Cold storage of *Leucospermum* cutflowers and *Leucadendron* greens

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously, in its entirety or in part, been submitted at any university for a degree.

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Date

SUMMARY

Quality of certain *Leucospermum* and *Leucadendron* cultivars after approximately 21 days shipping has been reported to be substandard due to 'drying out' of leaves and, in the case of Leucadendrons, involucral leaves. The nature of the symptoms of this 'drying out' and the conditions under which they form, viz. long exposures to low temperatures, has led us to hypothesize that these are symptoms of chilling injury (CI). Chilling injury, as far as we are aware, has not been documented on Leucospermums or Leucadendrons.

Typical CI symptomology is discussed and shown for *Leucospermum* 'Gold Dust', 'High Gold' and 'Succession' and for *Leucadendron* 'Chameleon', 'Laurel Yellow' and 'Safari Sunset'. The nature of CI symptoms for Leucospermums and Leucadendrons was generally membranous breakdown that manifested in some cases as a 'water soaked' appearance which, at a more advanced stage, was generally visible as 'dried out' patches on the leaves. In the case of the Leucadendrons CI was also visible on the immature involucral leaves which are more sensitive to chilling conditions than mature leaves. Dark discoloration of especially immature involucral leaves is also a symptom of CI. As water uptake of shoots with chilling injury is hindered the styles of the Leucospermums wilt. As can be expected, the lower the temperature below the threshold temperature and the longer the exposure the more severe the symptoms.

CI was recorded on cut flower shoots of *Leucospermum* 'Gold Dust', 'High Gold', 'Rigoletto', 'Succession' and 'Vlam' after 21 and 24 days storage at 1ºC. After 24 days storage the chilling injury was more severe than after 21 days storage in most cases. Each cultivar was pulsed with 5 ml per stem of a 2% (w/v) sugar solution of either lactulose, sucrose, glucose, fructose or mannose before storage. After storage, CI was recorded on day 0, 3, 7 and 10 of the vase phase. Of the cultivars tested 'Vlam' and especially 'Rigoletto' were more prone to chilling injury development. 'High Gold' and 'Vlam' shoots were pulsed with 0 (control), 1.5, 3 or 4% (w/v) solutions of either mannose or fructose. The best control of CI for both cultivars was achieved with 1.5%

(w/v) solution. Lower concentrations of mannose and fructose were tested on ‘High Gold’ shoots, with a 1% (w/v) solution giving the best control for both. At high concentrations signs of toxicity became evident directly after pulsing. ‘High Gold’ shoots were pulsed with 1% (w/v) solutions of mannose and fructose and sugar analyses were performed on shoots at different stages of storage and after 10 days in the vase. A slight increase in mannose and fructose was detectable in the stems of the shoots directly after pulsing but not in the leaves or the inflorescences. This is due to the low concentrations being used. The levels of all the carbohydrates decreased during the 21 days storage and more so during the vase phase of the flowering shoots. The fact that such low concentrations were effective in controlling chilling injury suggests that the sugars may have an effect other than on the osmotic potential.

Cut ‘flower’ shoots of *Leucadendron* ‘Chameleon’, ‘Laurel Yellow’ and ‘Safari Sunset’ were stored for 14, 21 and 28 days, at 1º, 3º and 5ºC and CI development recorded during the subsequent 10 day vase phase. ‘Laurel Yellow’ and ‘Safari Sunset’ showed signs of chilling injury on the leaves after 28 days storage at 3ºC or lower and ‘Safari Sunset’ stored for 21 days developed chilling injury during the vase phase. Immature involucral leaves were more sensitive to chilling injury than leaves. CI increased with longer exposure times and lower storage temperatures for all three cultivars evaluated. ‘Chameleon’ was the most chilling tolerant of the cultivars up to 21 days. At 5ºC chilling injury was low irrespective of cold storage duration but longer exposures to 1º and 3ºC resulted in increased chilling injury development during the vase phase. All three cultivars were pulsed with 5 ml per stem of a 1% (w/v) solution of lactulose, sucrose, glucose, fructose or mannose and stored for 14, 21 and 28 days at 1ºC. The sugars reduced chilling injury on the leaves for ‘Safari Sunset’ when stored for 28 days and, to a lesser extent, in ‘Chameleon’. The sugars failed to reduce chilling injury of the involucral leaves of ‘Chameleon’ and ‘Laurel Yellow’ whereas there was some control especially after 28 days for ‘Safari Sunset’. In some cases the sugar pulse exacerbated chilling injury. Chilling injury generally increased rapidly after storage during the first three days in the vase and then at a lower rate for the next seven days. *Leucadendron* ‘Chameleon’, ‘Laurel Yellow’ and ‘Safari Sunset’ ‘cut flower’ shoots were pulsed with a

1% (w/v) glucose solution. Expressed on a dry weight basis, an increase in glucose concentration was not detected. The reduction in chilling injury of leaves by a sugar pulse is speculated, as for the Leucospermums, to be as a result of their presence in the apoplast and not the symplast and that their presence there protects the membranes against chilling conditions in some way.

OPSOMMING

Die kwaliteit van sekere *Leucospermum* en *Leucadendron* kultivars na ongeveer 21 dae verskeping is waargeneem as substandaard as gevolg van die uitdroog van blare en, in die geval van *Leucadendrons*, die 'involucral' blare. Die aard van die simptome van hierdie uitdroging en die toestande waaronder dit plaasvind nl. lang periodes van blootstelling aan lae temperature, het ons tot die hipotese gebring dat hierdie simptome van koueskade is. Sover as wat ons bewus is, is koueskade nog nie gedokumenteer op *Leucospermums* of *Leucadendrons* nie.

Tipiese koueskade simptomologie word bespreek en gewys vir *Leucospermum* 'Gold Dust', 'High Gold' en 'Succession' en vir *Leucadendron* 'Chameleon', 'Laurel Yellow' en 'Safari Sunset'. Die koueskade simptome vir Leucospermums en Leucadendrons was oor die algemeen membraan afbraak wat 'n water deurdrenkte voorkoms tot gevolg gehad het wat in 'n meer gevorderde stadium sigbaar was as uitgedroogde kolle op die blare. In die geval van Leucadendrons was koueskade ook sigbaar op die onvolwasse 'involucral' blare wat meer sensitief is vir koue toestande as volwasse blare. Donker verkleuring van veral onvolwasse 'involucral' blare is ook 'n simptoom van koueskade. Aangesien wateropname van stele met koueskade verhinder word, verwelk die 'styles' van die *Leucospermums*. Soos verwag kan word hoe laer die temperature onder die drempel temperatuur en hoe langer die blootstelling, hoe meer ernstig die simptome.

Koueskade is aangeteken op gesnyde blomstele van *Leucospermum* 'Gold Dust', 'High Gold', 'Rigoletto', 'Succession' en 'Vlam' na 21 en 24 dae opberging by 1°C. Na 24 dae opberging was die koueskade meer ernstig as na 21 dae opberging in meeste gevalle. Elke kultivar het 5ml per steel van 'n 2% (g/v) suiker oplossing van laktolose, sucrose, glucose, fruktose of mannose voor opberging opgeneem. Na opberging is koueskade aangeteken op dag 0, 3, 7 en 10. Van die kultivars wat getoets is, was 'Vlam' en veral 'Rigoletto' meer geneig tot koueskade ontwikkeling. 'High Gold' en 'Vlam' stele is geplaas in oplossings van 0 (kontrole), 1.5, 3 of 4 % (g/v) oplossings van mannose of

fruktose. Die beste beheer van koueskade vir beide kultivars is deur die 1.5 (g/v) oplossing behaal. Laer konsentrasies van mannose en fruktose is getoets op ‘High Gold’ stele met ‘n 1% (g/v) mannose oplossing wat die beste beheer gegee het. Met hoë konsentrasies het tekens van toksisiteit sigbaar geword direk na opneem van die oplossing. ‘High Gold’ stele is geplaas in 1% (g/v) oplossings van mannose of fruktose en suiker analises is uitgevoer op stele by verskillende stadiums van opberging en na 10 dae in die vaas. ‘n Effense toename in mannose en fruktose is waargeneem in die stele van die blomme direk na opname van die oplossing, maar nie in die blare of die blomme nie. Dit is as gevolg van die lae konsentrasies wat gebruik is. Die vlakke van al die koolhidrate het afgeneem gedurende die 21 dae opberging en nog meer so gedurende die vaas periode van die blommende stele. Die feit dat sulke lae konsentrasies effektief is in die beheer van koueskade dui daarop dat die suikers ‘n effek het anders as op die osmotiese potensiaal.

Snyblomme van *Leucadendron* ‘Chameleon’, ‘Laurel Yellow’ en ‘Safari Sunset’ is opgeberg vir 14, 21 en 28 dae, by 1º, 3º en 5°C en koueskade ontwikkeling is aangeteken gedurende die opvolgende 10 dae vaas periode. ‘Laurel Yellow’ en ‘Safari Sunset’ het tekens gewys van koueskade op die blare na 28 dae opberging by 3°C of laer en ‘Safari Sunset’ opgeberg vir 21 dae het koueskade ontwikkel gedurende die vaas periode. Onvolwasse ‘involucral’ blare was meer sensitief vir koueskade as die blare. Koueskade het toegeneem met langer blootstellingstye en laer opbergins temperature vir al drie kultivars geëvalueer. ‘Chameleon’ was die mees koueverdraagsaam van die drie kultivars tot op 21 dae. By 5°C was laag ongeag van die koue opberging tydperk, maar langer blootstellings aan 1º en 3°C het gelei tot toename in koueskade ontwikkeling gedurende die vaas periode. Al drie kultivars is voorsien met 5ml per steel van ‘n 1% (g/v) oplossing van lactulose, sucrose, glucose, fruktose of mannose en opgeberg vir 14, 21 en 28 dae by 1°C. Die suikers het koueskade verminder op die blare van ‘Safari Sunset’ wanneer opgeberg vir 28 dae en, tot ‘n mindere mate, in ‘Chameleon’. Die suikers het egter nie koueskade verminder van die ‘involucral’ blare van ‘Chameleon’ en ‘Laurel Yellow’ nie, waar daar egter wel in ‘n mate beheer was veral na 28 dae vir ‘Safari Sunset’. In sommige gevalle het die voorsiening

van suiker die koueskade vererger. Koueskade het oor die algemeen vinnig toegeneem na opberging gedurende die eerste drie dae in die vaas en dan teen ‘n laer tempo vir die volgende sewe dae. *Leucadendron* ‘Chameleon’, ‘Laurel Yellow’ en ‘Safari Sunset’ snyblom stele is voorsien van ‘n 1% (g/v) glukose oplossing. Uitgedruk op ‘n droëmassa basis is ‘n toename in glukose konsentrasie nie waargeneem nie. Die afname in koueskade van blare deur die voorsiening van ‘n suiker oplossing is gespekuleer vir die *Leucospermums,* om ‘n resultaat te wees van hulle teenwoordigheid in die apoplas en nie die simplas nie, en dat die teenwoordigheid daar die membrane op ‘n manier beskerm teen koue toestande.

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1. Introduction

1. Introduction

Many *Proteaceae* species are exported as cut flower products to foreign markets and are popular worldwide due to their exotic nature. Progress in successful storage of various *Protea* cultivars has led to an increase in sea freight as opposed to air freight as the desired means of transport, which is much more cost effective and thus more profitable. Naturally, for similar reasons, it is desired that other genera of *Proteaceae* be shipped too e.g. Leucospermums and Leucadendrons.

It was observed that certain cultivars of cut 'flowers' of various fynbos species are negatively effected by shipping or storage conditions, those being long durations at low temperatures e.g. leaf desiccation of *Leucospermum cordifolium* after 21 days at 1ºC (Jones and Faragher, 1991). The nature of these symptoms e.g. membranous breakdown and the resulting appearance of 'dried out' patches or 'leaf desiccation' on the leaves, that develop on removal from storage and up to several days in the vase after storage conditions, suggests that the products may be undergoing a metabolic dysfunction as in chilling injury (CI) and not merely dehydration as previously thought. Since leaf desiccation does not occur when *Leucospermum* cut flowers are cold stored at 5°C (Jacobs, unpublished data) it appears that the desiccation may well be the result of CI.

During the primary response to chilling temperatures specific critical proteins and lipids may be affected (Steponkus, 1984) and during the secondary responses there is a loss of membrane integrity resulting in solute leakage (Kays and Paull, 2004; Campos et al, 2003). Lyons and Raison (1970) showed that membranes change from a supple liquid-crystalline phase to a solid gel phase at the temperature where CI occurred. Certain chilling sensitive plants or tissues have shown ability to harden against CI by being exposed to temperatures slightly above the threshold chilling temperature for a certain period of time before being subjected to chilling temperatures. It has been reported that in frost resistant plant cells there is an increase in the concentration of sugars during the winter which correlates with an increase in their 'hardening off' to the cold and frost (Levitt, 1978).

According to Coorts (1973) leaf sucrose and starch are used as substrates for respiration during storage and in the vase phase of the cut flower. During dry storage

and shipping of cut flowers, at low temperatures, dehydration occurs to a greater or lesser degree. Pulsing with sugar, increases the pool of non structural, metabolically active carbohydrates and may also maintain osmotic pressure (Halevy, 1976). A pre-storage pulse of cut *Leucadendron* 'Silvan Red' with sucrose protected the shoots during long term dry storage at 1ºC and improved subsequent vase life (Jones, 1991). These facts together suggest that sugar pulsing of cut 'flower' shoots may improve vase life and ability to withstand chilling conditions in some way.

The focus of this study was to determine whether certain cultivars of Leucospermums and Leucadendrons were, in fact, developing chilling injury, and to then evaluate the effectiveness of pulsing individual cut 'flower' shoots with sugars to try and control the chilling injury. Following on from this the ideal concentration of the most effective sugars was assessed in certain instances. Thereafter, sugar analyses were performed on pulsed and unpulsed shoots, and in the case of Leucospermums on pulsed shoots at different stages from directly after pulsing until the end of the vase phase, to try and understand sugar uptake and partitioning. It is hoped that the results might aid in improving postharvest handling and treatment of Leucospermums and Leucadendrons with the possibility of sea freight becoming a successful option for transport.

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2. Literature Review: Cold storage of cut flowers and greens with special reference to members of the family *Proteaceae*

Cold storage of cut flowers and greens with special reference to members of the family *Proteaceae*

2.1 Introduction

Various *Proteaceae* species are sold as cut flower products and are popular worldwide due to their exotic nature. South Africa, being in the Southern hemisphere, is ideally situated to provide the large European markets with fresh cut flowers during the Northern hemisphere winter, September to February, when supply from local markets is low. A major development in recent years is glucose pulsing of certain *Protea* products which significantly decreases leaf blackening and extends vase life (Stephens et al., 2001). This has resulted in a major increase in sea freight of Proteas as opposed to air-freighting which has been the main method of transport until recently. Shipping is beneficial as the transport costs are significantly lower. If tolerance of products to low temperatures for duration of transport is possible, it results in regulation of market supply to avoid a surplus of cut flowers on the market and extends availability of product, in the case of certain Leucospermums to Christmas when prices are higher. Certain cultivation practices of Leucospermums and Leucadendrons have enabled the delay of harvest, which has already resulted in extending the marketing period. An example of this is work done by Jacobs and Honeyborne (1978) where an understanding of the controlling factors of flower initiation led to the now common practice of disbudding or deheading of Leucospermums to delay flowering.

It is desirable that other genera of *Proteaceae* be shipped as opposed to air-freighted too e.g. Leucospermums and Leucadendrons. However, it has been observed that a large proportion of Leucadendrons and Leucospermums are negatively affected by shipping environments viz. long periods at low temperatures. Quality on arrival in Europe is often poor along with a decreased vase life. This, together with the nature of the symptoms e.g. ‘dried out’ patches that develop as a result of membranous breakdown, that can develop up to several days after removal from storage conditions, suggests that the products may be undergoing chilling injury (CI) and not just dehydration as previously thought.

This chapter will review published literature on; senescence of cut flowers, slowing the senescence process by various means focusing on temperature and sugar pulsing and some of the effects these have on storage of cut flowers including chilling injury.

2.2 Senescence of cut flowers

The vase life of cut flowers is limited but varies according to among other things, the species, stage of maturity and postharvest factors such as; food depletion, bacterial and fungal attack, wilting, bruising and crushing, temperature abuse, ethylene accumulation and poor quality water (Hardenburg et al., 1986). Generally, the petals have a shorter time between maturity to senescence and death than the leaves (Halevy and Mayak, 1979) and thus treatments are often aimed at improving petal and flower-head longevity. During petal senescence there are two main metabolic events which occur. The first is an increase in respiration and the second is hydrolysis of cellular components. The enzymatic changes which occur during petal senescence are mainly associated with these two events (Taiz and Zeiger, 2002).

Once a flowering shoot is harvested from the plant its water and carbohydrate supply are cut off and interrupted which lead to wilting and senescence. The most common causes of vase life termination of cut flowers are wilting and lack of full opening of flowers. This is not normal senescence, instead it is a sign of that the water uptake and water potential of the flower have decreased and contributes significantly to water imbalance. Bacteria in the vase solution contribute to the decline in water uptake but are not the only causes. Thus it is important to use an antibacterial holding solution to prevent bacterial growth and blockage of xylem vessels in the cut flowers (Halevy, 1976). Water potential can be improved by treating cut flowers with abscisic acid which induces stomatal closure, as transpiration continues mostly through the stomata, and by treating with cytokinins which promote water uptake (Halevy and Mayak, 1974a). Dry stored ‘Mercedes’ rose flowers (*Rosa hybrida* L.) had a longer vase life than when stored wet (Faragher et al., 1984) Thus, when dry cold storing cut flowers, by maintaining a high relative humidity transpiration is reduced, as is wilting (Hardenburg et al., 1986).

Cut flowers continue to respire at varying rates from harvest until senescence and death (Taiz and Zeiger, 2002). By pulsing with certain carbohydrates the substrates for respiration can largely be met (Nakahara et al., 1998). Carbohydrate application is used as standard practice in the cut flower industry and, in conjunction with maintaining the cold chain during handling, storage and transport, respiration is decreased and vase life extended (Hardenburg et al., 1986). As the flower senesces the membranes are negatively affected and eventually, with a combination of decreased water uptake and potential, a decreased respiration rate, and leaking solutes which results in the release of hydrolytic enzymes which eventually result in cell death, the flower wilts and brown discolouration on the leaves and petals may appear (Halevy and Mayak, 1979). Eventually vase life is terminated once the symptoms are visible to an unacceptable percentage.

If ethylene gas is present even in very small quantities, during handling, storage or transport, it can damage cut flowers by accelerating senescence. Typical symptoms include epinasty, leaf drop and yellowing, etc. Certain precautions to minimize ethylene damage can be utilized, e.g. ethylene scrubbers in conjunction with good air circulation during storage (Hardenburg et al., 1986; Taiz and Zeiger, 2002). Ethylene is an important postharvest factor with regard to vase life; however this chapter will not be focusing on ethylene.

2.2.1 Carbohydrates

Flower ontogeny has two distinct stages; firstly flower bud growth and development to full opening, and secondly maturation, wilting and senescence of the flowering bud (Halevy and Mayak, 1979). Sugars are the main substrate for respiration in flowers and are necessary for successful development from bud to senescing flower. Whether they are readily available is mainly dependent on the hydrolysis of polysaccharides, the rate of photosynthesis, translocation around the plant and respiration (Ho and Nichols, 1977). On the plant photosynthesis provides these carbohydrates but the rate of photosynthesis drops drastically for cut flowers due to, among other things, insufficient light levels. In certain instances if artificial light of the required intensity is applied to cut flowers post-harvest, there is an increase in photosynthesis and thus an increase in starch and sugars in the leaves e.g. *Protea eximia* (Bieleski et al., 1992). In cut flowers the free sugars are depleted which

initiates hydrolysis of sucrose, starch and eventually proteins to provide substrates for respiration. Coorts (1973) proved this hydrolysis of proteins by showing that protein breakdown is retarded by exogenously supplying sugars.

