

Leaf blackening and the control thereof in selected *Protea* species and cultivars

By

Nicole Elizabeth Windell

Thesis presented in partial fulfilment of the requirements for the degree of Masters of
Science (Agric) in the Faculty of Agriculture at Stellenbosch University



Supervisor: Dr. EW Hoffman

Department of Horticultural Science

Stellenbosch University

South Africa

Co-supervisor: Prof. G Jacobs

Department of Horticultural Science

Stellenbosch University

South Africa

March 2012

DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Signature:

Date:

Copyright © 2012 Stellenbosch University

All rights reserved

SUMMARY

Leaf blackening, a postharvest disorder which is characterized by a dark brown to black discoloration, is found in most commercially important *Protea* cut flower species and cultivars. As this disorder is known to increase with storage time, it is a major concern to the South African industry as the use of sea freight is increasingly preferred due to lower transport costs and a more favourable carbon footprint. The cause of leaf blackening has been strongly linked to a carbohydrate stress exerted by the large inflorescence, thus requiring the utilization of sugar bound polyphenols in the foliage, which when removed, can oxidize enzymatically or non-enzymatically.

A study where harvesting was done throughout the season as well as on selected days at 08:00, 12:00, 15:00 and 17:00, concluded that leaf blackening incidences in *Protea* cv. *Sylvia* stems varies significantly throughout the season, between years and even with the harvest time of day. Leaf blackening incidences increased from October onwards and remained high until February, before decreasing to acceptably lower levels towards March to May. Carbohydrate- and phenolic content together with water status of leaves at harvest was not able to accurately predict incidence of the associated leaf blackening. However, irrespective of the season of harvesting, leaf blackening was significantly lower when stems were harvested later in the day than compared to stems harvested in the morning. Low sucrose and high water content at these harvest times was positively correlated to high incidences of leaf blackening.

In a next study where uptake dynamics of glucose pulsing was investigated, *Protea* cv. *Sylvia* was harvested at different times throughout the day, dehydrated to various levels and pulsed with an increasing range of glucose concentrations. Pulsing solution uptake per stem was found to be highly influenced by these factors, as dehydration of stems and a harvest time later during the day both decreased stem water potential, which then increased pulse-solution uptake within a certain time period. The daily harvest time influenced transpiration, whilst pulse-solution uptake decreased with an increase in glucose pulse concentration.

When stems were pulsed pre-storage with an increasing range of glucose concentrations, not only did pulses of between 4.7 – 13.7% glucose significantly delayed the incidence of leaf blackening, but it also maintained a positive water balance longer in stems during vase life.

Ethanol or acetaldehyde vapour did not provide a viable alternative for reducing leaf blackening incidence in *Protea* cv. *Sylvia*, although a synergistic effect was found when ethanol vapour or pulsing was used in combination with glucose. A commercial verification trial disclosed that *Protea magnifica* and *Protea* 'Pink Ice' reacted more beneficial to ethanol vapour than was observed in 'Sylvia'.

This study confirms that carbohydrate availability within the *Protea* cut stem remains a key factor in the control of leaf blackening. Factors which assist in maintaining high internal carbohydrate levels, such as enhanced glucose pulse uptake or effective vase solution utilization will contribute to providing an optimum control of leaf blackening during vase life following long-term cold storage.

OPSOMMING

Loofblaarverbruining is 'n na-oes defek wat gekarakteriseer word deur 'n donker bruin na swart verkleuring wat voorkom in meeste kommersieël belangrike *Protea* snyblom spesies en kultivars. Hierdie defek is bekend daarvoor dat dit toeneem met stoortyd, dus is dit 'n groot kommer vir die Suid-Afrikaanse industrie, met toenemende gebruik van seevrag as vervoer keuse wat laer vervoer kostes en meer gunstige 'koolstof voetspoor' bevoordeel. Die oorsaak van loofblaarverbruining word sterk gekoppel aan 'n koolhidraat stres wat uitgeoefen word deur die groot bloeiwyse op die loofblare, waar suiker-gebonde polifenoliese verbindings ensiematies of nie-ensiematies geoksideer word met die verwydering van die suiker verbinding.

'n Studie waar geoes was regdeur die seisoen, sowel as op geselekteerde dae om 08:00, 12:00, 15:00 en 17:00, het bevind dat die voorkoms van loofblaarverbruining in stele van *Protea* kv. *Sylvia* aansienlik geskil regdeur die seisoen, tussen jare en selfs met die oes tyd gedurende die dag. Die voorkoms van loofblaarverbruining het toegeneem vanaf Oktober en het hoog gebly tot en met Februarie, voordat dit gedaal het tot aanvaarbare laer vlakke teen Maart, tot en met Mei. Koolhidraat-en fenoliese inhoud sowel as die water status van die blare by oes was onsuksesvol om die voorkoms van die gepaardgaande loofblaarverbruining akkuraat te voorspel. Loofblaarverbruining was egter aansienlik laer as stele geoes later in die dag teenoor stele geoes in die oggend, ongeag die seisoen van oes. Lae sukrose en 'n hoë water inhoud geassosieer met hierdie oes-tye was positief gekorreleer met 'n hoë voorkoms van loofblaarverbruining.

In 'n volgende studie waar die opname dinamika van glukose pulsing ondersoek was, is *Protea* kv. *Sylvia* stele geoes op verskillende tye dwarsdeur die dag, gedehidreer tot verskillende vlakke en met 'n toenemende reeks van glukose konsentrasies gepuls. Pulsoplossing opname per steel is sterk beïnvloed deur hierdie faktore, aangesien dehidrasie van die stele asook stele geoes later gedurende die dag die afname van steel waterpotensiaal veroorsaak het, terwyl die puls-oplossing opname versnel het binne 'n bepaalde tyd. Die tyd van oes beïnvloed ook transpirasie, terwyl vaas oplossing opname afgeneem met 'n toename in glukose puls konsentrasie.

Wanneer 'Sylvia' stele gepuls was voor stoor met 'n reeks van toenemende glukose konsentrasies, het nie net die puls van tussen 4.7 – 13.7% glukose aansienlik die voorkoms van loofblaarverbruining vertraag nie, maar dit het ook 'n positiewe water balans langer in stele gedurende die vaas lewe behou.

Nie etanol of asetaldheid dampe is bevind as geskikte alternatief vir glukose pulsing om die voorkoms van loofblaarverbruining in *Protea* kv. *Sylvia* te verlaag nie, alhoewel 'n sinergistiese effek waargeneem was wanneer etanol in kombinasie met glukose gebruik was. 'n Kommersieële

bevestigingstoetsing het bevind dat *Protea magnifica* en 'Pink Ice' meer voordeel uit 'n ethanol-damp behandeling kon trek teenoor 'Sylvia'.

Hierdie studie het bevestig die belangrikheid van koolhidraat beskikbaarheid in die *Protea* snyblom, vir beheer van loofblaarverbruining. Faktore wat die handhawing van hoë interne koolhidrate vlakke, soos bevorderde glukose puls opname of effektiewe vaas oplossing benutting sal bydra tot 'n optimal beheer van loofblaarverbruining tydens vaas lewe na langtermyn koue-opberging.

**Dedicated to my dad, mom, my three sisters and Jacobus
for always believing in me**

ACKNOWLEDGEMENTS

I am sincerely grateful to the following people and institutions:

My supervisor, Dr. Lynn Hoffman, for your shared passion in this study, great ideas, with seemingly endless possibilities and for the emotional support and your faith in me.

My co-supervisor, Prof. Gerard Jacobs, for your excellent insights, good advice and always seeing the 'big picture' with everything.

The National Research Fund (NRF) for their financial support in 2009; to the Protea Producers of South Africa (PPSA) and Productschap Tuinbouw (PT) as well as the Frank Batchelor Will Trust Grant for the financial support during the rest of my studies.

Technical and administrative staff in the Department of Horticultural Science, who was always there to help; to Tikkie, Gustav Lotzé and Renate Smit and for the 'girls', especially Shantel.

Marita van der Rijst for your statistical help.

Dr. Elke Crouch, for giving me a chance and for setting an example to always strive to become better in everything I do and for always smiling.

Dr. Elizabeth Rohwer, for your time and patience, always making the time to help and to just talk about anything and keeping me grounded.

My friends, keeping me relaxed and supporting me emotionally all the way.

My parents, Colin and Lucinda for your love, support and faith in me, as well as all my sisters, Vanessa, Colleen and Jaimé, for keeping me sane.

My soon to be family in-law, Koos, Chanelle and Bertus, for your added support and patience throughout this time.

Jacobus, for your endless love, patience and friendship, you've been a true pillar of support.

God, for your guidance in life and being the beacon of light in the dark.

TABLE OF CONTENTS

Declaration	i
Summary	ii
Opsomming	iv
Acknowledgements	vii
General introduction	1
1. LITERATURE REVIEW: A literature overview of the physiological and biochemical causes for leaf blackening in <i>Protea</i> and methods of controlling this disorder	4
1.1 Introduction	4
1.2 Factors influencing postharvest quality	6
1.2.1 <i>Flower maturity</i>	6
1.2.2 <i>Temperature</i>	7
1.2.3 <i>Light</i>	7
1.2.4 <i>Water balance</i>	8
1.2.5 <i>Carbohydrate stress</i>	10
1.2.6 <i>Ethylene</i>	13
1.2.7 <i>Mechanical damage and disease</i>	14
1.3 Biochemistry of leaf blackening	14
1.4 Treatments to control leaf blackening in <i>Protea</i>	16
1.4.1 <i>Temperature control</i>	16
1.4.2 <i>Girdling</i>	16
1.4.3 <i>Pulsing</i>	17
1.4.4 <i>Controlled atmosphere</i>	18
1.4.5 <i>Ethanol</i>	19
1.4.6 <i>Genetic selection and hybridization</i>	19
1.5 References	19
2. PAPER 1: Variation in leaf blackening of <i>Protea</i> cv. Sylvia cut flower stems over the season and with harvesting time	24
2.1 Introduction	25
2.2 Materials and methods	27
2.3 Results and discussion	32
2.4 Conclusion	39
2.5 References	40

2	<i>Tables</i>	42
2	<i>Figures</i>	45
3.	PAPER 2: The dynamics of glucose pulsing as a strategy to improve postharvest quality of <i>Protea</i> cv. <i>Sylvia</i> cut flower stems for long-term cold storage	55
3.1	<i>Introduction</i>	56
3.2	<i>Materials and methods</i>	58
3.3	<i>Results</i>	61
3.4	<i>Discussion</i>	66
3.5	<i>Conclusion</i>	69
3.6	<i>References</i>	70
3	<i>Tables</i>	73
3	<i>Figures</i>	75
4.	PAPER 3: Glucose pulse concentration affects vase-solution uptake dynamics and postharvest quality of long-term cold-stored <i>Protea</i> cv. <i>Sylvia</i> cut flower stems	79
4.1	<i>Introduction</i>	80
4.2	<i>Materials and methods</i>	82
4.3	<i>Results</i>	85
4.4	<i>Discussion</i>	88
4.5	<i>Conclusion</i>	90
4.6	<i>References</i>	91
4	<i>Tables</i>	94
4	<i>Figures</i>	96
5.	PAPER 4: Evaluating ethanol as a commercially viable treatment to control leaf blackening in selected <i>Protea</i> species	100
5.1	<i>Introduction</i>	101
5.2	<i>Materials and methods</i>	104
5.3	<i>Results</i>	108
5.4	<i>Discussion</i>	110
5.5	<i>References</i>	113
5	<i>Figures</i>	117
	General conclusion	123
	Appendix A: The efficacy of various postharvest treatments on the control of leaf blackening in <i>Protea magnifica</i> and <i>Protea</i> cv. <i>Sylvia</i>	127

The referencing and formatting style in this thesis is written according to the requirements, in general, of the *Postharvest Biology and Technology* journal.

GENERAL INTRODUCTION

Proteas and other Fynbos cut flower products are regarded to have a long vase life of up to two to three weeks (Jones et al., 1995). However, many of the *Protea* species, selections and cultivars are susceptible to a postharvest disorder called leaf blackening, which is characterized by the dark brown to black discoloration presented after harvest in the foliage. This disorder severely affects the quality and thus the vase life of these beautiful cut flowers. For this reason, some *Protea* have a high rejection rate when products reach their long-distance markets (Verhoogt, pers. comm., 2011).

Proteas are marketed internationally as an exotic cut flower in the niche product category. The industry is predominantly dependent on these international markets, as 75% of total *Protea* production products are exported annually (SAFEC report, 2011). A major challenge faced by the South African Fynbos industry to secure sustainable growth and to increase their current market share is to successfully circumvent problems associated with the transport distance required to the main export markets, which is primarily central Europe and the United Kingdom (PPECB, 2010). Increasingly over the past decade, retailers and exporters are pressurized to ship floral products via sea freight, rather than air freight, with rising fuel costs and the necessity to lower the carbon footprint being the main driving forces. Transport by sea freight places tremendous strain on maintaining superior postharvest quality of cut stems, as shipping via sea freight can take up to 21 or more days before reaching the European market, compared to three to five days via air freight. Leaf blackening incidence in *Protea* is known to increase with length of storage. Thus, to remain competitive and uphold the market demands for high quality produce with a long vase life, the control of leaf blackening is of the utmost importance.

Symptoms of leaf blackening and the factors leading to this disorder have been studied and reviewed extensively (Jones et al., 1995; Van Doorn, 2001), but still remain unclear. The extent and rate of leaf blackening in *Protea* stems postharvest varies widely between species (McConchie and Lang 1993a), clones within species, the maturity stage of the flower head, the time of year and even the time of day at which the stems are harvested (Paull and Dai, 1990). The central factor which correlates the strongest with the incidence in leaf blackening is the rapid decline in carbohydrate concentration in the leaves after harvest (McConchie et al., 1991; Bielecki et al., 1992; McConchie and Lang, 1993 a,b). This carbohydrate depletion is possibly caused by the strong sink associated with the developing flower head (Dai and Paull, 1995) which may initiate a sequence of events which leads to the development of leaf blackening (McConchie and Lang, 1993b). Leaf blackening is conceived to be caused by phenolic oxidation which then results in the characteristic black

discoloration typically expressed on the leaves. Providing exogenous sugars as a pulse (McConchie and Lang, 1993a, b) or in a holding solution (Stephens et al., 2005) has been successful to delay the onset of leaf blackening to an extent, but it does not eradicate the problem completely.

The aim of this study thus was to explore the correlation in *Protea* cv. Sylvia between the variation of postharvest leaf blackening incidence throughout the season and with harvest time during the day and the internal leaf quality parameters. Furthermore, the dynamics of the uptake process of a glucose pulse solution as influenced by various harvesting and pulsing protocols was studied as well as the influence that various glucose pulse concentrations may have on vase solution uptake and the water balance and longevity of the flowering stem. Finally, the efficacy of ethanol as an alternative treatment to glucose pulsing to reduce leaf blackening in *Protea* cvs. Pink Ice and Sylvia and *Protea magnifica* was re-evaluated, both in simulated and a commercial environment.

References

- Bieleski, R.L., Ripperda, J., Newman, J.P., Reid, M.S., 1992. Carbohydrate changes and leaf blackening in cut flower stems of *Protea eximia*. J. Am. Soc. Hortic. Sci. 117, 124 – 127.
- Dai, J.W., Paull, R.E., 1995. Source-sink relationship and *Protea* postharvest leaf blackening. J. Am. Soc. Hortic. Sci. 120, 475 – 480.
- Jones, R.B., McConchie, R., Van Doorn, W.G., Reid, M.S., 1995. Leaf blackening in cut *Protea* flowers. Hortic. Rev. 17, 173 – 201.
- McConchie, R.B., Lang, N.S., Gross, K.C., 1991. Carbohydrate depletion and leaf blackening in *Protea neriifolia*. J. Am. Soc. Hortic. Sci. 116, 1019 – 1024.
- McConchie, R.B., Lang, N.S., 1993a. Postharvest leaf blackening and preharvest carbohydrate status in three *Protea* species. Hortic. Sci. 28, 313 – 316.
- McConchie, R.B., Lang, N.S., 1993b. Carbohydrate metabolism and possible mechanisms of leaf blackening in *Protea neriifolia* under dark postharvest conditions. J. Am. Soc. Hortic. Sci. 118, 355 – 361.
- Paull, R.E., Dai, J.W., 1990. *Protea* postharvest leaf blackening: A problem in search of a solution. Acta Hortic. 264, 93 – 101.
- Perishable Products Export Control Board (PPECB), 2010. Export directory 2010. Malachite Design & Publishing, South Africa.
- South African Flower Export Council (SAFEC), 2011. Annual report. www.
- Stephens, I.A., Meyer, C., Holcroft, D.M., Jacobs, G. 2005. Carbohydrates and postharvest leaf blackening of Proteas. Hortic. Sci. 40, 181 – 184.

Van Doorn, W.G., 2001. Leaf blackening in *Protea* flowers: Recent developments. Acta Hortic. 545, 197 – 204.

LITERATURE REVIEW

A literature overview of the physiological and biochemical causes for leaf blackening in *Protea* and methods of controlling this disorder

1.1 Introduction

Proteaceae is a family indigenous to South Africa and Australia (Rebelo, 2001). Due to their uniquely large and attractive inflorescences, *Protea* cut flowers have been cultivated commercially since 1960 in South Africa (Vogts, 1982). *Protea* is also sold commercially, because of their general perceived long vase life of up to two to three weeks (Jones et al., 1995). Since then, many species of the *Proteaceae*, such as *Protea neriifolia*, *P. compacta*, *P. eximia* and *P. magnifica*, have become important cut flowers in the niche category to global markets.

However, *Protea* display a serious postharvest disorder – namely, blackening of the leaves (Jones et al., 1995). This can occur as soon as two to five days after harvest (Jones et al., 1995), which severely reduces the vase life quality and marketability of these cut flowers. Leaf blackening is particularly severe in selected species, such as *Protea neriifolia*, *P. eximia* and *P. compacta* (Jones et al., 1995; Van Doorn, 2001). Hybrids bred from these species, such as ‘Pink Ice’ (*P. compacta* x *P. susannae*) (Crick and McConchie, 1999) and ‘Sylvia’ (*P. eximia* x *P. susannae*) (Stephens, 2003) are seriously affected by this disorder. The degree to which leaf blackening occurs in these species varies widely between species (McConchie and Lang, 1993a) and even between clones of a species (Paull and Dai, 1990). It also varies over the season and between years (Jones et al., 1995). Van Doorn (2001) stated that other *Proteaceae* species and cultivars similarly show this disorder, but not as severe and consistent as in *Protea*. As such, leaf blackening has been reported in *Leucadendron* by Philosoph-Hardas et al. (2010), although this was an isolated study and might have been caused from a pathogenic origin. No (or little) leaf blackening have ever been reported in *Leucospermum* species (Van Doorn, 2001). This suggests that there may be many genetically pre-disposed factors in addition to collective environmental influences contributing to the development of leaf blackening within *Proteaceae* as a family.

The condition of leaf blackening has been described in detail by several authors in earlier studies (Paull et al., 1980; Ferreira, 1983; Brink and De Swart, 1986; De Swardt et al., 1987). Characterised by dark-brown to black discoloration on the leaves, De Swardt et al. (1987) categorised it into four distinct types, depending on the position of the leaves where it manifests first, namely leaf-tip, leaf-base, mid-rib area or lateral leaf margins (Fig. 1.1). But, because leaf

blackening rapidly spreads throughout the entire leaf blade, irrespective of the types described above, it may be the same process involved (Ferreria, 2005).

This disorder is often the single most prominent and important factor responsible for quality loss in this magnificent cut flower product. Similar factors affect the postharvest quality of *Protea* as in other commercial ornamentals (Reid, 2008). These main factors include: Flower maturity, temperature, light, water and food supply, ethylene, mechanical damage and disease. All these factors listed above can induce stress on the cut flower, if not adequately controlled. Thus, the suggested physiological cause of leaf blackening is thought to be the manifestation of some form of stress response (Jones et al., 1995). This stress response then leads to cellular membrane breakdown and the consequent substrate and enzyme interaction, resulting in the oxidation of polyphenol compounds, with the subsequent blackening of leaves (De Swardt, 1979; Paull et al., 1980; Whitehead and De Swardt, 1982; Ferreira, 1983).

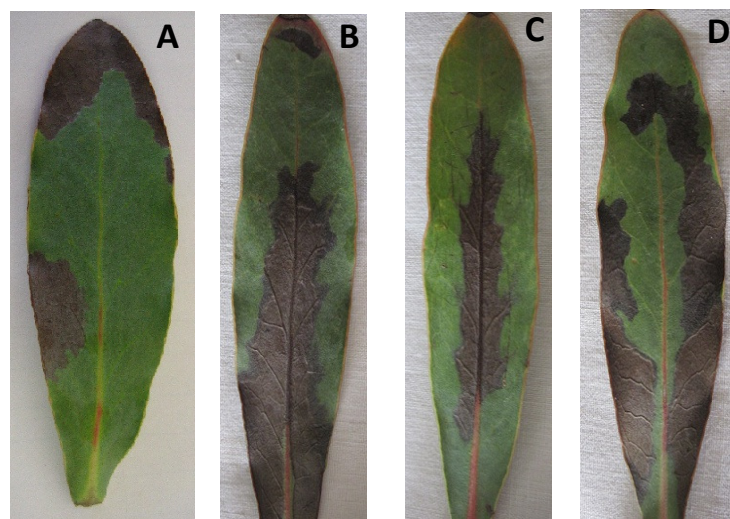


Fig. 1.1. An example of the various leaf positions where leaf blackening development can first manifest in *Protea* cv. Sylvia (*P. eximia* x *P. susannae*). **A.** leaf-tip; **B.** leaf-base; **C.** mid-rib; **D.** lateral leaf margins.

Although leaf blackening has extensively been reviewed by Jones et al. (1995) and later updated by Van Doorn (2001), this review aims to evaluate each of the factors and the underlying physiological mechanisms that may contribute to the development of postharvest leaf blackening in *Protea*. Also, studies concerning the biochemistry fundamental to the blackening process and lastly, methods for controlling leaf blackening will be discussed.

1.2 Factors influencing postharvest quality

1.2.1 Flower maturity

The minimum harvest maturity required for a cut flower to be harvested is at the stage where the flower buds still have the ability to fully open (with sufficient vase life) after transport (Kader, 2002; Reid, 2008). Harvesting flower buds at a more immature stage has the advantage of increasing packing density and decreasing risk for mechanical damage and desiccation (Reid, 2008). Roses and gladioli are harvested like this, because flower buds can be opened at a later stage with sugar pulsing, whilst both chrysanthemums and carnations are harvested at a more mature stage.

In *Protea* cut flowers, stems are harvested at the soft-tip stage (Export standards and requirements, 1997). At this stage, all florets are still enclosed but at the initial point where the bracts are just starting to retract from each other (Fig. 1.2). This closed, soft-tip stage is favoured for overseas export to minimize the risk of insects contained within an open inflorescence (Export standards and requirements, 1997). Paull and Dai (1990) reported that shoots harvested with immature inflorescences, where the involucral bracts have not yet contracted from the centre, had a higher incidence of leaf blackening than more mature and advanced inflorescences. This greater disposition to leaf blackening could most likely be linked to the significantly higher respiration rates that were measured in more immature inflorescences compared to mature, harvest-ready inflorescences (Ferreira, 1986). Joyce et al. (1995) also found lower respiration rates in mature stages of harvested *Grevillea* cv. *Sylvia* inflorescences compared to respiration rates measured of flowers in more immature stages.

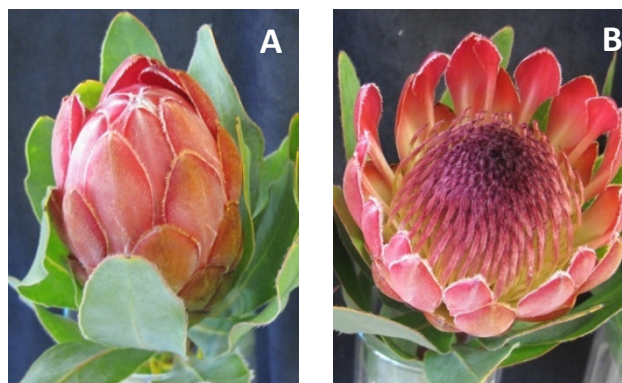


Fig. 1.2. The maturity stage of a *Protea* cv. *Sylvia* inflorescence showing (A) a more immature, but harvest-ready soft-tip stage where involucral bracts are just starting to retract from the centre and (B) a more mature and fully opened inflorescence, but not yet senesced.

1.2.2 Temperature

Respiration rates of cut flowers are generally quite high in comparison to other plant organs such as tubers. As there is a logarithmic increase in respiration with increased temperature, respiration is strongly correlated with the prevailing temperatures of the environment (Kader, 2002). Thus, cooling cut flowers as soon as possible after harvest significantly decreases the rate of senescence. For example, in roses and carnations, there were a 25 times increase in respiration rate when held at room temperature (20°C) compared to 0°C (Reid and Kofranek, 1980). Although low temperatures are beneficial in that respiration rate is significantly lowered under these conditions, some cut flowers originating from tropical or sub-tropical regions may need to be stored at a higher temperature to avoid chilling injury (Reid, 2008).

The onset and incidence rate of leaf blackening has been found to accelerate with an increase in vase life temperature (Jacobs and Minnaar, 1977a; Ferreira, 1983, 1986). Together with a rise in respiration rate (Ferreira 1983, 1986), membrane permeability also intensifies at high temperatures, leading to membrane disruption at supra-optimal temperatures (Jacobs, 1981), with the subsequent phenolic oxidation and the onset of leaf blackening.

Stephens et al. (2001) clearly showed that increasing storage temperature from 0°C to 10°C for a three day period resulted in an increased incidence of leaf blackening with a subsequent reduction in vase life of *Protea* cv. *Sylvia*. No typical chilling injury symptoms at low temperatures of approximately 0, 4.5, 7 and 10°C were reported for this cultivar (Stephens, 2003) or any other cultivar or species of *Protea* to date.

1.2.3 Light

In general, the presence of light during storage is not considered important, as most cut flowers are transported in the dark. There are exceptions where the absence of light can cause yellowing of the leaves in some cultivars of *Chrysanthemum*, *Alstroemeria* and *Lilium* when stored in the dark under higher temperatures (Reid, 2008).

In *Protea*, several studies reported that the development of leaf blackening decrease with the addition of postharvest lighting (Newman et al., 1990; Paull and Dai, 1990; McConchie et al., 1991; Jones and Clayton-Greene, 1992; Bielecki et al., 1992). Newman et al. (1990) observed leaf blackening to be significantly reduced in *P. eximia* stems when subjected to low levels of photosynthetically active radiation (PAR) of $15\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Leaf blackening was also reduced in *P. neriifolia*, *P. compacta* and *P. eximia* held under light (Jacobs and Minaar, 1977b; La Rue and La Rue,

1986). Exposing *P. neriifolia* to light intensities of $25\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ or above significantly inhibited the development of leaf blackening (Jones and Clayton-Greene, 1992). The incidence of leaf blackening considerably increased when 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was used to inhibit the photosynthetic electron transport chain of photosystem II in stems held under light. This finding distinctly links the importance of photosynthesis and the development of leaf blackening (Jones and Clayton-Greene, 1992).

From personal communications with growers, Van Doorn (2001) reported that leaf blackening was reduced where stems were held under incandescent lights in pack rooms as well as in cold rooms. Stems that were kept in the same environment, but enclosed in cardboard containers, blackened at an increased rate compared to stems kept in light. It is known that incandescent lamps emit considerable amounts of red light, which could be sufficient to saturate the phytochrome pigments within *Protea* leaves, converting it to the *Pr* form. In darkness phytochrome in the *Pr* form reverts to the active *Pfr* form, which may then cause a range of physiological responses, including leaf yellowing (Van Doorn and Van Lieburg, 1993). Van Doorn (2001) thus hypothesized that this conversion of phytochrome may also be important in the process of leaf blackening development, although to date no studies have investigated this possibility yet.

1.2.4 Water balance

Water loss or other forms of water stress is one of the main reasons for loss of postharvest quality in ornamentals, often visible as wilting (Halevy and Mayak, 1979; Reid and Kofranek, 1980). Cut flowers have a large surface area to weight ratio, therefore can potentially lose water rapidly (Kader, 2002). Lowering holding and storage temperatures reduces water loss, which is another major motivation for cooling cut flowers (Reid, 2008). After dry storage or long-term transportation, rehydration of cut flowers is possible, unless vascular blockage is present. This could be caused by air embolisms due to bacterial plugging, poor water quality or cellular metabolites. As water is transpired by the foliage and flowers of cut ornamentals, water is replaced through uptake from vase solution via the stem. Blockage however can cause more water to be lost from the system than what is being replaced, resulting in a disturbed water balance and the subsequent onset of water stress symptoms (Noordegraaf, 1999). Vascular blockage ultimately leads to a drastically reduced vase life (Halevy and Mayak, 1979; Reid, 2008).

Water stress has also been regarded as a major reason contributing to cell membrane damage and eventual leaf blackening development in *Protea* (De Swardt, 1979; Paull et al., 1980; Ferreira, 1983; Paull and Dai, 1990). However, Newman et al. (1990) and Reid et al. (1989) reported evidence

suggesting that this is not the case. Reid et al. (1989) found that placing a plastic bag over the inflorescence, thus reducing the water stress by inhibiting transpiration through the inflorescence, did not reduce leaf blackening compared to stems held without bags. In other cut flowers, water loss via the flowers or inflorescences only makes a small contribution to the total water loss (Haley and Mayak, 1979). However, the *Protea* inflorescence is substantially larger in proportion to the stem than in most cut flowers. Paull et al. (1980) was able to eliminate leaf blackening in *Protea* when the inflorescence on the stem was removed, and attributed it to reduced water demand by eliminating the flowering head. In a later study when Newman et al. (1990) girdled just below the inflorescence on the stem, leaf blackening was significantly reduced, but water uptake was unaffected. Reid et al. (1989) observed that a short period without water (10 hours at 20°C and 60% Relative Humidity) did not increase the incidence of leaf blackening in *P. eximia*.

In *P. neriifolia*, leaf blackening was reduced by re-cutting of the stem bases and replacing the holding solution daily throughout vase life (Du Plessis, 1978). Leucoanthocyanidins, which rapidly oxidize to form tannins in water, were reported to be a product leached from *Protea* stems into vase solution (De Swardt et al., 1987). The uptake of this high molecular weight polymerized tannin-like molecules then resulted in stem plugging during vase solution uptake, leading to the onset of leaf blackening (De Swardt et al., 1987). A reduction in leaf blackening incidence was reported when chemicals such as phenylmercury acetate (Masie, 1979) and lead acetate (Du Plessis, 1978; De Swardt, 1979) was used to precipitate these tannin compounds from the vase solution.

Jones et al. (1995) questioned these findings, as the role tannins play in the development of leaf blackening was not resolved. Clarity was not reached whether the reduced leaf blackening was due to the unblocking of the stem by precipitating the tannins or to a direct effect of the chemicals themselves. Many other compounds, such as proteins, carbohydrates and pectins are released from *Protea* stems in addition to tannins (De Swardt et al., 1987). Thus, microbial growth promoted by the presence of these leaching compounds can easily increase to amounts which would result in vascular blockage, with water stress and the subsequent development of leaf blackening as the outcome (Jones et al., 1995). However, when a range of anti-microbial agents which would prevent water stress from bacterial blockage were evaluated in different studies, no effect on the development of leaf blackening was reported (Van Doorn, 2001). Some of the compounds used included sodium hypochlorite (Bieleski et al., 1992), trichloro-isocyanuric acid, hydroxyquinoline citrate and silver nitrate (Van Doorn, 2001). Therefore Jones et al. (1995) and Van Doorn (2001) concluded that little evidence exists to support the concept that water stress is the primary cause of leaf blackening.

Another water-related link with increased leaf blackening involves the direct contact between *Protea* leaves and water in the form of condensation (Reid et al., 1989). Condensation usually occurs upon the interruption of the cold-chain during cold storage, where the cooled product is exposed to warmer temperatures (Van Doorn, 2001), without adequate time or conditions favouring evaporation. However, other than two reports by Van Doorn (2001) where leaf blackening is directly linked to condensation, no substantial scientific evidence has been represented to support this claim.

1.2.5 Carbohydrate stress (Food supply)

Sugars are the main substrate for respiration in flower metabolism and the availability thereof is regulated by starch and other polysaccharide hydrolysis, the rate of photosynthesis, translocation and respiration itself (Ho and Nichols, 1977). When harvested, cut flowers are limited to the existing reserves at that point in time in the cut shoot to sustain all required metabolic processes and to complete any further developmental processes.

When shoots with immature buds are favoured in harvesting for its various advantages, producers run the risk of depleting shoot reserves of those high respiring products at ambient or elevated temperatures. Such carbohydrate stress will lead to early and accelerated senescence (Noordegraaf, 1999). Roses, which are harvested at closed-bud stage, will double in their dry weight of the flower during expansion (Reid and Kofranek, 1980). Therefore, immature harvested flowers have a short vase life and poor quality when not provided with additional sugars.

Unlike fruits and vegetables, it is possible to provide additional reserves to cut flowers (Reid and Kofranek, 1980). This can be achieved by immersing cut flowering stems in water containing the required sugars and a biocide (to prevent microorganism growth). Sugar supplementation is used for bud-opening or to extend the vase life after storage, depending on the time applied and concentration used (Reid, 2004). Thus, in the cut flower industry, carbohydrate supplementation is a regular practise in many cut flowers to prepare stems for storage and for vase life extension (Halevy and Mayak, 1979, 1981; Goszczyńska and Rudnicki, 1988; Nowak et al., 1990).

The majority of studies on leaf blackening in *Protea* present evidence which conclude that postharvest leaf blackening is caused by a carbohydrate stress, mainly due to the depleting of leaf carbohydrates to sustain the continuous development of the inflorescence (Jones et al., 1995; Van Doorn, 2001).

Starch and sucrose have been identified in several studies to be the main metabolically active carbohydrates in *Protea* (McConchie et al., 1991; Bielecki et al., 1992; McConchie and Lang, 1993a,

b; Ferreira, 2005). Stephens (2003) however reported both fructose and glucose to occur in higher concentrations than sucrose within inflorescences and all three in similar amounts in the leaves at harvest of flowering stems of *Protea* cv. Sylvia.

Postharvest factors such as flower maturity, temperature, light and water stress contribute to quality loss and in the case of *Protea*, leaf blackening, can all be directly or indirectly linked to the depletion of carbohydrates. Harvesting at a more immature stage and storing *Protea* cut flowers at ambient or supra-optimal temperatures increases respiration rate and accelerates the depletion of carbohydrate reserves (Ferreira, 1986; Paull and Dai, 1990). Studies by McConchie et al. (1991) and Bielecki et al. (1992) both showed that starch and sucrose concentrations decline rapidly in the leaves of flowering stems held in the dark, compared to the increase in concentrations of these carbohydrates when held in light conditions with a PAR of $300\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ for both *P. neriifolia* and *P. eximia*. This finding illustrates that at sufficient light levels which can allow for net photosynthesis to occur, carbohydrates can be produced to sustain both the foliage and inflorescence development.

Analysis of foliage of several *Protea* cultivars revealed the presence of a polymeric soluble carbohydrate found in substantial concentrations, namely polygalatol (Bielecki et al., 1992; McConchie and Lang, 1993a, b). Polygalatol (1,5-anhydro-D-glucitol) is a sugar alcohol and a simple derivative of sorbitol (D-glucitol). After harvest, while sucrose, fructose, glucose and starch concentrations rapidly declined in *Protea* foliage, polygalatol remained relatively constant over time in both *P. neriifolia* and *P. eximia* (Bielecki et al., 1992; McConchie and Lang, 1993a). Ferreira (2005) found similar results for the hybrids *Protea* cv. Sylvia and Lady Di (*P. magnifica* x *P. compacta*), where all of the above mentioned carbohydrates declined, whilst polygalatol remained fairly constant directly after harvest and throughout long-term storage. This suggests that polygalatol does not contribute to the metabolically available carbohydrate pool. Bielecki et al. (1992) rather suggests that it might play an active role in osmotic buffering.

Carbohydrate depletion from *Protea* leaves and subsequently the stress contributing to leaf blackening has always been linked to the strong sink created by the inflorescence due to copious amounts of nectar produced (Mostert et al., 1980; Cowling and Mitchell, 1981; Ferreira, 1986; Paull and Dai, 1990; Dai, 1993). *Protea* nectar consists primarily of glucose, fructose, sucrose (in selected species) as well as xylose (Cowling and Mitchell, 1981; Van Wyk and Nicholson, 1995). Dai and Paull (1995) reportedly found that after applying radioactive C^{14} labelled sucrose to *P. neriifolia*, 50% of the radioactivity could be detected in the nectar after 24 hours. Furthermore, studies that include the removal of the flower head or girdling just below the inflorescence significantly reduced the development of leaf blackening (Paull et al., 1980; Brink and De Swardt, 1986; Reid et al., 1989; Paull and Dai, 1990; Newman et al., 1990; Dai and Paull, 1995; Stephens et al., 2001). All these studies

link sink-source relationships and photosynthate translocation from the foliage to the inflorescence with carbohydrate stress and the onset of leaf blackening (Newman et al., 1990; McConchie et al., 1991; McConchie and Lang, 1993a; Dai and Paull, 1995).

Studies on *Protea* 'Sylvia' in the Western Cape, South Africa, detected that the carbohydrate concentrations in flowering shoots tend to be significantly lower in early spring, following a typical Mediterranean winter (Hettasch et al., 2001). This period is typified by the rapid spring flush budbreak in August, followed by a growth flush extension from September to October, drawing on the already low carbohydrate reserve levels. In *Protea* 'Sylvia', a cultivar highly susceptible to leaf blackening, this vigorous spring flush growth occurs in a small space of time (Gerber et al., 2001). This spring flush extension period directly coincides with the time that *Protea* flowering stems on the plant are most susceptible to pre- and postharvest leaf blackening. Ferreira (2005) found that suppressing the spring flush growth with a plant growth regulator, Paclobutrazol ((2RS, 3RS)-1-(4-chlorophenyl)-4, 4-dimethyl-2-(1, 2, 4-triazol-1-yl) pentan-3-ol), trade-name 'Cultar', significantly reduced the postharvest development of leaf blackening by 39%, 14 days after treatment.

Providing exogenous sugars in the form of a pulse or in holding solution to extend vase life have provided additional proof for this carbohydrate stress hypothesis by extending *Protea* vase life in reducing or delaying the development of leaf blackening. However, results yielded varying and sometimes limited success. Earlier studies reported the efficacy of a very low concentration sucrose ($\leq 2\text{g.L}^{-1}$) provided in a holding solution to reduced leaf blackening effectively in *P. eximia* (Bieleski et al., 1992) and *P. neriifolia* (Brink and De Swardt, 1986; Brink, 1987; Paull and Dai, 1990; McConchie et al., 1991). Pulsing sucrose at much higher concentrations of 200g.L^{-1} for 24 hours (at 25°C) also reduced leaf blackening in *P. neriifolia* prior to storage (McConchie and Lang, 1993b).

Providing additional carbohydrates postharvest have been found to slow down the senescence process in many cut flowers, by sustaining cellular membrane integrity and mitochondrial function and by delaying the utilization of degrading proteins and other molecules in the general metabolism (Halevy and Mayak, 1979; Nowak et al., 1990). This could also be the reason for the delay in leaf blackening in *Protea* when supplemented with sugars. Haasbroek et al. (1987) observed that providing 3.5% sucrose in a holding solution containing *P. compacta* and *P. longiflora* stems while being exposed to gamma irradiation (to induce leaf blackening), leaf blackening was inhibited. In *Leucadendron* 'Silvan Red' (*L. laureolum* x *L. salignum*), providing a pulse of sucrose at 200g.L^{-1} for 24 hours at 1°C , protected stems from desiccation during a 42 day storage period at 1°C (Jones, 1995).

From the above studies, it is clear that carbohydrates are central to the process of leaf blackening, but each cultivar and species has a different response to carbohydrate supplementation,

for example, leaf blackening in *Protea magnifica* can not be successfully treated with carbohydrate supplementation. What exact role sugars play in these different cultivars and species to reduce leaf blackening requires further investigation.

1.2.6 Ethylene

Ethylene is a naturally occurring plant hormone which synchronizes senescence (Taiz and Zeiger, 2010). In climacteric fruit, ethylene facilitates ripening; which includes aspects such as the release of volatile aromas, the development of textures and starch breakdown (Tromp et al., 2005). But, being central to the process of senescence, ethylene accumulation can become detrimental to the postharvest shelf- or vase life of both fruit crops and ornamentals. Ethylene is produced by many ripening fruit and also is a by-product during combustion of organic material, such as gasoline and firewood (Kader, 2002). Even in very low concentrations (≥ 1 ppm), ethylene is detrimental to sensitive ornamentals, such as *Alstroemeria*, carnation, freesia, lily, orchids, snapdragons and sweet peas and may cause abscission of leaves and buds/flowers, petal blueing and senescence acceleration (Nowak et al., 1990).

In ethylene sensitive cut flowers, treatment with silver thiosulfate (STS) in the vase solution or as a pulse (Nowak et al., 1990), or with 1-methylcyclopropene (1-MCP) prior to storage provides inhibition or control over ethylene action (Kader, 2002). Storing cut flowers at low temperatures reduces ethylene sensitivity, together with the production thereof within flowers.

Although many cut flowers are affected by ethylene, there is little evidence of the role of ethylene in the process of leaf blackening in *Protea*. McConchie and Lang (1993b) found no relation to ethylene production from leaves and the occurrence of leaf blackening in a number of *Protea* species. Although data was not shown, Stephens (2003) also reported no difference in vase life in terms of leaf blackening when *Protea* 'Sylvia' was exposed to a continuous air flow containing ethylene ($50\mu\text{L.L}^{-1}$). Also, when Newman et al. (1990) and Bielecki et al. (1992) treated *P. eximia* cut stems with 4nmol silver triosulphate (STS), they found no reduction in leaf blackening compared to control stems. However, these studies were only done with one type of inhibitor (STS) and at a single concentration. On the contrary, Van Doorn (2001) suggested that the presence of fruit, such as apples, during postharvest storage and transport may have been reported to have a negative influence on leaf blackening occurrence in *Protea magnifica*, but have yet to be quantified.

1.2.7 Mechanical damage and Disease

Both mechanical damage from handling and lesions caused by disease before, during and after storage can have a major impact on the longevity and vase life potential of any perishable product (Reid, 2008).

In *Protea*, pre-harvest leaf blackening has been suggested to be caused by mechanical damage and fungal infection (Jones et al., 1995), where leaf blackening can be detected pre-harvest around a damaged area. Also, leaf blackening can be detected after storage on leaves where chaffing and handling damaged was inflicted prior to storage.

This incidence of leaf blackening is probably due to cellular and membrane damage, from physical damage, which then results in non-enzymatic phenolic oxidation, causing the discoloration (Jones et al., 1995).

1.3 Biochemistry of leaf blackening

Rapid carbohydrate depletion is currently hypothesized to be the main cause for stress that leads to the development of leaf blackening. However, connecting the depletion of carbohydrates to the reaction response of leaf blackening is still not fully understood (Van Doorn, 2001). Cell death is excluded, as constant respiration rates have been measured on blackened whole leaves (McConchie and Lang, 1993b) in comparison to pulsed, non-blackened leaves. But, as this was an isolated study, Van Doorn (2001) suggested that a 'false'-positive respiration rate could have been recorded, as oxidation reactions involved in leaf blackening could also have accounted for the measured respiration values. Following the process of carbohydrate depletion in plant cells, protein degradation is most often known to occur next, to substitute for depleted respiration substrates, subsequently leading to an accumulation of ammonium in the vacuoles (Halevy and Mayak, 1981). To date, no study has investigated this process and its possible relationship to leaf blackening in *Protea*.

Phenolics in plant cells are usually stored in the vacuoles or bound to other products in the cell walls after synthesis (Taiz and Zeiger, 2010). Phenolic compounds are known to be abundant in *Proteaceae* (Van Rheede van Oudtshoorn, 1963). These compounds are colourless in a reduced state, but turn brown or black when oxidized or polymerized (De Swardt, 1979). Thus, leaf blackening is ascribed to induced stresses, causing oxidation of these phenolic compounds (Paull et al., 1980; Ferreira, 1983). This oxidation process can occur either enzymatically or non-enzymatically

(Kader, 2002). Until now, it is still unclear which process of oxidation causes leaf blackening in *Protea* or if both processes are involved (Van Doorn, 2001).

Blackening in plants is often due to two main enzymes: peroxidase and polyphenol oxidase (PPO) (Van Doorn, 2001). Peroxidase and PPO activity was measured *in vitro* in *Protea* cv. Pink Ice (*P. compacta* x *P. susannae*) either held in darkness or 12 hours in light (McConchie et al., 1994). No increase or significant difference was found between treatments for levels of peroxidase activity. There was however a 10 fold increase in PPO activity for stems held in the light (which did not blacken in the recorded period). There was thus no correlation found between leaf blackening and the above-mentioned enzyme activity. PPO activity, measured *in vitro* in *P. neriifolia* leaves, was recorded to be high. When PPO activity was similarly measured in *Leucospermum*, no activity could be detected (Dai and Paull, 1997). Dai and Paull (1997) thus concluded that *Leucospermum*, which does not develop leaf blackening, might contain an inhibitor of *Protea* PPO. An *in vitro* measurement of PPO activity is not a true reflection of *in vivo* activity, because *in vitro* analysis requires the enzyme to be released from the chloroplast or peroxisomes (peroxidase) to react with its substrate (Van Doorn, 2001). Thus, membrane degradation or disruption is still required, to allow contact with the phenolics that are mainly stored in the vacuole (Jones et al., 1995). McConchie and Lang (1993b) found no convincing evidence that membrane degradation takes place before leaf blackening initiation. This conclusion was reached after no increase in oxidized glutathione, indicative of an oxidative stress, or malondialdehyde, a by-product of lipid peroxidation, also an indication of membrane integrity loss, was detected during the onset of leaf blackening. Although, there are other ways of measuring membrane degradation, thus such a conclusion can not be stated as fact.

For *Protea* species known to be susceptible to leaf blackening, many of the carbohydrates were reported to be bound to phenolics in the form of an O-glycoside ester (Perold, 1993). Perold (1993) thus suggested that a continuous demand from the large inflorescence sink may stimulate or induce cleavage of these sugar esters through the enzyme glucosidase. This enzymatic action releases the sugar, to be translocated to the inflorescence, while a reactive phenolic compound is exposed to be oxidized non-enzymatically in the presence of oxygen, resulting in leaf blackening. Activity of β -D-glucosidase also measured *in vitro*, was found to significantly increase just before the incidence of leaf blackening could be detected (Jones and Cass, 1996). Glucosidase activity also remained constant in leaves held under light, under which conditions blackening did not occur. Testing this hypothesis, Jones and Cass (1996) inhibited the glucosidase enzyme by supplying *Protea* cut flower stems with solutions containing ions of Zn^{2+} and Cu^{2+} or immersing the cut flowers in either a 20 or 50% ethanol solution and measuring the enzyme activity *in vitro*. Leaf blackening was

not reduced when zinc or copper was added, but was significantly delayed when ethanol was used. However, as ethanol inhibits numerous enzymes, this was not adequate proof that it is β -D-glucosidase in particular that is involved in the process of leaf blackening (Van Doorn, 2001).

Thus, many uncertainties still exist on the biochemical process underlying the onset and development of leaf blackening in *Protea*. Van Doorn (2001) suggested an ultra-structural analysis by means of transmission and scanning electron microscopy to further unravel this elusive process.

1.4 Treatments to control Leaf blackening in *Protea*

The primary motivation for determining the factors and understanding the processes involved in leaf blackening (as discussed above), or any postharvest disorder in a perishable crop, is to be able to develop methods to inhibit, control or reduce the disorder and thereby improve the quality and commercial longevity of that crop. The following methods have been suggested to provide some control of leaf blackening in *Protea*.

1.4.1 Temperature control

Cooling *Protea* cut flowers rapidly as soon as possible after harvest and packing reduces the respiration rate, and thus is an effective way to control leaf blackening (Van Doorn, 2001). Stephens (2003) confirmed that a reduction in leaf blackening can be achieved by decreasing storage temperatures. The optimum temperature suggested for storage is as close to 0°C as possible (Stephens, 2003), as no chilling injury has been reported for *Protea* stems to date.

