considered to be an indicator of the integrity of the reaction centre and light-harvesting complex of PSII, and thus reflects membrane damage or membrane alterations (DeEll *et al*., 1999). Lower temperatures can enhance membrane leakage and studies revealed that it appeared to be well correlated with Fv/Fm. DeEll *et al*. (1999) suspected that cold storage induced changes in the thylakoid membranes of plant tissues, result in a decreased exciton transfer efficiency of PSII, which seemed to be temperature dependent.

A few factors have to be considered, however, when interpreting chlorophyll fluorescence data. For example, the quantum yield is both time and temperature dependent. Thus, during ripening there may be a loss of photosynthetic competence per unit chlorophyll, leading to reduced PSII activity. In addition, there may be a decrease in chlorophyll content, which will affect all fluorescence measurements (DeEll *et al*., 1999). Light, temperature and nitrogen content in the fruit (Kingston, 1991) would also affect chlorophyll content and subsequently fluorescence measurements. Furthermore, it is reported that measurements may be affected by large variations among cultivars and even between fruit of the same cultivar, and thus would be influenced by sample size and ranges of fruit firmness and fluorescence (DeEll *et al*., 1999).

1.6 CONCLUSION

A review of the literature indicates that there are many pre- and postharvest factors that can contribute to the development of mesocarp discolouration and external chilling injury in fruit. Furthermore, these factors may or may not be related and may affect the physiology of the fruit in different ways. The fact that the severities of these disorders have not been reduced to acceptable levels, despite the volume of work that has already been conducted, is a reflection of the complexity of these problems. It is hoped that a further understanding of how the various factors may contribute to the disorders will aid future research, as well as provide acceptable protocols for shipping and storage, which will decrease the risk of disorders causing losses in the market place.