2.2.2 Membranes

During normal senescence cellular structures and macromolecules are broken down to be translocated from the senescing organ to actively growing regions which act as sinks (Taiz and Zeiger, 2002). Membrane breakdown could be expected as a manner in which to release these substances. During normal or forced aging or senescence there is a loss of membrane microviscosity or fluidity which reduces flexibility and thus leaks may occur. Membrane fluidity depends on various endogenous (e.g. fatty acyl desaturation, Ca^{2+}-cross linking and pH, etc.) and exogenous factors (e.g. salinity and temperature, etc.). This reduction in flexibility occurs as the membranes change from a liquid crystalline or fluid phase, to a gel or solid phase, as the phospholipid fatty tails in the lipid bilayer lose their freedom to move and become 'frozen'. Eventually, when the membranes become rigid and protein movement is halted the leaks start to occur resulting in membrane permeability and functions being impaired (Leshem, 1992). For example, when the tonoplast is disrupted and ion leakage occurs, the acidic contents of the vacuole mix with the normally neutral cytoplasm thus decreasing the pH of the cytoplasm. This decreases the efficiency of certain enzymes while allowing certain enzymes to operate more efficiently e.g. certain hydrolases or phospholipases (Borochov et al., 1978 cited by Marangoni et al., 1996) which eventually result in cell death (Halevy and Mayak, 1979).

2.3 Slowing senescence of cut flowers

There are many postharvest treatments that can be applied to retard the senescence process in cut flowers. Optimal handling procedures such as carbohydrate supplementation, antibacterial holding solutions, maintaining the cold chain and storage temperature manipulation, are examples of these. In this chapter we will concentrate mainly on carbohydrates and temperature.

2.3.1 Carbohydrate supplementation

A pre-storage pulse with exogenous sucrose has been shown to increase the longevity of cut flowers. Sucrose is used most often as the main ingredient in various pulsing solutions. Glucose and fructose are similarly effective (Halevy and Mayak, 1981; Halevy and Mayak, 1974b). Pulsing of cut flowers prior to shipment is a short-term treatment by the growers or exporters which refers to the uptake of chemical solutions through the stem, the effects of which should last for the whole shelf-life of the flowers when they are held in water (Halevy, 1976; Halevy and Mayak, 1974 a,b) This holds true if the flowers are shipped at the correct temperature. The sugar, among other things, replaces the depleted carbohydrates until completion of blooming, and maintains osmotic pressure (Halevy, 1976). Exogenously applied sucrose improves storage quality of cut flowers by replacing the carbohydrates that are depleted during storage at low temperatures (Goszcyńska and Rudnicki, 1988). By 'loading' stems of cut flowers with high concentrations of sugars and bactericides the flowers should have enough carbohydrates for their development and full opening.

Pulsed flowers generally take up more water after shipping than non-pulsed flowers. Floret cells were shown to absorb sugar which enabled better osmotic water uptake by the flowers (Halevy and Mayak, 1974b). Whitehead et al. (2003) suggested that sucrose's effect on the increased vase life of cut flowers was due more to its effect on metabolic processes as opposed to its effect as an osmoticum. The effects of sucrose on metabolism have been said to be its use as a substitute substrate for respiration (Nakahara et al. 1998), effect on amino acid metabolism (Eason et al., 2000), result in decreasing water loss during inflorescence senescence (Borohov et al., 1976), result in improved number of florets opening and increased vase life (Mor et al., 1984) and its effect on increasing the water content of petals of cut flowers and hindering membranous breakdown (Goszcyńska et al., 1990).

In many recorded instances ethylene has been shown to induce senescence. It is common practice to apply ethylene to accelerate ripening of fruit. During senescence of climacteric flowers there is a pre-climacteric rise in ethylene sensitivity and a climacteric rise in ethylene production during the later stages of senescence. Pulsing

of certain climacteric flowers at 22ºC with a 20% sucrose solution for 24 hours reduced ethylene sensitivity and increased longevity (Whitehead et al., 2003).

Cut flowers pulsed with high concentrations of sucrose (5-40%) accumulate the sucrose in the cells which increases the osmotic potential in the petals, allowing the flowers to absorb more water. The ability of the cut flower leaves to adjust osmotically varies with species and cultivars (Halevy, 1976). The petals can adjust osmotically more readily than green leaves and often the concentration of the pulsing solution has to be lower than optimal for the flower development and longevity as the leaves are generally more sensitive to higher concentrations than the flowers (Kofranek and Halevy, 1972; Halevy, 1976). The optimal concentration of a sugar pulse varies with the treatment and species or cultivar. Generally, the longer the exposure to the chemical solution, the lower the concentration required. Thus high concentrations are used for pulsing, intermediate concentrations for bud-opening and low concentrations for holding solutions (Halevy and Mayak, 1981).

Sucrose uptake at low concentrations is an active carrier mediated process whereas at higher concentrations the sucrose molecules enter the cells by diffusion down the concentration gradient (Taiz and Zeiger, 2002). Pulsing with too high a concentration of sucrose or at too high a temperature can cause damage to the flowers (Halevy and Mayak, 1981). In addition Halevy and Mayak (1979) showed that the primary site of excess sugar collection of exogenously applied sugars was the rose leaves, as the sugars were taken up and translocated in the same manner as naturally formed sugars, viz. leaves to petals. Thus, care must be taken when determining pulsing solution concentration so as not to have toxic effects on the leaves. In addition, if the concentration of the sugar is too high the volume of solution uptake decreases (Bravdo et al., 1974).

2.3.2 Temperature

Maintaining cut flowers at an optimal temperature during storage and handling is a fundamental postharvest factor. At increased temperatures, up to ±30ºC, the amounts of carbohydrates respired, which are the substrates for the respiration, increases (Rajapakse et al., 1994), along with increases in bacterial and fungal activity and acceleration in senescence. Moisture loss in cut flowers is directly related

to relative humidity's and temperature, thus temperature variations should be kept to a minimum (Halevy & Mayak, 1981). At low temperatures the respiration rate of the cut flower is decreased as the metabolic activities are slowed down as are bacterial and fungal activity (Taiz and Zeiger, 2002). This is obviously desirable for storage and handling. Storage temperature of cut flowers should be kept as low as possible (Halevy & Mayak, 1981). However, some cutflowers are chilling sensitive and are injured by storage at low temperatures, i.e. chilling injury.

2.4 Chilling injury

Chilling injury (CI) is described as a physiological injury to crops from tropical or subtropical origin (and some of generally temperate origins) due to exposure to temperatures above 0°C but below ±10°C, i.e. non freezing temperatures (Paull, 1990). This should not be confused with freezing injury as there is no ice-water phase transition. Once a product has experienced freezing, the rapid response once thawed suggests that the injury is not due to metabolic dysfunction as is the case with CI (Mazur, 1969; Mazur 1970).

Cut flowers are stored or shipped at low temperatures to retard senescence (Coorts, 1973). Temperature sensitive enzymes regulate respiration and their activity increases with an increase in temperature up to ± 30ºC. There is a significant negative linear relationship between respiration rate during storage and vase life after storage. This emphasizes the importance of maintaining temperatures as close to freezing point as possible during handling and transport without damaging the cut flowers, in order to optimize vase life (Çelikel and Reid, 2002).

2.4.1 Responses to chilling injury

The primary response to chilling temperatures is a direct stress and is generally considered to be physical i.e. changes in physical properties to cellular membrane composition and integrity, resulting in indirect injuries or dysfunctions. Two possible changes are suggested to occur as the primary response, viz. lipid changes and protein changes (Steponkus, 1984). Secondary responses are generally changes in physiological processes including increase in respiration rate, ethylene production rate change, decreases in protoplasmic streaming, increases in permeability and alteration of cellular structure e.g. loss of membrane integrity, leaking solutes, loss of

compartmentation and changes in enzyme activity (Lyons, 1973; summarised by Kays and Paull, 2004). For *Phalaenopsis* florets a symptom of chilling injury was early senescence. Increased ethylene production was noticed prior to floret senescence (Chaochia et al., 1999).

CI is a function of species sensitivity, temperature and duration of exposure (Paull, 1990). The plant or plant part sensitivity to CI varies with the species, cultivar, morphological and physiological condition at the time of exposure and stage of 'fruit ripening' (Lyons, 1973; Lyons et al., 1979; Sharples, 1980; Wang, 1982). In most fruits CI sensitivity increases to a maximum that coincides with the climacteric peak (Kosiyachinada and Young, 1976). CI susceptibility can be regulated in part by production conditions and mineral nutrition (Fidler et al., 1973).

CI damage may be cumulative in that a series of temperature abuses along the cold chain can add up to severe CI even though no one single time/temperature event was enough to cause injury. The threshold chilling temperature is the critical temperature below which the flowering shoot will develop CI if exposed for a long enough time periods. CI increases in severity as the temperature is decreased and the duration of exposure to critical temperatures is lengthened (Lyons, 1973).

CI can occur in the field, during handling, storage or transit, distribution, in retail stores or in the home (Kays and Paull, 2004). This research will deal with the prevention of CI during handling, storage or transit. CI can lower the quality of the product to such an extent that the product is rendered unsaleable. Of great concern is that the development of the symptoms is often slow and they only appear after the product has been removed from chilling temperatures, and the first person to encounter the visible damage is often the consumer. This is detrimental to the South African fynbos export industry. For certain chilling sensitive *Leucadendron* and *Leucospermum* species the symptoms that are thought to be CI have been reported after storage at 4°C for 21 days i.e. the general shipping temperature and duration to Europe.

A feature of CI is that it is reversible if exposed for a short enough time. The molecular changes which cause physiological dysfunctions at low temperatures can

be reversed if the affected tissue is raised to above the threshold chilling temperature before CI occurs (Creencia and Bramlage, 1971; Lieberman et al., 1958). Saltveit (2002) suggested that membranes damaged by chilling conditions may repair over time, decreasing the permeability caused by the CI, which could lead to re-absorption of the ions that had leaked out of the cell into the intercellular spaces. After a prolonged period at chilling temperatures the damage becomes visible as a result of internal breakdown of membranes. Symptoms include wilting, decay, loss of ability to take up water, discolouration, tissue collapse (pitting) and reduced disease resistance, etc. (Lyons, 1973; summarised by Kays and Paull, 2004).

2.4.2 Membranes and response to chilling injury

The membranous response and deterioration patterns of CI are similar in some ways to the response during senescence. A typical biological membrane is composed of 40% lipids, 60% proteins and up to 10% carbohydrates (composed of glycolipids and glycoproteins). Plant membranes are comprised of a fluid lipid bilayer, interspersed with proteins and sterols which influence the fluidity of the membrane. There are mostly noncovalent interactions between the lipids which make the membranes very flexible with fluid-like properties. Due to the fused ring structure sterols are not as flexible as most lipids (Horton et al., 1996; Taiz and Zeiger, 2002). Sterols act as stabilizers in the membranes and allow regular membrane functioning to occur over a large temperature range. The function of proteins in membranes is to a large extent transport, for example H^+-ATPase and Ca^{2+}-ATPase pumps. The membrane proteins are either attached peripherally or situated within the hydrophobic core of the lipid bilayer (Leshem, 1992).

A main cause of CI is thought to be the change in membrane permeability after exposure to temperatures below threshold chilling temperature. Chilled sweet potato root tissue leaked ions up to five times as much at 20ºC as healthy tissue (Lieberman et al., 1958). This ion leakage is most probably caused by physical phase changes in the membranes. Lyons and Raison (1970) reported that mitochondrial membranes change from a supple liquid-crystalline phase to a solid gel phase at chilling temperatures. Through electron spin resonance Raison et al. (1971) showed that the physical states of membranes are controlled in part by lipids and that the phase change occurs at the exact temperature where chilling injury occurs. Approximately

2-5% of the lipids in the chloroplast and mitochondria are believed to be involved in phase change (Raison and Orr, 1986). If only 2-5% are involved, the idea of certain 'domains' in the membranes forming pores or holes is a more likely hypothesis than the mass change of liquid-crystalline to gel phase throughout the entire membrane. Chilling sensitive plants generally have been shown to have a higher percentage of saturated as opposed to unsaturated fatty acids in the membranes than their chilling resistant counterparts (Lyons et al,. 1964). Thus membranes with a higher percentage of saturated fatty acid chains i.e. chilling sensitive plants, will solidify at higher temperatures than membranes with a lower percentage i.e. chilling resistant plants (Taiz and Zeiger, 2002). Lyons (1973) proposed that the phase change in the membranes where the lipids solidify will cause the membrane to contract resulting in cracks which would in turn lead to ion leakage and higher permeability. This is confirmed by work done by Saltveit (2002) who found that during exposure to chilling temperatures there is a subsequent increase in ion leakage rate when measured after chilling. As the membrane becomes more solid the proteins in the membranes can no longer function normally (Taiz and Zeiger, 2002). This change in the physical properties of the membrane's lipids and proteins is the primary response to the threshold temperature. The ultimate CI and dysfunction is the result of a progression of chronological events that follow a series of indirect injuries or dysfunctions (Lyons et al., 1979; Steponkus, 1984). These membrane phase changes don't occur in chilling-resistant species or at least they can maintain their liquid-crystalline state at lower temperatures.

The phase change results in an increased activation energy for membrane bound enzyme systems, which results in a decreased reaction rate, and thus an imbalance is created with non-membrane bound enzyme systems i.e. imbalance in metabolism. At this point there is cessation of protoplasmic streaming and coupled with other symptoms, a reduction in ATP supply. As a result, certain metabolites build up at the interface between the membrane bound/non-membrane bound systems. Once the concentrations of these metabolites have built up to dangerously high or toxic levels, CI symptoms start to appear which ultimately lead to the death of the cell and tissue (Lyons, 1973). This delay in appearance of the chilling injury symptoms is confirmed by Saltveit (2002) who reported that ion leakage rate only increased after chilling as a slow increase in the amount of leachable ions in the extra-cellular part of the tissue,

and an increase in the membrane permeability as the time of exposure to chilling temperatures increased.

Certain plants or tissues have shown ability to harden against chilling injury by being exposed to temperatures slightly above the threshold chilling temperature for a certain period of time before being subjected to chilling temperatures. This allows the plant or tissue to withstand chilling temperatures for longer before showing symptoms of chilling injury. For example, exposing cucumber cv. Pasendra plants to methodical decreases of temperatures before being subjected to chilling conditions acclimatized the plants to low temperatures and reduced CI. Thus, this low temperature hardening increased tolerance to chilling stress (Helmy et al., 1999). Different rates of chilling can affect the degree of chilling injury. This was shown too by Suzuki et al. (1998) who reported that Saintpaulia cv. Iceberg plants suffered 10% damage when temperatures of the plants were decreased slowly opposed to 60% damage when temperatures were decreased quickly. Lange and Cameron (1997) reported that postharvest chill-hardening of packaged sweet basil (*Ocimum basilicum*) was effective in increasing shelf life.

This ‘hardening’ also induces changes in composition of membrane lipids (Wang & Baker, 1979). Campos et al. (2003) concluded that during acclimation to chilling temperatures there was an increase in lipid synthesis, lower digalactosyldiacylglycerol (DGDG) to monogalactosyldiacylglycerol (MGDG) ratios (MGDG/DGDG) as a result of DGDG synthesis which results in increased membrane stability and changes in membrane unsaturation. In other words, plants try to adapt fluidity of the membranes at extremes of temperatures by, at low temperatures increasing the concentration of desaturation and conversely increasing the concentration of saturation at high temperatures, of glycerolipid fatty acyl chains (Raison et al., 1982).

Other treatments can increase chilling resistance too. For example temperature hardened flowering pot plants, *Clerodendrum speciosum* showed similar improved chilling resistance to plants treated with a foliar spray of 200 p.p.m. unionazale, paclobutrazol or ancymidol applied 10 days before chilling. Chilling-induced

membrane and protein degradation was inhibited by both treatments (Tamari et al., 1992).

2.4.3 Temperature manipulations

To counteract CI, it is logical to raise the temperature of the shipping container slightly above the threshold temperature, to avoid exposure to sub-critical temperatures but at increased temperatures the respiration rate increases (Rajapakse et al., 1994), bacterial and fungal activity increase and senescence is accelerated. Many different genera and cultivars of *Proteaceae* are generally shipped in the same container and thus it is necessary to keep the container temperature optimal for the majority of the products in the container, usually 4ºC. Since low temperature is the most effective means of extending storage life of fresh products (Lyons et al., 1979) this factor can't be compromised.

One option to avoid CI is intermittent warming. This involves storing the products below threshold temperatures, but periodically increasing the temperature of the products to above threshold temperature for a specified period, to allow the produce to recover, before lowering the temperature to below threshold temperature once again. These warm/cold cycles can be used over an extended period. This method is used successfully in the fruit industry for plums. It was shown that for peaches (*Prunus persica* L. Batsch cv. 'Paraguayo'), as a result of three intermittent warming cycles of 1 day at 20°C for every six days at 2°C, there was increased shelf life (Fernández-Trujillo & Artés, 1998). CI is presumed to be prevented during the warming period due to a combination of metabolisation of accumulated toxic compounds and re-establishment of necessary metabolites that have been respectively built up and depleted during the chilling, before any degenerative changes occur and recovery of the membranes i.e. returning to a fluid like state (Lyons, 1973; Jackman et al., 1988).

Logistically and practically it is difficult to manage intermittent warming and there is a constant problem of condensation on the products. Various species of *Proteaceae* are negatively affected by condensation. For example, leaf blackening is thought to be triggered by contact with condensation (Reid et al., 1989). To cool an entire container of *Proteaceae* cutflower products can take up to 24 hours depending on the

temperature differences between the initial temperature and the desired temperature. Similarly, to increase the temperature for a short time would be difficult to achieve, since efficacy of the containers to heat up or cool down the produce is limited. In order for the inner produce to reach the required temperature the outer produce would have to be warmed for longer than the optimum time, and subsequently would take a long time to all cool down. This could result in other undesirable factors such as accelerated senescence or increased fungal and bacterial attack, to which *Proteaceae* can be very susceptible etc. In the case of the peaches, there was an increase in wooliness and dryness of the cortical tissue (Fernández-Trujillo and Artés, 1998).

2.4.4 Sugars and chilling injury

The leaves of the Leucospermums seem to be more negatively affected by CI than the inflorescence. This could be explained by research done on other members of the *Proteaceae* family namely Proteas, where it was shown that carbohydrates of cut flowers are translocated to the inflorescence (sink) from the leaves (source) to be used in respiration to produce nectar (Bieleski et el., 1992; Dai and Paull, 1995). Dai and Paull (1995) found that inflorescence expansion, respiration, and most importantly nectar production are the primary sinks for the depletion of carbohydrates. In cut flowers this results in fewer carbohydrates in the leaves to protect them against CI. This supports the theory of translocation from the source to sink of carbohydrates and this could possibly be the case with Leucospermums and Leucadendrons.

The changes in the membranes which lead to CI may be prevented when protective compounds e.g. sugars, are present in the surroundings of the membranes during temperature and water stress. The sugars must be present in the protoplasm of the cell to be accessible to aid in protecting the membranes, not the vacuole (Jagendorf and Avron, 1958; Herber and Santarius, 1964). Santarius (1973) showed that the addition of sugars prior to water and temperature stress prevented the inactivation of phosphorylation and electron transport in thylakoid membranes in spinach. In addition he found that the protective effect of the sugars is a function of both their concentration and molecular weight. Trisaccharides were more effective than disaccharides, which in turn were more effective than monosaccharides.

Pulsing *Strelitzia reginae* flowers for 25 hours with a 40% sucrose pulse after cold storage at 10ºC improved vase life. The pulsing treatment increased the number of florets that opened (Finger et al., 2003). The developmental stage of the cut flower at harvest will influence the susceptibility to chilling injury and longevity. Developmental stage can play a role in CI sensitivity. For example, 'Fuerte' and 'Hass' avocados were most sensitive to chilling temperatures on the climacteric rise and climacteric peak (Kosiyachinada and Young, 1976). It has been reported that in frost resistant plant cells there is an increase in the concentration of sugars during the winter which correlates with an increase in their 'hardening off' to the cold and frost (Levitt, 1978). In evergreen plants, e.g. *Protea* 'Sylvia', carbohydrates are stored in the leaves as opposed to the stem (Hettasch et al., 2001). The above helps to explain why certain plants can withstand heavy frosts in winter but can be severely damaged by milder frosts in spring once dehardening has occurred (Herber and Santarius, 1964).

2.5 Effects of temperature and carbohydrate pulsing have on *Proteaceae* and related species

Cut kangaroo paw (*Angiozanthos* spp.), an Australian native cut flower, develops CI if stored at <2ºC for 4 weeks. It was shown that a relatively safe storage temperature for cut kangaroo paw flowers is between 2ºC and 5ºC. At 10ºC greater deterioration of the flowers has been found but this was as a result of increased senescence during storage at higher temperatures (Joyce and Shorter, 2000). The optimum temperature range for *Protea* storage was between 2 and 8ºC according to Paull et al. (1980). *Proteaceae* as a general rule are transported and stored at 4ºC. Proteas were found not to get CI when stored at 0º (Stephens et al., 2000). Jones and Faragher (1991) observed that after 21 days storage at 1ºC the vase life of *Leucospermum cordifolium* Salisb. Ex Knight was significantly decreased whilst *Leucospermum* 'Firewheel' retained a decent vase life of at least 7 days after storage. A decreased vase life is one symptom of CI and this suggests that there are differences between cultivars of the same genera (Leucospermums) in susceptibility to CI which corresponds with the literature. *Leucadendron* 'Silvan Red' showed a significantly reduced vase life after storage at 1ºC for 42 days, primarily as a result of leaf desiccation (Jones, 1995).