1.4.2 Girdling

Girdling, a process by which a ring of bark just below the inflorescence on the stem is removed, is a very effective way of delaying leaf blackening. This is affected by inhibiting inflorescence sink demands, thus reducing the rapid depletion of carbohydrates (Reid et al., 1989; Newman et al., 1990; Stephens et al., 2001). This option, usually performed with a knife or small blade, has become a trustworthy, although time consuming, commercial practice as a treatment for leaf blackening (Van Doorn, 2001). Van Doorn (2001) also suggests a few other girdling methods of applying in effect, such as using heat (electricity through wire or a type of laser device) to sever the phloem or to remove a ring of the uppermost leaves, which will in effect also separate the bark. Caution should be applied when using girdling, as removing the ring of bark can weaken the stem

below the inflorescence and increase the risk for breaking off the inflorescence throughout further packing and handling.

1.4.3 Pulsing

Pulsing with sugars has become essential to producing high quality *Protea* cut flowers that are susceptible to leaf blackening. This technique is especially effective for long term storage during transport (McConchie and Lang, 1993b). As reserve carbohydrates, in the form of starch, was shown to rapidly decrease within the first 24 hours after harvest (McConchie and Lang, 1993a; Ferreira, 2005); pulsing is required as soon as possible postharvest. Additional studies however showed certain *Protea* cultivars and species to respond better to glucose pulsing than sucrose (Stephens et al., 2001). Providing a holding solution of 1 or 2% glucose, after a cold storage period of 10 days, has provided significant reduction in leaf blackening in the cultivars 'Brenda' (*P. compacta* x *P. burchellii*), 'Cardinal' (*P. eximia* x *P. susannae*), 'Carnival' (*P. compacta* x *P. neriifolia*) and 'Sylvia' (*P. eximia* x *P. susannae*), but not in 'Pink Ice' (*P. compacta* x *P. susannae*) and 'Susara' (*P. magnifica* x *P. susannae*) (Meyer, 2003). Although, Ferreira (2005) reported that some *Protea* cultivars or species respond differently to a range of sugars, when provided as 2% holding solutions of either sucrose, fructose, glucose or galactose irrespective, glucose consistently gave the best results in all species or cultivars. The only caution in using pulsing as a technique to control leaf blackening is to avoid phytotoxicity within the leaves. Stephens (2003) and Meyer (2003) reported this phenomenon which could result from pulsing with too high concentrations of glucose, or for an extended period, where glucose could accumulate over time within the leaves to toxic levels. Phytotoxicity symptoms appear similarly to that of the leaf blackening disorder, with the exception that the lesions are more a light-brown colour than black and usually occur in the leaves at the distal end where the pulsing solution is administered (Fig. 1.3A). After storage though, phytotoxicity may also be detected as bract browning in the involucre bracts (Fig. 1.3B).

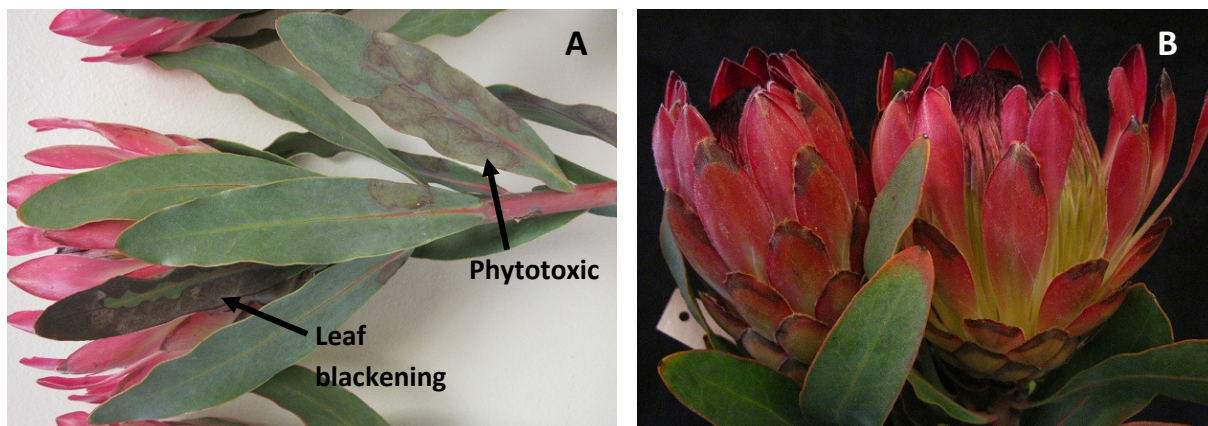


Fig. 1.3 *Protea* cv. Sylvia displaying leaf blackening and phytotoxic symptoms in the leaves (A) and after storage at the tips in the involucral bracts of the inflorescence (B).

Although providing an anti-microbial agent to the pulsing or holding solution is preferred in postharvest handling of most cut flowers (Van Doorn, 2001), Meyer (2003) found most of the *Protea* cultivars, to be sensitive to a standard concentration of hypochloride ($0.05\text{g}\cdot\text{L}^{-1}$), as leaf blackening was exaggerated in non-pulsed, control stems containing hypochloride compared to non-pulsed, controls kept just in water.

As pulsing stems is one of the most reliable technologies available thus far to reduce leaf blackening, further research is needed to refine these pulsing methods and investigate the pre- and postharvest factors driving the uptake of pulsing solutions, as leaf blackening is not constant between cultivars and species, or between seasons and even production years.

1.4.4 Controlled atmosphere (CA)

Many fruit crops are routinely cold-stored under controlled atmosphere, as this significantly suppresses respiration (Kader, 2002). Storing *P. neriifolia* stems in an atmosphere with low oxygen (1%) and higher carbon dioxide levels (5%) delayed leaf blackening during storage compared to controls (Jones and Clayton-Greene, 1992). However, Van Doorn (2001) reported that recent evidence suggests that upon removing cut flowers from CA, leaf blackening rapidly develops. In an experiment where the efficacy of ethanol vapour to control leaf blackening was evaluated, control stems kept in closed bags, also had decreased leaf blackening during vase life. This observation was ascribed by the authors to the accumulation of carbon dioxide from respiring stems, thus inhibiting respiration (Crick and McConchie, 1999).

1.4.5 Ethanol

There have been a few reports on the advantages using ethanol to reduce leaf blackening, either as a vapour (Crick and McConchie, 1999), dipped or held in an ethanol-containing solution (Jones and Cass, 1996). This was evaluated after significant reduction in apple scald was reported for scald-susceptible apples when treated either with an ethanol dip or vapour (Ghahamani and Scott, 1998). In an extended report, where Cannon and McConchie (2001) evaluated the efficacy of ethanol vapour and ethanol in pulsing or holding solutions as a method for controlling leaf blackening, a concentration range of between 6.5 – 7.0g ethanol vapour per kg stem fresh weight was found to be the most effective treatment for *Protea* cv. Pink Ice. However, results within treatments were highly variable and toxicity was eminent at higher concentrations (Cannon and McConchie, 2001). Although pulsing and holding solutions with ethanol could delay leaf blackening, it was accompanied by a loss in flower quality, making this technique unsuitable for commercial application to reduce leaf blackening (Cannon and McConchie, 2001). Further research is thus required to extend the use of ethanol on other *Protea* species and cultivars as well as to overcome practical barriers associated with the application of ethanol on a commercial scale.

1.4.6 Genetic selection and hybridization

Another long-term solution for producers to overcome leaf blackening is to cultivate species and cultivars that are selected to be less susceptible for leaf blackening. According to Van Doorn (2001), there is an ample amount of germ-plasm in the genus *Protea*, and thus breeding for reduced leaf blackening can be achieved. But until then, producers and exporters alike will have to rely on postharvest technology to control this serious disorder (Paull and Dai, 1990).

1.5 References

- Bieleski, R.L., Ripperda, J., Newman, J.P., Reid, M.S., 1992. Carbohydrate changes and leaf blackening in cut flower stems of *Protea eximia*. J. Am. Soc. Hort. Sc. 117, 124 – 127.
- Brink, J.A., 1987. The influence of the flower-head on leaf browning of cut *Protea neriifolia*. Protea News. 5, 11 – 12.
- Brink, J.A., De Swardt, G.H., 1986. The effect of sucrose in vase solution on leaf browning of *Protea neriifolia* R. Br. Acta Hortic. 185, 111 – 119.

- Cannon, A., McConchie, R., 2001. Controlling leaf blackening in *Protea*. A report for the Rural Industries Research and Development Corporation, RIRDC publication No. 01/098, Project No. US-89A, Australia.
- Cowling, R.M., Mitchell, D.T., 1981. Sugar composition, total nitrogen and accumulation of C₁₄ assimilates in floral nectaries of *Protea* species. J. S. Afr. Bot. 47, 743 – 750.
- Crick, S.G., McConchie, R., 1999. Ethanol vapour reduces leaf blackening in cut flower *Protea* 'Pink Ice' stems. Postharvest Biol. Technol. 17, 227 – 231.
- Dai, J-W., 1993. Postharvest leaf blackening in *Protea neriifolia* R. Br. Ph D. Diss., Univ. Hawaii at Manoa, Honolulu, Hawaii.
- Dai, J-W., Paull, R.E., 1995. Source-Sink relationship and *Protea* postharvest leaf blackening. J. Am. Soc. Hortic. Sc. 120, 475 – 480.
- Dai, J-W., Paull, R.E., 1997. Comparison of leaf susceptibility to enzymatic blackening in *Protea neriifolia* R. Br. and *Leucospermum* 'Rachel'. Postharvest Biol. Technol. 11, 101 – 106.
- De Swardt, G.H., Pretorius, J., Burger, L., 1987. The browning of foliage leaves in *Proteas* – a review. *Protea news* 6, 4 – 9.
- De Swardt, G.H., 1979. Blackening of *Protea* leaves. South African *Protea* Producers and Exporters Association (SAPPEX) Res. Paper 13. <http://www.sappex.org.za>
- Du Plessis, D.G.C., 1978. Leuko-antosianienbinders as beheermaatreël vir die voorkoming van bruinwording in *Protea* loofblare. M.Sc. Thesis, Randse Afrikaanse Univ., Johannesburg, South Africa.
- Export standards and requirements, 1997. Cut flowers and ornamental foliage. Part 3, Directorate food safety and quality assurance, pp. 30 – 51. <http://www.nda.agric.za>
- Ferreira, D.I., 1983. Prevention of browning of leaves of *Protea neriifolia* R. Br. Acta Hort. 138, 273 – 276.
- Ferreira, D.I., 1986. The influence of temperature on the respiration rate and browning of *Protea neriifolia* R. Br. inflorescences. Acta Hort. 185, 121 – 129.
- Ferreria, A., 2005. Further studies on leaf blackening in *Protea*. M.Sc. Thesis, Stellenbosch Univ., Stellenbosch, South Africa.
- Gerber, A.I., Theron, K.I., Jacobs, G., 2001. Synchrony of inflorescence initiation and shoot growth in selected *Protea* cultivars. J. Am. Soc. Hortic. Sci. 126, 182 – 187.
- Ghahamani, F., Scott, K.J., 1998. The action of ethanol in controlling superficial scald of apples. Aus. J. Agric. Res. 49, 199 – 205.
- Goszyńska, D.M., Rudnicki, R.M., 1988. Storage of cut flowers. Hortic Rev. 10, 35 – 62.

- Haasbroek, F.J., Rousseau, G.G., De Villiers, J.F., 1973. Effect of gamma-rays on cut blooms of *Protea compacta* R.Br., *P. longiflora* Lamarck and *Leucospermum cordifolium* Salisb. Ex Knight. *Agroplantae* 5, 33 – 42.
- Halevy, A.J., Mayak, S., 1979. Senescence and postharvest physiology of cut flowers. Part 1. *Hortic. Rev.* 1, 204 – 236.
- Halevy, A.J., Mayak, S., 1981. Senescence and postharvest physiology of cut flowers. Part 2. *Hortic. Rev.* 3, 59 – 143.
- Hettach, H.B., Theron, K.I., Jacobs, G., 2001. Dry mass allocation and carbohydrate allocation in successive growth flushes of *Protea* cultivars 'Sylvia' and 'Cardinal' shoots. *Acta Hortic.* 545, 215 – 225.
- Ho, L.C., Nichols, R., 1977. Translocation of ¹⁴C-sucrose in relation to changes in carbohydrate content in rose corollas cut at different stages of development. *Ann. Bot.* 41, 243 – 256.
- Jacobs, G., Minnaar, H.R., 1977a. Effect of temperature on the blackening of *Protea* leaves. *SAPPEX newsletter* 17, 20 – 24.
- Jacobs, G., Minnaar, H.R., 1977b. Effect of light on the blackening of *Protea* leaves. *SAPPEX newsletter* 18, 18 – 20.
- Jacobs, G., 1981. Post harvest handling of Proteas. In: P. Matthews (Editor), *The growing and marketing of Proteas*. ProteaFlora Enterprises, Melbourne, 40 – 52.
- Jones, R.B., 1995. Sucrose prevents foliage desiccation in cut *Leucadendron* 'Silvan Red' during cool storage. *Postharvest Biol. Technol.* 6, 293 – 301.
- Jones, R.B., Clayton-Greene, K.A., 1992. The role of photosynthesis and oxidative reactions in leaf blackening of *Protea neriifolia* R. Br. leaves. *Sci. Hortic.* 50, 137 – 145.
- Jones, R.B., Cass, A., 1996. The possible involvement of a β -D-glucosidase in postharvest leaf blackening in cut *Protea* flowers. *J. Int. Protea Assoc.* 31, 36 – 44.
- Jones, R.B., McConchie, R., Van Doorn, W.G., Reid, M.S., 1995. Leaf blackening in cut *Protea* flowers. *Hortic. Rev.* 17, 173 – 201.
- Joyce, D.C., Shorter, A.J., Joyce, P.A., Beal, P.R., 1995. Respiration and ethylene production by harvested *Grevillea* 'Sylvia' flowers and inflorescences. *Acta Hortic.* 405, 224 – 231.
- Kader, A., 2002. Postharvest technology of horticultural crops. *Postharvest handling systems: Ornamental crops* (Reid, M.S.). Third ed. 25, 315 – 325.
- La Rue, B.H., La Rue, R.L., 1986. Postharvest information from a *Protea* grower, shipper and importer's point of view. *Acta Hortic.* 185, 131 – 136.
- Masie, W.E., 1979. Fenielerkuri-asetaat as voorkomingsmiddel vir loofblaarverbruining by *Protea neriifolia* R. Br. M.Sc. Thesis, Randse Afrikaanse Univ., Johannesburg, South Africa.

- McConchie, R., Lang, N.S., 1993a. Carbohydrate metabolism and possible mechanisms of leaf blackening in *Protea neriifolia* under dark postharvest conditions. *J. Am. Soc. Hortic. Sc.* 118, 355 – 361.
- McConchie, R., Lang, N.S., 1993b. Postharvest leaf blackening and pre-harvest carbohydrate status in three *Protea* species. *HortSci.* 28, 313 – 316.
- McConchie, R., Lang, N.S., Gross, K.C., 1991. Carbohydrate depletion and leaf blackening in *Protea neriifolia*. *J. Am. Soc. Hortic. Sc.* 116, 1019 – 1024.
- McConchie, R., Lang, N.S., Lax, A.R., Lang, G.A., 1994. Re-examining polyphenol oxidase, peroxidase and leaf blackening activity in *Protea*. *J. Am. Soc. Hortic. Sc.* 119, 1248 – 1254.
- Meyer, C., 2003. Carbohydrates and leaf blackening in *Protea*. M.Sc. Thesis, Stellenbosch, Univ. Stellenbosch, South Africa.
- Mostert, D.P., Siegfried, W.F., Louw, G.N., 1980. *Protea* nectar and satellite fauna in relation to the food requirements and pollinating role of the Cape sugarbird. *S. Afr. J. Sc.* 76, 409 – 412.
- Newman, J.P., Van Doorn, W., Reid, M.S., 1990. Carbohydrate stress causes leaf blackening in *Proteas*. *Acta Hortic.* 264, 103 – 108.
- Noordegraaf, C.V., 1999. Problems of postharvest management in cut flowers. *Acta Hortic.* 482, 53 – 58.
- Nowak, J., Rudnicki, R.M., Duncan, A.A., 1990. Postharvest handling and storage of cut flowers, florist greens, and potted plants. Timber Press, Portland, Oregon.
- Paull, R.E., Dai, J-W., 1990. *Protea* postharvest black leaf, a problem in search of a solution. *Acta Hortic.* 264, 93 – 101.
- Paull, R.E., Goo, T., Criley, R.A., Parvin, P.E., 1980. Leaf blackening in cut *Protea eximia*: importance of water relations. *Acta Hortic.* 113, 159 – 166.
- Perold, G.W., 1993. Consistency and variation in metabolite patterns of South African Proteaceae: a chemical perspective. *S. Afr. J. Sc.* 89, 90 – 93.
- Philosoph-Hadas, S., Perzelan, Y., Rosenberger, S., Droby, S., Meir, S., 2010. *Leucadendron* 'Safari Sunset': Postharvest treatments to improve quality of cut foliage during prolonged sea shipment. *Acta Hortic.* 869, 207 – 217.
- Rebelo, T., 2001. SASOL Proteas, A field guide to the Proteas of Southern Africa. Second Edition, Fernwood Press, Vlaeberg, South Africa.
- Reid, M.S., 2004. The Commercial Storage of Fruits, Vegetables, and Florist and Nursery Stocks: Cut flowers and greens. *Agric. Handbook, USDA, ARS.* Nr. 66.
- Reid, M.S., 2008. Perishable Cargo manual: Handling of cut flowers for air transport. International Air Transport Association (IATA), 6th Ed., pp. 175 – 186.

- Reid, M.S., Kofranek, A.M., 1980. Postharvest physiology of cut flowers. *Chron. Hortic.* 20, 25 – 27.
- Reid, M.S., Van Doorn, W.G., Newman, J.P., 1989. Leaf blackening in *proteas*. *Acta Hortic.* 261, 81 – 84.
- Stephens, I.A., 2003. Leaf blackening of Proteas. Ph.D. Diss., Stellenbosch Univ., Stellenbosch, South Africa.
- Stephens, I.A., Holcroft, D.M., Jacobs, G., 2001. Low temperatures and girdling extend vase life of 'Sylvia' *proteas*. *Acta Hortic.* 545, 205 – 214.
- Taiz, L., Zeiger, E., 2010. *Plant physiology*. Fifth ed. Sinauer Associates, Inc. MA, U.S.A.
- Tromp, J., Webster, A.D., Wertheim, S.J., 2005. *Fundamentals of temperate zone tree fruit production: Fruit ripening & quality*. Buckhuys Publishers, Leiden, Netherlands.
- Van Doorn, W.G., 2001. Leaf blackening in *Protea* flowers: recent developments. *Acta Hortic.* 545, 197 – 204.
- Van Doorn, W.G., Van Lieburg, M.J., 1993. Interaction between the effects of phyto-chrome and gibberellic acid on the senescence of *Alstroemeria pelegrina* leaves. *Physiol. Plant.* 89, 182 – 186.
- Van Reede van Outshoorn, M.C.B., 1963. Distribution of phenolic compounds in some South African Proteaceae: a contribution to the chemotaxonomy of the family. *Planta Med.* 4, 399 – 406.
- Van Wyk, B-E., Nicholson, S.W., 1995. Xylose is a major nectar sugar in *Protea* and *Faurea*. *J. S. Afr. Sc.* 91, 151 – 152.
- Vogts, M., 1982. *South Africa's Proteaceae: know them and grow them*. C. Struik (Pty) Ltd., Cape Town, South Africa.
- Whitehead, C.S., De Swardt, G.H., 1982. Extraction and activity of polyphenoloxidase and peroxidase from senescing leaves of *Protea neriifolia*. *S. Afr. J. Bot.* 1, 127 – 130.

Variation in leaf blackening of *Protea* cv. *Sylvia* cut flower stems over the season and with harvesting time

Abstract

Leaf blackening is a postharvest disorder associated with most commercially important *Protea* species and cultivars. The expression of leaf blackening varies significantly between species and cultivars, even within selections of the same species, as well as during the season and time of day of harvesting. These variables that impact on leaf blackening influence the marketability of these niche products as consignments from the same production area could have vastly different quality outcomes. Unpredictability in leaf blackening incidences seriously affects the reliability of *Protea* as a cut flower as the interaction of these factors renders the trading of this species as risky to both exporters and retailers. *Protea* cv. *Sylvia* is a major commercially important cultivar, mainly because of its year-round flowering habit, relatively high yield and deep-red attractive flower head. However, one major disadvantage associated with 'Sylvia' is that it suffers severely from postharvest leaf blackening. The aim of this study was to record the variation in leaf blackening for this cultivar as expressed throughout the year as well as with various harvesting times during the day. The carbohydrate and phenolic content together with water status of the foliage subtending the inflorescence was determined in an attempt to correlate internal stem quality to the incidence of leaf blackening. Flowering stems were harvested throughout the seasons, from October 2008 until September 2010. At each harvest, five stem replicates were evaluated for leaf blackening, whilst the foliage of an additional five stems were analysed for total carbohydrate, phenolic and water content. In 2009, the orientation of the inflorescence on the plant at harvest was also recorded. On three harvesting dates in September, November and December 2009 stems were harvested at 08:00, 12:00 and 15:00 (+2 GMT), where after similar evaluation protocols and chemical analysis was followed as described for the seasonal study. Also, an extended harvest followed in February 2010, with two additional harvest times at 17:00 the previous day and at 10:00 on the day of harvest. Results from the seasonal study showed that leaf blackening is high early in the year and towards the end of the year with the lowest incidence recorded from April to August. Carbohydrate and phenolic content or water status of leaves at harvest was not able to accurately predict the incidence of the associated leaf blackening. Irrespective of the season of harvesting, leaf blackening was lower when harvested later in the day than compared to stems harvested in the morning. For these harvests low sucrose and reducing sugars and high water content at harvest was positively correlated to high incidences of leaf blackening. Despite this correlation of sucrose and water content (%) to leaf blackening, there was still a high degree of variation which could not be explained by the parameters measured. One of these variations could possibly be attributed to the bearing position of the flowering stem on the plant, as flowering stems which were not harvested terminally, had a lower incidence of leaf blackening. Further research is warranted to investigate other factors that could account for the variation associated with leaf blackening.

Keywords: Carbohydrates, total phenolics, polygalatol, harvesting temperature, water content

2.1 Introduction

Leaf blackening, a serious postharvest disorder displayed as a brown to black discoloration on the leaves (Jones et al., 1995) is expressed in most commercially important *Protea* cut flowers. This disorder renders the quality of these flowering stems commercially unacceptable, on both the local and export markets. In some instances, leaf blackening may already develop before harvest (Stephens, 2003) in the orchard, but mostly it develops within two to five days postharvest (Newman et al., 1990; McConchie et al., 1991; Jones and Clayton-Greene, 1992). As the main markets for the South African Fynbos industry is central Europe, United Kingdom (UK) and to a lesser extent North America and the Middle East (PPECB, 2010), Proteas are usually air freighted to these international markets to deliver a good quality product within the shortest time. However, *Protea* cut flowers do have a potentially long vase life of up to two to three weeks (Jones et al., 1995), provided that leaf blackening is not expressed in the foliage. Motivated by increasing pressure over the past few years from the major retailers and consumers alike to lower the carbon footprint and transport costs, *Protea* cut flowers are now increasingly being transported via sea freight. It poses an urgent challenge to researchers to find a solution to this persistent disorder, as shipping time increases from two to three days to approximately 19 to 21 days when using sea freight for South African Fynbos products.

Leaf blackening is not equally severe in all *Protea* species, selections or cultivars. The major *Protea* species of commercial importance that are susceptible to leaf blackening is reported to be *Protea eximia*, *P. neriifolia* and *P. compacta* (Jones et al., 1995; Van Doorn, 2001). Also, hybrid cultivars which share parentage to one of the species listed above are found to be similarly susceptible, such as *Protea* cv. Pink Ice (*P. compacta* x *P. susannae*) and a cultivar of particular importance for South African producers, *Protea* cv. Sylvia (*P. eximia* x *P. susannae*). The incidence, frequency and severity to which species or cultivars blacken is also reported to be highly varied, as McConchie and Lang (1993) found the development of leaf blackening to be most severe in *P. eximia*, intermediate in *P. neriifolia* and the lowest in the cultivar 'Pink Ice' (*P. compacta* x *P. susannae*) when comparing these species or cultivars. Even within clones leaf blackening development rates were found to vary considerably, as Paul and Dai (1990) reported significant differences in the leaf blackening and development between four *P. neriifolia* selections. In addition, it was also noted that leaf blackening incidence is higher at certain times in the year (Jones et al., 1995). This finding was reported in *P. neriifolia* stems cultivated in Hawaii, where leaf blackening accelerated during September (late summer, northern hemisphere) compared to stems harvested during October and November (autumn, northern hemisphere). Unpublished data from Van Doorn,

as cited in Jones et al. (1995), reported that the leaf blackening frequency from *Protea* flowers shipped from South Africa was highest in February (midsummer, southern hemisphere), but is reduced by April (autumn, southern hemisphere).

Perennial plants progress through various stages of vegetative and reproductive growth. In *Protea* species, vegetative growth occurs in flushes, where shoot growth is produced in growth spurts which is followed by a terminal-bud rest before another growth flush commences (Gerber et al., 2001; Hettach et al., 2001). These growth flushes are usually associated with the season in which it developed and are referred to as such. In *Protea* cv. Carnival (*P. compacta* x *P. neriifolia*), for example, four flushes occur within a growing season, with a spring flush, first summer, second summer and an autumn flush (Gerber et al., 2001; Hettach et al., 2001). For flower initiation, in most *Protea* species, at least two flushes are needed (Coetzee and Litteljohn, 2001). Thus, for *Protea* cv. Sylvia, which flowers year-round, there is always a combination of both new vegetative growth and inflorescences in various stages of development present on a single plant at any point in time. This poses a competitive relationship between sinks within the plant, especially during the vigorous and highly synchronized spring-flush budbreak following the dormant winter period.

Within South Africa, *Protea* cv. Sylvia is one of the top ten exported cut flowers despite the fact that it is well-known to be highly susceptible to leaf blackening development (SAPPEX, 2006). This cultivar is widely and extensively planted as it is one of the few *Protea* cultivars that exhibits a year-round flowering habit (Dürker, 1999). It is therefore possible to obtain a monthly income from 'Sylvia', as well as to supply the markets during the high demand periods of November to January, when flowers from other popular cultivars is not available from South Africa.

The exact cause of leaf blackening in *Protea* remains unclear despite several in-depth studies of this disorder (Jones et al., 1995; Van Doorn, 2001). Several researchers have shown a link between low carbohydrate reserves in the foliage and the development of leaf blackening (Brink and De Swardt, 1986; Reid et al., 1989; Newman et al., 1990; Paul and Dai, 1990; McConchie et al., 1991). Water stress and the role of phenolics have also been studied and reviewed (Jones et al., 1995; Van Doorn, 2001). However, no one study included a full report on a single cultivar or species throughout its flowering period to report the variation of leaf blackening occurrence over the entire export period. Similarly, the correlation between the internal quality of foliage as characterized by the carbohydrate-, water- and phenolic content at harvest with leaf blackening throughout the harvest period has not been studied.

This study aims to obtain a better understanding of the underlying cause(s) of leaf blackening, in order to assist the development of technologies towards the eradication or control of this disorder in *Protea* cv. Sylvia flowering stems. Thus the objective of this study was to observe and

record the incidence of leaf blackening for *Protea* cv. *Sylvia* throughout its entire production period as well as in relation to the time of harvest during the day, whilst the position of the flower at harvest on the plant was also considered. A further objective was to characterize and correlate the internal foliage quality status at harvest with the percentage leaf blackening. Internal quality parameters measured included the soluble sugars (sucrose and reducing sugars), starch levels, the presumably inert polyol-sugar, polygalatol, as well as the total phenolic- and water content.

2.2 Materials and Methods

2.2.1 Plant material

Dry-picked, harvest-ready (soft-tip stage), flowering stems of *Protea* 'Sylvia' (*P. eximia* x *P. susanna*) were harvested from a commercial farm located near Stellenbosch (33°55'S; 18°50'E) Western Cape, South Africa. Stems were collected on selected dates throughout 2008, 2009 and 2010 respectively, weather permitting, as no stems were harvested on rainy days. Stems harvested through the season were collected in the mornings between 08:00 and 10:00 (+2 GMT) on each harvesting date, whereas the harvest time for the daily harvest experiment is specified below. Within an hour after harvest stems were standardized (at the Department of Horticultural Science, Stellenbosch University) to a length of 40cm and 15 to 25 leaves, all selected from the subtending flush to the inflorescence.

2.2.2 Seasonal harvesting

Harvesting of stems in the seasonal study commenced in the first week of October 2008, where five stem replicates were randomly harvested to record the development of leaf blackening during vase life. A further five stems of similar maturity were selected for leaf analysis. Leaf sampling was done by removing five leaves from the flush subtending the inflorescence, starting from ± 5 cm below the floral head. Leaf samples were placed in sealed plastic bags and kept cold on ice during transport to the laboratory. Stems collected for leaf blackening evaluation were standardized for the vase life phase as described above.

Stems harvested in 2009 and 2010 were harvested from the same orchard as during 2008. Stems were harvested according to a completely randomized design and ten stem replicates were harvested at each harvest date and standardized for vase life evaluation as described above. Upon harvest, the position of the stem on the plant was classified according to three height levels: higher

than 1.5m; between 1.5 and 0.75m and lower than 0.75m and within four quadrants: North-East (NE); South-East (SE); South-West (SW) and North-West (NW), respectively.

2.2.3 Time of daily harvest

Four days differing mainly in average daily temperature were selected for harvesting: A 'cool' spring-day ($T_{\max} = 20^{\circ}\text{C}$; $T_{\text{avg}} = 14^{\circ}\text{C}$) on 4 September 2009; a 'warm' summer's day ($T_{\max} = 32^{\circ}\text{C}$; $T_{\text{avg}} = 23^{\circ}\text{C}$) on 3 November 2009, on a 'moderate' summer's day ($T_{\max} = 25^{\circ}\text{C}$; $T_{\text{avg}} = 24^{\circ}\text{C}$) on 10 December 2009 and a 'hot' summer's day ($T_{\max} = 35^{\circ}\text{C}$; $T_{\text{avg}} = 33^{\circ}\text{C}$) on 19 February 2010 (Figs. 2.1 B and C).

All stems ($n = 10$) harvested in 2009 were harvested from the same orchard, according to a completely randomized design at 08:00, 12:00 and 15:00 (+2 GMT). For the harvest on 19 February 2010 an extended two more harvest times were added at 17:00 the previous afternoon (18 February 2010) and at 10:00 on the day of harvest.

After standardization, as described above, stems harvested on 4 September and 3 November 2009 were pulsed with a 5.7% glucose solution for four hours. Stems harvested on 10 December 2009 was similarly pulsed with 5.7% glucose solution, but with the exception that during this trial stems were monitored until 10mL pulse-solution was removed. Stems harvested on 18 and 19 February 2010 were hydrated in tap water for an hour before dividing stems into two sub-sample groups for further processing.

After pulsing or hydration, stems harvested on 4 September 2009, 3 November 2009 and one of the sub-samples of stems harvested on 18 and 19 February 2010 were placed in tap water for vase life evaluation. Stems harvested on 10 December 2009 as well as the other sub-sample group harvested on 18 and 19 February 2010 were packed in standard S14 commercial flower storage cartons after pulsing. Each carton was individually wrapped with low density polyethylene black bags, to help prevent moisture loss during storage. Cartons were then placed in a cold room at 4°C ($\pm 1^{\circ}\text{C}$) for a 21 day storage period before vase life evaluation.

Temperature data was obtained from a weather station located near the harvesting farm, at Nietvoorbij ($33^{\circ}55'S$; $18^{\circ}54'E$), Stellenbosch, Western Cape in South Africa.

2.2.4 Vase life evaluation

In the seasonal study five freshly cut single stem replicates were placed in vases with tap water (after standardization as described above) to evaluate leaf blackening incidences. Leaf

blackening was recorded on day 5 and 10 of the evaluation period as the number of leaves with $\geq 10\%$ leaf blackening out of the total number of leaves per stem, and is presented as a percentage.

Stems harvested throughout the day on 4 September, 3 November 2009 as well as on 18 and 19 February 2010 were similarly evaluated as stems harvested in the seasonal study, with the exception that stems harvested on 10 December 2009 as well as those of 18 and 19 February were also evaluated after 21 days of cold storage at 4°C ($\pm 1^{\circ}\text{C}$).

2.2.5 Sample preparation for chemical analysis

On the harvesting dates and times throughout the season and day respectively, randomly selected five single stem replicates were prepared for chemical analysis, after standardization. Leaves from the flush subtending the inflorescence were defoliated, with the exception of stems harvested in 2008 where leaves were defoliated in the orchard. Total leaf fresh weight (g) was determined before leaves were stored in a -80°C freezer. After 24 hours, leaves of each stem replicate were freeze-dried for four days where after dry weight (g) was recorded, before leaves were milled (IKA-werke, Staufen, Germany) to a powder (sieve size = $500\mu\text{m}$) and kept at -20°C until analysis. Leaf water content was determined by subtracting the dry weight of the leaf from the fresh weight and then expressing this value as a percentage of the fresh weight.

2.2.6 Total phenolics, starch and soluble sugar preparation for HPLC extraction

To extract the soluble fraction containing the total phenolics a 100mg of milled sample was transferred to screw-top heating block tubes and extracted for 1 hour in 5mL 80% ethanol, whilst stirring on ice in a refrigerator at 4°C . The insoluble fraction was separated from the soluble fraction by centrifuging at 3500 rpm for 10min. After centrifugation, the supernatant containing the soluble fraction was transferred into individually marked vials and sealed. The pellet containing the insoluble fraction was extracted for an additional three cycles as described above, centrifuging after each extraction where after all supernatants were pooled and diluted to a final volume of 20mL total soluble extraction. All supernatants were passed through a $0.45\mu\text{m}$ filter before storing at -20°C until total phenolics could be determined.

The insoluble fraction was then further extracted for starch analysis by means of enzymatic hydrolysis. The pellet was heated to 60°C for 20 minutes on a heating block to facilitate complete ethanol evaporation before enzyme hydrolysis. A volume of 2mL of a 5mM sodium acetate buffer (pH 4.8) was added, mixed with a vortex, where after starch within the insoluble fraction was

gelatinized for 1 hour at 100°C. Samples were then cooled to 60°C before 2mL of amyloglucosidase enzyme (EC 3.2.1.3) (Fluka chemie, Buchs, Switzerland) (made in a 5mM sodium acetate buffer, pH 4.8; 15 IU) was added. Samples were incubated for a further 18 hours on a heating block at 60°C. After incubation, samples were immediately placed in a water bath for 5 minutes at 100°C in order to terminate any further hydrolysis by denaturing the enzyme. Samples were finally centrifuged for 5 minutes at 3500 rpm. The supernatant of the starch fraction was then stored at -20°C until starch analysis.

Individual soluble sugars were extracted from a 100mg dry leaf tissue sample in 1.5mL de-ionized water within a 2mL eppendorf re-closable tube, where after it was placed on ice in an ultrasonic bath for 10 minutes. The insoluble fraction was separated by centrifugation at 20 000 rpm for 15 minutes. Each sample supernatant was transferred to tubes (sealable) and heated in a warm bath at 100°C for 3 minutes. Supernatants were then immediately placed on ice for a further 30 minutes, where after samples were passed through a 0.45µm filter into marked HPLC specific vials.

2.2.7 Starch (determination)

Starch concentration was determined by the method described by Prado et al. (1998). For total starch analysis, 10µL of the 4mL insoluble fraction supernatant was diluted to 200µL with distilled water. Before analysis, a glucose standard range (of 0, 1, 2 and 4µg.mL⁻¹ glucose concentration) was included to construct a standard curve.

After the 200µL sample and standard aliquots were added to individual test tubes, 50µL 0.1M Potassium ferricyanide (Merck, Darmstadt, Germany) was added, followed by the addition of a 100µL of 0.05M NaOH and a 0.05M Na₂CO₃ solution. Samples were then mixed using a vortex and heated in a water bath at 100°C for 10 minutes. Samples were subsequently cooled down to room temperature (20°C ±1°C) before 1mL of 0.015M *o*-phenanthroline (Merck, Darmstadt, Germany) acidified in a 0.1M acetic acid solution was added. Samples were then heated once more in the water bath at 100°C for 10 minutes and cooled down again to room temperature (20°C ±1°C) to allow colour development to occur. Total starch was measured by reading absorbance at 505nm using a Cary 50, Bio UV-visible spectrophotometer (Varian, Varian Australia Pty Ltd., Victoria, Australia). Sample concentrations were quantified by means of the glucose standard curve and are expressed as mg per gram dry weight (DW) glucose equivalents.

2.2.8 Total phenolic content (determination)

Total phenolic content was determined according to the Folin-Ciocalteu method (Slinkard and Singleton, 1977). A 5 μ L aliquot of the 20mL soluble fraction extraction was diluted with 45 μ L distilled H₂O to make up a 50 μ L sample for analysis. A gallic acid concentration range, which included 0, 75, 125 and 250 μ g.mL⁻¹ gallic acid concentrations were used to construct a standard curve.

After sample and standard aliquots were pipetted into plastic cuvettes, 450 μ L 0.1M Folin-Ciocalteu colour reagent (Merck, Darmstadt, Germany) was added and samples were mixed by vortex. After 5 minutes, 500 μ L 0.528M (5.6%) sodium carbonate (Na₂CO₃) was added and vortexed. Samples were left at room temperature (20°C \pm 1°C) for 90 minutes to allow for colour development, before absorbance was measured at 750nm using a Cary 50, Bio UV-visible spectrophotometer (Varian, Varian Australia Pty Ltd., Victoria, Australia). Sample concentrations were quantified by the gallic acid standard curve and are expressed as mg per gram dry weight (DW) gallic acid equivalents.

2.2.9 Soluble sugar determination

Soluble sugars were separated on an Agilent 1100 HPLC system (Agilent technologies, Germany) equipped with a Refractive index detector. A Polyspher CHCA column (Merck, Germany) was used, running constantly at 80°C and eluted with filtered de-ionized H₂O, at a standard flow rate of 0.4mL.min⁻¹. Individual sugars were quantified against known standards of glucose, fructose, sucrose and polygalatol and are expressed in mg per gram leaf dry weight (DW).

2.2.10 Data classification and statistical analysis

Principle component analysis (PCA) and a forward stepwise Discriminant analysis (DA) were performed on data obtained throughout the seasonal study and within the different harvesting times per day. In order to perform the DA, data classification was required. Thus, for the seasonal study the months of the year were divided into four categories, based on the leaf blackening occurrence throughout the year. The months of January to April were grouped together as the summer leaf blackening period, May to July was identified as the low leaf blackening incidence period, with August and September signalling the start of leaf blackening occurrence, whilst October to December was associated with each other as the high leaf blackening incidence period. For stems harvested throughout the day, two types of DA was performed, classifying first between the four

months, September, November and December 2009 as well as February 2010, or classifying between the three harvesting times, 08:00, 12:00 and 15:00. Correlation analysis was also done between percent leaf blackening incidence and each chemical parameter. All calculations and modelling were performed using XLSTAT Version 2011.1.01 (Microsoft Inc., 2011). PCA bi-plot graphs are presented as the incorporation of both observational and variables with one figure to represent the distribution of observational data and variables according to the two main factors explaining the highest degree of variance of the data. The DA figures included an observations chart, which represents each observation on the factor axes as well as a variable chart, which shows the correlation of the data with the two principle factors. The summary of significant variables selected for stepwise DA and the confusion matrix for each analysis was also presented. In the seasonal study, only significant factors were presented in the stepwise DA charts.

Factorial analysis of variance (Factorial ANOVA) was used to calculate statistical differences between all data that resulted from observational and chemical analysis over the season, the different months and within the various harvest times throughout the day. All factorial ANOVAs were performed using STATISTICA version 10 (Statsoft, Inc., 2011). Means were separated by Fisher's Least Significant Differences (LSD) posthoc test, with significance level at $P < 0.05$.

Where covariates were included in statistical analysis, SAS version 9.1 (SAS Institute, Inc., 2000) was used.

2.3 Results and Discussion

2.3.1 Seasonal harvesting

Leaf blackening. The incidence of leaf blackening recorded throughout the 2008 to 2010 seasons were highly varied (Fig. 2.1A). The only dates where leaf blackening was recorded to be below 40%, after a 10 day evaluation without storage, was between weeks 9 (March) and 34 (August) (Fig. 2.1A). Weeks 40 (October) to 50 (December), a time when leaf blackening is known to be high, is well represented in the data. The highest incidence of leaf blackening during this period was between weeks 46 and 48 (November) for all three years. Monthly average temperatures reflect the same trend observed for leaf blackening where the period with the lowest temperatures also had the lowest incidence of leaf blackening; however, there were no significant correlation found ($r^2 = -0.245$; $p = 0.3268$) (Fig. 2.1B). This trend between temperature and leaf blackening was less evident between the daily average temperature and percentage leaf blackening over the season, which was also non-significant ($r^2 = -0.014$; $p = 0.9567$) (Fig. 2.1C). The frequency and

severity of leaf blackening for South African *Protea* cut flowers has been reported to be high in February, where after it is reduced by April (Van Doorn, unpublished data cited by Jones et al., 1995). In this study, leaf blackening in *Protea* cv. Sylvia is reported to increase in frequency towards the second half of the year, with the highest incidences found rather in November and December, than February.

Chemical analysis. Chemical analysis of the various major carbohydrates known to occur in *Protea* cv. Sylvia, showed differences over the season as well as from each other (Figs. 2.2A – D). The reducing sugar (glucose and fructose) concentrations (Fig. 2.2A) displayed an opposite trend to that of the sucrose concentrations (Fig. 2.2B), whereas no trend was found for polygalatol or starch concentrations throughout the seasons or between years (Figs. 2.2C and D). Polygalatol, a sugar-polyol and derivative of sorbitol which is present in high concentrations within *Protea*, remains therefore not only constant during vase life (Bieleski et al., 1992) and during storage (Ferreira, 2005), but also throughout the season as reported in this study.

The total phenolic content values for 2008 and 2009 remained relatively constant throughout the year, whilst values for 2010 were high at the start of the year from weeks 1 to 6, where after it declined towards week 40 (Fig. 2.2E). There were no correlation between total phenolic content and leaf blackening ($r^2 = -0.0002$; $p = 0.9999$), probably because of the great variation between years and the absence of data points for the last quarter of 2010.

Leaf water content for the subtending flush varied between 62% for the first quarter of the year to a slightly lower 55% for weeks 49 – 51. However, weekly irrigation is applied during the first half of the year which may have accounted for the slightly higher and constant values measured during that time.

When correlating the different carbohydrates, total phenolics and percentage water content of harvested stems to the corresponding incidence of leaf blackening, only sucrose and percentage water content was significantly correlated to the leaf blackening incidences recorded (Figs. 2.2B and F).

Besides correlation analysis, when monthly averages of all data points (observations) for all the variables measured (% leaf blackening, average month temperature, average day temperature, soluble and insoluble carbohydrates, total phenolic and water content) were submitted into the PCA bi-plot, the only significant correlation achieved by the PCA bi-plot from all the variables to the incidence of leaf blackening was also a positive correlation to sucrose ($r^2 = 0.528$; $p = 0.0201$) and a negative correlation to % water content ($r^2 = -0.659$; $p = 0.0021$). This suggests that increasing incidences of leaf blackening is linked to increased sucrose concentrations for stems at harvest whereas a lower percentage water content is accompanied by higher leaf blackening incidences (Fig.

2.3). However, for sucrose, this data does not fully support previous studies that showed a strong correlation between low carbohydrates and high incidences of leaf blackening. It is however important to note that McConchie and Lang (1993) indicated that inflorescence sink demand and oxidative substrate availability after harvest were much better related to the development of leaf blackening than the pre-harvest carbohydrate reserve status. For low water content, this significantly negative correlation could account for a stress response, which has been linked to leaf blackening previously (Jones et al., 1995; Van Doorn, 2001).

Another interesting observation that resulted from the PCA bi-plot is that the months were generally arranged clockwise within the bi-plot (Fig. 2.3). The top-right quadrant contained mostly observations from January to April, whereas the bottom-right quadrant displayed a concentration of May to August observations, followed by September to November observations in the bottom-lower quadrant, with a range of observations from October to January in the top-left quadrant (Fig. 2.3). It would seem as though the PCA bi-plot could separate or recognized seasonal differences as reported in Figs. 2.1 and 2.2.

When these same observations were submitted into a DA, with the four selected groups according to leaf blackening incidences, there were some separate distinctions between the groups on the observational chart (Fig. 2.4B) on the basis of the significant variables, the average day temperature, water content, polygalatol, starch and the reducing sugars measured (Table 2.1A), where these factors explained 90.91% of the total variance (Fig. 2.4B). Factor 1 could only partially distinguished stems harvested in October to December from stems harvested in May to July, whereas Factor 2 partially distinguished vertically between stems harvested in October to December from stems harvested in January to April (Fig. 2.4B). Stems harvested in May to July and August to September was not distinguishable by either Factor 1 or 2 (Fig. 2.4B). However, there was still a great deal of overlapping between the groups. The confusion matrix had a total of 89.5% correctly predicted sample groups, where miss-classified samples were found within the August to September, January to April and May to July harvested stems (Table 2.1B). When comparing the observational chart with that of the variable chart, stems harvested during January to April seem to be associated with day temperature and polygalatol, whereas stems harvested during October and December were associated with total starch (Fig. 2.4A). The only associated variable with stems harvested in May to July appear to be water content and reducing sugars; however this association was not strong (Fig. 2.4A).

In *Protea* cv. *Sylvia*, various stages of vegetative growth and reproductive development is represented at any point of time, as this cultivar flowers uniquely throughout the year (Gerber et al., 2001; Hettach et al., 2001). In this cultivar, the most energy-requiring period is directly after winter,

when highly synchronized bud-break occurs, followed by the rapid extension of the spring flush (Gerber et al., 2001). Hettach et al. (2001) has also shown a significant drop in carbohydrates of the leaves during this time. Ferreira (2005) has linked this carbohydrate depletion, due to vegetative demands, directly to leaf blackening. A significant lowering of leaf blackening incidence was observed when the spring flush development was retarded using a growth regulator, Paclobutrazol ((2RS, 3RS)-1-(4-chlorophenyl)-4, 4-dimethyl-2-(1, 2, 4-triazol-1-yl) pentan-3-ol), trade-name 'Cultar'. Inflorescences developing during this time are harvested during October and December, a time when leaf blackening is particularly severe. Leaf blackening could thus be associated with low foliage carbohydrate levels, although both PCA and DA results from this study suggest otherwise.

Harvesting position. When considering the harvesting position on the tree, there was a significant difference in leaf blackening of stems with respect to height ($p = 0.0006$) (Fig. 2.5A). The quadrant or side of the plant from which the stem was harvested however, did not significantly ($p = 0.8071$) influence leaf blackening of the associated stems (Fig. 2.5B). Leaf blackening (%), after a 10 day evaluation period, for stems harvested from the top or mid-section (Fig. 2.5 A) had almost double the amount leaf blackening compared to the stems harvested in the lowest position, irrespective of the season or month of harvesting. Situated on the lower parts of the plant, these stems are likely to grow slower, due to this more shaded position and further away from the translocation of photosynthates from the other source-stems, resulting in a longer development to harvest. However, most exported flowering stems are harvested above 0.75m on the plant, this factor probably would not contribute significantly to the variance found in leaf blackening over the season.

In addition to the chemical components measured in this study, which were selected because of their studied relevance known in literature to leaf blackening, various other physiological and even anatomical characteristics could influence leaf blackening incidence (Shtein et al., 2011). Shtein et al. (2011) not only found differences in xylem vessel characteristics, to be highly correlated to the loss of quality during vase life of *Dodonaea* cut branches, but also indicated the importance of stomatal and trichome density, as this anatomical characteristic directly influences transpiration rates. Hormones, such as abscisic acid (ABA) have also been shown to play a role in longevity during vase life, as it also influences stomatal conductance (Joyce and Jones, 1992). As none of these factors have been considered in this study, it is highly likely that an interaction of various factors in addition to carbohydrates and water content may contribute to the variation in leaf blackening observed over the season.