Stephens et al. (2000) showed that if cut flower stems were stored at 0ºC there were no differences in the carbohydrate concentrations in the leaves during storage as opposed to a marked difference between stems held at 0ºC, 4.5ºC, 7ºC, and 10ºC where there was a decrease in the carbohydrate concentration as the storage temperature was increased. This was said to be the result of increased respiration rates at higher temperatures. Expanding flower heads of various *Protea* cultivars were found to have a high respiration rate after harvest (Stephens et al., 2000; Dai & Paull, 1995; Ferreira, 1986), thus cooling the flowers as quickly as possible after harvest is critically important to slow *Protea* flower head development and reduce respiration (Newman et al., 1990). Joyce et al. (1995) found that 'Sylvia' flowers showed a lower respiration rate at the more mature stages of harvest.

In *P. neriifolia*, with a sucrose pulse of less than 12 hours, the leaves are the main sinks whilst if the pulse was for 18 hours the flower head is the main sink (Brink and De Swardt, 1986). This was further proved by Dai and Paull (1995) who found that when applying ^{14}C-sucrose to middle leaves of the cut flowers harvested at five different stages of development, more than 50% of the sucrose was found in the nectar 24 hours after harvesting. They proved that nectar production in proteas is a very strong carbohydrate sink. Several *Proteaceae* species have been shown to have nectar composed of fructose, glucose, sucrose and xylose (Cowling and Mitchell, 1981; Van Wyk and Nicolson, 1995 cited by Stephens et al., 2005). Flowering *Protea* shoots contained mainly fructose, glucose and generally smaller amounts of sucrose (Stephens et al., 2005). Glucose and fructose concentrations decreased faster than sucrose concentrations in *Freesia's* with the amount of carbohydrates available being strongly influenced by the stem length (Van Meeteren et al., 1995). Sucrose's uptake and metabolism was investigated by Kaltaler and Steponkus (1974). Cut roses (Rosa hybrida 'Red American Beauty') were placed in a modified Cornell solution (2% sucrose + 200 mg.L^{-1} 8-hydroxyquinone sulphate (8-HQS)) with a 10-hour photoperiod at 23ºC. The petal concentration of fructose and glucose increased but the sucrose concentration remained low suggesting sucrose was hydrolysed before reaching the petals. A 20% sucrose pulse on *P. neriifolia* for 24 hours resulted in an increase in the starch concentration after 48 hours which indicates that the sucrose had been incorporated into the starch reserves (McConchie and Lang, 1993). There is a double effect in pulsing certain Proteas with carbohydrates, namely glucose. After a glucose pulse vase life was significantly

extended and leaf blackening was significantly suppressed, thus the pulsed glucose is available for respiration and may, when present in high concentrations in the leaf, decrease hydrolysis of phenolic glycoside esters and thus decrease leaf blackening (Stephens et al., 2005).

Using pulsing or holding solutions at excessive carbohydrate concentrations were found to have negative effects. For example, a 3% sucrose vase solution caused leaf damage and increased leaf blackening in *P. neriifolia* (Brink and De Swardt, 1986), toxicity in the form of leaf spotting on *P. eximia* leaves was noted with supply of ≥1% sucrose solution in the holding solution (Newman et al., 1990), *P. neriifolia* stems showed an increase in leaf blackening after a pulse with sucrose concentrations over 7.5% (Paull and Dai, 1990). The sugar used in a pulse also seems to have a bearing on the resultant effect on the species and cultivar. McConchie and Lang (1993) found that a 20% sucrose pulse significantly reduced leaf blackening in *Protea neriifolia* whereas in contrast *Protea* 'Sylvia vase life was not significantly improved when pulsed with sucrose solutions of 10 and 20% (Stephens et al. 2001). However, a glucose holding solution of 2.5% significantly reduced leaf blackening in *Protea* 'Sylvia' and vase life was concluded due to flower head collapse after 20 days (Stephens et al., 2001). Jones (1995) pulsed *Leucadendron* 'Silvan Red' stems with 20% sucrose for 24 hours at 1ºC before 42 days dry storage at 1ºC with the result that leaf desiccation was prevented. When pulsed at greater than 10ºC with similar concentrations the effects were toxic which suggests that significantly more sucrose was directed to the leaves at higher temperatures. Thus, when pulsing Leucadendrons at higher temperatures the concentration of the pulse must be lower. He also showed that there was no fresh weight increase and that exogenously applied ^{14}C-sucrose was distributed mainly to the leaves and not to the flowerhead whilst pulsed labeled distilled water went mainly to the flowerhead. The total soluble sugar in the leaves was shown to decrease significantly in unpulsed stems but not so in pulsed stems. Thus Jones (1995) suggested that exogenously applied sucrose may prevent leaf desiccation by helping to maintain membrane integrity.

2.6 Conclusion

The hypothesis that the 'leaf desiccation' in Leucospermums and Leucadendrons is CI, is, to the best of our knowledge, a new concept. If the disorder is indeed CI and

pulsing with a sugar solution does control CI, shipping potential of these products will be greatly improved which will result in increased returns and extend the period of supply of the cut flowers to the Northern hemisphere markets.

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3. **Paper I:** Symptomology of Chilling Injury in Leucospermums and Leucadendrons

Symptomology of Chilling Injury in Leucospermums and Leucadendrons

Abstract

Chilling injury, as far as we are aware, has not been documented on Leucospermums or Leucadendrons. Typical visible chilling injury symptomology is discussed and shown for *Leucospermum* 'Gold Dust', 'High Gold' and 'Succession II' and for *Leucadendron* 'Chameleon', 'Laurel Yellow' and 'Safari Sunset'. The nature of chilling injury symptoms for Leucospermums and Leucadendrons was generally membranous breakdown that resulted in a 'water soaked' appearance which, at a more advanced stage, was visible as 'dried out' patches on the leaves. In the case of the Leucadendrons chilling injury was also visible on the immature involucral leaves which are more sensitive to chilling conditions than mature leaves and mature involucral leaves. As water uptake of shoots with chilling injury is hindered, the styles of the Leucospermums wilt. As can be expected, the lower the temperature below the threshold temperature and the longer the exposure the more severe the symptoms.

Introduction

Leucospermums. The inflorescence has many individual florets arranged on an involucral receptacle. The inflorescences are never borne terminally, rather in a distal axilliary position. The involucral bracts in this case are small and inconspicuous with coloured styles and perianth which form the inflorescence (Gerber, 2000; Rebelo, 2001).

Leucadendrons. The genus *Leucadendron* is dioecious, i.e. there are male and female plants. The female plants produce woody cones in which fruits are borne terminally on the shoots, and are more attractive and marketed on a larger scale than the male plants. The cone is, in some cases, surrounded by involucral leaves which generally change to an attractive colour e.g. red or yellow, during anthesis and then return to the original colour. *Leucadendron* stems are, depending on cultivar and species, marketed at different developmental stages viz. as greens, during anthesis when the large showy involucral leaves produced on the distal portion of the stem are

brightly coloured or as cones. Attractive leaves extend down the shoot (Gerber, 2000; Rebelo, 2001).

Chilling injury (CI) has long been observed on susceptible fresh produce. It was observed by exporters of cut 'flowers' of fynbos that certain cultivars of certain species are negatively effected by shipping conditions. The nature of these symptoms e.g. membranous breakdown and the resulting appearance of 'dried out' patches on the leaves, that develop on removal from storage and up to several days in the vase after storage conditions of long periods at low temperatures, suggests that the products may be undergoing a metabolic dysfunction as in CI and not merely dehydration as previously thought. As far as we are aware CI has not previously been documented on Leucospermums and Leucadendrons and it is the aim in this article to attempt to show examples of typical symptoms of CI on various susceptible cultivars of these fynbos products.

Plant material.

In the Spring of the 2003/2004 season, September to December, flowering shoots of 'Gold Dust' (*Ls. cordifolium*), 'High Gold' (*Ls. cordifolium* x *Ls. patersonii*) and 'Succession' (*Ls. lineare* x *Ls. cordifolium*) and in the Summer, February to April, shoots of 'Chameleon' (*Ld. laureolum* x *Ld. eucalyptifolium*), 'Laurel Yellow' (*Ld. laureolum* x *Ld. discolor*) and 'Safari Sunset' (*Ld. laureolum x Ld. salignum*), were harvested and packed as for export and transported to our laboratories within twenty-four hours. The stems were re-cut to similar lengths for each cultivar and the leaves removed from the proximal end leaving equal number of leaves per stem for each cultivar. The Leucospermums were stored for 21 days at 1ºC and the Leucadendrons were stored for 21 days at 1, 3 and 5ºC, in SAPPEX S14 fiberboard cartons with lids and sealed in black plastic bags to prevent drying out. After being removed from storage the shoots were re-cut and placed in vases with an antibacterial holding solution of Chrysal Clear Cut Food (Pokon Chrysal International at 10g.L^{-1}) for ten days at ambient temperature (20±2ºC).

Leucospermums. After exposure to chilling temperatures for a sufficiently long time period, 21 days, the Leucospermums develop grey-brown patches on the leaves. In a more advanced stage these patches dry out. In some cases no symptoms are

apparent immediately after removal from cold storage but symptoms develop after cold storage during the vase phase of the cut flowers. In these cases symptoms of CI generally appear first on the tips (Fig. 1a) of the leaves and progresses down the leaf with time (Fig. 1b & c). The CI symptoms increase, in most cases, rapidly in the first three days and then continue developing at a slower rate up to ten days during the vase phase.

Fig 1a. **Fig. 1b.** **Fig. 1c.**

Fig. 1a. Chilling injury is visible on the tips of the leaves of the 'Gold Dust' shoot that was stored for 21 Days at 1ºC and then held at ambient temperature for 10 days in the vase.

Fig. 1b. Chilling injury visible on the leaves of the 'High Gold' shoot directly after storage of 21 days at 1ºC.

Fig. 1c. Chilling injury visible on the leaves of the 'Succession II' shoot that was stored for 21 days at 1ºC and then held at ambient temperature for 8 days in the vase.

Water uptake of shoots with CI is impaired with the result that the styles and inflorescences wilt during the vase phase (Fig. 2).

Fig. 2. Wilted styles of a 'Succession II' inflorescence with chilling injury after being stored at 1ºC for 21 days and then held at ambient temperature for 8 days in the vase.

Leucadendrons. Similarly to Leucospermums, Leucadendrons are susceptible to CI and the symptoms become visible in the affected area as grey/brown areas of 'dried out' tissue (Fig. 3a & b). Immature inner involucral leaves of Leucadendrons are very sensitive to CI. Water uptake of shoots with severe CI of leaves is impaired.

Fig. 3a.

Fig. 3b.

Fig. 3a. Chilling injury visible on the leaves and inner bracts of 'Pisa' shoots that were stored for 21 days at 1ºC and subsequently held at ambient temperature for 10 days in the vase. Photo provided by G. Jacobs.

Fig. 3b. Severe chilling injury visible on the leaves and inner bracts of 'Safari Sunset' directly after being stored for 21 days at 2.5ºC. Photo provided by G. Jacobs.

The lower the temperature below the threshold, the more severe the CI (Fig. 4a–i).

Fig. 4a.

Fig. 4b.

Fig. 4c.

Fig. 4d

Fig. 4e.

Fig. 4f.

Fig. 4g.

Fig. 4h.

Fig. 4i.

Fig. 4a. ‘Safari Sunset’ shoots stored at 1ºC, **4b.** ‘Safari Sunset’ shoots stored at 3ºC, **4c.** ‘Safari Sunset’ shoots stored at 5ºC, **4d.** ‘Chameleon’ shoots stored at 1ºC, **4e.** ‘Chameleon’ shoots stored at 3ºC, **4f.** ‘Chameleon’ shoots stored at 5ºC, **4g.** ‘Laurel Yellow’ stored at 1ºC, **4h.** ‘Laurel Yellow’ stored at 3ºC and **4i.** ‘Laurel Yellow’ stored at 5ºC. Fig. (4a-i) were all held at ambient temperature for 10 days in the vase after 21 days storage.

The immature inner involucral leaves are often very susceptible to CI, and can be seen as brown water soaked patches on the inner bracts before drying out. (Fig. 5a&b).

Fig. 5a.

Fig. 5b.

Fig. 5a&b. Involucral leaves of 'Laurel Yellow' with chilling injury after 21 days storage at 1ºC. Photos provided by G. Jacobs.

Often the *Leucadendron* leaves and outer involucral leaves look undamaged but the inner involucral leaves are severely damaged, especially if there is soft new growth (Fig. 6a-f).

Fig. 6a.

Fig. 6b.

Fig. 6c.

Fig. 6d.

Fig. 6e.

Fig. 6f.

Fig. 6a. 'Safari Sunset' shoot with CI barely visible, **6b.** 'Safari Sunset' shoot with inner involucral leaves exposed, showing CI, **6c.** 'Chameleon' shoot with CI slightly visible, **6d.** 'Chameleon' shoot with inner involucral leaves exposed, showing CI, **6e.** 'Laurel Yellow' shoot with CI barely visible, **6f.** 'Laurel Yellow' shoot with inner involucral leaves exposed, showing CI.

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4. **Paper II:** Carbohydrate Supplementation and Cold Storage of Leucospermums and Resultant Chilling Injury

Carbohydrate Supplementation and Cold Storage of Leucospermums and Resultant Chilling Injury

Abstract

Chilling injury was recorded on cut flower shoots of *Leucospermum* 'Gold Dust', 'High Gold', 'Rigoletto', 'Succession II' and 'Vlam' after 21 and 24 days storage at 1ºC. After 24 days storage the chilling injury was more severe than after 21 days storage, in most cases. Each cultivar was pulsed with 5 ml per stem of a 2% (w/v) sugar solution of lactulose, sucrose, glucose, fructose or mannose before storage. After storage, flowers were held in Chrysal under ambient conditions (20±2ºC) and chilling injury was recorded on day 0, 3, 7 and 10. Of the cultivars tested, 'Vlam' and especially 'Rigoletto' were more prone to chilling injury development. 'High Gold' and 'Vlam' shoots were pulsed with 0 (control), 1.5, 3 or 4% w/v solutions of either mannose or fructose. 1.5% w/v gave the best control of chilling injury for both cultivars. Lower concentrations of mannose and fructose were tested on 'High Gold' shoots with a 1% w/v solution giving the best control for both. At high concentrations signs of toxicity became evident directly after pulsing. 'High Gold' shoots were pulsed with 1% w/v solutions of mannose and fructose, and sugar analyses were performed on shoots at different stages of storage and after 10 days in the vase. A slight increase in mannose and fructose was detectable in the stems of the shoots directly after pulsing but not in the leaves or the inflorescences. This is due to the low concentrations used. The levels of all the carbohydrates decreased during the 21 days storage and more so during the vase phase of the flowering shoots. The fact that such low concentrations were effective in controlling chilling injury suggests that the sugars may have an effect other than on the osmotic potential. The sugars may have an effect on maintaining membrane integrity as carbohydrate concentrations are low in the apoplast, as opposed to the symplast where sugar concentrations are relatively high.

Introduction

Many fresh produce develop CI during or after low temperature storage for extended periods. Desiccation of leaves occurred in a vase solution after *Leucospermum cordifolium* cut flowers were stored for 21 days at 1ºC (Jones and Faragher, 1991). Since leaf desiccation does not occur when *Leucospermum* cut flowers are cold stored at 5°C (Jacobs unpublished data) it appears that the desiccation is the result of chilling injury (CI). Typically, there are 60% proteins and 40% lipids in a biological membrane (Horton et al., 1996; Taiz and Zeiger, 2002). During the primary response to chilling temperatures specific critical proteins may be affected (Steponkus, 1984) and during the secondary responses there is a loss of membrane integrity resulting in solute leakage (Campos et al, 2003; Summarized by Kays and Paull, 2004). Lyons and Raison (1970) showed that membranes change from a supple liquid-crystalline phase to a solid gel phase at the temperature where CI occurs. As the membrane becomes more solid the proteins in the membranes can no longer function normally (Taiz and Zeiger, 2002). Saltveit (2002) concluded that during exposure to chilling temperatures there is an increase in subsequent ion leakage rate when measured after chilling, but not immediately. Instead there is a slow increase in the amount of leachable ions in the extra-cellular spaces of the tissue and an increase in the membrane permeability as the time of exposure to chilling temperatures increased. With increased permeability and ion leakage, a similar reaction to the browning reported as a result of enzymatic and/or air oxidation by Ribéreau-Gayon (1972) and Van Doorn (2001) is thought to occur as a result of CI.

Raison et al. (1971) showed that cell membranes of chilling resistant species don't undergo a phase change or can maintain their liquid-crystalline state at lower temperatures. Certain chilling sensitive plants or tissues have the ability to harden against CI by being exposed to temperatures slightly above the threshold temperature for a certain period before being subjected to chilling temperatures (Helmy et al., 1999; Suzuki et al., 1998). During this 'hardening', lipid compositional changes occur in the membranes (Wang and Baker, 1979). It has also been reported that frost resistant plant cells show an increase in sugar concentration during winter which correlates with their 'hardening off' to cold and frost (Levitt, 1978).

The leaves of the Leucospermums seem to be more negatively affected by CI than the inflorescence. According to Coorts (1973) leaf sucrose and starch are used as substrates for respiration during storage and in the vase phase of the cut flower. Pulsing with sugar, increases the pool of non structural metabolically active carbohydrates and may also maintain osmotic pressure (Halevy, 1976).

The purpose of this study was firstly to determine whether pulsing various *Leucospermum* cut flowers with sugars may decrease the incidence of CI, and secondly, determine the changes in the concentration of sugars during cold storage and thereafter in a holding solution at room temperature.

Materials and Methods

Plant Material.

In the 2003/2004 season, September to December, flowering shoots of 'Gold Dust' (*Ls cordifolium*), 'High Gold' (*Ls cordifolium* x *Ls patersonii*), 'Succession II' (*Ls lineare* x *Ls cordifolium*), 'Rigoletto' (*Ls cordifolium* x *Ls glabrum*) and 'Vlam' (*Ls cordifolium*) were harvested and packed as for export and transported to our laboratories within twenty-four hours. The stems were re-cut to similar lengths for each cultivar and the leaves were removed from the proximal end leaving equal numbers of leaves per stem for each cultivar. For Experiment 1 'Gold Dust' had ~ 23 leaves per shoot with a stem length of ~ 35 cm, 'Succession II' had 39±1 leaves per shoot with a stem length of 44±1 cm, Rigoletto had 16±1 leaves per shoot with a stem length of 36±1 cm, 'High Gold' had 31±1 leaves per shoot with a stem length of 46±1 cm and 'Vlam' had 15±1 leaves per shoot with a stem length of 39±1 cm. For Experiment 2 the 'High Gold' shoots had between 16 and 23 leaves per shoot with stem lengths of between 39 and 42 cm, while the 'Vlam' shoots had 13±1 leaves per shoot with stem lengths of 44±1 cm.

Pulsing of the shoots with a sugar solution was conducted at 23±2ºC under high-pressure sodium (HPS) lamps (400W, SON-T; Osram, Munich, Germany) that produce photoperiodic light at an intensity of approximately 110 $\mu mol.m^{-2}.s^{-1}$ to speed up the process of transpiration and thus the uptake of the sugar solution by the shoot. Shoots that were placed in tap water for the average time it took for the shoots

to take up 5 ml of the sugar solution served as the control. After pulsing the shoots were packed into SAPPEX S14 fiberboard cartons with lids and sealed in black plastic bags to prevent drying out. The cartons were stored for 21 (and 24, Experiment 1) days at 1ºC to simulate sea freight. After being removed from storage the shoots were re-cut and placed in vases with antibacterial holding solution of Chrysal Clear Cut Flower Food (Pokon Chrysal International at 10 $g.L^{-1}$), for 10 days at ambient temperature 20±2ºC. Immediately after cold storage and again at 3, 7 and 10 days during the vase life phase the number of leaves per shoot with symptoms of CI were counted.

Different sugars (Experiment 1).

Shoots of 'Gold Dust', 'High Gold', 'Succession II', 'Rigoletto' and 'Vlam' were pulsed with a 2% solution of lactulose, sucrose, glucose, mannose or fructose. Single shoots were used per treatment and treatments were repeated 8 times excepting 'Gold Dust' and 'High Gold' 24 days, and 'Vlam' 21 days where they were repeated 7 times and 'Vlam' 24 days where it was repeated 6 times.

Sugar concentrations (Experiment 2).

'High Gold' and 'Vlam' shoots were pulsed with 0 (control), 1.5, 3 or 4% solutions of either mannose or fructose. The experiment with 'High Gold' was repeated using mannose and fructose at 0, 0.5, 1.0, 1.5, 2.0 and 2.5%. Single shoots were used per treatment and treatments were repeated not less than 7 times.

Sugar metabolism (Experiment 3).

Twenty-four flower bearing 'High Gold' shoots were pulsed with either 1% mannose solution or 1% fructose solution. Sixteen control shoots were placed in tap water. Immediately after pulsing, 8 mannose and 8 fructose pulsed shoots as well as the two batches of 8 control shoots were separated into the leaves, stems and inflorescences and then freeze-dried. The remaining shoots were stored at 1ºC as described earlier. After 21 days of cold storage, 8 mannose and 8 fructose pulsed shoots were processed as described earlier and freeze-dried. The remaining shoots were placed in a holding solution of tap water and Chrysal Clear Cut Flower Food (Pokon Chrysal International at 10 $g.L^{-1}$) for 10 days whereafter they were processed

for freeze-drying as described earlier. Treatments were repeated 4 times and two shoots were used per replication.

Sugar analyses.

The freeze-dried samples were milled to a fine powder for the analysis of sucrose, glucose, fructose, inositol and, in the case of the mannose pulsed treatments, mannose was included in the analysis. Sugars were extracted from 0.2 g powder with a 5 ml solution of methanol, chloroform and water (MCW) for eighteen hours at ambient temperature (18±2ºC). MCW is comprised of 60% methanol, 25% chloroform, and 15% water, deionised through the Millipore water filtration system (Milli-Q Type 1 Reagent Grade Water System, Millipore Corporation, Bedford, USA). The extraction mixture was centrifuged (3 000 g_n, 5 min, 20±1ºC) and the supernatant was collected. One ml MCW was added to the residue, vortexed and then centrifuged (3 000 g_n, 5 min, 20±1ºC). The supernatant was again collected and added to the initial supernatant. To the pooled MCW-extract 1 ml chloroform was first added, followed by an additional 1 ml deionised water. The tube was shaken after each addition and finally centrifuged (3 000 g_n, 10 min, 20±1ºC) to separate the layers. The upper aqueous layer was collected and then evaporated to dryness under a rotary vacuum centrifuge (SC 210 A Speed Vac® Plus, Thermo Savant, Holbrook, NY).

The dried residue from the Savant was dissolved in 5 ml deionized water and put on an automatic shaker for ten minutes. After conditioning a C18 cartridge (which contained a preparative C18 bulk packing material, Wat 020594, Waters), firstly with methanol followed by deionised water, 1 ml of this sugar solution was cleaned by pulling it slowly through the C18 cartridge under a vacuum (VacMaster Sample Processing Station, International Sorbent Technology Ltd, Glamorgan, UK) into a 10 ml volumetric flask. The cartridge was washed with a further 4 aliquots of 2 ml each of deionised water. The eluate was made up to 10 ml by adding deionised water. The eluate was then further filtered through 0.45 µm filters (Millex-HV; Millipore Corporation, Bedford, USA) into vials ready for analysis by high performance liquid chromatography (Agilent 1100 Series HPLC; Agilent, Waldbronn, Germany). A Transgenomic™ ion exchange stainless steel column for the analysis of organic acids (300 x 7.8 mm) (model ICSep ICE-99-9850; Transgenomic, Omaha, NE) was

used with a Transgenomic™ guard column (model ICSep-ICE-GC-801; Transgenomic Inc, San Jose, CA) and was maintained at 30ºC. Sugars were separated using 4.25 mM H_2SO_4 at a flow rate of 0.5 ml.min^{-1}. A refractive index detector (model G1352A; Agilent) was used to detect separated sugars. An injection volume of 30 µl per sample was used.

Statistical analyses.

A complete randomized design was used. Analyses of variance for repeated measurements was performed on the data using the Statistica Program (Statistica 7, Statsoft, USA, 2005) and standard analysis of variance where applicable. Means were separated by Duncan's test ($P \leq 0.05$).

Results

Different sugars (Experiment 1).

Leaves of all cultivars not pulsed with sugars developed symptoms of CI during 21 days at 1°C which were exacerbated when exposure was increased to 24 days (Table 1). Of the cultivars tested 'Gold Dust' was the most tolerant of 1°C and 'Rigoletto' the least. All the sugars tested significantly reduced the incidence of CI in 'Gold Dust' and 'High Gold' after 21 as well as 24 days at 1°C. In the case of 'Succession II' only glucose, fructose and mannose reduced the incidence of CI when flowers were exposed for 21 days but not when storage period was extended to 24 days. Except for sucrose the other sugars tested reduced CI in 'Rigoletto' whereas none of the sugars reduced CI during cold storage in the case of 'Vlam'.

During the vase phase the incidence of CI increased rapidly in control shoots of 'Gold Dust'. All the sugars tested were effective in reducing the incidence CI during the vase phase of this cultivar (Fig. 1a & 1b). The incidence of CI, already high upon removal from cold storage for control shoots of 'High Gold' (Fig. 2a & 2b), increased only marginally during the vase phase. In contrast, sugar pulsed shoots had a low incidence of CI upon removal from cold storage, and although CI increased during the vase phase CI was significantly less than in control shoots. In the case of 'Succession II' the efficacy to reduce CI compared to control shoots appears to be sugar specific. Fructose appeared to be the most effective sugar to control the

development during the vase phase of 'Succession II' cold stored for either 21 or 24 days (Fig. 3a & 3b). None of the sugars was effective in controlling CI during the vase phase of 'Vlam' cold stored for 24 days whereas a modest effect was achieved when 'Vlam' was cold stored for 21 days only (Fig. 4a & 4b). Most of the leaves of 'Rigoletto' were injured after three weeks in cold storage especially the control (Fig 5a). However, some control by the sugars on removal from storage was clearly visible (Fig 5b). Poor control of CI was achieved during the vase phase irrespective of the sugar used for pulsing of this cultivar. Lactulose gave the best control and sucrose the poorest (Fig. 5c).

Sugar concentrations (Experiment 2).

Better control of CI during the vase phase of 'High Gold' and 'Vlam' was achieved when either mannose (fig. 6 & 8) or fructose (fig. 7 & 9) was used at 1.5 % as a pulsing sugar as compared to higher concentrations. When lower concentrations of mannose were tested (Fig. 10), 1.0% mannose appeared superior to lower or higher concentrations. All of the concentrations of mannose or fructose tested (Fig. 10 & 11) gave better results than the control. Both mannose and fructose, particularly at the higher concentrations of 3 and 4 %, gave a higher incidence of CI immediately after cold storage in 'Vlam' when compared to the control but the symptoms are possibly caused by the concentration being phytotoxic (Fig. 8 & 9). Possible signs of phytotoxicity are visible directly after pulsing when pulsed with concentrations of 4.5% fructose (Fig. 12a) and the effects thereof combined with CI are shown after 21 days storage at 1ºC (Fig. 12b & c).

Sugar metabolism (Experiment 3).

The concentration of inositol in control shoots of was lower in the stem than in the leaves or inflorescences and was also lower than any of the sugars measured (Table 2-7). The concentrations of glucose and fructose in control shoots were higher in the inflorescence than in the other shoot components. After pulsing with fructose or mannose, an increase in the concentration of these sugars was detected in the stems but not in the leaves or inflorescences (Table 2-7). Due to the slight overlap and the low mannose concentration, it was difficult to identify and quantify mannose. The presence of a slight amount of mannose was, however, detectable as a slight shoulder at the foot of the fructose peak. The retention time (rt) of this peak was the

same as that of a standard mannose solution. The levels of all the carbohydrates measured decreased during the 21 day cold storage period but even more so during the vase phase of the flowering shoots (Table 2-7).

Discussion and Conclusions

Drying out of leaves during cold storage at 1ºC as well as during the vase phase after cold storage was the general symptom of CI in *Leucospermum* in this study. This behaviour implicates loss of membrane integrity. The CI observed on the shoots stored for 24 days was higher than the shoots stored for 21 days (Table 1) and increased over the ten days in the vase (Fig. 1-5), which is typical of CI symptoms where the longer the exposure to chilling temperatures the more severe the symptoms and symptoms may intensify at room temperature after cold storage (Lyons, 1973). As the sensitivity to CI can vary according to the species, cultivar, plant part, morphological and physiological condition, temperature and duration of exposure, it can be expected to achieve varying sensitivity to CI between the different cultivars of Leucospermums and after the different exposure periods (Kays and Paull, 2004; Jones and Faragher, 1991). Of the cultivars tested ‘Vlam’ and in particular ‘Rigoletto’ were more prone to develop CI.

The efficacy of sugars to suppress CI, to the best of our knowledge, has not been reported before nor is there an obvious explanation for this effect. An increase in the concentration of either fructose or mannose after an uptake of 50 mg per stem by pulsing with a 1% solution of these sugars was detected in the stems but not the leaves or inflorescences. In contrast, an uptake of 500 mg glucose per stem by pulsing proteas resulted in doubling the leaf glucose content (Stephens et al., 2005). Furthermore, sugar concentrations decreased in all shoot parts during the cold storage and vase phase of ‘High Gold’ cutflowers (Table 2-7). Even if sugars were transported from the stem to the leaves during cold storage it is unlikely that osmotic potential would be greatly affected. Reasons other than a change in osmotic potential should therefore be considered to explain the reduction in CI by pulsing Leucospermums with sugars.

Compared to the symplast, sugars in the apoplast are present in low concentrations. According to Voitsekhovskaja et al. (2000) the concentration in the apoplasts of leaves varied from 0.3 to 6.0 mM (102 to 2052 mg/l) for sucrose and from 0.3 to 1.0 mM (54 to 180 mg/l) for hexoses. This is orders lower than a 2% (20000 mg/l) sugar pulsing solution. Higher concentrations of sugars in the apoplast, as a result of pulsing may therefore, in some way or another, cause membranes to be more tolerant of low temperatures and explain the lower incidence of CI. This may also explain the efficacy in reducing CI by sugars such as sucrose, glucose and fructose that are present in relatively high concentrations in the symplast but apparently not in the apoplast of *Leucospermum*. This line of reasoning is supported by the efficacy of small quantities (50 mg fructose or mannose) of sugar taken up per stem in reducing CI, but the apoplastic sugars may be significantly higher which is not reflected when expressed on a dry weight basis. In three of the cultivars evaluated ('Gold Dust', 'High Gold' and 'Rigoletto'), lactulose was superior to other sugars (Fig. 1, 2 & 5). This may be due to the inability of Leucospermums to metabolise lactulose.

There is, however, a number of inconsistencies that need to be addressed. Sugars were not as effective, or were ineffective in reducing CI in 'Succession II' and 'Vlam' (Fig. 3 & 4). In the case of 'Vlam' the lowest concentration tested was 1.5% for both fructose and mannose (Fig. 8 & 9). For both sugars 1.5% gave better control of CI than higher concentrations of 3 or 4%. Pulsing with lower concentrations of sugars may therefore improve the control of CI in 'Succession II' and 'Vlam'. In 'High Gold' pulsing with 2% mannose was effective in reducing CI in one case (Fig. 2) and not in another (Fig. 10). In the latter case 1% mannose was, however, effective.

In identifying the optimum concentration for pulsing it was noted that the leaves of Vlam pulsed with 3-4% fructose or mannose had a higher incidence of CI (Fig. 8 & 9). It is likely that in this case the leaf symptoms were due to the concentrations used being phytotoxic. Inflorescences were not affected by the high sugar concentration which correlates with work done by Halevy (1976) where he found that green leaves are more sensitive to high sugar concentrations as they have a lower ability to adjust osmotically than petals or inflorescences (Halevy, 1976). Using lower concentrations of mannose and fructose of 1.0 % appeared to give the best control of CI (Fig. 10 & 11).

The inflorescences had a higher concentration of sugars than the leaves (Table 2-7). The sugars in the inflorescences were composed predominantly of fructose and glucose and to a lesser extent sucrose, which correlates with previous research done on the nectar of proteas by Cowling and Mitchell (1981), Dai and Paull (1995), Stephens et al. (2001) and Meyer (2003). Dai and Paull (1995) and Bieleski et al. (1992) found that inflorescence expansion, respiration and nectar production, are the primary sinks for the depletion of carbohydrates, with nectar production being a major sink since, for example, *Protea neriifolia* produced on average 9.8 ml nectar per inflorescence and this may also be true for Leucospermums. The decrease in the sugars of the leaves and stem during cold storage and thereafter in the vase is possibly partly due to mobilization to the inflorescence.

In conclusion, flowering shoots pulsed with low concentrations of sugars had a lower incidence of CI upon removal from cold storage than control shoots and although CI increased during the vase phase CI was for the most part significantly less than in control shoots. This indicates that the sugar pulse helps to prevent CI although the mechanism by which it does this is uncertain. Although pulsing alone is not entirely effective as a preventative solution for CI, these results are very encouraging and a step forward towards successful long term cold storage of Leucospermums.

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Table 1. Incidence of CI in leaves of *Leucospermum* cultivars after either 21 or 24 days storage at 1ºC. Shoots pulsed with a zero or two percent solution of one of five different sugars; lactulose, sucrose, glucose, fructose and mannose.

	Gold Dust		High Gold		Succession II		Vlam		Rigoletto
	21 days	24 days	21 days	24 days	21 days	24 days	21 days	24 days	21 days
Control	1.4 a[z]	5.6 a	37.9 a	54.1 a	19.7 a	28.4 a	23.8 a	23.3 a	91.4 a
Lactulose	0.1 b	0.1 b	2.0 b	1.3 b	14.4 ab	18.8 a	3.6 a	30.0 a	25.8 c
Sucrose	0.1 b	0.7 b	1.2 b	1.7 b	10.3 abc	18.1 a	4.8 a	14.7 a	62.5 ab
Glucose	0.1 b	0.4 b	4.7 b	6.9 b	4.1 c	17.9 a	11.9 a	13.3 a	39.1 bc
Fructose	0.0 b	0.3 b	5.9 b	2.2 b	9.7 bc	12.5 a	3.6 a	8.9 a	27.3 c
Mannose	0.1 b	0.1 b	2.0 b	5.6 b	4.7 bc	15.0 a	11.9 a	11.1 a	31.3 bc

[z]Within columns, values with same the same letter are not significantly different at the 5% level, Duncan's Test.

Fig. 1a.

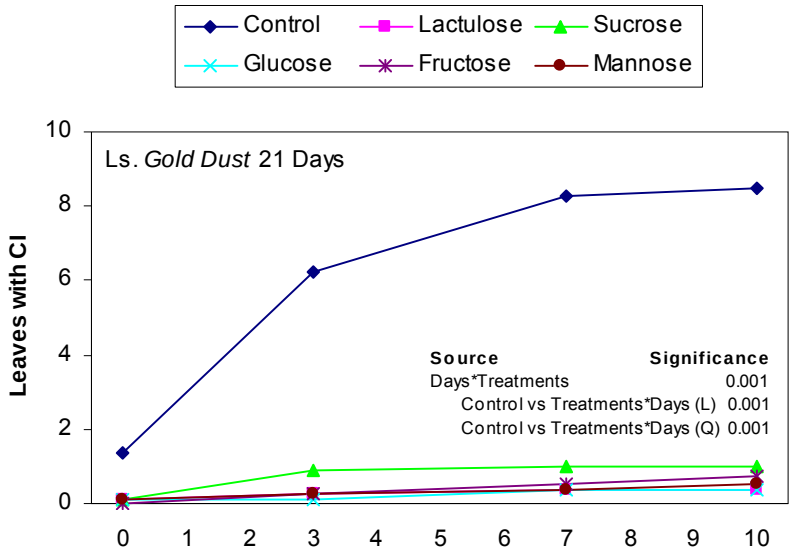


Fig. 1b.

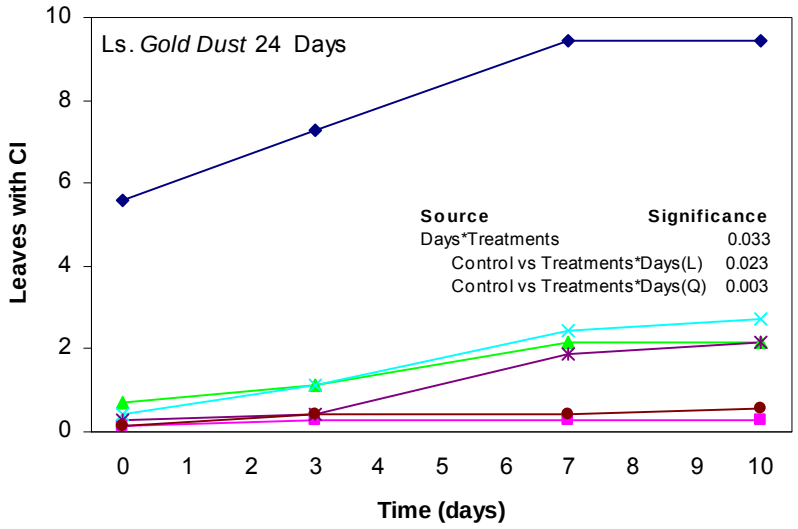


Fig. 1a&b. Number of leaves per stem of 'Gold Dust' (*L. cordifolium*) with chilling injury after either 21 or 24 days storage at 1ºC and the development thereof during the vase phase of 10 days at 22±1ºC, following a pre-storage pulse with a 2 % w/v sugar solution (uptake 5 ml/shoot) of lactulose, sucrose, glucose, fructose or mannose.

Fig. 2a.

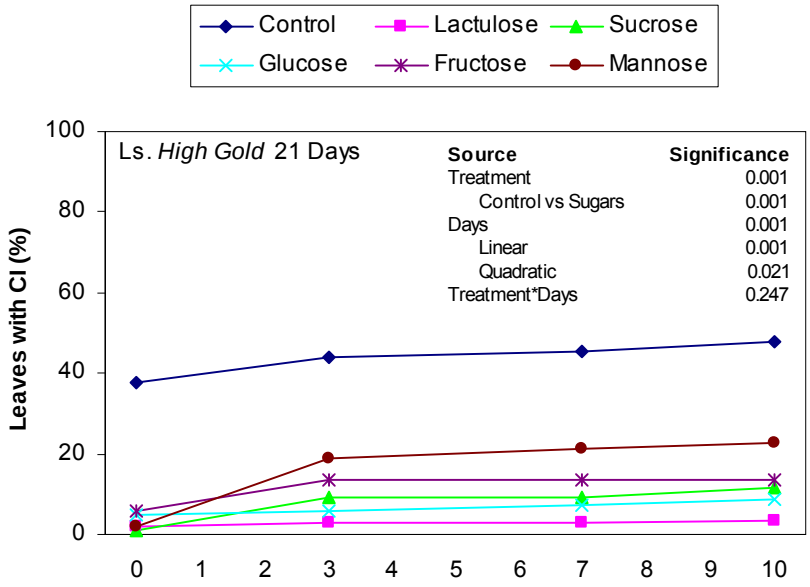


Fig. 2b.

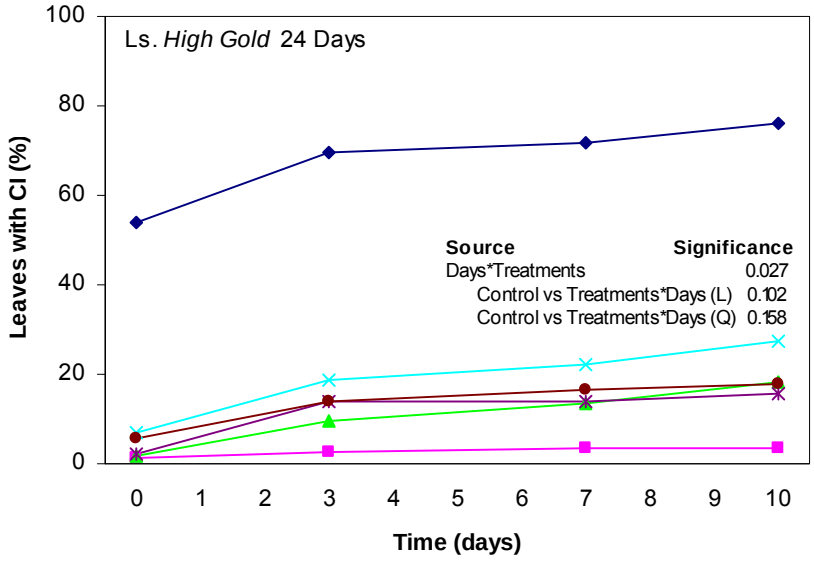


Fig. 2a&b. Leaves (%) per stem of 'High Gold' (*L. cordifolium* x *L. patersonii*) with chilling injury after either 21 or 24 days storage at 1ºC and the development thereof during a vase phase of 10 days at 22±1ºC, following a pre-storage pulse with a 2 % w/v sugar solution (uptake 5 ml/shoot) of lactulose, sucrose, glucose, fructose or mannose.