2.3.2 Time of daily harvest

Leaf blackening. Leaf blackening incidence also varied significantly with harvesting at different times throughout the day, both when stems were evaluated fresh or after cold-storage (Figs. 2.6A – D).

Stems that were evaluated fresh, after pulsing in September and November 2009 showed no significant interaction ($p = 0.2995$) between the month and the time of harvest throughout the day (Fig. 2.6A). The incidence of leaf blackening did also not differ significantly ($p = 0.3094$) between the two months of harvest (Fig. 2.6A). However, leaf blackening did differ significantly between times harvested during the day ($p = 0.0233$), where leaf blackening was lowest later in the day compared to mornings (Fig. 2.6A). When amount of glucose that accumulated with each pulsing treatment at harvest-time was used as a covariate, the lowest incidence of leaf blackening was observed still ($p = 0.0502$) when stems were harvested later in the day compared to early morning. The overall lower percent leaf blackening could be ascribed to the accumulation of carbohydrates by current photosynthesis, throughout the day, together with the addition of the pulsed glucose. Also, stems were not subjected to any storage. For stems harvested in February 2010, which was also evaluated fresh, but with the difference that stems were not pulsed, the same trend was observed where stems harvested in the afternoon had significantly lower leaf blackening ($p = 0.0031$) after 10 days evaluation than stems harvested during the morning (Fig. 2.6C). In this study, the lowest percentage leaf blackening was recorded in stems harvested at 12:00 and 15:00, both at $\pm 20\%$, whereas the highest percentage leaf blackening was recorded in stems harvested at 08:00 in the morning at $\pm 60\%$ (Fig. 2.6C). The advantage of an afternoon harvest is likely to be associated with the additional photosynthesis opportunity throughout the day as these stems are known to have a higher concentration of carbohydrates within the leaves when harvested (Paull and Dai, 1990).

The percent leaf blackening of stems that were harvested and pulsed on 10 December 2009 before storage for 21 days at 4°C ($\pm 1^{\circ}\text{C}$) differed ($p = 0.0527$) between the various times of harvest, where leaf blackening was higher with 51.1% in stems harvested in the morning (at 08:00) than stems harvested at 12:00 (28.9%) or 15:00 (33.9%) respectively (Fig. 2.6B). Percent leaf blackening values were generally relatively low after storage, likely due to the pulsing treatment of 10mL (5.7% glucose solution) before storage. Percent leaf blackening of stems harvested and stored in February 2010, as in the stems harvested and stored in December 2009, also showed a significant difference ($p = 0.0006$) between the various harvest time treatments. The lowest incidence of leaf blackening (after a 10 day evaluation period) was recorded in stems harvested in the late afternoon at 17:00 the previous day at 35% compared to the highest percent leaf blackening that was recorded for harvest

on the morning of the following day at 10:00, 12:00 and 8:00 with 87, 70 and 67.5% respectively (Fig. 2.6D).

Harvest-time experiments were not only selected to coincide with those known periods of high leaf blackening incidence (Fig. 2.1A), but also according to different harvesting temperatures. The harvest date of September 2009 was overall considered to be on a 'cool' day with an average daily temperature of $\pm 20^{\circ}\text{C}$, where stems harvested on 3 December 2009 were overall more on a 'mild' day with an average daily temperature of $\pm 24^{\circ}\text{C}$. The harvest dates in November 2009 and February 2010 were considered 'warm to hot' days with average daily temperatures of $\pm 32^{\circ}\text{C}$ and $\pm 33^{\circ}\text{C}$ respectively (Fig. 2.6E). However, irrespective of harvest date, temperatures on that day were always lowest in the morning at 08:00, compared to the rest of the day (Fig. 2.6F). Stems collected and evaluated from these different harvesting dates all conclude to a higher percent leaf blackening in the morning compared to later in the day. This finding is in agreement with one previous study that reported leaf blackening development to increase more rapidly in flowers harvested at 08:00 compared to flowers harvested in the afternoon for a *P. neriifolia* selection (Paull and Dai, 1990).

Chemical analysis. The trends or lack thereof observed in carbohydrate-, total phenolic- and water content for stems harvested in 2009 at each of the harvest times of 08:00, 12:00 and 15:00 (Figs. 2.7A – F) were in correspondence with that of stems harvested over the extended period of 17:00 the previous day to 08:00, 10:00, 12:00 and 15:00 the following day in February 2010 (Figs. 2.8A – F).

Reducing sugar concentrations recorded showed no significant interaction between months and time of harvest ($p = 0.3127$), but were higher ($p = 0.0555$) for stems harvested in the afternoon in September compared to early morning harvested stems (Fig. 2.7A). For the February 2010 harvested stems, reducing sugar concentrations were significantly ($p = 0.0321$) higher in those stems that were harvested after 12:00 ($\pm 18 \text{ mg.g}^{-1} \text{ DW}$), compared to the lower values measured in the morning harvested stems with $\pm 15 \text{ mg.g}^{-1} \text{ DW}$ (Fig. 2.8A). Reducing sugar content also declined significantly over the observed months ($p = 0.0001$), where September, November and December measured 13.4, 12.3 and 10.3 $\text{mg.g}^{-1} \text{ DW}$ respectively (Fig. 2.7A). Interestingly, the average reducing sugar concentration measured in February 2010 was considerably higher than recorded in any of the preceding three months.

There were no significant interactions between month and time harvested for any other chemical component analyzed, thus only main effects are discussed further. In sucrose, concentrations for all harvest dates increased significantly ($p < 0.0001$, Fig. 2.7B; $p < 0.0001$, Fig. 2.8B) throughout the day. The highest concentrations were recorded at 15:00 for all harvest dates (Fig. 2.7B), as well as at 17:00 for stems harvested the previous day in February 2010 (Fig. 2.8B).

Polygalatol or starch content however, as was reported for the seasonal study, did not vary significantly for any harvesting date or time (Figs. 2.7C and D; Figs. 2.8C and D).

For total phenolic content, stems harvested in December 2009 had measured consistently lower values than for stems harvested between the months of September and November (Fig. 2.7E). In stems harvested in February 2010, total phenolic content differed ($p = 0.0348$) over the harvesting times during the day where stems harvested at 10:00 am, were significantly lower than at any other harvest times (Fig. 2.8E).

Lastly, the percentage water content differed in leaves for the various harvest times and between months of harvests (Figs. 2.7F and 2.8F). Stems harvested in September had the highest water content within each harvest time, whilst estimated over the entire day, the water content was generally highest in the morning for stems harvested in 2009 (Fig. 2.7F). Water content was lowest in stems harvested in February 2010 in the afternoon from 12:00 to 15:00, although water content at 08:00 was not significantly different from that of stems measured at 17:00 the previous day (Fig. 2.8F). However, the water content may have been confounded by unknown irrigation scheduling.

When studied in isolation, trends observed for the chemical analysis for the respective harvesting months and times, proved to be varied and inconclusive. However, an analysis of this data by means of PCA and DA yielded more distinct associations. In PCA, samples' leaf blackening incidences were significantly correlated to harvest temperature ($r^2 = -0.489$; $p = 0.0002$), sucrose ($r^2 = -0.495$; $p = 0.0002$) and starch ($r^2 = 0.284$; $p = 0.0437$) (Fig. 2.9). Although these differences were significant, interpreting these differences is difficult, as no strong correlations could be found.

Using the stepwise DA, both the months of harvest as well as time of day showed a stronger association with parameters measured, compared to the seasonal study, as is seen in the summary stepwise analysis (Tables 2.2A and 2.3A). For analysis between the months, the two factors distinguishing between the groups explained 96.07% of the total variance between the observations. Factor 1 could distinguish stems harvested in February from the other harvest months, whilst factor 2 showed a difference in September-harvested stems from other months (Fig. 2.10B). The confusion matrix had a 92.2% correctly predicted sample groups, where only one or two samples were misclassified for stems harvested in September, November or December 2009 (Table 2.2B). In order to classify the various harvest times of day, using observations from all four months, there were more variables included which were significant in explaining the variance in the stepwise DA to group harvesting times than between the months (Table 2.3A). The two factors could explain a 100% variance when classifying the time of harvest (Fig. 2.10), where factor 1 contributed to 83.1% of the variance, distinguished between the morning harvested stems (08:00) and that of stems harvested later during the day (12:00 and 15:00), whereas factor 2 could only partially distinguish between

12:00 and 15:00 (Fig. 2.10D). Comparing the observational chart with the variable chart, stems harvested at 08:00 was more closely associated with water content, whereas stems harvested later in the day was more strongly associated with the carbohydrates, sucrose and reducing sugars.

2.4 Conclusion

From these findings, it can be concluded that leaf blackening in *Protea* cv. *Sylvia* varies throughout the season and between years. Leaf blackening incidences increased soon after winter (August) and had the highest incidence in November and December, before it declined to acceptably lower levels towards March to May. No internal quality parameter or other measured factors could predict or explain leaf blackening reliably and consistently over the seasons. PCA and DA charts only confirmed the observations that the flowering stems harvested during different times of the year can be classified into different groups, indicating that there are different factors defining the flowering shoot in that period. No single factor measured in this study at harvest, could be directly and consistently linked, with confidence, to the incidence of leaf blackening seasonally. Thus, different factors could contribute to leaf blackening and not only one single factor throughout the year. Other factors could also contribute to vase life and longevity or explain some of the seasonal variation found in plants, apart from internal chemical composition, as stem and leaf anatomy does change seasonally in plants.

Focussing on specific dates through the season, though, a stronger relationship between leaf blackening and carbohydrate content or water stress is indicated than with any other parameter studied. For *Protea* cut flowers, high incidence of leaf blackening is associated with a low carbohydrate status within the leaves, which is indicative for stems harvested in the mornings. Also, stems harvested early in the morning have shown higher water content, which results in a more turgid stem, that upon handling, have a greater chance of bruising and developing leaf blackening more easily. For stems harvested throughout the day, the main finding was that harvesting later in the day generally resulted in lowered leaf blackening incidences. This was attributed or correlated to higher carbohydrate status within the shoot. Even when pulsing at each harvest time throughout the day, leaf blackening incidence was still lower in the afternoon-harvested stems. Higher water content was also attributed to morning harvested stems, which also had the highest incidence of leaf blackening.

2.5 References

- Bieleski, R.L., Ripperda, J., Newman, J.P., Reid, M.S., 1992. Carbohydrate changes and leaf blackening in cut flower stems of *Protea eximia*. J. Am. Soc. Hortic. Sci. 117, 124 – 127.
- Brink, J.A., De Swardt, G.H., 1986. The effect of sucrose in vase solution on leaf browning of *Protea neriifolia* R. Br. Acta Hortic. 185, 111 – 119.
- Coetzee, J.H., Littlejohn, G.M., 2001. *Protea*: A floricultural crop from the Cape Floristic Kingdom. Hortic Rev. 26, 1 – 48.
- Dürker, G., 1999. Evaluation of export figures and prices in the 1997/1998 season in South Africa. Internal report. Dept. Horticultural Science, Stellenbosch University, South Africa.
- Ferreria, A., 2005. Further studies on leaf blackening of *Protea*. MSc Thesis, Stellenbosch University, South Africa.
- Gerber, A.I., Theron, K.I., Jacobs, G., 2001. Synchrony of inflorescence initiation and shoot growth in selected *Protea* cultivars. J. Am. Soc. Hortic. Sci. 126, 182 – 187.
- Hettach, H. B., Theron, K.I., Jacobs, G., 2001. Dry mass accumulation and carbohydrate allocation in successive growth flushes of *Protea* cultivar Sylvia and *Protea* cultivar Cardinal shoots. Acta Hortic. 545, 215 – 225.
- Jones, R.B., Clayton-Greene, K.A., 1992. The role of photosynthesis and oxidative reactions in leaf blackening of *Protea neriifolia* R. Br. leaves. Scientia. Hortic. 50, 137 – 145.
- Jones, R.B., McConchie, R., Reid, M.S., 1995. Leaf blackening in cut *Protea* flowers. Hortic. Rev. 17, 173 – 201.
- Joyce, D.C., Jones, P.N., 1992. Water balance of the foliage of cut Geraldton waxflower. Postharvest Biol. Technol. 2, 31 – 39.
- McConchie, R.B., Lang, N.S., Gross, K.C., 1991. Carbohydrate depletion and leaf blackening in *Protea neriifolia*. J. Amer. Soc. Hort. Sci. 116, 1019 – 1024.
- McConchie, R., Lang, N.S., 1993. Postharvest leaf blackening and pre-harvest carbohydrate status in three *Protea* species. HortScience 28, 313 – 316.
- Microsoft Inc., 2011. Office XLSTAT (Excel data analysis software), Version 1.01. <http://www.xlstat.com>
- Newman, J.P., Van Doorn, W., Reid, M.S., 1990. Carbohydrate stress causes leaf blackening in *Proteas*. Acta Hortic. 264, 103 – 108.
- Paull, R.E., Dai, J-W., 1990. *Protea* postharvest black leaf, a problem in search of a solution. Acta Hortic. 264, 93 – 101.
- PPECB (Perishable Products Export Control Board), 2010. Export directory: Part 3, All Flora. Malachite Press, Helderberg, South Africa. Section 2, pp. 79

- Prado, F.E., González, J.A., Boero, C., Sampietro, A.R., 1998. A simple and sensitive method for determining reducing sugars in plant tissues: Application to quantify the sugar content in Quinoa (*Chenopodium quinoa* Willd.) seedlings. *Phytochem. Analys.* 9, 58 – 63.
- Reid, M.S., Van Doorn, W.G., Newman, J.P., 1989. Leaf blackening in *proteas*. *Acta Hort.* 261, 81 – 84.
- SAPPEX (South Africa Protea Producers and Exporters Association), 2006. <http://www.sappex.org.za>
- Shtein, I., Meir, S., Riov, J., Philosoph-Hadas, S., 2011. Interconnection of seasonal temperature, vascular traits, leaf anatomy and hydraulic performance in cut *Dodonaea* 'Dana' branches. *Postharvest Biol. Technol.* 61, 184 – 192.
- Slinkard, K., Singleton, V.L., 1977. Total phenol analysis: Automation and composition and comparison with manual methods. *Am. J. Enol. Vitic.* 28, 49 – 55.
- Statistical analysis systems Institute Inc., 2000. SAS®/STAT User's guide, Version 9.1, SAS Institute Inc., SAS Campus drive, North Carolina 27513.
- Statsoft Inc., 2011. STATISTICA (data analysis software system), Version 10. <http://www.statsoft.com>
- Stephens, I.A., 2003. Leaf blackening of *Proteas*. PhD. Diss., Stellenbosch Univ., Stellenbosch, South Africa.
- Van Doorn, W.G., 2001. Leaf blackening in *Protea* flowers: Recent developments. *Acta Hort.* 545, 197 – 204.

Table 2.1 The summary of variables selected by a stepwise Discriminant Analysis (DA) performed on the measured and observed factors recorded throughout the season of 2008 to 2010 (A) on flowering stems of *Protea* cv. Sylvia and the confusion matrix indicating the percentage correctly predicted sample groups (B).

A

Variable IN/OUT	Status	Partial R ²	F	<i>p</i>
Day Temp (°C)	IN	0.537	13.132	< 0.0001
Water Content (%)	IN	0.506	11.254	< 0.0001
Polygalatol (mg.g ⁻¹ DW)	IN	0.338	5.441	0.004
Starch (mg.g ⁻¹ DW)	IN	0.273	3.882	0.018
Reducing Sugars (mg.g ⁻¹ DW)	IN	0.315	4.606	0.075

B

From \ To	Aug-Sep	Jan-Apr	May-Jul	Oct-Dec	Total	% correct
Aug-Sep	6	1	0	0	7	85.71%
Jan-Apr	0	8	1	1	10	80.00%
May-Jul	1	0	12	0	13	92.31%
Oct-Dec	0	0	0	8	8	100.00%
Total	7	9	13	9	38	89.47%

Table 2.2 The summary of variables selected by a stepwise DA performed on the measured and observed factors recorded between harvesting months (September, November and December 2009, as well as February 2010) (A) on flowering stems of *Protea* cv. Sylvia and the confusion matrix indicating the percentage correctly predicted sample groups (B)

A

Variable IN/OUT	Status	Partial R ²	F	<i>p</i>
Reducing Sugars (mg.g ⁻¹ DW)	IN	0.740	44.496	< 0.0001
Sucrose (mg.g ⁻¹ DW)	IN	0.557	19.278	< 0.0001
Total phenols (mg.g ⁻¹ DW)	IN	0.469	13.230	< 0.0001
Harvest Time Temp (°C)	IN	0.312	6.644	0.001
Leaf Blackening (% Day 10)	IN	0.475	12.956	< 0.0001
Water content (%)	IN	0.219	3.928	0.015

B

From \ To	Dec2009	Feb2010	Nov2009	Sep2009	Total	% correct
Dec2009	8	0	1	0	9	88.89%
Feb2010	0	15	0	0	15	100.00%
Nov2009	2	0	13	0	15	86.67%
Sep2009	1	0	0	11	12	91.67%
Total	11	15	14	11	51	92.16%

Table 2.3 The summary of variables selected by a stepwise DA performed on the measured and observed factors recorded between harvest times throughout the day (08:00, 12:00, 15:00) (A) on flowering stems of *Protea* cv. Sylvia and the confusion matrix indicating the percentage correctly predicted sample groups (B)

A

Variable IN/OUT	Status	Partial R ²	F	<i>p</i>
Harvest Time Temp (°C)	IN	0.503	24.288	< 0.0001
Daily Average Temp (°C)	IN	0.647	43.107	< 0.0001
Leaf Blackening (% Day 10)	IN	0.260	8.068	0.001
Sucrose (mg.g ⁻¹ DW)	IN	0.197	5.509	0.007
Water content (%)	IN	0.311	9.945	0.000
Polygalatol (mg.g ⁻¹ DW)	IN	0.139	3.481	0.040
Reducing Sugars (mg.g ⁻¹ DW)	IN	0.194	5.069	0.011

B

From \ To	12:00	15:00	8:00	Total	% correct
12:00	15	2	0	17	88.24%
15:00	2	15	0	17	88.24%
08:00	3	0	14	17	82.35%
Total	20	17	14	51	86.27%

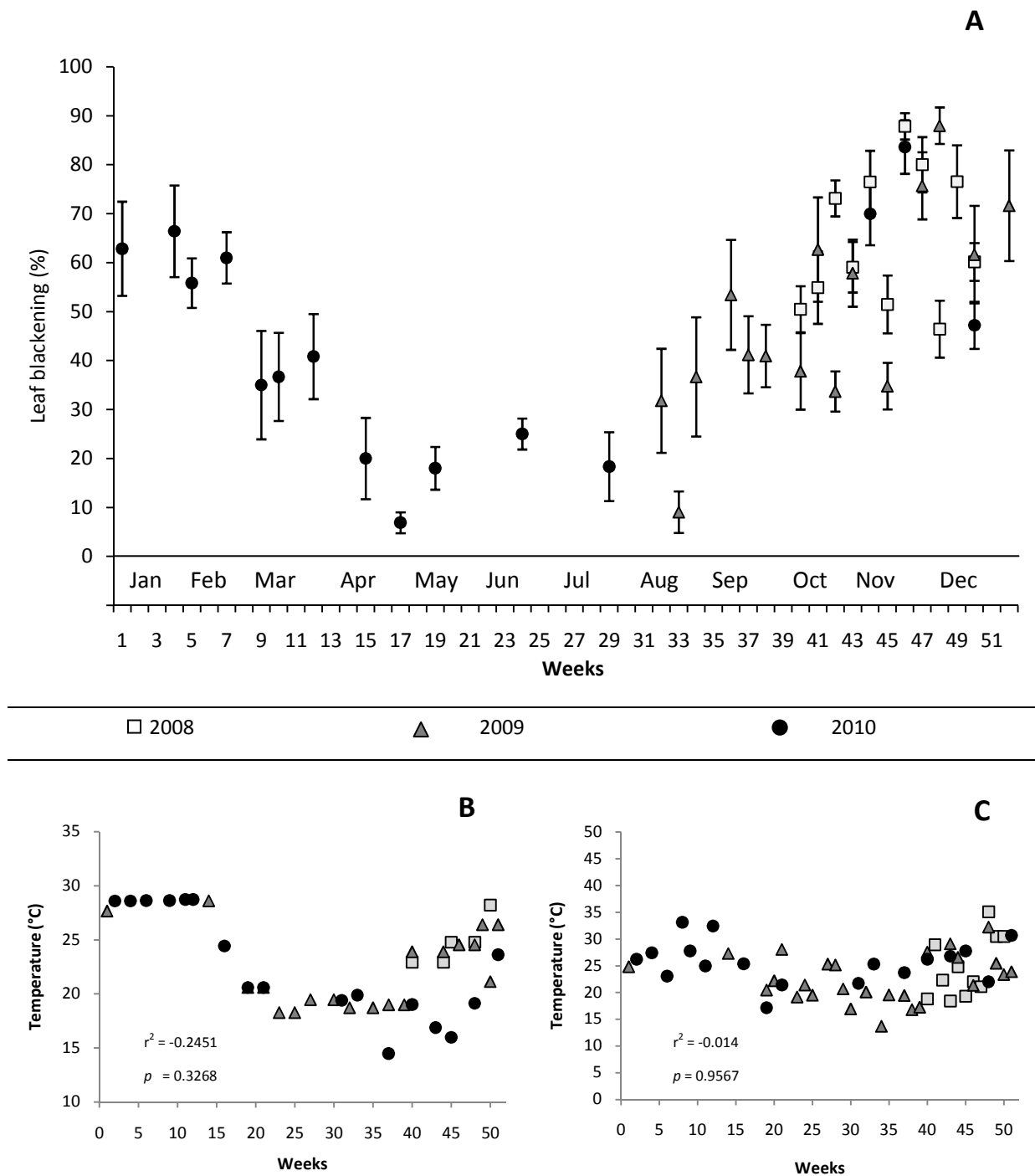


Fig. 2.1 The percentage (%) leaf blackening (A) recorded after a 10 day fresh evaluation period on *Protea* cv. *Sylvia* flowering stems throughout the year in 2008, 2009 and 2010. The corresponding average monthly temperatures (B) and daily temperatures (C) for the same dates obtained from Nietvoorbij (Stellenbosch, SA) weather station. The R^2 -value and corresponding p -value indicates the correlation and significance between leaf blackening incidence and measured temperatures.

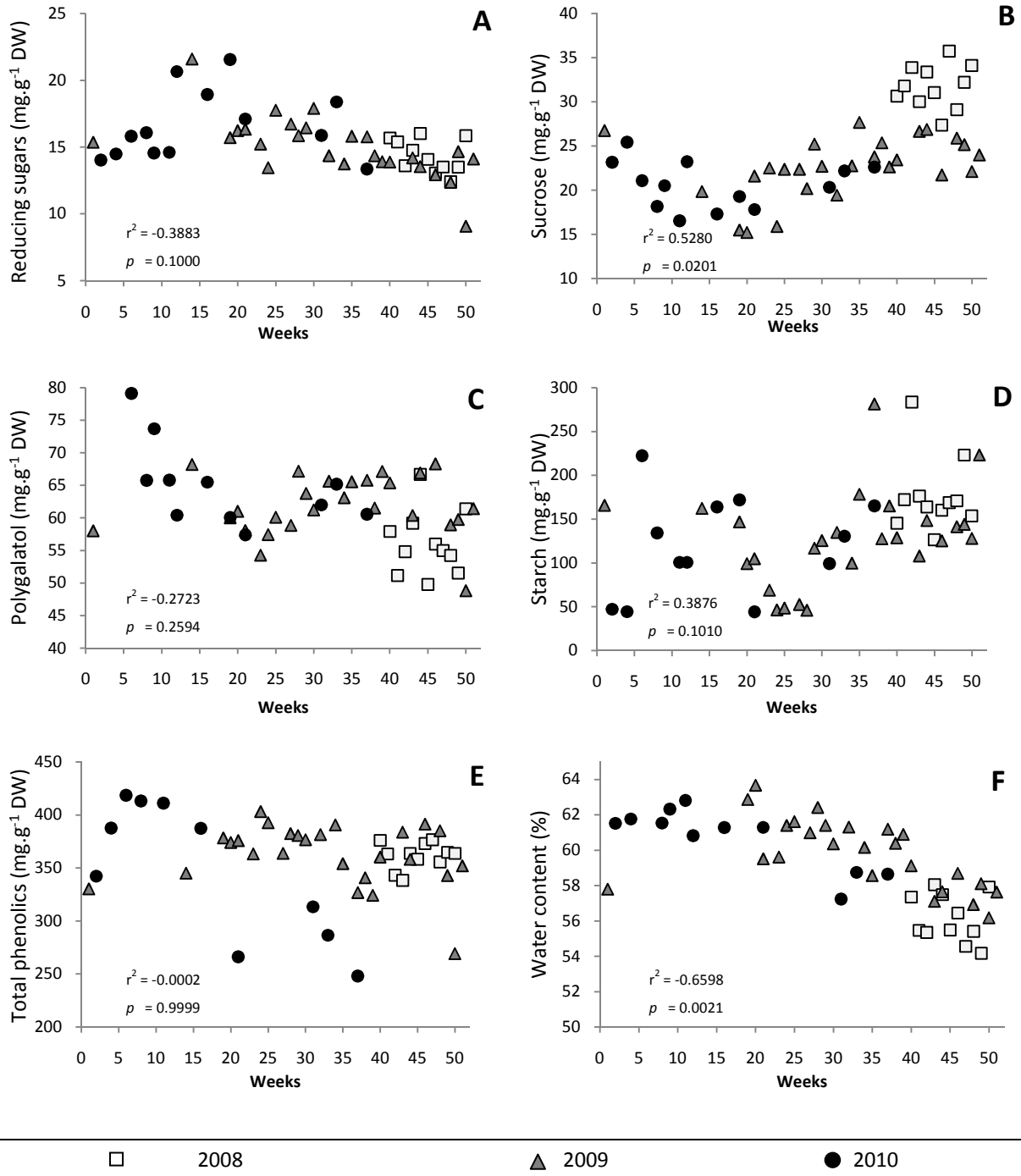


Fig. 2.2 The total reducing sugars (A), sucrose (B), polygalatol (C) and starch (D) and total phenolic content (E) expressed as $\text{mg}\cdot\text{g}^{-1}$ DW as well as the percentage (%) water content (F) measured at each harvest date for stems harvested throughout the year of 2008, 2009 and 2010. The R^2 -values and corresponding p -values in each graph indicate the correlation and significance between leaf blackening incidence and the different carbohydrate-, phenolic- or water content measured.

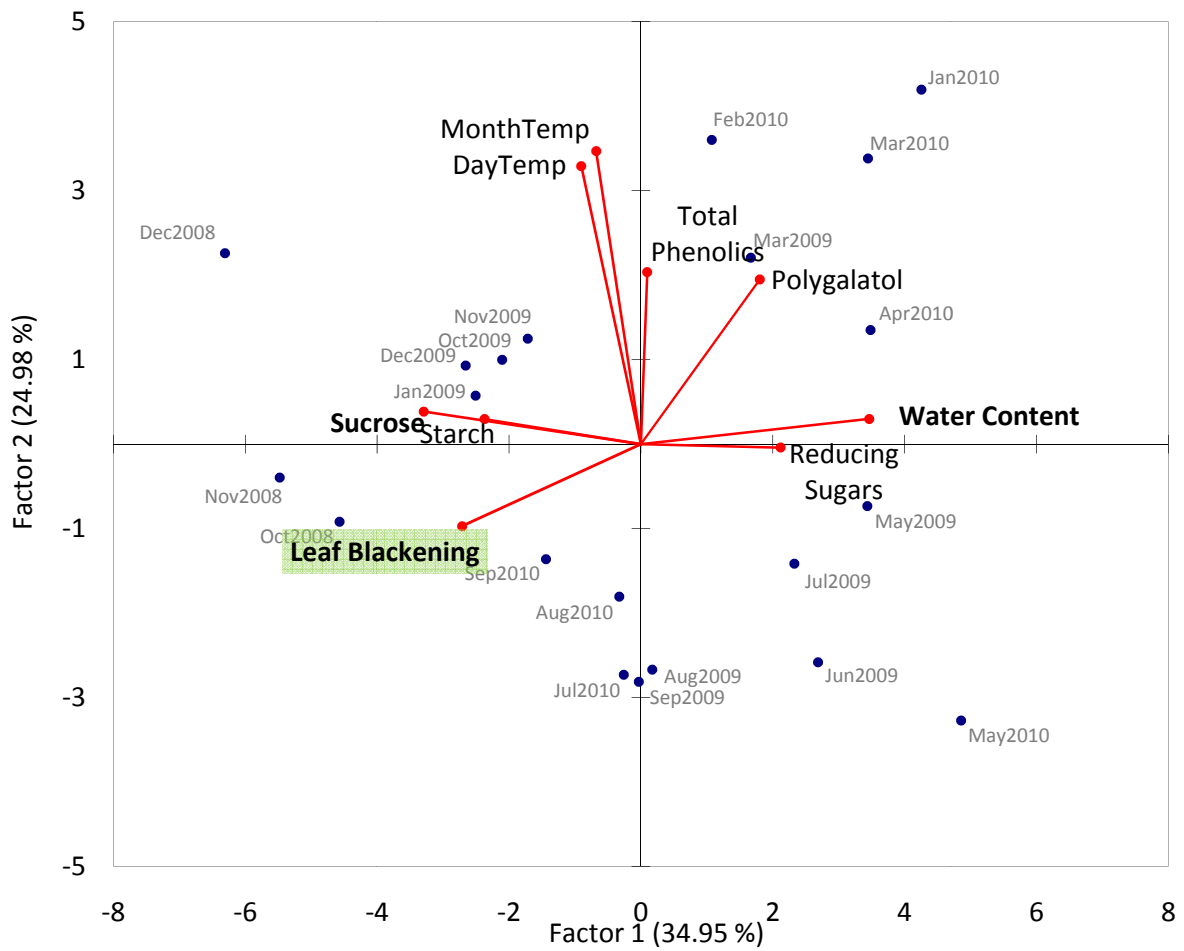


Fig. 2.3 The variables (significantly correlated factors are highlighted in **bold** in terms of leaf blackening) and observations (month-year) of a principle component analysis (PCA) bi-plot chart for *Protea* cv. *Sylvia* flowering stems harvested throughout 2008 – 2010 using all the observational and measured factors. Percentages (%) indicate the corresponding percentage of variance described by each factor.

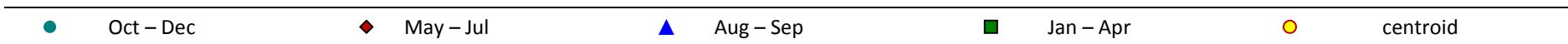
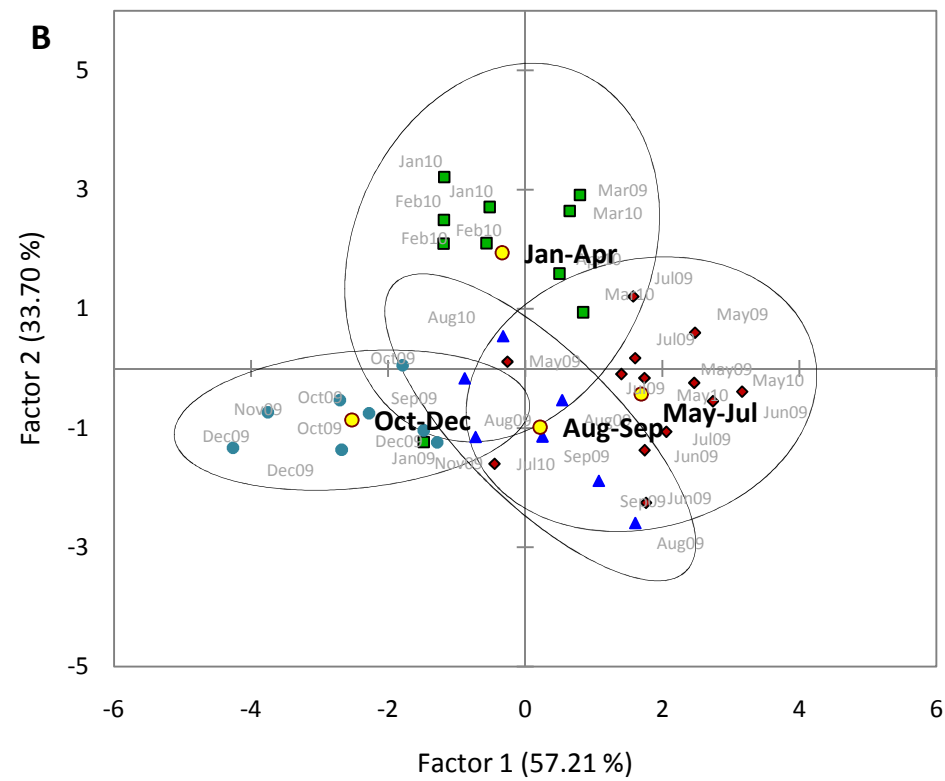
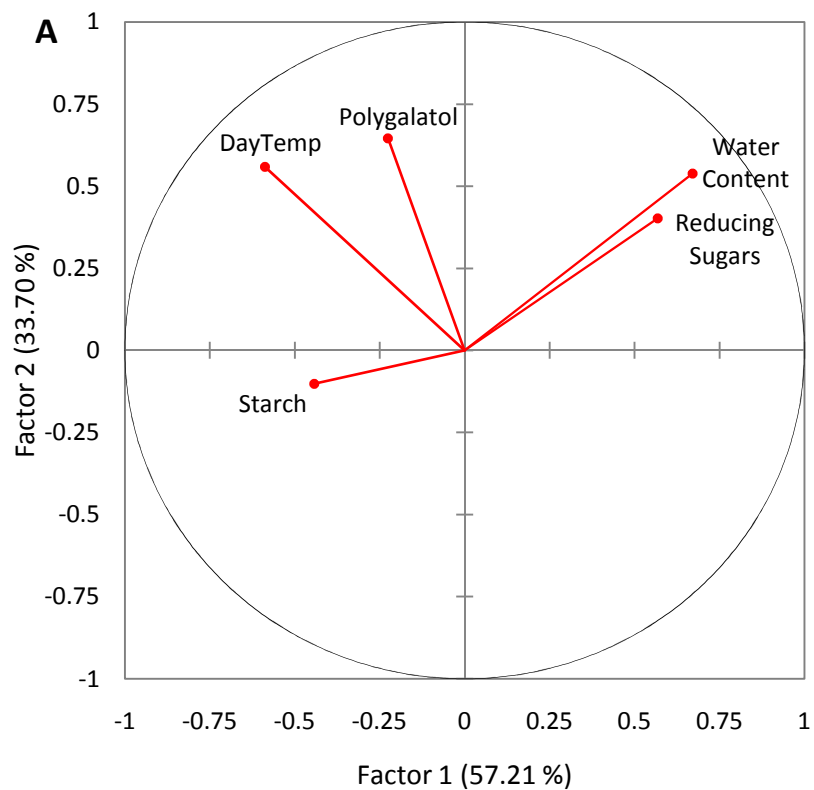
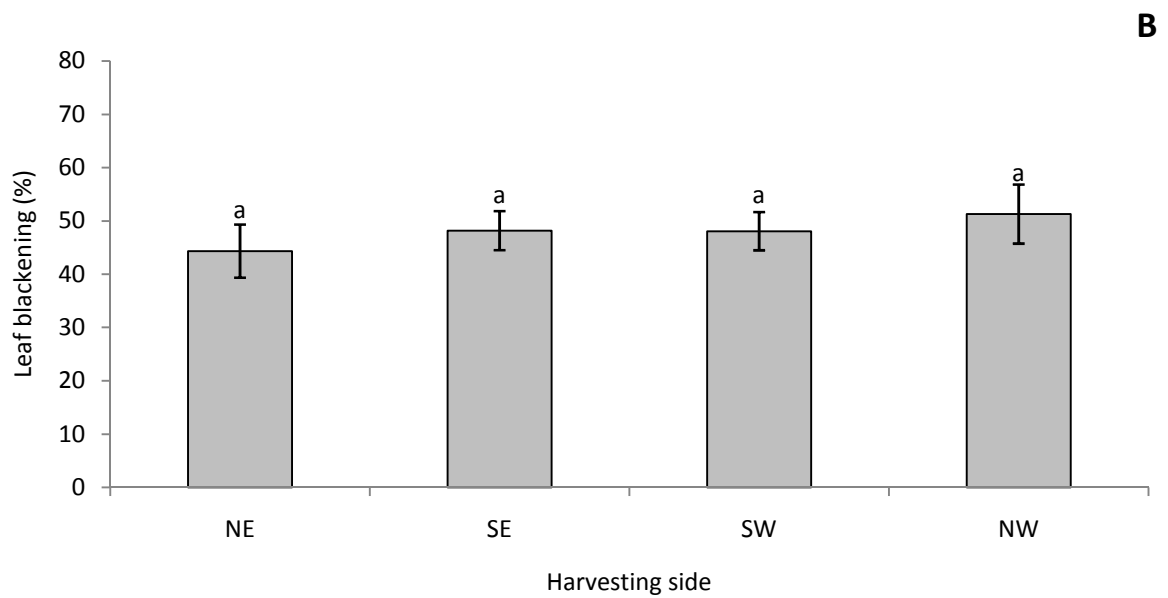
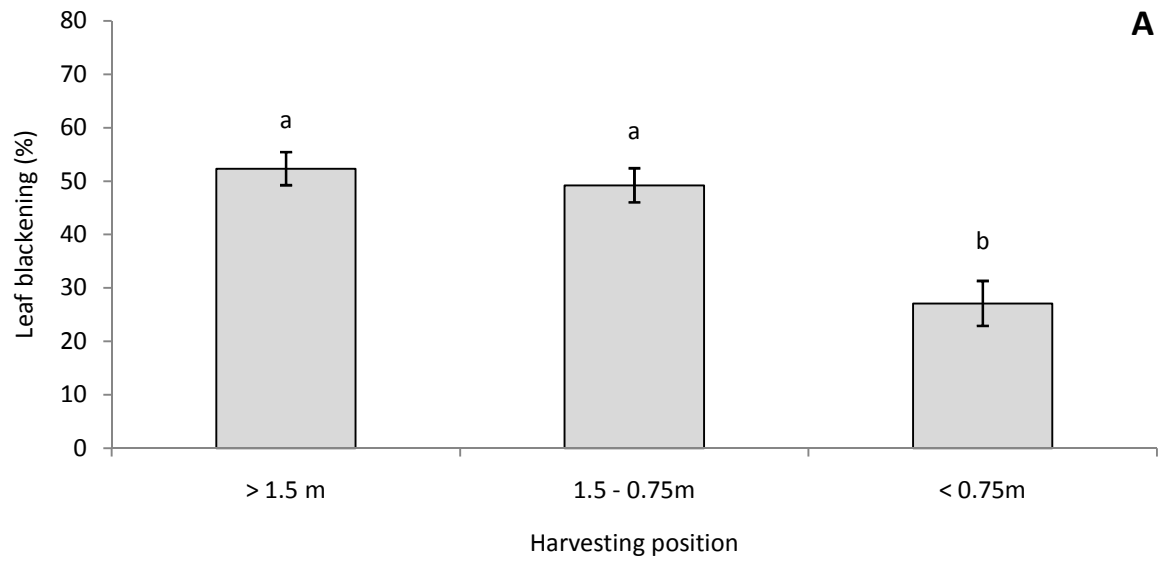


Fig. 2.4 Variables significantly selected for stepwise Discriminant Analysis (DA)(A) and Observations (B) charts for *Protea* cv. *Sylvia* flowering stems harvested throughout 2008 – 2010 using all the observational and measured factors in a DA. Percentages indicate the corresponding percentage of variance described by each factor.



ANOVA	Main Effects	F	p
A	Harvesting height	7.847	0.0006
B	Harvesting quadrant	0.584	0.6263

Fig. 2.5 The percentage (%) leaf blackening for *Protea* cv. *Sylvia* stems harvested throughout the year of 2009, harvested at different heights (A) or different sides (B) of the tree. Vertical error bars indicate the Standard Error of the mean for each data point. Different letters indicate a significant difference at the $P < 0.05$ level.

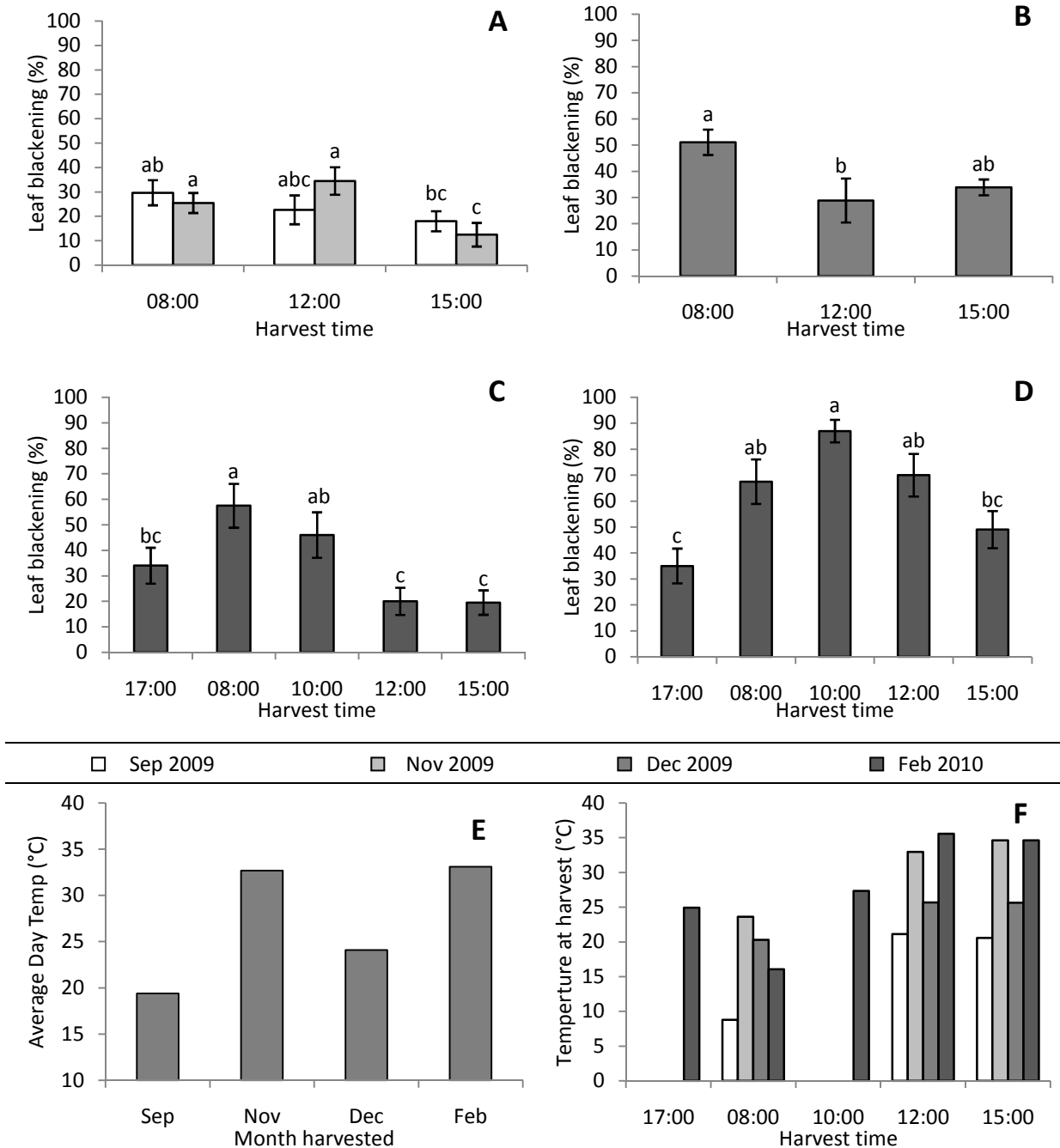


Fig. 2.6 The percentage (%) leaf blackening recorded after a 10 day evaluation period of *Protea* cv. Sylvia flowering stems when evaluated fresh (A and C) in September 2009 (n = 15), November 2009 and February 2010 (n = 10) respectively or after storage of 21 days at 4°C (±1°C) for stems harvested in December 2009 and February 2010 (n = 10) (B and D). The daily average temperatures (E) and harvest time temperatures (F) for the each harvest date were obtained from Nietvoorbij (Stellenbosch, SA) weather station. Vertical error bars indicate the Standard Error of the mean for each data point. Different letters indicate a significant difference at the $P < 0.05$ level.

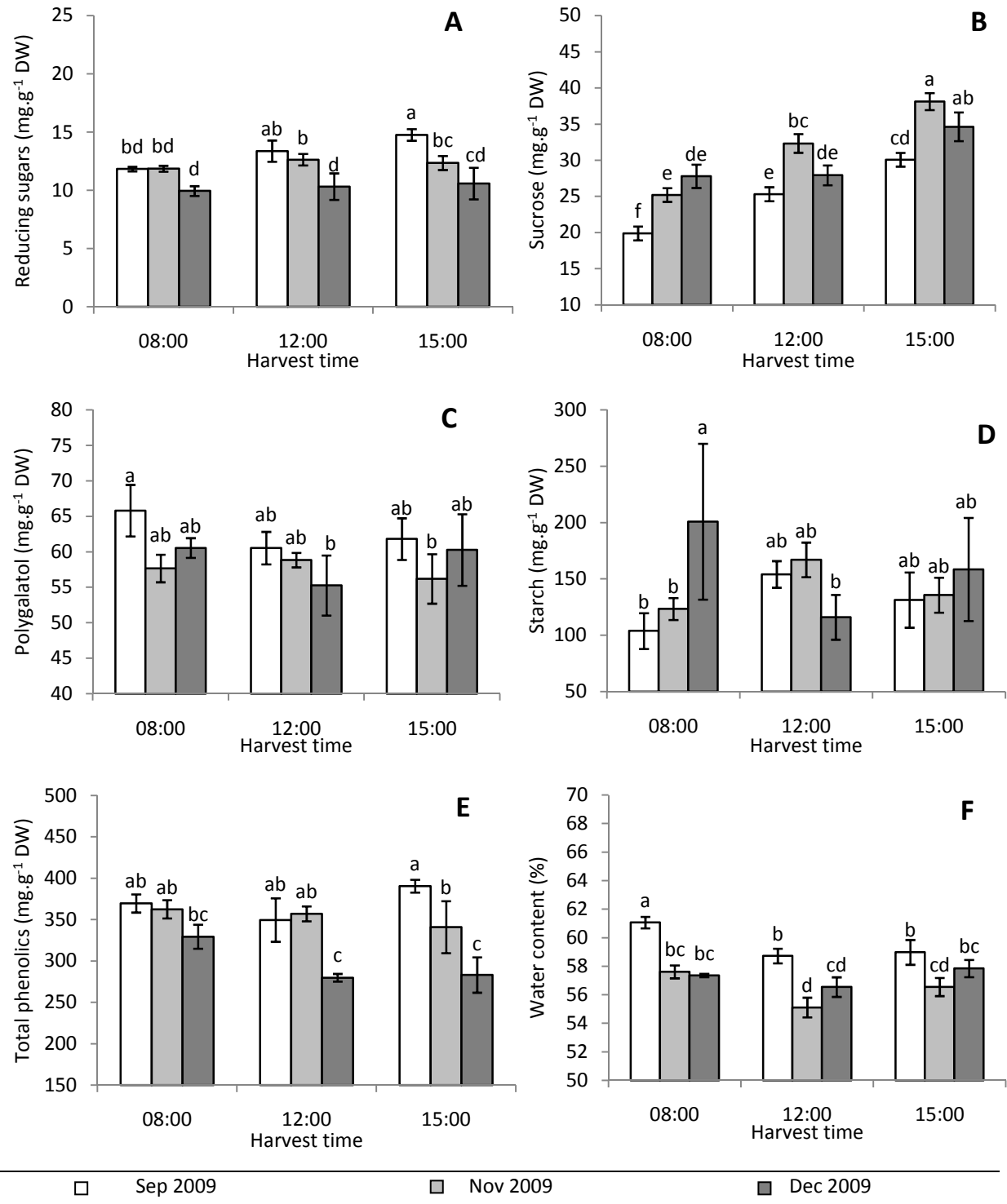


Fig. 2.7 The total reducing sugars (A), sucrose (B), polygalatol (C), starch (D) and total phenolic content (E) expressed as mg.g⁻¹ DW as well as the percentage (%) water content (F) measured in *Protea* cv. *Sylvia* foliage at each harvest time for the corresponding stems harvested at 08:00, 12:00 and 15:00 for September (n = 5), November (n = 5) and December (n = 3) 2009 respectively. Vertical error bars indicate the Standard Error of the mean for each data point. Different letters indicate a significant difference at the $P < 0.05$ level.