Fig. 3a.

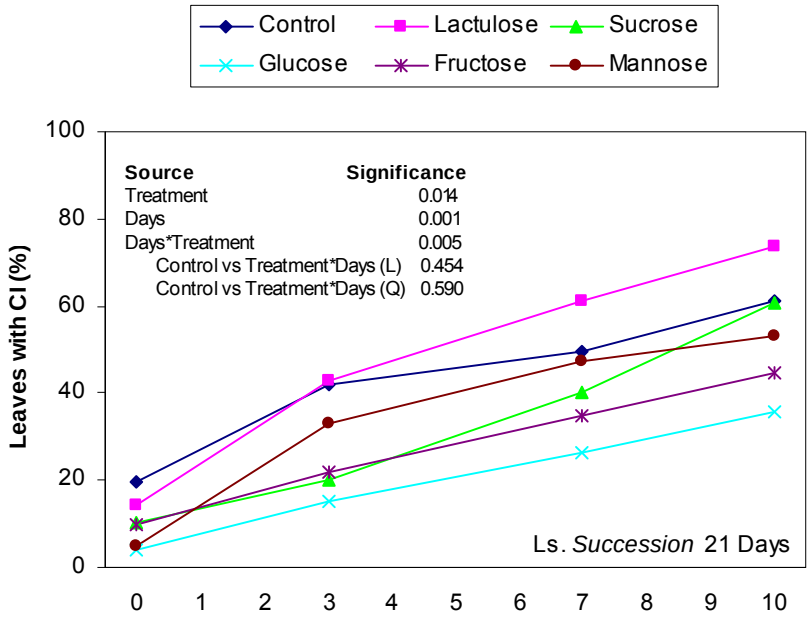


Fig. 3b.

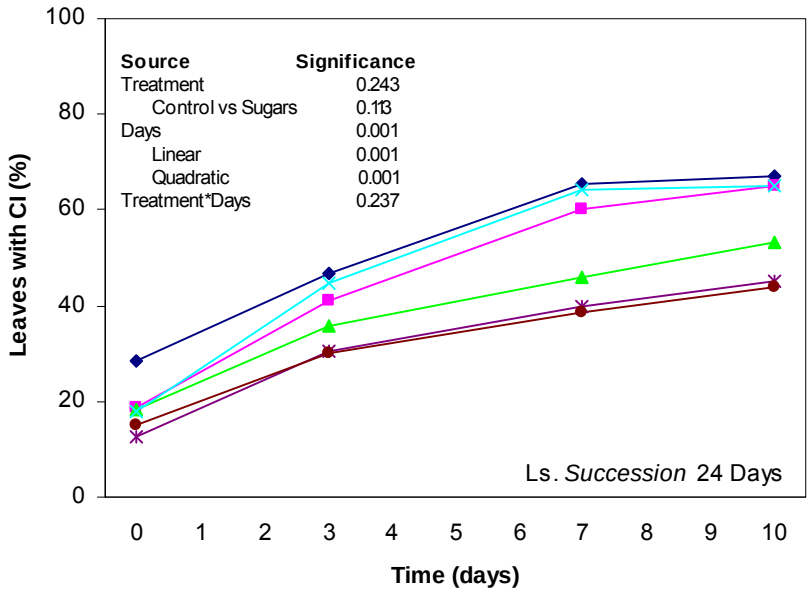


Fig. 3a&b. Leaves (%) per stem of 'Succession II' (*L. lineare* x *L. cordifolium*) with chilling injury after either 21 or 24 days storage at 1ºC and the development thereof during a vase phase of 10 days at 22±1ºC, following a pre-storage pulse with a 2 % w/v sugar solution (uptake 5 ml/shoot) of lactulose, sucrose, glucose, fructose or mannose.

Fig. 4a.

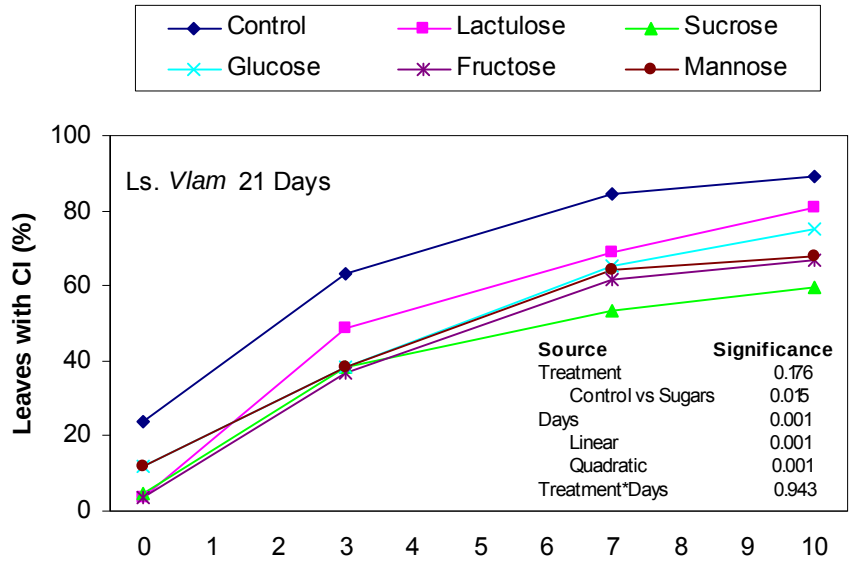


Fig. 4b.

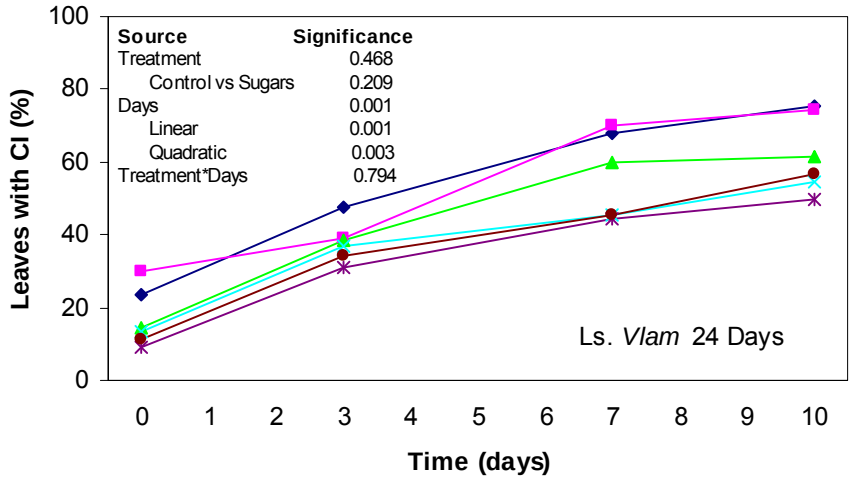


Fig. 4a&b. Leaves (%) per stem of 'Vlam' (*L. cordifolium*) with chilling injury after either 21 or 24 days storage at 1ºC and the development thereof during a vase phase of 10 days at 22±1ºC, following a pre-storage pulse with a 2% w/v sugar solution (uptake 5 ml/ shoot) of lactulose, sucrose, glucose, fructose or mannose.

Fig. 5a.

Fig. 5b.

Fig. 5a. ‘Rigoletto’ shoots with chilling injury stored at 1ºC for 21 days and then held at 22±1ºC for 24 hours in the vase at ambient temperature.

Fig. 5b. ‘Rigoletto’ shoots pulsed with 5 ml per stem 2 % w/v mannose solution and then stored at 1ºC for 21 days and then held at 22±1ºC for 24 hours in the vase at ambient temperature.

Fig. 5c.

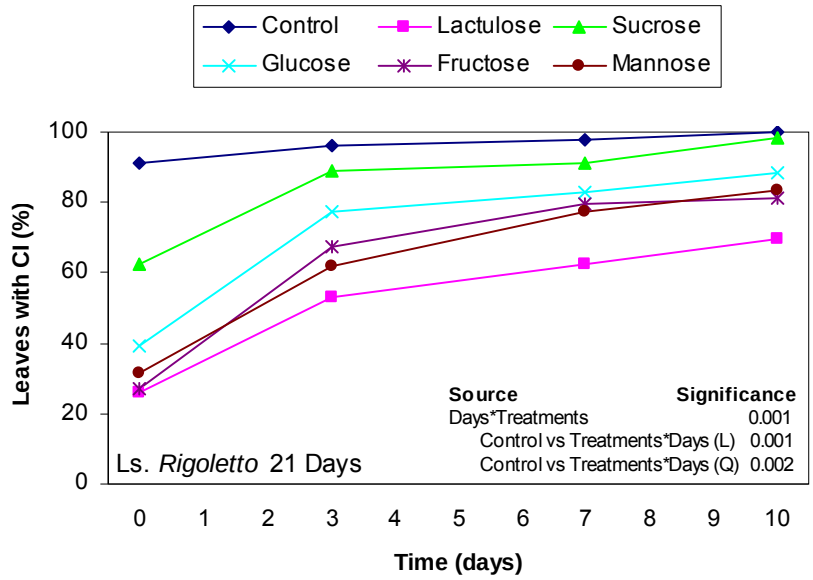
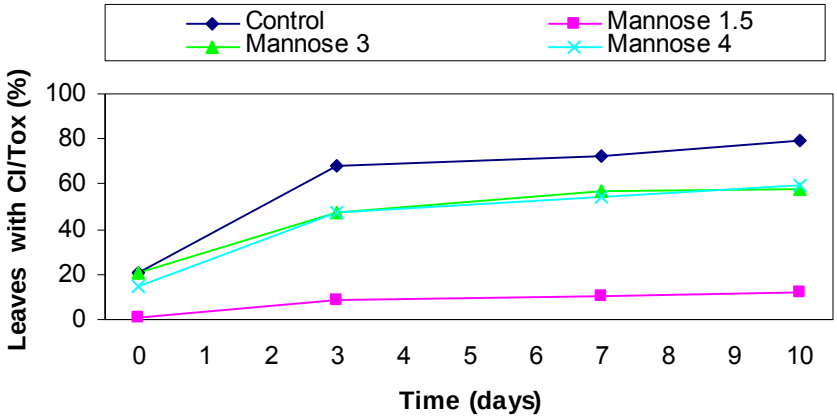
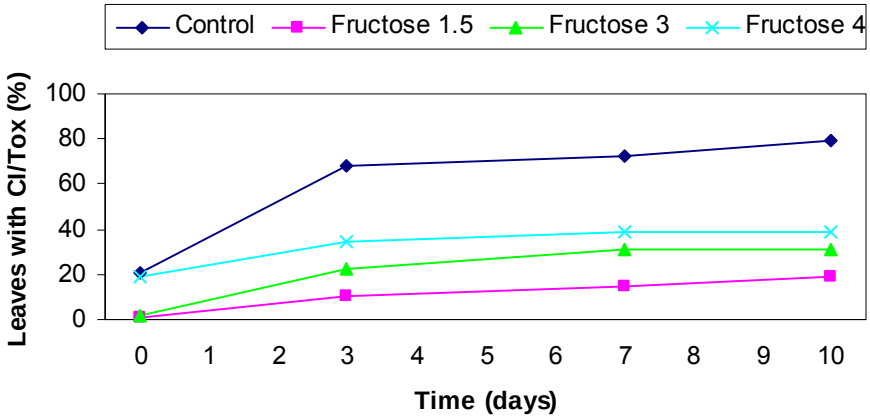


Fig. 5c. Leaves (%) per stem of 'Rigoletto' (*L. cordifolium* x *L. glabrum*) with chilling injury after either 21 or 24 days storage at 1ºC and the development thereof during a vase phase of 10 days at 22±1ºC, following a pre-storage pulse with a 2 % w/v sugar solution (uptake 5 ml/shoot) of lactulose, sucrose, glucose, fructose or mannose.



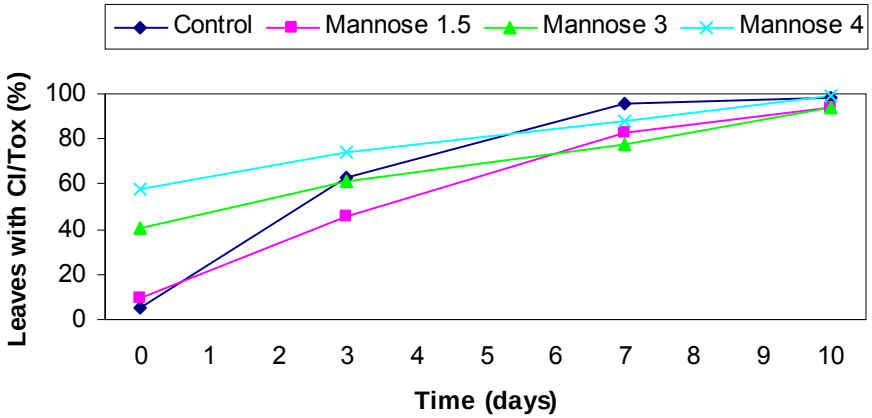
Source	Significance
Treatment	0.005
Days	0.001
Treatment*Days	0.001
Conc. Linear*Days Linear	0.757
Conc. Linear*Days Quadratic	0.524
Conc. Quadratic*Days Linear	0.002
Conc. Quadratic*Days Quadratic	0.016

Fig. 6. The effect of different concentrations of mannose pulse solutions, 0 (control), 1.5, 3 and 4% w/v (uptake 5 ml/shoot) on the development of chilling injury and signs of toxicity on 'High Gold' shoots in the vase over ten days, at 22±1ºC, after being stored for 21 days at 1ºC.



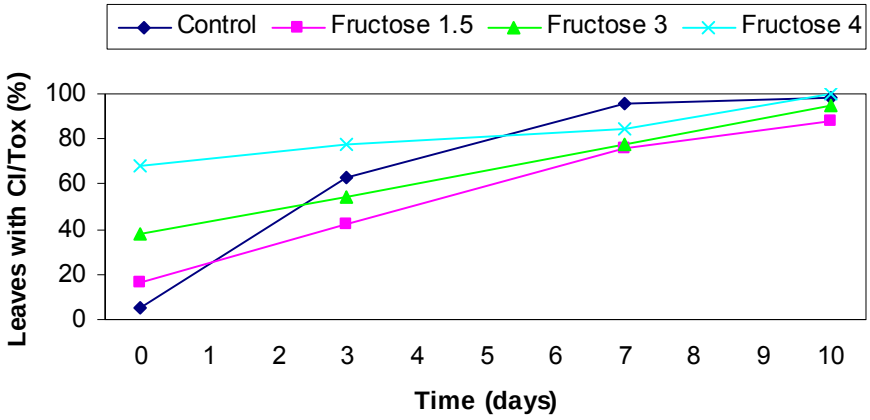
Source	Significance
Treatment	0.002
Days	0.001
Treatment*Days	0.001
Conc. Linear*Days Linear	0.008
Conc. Linear*Days Quadratic	0.082
Conc. Quadratic*Days Linear	0.068
Conc. Quadratic*Days Quadratic	0.037

Fig. 7. The effect of different concentrations of fructose pulse solutions, 0 (control), 1.5, 3 and 4 % w/v (uptake 5 ml/shoot) on the development of chilling injury and signs of toxicity on 'High Gold' shoots in the vase over ten days, at 22±1ºC, after being stored for 21 days at 1ºC.



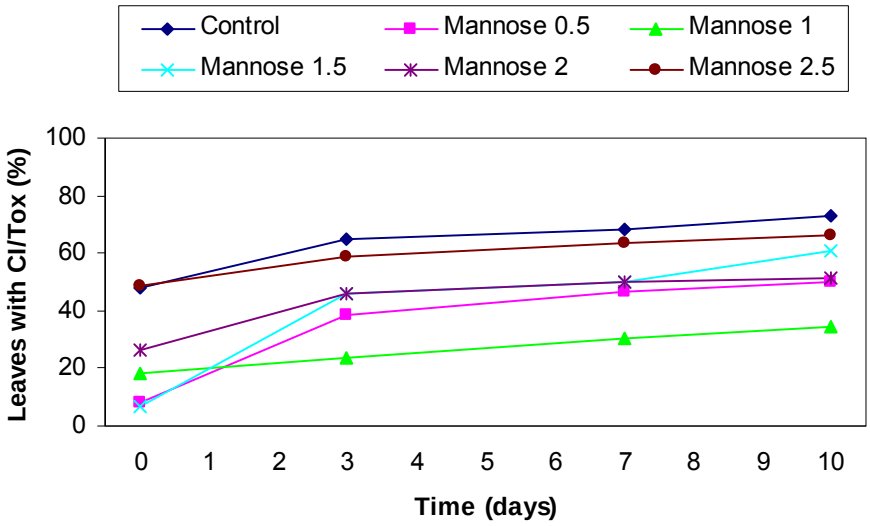
Source	Significance
Treatment	0.010
Days	0.001
Treatment*Days	0.001
Conc. Linear*Days Linear	0.001
Conc. Linear*Days Quadratic	0.001
Conc. Quadratic*Days Linear	0.268
Conc. Quadratic*Days Quadratic	0.154

Fig. 8. The effect of different concentrations of mannose pulse solutions, 0 (control), 1.5, 3 and 4 % w/v (uptake 5 ml/shoot) on the development of chilling injury and signs of toxicity on 'Vlam' shoots in the vase over ten days, at 22±1ºC, after being stored for 21 days at 1ºC.



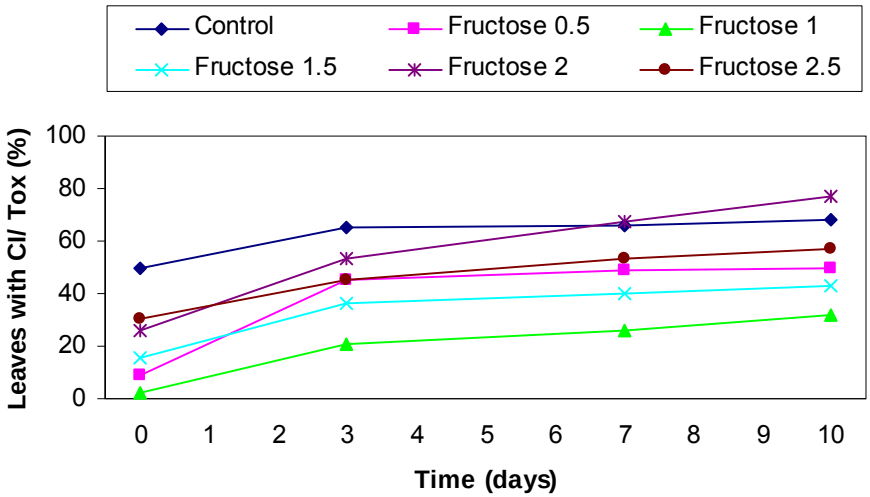
Source	Significance
Treatment	0.003
Days	0.001
Treatment*Days	0.001
Conc. Linear*Days Linear	0.001
Conc. Linear*Days Quadratic	0.001
Conc. Quadratic*Days Linear	0.353
Conc. Quadratic*Days Quadratic	0.044

Fig. 9. The effect of different concentrations of fructose pulse solutions, 0 (control), 1.5, 3 and 4% w/v (uptake 5 ml/shoot) on the development of chilling injury and signs of toxicity on 'Vlam' shoots in the vase over ten days, at 22±1ºC, after being stored for 21 days at 1ºC.



Source	Significance
Treatment	0.592
Days	0.001
Treatment*Days	0.002
Conc. Linear*Days Linear	0.490
Conc. Linear*Days Quadratic	0.649
Conc. Quadratic*Days Linear	0.099
Conc. Quadratic*Days Quadratic	0.342
Conc. Cubic*Days Quadratic	0.505

Fig. 10. The effect of different concentrations of mannose pulse solutions, 0 (control), 0.5, 1, 1.5, 2 and 2.5 % w/v (uptake 5 ml/shoot) on the development of chilling injury and signs of toxicity on 'High Gold' shoots in the vase over ten days, at 22±1ºC, after being stored for 21 days at 1ºC.