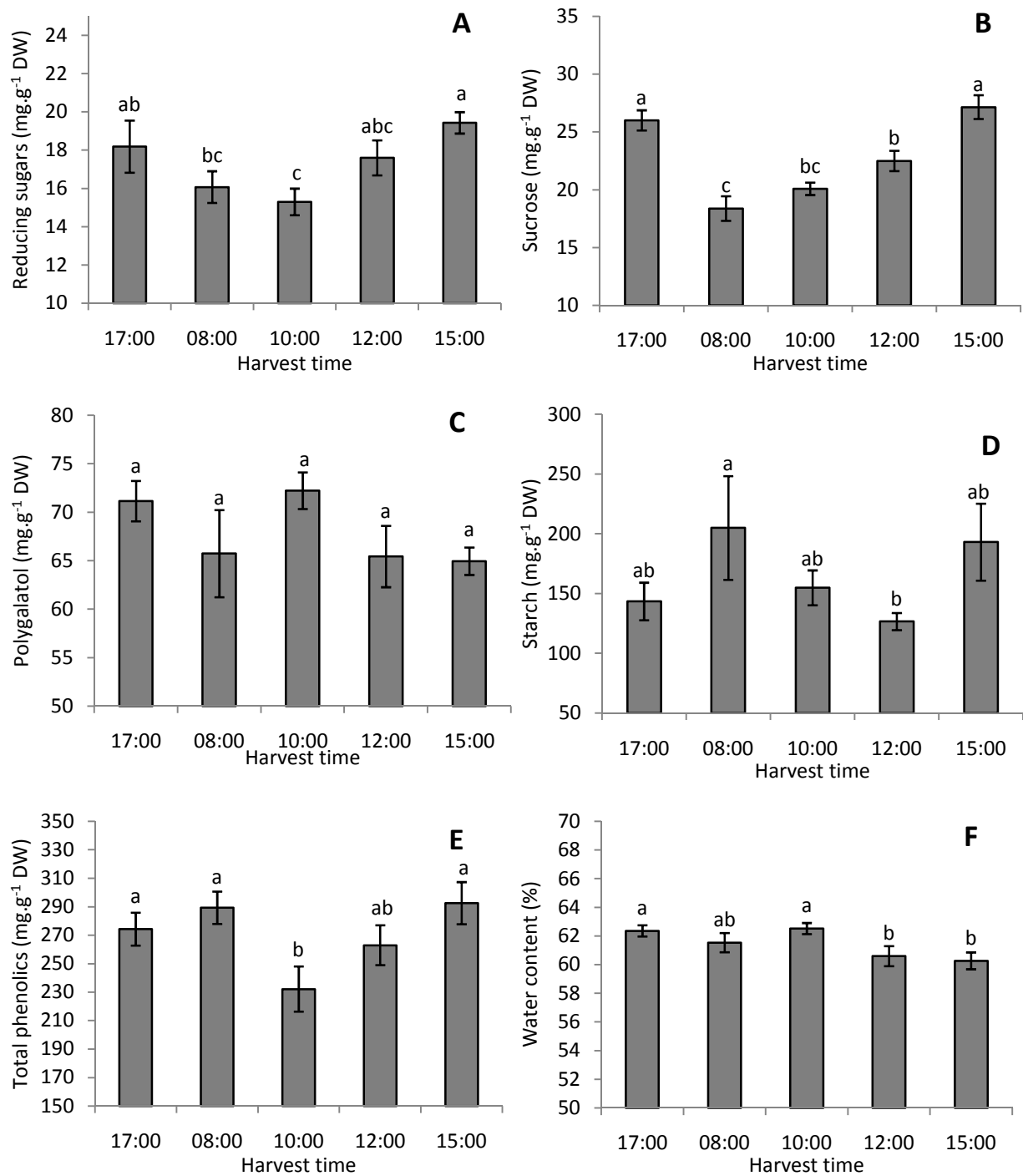


Fig. 2.8 The total reducing sugars (A), sucrose (B), polygalatol (C), starch (D) and total phenolic content (E) expressed as mg.g⁻¹ DW as well as the percentage (%) water content (F) measured in *Protea* cv. *Sylvia* foliage at each harvest time for the corresponding stems harvested at 17:00 the previous day and 08:00, 10:00, 12:00 and 15:00 in February 2010 (n = 5). Vertical error bars indicate the Standard Error of the mean for each data point. Different letters indicate a significant difference at the $P < 0.05$ level.

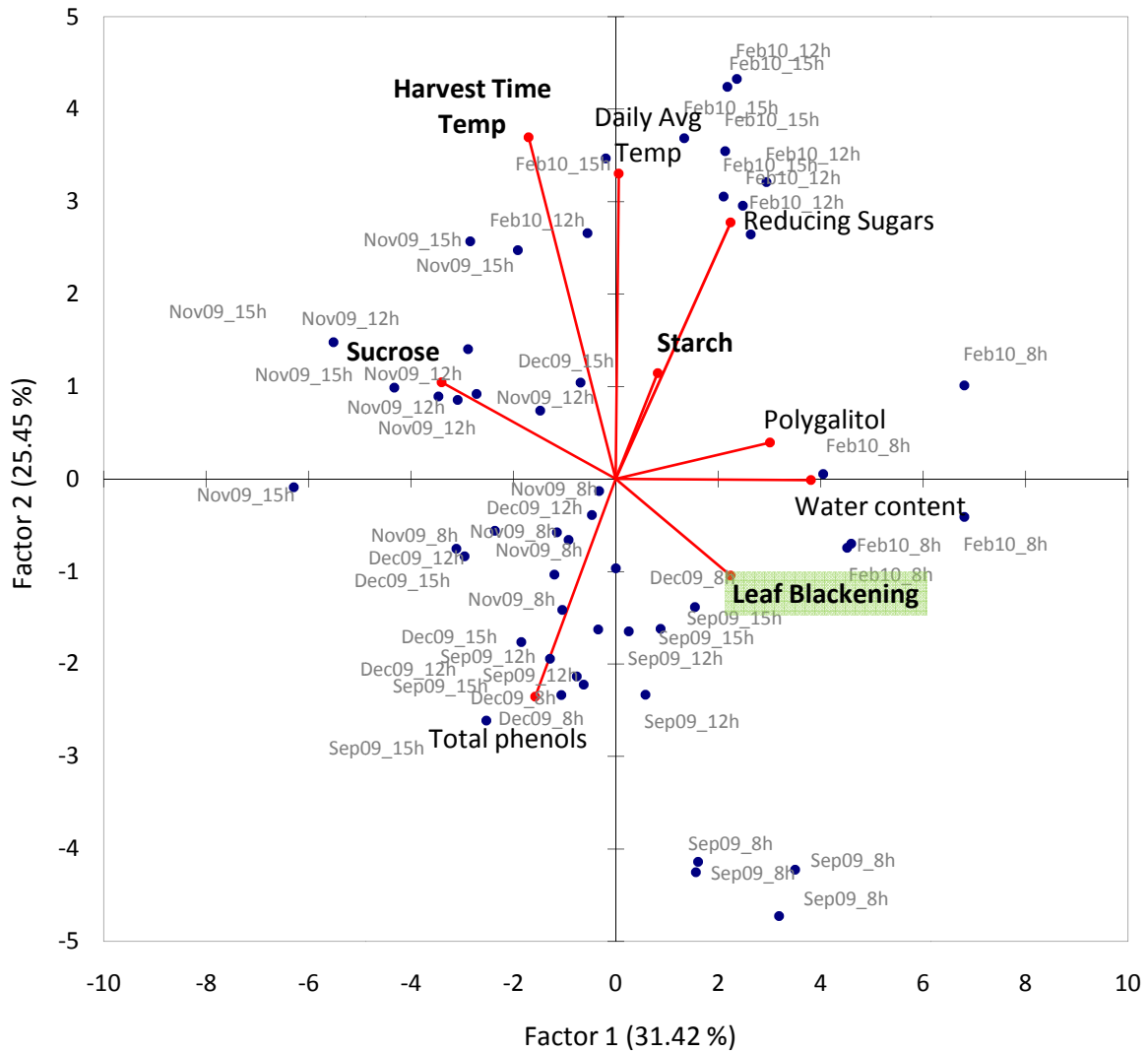


Fig. 2.9 The variables (significantly correlated factors are highlighted in **bold** in terms of leaf blackening) and observations (month-time average of all replicates) of a principle component analysis (PCA) bi-plot chart for *Protea* cv. *Sylvia* flowering stems harvested throughout the day in September, November and December 2009 and February 2010 using all the observational and measured factors. Percentages indicate the corresponding percentage of variance described by each factor.

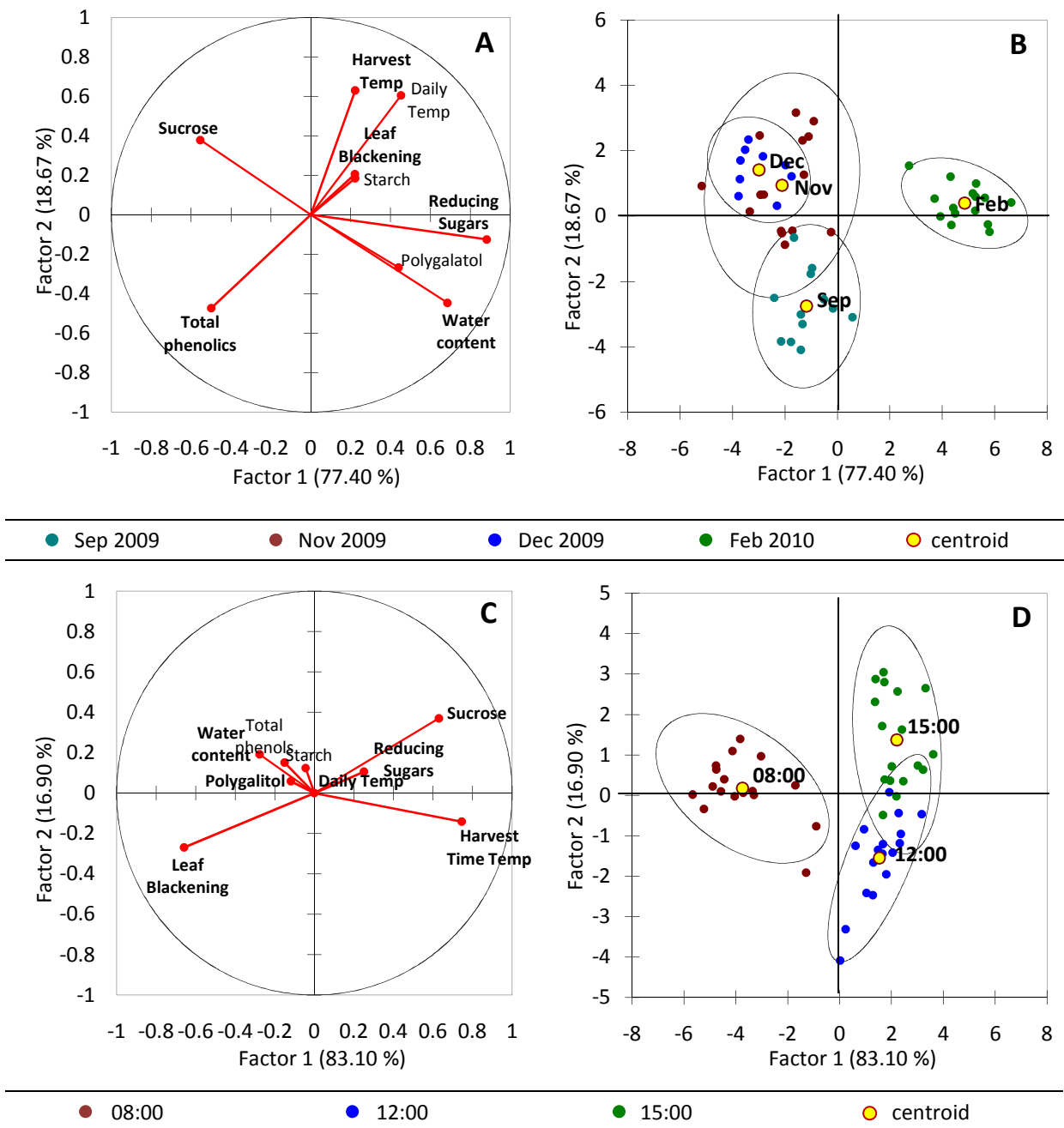


Fig. 2.10 Variables (significantly selections for stepwise Discriminant Analysis (DA) is highlighted in **bold**) (A) and observational (B) charts for *Protea* cv. *Sylvia* flowering stems harvested at different months of 2009 and 2010 and the variables (C) and observational (D) charts for the same harvest dates, but grouped according to harvesting time throughout the day. Percentages (%) indicate the corresponding percentage of variance described by each factor.

The dynamics of glucose pulsing as a strategy to improve postharvest quality of *Protea* cv. *Sylvia* cut flower stems during a long-term cold storage regime

Abstract

Leaf blackening is a serious postharvest disorder that manifests itself in many commercially important cultivars of *Protea* cut flowers. The current postharvest treatment used to delay the incidence of leaf blackening is pulsing with a 6 – 10% glucose solution. Time is an accepted guideline provided in pulsing protocols, but factors such as the vapour pressure differential between flowering stems and the environment (transpiration) and the differential of the water potential of the stem and that of the osmotic potential of the solution may seriously impact on the rate and volume of pulse uptake within a certain set of time. Flowering stems of *Protea* cv. *Sylvia* were harvested at 08:00, 12:00 and 15:00 (+2 GMT) respectively on both a relatively 'cool' (20°C maximum temperature) and 'warm' (32°C maximum temperature) day, or were dehydrated by 0, 5, 10 and 15% or pulsed with a glucose concentration solution range of 0, 4.8, 7.1, 9.4, 13.8 and 17.7% respectively for four hours. Stem and pulse-solution weight was used to calculate the solution uptake, water retained and glucose accumulated by the stem as well as to estimate transpiration. Stems harvested at 08:00 absorbed significantly less solution after four hours of pulsing than stems harvested later in the day. Similarly, pulse uptake volumes increased with a decrease in stem hydration from 100% to 85% hydration. Furthermore, solution uptake decreased with an increase in glucose concentration. Despite lower uptake volumes at the higher glucose concentration range, high pulse concentrations lead to an increase in glucose accumulation in stems after the first hour of pulsing. Decreased water potential, as is likely to be present in dehydrated stems or stems harvested later in the day experienced a higher potential deficit between the pulse solution and the stem. This could explain the increase in solution uptake within the first hour of pulsing, where after equilibrium was reached. Transpiration also contributed to an increased solution uptake, especially on 'warm' days, driven by a higher water vapour pressure deficit between flowering stems at field temperatures and the cooler laboratory environment. Pulsing of *Protea* cv. *Sylvia* flowering stems should therefore be closely monitored, as these different factors play an important role in the rate of pulse uptake and if not controlled, could result in toxic or inadequate levels of glucose uptake. Correct pulsing protocols are of key importance to control the development and incidence of leaf blackening successfully during long term cold storage which is required in the sea freight period to distant markets.

Keywords: Leaf blackening, dehydration stress, harvest time, cut flowers, transpiration, water potential

3.1 Introduction

Leaf blackening is a serious postharvest condition that manifests in most *Protea* cut flowers as a discoloration developing on leaves within 3 – 5 days after harvest (Jones et al., 1995). This disorder reduces the vase life drastically and negatively impacts on the visual appearance of the product. Leaf blackening as a postharvest disorder remains the main constraint to the export of these niche products (Van Doorn, 2001) as it may be the single most important factor responsible for an entire consignment rejection (Verhoogt, pers. comm., 2011).

Leaf blackening affects most of the major commercially cultivated *Protea* species such as *Protea eximia*, *P. compacta* and *P. neriifolia*, as well as some of the important hybrid cultivars derived from one of the above listed species such as 'Sylvia' (*P. eximia* x *P. susannae*) or 'Pink Ice' (*P. compacta* x *P. susannae*) (Jones et al. 1995; Van Doorn, 2001). Jones et al. (1995) and Van Doorn (2001) extensively reviewed a number of studies that focused on the role of environmental factors as well as the physiological mechanisms underlying leaf blackening. A link between low carbohydrate levels in the leaves (that subtend and support the large inflorescence) and the onset and incidence of leaf blackening have been pointed out by several researchers (Newman et al., 1989; McConchie et al., 1991, 1994; Bielecki et al., 1992; McConchie and Lang, 1993). One of the processes linked to leaf blackening is the continued development of the inflorescence after harvest which involves vast amounts of nectar production (Mostert et al., 1980), resulting in a major carbohydrate sink within the harvested stem. Biochemically, it has been hypothesized that freely available carbohydrates become rapidly depleted in the leaves that support the developing inflorescence. Therefore other carbohydrate sources, such as sugar-bound polyphenols are cleaved to release the sugars, thus exposing polyphenols for enzymatic or non-enzymatic oxidation, which then leads to the discoloration typical of leaf blackening (Jones et al. 1995).

The South African Fynbos industry relies predominantly on the European export market (Dorrington, 2008). Increasing pressure from retailers to lower transport costs and carbon footprints has led to a switch in the use of sea freight as the alternative transportation method to air freight. Although cold chain management can be better controlled during sea freight, the time of transport via sea freight to Europe takes approximately 21 days compared to the 2 – 3 days required for air freight. This extended postharvest cold storage period significantly increases the risk for a higher incidence in leaf blackening.

The collective outcome for all leaf blackening methods currently used is to lower or limit the respiration rate throughout the cold storage- and transport period in order to delay the onset or relieve the degree of leaf blackening development. Fuming or pulsing *Protea* cut flower stems with

ethanol has proven to be effective to lower leaf blackening (Crick and McConchie, 1999; Cannon and McConchie, 2001), but is a costly method and may yield inconsistent results under temperature fluctuations (Cannon and McConchie, 2001). Girdling (removal of a ring of bark) of the stem just below the inflorescence can physically prevent the flower from depleting foliage sugar reserves (Reid et al. 1989; Newman et al. 1989; Stephens et al., 2001). As handling should be kept to a minimum, girdling is not only highly labour intensive as well as time consuming, but producers also run the risk of weakening the flower stem, which could snap off during further handling and packing, or cause a point for fungal infection. Lastly, providing a concentrated carbohydrate supplementation through pulsing soon after harvest, whereby the reserve carbohydrate pool can be replenished, has provided the most constant and reliable control of leaf blackening throughout the entire vase life (Jones et al., 1995; Van Doorn, 2001; Meyer, 2003; Stephens et al., 2005).

Pulsing with 6 to 10% glucose solution for 24 hours have been found to provide a significant delay in leaf blackening in *Protea* cv. Sylvia (Stephens et al., 2001; Stephens, 2003) to the extent that it is used commercially in South Africa on susceptible cultivars, mainly when the incidence of leaf blackening is most severe, throughout spring up until early summer (August – November). Although this approach can be successfully executed on small scale, there are still some major commercial constraints to using this method effectively. A serious disadvantage in glucose pulsing is the unpredictable rates of pulse uptake, as these rates could be influenced by the water relations within the stem; which in turn would be affected by the micro-climatic conditions immediately prior to harvest, such as a recent rain shower or irrigation. In addition, the concentration of the pulsing solution (glucose) in relation to the water relations within the stem could also influence the accumulation capacity of pulse solution in the stem. Due to the variation in uptake rates throughout the season, between processing areas and also between cultivars, to date, no optimum pulsing time has been recommended to producers for commercial use on any *Protea* cultivar.

In the intact plant, a water potential gradient is the driving force in the transport of water from the soil as well as that of solutes, hormones and other chemicals within the plant (Lambers, 2008; Taiz and Zeiger, 2010). Similarly, the water potential and water status of a cut flower stem is the driving force for the uptake of pulse or vase solutions. In turn, the osmotic potential of the pulse or vase uptake solution also strongly influences both the ability and the rate at which a solution is acquired by the stem. This principle was effectively demonstrated through measuring water/solution flow within isolated rose stem segments (Durkin, 1979). In addition, a transpiring stem will also influence the pulse or vase solution uptake dynamics. As the harvest time of cut flowers is known to affect the immediate carbohydrate pool and water status (Dole and Wilkins,

2005) which would impact in turn on the osmotic and water potential of the stem, even the time of harvest may contribute to variation in the solution uptake ability of cut stems.

In most cut flowers, high carbohydrate levels are strongly linked to the 'delay' of senescence or the longevity of a cut flower's vase life (Halevy and Mayak, 1981). Carbohydrates play an important role in the maintenance of respiration and other functions needed to ensure postharvest inflorescence longevity (Nowak and Rudnicki, 1990). Previous studies have shown that pulsing with sugars improves cell integrity, and thus function, and water balance or status within cut flowers as well as contributing to an osmotic adjustment within the flowers which increases longevity (Halevy and Mayak, 1979).

The aim of this study was to investigate the dynamics of the uptake process of a glucose pulse solution as influenced by varying hydration levels of the flowering stem, or under different harvest times and conditions during the day and by exposing the cut flower stems to a range of glucose pulse concentrations. A greater understanding of the contribution of these respective factors will assist in constructing guidelines to producers on the implementation of glucose pulsing protocols to effectively control the development of leaf blackening in *Protea* cv. *Sylvia*.

3.2 Materials and methods

3.2.1 Plant material and Pulsing Solutions

Dry-picked, harvest-ready (soft-tip stage), flowering stems of *Protea* 'Sylvia' (*P. eximia* x *P. susannae*) were collected from a commercial farm located near Stellenbosch (33°55'S; 18°50'E), Western Cape, South Africa. Within an hour of harvest, stems were standardized to a length of 40cm, with 20 – 30 leaves per stem at the Department of Horticulture, Stellenbosch University. The study was conducted over two seasons, during the high leaf blackening incidence period, with the 'dehydration stress' experiment being conducted in August 2009, the 'harvest time' experiments conducted in September and November 2009 and the 'glucose concentration' experiment conducted in November 2010. All experiments were harvested in the morning (08:00), except for the 'harvest time' experiments, which also included a harvest at midday (12:00) and in the afternoon (15:00).

The glucose pulsing solutions used for all experiments were made up according to molar concentration (M) values, as all uptake measurements were quantified by weight and volume. In the 'dehydration stress' and both 'harvest time' experiments, a glucose molarity of 0.32M was used, which is equivalent to a 5.7% glucose solution. For the experiment where a glucose concentration

range was used the molarity values ranged from 0, 0.27, 0.40, 0.52, 0.76 and 0.98M which is equivalent to 0, 4.8, 7.1, 9.4, 13.7 and 17.7% glucose solutions respectively.

During each pulsing experiment, vases were covered with Parafilm™, with a small hole pierced to allow for the insertion of the stem, in order to reduce loss of water through evaporation. Stems were pulsed in an evaluation room with temperatures of 18°C ($\pm 2^\circ\text{C}$), light/dark cycles of 10/14 hours with $15\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light levels and a relative humidity of 60% ($\pm 10\%$).

3.2.2 Factors evaluated for study of glucose pulsing dynamics

3.2.2.1 Dehydration stress

To study the role of dehydration stress on the uptake of pulse solution, stems were harvested on 25 August 2009, weighed after standardizing (as described above) and placed in tap water to fully hydrate overnight at room temperature ($20^\circ\text{C} \pm 2^\circ\text{C}$). After 22 hours, at presumably full hydration, stems were again weighed and allocated, according to a completely randomized design, to various dehydration treatment groups, with 8 single replicates per treatment. The dehydration treatments implicated that all stems after removal from the overnight tap water, were left dry (outside in semi-shade) until stems had lost 0, 5, 10 or 15% of their weight to achieve a hydration status of 100, 95, 90 or 85% respectively. On reaching the required state of dehydration stress, stems were then pulsed with a 5.7% glucose solution for 4 hours, whilst both the stem and the respective pulse-solutions were weighed hourly.

3.2.2.2 Harvest time

To determine the impact of the time of harvest on pulse uptake, 10 stem single replicates were harvested according to a complete randomized design at 08:00, 12:00 and 15:00 (+2 GMT) and standardized as described above. Such a harvest was done both on a 'cool' spring-day (4 September 2009) with an average temperature of $\pm 14^\circ\text{C}$ and maximum temperatures reaching 20°C and on a 'warm' summer's day (3 November 2009) when the average temperature was $\pm 23^\circ\text{C}$ with maximum temperatures reaching 32°C . After grading all stems (at each harvest time) and their respective pulse solution was weighed, immediately prior to pulsing. Stems were then pulsed for four hours with 5.7% glucose solution, whilst both the stems and pulse-solutions were weighed hourly throughout the pulsing period. In addition, temperature and relative humidity weather data was obtained from a weather station located near the harvesting farm, at Nietvoorbij ($33^\circ 55' \text{S}$; $18^\circ 54' \text{E}$),

Stellenbosch, Western Cape in South Africa and verified by means of a LogTag® (LogTag Recorders, Auckland, New Zealand) logger which recorded the temperature and relative humidity on site, to determine the vapour pressure deficit.

3.2.2.3 Glucose concentration range

In this experiment, pulse solution uptake rates within a glucose concentration range were compared. Stems that were harvested at 08:00 on 10 November 2010 were weighed after grading, immediately prior to pulsing. Stems were allocated into groups of 6 single stem replicates each to treatment according to a randomized complete design and pulsed for four hours. The following glucose concentration range: 0% glucose (control); 4.8; 7.1; 9.4; 13.7 and 17.7% respectively were used. Stems and pulsing solutions were weighed hourly throughout the entire uptake period.

3.2.3 Calculations

In each experiment, both the solution weight and stem weight was measured hourly for the four hours of pulsing, also to include the starting weights. From these values were calculated: Solution uptake; glucose and water uptake; water retained in the stem; transpiration by the stem as well as the contribution of water retained and transpired to the glucose accumulation in the stem.

Solution uptake was directly measured as the weight of solution lost from the pulsing solution. The fraction of glucose uptake was calculated by multiplying the corresponding molar concentration with the solution absorbed. Water uptake was determined as the fraction that remained after the calculated glucose uptake was subtracted from the solution uptake.

Water retained by the stem was calculated by subtracting the amount of glucose taken up from the stem weight differences measured over the four hour period. Similarly, transpiration was calculated by subtracting the water retained by the stem from the water removed from the pulsing solution.

The amount of glucose accumulated by the stem which could be contributed to water retained and transpiration were calculated by multiplying the weighted water retained or transpired with that of the relevant glucose solution concentration.

For stems collected for the harvest time experiment, the vapour pressure deficit was calculated by determining the vapour pressure of the cut flower stem and its immediate environment in the field, at harvest, and also after the first and last hour in the laboratory during the pulsing period. The vapour pressure, measured in mbar, of the cut flower stem and its environment

was determined from a psychrometric chart using the temperature and relative humidity obtained from the weather data, as well as the LogTag® temperature and humidity logger. The vapour pressure deficit is then calculated as the vapour pressure difference between the inside of the cut flower stem (assumed that it is at 100% relative humidity at that specific temperature) and the environment.

3.2.4 Statistical analysis

One-way analysis of variance (ANOVA) was used to calculate statistical differences between treatments at the end of the four hour pulsing period. Repeated measures analysis of variance (RANOVA) was used to analyse the interaction between treatments with respect to the rate of solution uptake, glucose uptake as well as water retained and transpired. All statistical analysis was performed using STATISTICA version 10 (Statsoft, Inc., 2011). Means were separated by Fisher's Least Significant Difference (LSD) test, with a significance level at $P < 0.05$.

3.3 Results

3.3.1 Dehydration stress

Fully hydrated stems (controls), which were pulsed immediately after rehydration, took up the least amount of pulse-solution compared to all other dehydrated treatments (Table 3.1). After the four hour pulsing period, solution uptake significantly increased with the level of dehydration (Table 3.1). Glucose accumulation was also significantly different between hydration treatments after the four hour pulse, where more than $1\text{g glucose}\cdot\text{stem}^{-1}$ was accumulated at the lowest hydration treatment of 85%, compared to $0.2\text{g glucose}\cdot\text{stem}^{-1}$ for fully hydrated stems (Table 3.1).

Similarly, there was a significant difference between the hydration level and the amount of water retained after four hours (Table 3.1). No water was retained within the stem in fully hydrated stems, whereas increasing amounts of ± 4 , 11 and $15\text{g water}\cdot\text{stem}^{-1}$ were retained in the 95, 90 and 85% hydration treatments respectively. Likewise, there was a significant difference between hydration treatments for nett transpiration ($p = 0.024$) after the four hour pulse, however the differences were less pronounced between treatments, which ranged between 3.1 and $4\text{g}\cdot\text{stem}^{-1}$ (Table 3.1 and Fig. 3.1D).

The glucose that accumulated via the water retained also increased progressively with an increase in dehydration, whereas the glucose that accumulated via the water transpired was similar,

with no significant difference between the glucose amounts accumulated for the different dehydration treatments ($p > 0.05$).

There was a significant interaction between nett solution uptake ($p < 0.0001$), as well as nett glucose ($p < 0.0001$) uptake over the entire pulsing period (Figs. 3.1A and B). Both pulse solution and glucose nett uptake was higher for the 85 and 90% hydrated stems, whilst a lower uptake was recorded for the 95% and fully hydrated stems (Figs. 3.1A and B). Similarly, there was a significant interaction for the water retained between treatments over the four hour period ($p < 0.0001$) (Fig. 3.1C). No nett water was retained in fully hydrated stems over the entire pulsing period compared to the moderate nett uptake for the 95% hydration treatment and with steep uptake volumes within the first hour for both the 90 and 85% hydrated stems (Fig. 3.1C). Nett transpiration by treatment stems over the entire pulsing period resulted in a significant interaction ($p = 0.0240$) over time (Fig. 3.1D). Transpiration within the first hour varied to different extents between hydration treatments, where after a gradual increase was observed in all hydration levels (Fig. 3.1D).

3.3.2 Harvest time (cool day)

Stems harvested on a 'cool' day, with maximum temperatures reaching only 20°C, showed a significant difference ($p < 0.001$) between solution, water and glucose uptakes in stems harvested in the morning (08:00) compared to stems harvested later in the day, at 12:00 and 15:00, which did not differ from each other (Table 3.1). After four hours, only 2.75g pulse solution was absorbed in the morning-harvested stems, compared to the almost doubled amount of 5.06g and 5.57g for midday (12:00) and afternoon (15:00) harvested stems respectively (Table 3.1). The corresponding amount of glucose uptake calculated after four hours followed the same significant pattern (Table 3.1).

Water retained by the stems of the different harvesting times also were significantly less for stems harvested in the morning compared to later in the day ($p < 0.001$). However, when nett transpiration was calculated, there were no significant difference between the various harvest times ($p > 0.05$) (Table 3.1).

Similarly, the glucose that accumulated via the water retained after the four hour pulsing period were significantly different ($p < 0.001$) between stems harvested in the morning (0.04g) compared to the stems harvested later in the day, at 0.19 and 0.20g respectively (Table 3.1). This glucose accumulation trend is in concurrence with the amount of water retained. Similar to the hydration experiment, there was no significant difference in glucose accumulated via transpiration ($p > 0.05$) between harvest times, where the transpiration accounted for approximately ± 0.11 g glucose in all treatment stems (Table 3.1). Thus, stems harvested in the morning accumulated more

glucose via transpiration, whereas stems harvested later in the day both acquired more glucose via water retained than via transpiration (Table 3.1).

Stems harvested at 08:00 in the morning had a low vapour pressure deficit, which increased from the field to the laboratory where pulsed, whereas stems harvested later in the day at 12:00 and 15:00 respectively remained relatively constant (Table 3.2).

Following the net uptake over the four hours of pulsing, except for the uptake driven by transpiration, there was a significant interaction between solution uptake ($p < 0.0001$), glucose uptake ($p < 0.0001$) and water retained ($p < 0.0001$), and the pulsing period (Figs. 3.2A to D). For solution and glucose uptake, stems harvested in the morning had slower, but consistent increase over the entire pulsing time, whereas stems harvested later in the day had an initially high uptake within the first hour, where after uptake continued, but according to a similar consistent trend (Figs. 3.2A and B). However, a significant interaction for the water retained over the pulsing time could clearly be seen between treatments, as stems harvested in the morning only retained water in the first hour where after it remained constant, compared to stems harvested later in the day, where net water retention was much higher in that first hour, but which also remained fairly constant there after (Fig. 3.2C).

Water loss via transpiration gradually increased throughout the pulsing period. Although stems harvested in the morning had a more steady loss, there were no difference between the harvesting times, and therefore there was no significant interaction (Fig. 3.2D).

3.3.3 Harvest time (warm day)

Stems harvested on a warm day in November 2009, with maximum temperatures reaching 32°C, also displayed a significant difference ($p < 0.001$) between treatments in terms of solution uptake (Fig. 3.3A). Pulse uptake on a 'warm' day differed from that of a 'cool' day in that significant differences were recorded for solution uptake between all harvest time treatments and not just between the morning- and that of stems harvested later in the day (Table 3.1). Similarly to stems harvested on a cool day, stems harvested in the morning at 08:00 accumulated significantly less solution (8.51g solution.stem⁻¹) compared to the 11.78 and 10.35g solution.stem⁻¹ that was accumulated by stems harvested at 12:00 and 15:00 respectively (Table 3.1).

A larger volume of solution uptake for the 'warm' day harvested stems, resulted in a higher glucose accumulation after four hours (Table 3.1). Stems harvested at 12:00 acquired the highest amount of glucose (0.67g.stem⁻¹) after four hours of pulsing with 5.7% glucose (Table 3.1).

Similarly to a 'cool' day, there was a significant difference ($p < 0.001$) between water retained in the stems, where stems harvested in the morning retained $<0.5\text{g water.stem}^{-1}$ after four hours, compared to stems harvested later in the day, retaining $\pm 3\text{g water.stem}^{-1}$ (Table 3.1). However, for stems harvested on a 'warm' day, the water transpired was significantly different ($p = 0.008$), where the lowest transpiration was recorded in stems harvested at 15:00, compared to stems harvested earlier in the day (Table 3.1).

Glucose uptake linked to water retention on a 'warm' day resulted in almost an identical trend as recorded for glucose uptake explained via water retained on a 'cool day' (Table 3.1). The glucose uptake explained via transpiration followed the same pattern as noticed for the water transpired (Table 3.1). There was a significant difference ($p = 0.008$) in glucose accumulation via transpiration in stems harvested at 15:00 compared to earlier in the day, where the 08:00 and 12:00 harvested stems accumulated $\pm 0.5\text{g}$ via transpiration, whilst stems harvested at 15:00 accumulated $\pm 0.4\text{g}$ (Table 3.1).

A greater vapour pressure deficit was calculated for stems harvested later in the day when compared to stems harvested in the morning, on both harvesting days in September and November 2009 (Table 3.2). Although the vapour pressure deficit decreased by the start of pulsing, stems harvested at 12:00 and 15:00 in November 2009 still had more than double the vapour pressure deficit than stems that were harvested in the morning (Table 3.2). However, by the end of the pulsing period, vapour pressure deficits calculated were the same for all harvest times, irrespective of harvest date (Table 3.2).

Similar to the hydration experiment, there was significant interaction for solution ($p < 0.0001$) and glucose uptake ($p < 0.0001$), as well as for water retained ($p < 0.0001$) and transpired ($p < 0.0001$) over the four hour pulsing period (Figs. 3.3A to D). The nett uptake of both solution and glucose within the first hour was different than the uptake thereafter (Fig. 3.3A and B). Differences in uptake were also evident between harvest times, where the highest uptake was recorded in stems harvested at 12:00 and the lowest at 08:00 (Figs. 3.3A and B). For stems harvested at 08:00, the most water was retained within the first hour after pulsing commenced, where after more water was lost than gained by the last hour of pulsing (Fig. 3.3C). In comparison, stems harvested later in the day, had an increased higher amount of water retained within the first hour, where after water was not retained further during the pulse period (Fig. 3.3C). The lowest nett transpiration was recorded for stems harvested at 15:00, compared to the initially much higher transpiration for stems harvested at 12:00 (Fig. 3.3D).

3.3.4 Glucose concentration range

Solution uptake after four hours decreased progressively with the increase in pulse solution concentration (Table 3.1). Control stems accumulated significantly ($p < 0.05$) more pulse solution than stems pulsed with the highest glucose concentration (17.7%), after four hours (Table 3.1). Although this resulted in a significant difference between the amounts of glucose accumulated within all stems of the various pulse-concentration treatments after four hours, glucose accumulation still increased with an increase in glucose pulse-concentration (Table 3.1). Stems exposed to the lowest pulse-concentration of 4.8% only accumulated 0.6g glucose compared to stems treated with the highest concentration of 17.7% which accumulated almost 2g of glucose during the pulse-period (Table 3.1).

On the contrary, water retained within the stems after four hours significantly decreased ($p < 0.001$) with an increase in glucose pulse-concentration (Table 3.1). There was a significant difference in the amount of water transpired between stems pulsed with 9.4% glucose after four hours compared to all the other treatments (which did not significantly differ from each other) (Table 3.1).

Although the water retained decreased with an increase in pulsing glucose-concentration, the glucose uptake associated with the water retained increased with an increase of pulse concentration (Table 3.1). The glucose uptake explained via the water transpired also increased with pulse concentration increase, although the amount of water that was transpired was similar after four hours in all treatments (except for 9.4% concentration treatment) as mentioned above.

Similar uptake trends were observed for stems pulsed with a range of glucose concentrations as was recorded for all of the previous discussed experiments (Figs. 3.1 to 3.3). The highest nett uptake of solution and glucose were in the first hour of pulsing, where after it decreased gradually (Figs. 3.4A and B). The nett glucose uptake continued to increase after two hours of pulsing for the three highest concentrations, whereas the rate of the three lowest glucose pulse-concentrations decreased considerably (Fig. 3.4B).

Water retention in the stems occurred in the first hour of uptake for all treatments, where after no difference was observed over time for the remaining three hours (Fig. 3.4C). A slight water loss was even recorded within the last hour of pulsing in stems exposed to the highest glucose pulse-concentration (17.7%) (Fig. 3.4C). The rate of transpiration throughout the pulsing period remained constant for all treatments (Fig. 3.4D), with the exception of stems pulsed with 9.4% glucose solution where transpiration was recorded to be higher.

3.4 Discussion

Sugar pulsing is recommended in many cut flower species in order to extend their vase life quality (Nowak and Rudnicki, 1990). This is especially applicable in the case of *Protea* cv. *Sylvia*, which is a commercially important fynbos cut flower. 'Sylvia' is widely planted specifically because of its year-round flowering habit, despite that severe leaf blackening incidence is known to occur during spring and early summer (southern hemisphere).

Methodology for pulsing cut flowers in most studies refer only to the duration of pulsing which then serves as the only measure for solution uptake (Halevy and Mayak, 1979; Reid, 2004). Most existing reports on sugar pulsing in *Proteaceae* cut flower products, such as kangaroo paw (Teagel et al., 1991) and various *Protea* species and cultivars (Stephens et al., 2001); as well as other cut flower research such as in roses (Doi and Reid, 1995) and lilies (Almeida et al., 2011) only report the time of pulsing, with no reference made to the volume of solution that is accumulated during this time.

Solution uptake is controlled primarily by two main factors: the differential between the vapour pressure of the flowering stem and its immediate environment (transpiration) and secondly, the differential between the water potential of the stem and the osmotic potential of the pulsing solution. Therefore pulsing time as a measure of uptake is of little use as the extent of these factors may vary considerably with the current water balance of the flowering stem and the nature of the pulse solution.

3.4.1 Transpiration

The vapour pressure differential between the flowering stem and the immediate environment is the driving force for transpiration. The contribution of transpiration to solution uptake and thus the glucose accumulation by the stem during pulsing is highly dependent on this vapour pressure deficit. After harvest, changes in water potential are still prevalent as transpiration continues (Rogers, 1973).

Accumulation of glucose in *Protea* cv. 'Sylvia' stems were partly governed by the water transpired during a four hour period of pulsing with a glucose solution (Table 3.1). Water lost by transpiration between the four experiments described in this paper varied from less than 2.0g.stem⁻¹ for flowers picked on a 'cool' day (September 2009) compared to as much as 7.45g.stem⁻¹ on a 'hot' day (November 2009), indicating that the conditions that controlled transpiration (vapour pressure differential) differed greatly between the respective experimental days (Table 3.1).

Harvesting on a sunny day, as was experienced in November 2009, when the air temperature reached 32°C, the cut flower temperature was most likely substantially higher than the ambient temperature of the pulsing room at 18°C ($\pm 2^\circ\text{C}$). The large vapour pressure deficit between the cut flower and the environment (23mbar) then drives the higher nett transpiration for the first hour of pulsing (Fig. 3.3D) much stronger than thereafter (Table 3.2). Once the stem and the pulsing environment reached a temperature equilibrium, transpiration proceeded slower, as the vapour pressure difference between the cut flowers and the environment (vapour pressure deficit) were now much lower (8.6mbar), which explains the significant interaction ($p < 0.001$) found in Fig. 3.3D.

For stems collected under cooler conditions in September 2009 when temperatures peaked at 20°C, the nett transpiration (Fig. 3.2D) was much lower where only 1.9g.stem⁻¹ transpired after four hours (Table 3.1). This observation can be ascribed to a lower vapour pressure deficit between the cut flowers and the pulsing room (1.5 and 8.6mbar) at a temperature of 18°C ($\pm 2^\circ\text{C}$) and relative humidity of 60% ($\pm 10\%$). In fact, no transpiration occurred during the first hour of pulsing for stems harvested on a 'cool' day at noon or mid-afternoon (Fig. 3.2D). Apparently the forces that retained the water within the stem under these harvesting and pulsing conditions were greater than the forces driving transpiration.

Transpiration is not affected by the concentration of glucose pulsing solution; however, for stems pulsed with 9.4% glucose, a higher transpiration rate was recorded. In this treatment, 7.44g water.stem⁻¹ was transpired compared to the $\pm 5\text{g water}\cdot\text{stem}^{-1}$ transpired by stems treated with the other concentrations (Table 3.1; Fig. 3.4D). This is difficult to explain, as there was no significant difference ($p = 0.229$) between treatments when values from the 9.4% concentration treatment is removed from the analysis (data not shown).

The contribution of the transpiration rate to the uptake of solution between differently dehydrated stems could be explained by the fact that stems were placed for different lengths of time outdoors without water to obtain the various levels of dehydration. Although temperature and humidity during dehydration was not measured, this may have inevitably lead to stem temperatures varying between treatments during the first hour of pulsing when compared to the inside laboratory conditions (Fig. 3.1D), which could have contributed to different vapour pressure differences. From these results it is apparent that transpiration by itself own is seldom adequate to affect the uptake of 10ml solution by 'Sylvia' flowering stems during a four hour pulsing period.

3.4.2 Shoot water potential

The difference between the water potential of the flowering shoot and that of the osmotic potential of the pulsing solution is the second driving force for solution uptake and thus glucose accumulation in the stem. Picking flowers early in the morning and into water is mostly recommended, as cell turgidity is at its most optimum during this time (Nowak and Rudnicki, 1990). Hydration stress is a major concern for cut flowers sensitive to dehydration/wilting, such as roses which suffer easily from bent-neck (Van Doorn, 1997). In cultivars where pulsing of sugars are required as a treatment, this procedure occurs soon after harvest, following the grading of the stems. When this protocol is followed, the contribution of the water potential of the stem in driving solution uptake of these hydrated cut flowers is minimal.

No or very little water ($0.5\text{g}\cdot\text{stem}^{-1}$) was retained by fully hydrated stems (Fig. 3.1C) or stems harvested early in the morning (Figs. 3.2C and 3.3C), with the result that the accumulation of glucose attributed to the stem water potential for these stems was minimal (Table 3.1). This indicates that the water potential of the stem was not below the osmotic potential of the solution. Solution uptake increased in stems with progressive hydration levels over a four hour period, from $\pm 2.5\text{g}\cdot\text{stem}^{-1}$ for stems at a hydration level of 100% to $\pm 20\text{g}\cdot\text{stem}^{-1}$ for stems at a hydration level of 85% (Fig. 3.1A). From that, water retained after a four hour pulsing period increased from 0 to $14\text{g}\cdot\text{stem}^{-1}$ with a decrease in the level of stem hydration from 100 to 85% respectively (Fig. 3.1C). Similarly, the weight of water retained increased from less than 0.5g to more than $3\text{g}\cdot\text{stem}^{-1}$ when flower picking was delayed from early to mid-day or later (Table 3.1). The decrease in the water potential of the stems with increasing degree of dehydration or in stems harvested at noon or mid-afternoon contributed to a significant interaction ($p < 0.001$) for the rate of uptake of the solution. This in turn contributed to a significant interaction ($p < 0.001$) for rate of glucose accumulation in the stems (Figs. 3.1 to 3.3A; 3.1 to 3.3B). It is clear that low stem water potential that develop during the course of a day can contribute significantly to the uptake of glucose pulsing solutions.

Irrespective of the level of dehydration, most of the water retained by the stem occurred within the first hour (Fig. 3.1C). The difference between the water potential of the stem and the osmotic potential of the solution decreased with an increase in water retained by the stem. This difference then explains the slower rate of water retention by the stem, following the first hour of pulsing, as well as the significant ($p < 0.001$) interaction between pulsing time and the level of dehydration (Fig. 3.1C). When stems experienced moderate levels of water stress the water potential of the stem and that of the osmotic potential of the pulsing solution reach equilibrium or

nearly so within one hour of pulsing. No or little further water was retained by the stem after the first hour of pulsing (Figs. 3.2 to 3.4C).

The difference between the osmotic potential of the glucose pulsing solution and the water potential of the stem decreased with an increase in glucose concentration. Similar results were recorded in early studies with sucrose pulsing of roses (Marousky, 1969). The water retained decreased significantly ($p < 0.001$) from 9.12g to 3.58g.stem⁻¹ with an increase in the osmotic potential of the pulsing solution by increasing the glucose concentration from 0 to 17.7% (Fig. 3.4C); concurrently the glucose accumulated in stems also increased significantly ($p < 0.0001$) from 0 to 0.72g per stem (Table 3.1).

Irrespective of the glucose concentration though, most of the water retained by the stem was established in the first hour of pulsing, indicative of the difference between the water potential of the stem and the osmotic potential of the pulsing solution (Fig. 3.4C). This was clearly seen in the hydration and harvesting time experiments, explaining the significant interaction obtained. After the first hour of pulsing, no or little further increase in water retained by the stem occurred, as the water potential of the stem approximated the osmotic potential of the pulsing solution (Fig. 3.4C). Although this was true, the initial difference between the water potential of the stem and that of the osmotic potential of the pulsing solution resulted in a significant difference in solution uptake between treatments after the first hour and subsequently the ultimate contribution of glucose uptake after four hours of pulsing (Table 3.1; Figs. 3.4A and B). The increase in glucose concentration lead to a linear increase in the amount of glucose accumulated in the stems after four hours, although the opposite was true for water retained (Table 3.1). This confirms findings reported by Meyer (2003), who found that pulsing different glucose concentrations until 10mL was removed required varied pulsing times to reach the 10mL of uptake. Stems pulsed with lower concentrations obtaining their required volumes much faster (2 – 4 hours) than stems pulsed with higher concentrations (7 – 10 hours).

3.5 Conclusion

When considering solution uptake dynamics recorded in this study, it would seem that the first hour of uptake is most critical time. Although harvesting later in the day allows for less time to process stems, it facilitates a faster and more efficient uptake because of higher water potential differences as well as an increased vapour pressure deficit which is created by the more pronounced temperature difference between the stem and pulsing room. Stems harvested later during the day

also benefit from higher carbohydrate status at harvest (Rogers, 1973), an internal quality parameter of particular importance in the cut flower *Protea* (Paull et al., 2003).