Source	Significance
Treatment	0.010
Concentration Linear	0.874
Concentration Quadratic	0.003
Days	0.001
Days Linear	0.001
Days Quadratic	0.001
Days*Treatment	0.076
Conc. Linear*Days Linear	0.209
Conc. Linear*Days Quadratic	0.384
Conc. Quadratic*Days Linear	0.303
Conc. Quadratic*Days Quadratic	0.516

Fig. 11. The effect of different concentrations of fructose pulse solutions, 0 (control), 0.5, 1, 1.5, 2 and 2.5 % w/v (uptake 5 ml/shoot) on the development of chilling injury and signs of toxicity on 'High Gold' shoots in the vase over ten days, at 22±1ºC, after being stored for 21 days at 1ºC.

Fig. 12a. **Fig. 12b.** **Fig. 12c.**

Fig. 12a. 'High Gold' shoot with signs of toxicity on the edges of the leaves directly after pulsing with 5 ml of a 4.5% w/v fructose solution.

Fig. 12b. 'High Gold' shoot with signs of toxicity and chilling injury after being pulsed with 5 ml of a 4.5% w/v fructose solution and then stored for 21 days at 1ºC and then held for 2 days at ambient temperature in the vase.

Fig. 12c. Close-up of 'High Gold' leaves with toxicity and chilling injury after being pulsed with 5 ml of a 4.5% w/v fructose solution and then stored for 21 days at 1ºC and then held for 2 days at ambient temperature in the vase.

Table 2. Mean sugar concentrations (mg/g) in the leaves of 4 reps of 2 inflorescences per rep of control and of pulsed inflorescences with a 1% w/v fructose solution, 5 ml/stem, of 'High Gold' (Ls. *cordifolium* x Ls. *patersonii*) at ambient conditions. Stored for 0, 21 days and 21 days plus 10 days in the vase.

Treatments	Sucrose	Glucose	Inositol	Fructose
Control	11.7a[g]	7.64a	3.21a	13.1a
Pulsed	12.1a	9.13a	3.34a	16.0a
21 Days	7.41b	4.60b	3.66a	9.04b
21 Days+10D	2.06c	2.11c	0.412b	5.11c
Pr>F	<.001	<.001	<.001	<.001

[g]Means (n=4) with different subscripts differ significantly at the 5% level, Duncan's Test.

Table 3. Mean sugar concentrations (mg/g) in the stems of 4 reps of 2 inflorescences per rep of control and of pulsed inflorescences with a 1% w/v fructose solution, 5 ml/stem, of 'High Gold' (Ls. *cordifolium* x Ls. *patersonii*) at ambient conditions. Stored for 0, 21 days and 21 days plus 10 days in the vase.

Treatments	Sucrose	Glucose	Inositol	Fructose
Control	14.6a[g]	15.4a	0.525a	12.2a
Pulsed	15.8a	16.2a	0.563a	14.1b
21 Days	9.05b	10.3bc	0.469a	9.56c
21 Days+10D	4.08c	9.07c	0.256b	8.18dc
Pr>F	<.001	<.001	<.001	<.001

[g]Means (n=4) with different subscripts differ significantly at the 5% level, Duncan's Test.

Table 4. Mean sugar concentrations (mg/g) in the inflorescences of 4 reps of 2 inflorescences per rep of control and of pulsed inflorescences with a 1% w/v fructose solution, 5 ml/stem, of 'High Gold' (Ls. *cordifolium* x Ls. *patersonii*) at ambient conditions. Stored for 0, 21 days and 21 days plus 10 days in the vase.

Treatments	Sucrose	Glucose	Inositol	Fructose
Control	8.58a[g]	36.3a	2.13a	35.0a
Pulsed	9.61a	35.9a	2.08a	35.1a
21 Days	9.74a	27.7b	2.18a	28.5b
21 Days+10D	3.50b	15.1c	1.56a	19.4c
Pr>F	<.001	<.001	<.293	<.001

[g]Means (n=4) with different subscripts differ significantly at the 5% level, Duncan's Test.

Table 5. Mean sugar concentrations (mg/g) in the leaves of 4 reps of 2 inflorescences per rep of control and of pulsed inflorescences with a 1% w/v mannose solution, 5 ml/stem, of 'High Gold' (Ls. *cordifolium* x Ls. *patersonii*) at ambient conditions. Stored for 0, 21 days and 21 days plus 10 days in the vase.

Treatments	Sucrose	Glucose	Inositol	Mannose	Fructose
Control	11.2ab[g]	9.88a	2.44b	-	16.9a
Pulsed	13.6a	7.74ab	3.14a	-	13.6ab
21 Days	8.48b	4.84b	2.86ab	-	9.81b
21 Days+10D	1.37c	1.19c	0.239c	-	3.22c
Pr>F	0.001	0.001	0.001	-	0.001

[g]Means (n=4) with different subscripts differ significantly at the 5% level, Duncan's Test.

Table 6. Mean sugar concentrations (mg/g) in the stems of 4 reps of 2 inflorescences per rep of control and of pulsed inflorescences with a 1% w/v mannose solution, 5 ml/stem, of 'High Gold' (Ls. *cordifolium* x Ls. *patersonii*) at ambient conditions. Stored for 0, 21 days and 21 days plus 10 days in the vase.

Treatments	Sucrose	Glucose	Inositol	Mannose	Fructose
Control	15.5a[g]	19.0a	0.48a	Trace	14.9a
Pulsed	15.3a	14.9ab	0.48a	1.23	12.5b
21 Days	10.3b	11.3bc	0.40ab	Trace	11.8b
21 Days+10D	4.45c	10.9bd	0.26bc	Trace	9.44c
Pr>F	0.001	0.064	0.008	-	0.002

[g]Means (n=4) with different subscripts differ significantly at the 5% level, Duncan's Test.

Table 7. Mean sugar concentrations (mg/g) in the inflorescences of 4 reps of 2 inflorescences per rep of control and of pulsed inflorescences with a 1% w/v mannose solution, 5 ml/stem, of 'High Gold' (Ls. *cordifolium* x Ls. *patersonii*) at ambient conditions. Stored for 0, 21 days and 21 days plus 10 days in the vase.

Treatments	Sucrose	Glucose	Inositol	Mannose	Fructose
Control	7.74a	31.9ab	1.08ab	trace	30.1ab
Pulsed	8.80a	35.7a	1.43a	trace	34.4a
21 Days	8.19a	24.7b	1.18ab	trace	24.3b
21 Days+10D	2.23b	9.91c	0.519b	trace	12.1c
Pr>F	0.001	0.001	0.161	-	0.001

[g]Means (n=4) with different subscripts differ significantly at the 5% level, Duncan's Test.

5. **Paper III:** Cold Storage of *Leucadendron* ‘Safari Sunset’, ‘Laurel Yellow’ and ‘Chameleon’

Cold Storage of *Leucadendron* 'Safari Sunset', 'Laurel Yellow' and 'Chameleon'

Abstract

Cut 'flower' shoots of *Leucadendron* 'Chameleon', 'Laurel Yellow' and 'Safari Sunset' were stored for 14, 21 and 28 days, at 1, 3 and 5ºC and chilling injury development recorded during the subsequent 10 day vase phase. 'Laurel Yellow' and 'Safari Sunset' showed signs of chilling injury on the leaves after 28 days storage at 3ºC or lower and 'Safari Sunset' stored for 21 days developed chilling injury during the vase phase. Immature involucral leaves were more sensitive to chilling injury than leaves. CI increased with longer exposure times and lower storage temperatures for all three cultivars evaluated. 'Chameleon' was the most chilling tolerant out of the three cultivars up to 21 days. At 5ºC chilling injury was low irrespective of cold storage duration but longer exposures to 1 and 3ºC resulted in increased chilling injury development during the vase phase. All three cultivars were pulsed with 5 ml/stem of a 1% w/v solution of lactulose, sucrose, glucose, fructose or mannose and stored for 14, 21 and 28 days at 1ºC. The sugars reduced chilling injury on the leaves of 'Safari Sunset' when stored for 28 days and to a lesser extent in 'Chameleon'. The sugars failed to reduce chilling injury of the involucral leaves of 'Chameleon' and 'Laurel Yellow' whereas there was some control especially after 28 days for 'Safari Sunset'. In some cases the sugar pulse exacerbated chilling injury. Chilling injury generally increased rapidly after storage during the first three days in the vase and then at a lower rate for the next seven days. *Leucadendron* 'Chameleon', 'Laurel Yellow' and 'Safari Sunset' 'cut flower' shoots were pulsed with a 1% w/v glucose solution. Expressed on a dry weight basis an increase in glucose concentration was not detected. The reduction in chilling injury of leaves by a sugar pulse is speculated to be as a result of their presence in the apoplast and not the symplast and that their presence there protects the membranes against chilling conditions in some way.

Introduction

Quality of certain *Leucadendron* cultivars after cold storage for 21 days has been reported to be substandard due to 'drying out' of leaves and involucral leaves. However, the nature of the symptoms of this 'drying out' and the conditions under which they develop, viz. long exposures to low temperatures led us to hypothesize that these are symptoms caused by chilling injury (CI). During CI, biological membranes composed typically of two thirds proteins (Taiz & Zeiger, 2002), change to a solid gel phase from a liquid crystalline phase at or below the temperature where CI occurs (Lyons & Raison, 1970). There are two responses during CI, the primary and secondary response. The primary response consists of physical changes to the properties of the membrane lipids and proteins that further result in the secondary responses of indirect injuries or dysfunctions including membrane leakage (Steponkus, 1984). Saltveit (2002) reported that the ion leakage rate does not increase immediately in chilling sensitive tissue when it is exposed to chilling conditions. Dark discoloration may also result from CI. A group of flavonoids, leuco-anthocyanins found in plant cells, undergo browning after enzymatic and/or air oxidation (Ribéreau-Gayon, 1972; Van Doorn, 2001). A similar process is thought to occur after increased permeability due to CI.

Chilling resistant species avoid phase change by maintaining their liquid-crystalline state at low, normally chilling temperatures (Raison et al., 1971). Certain chilling sensitive plants or tissues have shown ability to harden against CI by being exposed to temperatures slightly above the threshold chilling temperature for a certain period of time before being subjected to chilling temperatures. This allows the plant or tissue to withstand chilling temperatures for longer before showing symptoms of CI (Helmy et al., 1999; Suzuki et al., 1998). This hardening induces changes in lipid composition in the membranes (Wang & Baker, 1979). It has been reported that in frost resistant plant cells there is an increase in the concentration of sugars during the winter which correlates with an increase in their 'hardening off' to the cold and frost (Levitt, 1978). The above helps to explain why certain plants can withstand heavy frosts in winter but can be severely damaged by milder frosts in spring once dehardening has occurred (Herber &

Santarius, 1964). By exposing cucumber cv. Pasendra plants to methodical decreasing of temperatures before subjecting them to chilling conditions acclimatized the plants to low temperatures and reduced CI. Thus, low temperature hardening increased tolerance to chilling stress (Helmy et al., 1999). Lange and Cameron (1997) reported that postharvest chill-hardening of packaged sweet basil (*Ocimum basilicum*) was effective in increasing shelf life. Different rates of chilling can affect the degree of CI. This was shown too by Suzuki et al. (1998) who reported that Saintpaulia cv. Iceberg plants suffered 10% damage when temperatures of the plants were decreased slowly as opposed to 60% damage when temperatures were decreased quickly.

Variations between cultivars to susceptibility to CI can be expected. Work done by Jones and Faragher (1991) showed that after storage of 'Silvan Red' for 21 days at 1ºC, the vase life had decreased by 11 percent. A pre-storage pulse of cut *Leucadendron* 'Silvan Red' with sucrose protected the shoots during long term dry storage at 1ºC and improved subsequent vase life (Jones, 1991).

The decreased 'vase-life' and 'drying-out' of the leaves and involucral leaves of Leucadendrons we hypothesized to be CI. Our aim in this research was to determine for a few susceptible cultivars that it was indeed CI, to try to establish at what critical temperature and after how long CI occurs, and then to attempt to control the problem by pulsing shoots with various sugars. Once this had been completed sugar analyses would be performed on pulsed and non-pulsed shoots to try and understand sugar uptake and partitioning by the shoots.

Materials and Methods

Plant material.

In the 2003/2004 season, February to April, 'bearing' shoots of 'Chameleon' (*Ld. laureolum* x *Ld. eucalyptifolium*), 'Laurel Yellow' (*Ld. laureolum* x *Ld. discolor*) and 'Safari Sunset' (*Ld. laureolum* x *Ld. salignum)* were harvested and packed as for export and transported to our laboratories within twenty-four hours. The stems were re-cut to

similar lengths for each cultivar and the leaves were removed from the proximal end leaving equal numbers of leaves per stem for each cultivar. For Experiment 1 'Chameleon' had 20±1 leaves and 15±1 involucral leaves per shoot with a stem length of 50±1 cm, 'Laurel Yellow' had 12±1 leaves and 11±1 involucral leaves per shoot with a stem length of 50±1 cm and 'Safari Sunset' had 22±1 leaves for 14 and 21 days and 28±1 leaves for 28 days, 9±1 involucral leaves per shoot and a stem length of 50±1 cm. For Experiment 2 the 'Chameleon' shoots had 19±1 leaves and 15±1 involucral leaves per shoot with stem lengths of 47±1 cms, while the 'Laurel Yellow' shoots had 11±1 leaves and 11±1 involucral leaves per shoot with stem lengths of 37±1 cm and for 'Safari Sunset' shoots there were 37±1 leaves, 9±1 involucral leaves and a stem length of 55±1 cm for the shoots stored for 14 and 21 days and for the shoots stored for 28 days there were 42±1 leaves, 11±1 involucral leaves and stem lengths of 76±1cm.

Pulsing of the shoots (Experiment 2 and 3) with a sugar solution was conducted at 23±2ºC under high-pressure sodium (HPS) lamps (400W, SON-T; Osram, Munich, Germany) that produce photoperiodic light at an intensity of approximately 110 $\mu mol.m^{-2}.s^{-1}$ to speed up the process of transpiration and thus the uptake of the sugar solution by the shoot. Shoots that were placed in tap water for the average time it took for the shoots to take up 5 ml of the sugar solution served as the control. After pulsing the shoots were packed into SAPPEX S14 fiberboard cartons with lids and sealed in black plastic bags to prevent drying out. The cartons were stored for 14, 21 and 28 days at 1, 3 and 5ºC (Experiment 1) and at 1ºC (Experiment 2). After being removed from storage the shoots were re-cut and placed in vases with antibacterial holding solution of Chrysal Clear Cut Flower Food (Pokon Chrysal International at 10 $g.L^{-1}$), for 10 days at ambient temperature 20±2ºC. Immediately after cold storage and again at 3, 7 and 10 days during the vase phase the number of leaves and the number of involucral leaves per shoot with symptoms of chilling injury were counted.

Storage period and temperature (Experiment 1).

Seventy two shoots of 'Chameleon', 'Laurel Yellow' and 'Safari Sunset', were stored for 14, 21 or 28 days at 1, 3 or 5ºC without being pulsed. Single shoots were used per treatment and treatments were repeated 8 times.

Different sugars (Experiment 2).

'Chameleon', 'Laurel Yellow' and 'Safari Sunset' shoots were pulsed with 0 (control), or 1% solutions of lactulose, sucrose, glucose, fructose or mannose and stored at 1ºC for 14, 21 and 28 days. Single shoots were used per treatment and treatments were repeated 8 times.

Sugar uptake and partitioning (Experiment 3).

Bearing 'Chameleon', 'Laurel Yellow' and 'Safari Sunset' shoots were pulsed with a 1% glucose solution. Control shoots of each cultivar were placed in tap water. Immediately after pulsing the pulsed shoots as well as the three batches of 12 control shoots were separated into the leaves, stems and inner bracts and then freeze-dried. Treatments were repeated 4 times and three shoots were used per replication.

Sugar analyses.

The freeze-dried samples were milled to a fine powder for the analysis of sucrose, glucose, fructose, and an unidentified substance was included in the analysis. Sugars were extracted from 0.2 g powder with a 5 ml solution of methanol, chloroform and water (MCW) for eighteen hours at ambient temperature 18±2ºC. MCW is comprised of 60 % methanol, 25 % chloroform, and 15 % water, deionised through the Millipore water filtration system (Milli-Q Type 1 Reagent Grade Water System, Millipore Corporation, Bedford, USA). The extraction mixture was centrifuged (3 000 g_n, 5 min, 20±1ºC) and the supernatant was collected. One ml MCW was added to the residue, vortexed and then centrifuged (3 000 g_n, 5 min, 20±1ºC). The supernatant was again collected and added to the initial supernatant. To the pooled MCW-extract 1 ml chloroform was first added, followed by an additional 1 ml deionised water. The tube was shaken after each addition and finally centrifuged (3 000 g_n, 10 min, 20±1ºC) to separate the layers. The

upper aqueous layer was collected and then evaporated to dryness under a rotary evaporated/vacuum centrifuge (SC 210 A Speed Vac® Plus, Thermo Savant, Holbrook, NY).

The dried residue from the Savant was dissolved in 5 ml deionized water and put on an automatic shaker for ten minutes. After conditioning a C18 cartridge (which contained a preparative C18 bulk packing material, Wat 020594, Waters), firstly with methanol followed by deionised water, 1 ml of this sugar solution was cleaned by pulling it slowly through the C18 cartridge under a vacuum (VacMaster Sample Processing Station, International Sorbent Technology Ltd, Glamorgan, UK) into a 10 ml volumetric flask. The cartridge was washed with a further 4 aliquots of 2 ml each of deionised water. The eluate was made up to 10 ml by adding deionised water. The eluate was then further filtered through 0.45 µm filters (Millex-HV; Millipore Corporation, Bedford, USA) into vials ready for analysis by high performance liquid chromatography (Agilent 1100 Series HPLC; Agilent, Waldbronn, Germany). A Transgenomic™ ion exchange stainless steel column for the analysis of organic acids (300 x 7.8 mm) (model ICSep ICE-99-9850; Transgenomic, Omaha, NE) was used with a Transgenomic™ guard column (model ICSep-ICE-GC-801; Transgenomic Inc, San Jose, CA) and was maintained at 30ºC. Sugars were separated using 0.00425 M (or 4.25 mM) H_2SO_4 at a flow rate of 0.5 $ml.min^{-1}$. A refractive index detector (model G1352A; Agilent) was used to detect separated sugars. An injection volume of 30 µl per sample was used.

Statistical analysis.

A complete randomized design was used. Repeated measures analyses of variance was performed on the data using the Statistica Program (Statistica 7, Statsoft, USA, 2005) and standard analysis of variance where applicable. Means were separated by Duncan's test ($P \leq 0.05$).

Results

Storage period and temperature (Experiment 1).

Upon removal from cold storage CI was evident on leaves of 'Safari Sunset' and 'Laurel Yellow' but in the case of 'Chameleon' symptoms did not develop (Table 1). Immature involucral leaves were more susceptible to low temperatures than leaves (Table 2). CI increased with longer exposure times and lower temperatures for the cultivars evaluated. Up to 21 days storage 'Chameleon' appeared more tolerant of low temperatures than 'Safari Sunset' and or 'Laurel Yellow'.

The incidence of CI increased during the vase phase for leaves of 'Safari Sunset' (Fig. 1a & b). CI was low at 5ºC irrespective of the cold storage duration tested but longer exposures to 3 and 1ºC resulted in an increased rate of CI development as time in the vase progressed. 'Laurel Yellow' leaves did not develop CI during the vase phase when cold stored for 3 weeks or less. However when cold stored for 28 days the results were erratic (Fig. 2a). Very little CI developed on leaves during the vase phase when 'Laurel Yellow' was cold stored at 1ºC but CI developed rapidly when stored at 3ºC and to a lesser degree when stored at 5ºC.

CI of immature involucral leaves developed rapidly during the vase phase in all three cultivars evaluated (Fig. 3-5). CI increased during the vase phase irrespective of storage temperature or duration except for 'Safari Sunset' stored at 5ºC and 'Chameleon' stored at 1ºC for 28 days. In the latter case CI was already high upon removal from cold storage. The 3ºC cold room started malfunctioning somewhere during the 21 to 28 day period in Experiment 1. The results obtained with 28 days at 3ºC should therefore be considered as compromised.

Different sugars (Experiment 2).

Upon removal from cold storage leaves of 'Safari Sunset' stored for 28 days had CI (Fig. 6c). During the vase phase CI of leaves of 'Safari Sunset' increased rapidly during the first 3 days stored for 28 days (Fig. 6c) but not with shorter storage periods. All the

sugars tested reduced the incidence of CI in this case. A high percentage of the involucral leaves had CI upon removal from storage which intensified during the vase phase in the case of 'Safari Sunset' (Fig. 9a-c). With the exception of glucose, pulsing with the other sugars tested reduced CI to various degrees (Fig. 9a-c).