The risk however exists that glucose toxicity can also occur if stems are pulsed at supra-optimal glucose concentration (Stephens et al., 2005; Ferreria, 2005), or under extended uptake times that will allow high levels of glucose to accumulate. Thus, shorter pulsing times will lower the risk of glucose toxicity and allows for faster processing and grading.

In conclusion, when glucose pulsing is used in *Protea* cv. Sylvia to control leaf blackening, stems are recommended to be harvested later in the day and not be placed directly in water after harvest. It is also recommended that stems be pulsed with an intermediate glucose concentration of between 7 – 15% for between 1 – 2 hours. However, based on pulsing environments, this pulsing time may differ between various pack houses.

3.6 References

- Almeida, E.F.A., de Oliveira Paiva, P.D., de Oliveira Lima, L.C., Silva, F.C., Nogueira, A., 2011. Calla lily inflorescences postharvest: Pulsing with different sucrose concentrations and storage conditions. *Ciênc. Agrotec. Lavras*, 35, 657 – 663.
- Bieleski, R.L., Ripperda, J., Newman, J.P., Reid, M.S., 1992. Carbohydrate changes and leaf blackening in cut flower stems of *Protea eximia*. *HortScience* 117, 124 – 127.
- Cannon, A., McConchie, R., 2001. Controlling leaf blackening in *Protea*: A report for the Rural Industries Research and Development Corporation. Publication No 01/098, 1 – 21.
- Crick, S.G., McConchie, R., 1999. Ethanol vapour reduces leaf blackening in cut flower *Protea* 'Pink Ice' stems. *Postharvest Biol. Technol.* 17, 227 – 231.
- Doi, M., Reid, M.S., 1995. Sucrose improves the postharvest life of cut flowers of a Hybrid *Limonium*. *HortScience* 30, 1058 -1060.
- Dole, J.M., Wilkins, H.F., 2005. Floriculture: Principles and species. Second Edition. Prentice-Hall International, Inc., Upper Saddle River, NJ.
- Dorrington, P., 2008. Country profile of South Africa presentation by chairman of South African Protea Producers Exporters (SAPPEX), 9th International Protea Association (IPA) symposium, Sept 2008, Stellenbosch, South Africa.
- Durkin, D.J., 1979. Some characteristics of water flow through isolated rose stem segments. *J. Am. Soc. Hortic. Sci.* 104, 777 – 783.
- Ferreira, A., 2005. Further studies on leaf blackening of *Protea*. MSc thesis, Stellenbosch University, South Africa.

- Halevy, A.H., Mayak, S., 1979. Senescence and postharvest physiology of cut flowers, Part 1. *Hortic. Rev.* 3, 204 – 226.
- Halevy, A.H., Mayak, S., 1981. Senescence and postharvest physiology of cut flowers, Part 2. *Hortic. Rev.* 7, 59 – 143.
- Jones, R.B., McConchie, R., Reid, M.S., 1995. Leaf blackening in cut *Protea* flowers. *Hortic. Rev.* 17, 173 – 201.
- Lambers, H, 2008. *Plant physiological ecology*. Springer, New York, USA.
- Marousky, F.J., 1969. Vascular blockage, water absorption, stomatal opening and respiration of cut 'Better times' roses treated with 8-Hydroxyquinoline citrate and sucrose. *HortScience* 94, 223 – 226.
- McConchie, R., Lang, N.S., Gross, K.C., 1991. Carbohydrate depletion and leaf blackening in *Protea neriifolia*. *J. Am. Soc. Hortic. Sci.* 116, 1019 – 1024.
- McConchie, R., Lang, N.S., 1993. Carbohydrate metabolism and possible mechanisms of leaf blackening in *Protea neriifolia* under dark postharvest conditions. *J. Am. Soc. Hortic. Sci.* 118, 355 – 361.
- McConchie, R., Lang, N.S., Lax, A.R., Lang, G.A., 1994. Reexamining polyphenol oxidase, peroxidase and leaf blackening activity in *Protea*. *J. Am. Soc. Hortic. Sci.* 119, 1248 – 1254.
- Meyer, C. 2003. Carbohydrates and leaf blackening of *Protea*. MSc thesis, Stellenbosch University, South Africa.
- Mostert, D.P., Siegfried, W.R., Louw, G.N., 1980. *Protea* nectar and satellite fauna in relation to the food requirements and pollinating role of the Cape sugarbird. *S. Afr. J. Sci.* 76, 406 – 412.
- Newman, J.P., Van Doorn, W.G., Reid, M.S., 1989. Carbohydrate stress causes leaf blackening in *Proteas*. *Acta Hortic.* 264, 103 – 103.
- Nowak, J., Rudnicki, R.M., 1990. *Postharvest handling and storage of cut flowers, florist greens and potted plants*. Portland Timber Press, Place.
- Paull, R.E., Jaroenkit, T., Chen, N.J., 2003. *Protea*, a cut woody ornamental: Diversity and variable postharvest responses. *Acta Hortic.* 602, 127 – 132.
- Reid, M.S., 2004. The Commercial Storage of Fruits, Vegetables, and Florist and Nursery Stocks: Cut flowers and greens. *Agriculture Handbook, USDA, ARS. Nr.* 66.
- Reid, M.S., Van Doorn, W.G., Newman, J.P., 1989. Leaf blackening in *Proteas*. *Acta Hortic.* 261, 81 – 84.
- Rogers, M.N., 1973. An historical and critical review of postharvest physiology research on cut flowers. *HortScience* 8, 189 – 194.

- Statsoft Inc., 2011. STATISTICA (data analysis software system), Version 10.
<http://www.statsoft.com/>
- Stephens, I.A., Holcroft, D.M., Jacobs, G., 2001. Low temperature and girdling extend vase life of 'Sylvia' proteas. *Acta Hortic.* 545, 205 – 214.
- Stephens, I.A., 2003. Leaf blackening in Proteas. Ph.D Dissertation. Stellenbosch University, South Africa.
- Stephens, I.A., Holcroft, D.M., Jacobs, G., 2005. Postharvest treatments to extend vase life of selected *Proteaceae* cut flowers. *Acta Hortic.* 602, 155 – 159.
- Taiz, L., Zeiger, E., 2010. Plant physiology. Fifth Edition. Sinauer Associates, Inc., Sunderland, MA, USA.
- Teagel, S., White, J., Sedgley, M., 1991. Post-harvest vase life of cut flowers of three cultivars of kangaroo paw. *Sci. Hortic.* 48, 277 – 284.
- Van Doorn, W.G., 1997. Water relations of cut flowers. *Hortic. Rev.* 18, 1 – 85.
- Van Doorn, W.G. 2001. Leaf blackening in *Protea* flowers: Recent developments. *Acta Hortic.* 545, 197-204.

Table 3.1

The amount (g) of pulse-solution, water and glucose uptake for flowering stems of *Protea* cv. Sylvia after 4 hours of pulsing following exposure to various levels of hydration (%), harvested at different times during a 'cool' and 'hot' day or when subjected to a glucose concentration pulse range. The water retained or transpired by the stem and the amount of glucose accumulated in the stem was calculated from the solution, water and glucose uptake values.

Trial	Treatment	Solution Uptake.stem ⁻¹		Water Uptake.stem ⁻¹		Water Retained.stem ⁻¹		Water Transpired.stem ⁻¹		Glucose Uptake.stem ⁻¹		Glucose from water Retained.stem ⁻¹		Glucose from water Transpired.stem ⁻¹	
		g		g		g		g		g		g		g	
Level of hydration (%)	100	3.94 ± 0.11	d ^z	3.71 ± 0.11	d	-0.04 ± 0.09	d	3.75 ± 0.13	ab	0.22 ± 0.01	d	0.00 ± 0.01	d	0.23 ± 0.01	a
	95	7.81 ± 0.46	c	7.37 ± 0.44	c	4.25 ± 0.46	c	3.13 ± 0.08	b	0.44 ± 0.03	c	0.25 ± 0.03	c	0.19 ± 0.00	a
	90	15.00 ± 0.65	b	14.15 ± 0.62	b	11.03 ± 0.47	b	3.13 ± 0.35	b	0.85 ± 0.04	b	0.66 ± 0.03	b	0.19 ± 0.02	a
	85	19.81 ± 0.98	a	18.69 ± 0.92	a	14.63 ± 0.65	a	4.06 ± 0.31	a	1.12 ± 0.06	a	0.88 ± 0.04	a	0.24 ± 0.02	a
<i>LSD</i> ^y		1.84		1.73		1.34		0.71		0.10		0.08		0.04	
Harvest time Sep2009 (T _{max} : 20°C)	08:00	2.75 ± 0.22	b	2.60 ± 0.20	b	0.68 ± 0.08	b	1.92 ± 0.21	a	0.16 ± 0.01	b	0.04 ± 0.00	b	0.12 ± 0.01	a
	12:00	5.06 ± 0.24	a	4.78 ± 0.22	a	3.11 ± 0.20	a	1.66 ± 0.18	a	0.29 ± 0.01	a	0.19 ± 0.01	a	0.10 ± 0.01	a
	15:00	5.57 ± 0.18	a	5.26 ± 0.17	a	3.35 ± 0.14	a	1.91 ± 0.20	a	0.32 ± 0.01	a	0.20 ± 0.01	a	0.11 ± 0.01	a
<i>LSD</i>		0.63		0.58		0.38		0.58		0.04		0.02		0.04	
Harvest time Nov2009 (T _{max} : 32°C)	08:00	8.51 ± 0.42	c	8.03 ± 0.39	c	0.37 ± 0.07	b	7.66 ± 0.40	a	0.48 ± 0.02	c	0.02 ± 0.00	b	0.46 ± 0.02	a
	12:00	11.78 ± 0.37	a	11.11 ± 0.35	a	2.93 ± 0.23	a	8.18 ± 0.35	a	0.67 ± 0.02	a	0.18 ± 0.01	a	0.49 ± 0.02	a
	15:00	10.35 ± 0.17	b	9.76 ± 0.16	b	3.26 ± 0.30	a	6.50 ± 0.33	b	0.59 ± 0.01	b	0.20 ± 0.02	a	0.39 ± 0.02	b
<i>LSD</i>		0.97		0.92		0.64		1.05		0.06		0.04		0.06	
Glucose pulsing solution (%)	0	13.65 ± 0.64	a	13.65 ± 0.64	a	9.12 ± 0.66	a	4.53 ± 0.24	b	0.00 ± 0.00	f	0.00 ± 0.00	d	0.00 ± 0.00	d
	4.8	12.57 ± 0.75	ac	11.97 ± 0.72	a	7.26 ± 0.64	b	4.71 ± 0.27	b	0.60 ± 0.04	e	0.36 ± 0.03	c	0.24 ± 0.01	c
	7.1	12.90 ± 0.80	ab	12.00 ± 0.75	a	6.93 ± 0.46	b	5.07 ± 0.51	b	0.90 ± 0.06	d	0.53 ± 0.03	b	0.38 ± 0.04	c
	9.4	13.97 ± 0.68	a	12.70 ± 0.62	a	5.25 ± 0.44	c	7.44 ± 0.89	A	1.27 ± 0.06	c	0.53 ± 0.04	b	0.74 ± 0.09	b
	13.6	11.39 ± 0.57	bc	9.90 ± 0.49	b	4.90 ± 0.13	cd	5.00 ± 0.54	b	1.49 ± 0.07	b	0.74 ± 0.02	a	0.75 ± 0.08	b
	17.7	10.82 ± 0.51	c	9.02 ± 0.43	b	3.58 ± 0.19	d	5.44 ± 0.33	b	1.80 ± 0.09	a	0.72 ± 0.04	a	1.09 ± 0.07	a
<i>LSD</i>		1.93		1.78		1.35		1.49		0.17		0.09		0.17	

^z Different letters within columns indicate a significant difference between treatments at the 5% level.

^y Least significant difference (LSD) values for each trial at the 5% significance level.

Table 3.2

The harvesting conditions (temperature and relative humidity) and the calculated vapour pressure deficits (mbar) for *Protea* cv. *Sylvia* stems at the start and end of the pulsing period when harvested in September 2009 ('cool' day, $T_{\max} = 20^{\circ}\text{C}$) and November 2009 ('warm' day, $T_{\max} = 33^{\circ}\text{C}$) at the respective harvest times throughout the day and at the start and end of the pulsing period. The laboratory environment was constant at 18°C ($\pm 2^{\circ}\text{C}$) and 60% ($\pm 10\%$) relative humidity during pulsing times.

Trial	Harvest time	HARVESTING CONDITION		VAPOUR PRESSURE DEFICIT		
		Harvest temperature	Harvest relative humidity	Field	Laboratory Time 0	Laboratory Time 4
		$^{\circ}\text{C}$	%	mbar	mbar	mbar
September 2009	08:00	8.8	97.8	0.2	1.5	8.6
	12:00	20.1	60.9	8	8.6	8.6
	15:00	20.6	56.6	10	8.6	8.6
November 2009	08:00	20.3	63.8	8	8.6	8.6
	12:00	33.0	27.2	41	23.5	8.6
	15:00	32.0	26.4	34	23	8.6

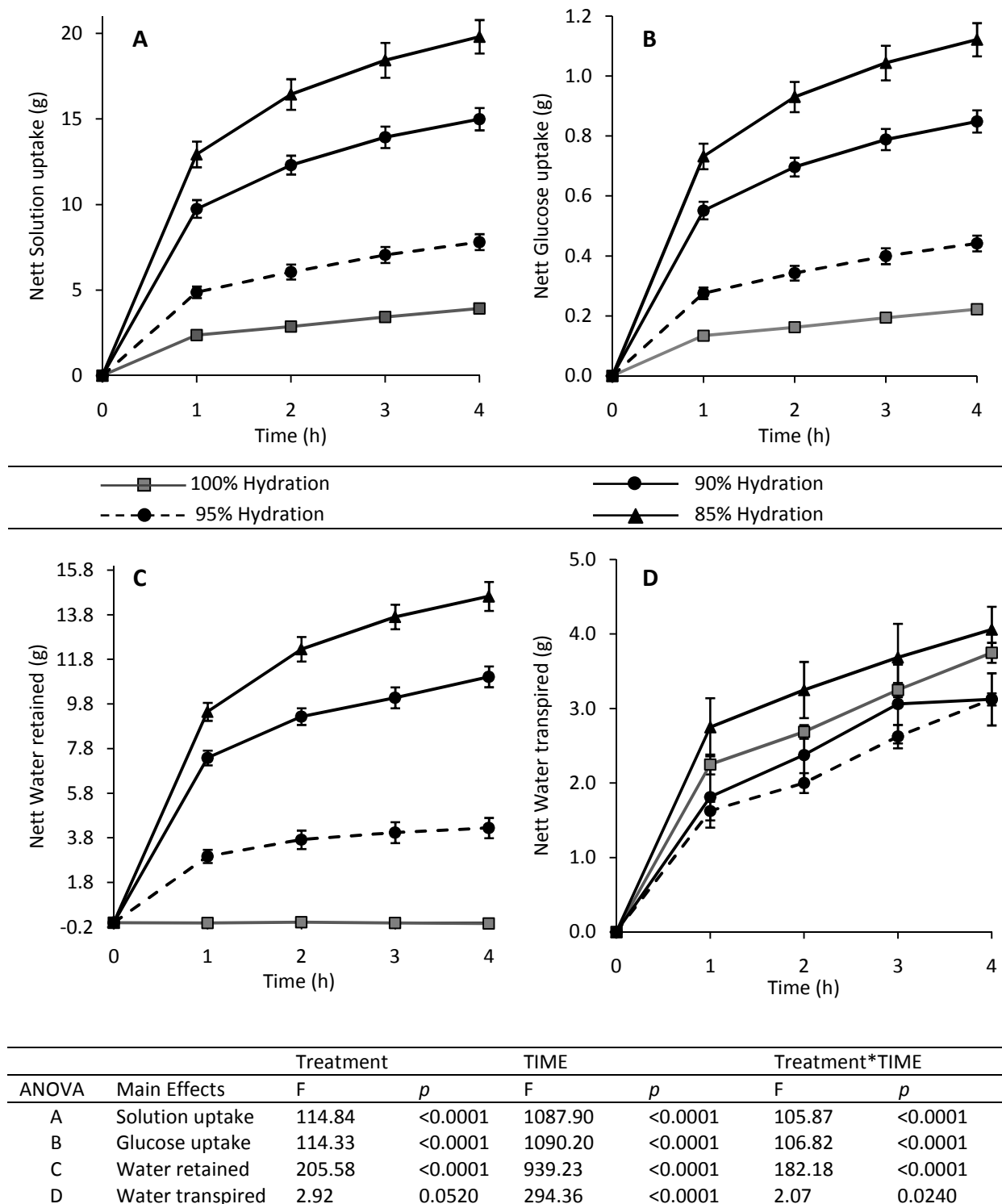


Fig. 3.1. The rate of glucose pulse-solution (5.7%) uptake (A), glucose accumulated (B) as well as the amount of water retained (C) and transpired (D) in flowering stems of *Protea cv. Sylvia* over a four hour pulsing period, following an overnight hydration and then various degrees of dehydration treatments prior to pulsing (n = 8). Vertical error bars indicate the Standard Error of the mean for each data point.

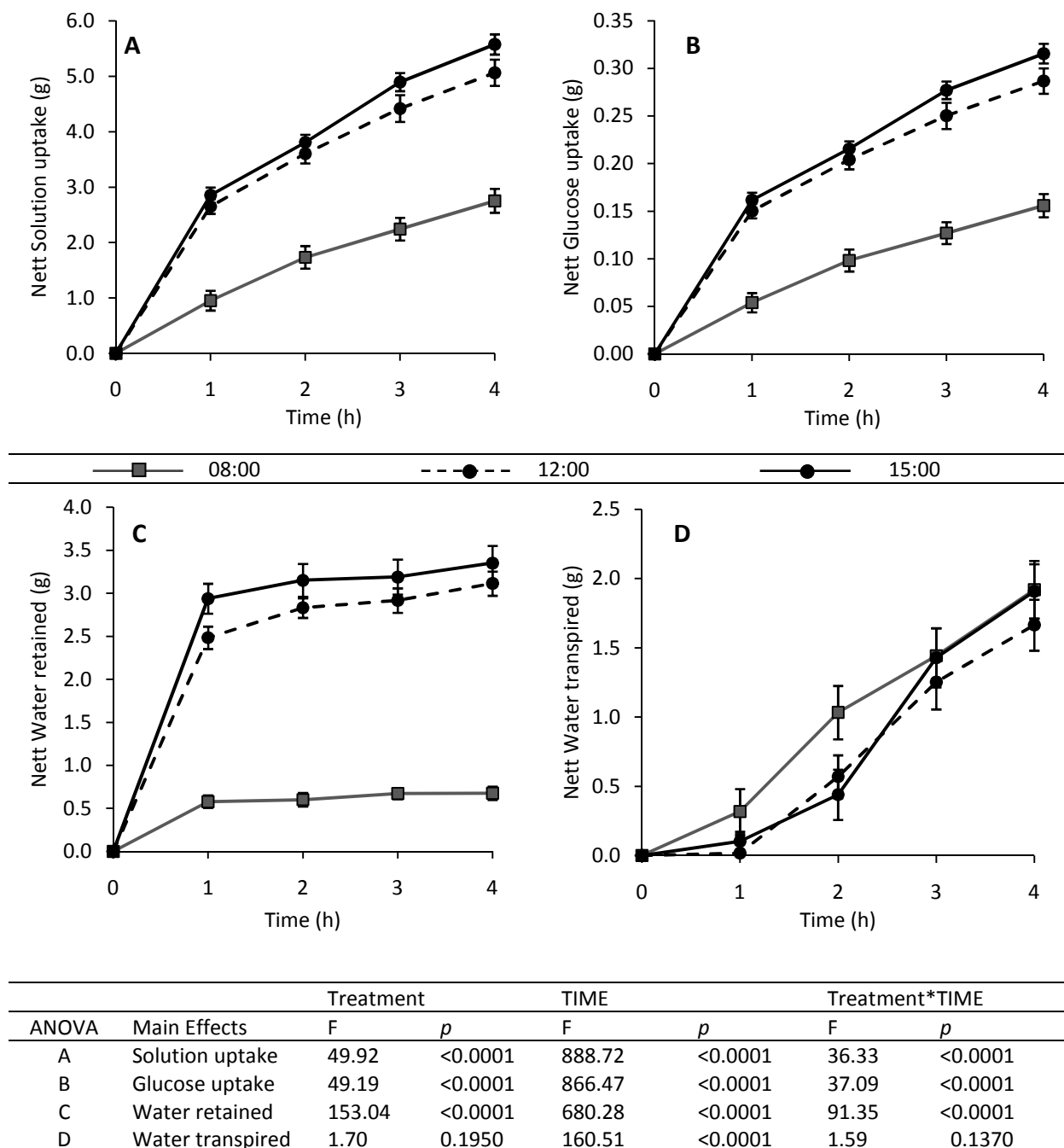


Fig. 3.2. The rate of glucose pulse-solution (5.7%) uptake (A), glucose accumulated (B) as well as the amount of water retained (C) and transpired (D) in flowering stems of *Protea cv. Sylvia* over a four hour pulsing period, following 1 hour after harvest 08:00, 12:00 and 15:00 respectively on 4 September 2009 ($T_{\max} = 20^{\circ}\text{C}$; $T_{\text{avg}} = 14.7$) ($n = 10$). Vertical error bars indicate Standard Error of the mean for each data point.

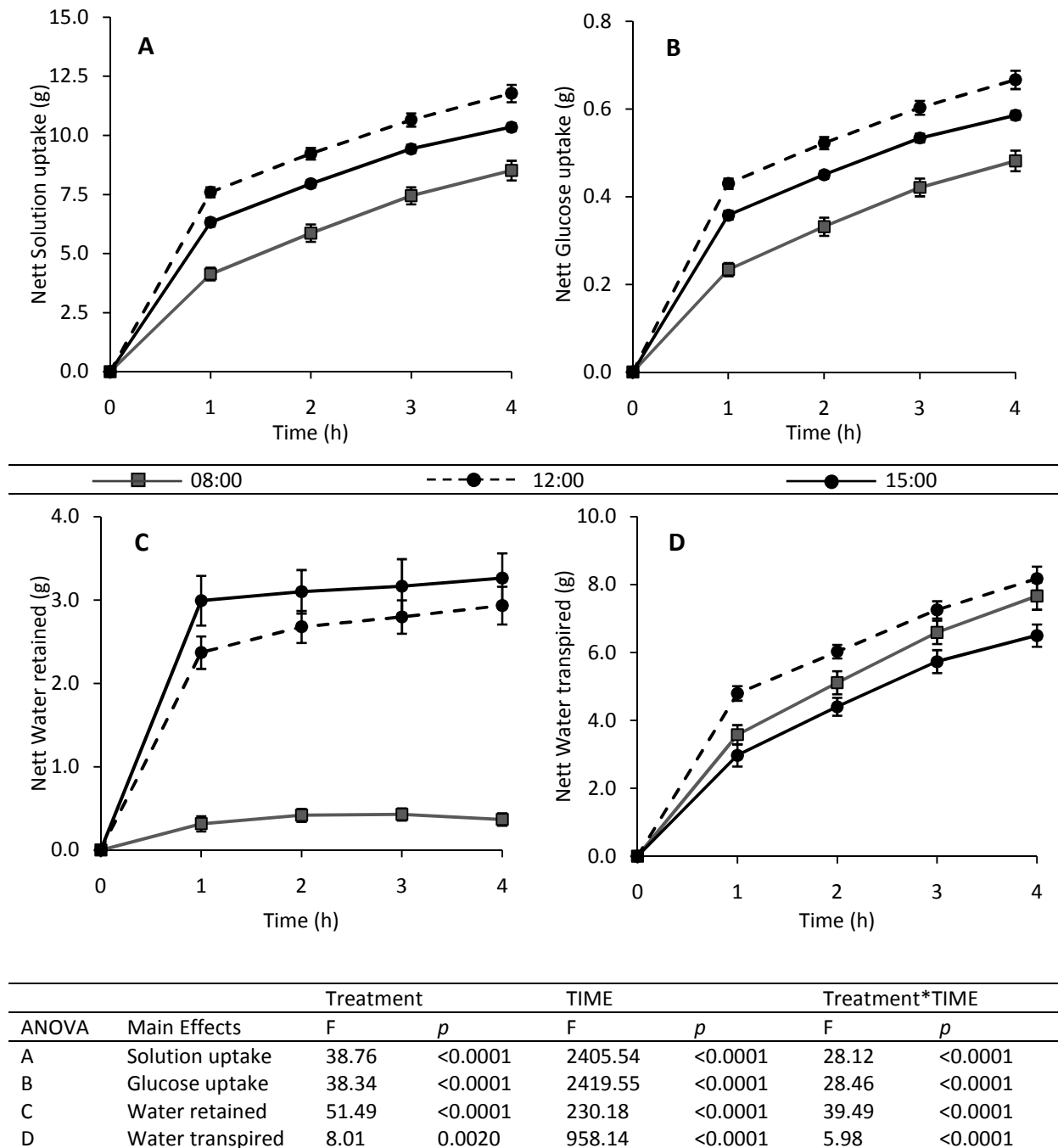


Fig. 3.3. The rate of glucose pulse-solution (5.7%) uptake (A), glucose accumulated (B) as well as the amount of water retained (C) and transpired (D) in flowering stems of *Protea cv. Sylvia* over a four hour pulsing period, following 1 hour after harvest 08:00, 12:00 and 15:00 respectively on 3 November 2009 ($T_{max} = 32^{\circ}\text{C}$, $T_{avg} = 23.7^{\circ}\text{C}$) ($n = 10$). Vertical error bars indicate Standard Error of the mean for each data point.

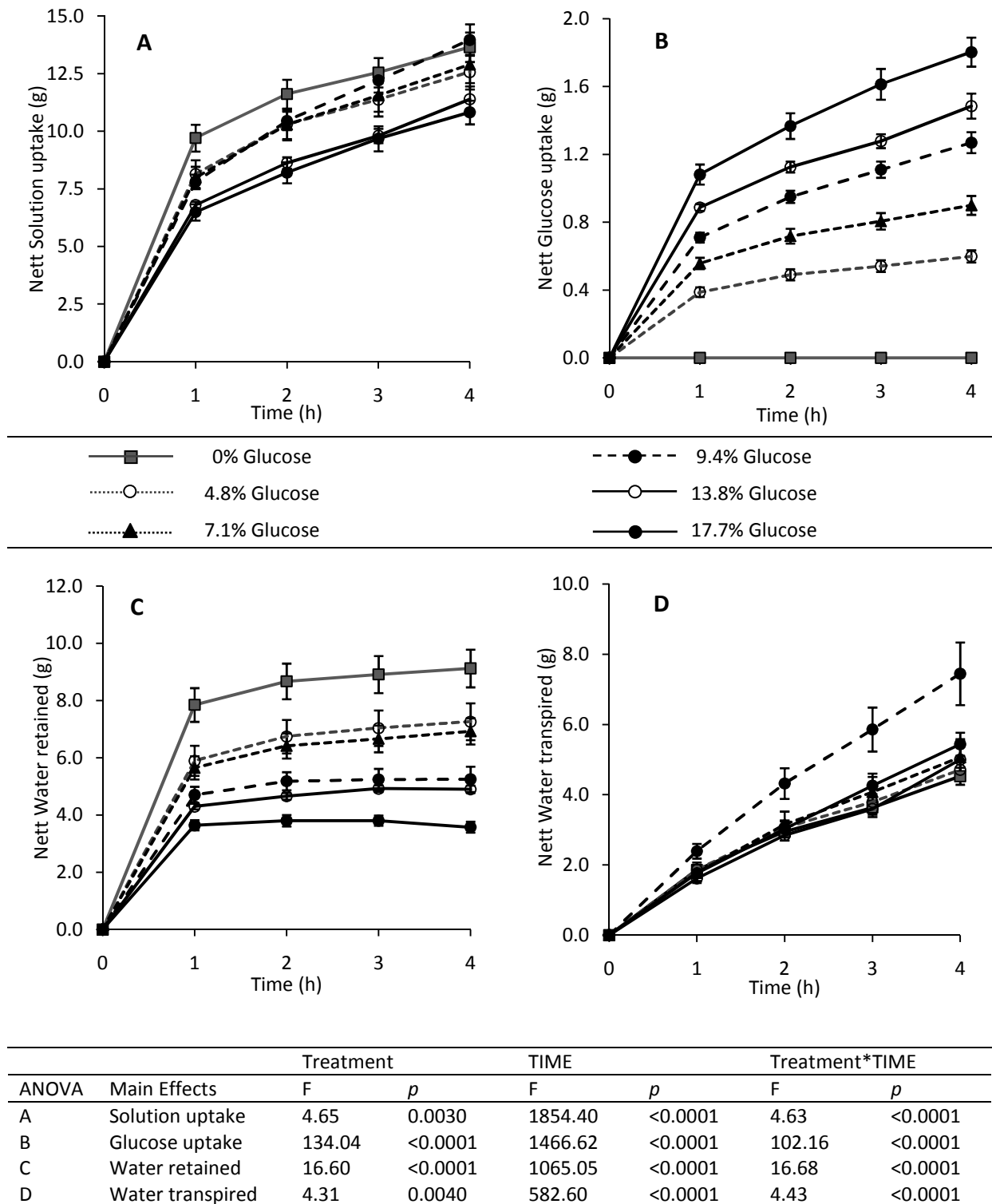


Fig. 3.4. The rate of glucose pulse-solution uptake (A), glucose accumulation (B) as well as the amount of water retained (C) and transpired (D) in flowering stems of *Protea cv. Sylvia* over a four hour period when pulsed with a range (0 – 17.7%) of glucose solution concentrations, following 1 hour after harvest (n = 6). Vertical error bars indicate the Standard Error of the mean for each data point.

Glucose pulse concentration affects vase-solution uptake dynamics and postharvest quality of long-term cold-stored *Protea* cv. *Sylvia* cut flower stems

Abstract

Subject to increasing consumer and market pressures that favours lower carbon footprints and product prices, the South African fynbos industry has increased exports of *Proteaceae* cut flowers by sea freight. This change in preferred transport method increases the risk for susceptible *Protea* cultivars of developing leaf blackening during these long-term periods of cold-storage. Pulsing with glucose has been shown to delay the onset of leaf blackening and to extend the vase life by supplying the carbohydrates required for further flower maintenance and development during vase life. The aim of this study was to evaluate the effect of different pre-storage glucose concentrations pulse treatments on the water balance during vase life and its related effect on the postharvest quality of *Protea* cv. *Sylvia* (*P. eximia* x *P. susannae*) stems when held in a vase solution containing a low concentration of glucose. Harvest-ready stems were pulsed with 0, 4.7, 7.1, 9.4, 13.7 and 17.7% glucose respectively for four hours or until ± 12 mL were absorbed. Stems were subsequently stored for 21 days at 4°C (± 1 °C) to simulate sea freight conditions. After storage, stem fresh weight and that of the solution removed during vase life was recorded and used to calculate the ratio of water that was retained and/or transpired by the stems respectively. In one of the experiments, stomatal conductance was also measured in addition to the other parameters. The incidence of leaf blackening and flower quality was recorded throughout 7 days of vase life. Pulsing stems pre-storage with a glucose concentration between 4.7 and 9.4% until ± 10 mL is absorbed resulted in the least amount of leaf blackening ($\pm 35 - 50\%$) compared to controls ($\pm 100\%$) and produced the highest marketable flower quality. Water balance was maintained the best under these optimum glucose concentrations, resulting from a combination of water that is retained longer and a lower transpiration rate during vase life compared to stem pulsed with other glucose concentrations or the control. Providing a vase life solution containing glucose delayed leaf blackening development and extended flower quality. This was however only the case if stems were pulsed with glucose prior to storage. Stomatal conductance data suggested that the glucose pulse may play a role in protecting stomatal functionality and therefore assist in delaying and lower the incidence of leaf blackening by maintaining a positive water balance for an extended period.

Keywords: Vase life, stomatal conductance, cut flowers, *Proteaceae*, carbohydrates, holding solution

4.1 Introduction

The cut flower market has become fully globalized, as the major consumer markets such as the USA, Europe and Japan are supplied from producers all over the world (Reid, 2001). Approximately 55% of all cut flower products exported from South Africa for the 2010 season could be contributed to the Fynbos Industry (SAFEC report, 2011) of which 60% is calculated to be *Protea* cut flower stems alone (PPSA producer survey results, 2011). The major export destinations for the past five seasons were Central Europe (67%), United Kingdom (16%), middle and east Mediterranean countries (6%) as well as the middle and east Asia (5%) (PPECB, 2010). Thus, all current markets for South African *Protea* cut flowers are logistically far and therefore distinct postharvest challenges exist in offering a high-quality product to these discerning markets.

Increasing pressure from leading retailers to lowering the carbon footprint impact on the environment has been pushing for the increasing use of sea freight as an alternative to air freight (Dorrington, 2008). Excluding this environmental drive, sea freight also provides the capacity for exporting larger volumes of product at peak times, whilst decreasing transport costs. The use of sea freight however, extends transport time from the two to three days required for air freight to more than 21 days. A product of similar or better quality than delivered by competitors such as Portugal, the Canary Islands and Israel which have a much shorter transport distance to the same markets is essential for the South African Fynbos industry to retain its current market shares.

There are several factors contributing to the longevity of a cut flower and thus its ability to reach the expected vase life. These factors include: stage of maturity at harvest, water quality, temperatures throughout the handling chain and during vase life, available food reserves, prevailing light levels and ethylene (Kader, 2002).

Leaf blackening in *Protea* is a physiological postharvest disorder that places a serious constraint on maintaining the quality of this otherwise magnificent cut flower. Whole consignment rejections can be ascribed to this postharvest disorder alone, especially for a highly susceptible cultivar such as *Protea cv. Sylvia* (Verhoogt, pers. comm., 2011), which is cultivated widely in South Africa due to year-round flowering habit and availability. Insufficient carbohydrate reserves to support the high respiration rates of the large developing *Protea* inflorescence (Ferreira, 1986) has been ascribed as the underlying cause of leaf blackening by a number of researchers as reviewed by Jones et al. (1995). Subsequently sugar polyphenols are targeted and sugars are cleaved off which expose phenols to be oxidized enzymatically or non-enzymatically (Perold, 1993; McConchie and Lang, 1993a).

Many studies have shown a significant improvement in the vase life for a wide range of cut flowers with sugar supplementation (Halevy and Mayak, 1979; Goszczynska and Rudnicki, 1988). Pulsing with sucrose in particular have shown to extend the vase life for calla lily (Almeida et al., 2011), carnation and freesia (Weerts, 2008) and gladiolus (Singh and Kumar, 2009). Carbohydrates are the energy source for all metabolic reactions in living cells, including respiration. Therefore, providing an external carbohydrate supply assists in maintaining cell integrity and extends functionality once cut flowers are removed from the primary source, the intact plant (Halevy and Mayak, 1981).

Postharvest water stress has also been highly associated with the onset of flower quality deterioration (Van Doorn, 1997). Pulsing with sugars has been found to improve hydration in cut stems, possibly through osmotic adjustment (Halevy and Mayak, 1979). The most effective method currently used to alleviate or delay the onset of leaf blackening in *Protea* cut flowers is pulsing with sugars prior to storage and/or transport (McConchie and Lang, 1993a; Van Doorn, 2001; Stephens, 2003).

Sucrose is the preferred carbohydrate provided as a pulse in cut flowers, as it is a non-reducing transport sugar (Reid, 2004). However, in *Protea* it has been found that pulsing with glucose is more effective to control leaf blackening than sucrose (Stephens et al., 2001a). The exact reason for this is unknown; however Stephens (2003) speculated that sucrose cannot be utilized in *Protea* cv. *Sylvia*, either because of limitations in the enzymes required for cleaving sucrose (invertase or sucrose synthase), or of a limited number of sucrose transporters available to facilitate uptake. Another simpler explanation is related to the cost of nectar production which has been correlated positively to leaf blackening in previous studies (Paull and Dai, 1990; Dai, 1993). The hybrid parents of *Protea* cv. *Sylvia* namely *P. eximia* and *P. susannae* are known to also have high concentrations of glucose and fructose in their nectar in addition to xylose (Van Wyk and Nicholson, 1995). Stephens (2003) suggested that nectar production by the inflorescence could be supplemented by provided external glucose sources, so that the need to deplete existing leaf resources could be minimised and thus delaying the onset of leaf blackening.

In the previous study (Paper 2), different glucose concentrations lead to varied uptake dynamics of the pulse solution. The aim of this study was to evaluate the influence of these different glucose pulse concentrations on vase life solution uptake dynamics and subsequently the impact of this on the postharvest quality following long-term cold storage conditions.

4.2 Materials and Methods

4.2.1 Plant material

Dry-picked, harvest-ready (soft-tip stage), flowering stems of *Protea* 'Sylvia' (*P. eximia* x *P. susannae*) were collected between 08:00 and 10:00 from a commercial farm located near Stellenbosch (33°55'S; 18°50'E) Western Cape, South Africa on 14 September 2010, 10 November 2010 and 18 July 2011 respectively. Within an hour after harvest, stems were standardized to a length of 40cm and with 20 – 30 leaves per stem at the Department of Horticultural Science, Stellenbosch University.

4.2.2 Pre-storage treatments

Stems harvested on 14 September 2010 were used to evaluate the influence of the holding solution on the postharvest quality of glucose-pulsed stems. Harvested stems were allocated to 3 treatment groups of 16 replicates each in a randomized design and pulsed with glucose concentrations made according to molality (Paper 2, p. 58: 0, 0.52, 0.76M) in a concentration range of 0, 9.4 and 13.7% respectively, until 10mL was removed from the pulse-solution.

Subsequently, two experiments were designed to evaluate the effect of glucose pulse concentrations on the vase solution uptake dynamics. Stems harvested on 10 November 2010 were allocated into groups of 6 replicates per treatment, according to a completely randomized design and pulsed for four hours with the following glucose concentrations made up as described above in a concentration range: 0% glucose (control), 4.7, 7.1, 9.4, 13.7 and 17.7% respectively. Similarly, stems that were harvested on 18 July 2011 were allocated into groups of 8 replicates each to treatments, according to a complete randomized design and pulsed for 20 hours with the same glucose concentrations used in the experiment of November 2010, only omitting the 7.1% glucose pulse treatment, until 12mL (± 0.4 mL) were removed from the pulse-solution.

All stems were weighed immediately after pulsing and packed in standard S14 commercial flower storage cartons. Each carton was individually wrapped with low density polyethylene black bags, to help prevent moisture loss during storage. Cartons were then placed in a cold room at 4°C (± 1 °C) for a 21 day storage period.

4.2.3 *Post storage procedures*

For stems harvested on 14 September 2010, the three glucose concentration treatment groups were divided immediately after storage each into a further two groups, with eight stem replicates per treatment combination. The treatment groups consisted either of tap water (control) or tap water containing a 1.5% Chrysal Clear Professional 3™ (Pokon Chrysal, UK) (CCProf3) solution as a holding solution. Stem evaluation was done on day 0 and 5 of vase life.

After storage, individual stem weights were immediately recorded for all treatments conducted on 10 November 2010 and on 18 July 2011. Stems from the July 2011 experiment were re-cut by 2cm and weighed again, to determine if uptake was compromised after storage for stems harvested on 10 November 2010. Vase solutions used consisted of a 1.5% CCProf3 holding solution. Stems were then placed individually into a vase, where after both stem and vase-solution was weighed consecutively on day 1; 2; 5 – 7 for stems harvested in November 2010 and on day 2; 4; 6 and 7 for stems harvested in July 2011.

Throughout each experiment, vase openings were sealed with Parafilm™, with only a small hole pierced for the stem, to prevent evaporation from the vase solution surface. Recordings were standardized to the same time each day at 14:00. Stems were kept in an evaluation room with temperatures of 18°C ($\pm 2^\circ\text{C}$), light/dark cycles of 10/14 hours with $15\mu\text{mol.m}^{-2}.\text{s}^{-1}$ light levels and a relative humidity of 60% ($\pm 10\%$).

4.2.4 *Post harvest quality and stomatal conductance*

Leaf blackening was recorded for each stem (on the same days as weights were recorded) as the number of leaves with $\geq 10\%$ leaf blackening out of the total number of leaves per stem, presented as a percentage. Flower quality was assessed for each stem according to the observation scoring rating index (1 – 5; where 1 = very poor quality and 5 = top quality) following the same principle set by the Dutch flower assessment protocol (Floral solutions, 2006). Loss of flower quality included the incidence of bract browning, involucre bract curling as well as floret collapse.

In the experiment conducted in July 2011, stomatal conductance ($g^s = \text{mmol.m}^{-2}.\text{s}^{-1}$) was measured in addition to the recording of leaf blackening, using a PMR-5 steady state porometer fitted with an EGM-4 environmental gas monitor (PP systems, Amesbury, MA, USA). Measurements were taken on a standard leaf area of 1.25cm^2 and at a flow rate of 50mL.min^{-1} at 20°C ($\pm 1^\circ\text{C}$). The abaxial side of the upper-most leaf on the flush that subtends the inflorescence and that has not yet blackened was selected for each recording date, on 8 randomly selected replicate stems.

For stems harvested on 14 September 2010, leaf blackening was recorded and flower quality was assessed as described above.

4.2.5 Calculations

The vase life solution uptake dynamics was calculated similarly to the uptake of pulsing solutions presented in Paper 2 (p. 60), except that the time factor was not over four hours as in pulsing treatments, but over seven days of vase life. Also, the glucose accumulated over this period per mL was the same for all treatments, as 1.5% CCProf3 provides 5.6g glucose per 100mL holding solution.

In both experiments, where holding solution uptake dynamics was studied, the vase life solution weight and stem weight was measured at the same time during each evaluation day throughout the vase life. The weights at the start of vase life were also recorded. From these values were calculated: total vase solution uptake; glucose (provided through CCProf3) accumulation in the stem; as well as water retained and transpired by the stem.

Holding solution uptake was directly measured as the weight of solution lost from the vase. The fraction of glucose uptake was calculated by multiplying the corresponding molarity concentration of CCProf3 with the solution weight removed from the vase.

Water retained by the stem could be calculated by subtracting the amount of glucose accumulated from the stem weight differences measured over the vase life period. Similarly, transpiration was calculated by subtracting the water retained by the stem from the water removed from the vase life solution.

4.2.6 Statistical analysis

One-way analysis of variance (ANOVA) was used to obtain statistical differences between treatments at the date of vase life termination (day 5 or 7) for the various parameters. Data presented as percentages (leaf blackening) was transformed using log transformations prior to the ANOVA analysis. Repeated measures analysis of variance (RANOVA) was used to analyse the interaction between treatments and time (vase life). All statistical analysis was performed using Statistica version 10 (Statsoft, Inc., 2011). Means were separated by Fisher's Least Significant Difference (LSD) test at significance level at $P < 0.05$. Where covariates were included in statistical analysis, SAS version 9.1 (SAS Institute, Inc., 2000) was used.

4.3 Results

4.3.1 Glucose pulse concentration effects on vase solution uptake dynamics

Stem fresh weight lost during the 21 days storage period was on average 6.4% for all treatment stems, irrespective of glucose pulse concentration (Table 4.1). The only exception noted was in stems harvested in July 2011 and pulsed with the highest concentration (17.7%) glucose, which only lost 4.8% of its fresh weight during storage.

The two lowest glucose concentrations (4.8 and 7.1%) used to pulse stems harvested in November 2010 and the three mid-range glucose concentration treatments (4.8, 9.4 and 13.7%) applied to stems harvested in July 2011 was the most effective treatment in preparing stems to utilize the vase solution (Table 4.1).

Glucose had a significant ($p < 0.0001$) effect on the ability for the pulsed stem to retain water or not (Table 4.1; Figs. 4.1A and 4.2A). In both experiments, control stems were unable to retain any water after storage and consistently continued to lose water content, even though vast amounts of solution were taken up from the vase (Table 4.1). When considering nett water retention over the entire evaluation period, an interaction between time and treatment is noted in the first experiment (Fig. 4.1A). Stem weight recovered to almost its original weight before storage for all treatments except the control for stems harvested on 10 November 2010, that is, the $\pm 10\text{g}$ lost during storage was regained within the first day of uptake (Fig. 4.1A). Thereafter however, the ability to retain water decreased differentially between the glucose-pulsed and the control stems (Fig. 4.1A). Water retention and content declined rapidly in control stems, whereas all glucose pulsed stems showed a much slower decline in water retention. The only exception was again that of the 17.7% glucose-pulsed stems that declined more rapidly than the other treatments towards the end of vase life, but still retained more water than the control stems (Table 4.1; Fig. 4.1A).

In the experiment conducted in July 2011, again a significant interaction ($p < 0.0001$) between treatments over time illustrate the impact of the different glucose pulsing concentrations to differentially affect the ability of stems to maintain functional water uptake (Fig. 4.2A). Both the controls and stems pulsed with the highest glucose concentration (17.7%) were unable to retain water, from the start of vase life evaluation (Fig. 4.2A). The lowest glucose concentration treatments maintained its water status and water content for two days, before a decline set in (Fig. 4.2A). The other two glucose pulse concentrations (9.4 and 13.7%) were able to recover half their weight lost during storage within the first two days of evaluation, where after a slow decrease in water content was recorded up to day 6 of vase life, followed by a nett water loss (Fig. 4.2A).

A significant interaction between nett transpiration over the vase life evaluation period was observed for stems harvested in November 2010 and July 2011 (Figs. 4.1B and 4.2B). Transpiration rates after storage were highest for control stems during the first few days of vase life in both experiments (Figs. 4.1B and 4.2B), although more pronounced so for stems harvested in November 2010. In the November harvested stems, the two highest glucose pulse concentrations (13.7 and 17.7%) had the lowest nett transpiration of all treatments (Fig. 4.1B). For the stems harvested in July 2011, only stems pulsed with the highest glucose concentration (17.7%) had a nett transpiration which remained consistently lower than in the other treatments (Fig. 4.1B). In these stems, the nett transpiration remained constant for the 4.8, 9.4 and 13.8% glucose pulsed stems, until day 6, where after a decline was observed in all treatments during vase life (Fig. 4.2B).

Corresponding with the vase solution uptake, the glucose contribution from the holding solution during vase life could be calculated. Throughout the vase life evaluation period, a total of approximately 1.0g glucose was accumulated by the 4.8, 7.1 and 9.4% glucose pulsed stems in the November 2010 harvested stems (Table 4.1). Control stems however only accumulated 0.8g of glucose. The two highest pulse concentration treatments of 13.7 and 17.7% accumulated similar amounts to controls with 0.8g and 0.7g glucose respectively (Table 4.1). In the July 2011 harvested stems, glucose uptake after seven days was lower at ± 0.7 g for the mid-range glucose concentrations pulse treatments. Still, control stems only accumulated 0.6g of glucose, whilst the highest glucose pulse concentration (17.7%) stems significantly ($p < 0.0308$) accumulated the least amount of glucose at only $0.5\text{g}\cdot\text{stem}^{-1}$ (Table 4.1).

4.3.2 Post-storage vase life quality observations

Leaf blackening was significantly lower ($p < 0.0001$) in glucose-pulsed stems compared to control stems (Table 4.1). For the stems harvested in November 2010, leaf blackening was already at 44% compared to the 2 – 10% recorded for the glucose-pulsed treatments at day 0 evaluation (Table 4.1). In the stems harvested in July 2011, leaf blackening for control stems on day 0 of evaluation reached values as high as 87% (Table 4.1). Even in glucose-pulsed stems, leaf blackening levels were recorded at between 25 – 38%, where the lowest incidence was observed for the 13.7% glucose-pulsed stems, displaying a 25% leaf blackening (Table 4.1).

For stems harvested in November 2010, leaf blackening in the control stems increased to 98% on day 7, which was significantly higher ($p < 0.0001$) than in glucose-pulsed stems, where leaf blackening remained below $\pm 40\%$ (Table 4.1). For stems harvested in July 2011, there was a similar difference between treatments ($p < 0.0001$) on day 7 of vase life, although much higher leaf

blackening was recorded in all evaluated stems. Control stems were all 100% blackened, while leaf blackening incidences varied between 50 – 70% for the glucose-pulsed stems (Table 4.1).

For stems harvested in November 2010, a significant interaction ($p < 0.0001$) in leaf blackening of treatments was observed over the evaluation period ($p < 0.0001$) (Fig. 4.3A). Leaf blackening onset rates were highest for the control stems during the first two days. For the glucose pulsed stems, with the exception of the highest pulse concentration, the rate at which leaf blackening developed, gradually increased over the first two days, where after it stabilized (Fig. 4.3A). In the stems harvested in July 2011, the rate of leaf blackening onset in the controls was not as steep as leaf blackening was already close to 100% (Fig. 4.3A). A similar rate of leaf blackening development was observed for the glucose-pulsed stems, therefore no significant interaction between treatments over time was reported (Fig. 4.3B). Leaf blackening increased in all treatments over time. The control stems had consistently higher leaf blackening than the glucose-pulsed stems (Fig. 4.3B).

Flower quality, for stems harvested in November 2010, all had a top scoring index of 5, irrespective of treatment, on day 0 of vase life. By the end of vase life evaluation, flower quality was maintained at a significantly higher ($p = 0.0022$) score of ≥ 3 (considered marketable) for all glucose pulsed stems, with the exception of stems pulsed with 17.7% glucose (Table 4.1). The flowers of those stems, together with flowers of the control group lost quality rapidly and were considered non-marketable at the end of the evaluation period (Table 4.1).

4.3.3 Holding solution

Glucose pulsing improved both foliage and flower quality on day 0 of vase life for stems harvested in September 2010, similar to that reported in the previous experiments. Pulsed stems had a significantly ($p < 0.0001$) lower incidence of leaf blackening at approximately 10% compared to control stems that showed a much higher leaf blackening percentage of 75% (Table 4.2). Flower quality was also significantly better ($p < 0.0001$) in pulsed stems compared to control stems (Table 4.2).

As vase life progressed, with stems exposed to either water or the glucose-containing CCProf3 as holding solution, significant differences between treatments emerged. Control stems had a 100% leaf blackening and the lowest flower quality rating by day 7 of vase life, irrespective of the type of holding solution (Table 4.2). However, using CCProf3 as holding solution was advantageous to stems pulsed with 9.4% glucose, as leaf blackening was significantly higher, whilst flower quality declined considerably to barely saleable for stems held only in water (Table 4.2). For stems pulsed with 9.4% glucose and held in CCProf3, leaf blackening only increased to 22% after seven days and flower

quality remained high at a rating of 4 (Table 4.2). This benefit offered by the CCProf3 holding solution was not evident in the 13.8% pulsed glucose concentration stems, where additional glucose was provided, as no significant difference in leaf blackening or flower quality was noted (Table 4.2).

4.3.4 Stomatal conductance

On the first day after storage (day 0 of vase life), stomatal conductance differed significantly ($p < 0.0001$) between treatments (Fig. 4.4A). The highest stomatal conductance was recorded in the control stems ($68\text{mmol}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$), followed by a decline in the 4.8% glucose pulsed stems, with the lowest values recorded in the upper glucose concentration pulse range of 9.4 – 17.7%. Similarly, leaf blackening was recorded to be significantly higher in the control stems with the highest stomatal conductance's, in comparison to the glucose pulsed stems (Fig. 4.4A).

When evaluating stomatal conductance measurements, a significant interaction ($p < 0.0001$) was recorded between treatments over the evaluation period (Fig. 4.4B). Stomatal conductance was higher in control stems for the first three days, before declining to similar values of glucose-pulsed stems, which were below $40\text{mmol}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$ from the first day of vase life (Fig. 4.4B). After three days, stomatal conductances in all glucose pulsed stems were below $10\text{mmol}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$, whereas control stems remained slightly higher ($28\text{mmol}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$) (Fig. 4.4A).

4.4 Discussion

Pulsing flowering stems of *Protea* cv. *Sylvia* with an optimum volume and concentration of glucose immediately after harvest provides delayed onset of leaf blackening and maintains flower quality, even when subjected to long-term cold storage. This study confirms similar results from previous studies on pulsing *Protea* with glucose (Stephens et al., 2001a; Meyer, 2003; Ferreira, 2005; Stephens et al., 2005). The concentration of glucose chosen as a pulse treatment for *Protea* cv. *Sylvia* stems before storage, however not only influenced the postharvest leaf and flower quality, but also the uptake dynamics of the holding solution during vase life.

Reduced ability to take up water or solution relates to a loss in water balance in cut flowers, with the subsequent acceleration of senescence (Van Doorn, 1997). The specific glucose concentration of the pulse solution had a remarkable influence on the uptake capacity of the flowering stems of *Protea* cv. *Sylvia* from the holding solution, after long-term cold storage. The ability of the flowering stems to restore the loss of fresh weight, which occurred during the cold storage period, by absorbing and retaining water during the first day in the holding solution,

depended strongly on the glucose concentration used for the pre-storage pulsing and which month (season) the flowers were harvested in.

For stems harvested in November, pulsing with glucose ranging from 4.8 to 17.7% prior to storage allowed water lost during storage to be almost completely recovered during early vase life. This implies that membranes were still intact and vase solution was accessible to the stem, thus cellular function and water balance could be maintained. This could be ascribed to a lower water potential of the cells in the flowering stems than that of the holding solution, leading to an osmotically driven uptake of vase solution. In rose cut flowers, water loss was also delayed by providing a sucrose-containing pulse before vase life (Ichimura and Shimizu-Yumoto, 2007). Previous studies have shown that carbohydrate reserves decrease rapidly in *Protea neriifolia* kept in the dark at ambient or low temperatures (Bieleski et al., 1992; McConchie and Lang 1993a,b). Even in pulsed *Protea* cv. Sylvia stems, a study showed that total carbohydrates, also consisting of glucose, decreased to very low levels after 21 days cold storage (Ferreira, 2005). Such low levels of glucose thus could not affect the osmotic potential of the stem. Alternatively, the concentration of polygalatol, a major carbohydrate in *Protea* leaves, in these studies, whether stored or not, was not affected. Polygalatol is a sugar-polyol and derivative of sorbitol found in large concentrations in *Protea* cut flowers and was suggested to play a role in osmotic balance as it does not form part of the carbohydrate pool (Bieleski et al., 1992). For this reason, the possibility that the osmotic potential of control stems was lower than that of the holding solution and that plasmolysis occurred, seems unlikely. After day one in the holding solution, flowering stems pulsed with glucose concentration ranging from 4.8 to 9.4% was able to maintain their water status to a large degree. However, over the next 6 days a decrease in water retained by the stems indicated that membrane integrity was increasingly compromised, even more so for the high glucose-pulse concentration treatment of 17.7%. Membrane integrity might have been compromised to a greater extent in stems harvested in July, as greater loss of water from those stems was observed, probably due to loss of stomatal control. This could be noted from the first day of vase life, as July-harvested stems failed to restore the water lost during storage to the same degree as was possible in November-harvested stems.

Irrespective of whether flowering stems were harvested in July or November, a significant difference in leaf blackening after 21 days cold-storage was observed between pulsed and non-pulsed stems. During cold storage, membrane integrity of non-pulsed stems seemed to be affected and severe leaf blackening developed during this period. Although McConchie and Lang (1993b) found no evidence of membrane degradation preceding the incidence of leaf blackening, the difference in the ability of pulsed and control stems to maintain water balance after storage strongly

suggests a causal relationship between membrane integrity and leaf blackening. Pulsing with glucose prior to cold-storage assisted in maintaining cellular membrane functionality and this is evident by the lower incidence of leaf blackening on the removal of flowering stems from cold storage as well as in the successful maintenance of the water relations within the stem.

The post-storage reduction in stomatal conductance observed in pre-cold storage glucose pulsed stems in this study suggests again the role glucose plays in the maintenance of cellular function. This might also explain the more rapid transpiration observed for control stems during the first two days in the holding solution. Sugars have been proposed to assist in the control of stomatal closure and therefore reduce water loss from stems (Marousky, 1969). However, our data does not support any relationship between the net transpiration and stomatal conductance measured. This could be because most water lost via transpiration occurs from the inflorescence according to previous studies (Ferreira, 1986; Stephens, 2003) and not the leaves, where stomatal conductance was measured.

Providing a holding solution or preservative after storage is commonly known to increase the vase life for most cut flowers (Halevy and Mayak, 1981; Goszczynska and Rudnicki, 1988). Similarly, in *Protea*, Meyer (2003) reported that the presence of glucose in the holding solution reduced leaf blackening of cold-stored *Protea*. The holding solution used in this study did provide a delay in leaf blackening and extended flower quality after storage, but only when a pulse was provided prior to storage. This can be explained in that glucose accumulation via vase life solution did not provide an adequate amount of carbohydrates to delay or suppress leaf blackening as calculated amounts of only 0.1 – 0.2g glucose accumulated per day.

Another observation made from the stems harvested in July 2011 is that pulsing glucose pre-cold storage was not sufficient to delay leaf blackening to meet commercial export criteria. Taking into account the respiration rate at different temperatures (Q_{10} factor), storage at 0 or 1°C would probably have resulted in lower incidence of leaf blackening and better flower quality compared to storage at 4°C as was done in this study. Stem respiration rates in *Grevillea* species doubled ($Q_{10} = 2.06$) from when storing at 5°C compared to 0°C as a storage temperature (Joyce et al., 2000). Thus, when leaf blackening incidences is higher for *Protea* cv. Sylvia, such as the case during August to November (southern hemisphere), temperature control may be just as important as pulsing.

4.5 Conclusion

The efficacy of pre-storage glucose pulsing to reduce the incidence of leaf blackening during cold storage as well as during the vase life period of *Protea* cv. Sylvia by providing additional

carbohydrate, is in agreement with studies done previously, including those cited in some extensive reviews (Jones et al., 1995; Van Doorn, 2001; Stephens et al., 2001a; Stephens et al., 2005). There is little doubt that the presence of externally provided glucose in *Protea* cultivars, which respond to glucose as a pulse or holding solution, such as *Protea* cv. Sylvia (Stephens et al., 2001a), is effective to reduce leaf blackening. The reason that some growers experience no or little reduction in leaf blackening after pulsing is most likely due to inadequate uptake of glucose during the pulsing process, which is explained in detail in Paper 2. An accumulation of approximately 1.0g glucose per stem appears to be the optimum dosage to reduce the development of leaf blackening during long term cold storage of 21 days in *Protea* cv. Sylvia. Thus, not only does the glucose pulsing help to sustain further development, it probably also assists in maintaining the cell integrity required for a positive water balance to help counteract the stresses of vase life, and thus subsequently the development of leaf blackening.

Also, substituting water with a glucose-containing solution during vase life after cold storage, further assists to maintain both leaf and flower quality. It is recommended that, even though pulsed before storage, sea-freighted flowers be placed in a holding solution containing 1.5% glucose immediately after unpacking. The holding solution should in addition to glucose also contain a suitable preservative, which is not hypochloride, as this can exaggerate leaf blackening development significantly (Meyer, 2003).

4.6 References

- Almeida, E.F.A., Paiva, P.D de O., Lima, L.C de O., 2011. Calla lily inflorescences postharvest: Pulsing with different sucrose concentrations and storage conditions. *Ciênc. Agrotec. Lavras.* 35, 657 – 663.
- Bieleski, R.L., Ripperda, J., Newman, J.P., Reid, M.S., 1992. Carbohydrate changes and leaf blackening in cut flower stems of *Protea eximia*. *J. Am. Soc. Hortic. Sci.* 117, 124 – 127.
- Dai, J-W., 1993. Postharvest leaf blackening in *Protea neriifolia* R. Br. Ph.D. Dissertation, University of Hawaii, Honolulu, HI.
- Dorrington, P., 2008. Country profile of South Africa presentation by chairman of South African Protea Producers Exporters (SAPPEX), 9th International Protea Association (IPA) symposium, Sept 2008, Stellenbosch, South Africa.
- Ferrera, D.I., 1986. The influence of temperature on the respiration rate and browning of *Protea neriifolia* R. Br. Inflorescences. *Acta Hortic.* 185, 121 – 125.
- Ferrera, A., 2005. Further studies on leaf blackening of *Protea*. MSc Thesis, Stellenbosch University, South Africa.

- Floral solutions, 2006. Protocol for cut flower vase life tests. Pokon & Chrysal International BV, Naarden, Netherlands. pp. 8.
- Goszcynska, D.M., Rudnicki, R.M., 1988. Storage of cut flowers. *Hortic. Rev.* 10, 35 – 62.
- Halevy, A.H., Mayak, S., 1979. Senescence and postharvest physiology of cut flowers, Part 1. *Hortic. Rev.* 1, 204 – 226.
- Halevy, A.H., Mayak, S., 1981. Senescence and postharvest physiology of cut flowers, Part 2. *Hortic. Rev.* 3, 59 – 143.
- Ichimura, K., Shimizu-Yumoto, H., 2007. Extension of the vase life of cut roses by treatment with sucrose before and during simulated transport. *Bull. Natl. Inst. Flor. Sci.* 7, 17 – 27.
- Jones, R.B., McConchie, R., Reid, M.S., 1995. Leaf blackening in cut *Protea* flowers. *Hortic. Rev.* 17, 173 – 201.
- Joyce, D.C., Meara, S.A., Hetherington, S.E., Jones, P., 2000. Effects of cold storage on cut *Grevillea* 'Sylvia' inflorescences. *Postharvest Biol. Technol.* 18, 49 – 56.
- Kader, A., 2002. Postharvest technology of horticultural crops. Postharvest handling systems: Ornamental crops (Reid, M.S.). Third Ed. 25, 315 – 325.
- Marousky, F.J., 1969. Vascular blockage, water absorption, stomatal opening and respiration of cut 'Better times' roses treated with 8-Hydroxyquinoline citrate and sucrose. *HortScience* 94, 223 – 226.
- McConchie, R., Lang, N.S., 1993a. Postharvest leaf blackening and pre-harvest carbohydrate status in three *Protea* species. *HortScience* 28, 313 – 316.
- McConchie, R., Lang, N.S., 1993b. Carbohydrate metabolism and possible mechanisms of leaf blackening in *Protea neriifolia* under dark postharvest conditions. *J. Am. Soc. Hortic. Sc.* 118, 355 – 361.
- Meyer, C., 2003. Carbohydrates and leaf blackening of *Protea* cut flowers. MSc thesis, Stellenbosch University, South Africa.
- Paull, R.E., Dai, J-W., 1990. *Protea* postharvest black leaf, a problem in search of a solution. *Acta Hortic.* 264, 93 – 101.
- Perishable Products Export Control Board (PPECB) Export directory 2010. Part 3: All Flora. Malachite Press, Helderberg, South Africa. Section 2, pp. 79
- Perold, G.W., 1993. Consistency and variation in metabolite patterns of South African Proteaceae: a chemical perspective. *SA J. Sci.* 89, 90 – 93.
- Protea Producers of South Africa (PPSA), 2011. PPSA Producers Survey results. (Edit: Kotzé, M) <http://www.sappex.org.za>
- Reid, M.S., 2001. Advances in shipping and handling of ornamentals. *Acta Hortic.* 542, 277 – 284.

- Reid, M.S., 2004. The Commercial Storage of Fruits, Vegetables, and Florist and Nursery Stocks: Cut flowers and greens. Agric. Handbook, USDA, ARS. Nr. 66.
- Reid, M.S., 2008. Perishable cargo manual: Handling of cut flowers for air transport. International Air Transport Association (IATA), Sixth Ed., pp. 175 – 186.
- Singh, A., Kumar, J. 2009. Influence of sucrose pulsing and sucrose in vase solution on flower quality of modified atmosphere low temperature (MALT)-stored gladiolus cut spikes. Acta Hortic. 847, 129 – 138.
- South African Flower Export Council (SAFEC) report, 2011. A development strategy for the South African floriculture industry. Compiled by Prof. H. Boshoff of Futures Trust, May 2011. www.safflower.co.za
- Statistical Analysis systems Institute Inc., 2000. SAS®/STAT User's guide, Version 9.1, SAS Institute Inc., SAS Campus drive, North Carolina 27513.
- Statsoft Inc., 2011. STATISTICA (data analysis software system), Version 10. <http://www.statsoft.com>
- Stephens, I.A., 2003. Leaf blackening of Proteas. Ph.D Dissertation, Stellenbosch University, South Africa.
- Stephens, I.A., Jacobs, G., Holcroft, D.M., 2001a. Glucose prevents leaf blackening in 'Sylvia' proteas. Postharvest Biol. Technol. 23, 237 – 240.
- Stephens, I.A., Holcroft, D.M., Jacobs, G., 2001b. Low temperatures and girdling extend vase life of 'Sylvia' proteas. Acta Hortic. 545, 205 – 214.
- Stephens, I.A., Meyer, C., Holcroft, D.M., Jacobs, G., 2005. Carbohydrates and postharvest leaf blackening of Proteas. HortScience 40, 181 – 184.
- Van Doorn, W.G., 1997. Water relations of cut flowers. Hortic. Rev. 18, 1 – 85.
- Van Doorn, W.G., 2001. Leaf blackening in *Protea* flowers: Recent developments. Acta Hortic. 545, 197 – 204.
- Van Wyk, B.E., Nicholson, S.W., 1995. Xylose is a major nectar sugar in *Protea* and *Faurea*. SA J. Sci. 91, 151 – 153.
- Weerts, J.A., 2008. The effect of sucrose-pulsing on cut carnation and freesia flowers. MSc Thesis. University of Johannesburg, South Africa.

Table 4.1

The glucose pulse and vase solution uptake dynamics recorded in cut flower stems of *Protea* cv. *Sylvia* when pulsed with a range of glucose concentrations in November 2010 and July 2011 respectively, prior to storage of 21 days at 4°C (±1°C). The amount (g) of glucose accumulated with pulsing, but prior to cold storage as well as the amount of stem weight loss during cold storage was recorded before vase life evaluation. The amount (g) of vase solution (1.5% Chrysal clear Professional3™) uptake and equivalent glucose accumulated thereof as well as variation in stem weight (g/%) was recorded on the termination of vase life (day 7). Percentage (%) leaf blackening was determined at day 0 and 7 of vase life. Flower quality was also observed on the final day of vase life for stems evaluated in November 2010.

Trial	Glucose pulse concentration	Glucose accumulated (pre-storage pulsing)	Stem weight loss during storage	Vase Solution Uptake (Day 7)	Variation in Fresh stem weight (Day 7)	Vase life glucose accumulated (Day 7)	Leaf blackening (Day 0)	Leaf blackening (Day 7)	Flower quality (Day 7)
	%	g	g / (%)	g	g/ (%)	g	%	%	(score)
Nov 2010	0	0.0 ± 0.0	11.3 (6.4) a ^z	155.4 bc	-13.9 (91.5) c	0.8 bc	44.4 a	98.9 a	1.7 bc
	4.8	0.6 ± 0.0	11.0 (6.2) a	204.0 a	4.7 (102.9) ab	1.1 a	10.6 b	27.2 bc	3.7 a
	7.1	0.9 ± 0.1	12.4 (6.7) a	190.8 a	6.8 (104.1) a	1.0 a	5.0 bc	38.3 bc	3.5 a
	9.4	1.3 ± 0.1	11.5 (6.5) a	169.8 ab	7.8 (105.0) a	1.0 ab	7.2 bc	36.7 bc	3.0 ab
	13.7	1.5 ± 0.1	12.9 (7.7) a	137.7 cd	6.4 (104.1) ab	0.8 cd	5.0 bc	26.7 c	3.3 a
	17.7	1.8 ± 0.1	13.0 (7.9) a	114.6 d	-3.4 (98.0) b	0.7 d	2.8 c	40.6 b	1.2 c
ANOVA	F		0.18	7.75	6.44	7.75	8.44	76.54	4.89
	P		0.9692	<0.0001	0.0004	<0.0001	<0.0001	<0.0001	0.0022
	%	g	g/ (%)	g	g/ (%)	g	%	%	(score)
Jul 2011	0	0.0 ± 0.0	10.2 (6.2) a	107.5 ab	-20.5 (86.1) b	0.6 ab	87.5 a	100.0 a	_y
	4.8	0.6 ± 0.0	10.6 (6.9) a	126.6 a	-5.3 (96.2) a	0.7 a	36.6 b	60.0 bc	-
	9.4	1.0 ± 0.0	10.0 (6.4) ab	139.8 a	0.2 (100.1) a	0.8 a	25.6 b	50.6 c	-
	13.7	1.6 ± 0.1	11.7 (7.2) a	120.1 a	-4.5 (96.9) a	0.7 a	32.5 b	58.1 bc	-
	17.7	2.0 ± 0.3	7.5 (4.9) b	84.2 b	-21.2 (85.1) b	0.5 b	38.1 b	71.8 b	-
ANOVA	F		2.83	3.02	12.89	3.02	20.10	15.66	
	p		0.0421	0.0311	<0.0001	0.0308	<0.0001	<0.0001	

^z Different letters in a column indicate a significant difference for treatments at the 5% level.

^y Data not available

Table 4.2

The percentage (%) leaf blackening and flower quality rating recorded in flowering stems of *Protea* cv. *Sylvia* at day 0 and 7 of vase life, where stems were held in either tap water or a 1.5% Chrysal clear Professional 3™ (CCProf3) vase solution (1.5%) during the evaluation period. Stems were harvested in September 2010, where after they were pulsed with a range of glucose concentrations prior to cold storage for 21 days at 4°C (±1°C).

VASE LIFE (DAY 0)				
Trial	Glucose pulse concentration (%)		Leaf blackening (%)	Flower quality (score) ^x
Sept 2010	0		75.8 a ^z	3.3 b
	9.4		9.4 b	4.8 a
	13.8		11.9 b	4.5 a
ANOVA	F		53.482	13.953
	p		<0.0001	<0.0001
VASE LIFE (DAY 7)				
Trial	Glucose pulse concentration (%)	Vase holding solution	Leaf blackening (%) ^y	Flower quality (score)
Sept 2010	0	H ₂ O	100.0 a	1.0 d
	0	CCProf3	100.0 a	1.0 d
	9.4	H ₂ O	35.8 b	2.8 c
	9.4	CCProf3	22.5 c	4.3 a
	13.8	H ₂ O	40.0 b	3.5 b
	13.8	CCProf3	24.6 bc	3.4 bc
ANOVA	F		52.298	36.887
	p		<0.0001	<0.0001

^z Different letters in a column indicate a significant difference between treatments at the 5% level

^y Leaf blackening 'day 0' was included as a covariate

^x Flower quality scores were determined upon the following criteria (Floral solutions, 2006):

- | | |
|---|--|
| 1 | Very poor quality, very big quality problems, all consumers will discard the flowers |
| 2 | Poor quality, big quality problems, most consumers will discard the flowers |
| 3 | Reasonable quality, moderate quality problems, most consumers will not yet discard flowers |
| 4 | Good quality, small quality problems, all consumers will not discard the flowers |
| 5 | Very good quality, no quality problems |

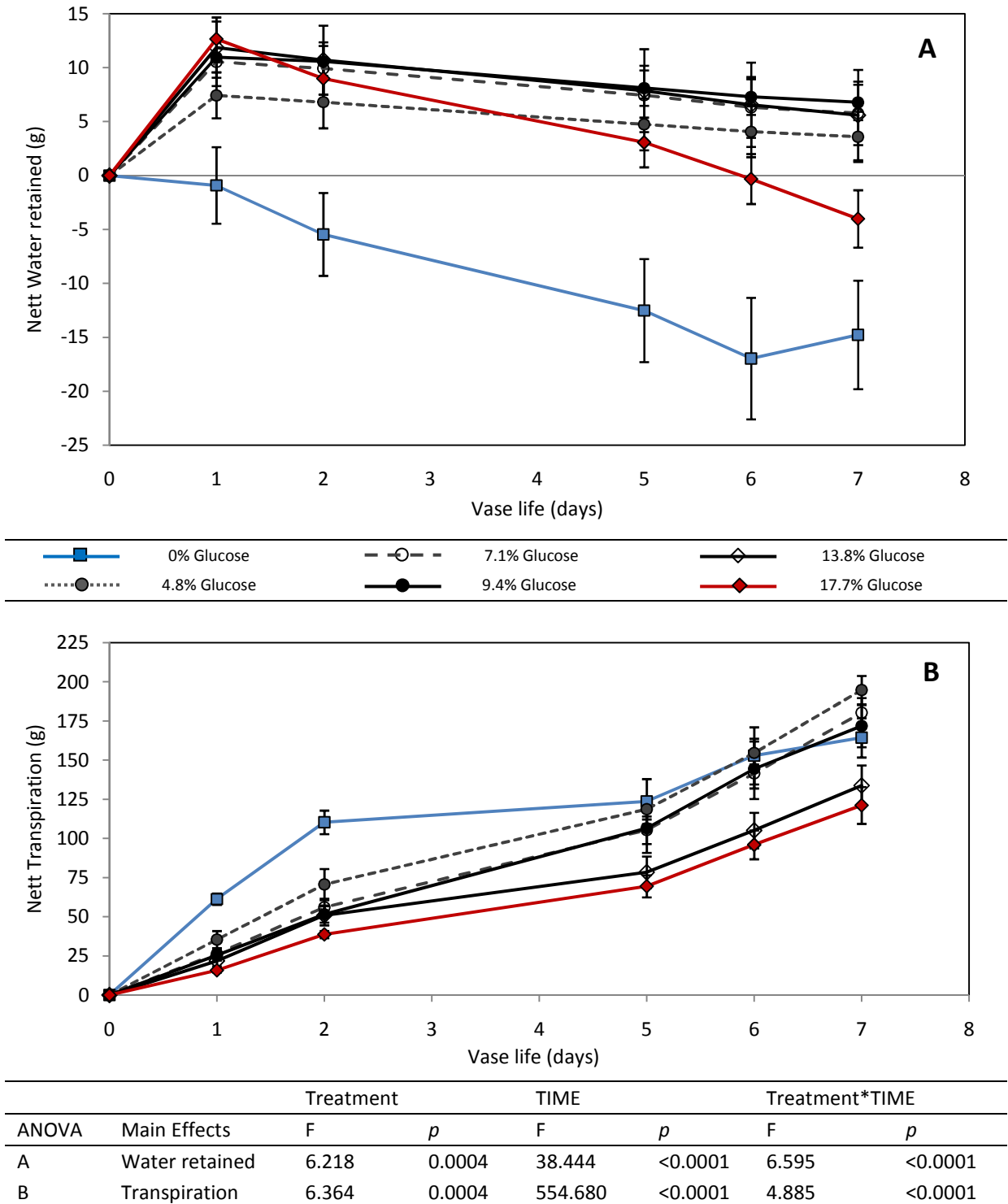


Fig. 4.1 The nett water retained (A) by the stems and the calculated nett transpiration (B) (during the vase life period) of flowering stems of *Protea* cv. *Sylvia* harvested in November 2010, following cold storage at 4°C (±1°C) for 21 days (n = 6). Vertical error bars indicate the Standard Error of the mean for each data point.

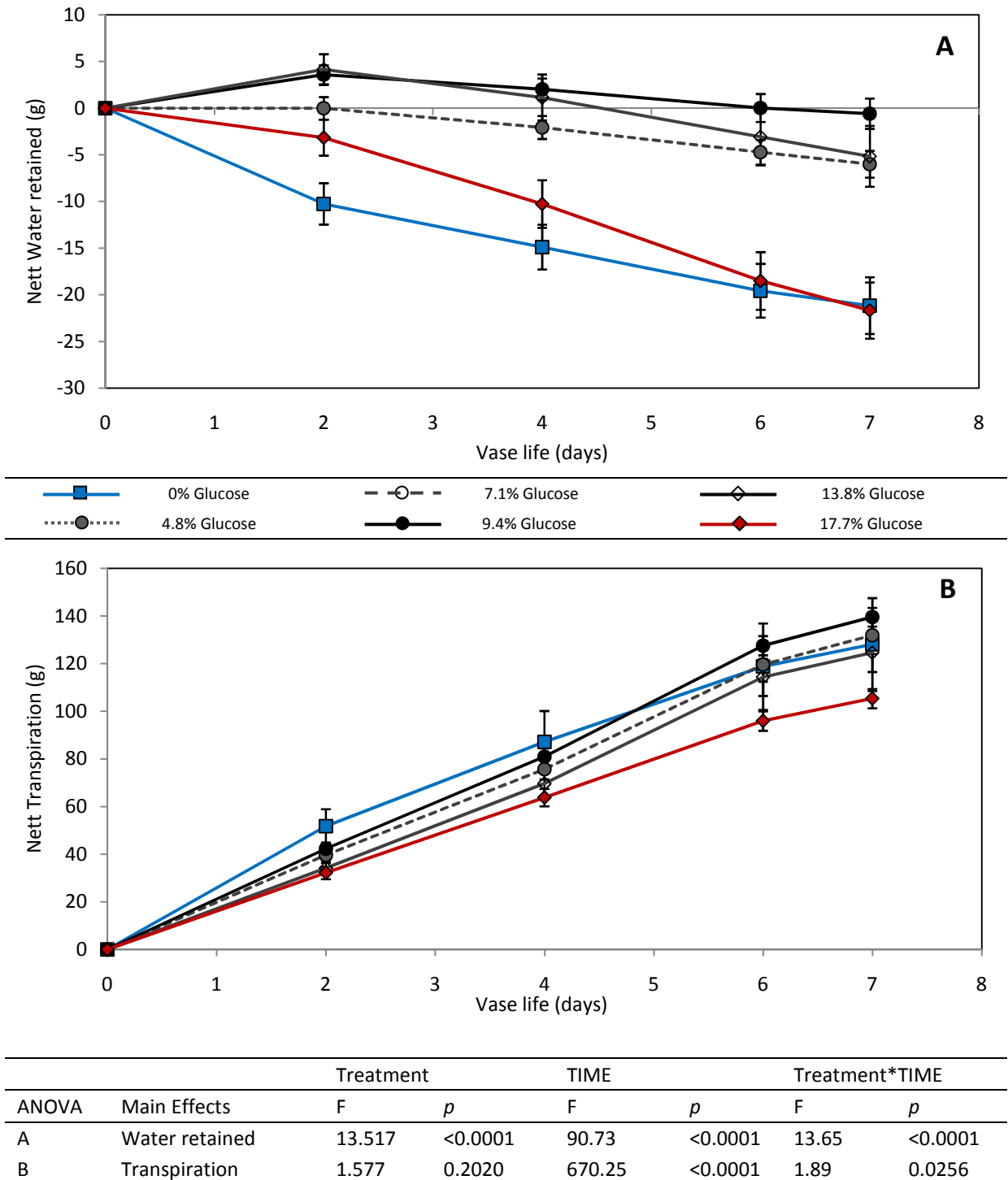
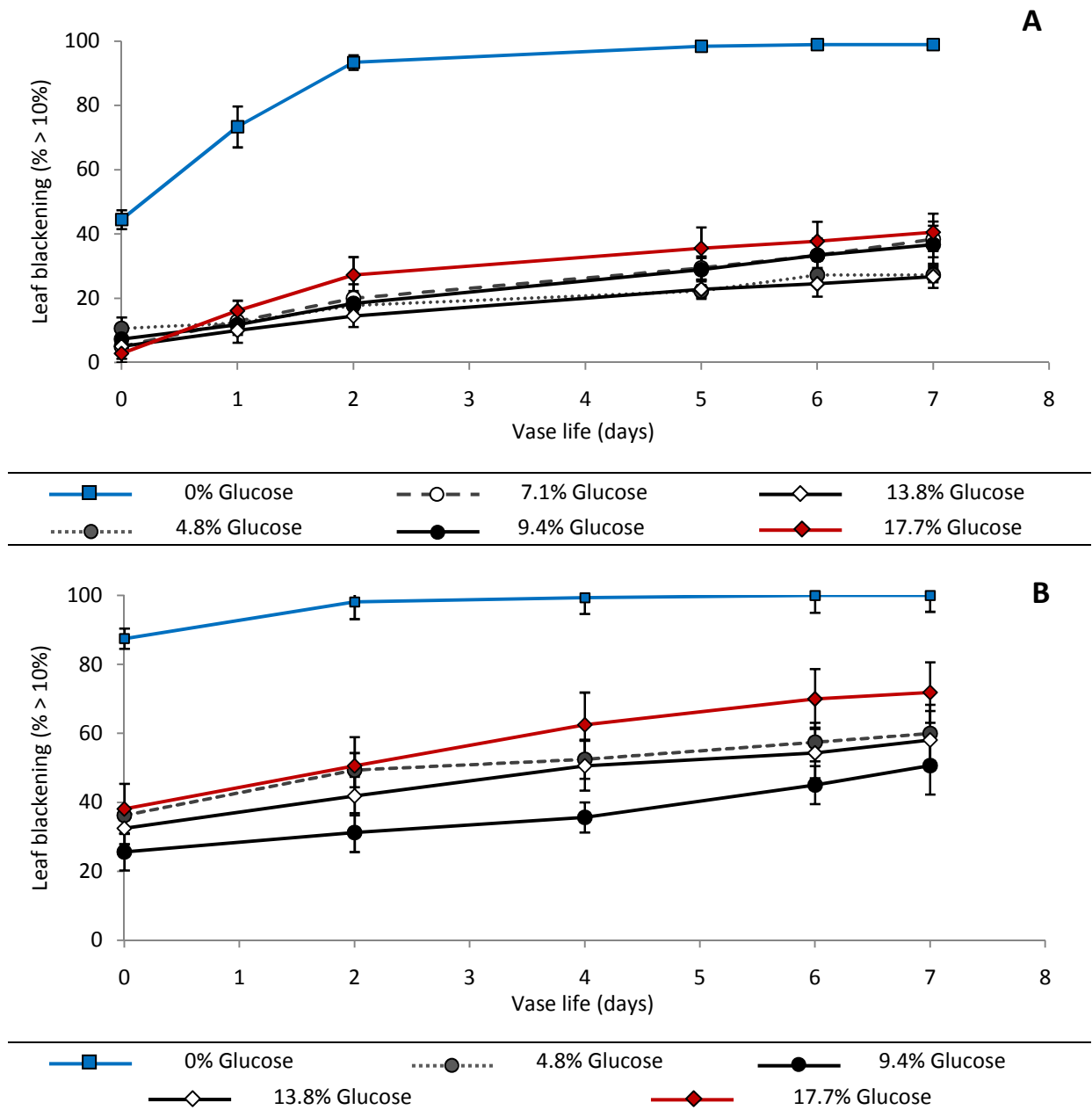
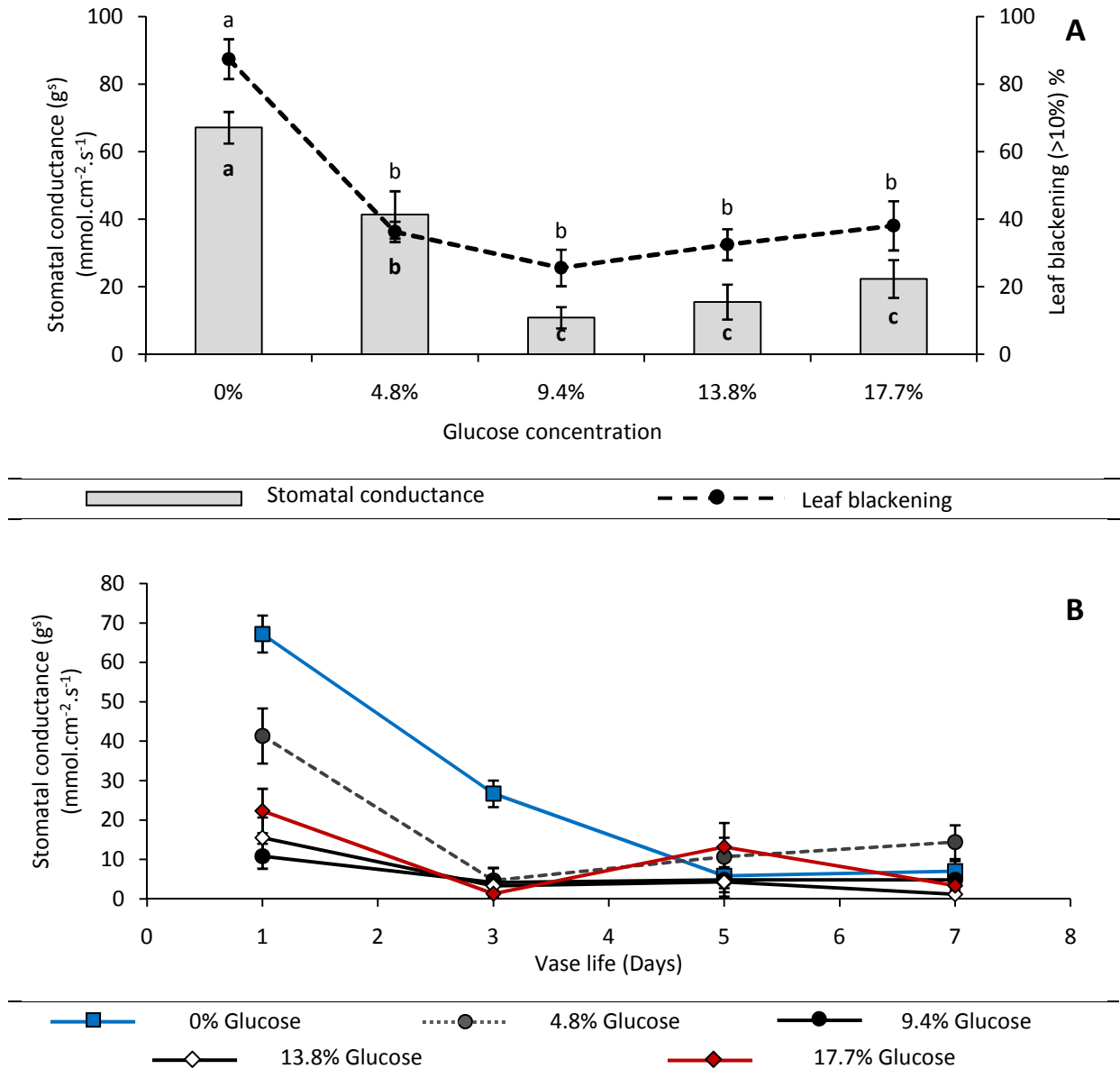


Fig. 4.2 The nett water retained (A) by the stems and the calculated nett transpiration (B) (during the vase life period) of flowering stems of *Protea* cv. *Sylvia* harvested in July 2011, following cold storage at 4°C (±1°C) for 21 days (n = 6). Vertical error bars indicate the Standard Error of the mean for each data point.



		Treatment		TIME		Treatment*TIME	
ANOVA	Main Effects	F	p	F	p	F	p
A	Leaf blackening (Nov 2010)	42.66	<0.0001	178.70	<0.0001	4.56	<0.0001
B	Leaf blackening (Jul 2011)	28.86	<0.0001	49.43	<0.0001	1.58	0.0811

Fig. 4.3 The percentage (%) leaf blackening in *Protea* cv. *Sylvia* flowering stems harvested in November 2010 (A) and July 2011 (B) respectively. Stems were pulsed with a range of glucose concentrations prior to a storage period of 21 days at 4°C (±1°C), where after stems were evaluated in 1.5% Chrysal clear Professional3™ holding solution (n = 6 or 8 respectively) for 7 days. Vertical error bars indicate the Standard Error of the mean for each data point.



	Treatment		TIME		Treatment*TIME	
Main effects	F	p	F	p	F	p
A Stomatal conductance (Day 0)	19.22	<0.0001				
Leaf blackening (Day 0)	20.10	<0.0001				
B Stomatal conductance (Day 0)	14.11	<0.0001	54.80	<0.0001	9.41	<0.0001

Fig. 4.4 (A) The incidence of leaf blackening (%) and stomatal conductance on day 0 of vase life for the flowering stems of *Protea* cv. *Sylvia* exposed to a glucose pulse concentration range prior to storage and held in 1.5% Chrysal clear Professional3™ solution during evaluation. (B) The stomatal conductance measured in flowering stems of *Protea* cv. *Sylvia* over a 7-day vase life period, using a 1.5% Chrysal clear Professional 3™ as holding solution. Stems were harvested in July 2011 and pulsed with a glucose concentration range, prior to cold storage of 21 days at 4°C (±1°C). Vertical error bars indicate the Standard Error of the mean for each data point.

Evaluating ethanol as a commercially viable treatment to control leaf blackening in selected *Protea* species

Abstract

Leaf blackening is a postharvest disorder, typical of many *Protea* species and cultivars. As this disorder results in undesirable dark-brown to black discoloration on leaves, it places a serious constraint on the export potential of this niche product produced by the South African fynbos industry. Furthermore, South Africa is geographically far removed from all important international floricultural markets and additional pressure from retailers to transport flowers by sea presents further challenges to producers to remain competitive by delivering high quality cut flower stems to a discerning market. The cause of leaf blackening has been strongly linked to a carbohydrate stress exerted by the large inflorescence, thus requiring the utilization of sugar bound polyphenols in the foliage, which when removed, can oxidize enzymatically or non-enzymatically. Thus, pulsing with glucose has been to date the method of choice to alleviate leaf blackening development. Ethanol vapour has been shown to reduce scald in apples. A study on *Protea* cv. Pink Ice has shown a reduction in leaf blackening with ethanol treatment, but stems were evaluated without long term cold storage. This study aims to investigate ethanol as an alternative method in the control of leaf blackening in selected *Protea* species under long term cold storage and to compare its efficacy to currently used methods. Stems of *Protea* cv. Pink Ice and *Sylvia* were exposed to a range of ethanol vapour concentrations based on stem fresh weight, various periods of cold storage, as well as to pulsing with ethanol alone and in combination with glucose. Simulated results were verified by means of a commercial trial, where ethanol-treated stems were sea freighted to the Netherlands for evaluation. The use of ethanol as either a vapour or pulse treatment was found to vary widely in different *Protea* species. For both *Protea magnifica* (commercial trial) and 'Pink Ice', some reduction in leaf blackening was achieved with ethanol treatment, but no significant reduction in leaf blackening was found for *Protea* cv. *Sylvia*. Any treatment combination that included glucose-pulsing consistently resulted in a better quality product than with ethanol treatments alone. This finding was also confirmed in the commercial trial. Ethanol and glucose in combination had a synergistic effect and performed better than glucose alone. In conclusion, ethanol as an alternative treatment did not provide reliable results to achieve the same effect as pulsing with glucose, and can only be recommended as an additional treatment in combination with glucose pulsing. Thus, as yet, ethanol vapour or pulsing alone is not a commercial alternative to pulsing with glucose in the control of leaf blackening in *Protea*.

Keywords: Ethanol vapour, ethanol pulsing, glucose pulsing, acetaldehyde, cold storage, *Protea* cv. *Sylvia*, *Protea* cv. Pink Ice, *Protea magnifica*

5.1 Introduction

The South African Fynbos industry relies predominantly on the export market. Earning \$40 million in 2007, Fynbos products make an important contribution to the gross domestic product (GDP) which is essential to secure economic growth for South Africa (Dorrington, 2008). The main export markets for *Protea*, one of the major South African Fynbos products, are currently Central Europe (67%), followed by the United Kingdom (16%), the middle- and east- Mediterranean countries (6%) as well as middle- and east Asia (5%)(PPECB, 2010). These long distance market destinations put additional pressure on the South African fynbos industry to provide a superior quality product that will compete with located producers, such as Portugal, the Canary Islands and Israel that are much closer to the same markets.

Leaf blackening is a postharvest disorder found in certain *Protea species* and cultivars such as *Protea* cv. Pink Ice (*P. compacta* x *P. susannae*) (Crick and McConchie, 1999) and *Protea* cv. Sylvia (*P. eximia* x *P. susannae*) (Stephens et al., 2001a; Meyer, 2003; Ferreira, 2005). Leaf blackening symptoms, which are seen as distinctly visible brown to black discolorations on the leaves (Brink and De Swardt, 1986) can occur as soon as 3 – 5 days after harvest. This undesired blackening can drastically reduce the vase life of *Protea*, making it unsuitable for marketing (Jones et al., 1995). For this reason, leaf blackening in *Protea* cut flowers renders it a risky product and may be the single most important reason for an entire consignment rejection (Verhoogt pers. comm., 2011). Recently, increasing air freight costs (FreshInfo, 2011) and the need to minimize carbon footprints has led to increasing pressure from exporters and retailers on the *Protea* Industry to use sea freight as an alternative transport to air freight. A major disadvantage of this alternative mode of transport is the extended delivery time and thus the increasing risk for leaf blackening development. Transport time of sea freight vs. air freight increases drastically for this highly perishable product from a few days (3 to 5 days) to a few weeks (± 21 days).

Currently, the main causal link to leaf blackening is carbohydrate stress, causing sugar-bound polyphenols to be utilized, thus exposing the phenols to enzymatic or non-enzymatic oxidation. This stress is strongly associated with the sink demands of the flowering stem (Reid et al., 1989; Newman et al., 1990; McConchie et al., 1991; Jones and Clayton-Green, 1992; Bieleski et al., 1992; McConchie and Lang, 1993; Dai and Paul, 1995).

Although postharvest carbohydrate supplementation, through pulsing as well as girdling, have been shown to significantly delay the onset of leaf blackening in susceptible *Protea* species (Brink and De Swardt, 1986; Paull and Dai, 1990; McConchie et al., 1991; Bieleski et al., 1992; Stephens et al., 2001a, b, 2005), both these methods are time-consuming and difficult to successfully implement

on a commercial scale. Therefore the scope exists for alternative or additional methods that will ensure acceptable postharvest quality in *Protea* cut flower stems after prolonged cold storage of 21 to 24 days and which can be implemented with ease at the producer or exporters level.

Ethanol vapour was found to control superficial scald in apples (Scott et al., 1995; Ghahramani and Scott, 1998) by reducing α -farnesene to conjugated trienes, which play a key role in scald development. In apples, this was considered an exciting finding, as ethanol as a treatment could possibly replace the use of the antioxidant, diphenylamine (DPA), as it is currently one of few treatments available to successfully prevent superficial scald (Ghahramani and Scott, 1998). DPA is increasingly banned due to its toxic and environmental concerns (Environmental Protection Agency: DPA factsheet, 1998). Using ethanol to control superficial scald in apple has the advantage of being labelled as a 'green' product that is not harmful, metabolizes easily and is easy to apply (Chervin et al., 2001).

Ethanol is a naturally occurring product produced in plants from pyruvate through acetaldehyde formation (Smagula and Bramlage, 1977). In horticultural crops, ethanol is usually associated with anaerobic respiration and has been shown to affect various metabolic reactions in plant cells, through the process of inhibiting or accelerating senescence, depending on the concentrations present (Pesis, 2005). Thus studies on other horticultural crops, such as broccoli (Mori et al., 2009), cherries (Vidrih et al., 1998), peaches (Polenta et al., 2005), persimmons (Oshida et al., 1996) and raspberries (Wang, 2003) have been subjected to ethanol application, however with varying degrees of success to increase shelf or storage life. Ethanol vapour or pulsing treatments have also been extended to ornamental longevity studies, such as on *Alstroemeria* (Bazaz and Tahraniifar, 2011), *Anthurium* (Thawiang et al., 2007), *Bougainvillea* (Sharif Hossain, 2007), carnations (Wu et al., 1992; Podd and Van Staden, 1999) as well as on chrysanthemums (Petridou et al., 2001). In most of these above mentioned studies, where successful, mode of action was attributed to ethylene inhibition (Petridou et al., 1999; Podd and Van Staden, 1999). However, successful results found in *Protea* cv. Pink Ice suggested ethanol vapour reduces the activity of polyphenol oxidase (PPO) (Crick and McConchie, 1999), which according to Whitehead and de Swardt (1982), is thought to be involved in the blackening process of *Protea* leaves.

Ethanol vapour was found to significantly suppress leaf blackening of *Protea* 'Pink Ice' held at 20°C compared to the control, when stems were exposed to an optimum concentration of 5.6g ethanol.kg⁻¹ fresh weight (Crick and McConchie, 1999). Cannon and McConchie (2001), evaluated ethanol as a postharvest treatment extensively, with respect to a vapour concentration range, as well as other alcohols, including butanol and propanol, and different ethanol releasing agents in their study. Ethanol was also evaluated for its efficacy as both a holding and pulse solution (Cannon

and McConchie, 2001), where ethanol pulsing concentrations as high as 40% was tested. Conclusions from this study were that leaf blackening reduction was best obtained in *Protea* cv. Pink Ice at a vapour concentration of 6 – 7g ethanol.kg⁻¹ fresh weight, which was slightly higher than previously reported by Crick and McConchie (1999). Where ethanol was used, both as a pulsing and holding solution, flower quality was drastically reduced (Cannon and McConchie, 2001). These reports led to a preliminary study to determine the efficacy of ethanol to reduce leaf blackening in cut *Protea* stems during prolonged cold storage as would be required for sea freight to European markets (Windell et al., 2010). In this study, the most optimum concentration for 'Pink Ice' was found to be 2.5g ethanol.kg⁻¹ fresh weight when stored for 14 days at 4°C. However, longer storage durations resulted in decreased efficacy, as leaf blackening rapidly increased over storage time. Also, Windell et al. (2010) showed ethanol vapour to be less effective in cultivars other than 'Pink Ice' to control leaf blackening. This aspect requires further investigating, if ethanol is to be considered as an alternative commercial treatment to glucose pulsing in the control of leaf blackening in *Protea*.