Leaves of 'Laurel Yellow' did not develop CI during storage up to 21 days or during the vase phase. CI developed during the vase phase when the product was stored for 28 days (Fig. 7) and most of the sugars tested reduced CI. In contrast, CI of involucral leaves developed during storage which intensified during the vase phase and pulsing with sugars did not reduce CI (Fig. 10a-c).

Leaves of 'Chameleon' developed CI during cold storage when the period exceeded 14 days (Fig. 8a & b). CI of leaves increased further during the vase phase and most sugars tested reduced CI. Involucral leaves developed CI during cold storage of 28 days but not for shorter storage periods which intensified during the vase phase and pulsing with sugars did not reduce CI (Fig. 11a-c).

Sugar uptake and partitioning (Experiment 3).

The sucrose concentration in the control shoots was higher in the leaves than in any other shoot components (Table 3). For 'Chameleon' and 'Laurel Yellow' the glucose and fructose concentration of control shoots was highest in the involucral leaves whereas for 'Safari Sunset' they were highest in the stems. Glucose concentrations did not increase significantly in any of the shoot parts analysed for the three cultivars tested as a result of glucose pulsing (Table 3).

Discussion and Conclusions

'Drying out' and browning of the leaves, and to a greater extent the involucral leaves, were the most conspicuous symptom when Leucadendrons were cold stored for extended periods. These symptoms for both leaves and involucral leaves developed in most cases more extensively during the vase phase (Fig. 1-5) at 20±2ºC rather than

during the cold period itself. They also increased with longer cold storage periods and increased with decreasing temperatures, with the exception of 1ºC for 28 days in 'Laurel Yellow' (Fig. 2), These all indicate that CI is the cause thereof. Furthermore, during CI membranes have been shown to change from a supple liquid-crystalline phase to a solid gel phase at chilling temperatures (Lyon and Raison, 1970) which causes ion leakage (Lieberman et al., 1958). The resultant diffusion of phenolic compounds and air and/or enzymatic oxidation of certain flavonoids can result in tissue browning that was in particular evident in immature involucral leaves (Ribéreau-Gayon, 1972; Van Doorn, 2001). The tissue damage may then lead to a dried out appearance. It then becomes logical to conclude that these are as a result of CI.

Leaves were less prone to developing CI than the inner involucral leaves of the cultivars tested. This is possibly related to differences in the degree of hardening off. Developmental stage can play a role in CI sensitivity. For example, 'Fuerte' and 'Hass' avocados were most sensitive to chilling temperatures on the climacteric rise and climacteric peak. If 'Hass' were stored post climacteric they could be stored at the same temperature viz. 2ºC for 11 more days than at the climacteric peak or during the climacteric rise (Kosiyachinada & Young, 1976). Sensitive plants that have been acclimatized to low temperatures are more tolerant to chilling temperatures than plants that have not undergone hardening and will be less severely affected by chilling temperatures (Helmy et al., 1999). As opposed to softer involucral leaves, older leaves show less CI which indicates that older leaves of Leucadendrons are 'harder' than new involucral leaves.

Leucadendrons, depending on cultivar, can be marketed in different developmental stages viz. (a) as greens when the degree of hardening off of leaves and involucral leaves will be a function of the time after cessation of elongation growth of shoots, (b) during the phase of anthesis or (c) as a cone post anthesis. Since this work was done on greens it will be useful to determine the susceptibility for CI for the different developmental stages of the commercial cultivars. This will apply especially to cultivars marketed as greens. Furthermore since hardening may also be a function of climatic

and cultural factors their relationship to CI sensitivity should be quantified in order to develop practices that minimize the risk of CI.

The sugars were not as effective in controlling CI as for Leucospermums (Paper II). There was control of CI by the sugars in the leaves (Fig. 6-8) but no apparent control in the involucral leaves (Fig. 9-11) except for 'Safari Sunset' 28 days (Fig. 9c). The relatively poor control of CI with sugar pulsing may be concentration related as was shown to be the case in Leucospermums. As in the case of Leucospermums the effect of sugars to suppress CI has no obvious explanation and we speculate that the positive effects of the sugars are due to their presence in the apoplast and not the symplast. By pulsing with a 1% glucose solution, 50 mg were administered per stem in our experiment. However this was not reflected by an increase in the concentration, expressed on a dry weight basis for glucose in any of the shoot parts (Table 3). We therefore conclude that too little glucose have been taken up during pulsing to affect the osmotic potential of the symplast. Other explanations should therefore be considered to explain the efficacy of sugar pulsing in reducing CI. Voitsekhovskaja et al. (2000) found very low concentrations of sugars in the apoplast of leaves varying from 0.3 to 6.0 mM (102 to 2052 mg/l) for sucrose and from 0.3 to 1.0 mM (54 to 180 mg/l) for hexoses. By pulsing with a 1% glucose solution (10 000 mg/l) may have increased the apoplastic concentration of sugars to a level that in some or other way protected the membranes against low temperatures and thus decrease the incidence of CI. Since pulsing with 1% sugar solution exacerbated CI of involucral leaves in a number of cases lower concentrations should be tested before commercial recommendations can be made.

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Table 1. Effect of storage temperature and duration on the incidence of CI expressed as percentage leaves affected for three *Leucadendron* cultivars on removal from storage.

	Storage days and Temperature								
	14 Days			21 Days			28 Days		
	1ºC	3ºC	5ºC	1ºC	3ºC	5ºC	1ºC	3ºC	5ºC
Safari Sunset	0.0a[z]	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	4.0b	0.0ab
Laurel Yellow	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	2.1a	0.0a	0.0a
Chameleon	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a

[z]Values within cultivar and days storage with different subscripts differ significantly at the 5% level, Duncan's Test.

Table 2. Effect of storage temperature and duration on the incidence of CI expressed as percentage shoots with involucral leaves affected for three *Leucadendron* cultivars on removal from storage.

	Storage days and Temperature								
	14 Days			21 Days			28 Days		
	1ºC	3ºC	5ºC	1ºC	3ºC	5ºC	1ºC	3ºC	5ºC
Safari Sunset	15.3a[z]	5.6a	0.0a	22.2a	20.8a	2.8a	43.1a	30.5a	0.0b
Laurel Yellow	44.3a	38.6ab	27.3b	52.3a	51.1a	28.4b	66.0a	47.7ab	33.0b
Chameleon	0.0a	0.0a	0.0a	12.5a	0.0b	0.0b	64.2a	3.3b	0.0b

[z]Values within cultivar and days storage with different subscripts differ significantly at the 5% level, Duncan's Test.

Fig. 1. Development of CI during the vase phase of 'Safari Sunset' (expressed as a percentage of leaves affected) after storage at 1, 3 or 5ºC for 21 days (a) or 28 days (b).

Fig. 1a

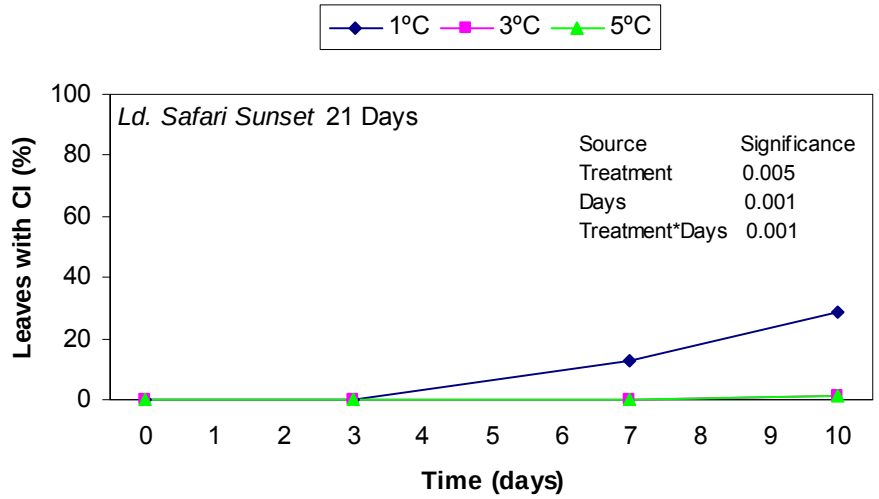


Fig. 1b

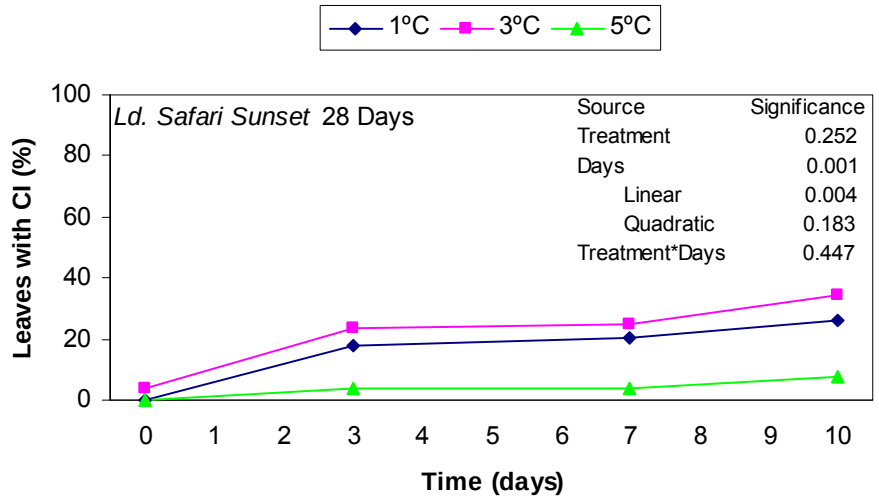


Fig. 2a. Development of CI during the vase phase of 'Laurel Yellow' (expressed as a percentage of leaves affected) after storage at 1, 3 or 5ºC for 28 days.

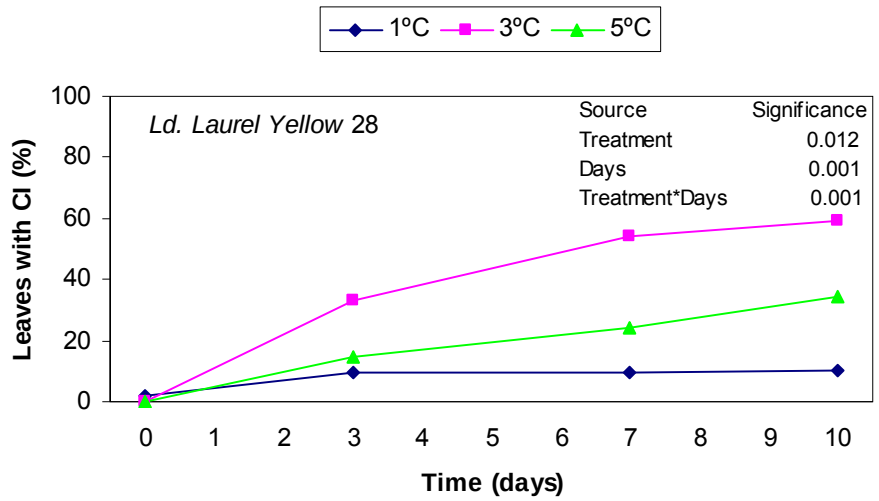
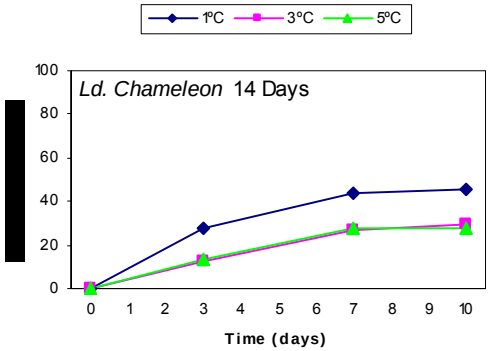


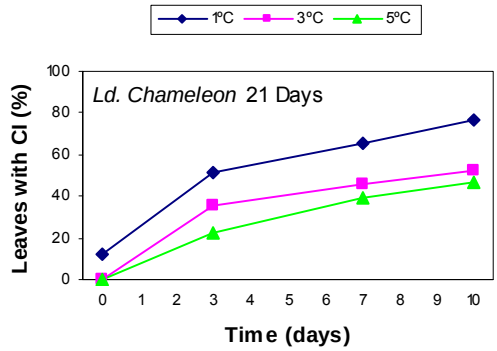
Fig. 3. Development of CI during the vase phase of 'Chameleon' (expressed as a percentage of involucral leaves affected) after storage at 1, 3 or 5ºC for 14 (a), 21 (b) or 28 days (c).

Fig. 3a



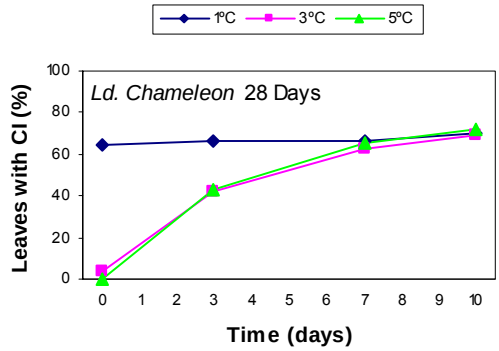
Source	Significance
Treatment	0.277
Days	0.001
Days Linear	0.001
Days Quadratic	0.002
Treatment*Days	0.371

Fig. 3b



Source	Significance
Treatment	0.001
Days	0.001
Days Linear	0.001
Days Quadratic	0.001
Treatment*Days	0.321

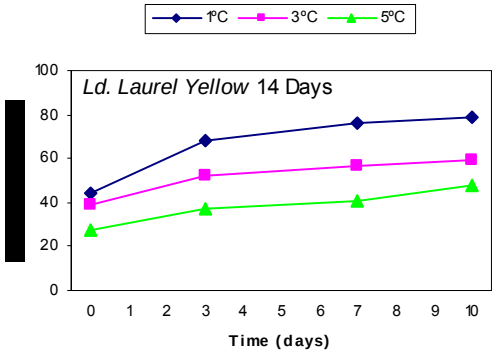
Fig. 3c



Source	Significance
Treatment	0.013
Days	0.001
Treatment*Days	0.001

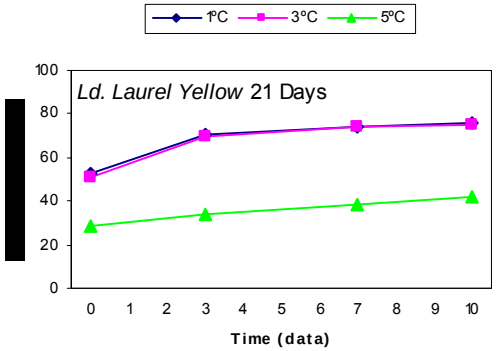
Fig. 4. Development of CI during the vase phase of 'Laurel Yellow' (expressed as a percentage of involucral leaves affected) after storage at 1, 3 or 5ºC for 14 (a), 21 (b) or 28 days (c).

Fig. 4a



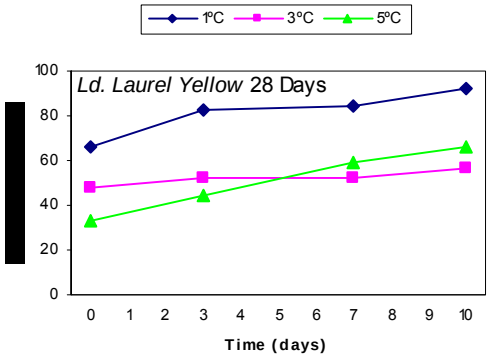
Source	Significance
Treatment	0.005
Days	0.001
Treatment*Days	0.051

Fig. 4b



Source	Significance
Treatment	0.001
Days	0.001
Treatment*Days	0.012

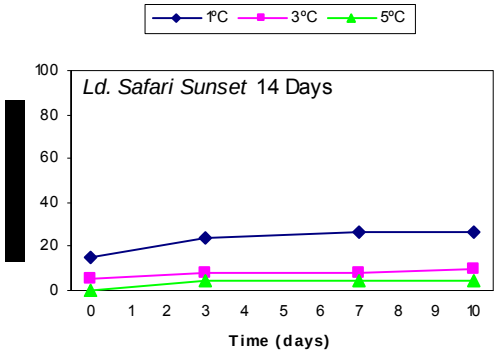
Fig. 4c



Source	Significance
Treatment	0.003
Days	0.001
Treatment*Days	0.001

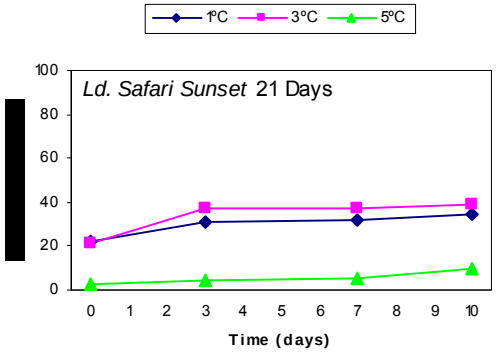
Fig. 5. Development of CI during the vase phase of 'Safari Sunset' (expressed as a percentage of involucral leaves affected) after storage at 1, 3 or 5ºC for 14 (a), 21 (b) or 28 days (c).

Fig. 5a



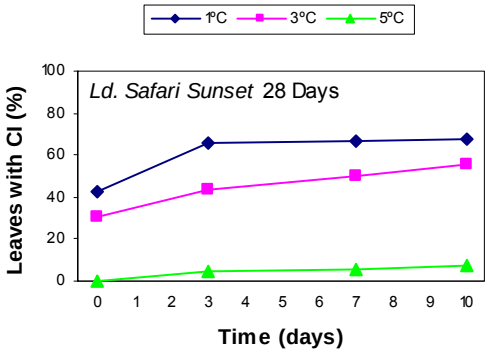
Source	Significance
Treatment	0.099
Days	0.001
Days Linear	0.006
Days Quadratic	0.050
Treatment*Days	0.388

Fig. 5b



Source	Significance
Treatment	0.032
Days	0.001
Treatment*Days	0.023

Fig. 5c



Source	Significance
Treatment	0.001
Days	0.001
Days Linear	0.001
Days Quadratic	0.002
Treatment*Days	0.063

Fig. 6a, b & c. Leaves (%) per stem of 'Safari Sunset' (*Ld. laureolum* x *Ld. salignum*) with chilling injury after 14, 21 or 28 days storage at 1ºC and the development thereof during a vase phase of 10 days at 22ºC, following a pre-storage pulse with a 1% w/v sugar solution (uptake 5 ml/shoot) of lactulose, sucrose, glucose, fructose or mannose.

Fig. 6a

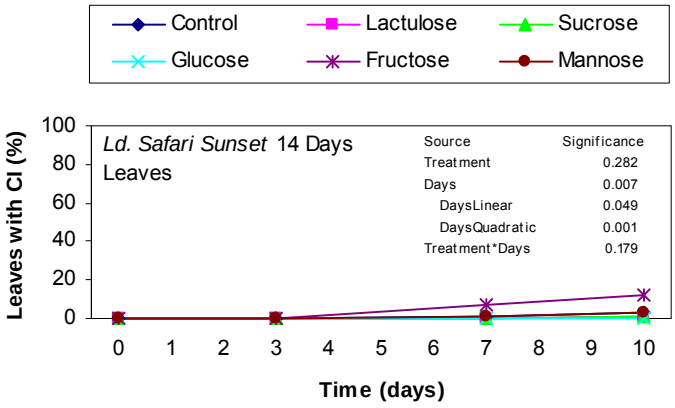


Fig. 6b

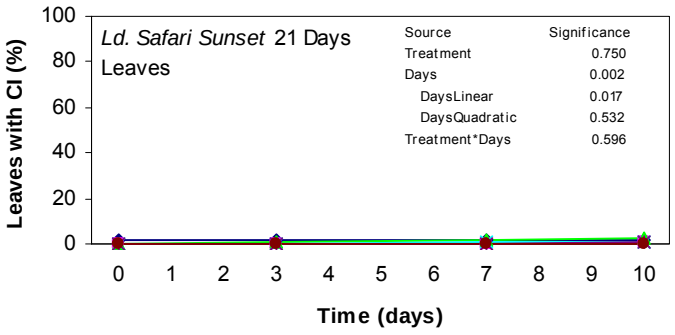


Fig. 6c

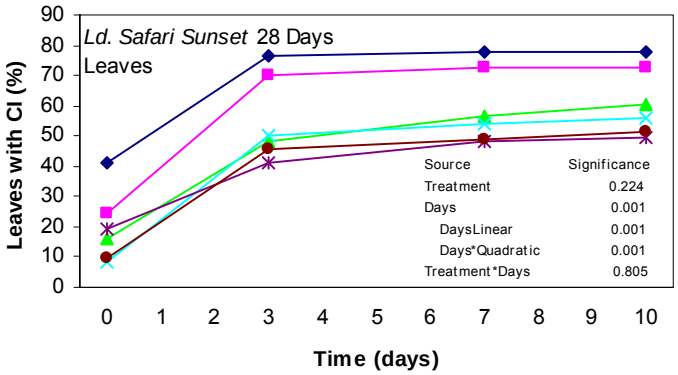


Fig. 7. Leaves (%) per stem of 'Laurel Yellow' (*Ld. laureolum* x *Ld. discolor*) with chilling injury after 28 days storage at 1ºC and the development thereof during a vase phase of 10 days at 22ºC, following a pre-storage pulse with a 1% w/v sugar solution (uptake 5 ml/shoot) of lactulose, sucrose, glucose, fructose or mannose.

Fig. 7

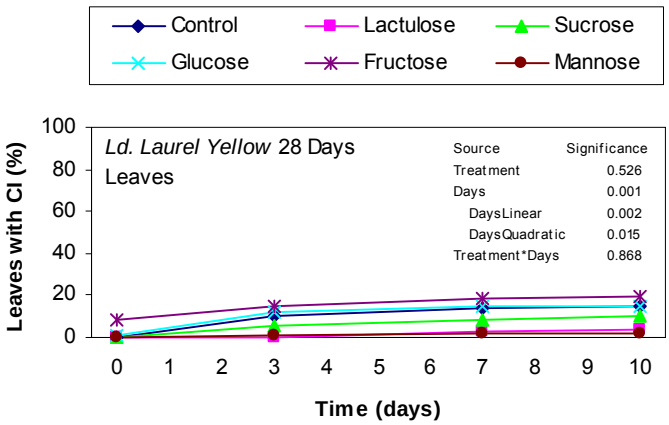


Fig. 8a & b. Leaves (%) per stem of 'Chameleon' (*Ld. laureolum* x *Ld. eucalyptifolium*) with chilling injury after either 21(a) or 28(b) days storage at 1ºC and the development thereof during a vase phase of 10 days at 22ºC, following a pre-storage pulse with a 1% w/v sugar solution (uptake 5 ml/shoot) of lactulose, sucrose, glucose, fructose or mannose.

Fig. 8a

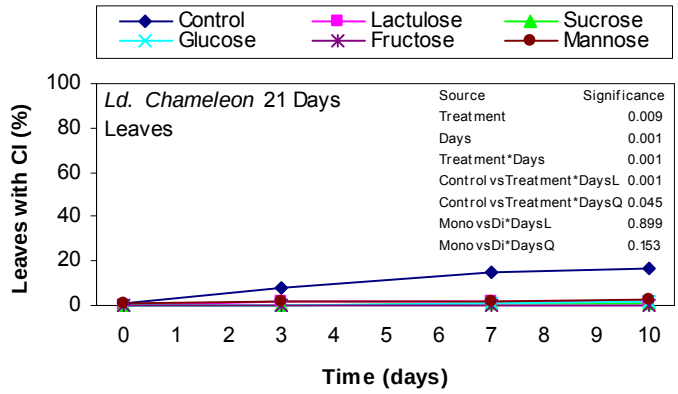


Fig. 8b

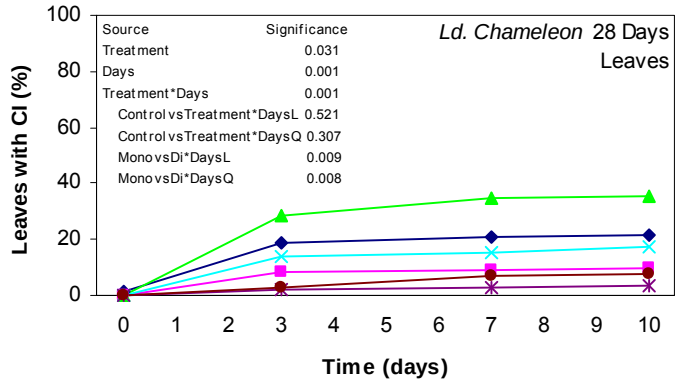


Fig. 9a, b & c. Involucral leaves (%) per stem of 'Safari Sunset' (*Ld. laureolum* x *Ld. salignum*) with chilling injury after 14, 21 or 28 days storage at 1ºC and the development thereof during a vase phase of 10 days at 22ºC, following a pre-storage pulse with a 1 % w/v sugar solution (uptake 5 ml/shoot) of lactulose, sucrose, glucose, fructose or mannose.

Fig. 9a

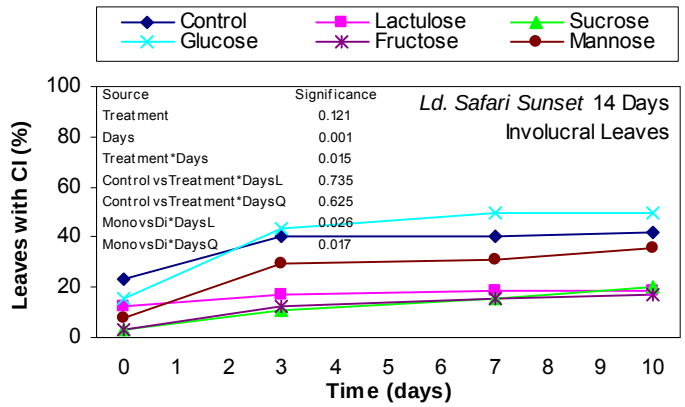


Fig. 9b

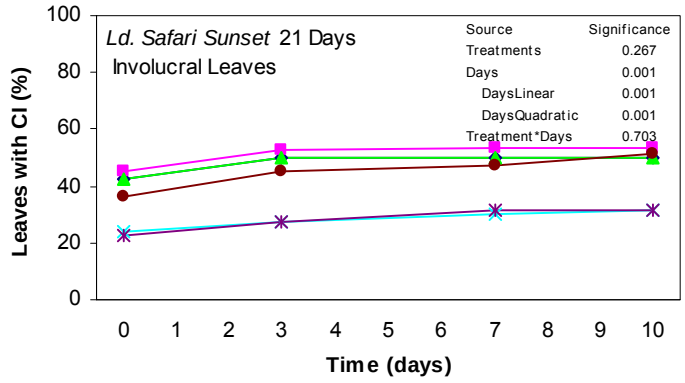


Fig. 9c

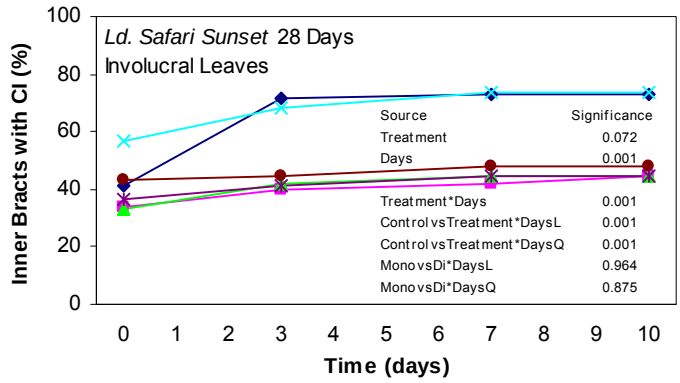


Fig. 10a, b & c. Involucral leaves (%) per stem of 'Laurel Yellow' (*Ld. laureolum* x *Ld. discolor*) with chilling injury after either 14, 21 or 28 days storage at 1ºC and the development thereof during a vase phase of 10 days at 22ºC, following a pre-storage pulse with a 1% w/v sugar solution (uptake 5 ml/shoot) of lactulose, sucrose, glucose, fructose or mannose.

Fig. 10a

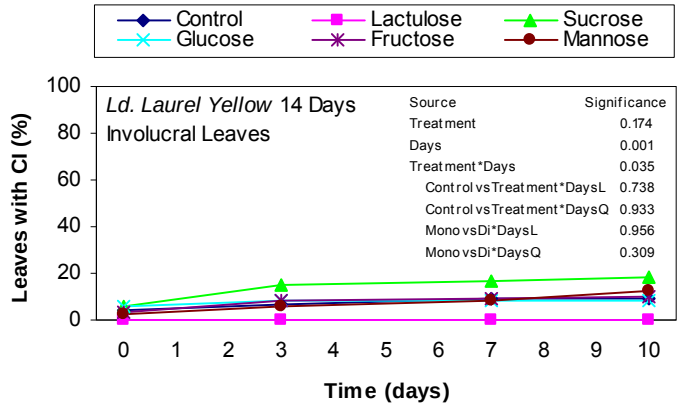


Fig. 10b

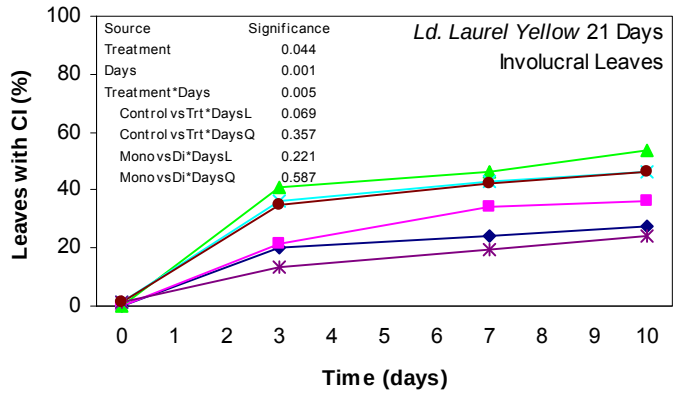


Fig. 10c

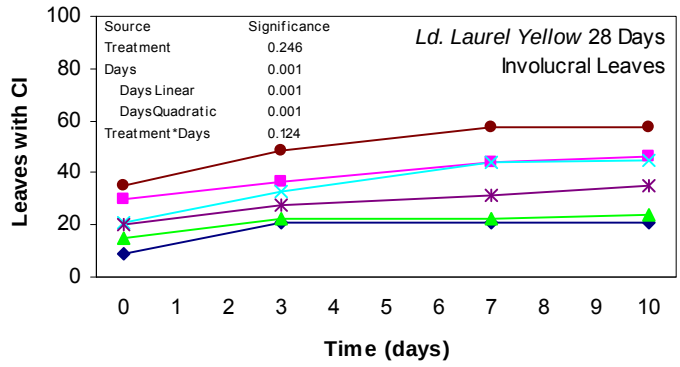


Fig. 11a, b & c. Involucral leaves (%) per stem of 'Chameleon' (*Ld. laureolum* x *Ld. eucalyptifolium*) with chilling injury after 14, 21 or 28 days storage at 1ºC and the development thereof during a vase phase of 10 days at 22ºC, following a pre-storage pulse with a 1% w/v sugar solution (uptake 5 ml/shoot) of lactulose, sucrose, glucose, fructose or mannose.

Fig. 11a

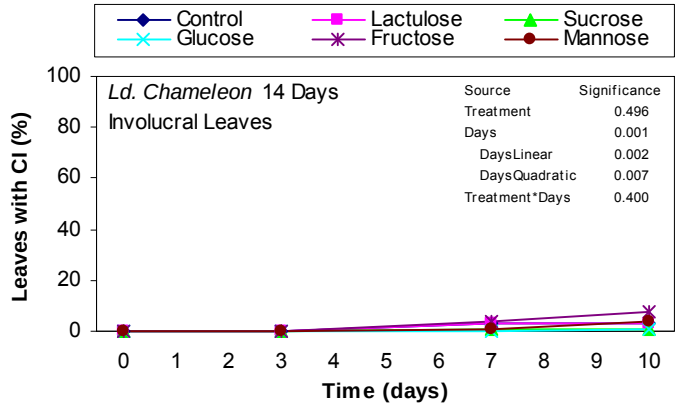


Fig. 11b

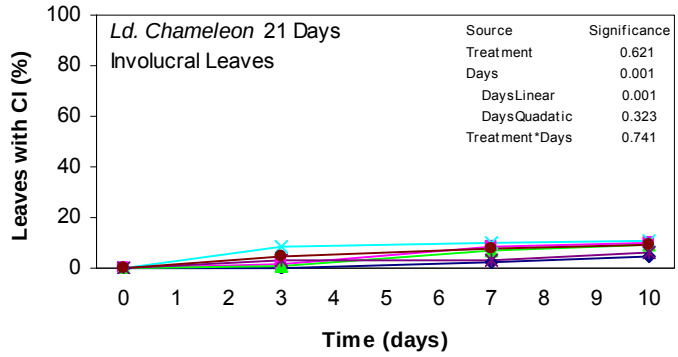


Fig. 11c

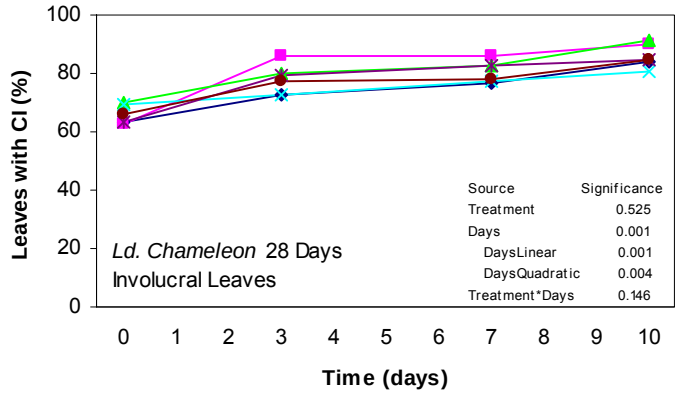


Table 3. Sugar concentration in leaves, involucral leaves and stems after uptake of 5 ml of a 1% glucose solution for three *Leucadendron* cultivars.

Cultivar and Shoot Part	Sucrose		Glucose		Fructose	
	Control	Pulsed	Control	Pulsed	Control	Pulsed
'Safari Sunset'						
Leaves	13.6 a[z]	15.2 a	5.2 a	7.6 a	2.6 a	3.6 b
Involucral Leaves	5.2 a	6.1 a	8.0 a	9.1 a	3.6 a	4.2 a
Stem	10.4 a	11.2 a	16.8 a	17.8 a	5.3 a	5.5 a
'Laurel Yellow'						
Leaves	21.1 a	20.6 a	11.9 a	11.9 a	4.9 a	5.8 a
Involucral Leaves	7.7 a	7.8 a	17.2 a	19.4 a	6.0 a	7.3 b
Stem	11.8 a	11.3 a	6.2 a	7.4 a	5.9 a	5.7 a
'Chameleon'						
Leaves	20.9 a	22.0 a	6.0 a	6.0 a	6.1 a	6.8 a
Involucral Leaves	4.7 a	6.9 a	17.6 a	20.4 a	11.8 a	12.3 a
Stem	8.5 a	11.3 b	11.9 a	15.6 a	8.8 a	9.5 a

[z]Values within sugar type and shoot part followed by different letters differ significantly at $P \leq 0.05$, Duncan's Test.

6. General Conclusions

General Conclusions

During handling, shipping and storage of *Leucospermum* cut flowers and *Leucadendron* greens, maintenance of low temperatures is essential. Low temperatures for prolonged periods have been reported to have negative effects on the cut flowers and greens.

'Drying out' of leaves during cold storage at 1ºC as well as during the vase phase after cold storage was the general symptom of chilling injury in *Leucospermum* in this study**.** This behaviour implicates loss of membrane integrity. The CI observed on the shoots stored for 24 days was higher than the shoots stored for 21 days and increased over the ten days in the vase. This is typical of CI symptoms where the longer the exposure to chilling temperatures the more severe the symptoms and symptoms may intensify at room temperature after cold storage. As the sensitivity to CI can vary according to the species, cultivar, plant part, morphological and physiological condition, temperature and duration of exposure, it can be expected to achieve varying sensitivity to CI between the different cultivars of Leucospermums and after the different exposure periods. Of the cultivars tested 'Vlam' and in particular 'Rigoletto' were more prone to develop CI than 'Gold Dust', 'High Gold' and 'Succession II'.

Leucospermums pulsed with 2 % sugar solutions controlled chilling injury with varying degrees of success. The efficacy of sugars to suppress CI in Leucospermums, to the best of our knowledge, has not been reported before nor is there an obvious explanation for this effect. An increase in the concentration of either fructose or mannose after an uptake of 50 mg per stem by pulsing with a 1% solution of these sugars was detected in the stems but not the leaves or inflorescences. Furthermore sugar concentrations decreased in all shoot parts during the cold storage and vase phase of 'High Gold' cut flowers. Even if sugars were transported from the stem to the leaves during cold storage it is unlikely that osmotic potential would be greatly affected. Reasons other than a change in osmotic potential should therefore be considered to explain the reduction in CI by pulsing Leucospermums with sugars.

Compared to the symplast, sugars in the apoplast are present in low concentrations and if pulsed with a 1% solution it would increase the concentration of apoplastic sugars greatly, relatively speaking. A higher concentration of sugars in the apoplast, as a result of pulsing may therefore, in some way or another, cause membranes to be more tolerant of low temperatures and explain the lower incidence of CI. This may also explain the efficacy in reducing CI by sugars such as sucrose, glucose and fructose that are present in relatively high concentrations in the symplast but apparently not in the apoplast of Leucospermums. This line of reasoning is supported by the efficacy of small quantities (50 mg fructose or mannose/stem) of sugar taken up per stem in reducing CI but the apoplastic sugars may be significantly higher which is not reflected when expressed on a dry weight basis. In three of the cultivars evaluated (‘Gold Dust’, ‘High Gold’ and ‘Rigoletto’), lactulose was superior to other sugars. This may be due to the inability of Leucospermums to metabolise lactulose.

When ‘Vlam’ and ‘High Gold’ were pulsed with 3-4% fructose or mannose solutions a higher incidence of CI was recorded. It is likely that in these cases the leaf symptoms were due to the concentrations used being phytotoxic.

Sugars were not as effective or were ineffective in reducing CI in ‘Succession II’ and ‘Vlam’. Pulsing with lower concentrations of sugars may therefore improve the control of CI in ‘Succession II’ and ‘Vlam’. In ‘High Gold’ pulsing with 2% mannose was effective in reducing CI in one case and not in another. In the latter case 1% mannose was, however, effective.

‘Drying out’ and browning of the leaves, and to a greater extent the involucral leaves were the most conspicuous symptom when Leucadendrons were cold stored for extended periods. Symptoms in Leucadendrons developed similarly to that in Leucospermums and increased similarly with longer cold storage periods and decreasing temperatures. Leaves were less prone to developing CI than the involucral leaves of the cultivars tested possibly related to differences in the degree of hardening off. Older leaves show less CI than softer involucral leaves which indicates that older

leaves of Leucadendrons are ‘harder’ than new involucral leaves. Leaves of ‘Safari Sunset’ appeared to be more susceptible to CI than leaves of the other cultivars tested viz. ‘Laurel Yellow’ and ‘Chameleon’. Cultivars differ in the concentration of sugars in the leaves as well as the in the partitioning between stem and leaves. Involucral leaves of ‘Laurel Yellow’ were more susceptible to CI followed by ‘Safari Sunset’ with ‘Chameleon’ apparently the most resistant. It is unknown whether the involucral leaves differed in their degree of hardening off since the time of cessation of elongation growth of the shoots were not recorded.

Leucadendrons, depending on cultivar, can be marketed in different developmental stages viz. (a) as greens when the degree of hardening off of leaves and involucral leaves will be a function of the time after cessation of elongation growth of shoots, (b) during the phase of anthesis or (c) as a cone post anthesis. It will be useful to determine the susceptibility for CI for the different developmental stages of the commercial cultivars. This will apply especially to cultivars marketed as greens. Furthermore, since hardening may also be a function of climatic and cultural factors their relationship to CI sensitivity should be quantified in order to develop practices that reduce CI sensitivity.

The sugars were not as effective in controlling CI as for Leucospermums. There was control of CI by the sugars in the leaves but no apparent control in the inner bracts except for ‘Safari Sunset’ stored for 28 days. The relatively poor control of CI with sugar pulsing may be concentration related as was shown to be the case in Leucospermums. As in the case of Leucospermums the effect of sugars to suppress CI has no obvious explanation and we speculate that the positive effects of the sugars are due to their presence in the apoplast and not the symplast. Pulsing with a 1% glucose solution (10 000 mg/l) may have increased the apoplastic concentration of sugars to a level that in some or other way protected the membranes against low temperatures and thus decreased the incidence of CI. Since pulsing with 1% sugar solution exacerbated CI of involucral leaves in a number of cases lower concentrations should be tested before commercial recommendations can be made.