Another, and related naturally occurring plant product, acetaldehyde, has been linked to horticultural crops in a similar manner to ethanol, especially as acetaldehyde action relies on much lower concentrations than ethanol, and can be converted to ethanol (Pesis, 2005). Although the metabolic role of acetaldehyde postharvest is unclear, acetaldehyde, similar to ethanol, is able to transform phenolics as it is capable of polymerizing tannins in astringent crops, such as bananas and persimmons (Pesis, 2005). Also, at certain concentrations, acetaldehyde and ethanol inhibits or delays the senescence process in cut flowers, such as carnations, where its efficacy has similarly been linked to the role it plays in reducing ethylene production (Podd and Van Staden, 1999).

The aim of this study was thus to re-evaluate the use of ethanol to control leaf blackening in the highly susceptible *Protea* cv. 'Sylvia' and to compare the optimum ethanol concentration required for 'Sylvia' when subjected to long term cold storage to that of a cultivar known to be responsive to ethanol treatment, such as 'Pink Ice'. Ethanol applications either as a vapour or as a pulse alone and in combination with glucose pulsing were compared to glucose pulsing as the standard treatment; with or without a vase holding solution. Acetaldehyde as an alternative to ethanol vapour in the control of leaf blackening in 'Sylvia' was also investigated. Finally, simulated cold-storage leaf blackening incidence on ethanol-treated stems were verified by means of a commercial trial on stems of 'Sylvia' and *Protea magnifica* that was sea freighted from Cape Town to the Netherlands over a 24-day period.

5.2 Materials and Methods

5.2.1 Vapour Experiments

5.2.1.1 Plant material

Commercially packed flowering stems of *Protea* 'Pink Ice' (*P. compacta* x *P. susannae*) and *Protea* 'Sylvia' (*P. eximia* x *P. susannae*) were used for the vapour experiments. Standard S14 commercial cartons containing graded stems of 40 to 50cm stem length and with leaves only present on the subtending flush were obtained from a local exporter (Bergflora), which in turn sourced the stems from different commercial farms throughout the Western Cape, South Africa. Stems used for the ethanol concentration range experiment were sourced in March 2009; whereas for the storage duration experiment, stems were sourced in June 2009. Stems used in the acetaldehyde and glucose pulsing experiment prior to storage were harvested in October 2008, whilst stems used for pulsing with ethanol and in the glucose experiments were harvested in July 2010. Lastly, the stems exposed to the various holding solutions were sourced during October and November 2009, via Bergflora exporters.

5.2.1.2 Ethanol concentration range

To re-evaluate and establish an optimum concentration for ethanol vapour treatment on *Protea* cv. Sylvia, a concentration range similar to that of a previous study (Windell et al., 2010) was used. Stems of each treatment group were weighed to determine the appropriate ethanol treatment concentration, as it is based on stem fresh weight. Each treatment unit weighed approximately 10kg and consisted of approximately ± 50 *Protea* 'Sylvia' or ± 60 'Pink Ice' stems respectively. Ethanol treatments included: No ethanol (control) and then an increasing concentration range of 0, 1, 3 and 5g.ethanol kg⁻¹ fresh weight respectively.

The appropriate amount of ethanol (95% Analytical reagent grade, Merck, Darmstadt, Germany) required per treatment was applied to two sponges that were each placed in an open top plastic container which was positioned central to all stems within the carton. Immediately after ethanol application, cartons were closed and individually wrapped with cling-on plastic (Gladwrap®), followed by low density polyethylene black bags. Cartons were then stored in a cold room at 2°C ($\pm 1^\circ\text{C}$) to simulate shipping conditions for only a 14-day period. After 3 days, the cling-on plastic wrapping was removed when ethanol evaporation was estimated to be completed, in order to prevent anaerobic respiration, whilst polyethylene bags were retained to prevent excessive moisture loss from stems during cold-storage.

After storage, 15 single stem replicates were randomly chosen for vase life evaluation. Stems were kept in an evaluation room with temperatures of 18°C ($\pm 2^\circ\text{C}$), light/dark cycles of 10/14 hours with $15\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light levels (produced by fluorescent bulbs) and a relative humidity of 60% ($\pm 10\%$). Stems were held in tap water during the vase life period and were evaluated for leaf blackening on day 0 and 5 of vase life. Leaf blackening was recorded as the number of leaves with $\geq 10\%$ leaf blackening out of the total number of leaves per stem and is presented as a percentage.

5.2.1.3 Storage duration

To determine the effect of storage duration on ethanol vapour efficacy, the ethanol concentration range experiment was repeated with both *Protea* cvs. Pink Ice and Sylvia, but with the exception that the $5\text{g}\cdot\text{ethanol}\cdot\text{kg}^{-1}$ treatment was omitted from this experiment. Treatment cartons were wrapped and stored as described above, but stems were stored for either a 14 or 21 day period at 2°C ($\pm 1^\circ\text{C}$).

After each storage period, five and ten single stem replicates were randomly selected from treatment groups for vase life evaluation for *Protea* cvs. Sylvia and Pink Ice respectively. Leaf blackening was similarly recorded on days 0 and 5 of the evaluation period, as described above.

5.2.1.4 Acetaldehyde

The efficacy of acetaldehyde vapour to control leaf blackening was evaluated for *Protea* cv. Sylvia stems, using the same experimental layout as for the ethanol concentration range experiment, except that ethanol concentrations were replaced with acetaldehyde applications of 0 (control), 0.05, 0.1, 0.25 and $0.5\text{g}\cdot\text{acetaldehyde}\cdot\text{kg}^{-1}$ fresh weight. Stems were then stored at 4°C ($\pm 1^\circ\text{C}$), similar to some commercial constraints that products can not be stored at optimum temperature, for a 14 day cold storage transport simulation period.

After storage, 5 single stem replicates were randomly selected from each treatment group and placed in tap water for the vase life evaluation period. Leaf blackening was similarly recorded on days 0 and 5 of evaluation period, than described for the above experiments.

5.2.2 Pulsing Experiments

5.2.2.1 Plant material

Dry-picked, harvest-ready ('soft-tip' stage), flowering stems of *Protea* 'Sylvia' (*P. eximia* x *P. susannae*) that were used in the pulsing experiments were collected from a commercial farm located near Stellenbosch ($33^\circ 55'S$; $18^\circ 50'E$), Western Cape, South Africa. Only stems used in the trial where

a holding solution containing a low concentration of glucose was evaluated for its efficacy to assist in leaf blackening control in conjunction with ethanol, were sourced from a local commercial exporter, Bergflora. Within an hour after harvest or collection from the exporter, stems were standardized to a length of 40cm, leaving 20 to 30 leaves on the subtending flush to the inflorescence, at the Department of Horticulture, Stellenbosch University.

5.2.2.2 *Glucose pulsing prior to ethanol vapour treatment*

After standardization, stems of *Protea* cv. *Sylvia* were either pulsed with distilled water (control) or with a 5.7% glucose pulse solution until 10mL were absorbed per stem. Immediately after pulsing, 5 single stem replicates of each pulsing treatment was allocated to ethanol vapour treatment groups that consisted either of a 'no ethanol treatment' (control) or an application of 1.5g ethanol.kg⁻¹ fresh weight. After treatments were applied, stems were wrapped in a similar manner to that described previously and were then stored at 2°C (±1°C) for a 21 day period.

After storage, leaf blackening was recorded for both the pulse and vapour treatment combinations in tap water on days 0 and 5 of the evaluation period.

5.2.2.3 *Ethanol pulsing vs. glucose pulsing*

Determining the efficacy of ethanol pulsing as oppose to the ethanol vapour treatment and that of glucose pulsing, *Protea* cv. *Sylvia* stems were standardized and treated with the following pulse solutions: distilled H₂O (control), a pure 1% or 2% ethanol solution, a 9.4% glucose solution as well as with a 1% or 2% ethanol solution made up in a 9.4% glucose solution. All stems were pulsed until 10mL was removed from each treatment solution. Immediately after pulsing, stems were packed in commercial S14 cartons and individually wrapped with a low density polyethylene black plastic bag, to reduce moisture loss during storage. Cartons were stored for a 21 day simulated transport period at 2°C (±1°C).

After storage, 10 single stem replicates were randomly removed from each treatment group and placed in tap water for the evaluation period. Leaf blackening was recorded on days 0, 1, 3 and 5 of the evaluation period, as described previously.

5.2.3 *Vase life holding solution*

To evaluate the impact a vase life holding solution containing a low concentration of glucose may have in combination with an ethanol treatment to control of leaf blackening, the vapour and ethanol pulsing experiments were repeated in *Protea* cv. *Sylvia*. For stems exposed to ethanol

vapour, a 0 (control), 1 and 3g ethanol.kg⁻¹ fresh weight was used and for stems pulsed, distilled H₂O (control), a 2% ethanol solution, a 5.7% glucose solution and a 2% ethanol made up in a 5.7% glucose solution was pulsed until 10mL was removed from each treatment solution. Vapour-exposed stems were packed and wrapped similar to that described for the ethanol vapour experiments in 5.2.1.2, whereas pulsed stems were only wrapped in low density polyethylene black bags during storage. Stems were then stored for a 21 day simulated transport period at 2°C (±1°C).

After storage, 10 single stem replicates for the vapour experiment and 5 single stem replicates for the pulsing experiment were either placed in tap water or in a 1.5% commercial vase holding solution, Chrysal clear Professional3™ (Pokon Chrysal, UK) (CCProf3) for the whole evaluation period. Leaf blackening was recorded after 3 days for stems exposed to vapour treatments and after 5 days for stems pulsed before storage, during the vase life evaluation period.

5.2.4 Commercial Verification

The commercial use of ethanol vapour alone and in combination with glucose pulsing and girdling to control leaf blackening in *Protea* was repeated twice by means of sea freight containers shipped from Cape Town to the Netherlands to verify cold-room storage simulations. *Protea magnifica* and *Protea* cv. *Sylvia* was used for these trials, where *P. magnifica* stems were treated with 3g ethanol.kg⁻¹ vapour only, while 'Sylvia' stems were treated with a 5.7% glucose pulse, a 3g ethanol.kg⁻¹ vapour treatment, girdling or a combination of the listed treatments. For details, see 'Materials and methods' (p. 131) in Appendix A.

5.2.5 Statistical Analysis

One-way and factorial analysis of variance (ANOVA) was used to calculate statistical differences between treatments at the assessment dates during vase life (day 0 or 5). Repeated measures analysis of variance (RANOVA) was used to analyse the interaction between treatments and time (vase life). Percentages determined for leaf blackening were Log-transformed prior to analysis, but data is presented as percentages. Means were separated by Fisher's Least Significant Difference (LSD) test at significance level at $P < 0.05$. All statistical analysis was performed using Statistica version 10 (Statsoft, Inc., 2011).

5.3 Results

5.3.1 Vapour Experiments

5.3.1.1 Ethanol concentration range

Upon removal (day 0), after the 14-day cold storage simulation at 2°C, stems from all ethanol concentration treatments for both *Protea* cvs. Pink Ice and Sylvia had low leaf blackening incidence of <15% (Fig. 5.1A). No interaction between ethanol concentration and cultivar was observed, as well as for percentage leaf blackening between treatment concentrations or between cultivars (Fig. 5.1A). On day 5 of evaluation, no significant interaction ($p = 0.4626$) for percentage leaf blackening between ethanol concentration and cultivar was observed (Fig. 5.1B). However, there was a significant difference in percentage leaf blackening for both cultivar ($p < 0.0001$) and concentration ($p = 0.0247$). Percent leaf blackening was the least at 1g ethanol.kg⁻¹ fresh weight in *Protea* cv. 'Pink Ice' at 6.5% compared to a 26.7% leaf blackening recorded for *Protea* cv. Sylvia at the same concentration treatment respectively. Upon removal, ethanol toxicity was observed in the foliage of stems exposed to higher ethanol concentrations (Fig. 5.1C).

5.3.1.2 Storage duration

Results obtained when investigating the impact of longer storage durations on the development of leaf blackening when stems were treated with an ethanol concentration range showed that the longer storage affected *Protea* cv. Pink Ice more severely than 'Sylvia' (Figs. 5.2A to D). There was a significant interaction in the percent leaf blackening between the ethanol concentrations used and the storage duration for *Protea* cv. Pink Ice upon removal on day 0 of vase life (Fig. 5.2A). It is illustrated most evidently in control stems where leaf blackening incidence was ten fold higher at ±3% compared to 30% for the storage duration of 14 days and 21 days respectively. However, by day 5 of vase life percent leaf blackening was high with no significant difference between any of the ethanol concentrations or storage durations (Fig. 5.2B). In *Protea* cv. Sylvia though, no interaction or significant differences in percent leaf blackening was recorded between or within storage duration or ethanol vapour concentration treatments.

5.3.1.3 Acetaldehyde

When evaluating the use of acetaldehyde vapour as an alternative treatment to ethanol vapour in 'Sylvia' stems, no interaction for percent leaf blackening between concentration and evaluation day was observed (Fig. 5.3). Leaf blackening significantly increased from day 0 of vase life

with leaf blackening ranging from 4 and 11% to between 40 and 70% by day 5. However, there were no significant differences in leaf blackening of stems between the various acetaldehyde concentrations (Fig. 5.3).

5.3.2 Pulsing Experiments

5.3.2.1 Glucose pulsing prior to ethanol vapour treatment

Providing a glucose pulse prior to an ethanol vapour treatment, resulted in no significant interaction or difference between treatments (Figs. 5.4A and B). Leaf blackening incidences upon removal (day 0) were lower, although not significant, for pulsed stems compared to the controls (Fig. 5.4A), whereas pulsed-only or control stems exposed to an ethanol vapour of 1.5g ethanol.kg⁻¹ fresh weight had similar incidences of leaf blackening (Fig. 5.4A). Leaf blackening increased from day 0 to 5 of vase life, but still with no interaction or significant difference detected between or within treatments (Fig. 5.4B).

5.3.2.2 Ethanol pulsing vs. glucose pulsing

Significant interactions ($p < 0.0001$) emerged in percent leaf blackening between the various ethanol and glucose-pulsed treatments over the vase life period (Fig. 5.5). Upon removal, leaf blackening was <20% for all stems that contained glucose in the pulse solution, whereas stems pulsed with water (control) or either 1% or 2% ethanol, had >50% leaf blackening (Fig. 5.5). Also, the development rate of leaf blackening was much slower in stems containing glucose, compared to controls or stems pulsed with ethanol. At the termination of the evaluation period, day 5, there was a significant difference between treatments, where both controls and ethanol treatments were fully blackened at 100%, which differed significantly ($p < 0.0001$) from the 9.4% glucose treatment as well as from 1% ethanol in combination with glucose together with the 2% ethanol in combination with glucose (Fig. 5.5). The latter treatment had a significantly lower percent leaf blackening of 36% by day 5 of vase life (Fig. 5.5).

5.3.3 Vase life holding solution

There were significant differences in percent leaf blackening (after 3 or 5 days of vase life) for the stems subjected to the various vapour and pulsing treatments and then held in either tap water or a commercial vase holding solution (Figs. 5.6 A and B). No significant interaction ($p = 0.0696$) in percent leaf blackening was recorded between the vapour concentration treatment and the choice

of vase life holding solution. However, percent leaf blackening was consistently lower when stems were held in the commercial CCProf3 holding solution in comparison to tap water as a holding solution (Fig. 5.6A). Also, a significantly lower incidence of leaf blackening was found in stems treated with 1g.kg^{-1} and 3g.kg^{-1} ethanol vapour concentration as opposed to stems which received no ethanol vapour (Fig. 5.6A).

A significant interaction ($p = 0.0140$) in percent leaf blackening between pulsing treatments and the choice of holding solution during vase life was recorded (Fig. 5.6B). In controls, percent leaf blackening of below 10% was recorded in the CCProf3 holding solution, compared to stems held in tap water where leaf blackening was significantly higher. Stems pulsed with 2% ethanol however, had leaf blackening percentages of between 30 and 40%, regardless of holding solution (Fig. 5.6B). To the contrary, leaf blackening in glucose-pulsed stems were very low (below 5%), irrespective of holding solution (Fig. 5.6B). Stems pulsed with the 5.7% glucose and 2% ethanol combination had lower incidences of leaf blackening when held in CCProf3 rather than in tap water (Fig. 5.6B).

5.3.4 Commercial evaluation

Leaf blackening was significantly reduced in stems of *Protea magnifica* of both consignments when treated with a concentration of $3\text{g.ethanol kg}^{-1}$ fresh weight.

For *Protea cv. Sylvia*, all treatments which included glucose pulsing displayed less leaf blackening compared to the control or ethanol-treated stems, in both consignments (Appendix A, Figs. 11 and 12). In the first consignment, leaf blackening was best controlled throughout vase life for stems that received either a glucose pulse or a treatment combination that include girdling, ethanol vapour and glucose pulsing. In the second consignment the same treatment proved most beneficial to suppress leaf blackening development, but this benefit dissipated by day 3 of vase life, where upon all stems, irrespective of treatment were considered unmarketable (Appendix A, Fig. 22).

In addition to the effect ethanol had on leaf blackening in the foliage, pulsing with ethanol at 2% also impacted on the flower quality. Flowers dehydrated to such an extent that stems were unmarketable, already early during vase life (Appendix A, Fig. 19).

5.4 Discussion

Exposing *Protea cv. Pink Ice* to an ethanol vapour concentration range, whilst storing for 14 days at 4°C , revealed the optimum concentration for reducing leaf blackening to be approximately

half (Windell et al., 2010) of that suggested for 'Pink Ice' at room temperature of 20°C by Crick and McConchie (1999;) and Cannon and McConchie (2001). This concentration was also applicable in this study for *Protea* cv. Sylvia, although the effectiveness of the vapour seemed less pronounced. In both the studies of Crick and McConchie (1999) and Cannon and McConchie (2001), each stem was enclosed in its own bag where stems were individually exposed to the required ethanol dosage. Testing whether this treatment is as effective when applied to a commercial carton of stems, the study by Windell et al. (2010) and reported here differed from that above in that all stems were weighed collectively, where after ethanol vapour was applied to the group as a whole, as would probably be done in a commercial venture.

The volatility of ethanol during the low temperatures at which 'Sylvia' stems were stored in this study proved to be sufficient to effectively reach all stems. Although a high degree of variation was observed between treatments, ethanol is highly volatile, and at 0°C, Ghahramani and Scott (1999) reported that absorption of ethanol by apples (5.6g ethanol.kg⁻¹ fresh weight) held in an ethanol permeable polyethylene bag (50µm density) was extremely high, compared to ethanol without apples. This variation could also be due to the fact that stems were sourced from different farms and under different picking and packing regimes.

Higher ethanol concentrations per stem fresh weight, as is recommended by Crick and McConchie (1999) proved to be phytotoxic under application methods, and storage temperature and durations used in this study. This phytotoxicity could be due to additionally induced ethanol and acetaldehyde accumulation under low temperatures (Pesis, 2005). Studies have shown that ethanol and acetaldehyde are both produced and will accumulate during stress conditions, such as wounding, freezing or chilling injury or water stress, even when sufficient O₂ concentration is present (Kimmerer et al., 1982; Pesis, 2005). Although, no study has shown that *Protea* suffers from chilling injury (Stephens, 2003; Van Doorn, 2001), low temperatures together with dry and extended storage could all induce a form of stress. As no ethanol content was measured in the leaves in this study, the effect of long-term cold-storage in terms of internal ethanol production cannot be confirmed.

Pulsing with ethanol, where the release rate is easier controlled than with the vapour, was found to severely dehydrate the flowering stems, especially that of the flower bracts. As ethanol pulsing reduced the quality of the main point of interest, the flower, the goal of improving vase life by controlling leaf blackening, served no further purpose. Cannon and McConchie (2001) similarly reported a lowered flower quality in stems pulsed with ethanol at all of the concentrations evaluated.

When pulsing with a low ethanol concentration (1 – 2%) in combination with a glucose solution, a synergistic effect was observed, where both the longevity of the flowers as well as a delay in leaf blackening was achieved. This treatment was also significantly better in terms of leaf blackening control than when stems were only pulsed with glucose. When ethanol or acetaldehyde concentrations become too high, it has an opposite effect on the metabolism of the plant, by increasing respiration and further inducing aerobic glycolysis (Janes and Frenkel, 1978; Pesis, 2005), thus accelerating senescence and decreasing carbohydrate reserves. The addition of glucose could play a role in either reducing the carbohydrate stress resulting from increased respiration due to the ethanol treatment or offer some other form of metabolic support, such as an osmotic aid to counteract the dehydrating properties of ethanol.

Storage duration and product quality in flowers are inversely correlated, as increased storage duration invariably leads to a reduced product quality as affected by progressive senescence and cellular stress. For *Protea*, where product quality is largely determined by the severity and rate of leaf blackening development, this statement is just as true. In this study, optimum storage and optimal ethanol vapour concentration of 1 to 2.5g.ethanol kg⁻¹ fresh weight had the least amount of leaf blackening after 2 to 3 weeks storage, at day 0, when removed from cold storage. However, thereafter leaf blackening rapidly increased by day 3 to such an extent that the stems were unmarketable. The process of leaf blackening can apparently not be inhibited or delayed by ethanol vapour or pulsing to acceptable levels under long-storage durations as is required for sea freight to European markets. These findings are similar to previously found in the study of Windell et al. (2010). It was reported that ethanol had a significant effect on reducing leaf blackening for *Protea* cv. Pink Ice, if stored for only 14 days, but when storage duration was increased to 21 or 28 days, ethanol efficacy diminished and leaf blackening was unacceptably high early in the vase life period.

Phytotoxicity in stems was evident in the acetaldehyde vapour treatments as the process of leaf blackening was clearly enhanced and accelerated. Despite the low concentrations used, concentrations may still have been supra-optimal, as acetaldehyde concentrations of as little as 0.1g acetaldehyde.kg⁻¹ fresh weight have been found to induce postharvest disorders in apples and even more so in pears (Smagula and Bramlage, 1977). As mentioned earlier, acetaldehyde at sufficiently high concentrations will increase respiration (Pesis, 2005) which will then utilize the vital carbohydrate reserves required for further flower development in *Protea* (Jones et al., 1995). In this study, however, stems exposed to acetaldehyde vapour were stored at a higher temperature of 4°C, rather than 2°C, which could also explain some of the accelerated leaf blackening development during storage. Furthermore, acetaldehyde application, although observed as leaf blackening, was mostly due to phytotoxic symptoms.

In conclusion, ethanol vapour as a postharvest treatment to control leaf blackening development is not suitable to be used as a substitute for glucose pulsing. It should rather be considered as an additional treatment to glucose pulsing, as was demonstrated in the commercial verification trial. Ethanol is very volatile, with a narrow optimum concentration range, loses its efficacy with long-term cold-storage and has a dehydrating effect on the inflorescence. Also, if applied at too high concentration, ethanol may lead to a faster depletion of carbohydrate reserves due to enhanced respiration, which will then aggravate leaf blackening incidences. Although there are a few advantages to the application of ethanol, the highly regulated commercial application thereof and the management of such an investment in a flammable and expensive product, that only reduces leaf blackening incidence under certain, sometimes unknown, circumstances outweighs the more reliable methods of glucose pulsing or girdling, even though being more labour intensive and time-consuming.

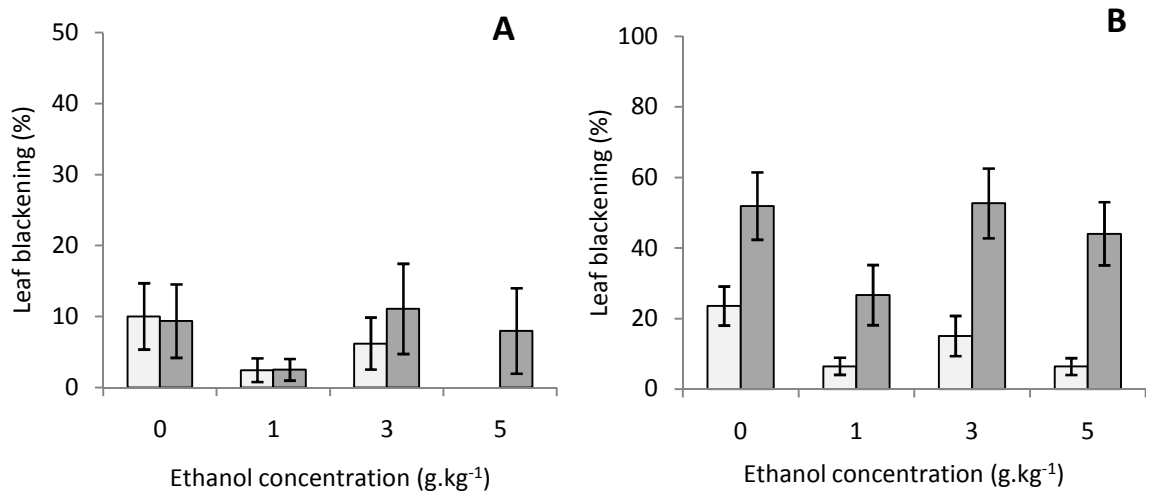
5.5 References

- Bazaz, A.M., Tehranifar, A., 2011. Effect of ethanol, methanol and essential oils as novel agents to improve vase-life of *Alstroemeria* flowers. *J. Biol. Environ. Sci.* 5, 41 – 46.
- Bieleski, R.L., Ripperda, J., Newman, J.P., Reid, M.S., 1992. Carbohydrate changes and leaf blackening in cut flower stems of *Protea eximia*. *J. Am. Soc. Hortic. Sci.* 117, 124 – 127.
- Brink, J.A., De Swardt, G.H., 1986. The effect of sucrose in vase solution on leaf browning of *Protea neriifolia* R. Br. *Acta Hortic.* 185, 111 – 119.
- Cannon, A., McConchie, R., 2001. Controlling leaf blackening in *Protea*. A report for the Rural Industries Research and Development Corporation, RIRDC publication No. 01/098, Project No. US-89A, Australia.
- Chervin, C., Westercamp, P., Raynal, J., André, N., Bonneau, A., 2001. Ethanol vapour treatment against apple scald: How to start a research program. *Acta Hortic.* 553, 257 – 260.
- Crick, S.G., McConchie, R., 1999. Ethanol vapour reduces leaf blackening in cut flower *Protea* 'Pink Ice' stems. *Postharvest Biol. Technol.* 17, 227 – 231.
- Dai, J-W., Paull, R.E., 1995. Source-Sink relationship and *Protea* postharvest leaf blackening. *J. Am. Soc. Hort. Sc.* 120, 475 – 480.
- Dorrington, P., 2008. Country profile of South Africa presentation by chairman of South Africa Protea Producers Exporters (SAPPEX), 9th International Protea Association (IPA) symposium, Sept 2008, Stellenbosch, South Africa.
- Environmental Protection Agency (EPA), 1998. Diphenylamine (DPA) Factsheet. <http://www.epa.gov>

- Ferreira, A., 2005. Further studies on leaf blackening of *Protea*. MSc Thesis, Stellenbosch University, South Africa.
- Fresh Info weekly Newsletter, December 2011. Fresh Produce Journal. <http://www.freshinfo.com>
- Ghahamani, F., Scott, K.J., 1998. The action of ethanol in controlling superficial scald of apples. *Aus. J. Agric. Res.* 49, 199 – 205.
- Janes, H.W., Frenkel, C., 1978. Promotion of softening process in pear by acetaldehyde independent of ethylene action. *J. Am. Soc. Hortic. Sci.* 103, 397 – 400.
- Jones, R.B., Clayton-Greene, K.A., 1992. The role of photosynthesis and oxidative reactions in leaf blackening of *Protea neriifolia* R. Br. Leaves. *Sci. Hortic.* 50, 137 – 145.
- Jones, R.B., McConchie, R., Van Doorn, W.G., Reid, M.S., 1995. Leaf blackening in cut *Protea* flowers. *Hortic. Rev.* 17, 173 – 201.
- Kimmerer, T.W., Kozlowski, T.T., 1982. Ethylene, ethane, acetaldehyde, and ethanol production by plants under stress. *Plant Physiol.* 69, 840 – 847.
- McConchie, R.B., Lang, N.S., Gross, K.C., 1991. Carbohydrate depletion and leaf blackening in *Protea neriifolia*. *J. Am. Soc. Hort. Sci.* 116, 1019 – 1024.
- McConchie, R.B., Lang, N.S., 1993. Carbohydrate metabolism and possible mechanisms of leaf blackening in *Protea neriifolia* under dark postharvest conditions. *J. Am. Soc. Hort. Sci.* 118, 355 – 361.
- Meyer, C. 2003. Carbohydrates and leaf blackening of *Protea* cut flowers. MSc thesis, Stellenbosch University, South Africa.
- Mori, T., Terai, H., Yamauchi, N., Suzuki, Y., 2009. Effects of postharvest ethanol vapour treatment on the ascorbate-glutathione cycle in broccoli florets. *Postharvest Biol. Technol.* 52, 134 – 136.
- Newman, J.P., Van Doorn, W.G., Reid, M.S., 1990. Carbohydrate stress causes leaf blackening. *J. Int. Protea Assoc.* 17, 26 – 31.
- Oshida, M., Yonemori, K., Sugiura, A., 1996. On the nature of coagulated tannins in astringent-type persimmon fruit after an artificial treatment of astringency removal. *Postharvest Biol. Technol.* 8, 317 – 327.
- Paul, R.E., Dai, J.W., 1990. *Protea* postharvest leaf blackening: A problem in search of a solution. *Acta Hortic.* 264, 93 – 101.
- Perishable Products Export Control Board (PPECB) Export directory 2010. Part 3: All flora. Malachite Press, Helderberg, South Africa. Section 2, pp. 79.
- Pesis, E., 2005. The role of the anaerobic metabolites, acetaldehyde and ethanol, in fruit ripening, enhancement of fruit quality and fruit deterioration. *Postharvest Biol. Technol.* 37, 1 – 19.

- Petridou, M., Voyiatzi, C., Voyiatzis, D., 1999. Aspirin, methanol and some antibacterial compounds prolong the vase life of cut carnations. *Adv. Hortic. Sci.* 13, 161 – 164.
- Petridou, M., Voyiatzi, C., Voyiatzis, D., 2001. Methanol, ethanol and other compounds retard leaf senescence and improve the vase life and quality of cut chrysanthemum flowers. *Postharvest Biol. Technol.* 23, 79 – 83.
- Podd, L.A., Van Staden, J., 1999. The use of acetaldehyde to control carnation flower longevity. *Plant Growth Regul.* 28, 175 – 178.
- Polenta, G., Budde, C., Murray, R., 2005. Effects of different pre-storage anoxic treatments on ethanol and acetaldehyde content in peaches. *Postharvest Biol. Technol.* 38, 247 – 253.
- Reid, M.S., Van Doorn, W.G., Newman, J.P., 1989. Leaf blackening in proteas. *Acta Hortic.* 261, 81 – 84.
- Scott, K.J., Yuen, C.M.C., Ghahramani, F., 1995. Ethanol vapour – a new anti-scald treatment for apples. *Postharvest Biol. Technol.* 6, 201 – 208.
- Sharif Hossain, A.B.M., Boyce, A.N., Osman, N., 2007. Postharvest quality, vase life and photosynthetic yield (Chlorophyll fluorescence) of Bougainvillea flower by applying ethanol. *Au. J. Basic App. Sci.* 1, 733 – 740.
- Smagula, J.M., Bramlage, W.J., 1977. Acetaldehyde accumulation: Is it a cause of physiological deterioration of fruits? *HortScience* 12, 200 – 203.
- Statsoft Inc., 2011. STATISTICA (data analysis software system), Version 10. <http://www.statsoft.com>
- Stephens, I.A., 2003. Leaf blackening of Proteas. PhD. Diss., Stellenbosch University, Stellenbosch, South Africa.
- Stephens, I.A., Jacobs, G., Holcroft, D.M., 2001a. Glucose prevents leaf blackening in 'Sylvia' proteas. *Postharvest Biol. Technol.* 23, 237 – 240.
- Stephens, I.A., Holcroft, D.M., Jacobs, G., 2001b. Low temperature and girdling extend vase life of 'Sylvia' Proteas. *Acta Hortic.* 545, 205 – 214.
- Stephens, I.A., Meyer, C., Holcroft, D.M., Jacobs, G. 2005. Carbohydrates and postharvest leaf blackening of Proteas. *J. Hortic. Sci.* 40(1), 181 – 184.
- Thawiang, N., Buanong, M., Kanlayanarat, S., 2007. Effect of ethanol on postharvest quality of cut anthurium flowers (*Anthurium andraeanum* L. cv. Marshall). *Acta Hortic.* 755, 419 – 423.
- Van Doorn, W.G., 2001. Leaf blackening in *Protea* flowers: Recent developments. *Acta Hortic.* 545, 197 – 204.
- Vidrih, R., Zavrtnik, M., Hribar, J., 1998. Effect of low O₂, high CO₂ or added acetaldehyde and ethanol on postharvest physiology of cherries. *Acta Hortic.* 468, 695 – 703.

- Wang, C.Y., 2003. Maintaining postharvest quality of raspberries with natural volatile compounds. *Int. J. Food Sci. Technol.* 38, 869 – 875.
- Whitehead, C.S., de Swardt, G.H., 1982. Extraction and activity of polyphenoloxidase and peroxidase from senescing leaves of *Protea neriifolia*. *S. Afr. J. Bot.* 1, 127 – 130.
- Windell, N.E., Stephens, I.A., Jacobs, G., Hoffman, L., 2010. Effect of ethanol vapour on leaf blackening of selected *Protea* species. *Acta Hort.* 869, 135 – 139.
- Wu, M-J., Zacarias, L., Saltveit, M.E., Reid, M.S., 1992. Alcohols and carnation senescence. *HortScience* 27, 136 – 138.



		□ <i>Protea</i> cv. Pink Ice		■ <i>Protea</i> cv. Sylvia			
		Concentration		Cultivar		Concentration*Cultivar	
ANOVA	Main Effects	F	p	F	p	F	p
A	Leaf blackening (Day 0)	1.284	0.2833	0.599	0.4404	1.211	0.3093
B	Leaf blackening (Day 5)	3.242	0.0247	25.619	<0.0001	0.863	0.4626



Fig. 5.1 The percentage (%) leaf blackening recorded at day 0 (A) and 5 (B) of vase life after flowering stems of *Protea* cv. Pink Ice (n = 15) and Sylvia (n = 15) were exposed to 0 (control), 1, 3 and 5g ethanol.kg⁻¹ fresh weight concentrations during a storage period of 14 days at 2°C (±1°C). Vertical error bars indicate standard error of the mean for each data point. Also, an example of typical ethanol phytotoxicity symptoms (C) in the foliage of *Protea* cv. Sylvia observed when stems are fumed with ethanol vapour concentrations. This differs from ‘normal’ leaf blackening symptoms of black discoloration, starting from a specific point and not overall. Acetaldehyde phytotoxicity symptoms have a similar appearance.

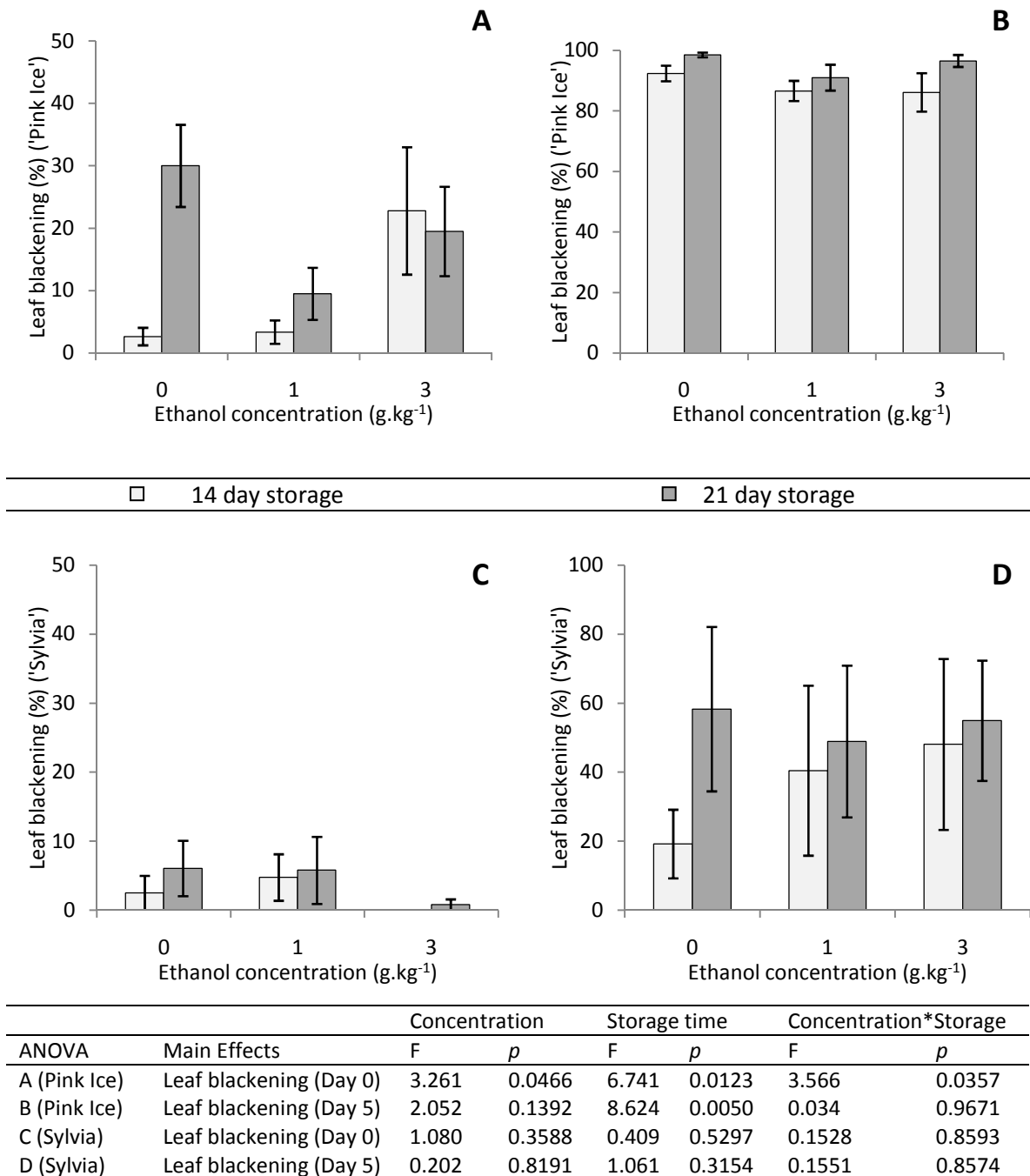


Fig. 5.2 The percentage (%) leaf blackening in flowering stems of *Protea* cv. Pink Ice (A and B) (n = 5) or 'Sylvia' (C and D) (n = 10) evaluated on day 0 (A and C) and 5 (B and D) of vase life after exposure to 0 (control), 1 and 3g ethanol.kg⁻¹ fresh weight during a storage period of 14 or 21 days respectively at 2°C (±1°C). Vertical error bars indicate standard error of the mean at each data point.

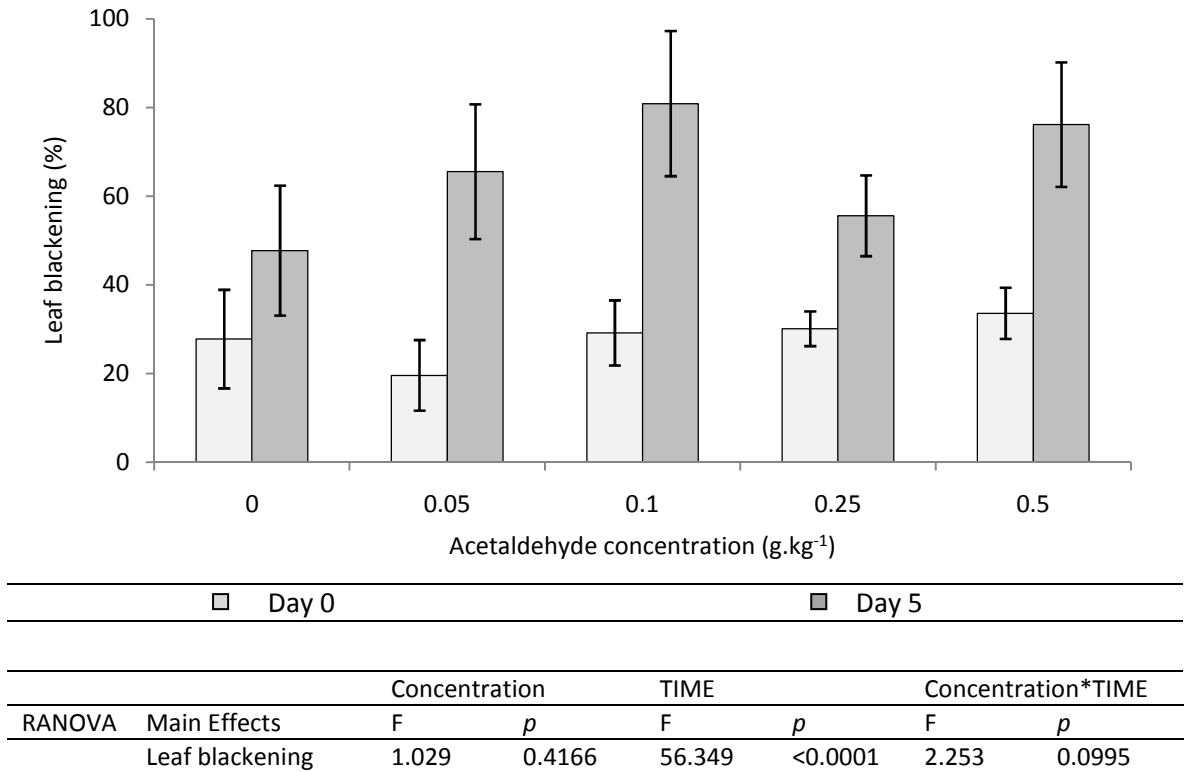


Fig. 5.3 The percentage (%) leaf blackening in *Protea* cv. *Sylvia* recorded on day 0 and 5 of vase life after exposure to an acetaldehyde concentration range for 14 days of cold storage at 4°C. Vertical error bars indicate standard error of the mean at each data point.

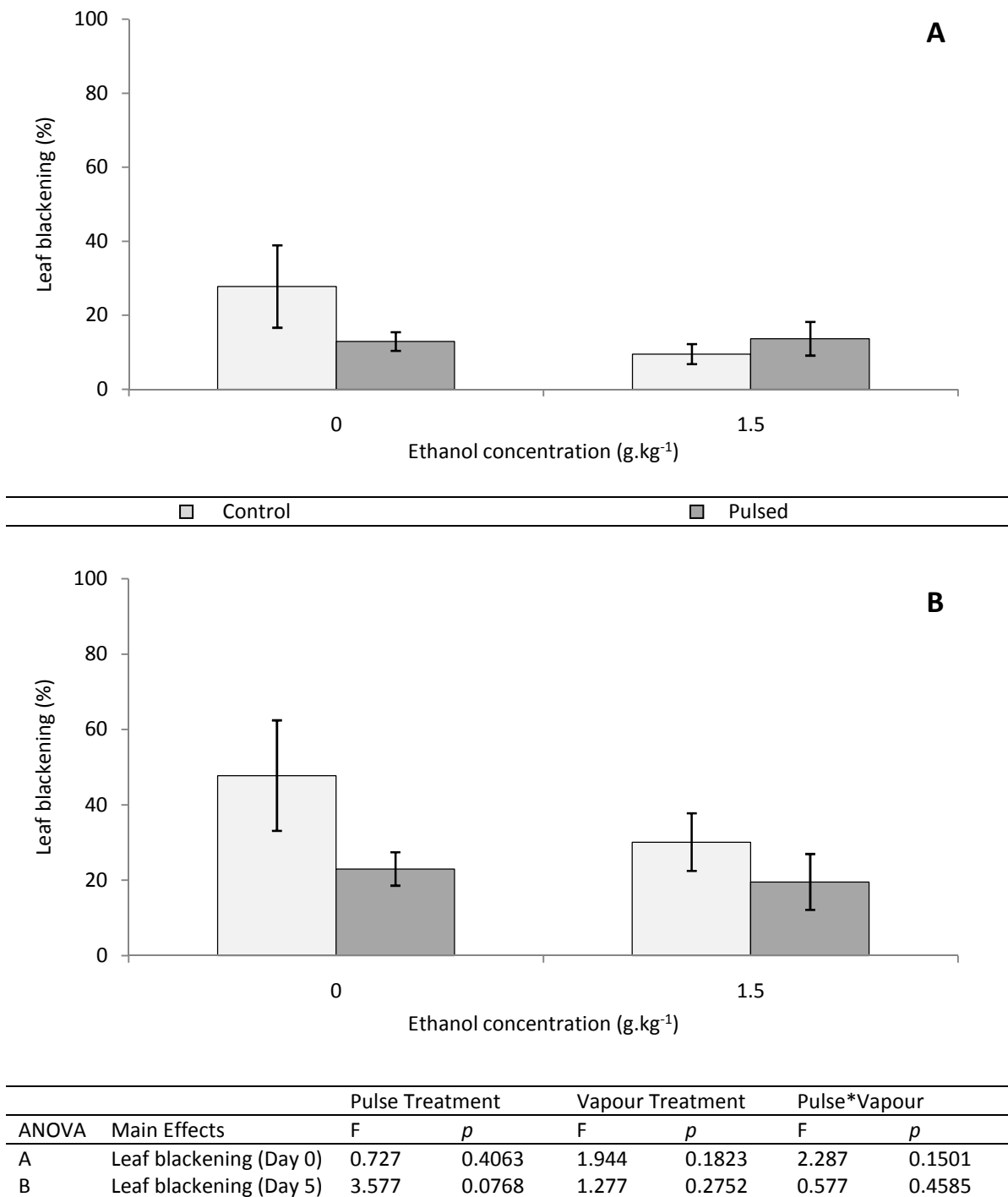
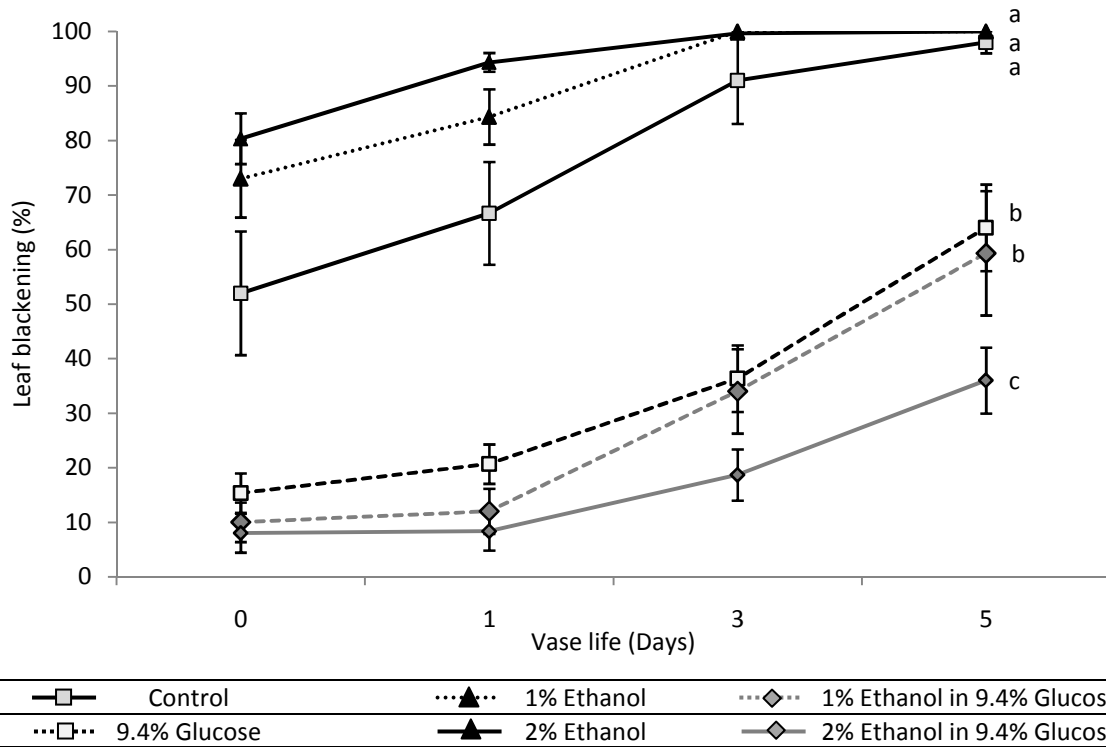


Fig. 5.4 The percentage (%) leaf blackening recorded on day 0 (A) and 5 (B) of vase life in *Protea* cv. *Sylvia* flowering stems after pulsing with 5.7% glucose solution and storing with (1.5g ethanol.kg⁻¹) or without (0g ethanol.kg⁻¹) ethanol vapour treatment for 21 days at 4°C (±1°C). Vertical error bars indicate the standard error of the means for each data point.



RANOVA	Main Effects	Treatment		TIME		Treatment*TIME	
		F	p	F	p	F	p
	Leaf blackening	43.692	<0.0001	155.128	<0.0001	3.506	<0.0001

Fig. 5.5 The percentage (%) leaf blackening in *Protea* cv. *Sylvia* flowering stems over a 5-day vase life period, after stems were pulsed with either 9.4% glucose, 1 or 2% ethanol or a combination thereof until 10mL pulse solution absorbed, where after stems were stored for 21 days at 2°C (±1°C). Vertical error bars indicate the standard error of the means for each data point. Different letters on day 5 of the evaluation period indicate a significant difference at the 5% level.

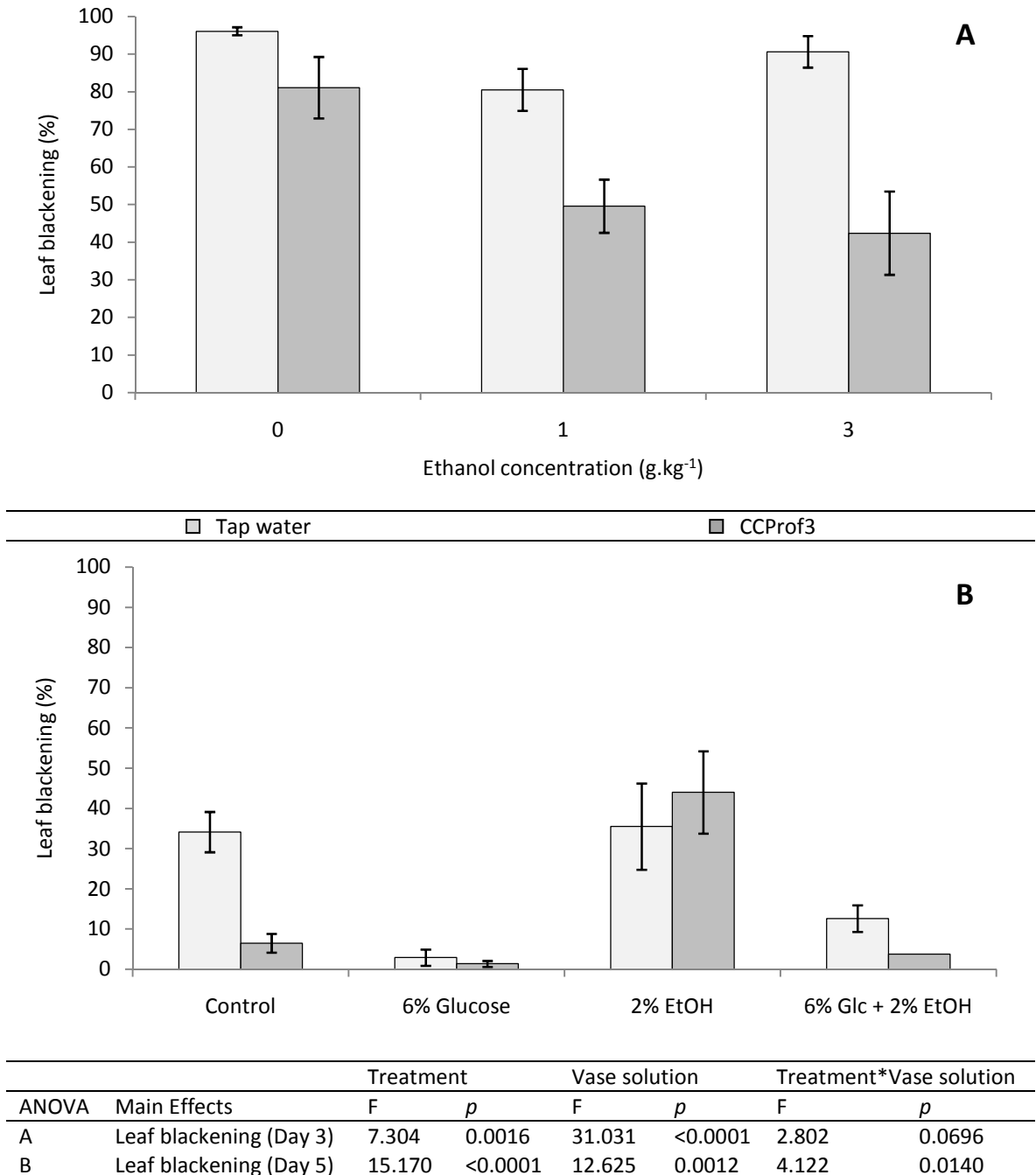


Fig. 5.6 The percentage (%) leaf blackening in *Protea* cv. *Sylvia* flowering stems after 3 or 5 days vase life where stems were either held in tap water or in a 1.5% Chrysal Clear Professional 3™ (CCProf3) holding solution following an ethanol fuming concentration treatment (A) or an 2% ethanol, 5.7% glucose and an ethanol-glucose combination pulsing treatment (B) stored for 21 days storage at 2°C (±1°C). Vertical error bars indicate standard errors of the means for each data point.

GENERAL CONCLUSION

Leaf blackening in *Protea* cut flower stems is a major concern for the South African Fynbos industry, especially under increasing pressure from rising air freight costs and retailers' concern for a lower carbon footprint which shifts the shipping of these products towards sea freight rather than air freight. Sea freight transport extends the time of storage from 3 to 5 days, as required for air freight, to up to 21 days. Although the cold chain can be better maintained in sea freight containers, leaf blackening incidences are known to increase with storage duration. As a result, the use of effective postharvest treatments is essential to ensure that products of consistently high postharvest quality can be offered to the discerning European markets all year round.

The aim of this study was to obtain a better understanding of the underlying mechanisms and possibly predicting factors that may cause this disorder, in order to optimize the current practices or introduce new methods to control leaf blackening in *Protea*. Thus, the objective was to record the seasonal and daily variation in *Protea* cv. *Sylvia* in terms of leaf blackening incidence and measure the corresponding foliage internal carbohydrate-, phenolic- and water content in order to correlate the leaf blackening incidences to the factors measured. Furthermore, to investigate some of these environmental factors that influence the main method currently used to control leaf blackening incidence, glucose pulsing, so that recommendations for optimum pulsing time may be suggested, especially for more effective control during long term cold storage. Also, because the concentration of glucose pulsed has been found to influence the degree of leaf blackening incidence, an additional objective was to investigate the other vase life dynamics affected by glucose pulse concentrations. In addition to the glucose pulsing optimization study, ethanol was also re-evaluated as an alternative method to reduce leaf blackening incidence, because ethanol has not yet been tested for long-term cold stored stems. A commercial trial using sea freight from South Africa to the Netherlands was done to verify ethanol studies where storage was simulated in a cold room. This study included a combination of glucose pulsing and girdling treatments in addition to ethanol fuming and pulsing, so that a commercial recommendation could be made for sea-freighted cut flower stems *Protea magnifica* and *Protea* cv. *Sylvia*.

Leaf blackening incidence was highly varied throughout the season in *Protea* cv. *Sylvia*, with the maximum incidences of leaf blackening found from October up until March, before declining by April and remaining low until September (Paper 1). From the chemical leaf analysis done throughout the season, the incidence of leaf blackening was significantly correlated to an increase in sucrose concentration and a decrease in percent water content. This then implies that free-carbohydrate availability, in the form of sucrose, does not necessarily provide the adequate amounts needed to

lower incidence of leaf blackening. Results obtained in this study suggest that different factors may influence leaf blackening incidences within the respective seasons and that one single factor might be more associated with leaf blackening occurrence in early summer, than in spring. Investigation into pre-harvest cultivation practices and the prevailing environmental conditions present during flush extension and flower development is required to explain the unaccounted variances reported by the Principle component- and Discriminant analysis plots. Investigation into the anatomical changes that might influence the differentiation and expansion of cells within stems and leaves under certain environmental conditions and to include other internal factors, such as the hormone ABA or other specific enzymes involved is needed to relate these differences to leaf blackening. *Protea cv. Sylvia* was chosen for this study because of its year-round flowering habit and high incidence of developing leaf blackening. However, the outcome for other *Protea* cultivars or species, because of different genetic, anatomical, soil or environmental conditions might lead to different findings and associations with leaf blackening incidences.

The soluble carbohydrate content of foliage correlated well with the leaf blackening incidences reported in stems harvested at different times throughout the day (Paper 1), although the opposite was found for seasonal harvested stems. Sucrose and reducing sugars increased from morning to afternoon harvests as current photosynthates accumulated, whilst the incidence of leaf blackening decreased accordingly. Even when harvested stems were glucose-pulsed at each harvest time, leaf blackening incidence was still lower in late-afternoon harvested stems than those harvested early-morning. Leaf water content was linked to leaf blackening development as stems harvested in the morning, when turgor pressure is generally higher, also had the most leaf blackening incidence. From these findings, it is recommended that *Protea cv. Sylvia* be harvested rather later within a day, rather than in the morning.

The level of stem hydration, the time of harvesting and the glucose concentration of the pulse solution was shown in the next study to all strongly influence the amount of pulse-solution that can be absorbed by the stem in a given time period (Paper 2). Stem dehydration was found to decrease stem water potential, which will facilitate enhanced solution uptake. Also, the glucose concentration used for pulsing increases osmotic potential of the pulse solution, which in turn decreases uptake along with increasing concentration. Thus, when harvesting later in the day, the combination of a higher transpiration rate and the lower water potential of the stem, resulted in faster uptake ability. In commercial practice, as well as in the literature, the general protocol for pulsing cut flowers is given as a certain set of time. However, in this study, these findings illustrate that hydration, vapour pressure deficit between the cut flower and the pulsing environment and even pulsing concentration influences the rate of uptake. These factors are of major importance

when pulsing, as conditions leading to inadequate or toxic amounts of pulsing solution absorbed will negatively impact the storability and vase life of *Protea* cv. *Sylvia* stems.

Although additional carbohydrates are mainly supplied to support inflorescence development during storage and vase life, glucose pulsing has also been shown to offer additional properties other than to lower leaf blackening incidences (Paper 3). Glucose concentrations of between 4.7 and 13.7% were able to sustain a positive water balance for longer during vase life after long-term cold storage, compared to no pulsing or pulsing with higher concentrations. This in turn, assisted in the delay of leaf blackening for longer periods. Furthermore, in this study, the addition of a vase life preservative containing low concentrations of glucose significantly contributed to further delay leaf blackening development after long-term cold storage, provided stems were pulsed before storage.

Upon measuring stomatal conductance, stems pulsed with glucose also had significantly lower stomatal conductance. This suggests a role of glucose in the maintenance of leaf cellular function under stress conditions such as long-term cold storage, not just as a carbohydrate supply to inflorescences. Research to determine the role of stomatal control and leaf blackening requires further investigation to address the involvement of glucose in stomata and comparing it to other stomatal closing agents, such as ABA or potassium chloride salts.

Ethanol as an alternative treatment to glucose pulsing for the control of leaf blackening was considered a few times in *Protea* cut flowers, because of its easier application and reduced requirement for labour (Paper 4). However, these studies offered no further insights into the mechanism or efficacy of ethanol to control leaf blackening. In this study, although ethanol treatment did provide some level of control in leaf blackening incidence for *Protea magnifica* and the cultivar 'Pink Ice', no significant added benefit was found for *Protea* cv. *Sylvia*. It was also reported that ethanol had a dehydrating effect on the *Protea* cut flowers. Whether this dehydration was also evident in the foliage and may have played a part in the lowered incidence of leaf blackening, as low water content was found to correlate with lower leaf blackening incidences, is still unclear. Thus, until the exact mechanism of ethanol in the reduction of leaf blackening in *Protea* is understood, progress to control leaf blackening by means of ethanol in 'Sylvia' and other cultivars will be hampered.

Providing a glucose pulse in combination with either ethanol vapour or as a pulse had a synergistic effect in the control of leaf blackening. The ethanol vapour concentration range considered optimal is narrow (1 – 3g ethanol.kg⁻¹ fresh weight). The release and application of ethanol as a vapour thus requires careful control to not exceed this limit, as higher dosage will result in phytotoxicity of the foliage and dehydration of the flower. The commercial trial verified results found in the simulated cold-storage trials done in this study. Thus, currently the use of ethanol

pulsing or vapour as a commercial alternative to glucose pulsing is not recommended, when used under similar storage conditions and applications methods.

In conclusion, leaf blackening in *Protea* remains a challenging postharvest disorder. From the seasonal and daily harvesting study, it is apparent that leaf blackening is more complex than just a lack of carbohydrates within the stems. The at-harvest external environment and internal status of a flowering *Protea* stem highly influences its leaf blackening incidence predisposition and should be considered when harvesting. Pulsing with glucose remains the most reliable postharvest treatment to counteract the stresses after harvesting inducing the development of leaf blackening. The success of glucose lies in the practical application thereof for pulsing and therefore specific refined protocols is needed for other cultivars.

APPENDIX A

Commercial Verification Trial Report

October 2009



**THE EFFICACY OF VARIOUS POST HARVEST TREATMENTS ON
THE CONTROL OF LEAF BLACKENING IN
PROTEA MAGNIFICA AND *PROTEA* CV. SYLVIA**



Dept. Horticultural Science
Stellenbosch University
October 2009



Executive Summary

Leaf blackening is one of the major postharvest constraints in the commercial export of several species of *Protea*. Species highly susceptible to leaf blackening include *Protea magnifica*, *P. eximia*, *P. compacta* and *P. neriifolia*, but also cultivars derived from one of these species, such as 'Lady Di', 'Sylvia' and 'Pink Ice' (Van Doorn, 2001).

Many studies have linked carbohydrate stress to the onset of leaf blackening (Jones et al., 1995), though the exact mechanism involved are still debated. The current methods for control of leaf blackening are girdling just below the flower head or pulsing with sugars. These methods have proven to offer some control over the development of leaf blackening. However, the application of the existing protocols may not be easy to execute on a commercial scale. A preliminary study of Crick and McConchie (1999) found that leaf blackening in 'Pink Ice' was reduced by applying ethanol vapour as a treatment to the foliage, whilst a more in-depth study by Cannon and McConchie (2001) confirmed results from that earlier report and suggested the use of ethanol vapour as an alternative method to treat leaf blackening in *Protea*.

Thus, the aim of this study was to verify results from simulated studies using cold storage in a commercial sea freight shipping container travelling from Cape Town to Rotterdam, in the Netherlands. The efficacy of ethanol vapour treatment to reduce leaf blackening in both susceptible cultivars and species was compared to the current alternatives of glucose pulsing or girdling of stems.

For *Protea magnifica*, two different stocks (of seedling populations) were sourced from the same farm, whereas *Protea* cv. *Sylvia* were sourced elsewhere. For *Protea magnifica*, treatments consisted of a control (no ethanol) and an ethanol vapour application of 3g ethanol. kg⁻¹ fresh weight was used, whereas treatments applied to 'Sylvia' included: no ethanol (control), an ethanol application at 3g ethanol. kg⁻¹ fresh weight, a glucose pulse of 5.7%, and two treatment combinations of 5.7% glucose and ethanol (3g ethanol. kg⁻¹ fresh weight), as well as girdling prior to a glucose pulse and ethanol (3g ethanol. kg⁻¹ fresh weight) treatment. After treatment, stems were packed in containers at 2°C at the premises of Bergflora exporters at Cape Town International Airport, before being sea freighted to the Netherlands. A similar experiment where both *Protea magnifica* and 'Sylvia' were again included was repeated two weeks later. Upon removal, stems were evaluated by the number of leaves that had to be removed due to > 10% blackening, whilst *Protea* cv. *Sylvia* was assessed further during vase life for leaf blackening development.

In *Protea magnifica*, applying ethanol vapour at 3g.kg⁻¹ fresh weight successfully reduced the number of stems with >10% leaf blackening and in the second consignment the application of the ethanol vapour treatment was confirmed to increase the number of marketable stems.

In the first consignment of *Protea* cv. 'Sylvia', on arrival, all stems treated with glucose or that were girdled had lower leaf blackening than observed in the controls or ethanol treatment. However, during vase life the optimum treatment emerged as the treatment combination of girdling, glucose pulsing and ethanol vapour application. Stems exposed to ethanol vapour only were comparable or had higher leaf blackening incidences when compared to the control. In the second consignment, the three-treatment combination of girdling, glucose pulsing and ethanol vapour again produced the highest amount of saleable stems upon arrival. However, thereafter both leaf blackening and flower quality decreased drastically in all treatments, to a level where no stems were saleable by day 3 of vase life.

To conclude: Studies on *Protea magnifica* and *Protea* cv. *Sylvia* stems, from two commercial consignments of 3-weeks cold storage, between Cape Town and the Netherlands during Sept – Oct 2009, showed that ethanol vapour alone as a commercial treatment to reduce leaf blackening is not viable. Ethanol vapour at 3g.kg⁻¹ fresh weight reduced the incidence of leaf blackening in *Protea magnifica* upon arrival in comparison to controls, but percent leaf blackening was still unacceptably high. The initial control of leaf blackening observed upon arrival for ethanol-treated *Protea* cv. *Sylvia* stems dissipated early during vase life, offering no real long-term benefit, but with the risk of introducing phytotoxicity.

A treatment combination that included girdling, glucose and ethanol pulsing in 'Sylvia' stems provided the best results upon arrival, but could not prevent the onset of severe leaf blackening by day 3 of vase life, rendering the stems unmarketable.

1. Introduction

Blackening of leaves is considered a major postharvest disorder in several *Protea* species as it has a negative effect on product quality and is the most common cause of *Protea* consignment rejection.

Leaf blackening is particularly important in species such as *Protea magnifica*, *P. eximia*, *P. compacta* and *P. neriifolia*, and cultivars derived from affected species, such as 'Lady Di', 'Sylvia', 'Cardinal', and 'Pink Ice' (Van Doorn, 2001). The selection of *Protea* cultivars such as 'Sylvia' (*P. eximia* × *P. susannae*) for its year round flowering habit has resulted in significant plantings being made in South Africa, with the intention to maximise production within the premium European marketing window of September to January. However, production at this time has been complicated by an increased disposition to leaf blackening, which negatively impacts profit margins.

Leaf blackening is defined as regions of dark brown to black discolorations appearing in various regions of the *Protea* leaf. Leaf blackening may occur within several hours of harvest, although more commonly it will present within two to five days postharvest.

Although water stress was previously linked as a main cause of leaf blackening, stronger evidence ascribes this disorder to a lack of carbohydrates in the leaves. The leaves apparently provide a source of carbohydrates to the flower head, as was shown in experiments on girdling or when using darkness as opposed to additional light. The actual biochemical mechanism of leaf blackening remains unclear. It is hypothesised that liberation of sugar components for the use by the flower head results in highly reactive free phenol which is oxidized non-enzymatically.

Control of leaf blackening in *Protea* has mainly been offered by means of pulsing stems with carbohydrates. However, although sugar pulsing solutions, in particular glucose have been beneficial in suppressing leaf blackening (Stephens et al., 2001; Stephens et al. 2005), the accurate application of this technology is problematic. Vase life correlated well with girdling and maintenance of low-temperature storage (0°C). Girdling was therefore recommended by Stephens et al., 2000 to prolong vase life of 'Sylvia' commercially. Again, the large-scale application of girdling is labour intensive and not practical for most producers. Alternative control measures such as ethanol vapourization of stems (Crick and McConchie, 1999; Cannon and McConchie, 2001) thus warrants further investigation.

The aim of the project is to gain a more in-depth understanding of factors that may contribute to the development of leaf blackening in *Protea* and to develop post-harvest technologies that may offer additional control of leaf blackening, especially under conditions of long term storage and transport. This objective of this study is to determine the efficacy of glucose pulsing, girdling and ethanol fumigation as well as a combination of these treatments to control leaf blackening in *Protea magnifica* and *Protea* cv. 'Sylvia' under commercial long term cold storage and transport conditions.

2. Materials and methods

Protea magnifica

Plant material: *Protea magnifica* stems were obtained from Willowgreen farm, South Africa as stock nrs. 418 and 421, representing two picking dates in week 39 of 2009, with stock nr. 418 harvested approximately 5 days prior to stock nr. 421.

Postharvest treatment: After harvesting stems were graded, cooled and transported to Bergflora Exporters, Cape Town International Airport, where stems were either left untreated (control) or treated with ethanol (30ml of 95% ethanol per 10kg fresh weight). Each treatment and picking date was replicated twice to account for the variation expected in *P. magnifica* stems derived from seedling populations.

Protea* cv. *Sylvia

Plant material: *Protea* cv. *Sylvia* stems were harvested at the commercial soft-tip stage on Arnelia Farm, South Africa on two harvesting dates, 28 September and 5 October 2009.

At each harvest date stems were transported to the packing shed where completely randomized selected stems received one of various treatments, either within 30 minutes from harvesting and/or on the following day (29 September or 6 October 2009 respectively), after cooling and transportation to Bergflora Exporters (Table 1).

Temperature ($\pm 1^{\circ}\text{C}$) and relative humidity (98 – 100%) within all boxes during transportation was monitored by means of LogTag data loggers (LogTag Recorders Ltd., Auckland, New Zealand) which were inserted with the stems at packing at Arnelia, Hopefield for 'Sylvia' or at Bergflora Exporters for *Protea magnifica*.

Storage and export: All stems were cold-stored (4°C) at Bergflora until shipment to Aalsmeer. Stems of 'Sylvia' harvested on 28 September and all *Protea magnifica* stems from stock 418 and 421 were transported by the Santa Carolina (CRLU 3604577, X118232; 20 foot, palette nr. 10, week 40) to the Netherlands. The consignment arrived in Rotterdam, Netherlands on 21 October and were received, unpacked and assessed at Oudendijk Imports, Aalsmeer, on 22 October 2009.

Table 1. Various postharvest treatments used on Protea cv. Sylvia stems to evaluate the efficacy of these treatments to control the development of leaf blackening.

Treatment		Girdling	Glucose Pulsing	Ethanol	Comment
1	Control (no treatment)	None	None	None	Stem were placed in water, then graded and packed; S14 box size
2	Glucose only	None	10ml 5.7% glucose/stem within 30 minutes from harvest	None	Stems were graded packed immediately after pulsing; S14 box size
3	Ethanol only	None	None	30ml of 95% ethanol per 10kg fresh weight	Untreated stems were exposed to ethanol on 29 Sept., following cooling and transport; S30 box size
4	Glucose & Ethanol	None	10ml 5.7% glucose/stem within 30 minutes from harvest	30ml of 95% ethanol per 10kg fresh weight	Glucose pulsed stems were exposed to ethanol on 29 Sept., following cooling and transport; S30 box size
5	Girdle, glucose & ethanol	Girdling immediately prior to pulsing	10ml 5.7% glucose/stem within 30 minutes from harvest	30ml of 95% ethanol per 10kg fresh weight	Girdled and glucose pulsed stems were exposed to ethanol on 29 Sept., following cooling and transport; S30 box size

Quality Assessments: The assessment of stems entailed the following: stems were categorized as either below (<), equal and above (\geq) 10% leaf blackening of the entire stem, where after all blackened (\geq 10%) leaves were removed by hand and counted collectively per box of stems per treatment. A vase life test of 7 days was then conducted for 'Sylvia' on randomly selected 10 stems per treatment where the number of blackened leaves (\geq 10%) was recorded on day 1, 3, 5 and 7 of vase life.

Flower scoring index: Where flowers were scored, the index was given as set out by the Flower quality scores determined upon the following criteria (Floral solutions, 2006):

-
- 1 Very poor quality, very big quality problems, all consumers will discard the flowers
 - 2 Poor quality, big quality problems, most consumers will discard the flowers
 - 3 Reasonable quality, moderate quality problems, most consumers will not yet discard flowers
 - 4 Good quality, small quality problems, all consumers will not discard the flowers
 - 5 Very good quality, no quality problems
-

3. Results and discussion

Protea magnifica:

First consignment on 'Santa Carolina' harvested in week 39-40 of 2009: On the first assessment opportunity, immediate after arrival and unpacking, 'Magnifica' stems treated with ethanol showed lower leaf blackening incidences than observed in the control. This was more noticeable in stock 418 that were approximately 5 days longer in storage than stock 421, as more stems in stock 418 had less than 10% leaf blackening (Fig. 1). When the total number of leaves were compared that was removed per treatment per box, less leaves were removed in stock 421 with the ethanol treatment (Fig. 2).

When the total number of blackened leaves that were removed per stem was counted, less leaves were removed with the ethanol treatment, except for in stock 418 (Fig. 3), where the group with more than 10% blackened leaves had a significantly higher number of blackened leaves removed. These damaged leaves then lead to more leaves in total being removed from the ethanol treatment in stock 418 compared to the control (Fig. 4). Figures 5-7 provide photographic evidence of the development of leaf blackening in stock 418 and 421 'Magnifica' stems, as well as other quality defects noted (Fig. 8).

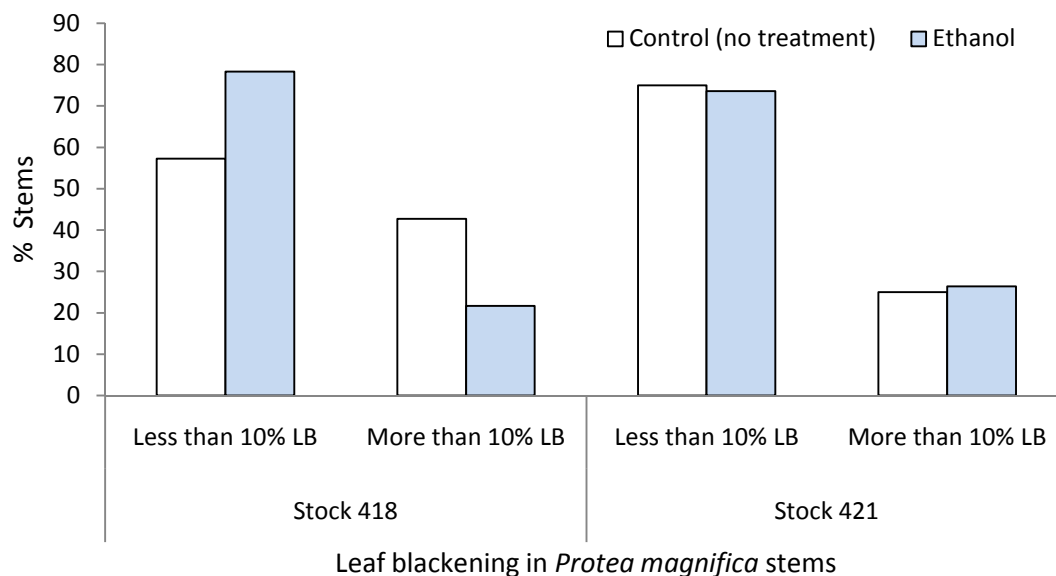


Fig. 1. Percentage stems of *Protea magnifica* from stock 418 and 421 harvested during week 39 of 2009, showing either <10% leaf blackening (LB) or \geq 10% leaf blackening (LB) when untreated (control) or subjected to a postharvest ethanol treatment at 3g ethanol. kg⁻¹ fresh weight.

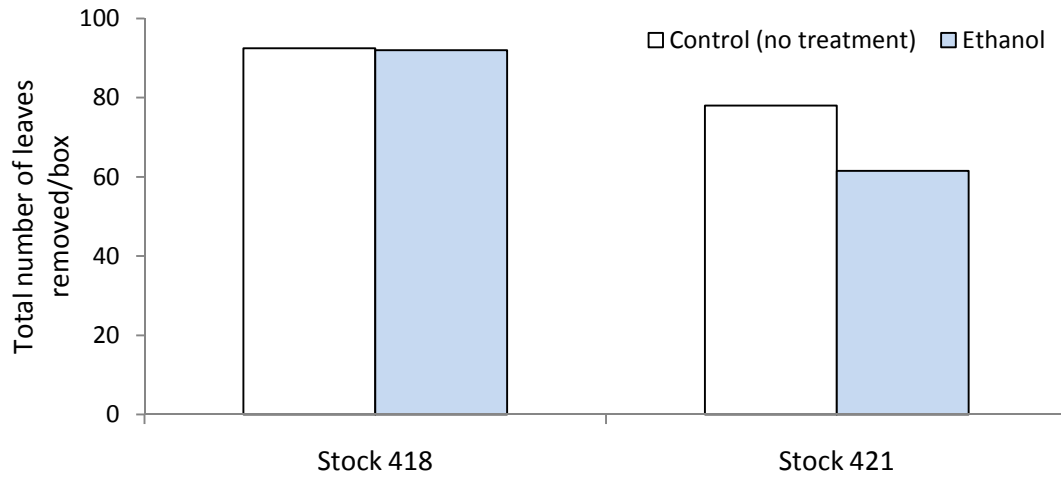


Fig. 2. Total number of leaves removed per box of *Protea magnifica* from stock 418 and 421 harvested during week 39 of 2009, when untreated (control) or subjected to a postharvest ethanol treatment at 3g ethanol. kg⁻¹ fresh weight.

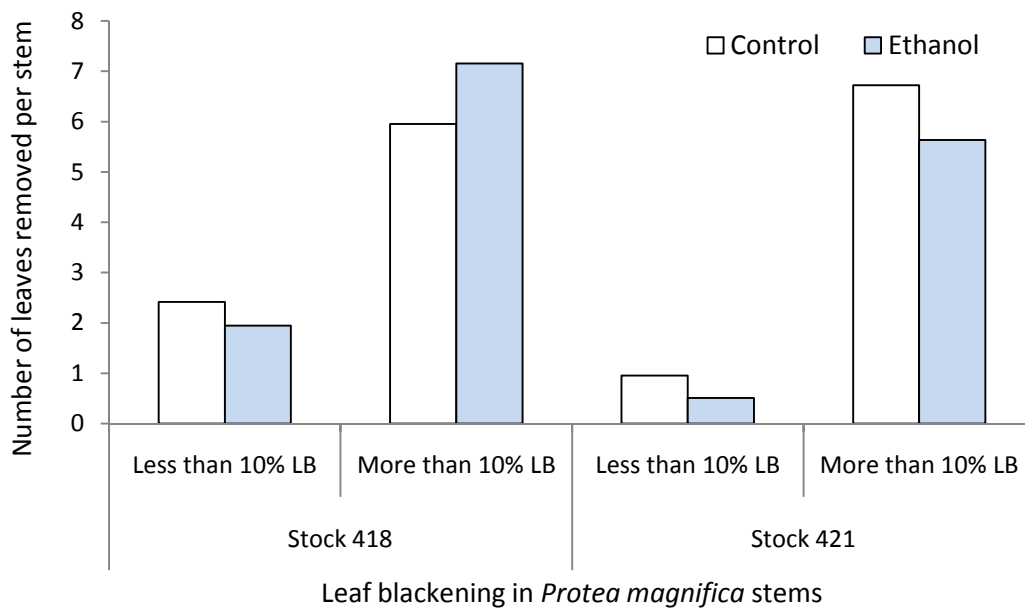


Fig. 3. Number of leaves removed per stem per leaf blackening category of *Protea magnifica* from stock 418 and 421 harvested during week 39 of 2009, when untreated (control) or subjected to a postharvest ethanol treatment of 3g ethanol. kg⁻¹ fresh weight.

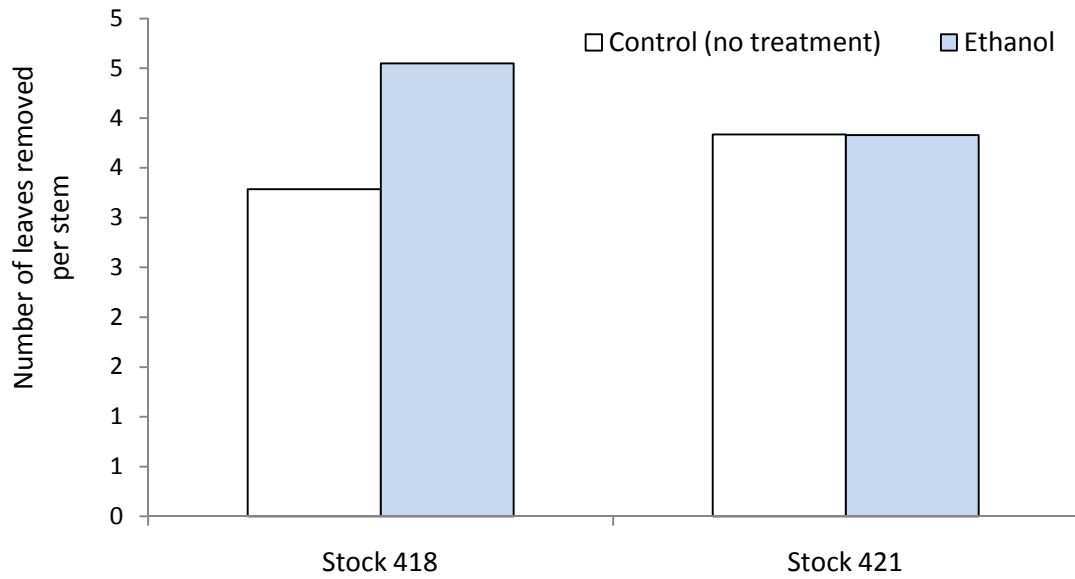


Fig. 4. Number of leaves removed per stem of *Protea magnifica* from stock 418 and 421 harvest in week 39 of 2009, when untreated or subjected to a postharvest ethanol treatment of 3g ethanol. kg⁻¹ fresh weight.

Protea magnifica stock 418

A. Control (no treatment)



B. Ethanol treatment



Protea magnifica stock 421

A. Control (no treatment)



B. Ethanol treatment



Fig. 5. Photographic evidence of *Protea magnifica* stem quality, stock 418 and 421 harvested in week 39 of 2009, representing two picking dates 5 days apart, at unpacking, after a postharvest ethanol treatment of 3g.kg⁻¹ fresh weight and 3 weeks cold storage at ±1°C and ±98% RH.

Protea magnifica stock 418

Control (no treatment)

Selection of stems with <10% leaf blackening



Ethanol treatment

Selection of stems with <10% leaf blackening



Control (no treatment)

Selection of stems with $\geq 10\%$ leaf blackening



Ethanol treatment

Selection of stems with $\geq 10\%$ leaf blackening



Fig. 6. Photographic evidence of *Protea magnifica* stem quality (stock 418) showing a selection of stems with less than (<) 10% leaf blackening (top) as well as with more than (\geq) 10% leaf blackening (bottom), after postharvest ethanol treatment at $3\text{g}\cdot\text{kg}^{-1}$ fresh weight and 3 weeks cold storage at $\pm 1^\circ\text{C}$ and $\pm 98\%$ RH.

Protea magnifica stock 421

Control (no treatment)

Selection of stems with <10% leaf blackening



Ethanol treatment

Selection of stems with <10% leaf blackening



Control (no treatment)

Selection of stems with \geq 10% leaf blackening



Ethanol treatment

Selection of stems with \geq 10% leaf blackening



Fig. 7. Photographic evidence of *Protea magnifica* stem quality (stock 421) showing a selection of stems with less than (<) 10% leaf blackening (top) as well as with equal or more (\geq) than 10% leaf blackening (bottom), after postharvest ethanol treatment at 3g.kg⁻¹ fresh weight and 3 weeks cold storage at $\pm 1^{\circ}\text{C}$ and $\pm 98\%$ RH.

P. magnifica stem showing fungal infections on the leaf and inflorescence base



P. magnifica inflorescence showing darkening of the tips of the lower involucral bracts ('dirty finger nail syndrome')



P. magnifica stem showing leaf blackening developing from the mid-rib of the leaf



Selected stems of *P. magnifica* exhibiting alternative types of leaves not showing any incidence of leaf blackening



Fig. 8. *Protea magnifica* stems showing various aspects of defects in quality (top and bottom left) as well as a selection of stems with leaves not prone to leaf blackening (bottom, right).

***Protea magnifica*:**

Second consignment on 'Mol Cullinan' that was harvested in week 41 of 2009: Data obtained from 'Magnifica stems' that was harvested during week 41 and assessed on arrival (5 November) at Oudendijk Import support data obtained from stems harvested in week 39 – 40 (Fig. 1), where less stems had blackened leaves in the ethanol treated stems, and more stems were considered saleable when treated with ethanol (Fig. 9). The beneficial effect of ethanol may be more apparent in stems that were stored for a 3-week period (second consignment, Fig. 9) than for stems that were stored for a shorter period (stock 421, Fig. 1).

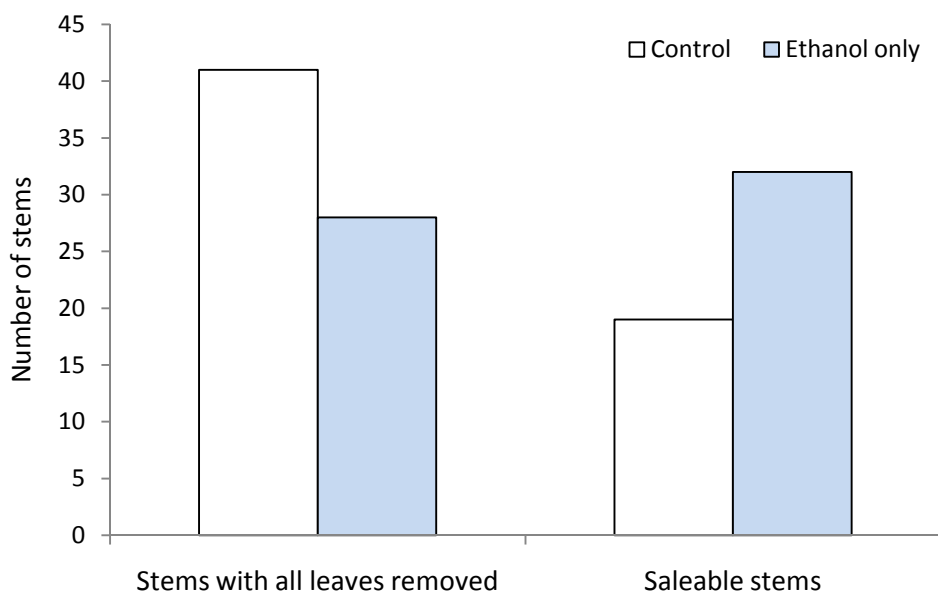


Fig. 9. Number of *Protea magnifica* stems (harvested week 41) that were considered saleable or with all leaves removed due to leaf blackening on arrival (5 November 2009, day 1 assessment) at Oudendijk Imports. Stem were either treated postharvest with ethanol at 3g.kg⁻¹ fresh weight prior to shipping or were left untreated (control).

Protea magnifica (Control-no treatment)



Protea magnifica (Ethanol treatment)



Fig. 10. Photographic evidence of 2 boxes of *Protea magnifica* stems harvested in week 41, where after the stems were either left untreated (control) or treated with ethanol postharvest at 3g.kg⁻¹ fresh weight, before shipping at $\pm 1^{\circ}\text{C}$ and $\pm 98\% \text{RH}$.

Protea cv. Sylvia:

First consignment on 'Santa Carolina' (harvested week 40 of 2009): In *Protea cv. Sylvia*, stems treated with glucose, glucose and ethanol, and stems that were girdled, and then treated with glucose and ethanol consecutively showed improved control of leaf blackening compared to the untreated control. Stems treated with ethanol only showed significantly more stems with $\geq 10\%$ leaf blackening than in any of the other treatments or control (Fig. 11).

When considering the number of leaves removed per stem per leaf blackening category (Fig. 12) or the total number of leaves removed per stem (Fig. 13), the ethanol and control treatment had the most leaves removed. Within the glucose treatment group, the girdle-, glucose- and ethanol treatment had the lowest number of leaves removed per stem, whereas more leaves were removed in the glucose-only treatment, which was followed by the combined glucose and ethanol treatment. On day 1 of the vase life assessment, more leaves were removed from the control than from any other treatment (Fig. 14, 15). Thereafter, however, the ethanol treatment had significantly more leaves removed compared to any of the glucose treatments (glucose only; glucose and ethanol; girdling, glucose and ethanol). On day 4 and 7, unacceptable high numbers of leaves with $\geq 10\%$ leaf blackening were removed from both the control and ethanol treated stems, whereas in stems where the treatment included glucose, leaf blackening was controlled at an acceptable level.

Fig. 16 and Fig. 17 provide photographic evidence of the 'Sylvia' stem quality on arrival at Oudendijk Imports and on day 4 of vase life assessment respectively. Fig. 18, 19 and 20 show 'Sylvia' stems for the different postharvest treatments on day 7 of vase life assessment.

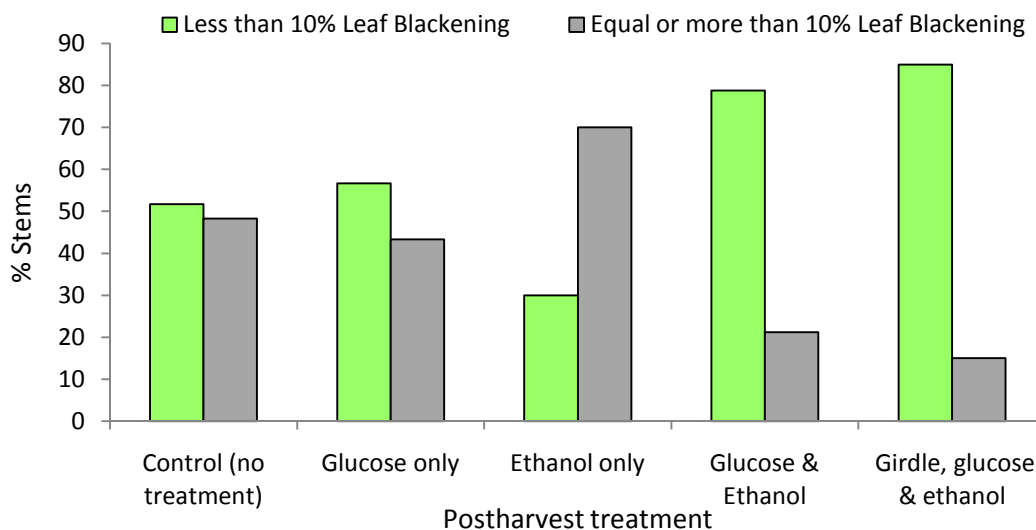


Fig. 11. Percentage stems of *Protea cv. Sylvia* showing either $< 10\%$ leaf blackening or $\geq 10\%$ leaf blackening when subjected to various postharvest treatments prior to shipping at $\pm 1^\circ\text{C}$ and $\pm 98\%$ RH for 3 weeks.

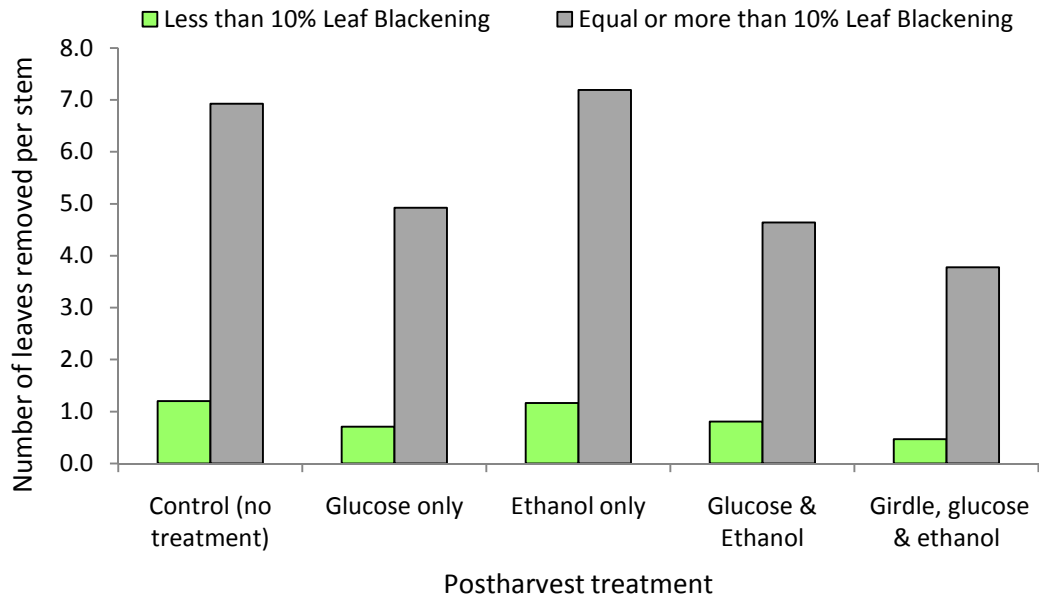


Fig. 12. The number of leaves removed per stem per leaf blackening category of *Protea* cv. *Sylvia* showing either < 10% leaf blackening or $\geq 10\%$ leaf blackening when subjected to various postharvest treatments prior to shipping at $\pm 1^\circ\text{C}$ and $\pm 98\%$ RH for 3 weeks.

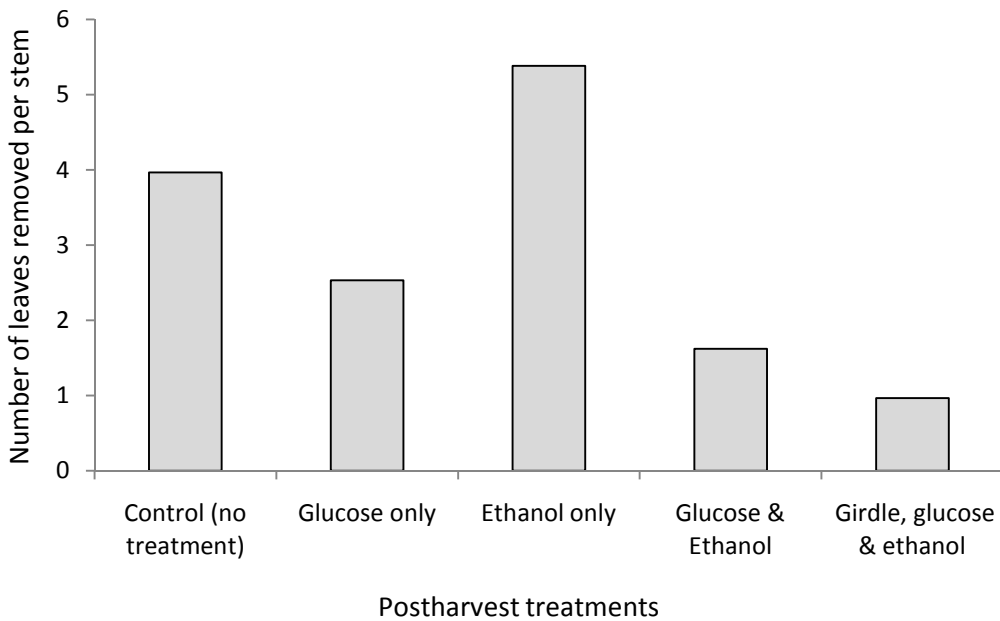


Fig. 13. The number of leaves showing $\geq 10\%$ leaf blackening removed per stem of *Protea* cv. *Sylvia* when subjected to various postharvest treatments prior to shipping at $\pm 1^\circ\text{C}$ and $\pm 98\%$ RH for 3 weeks.

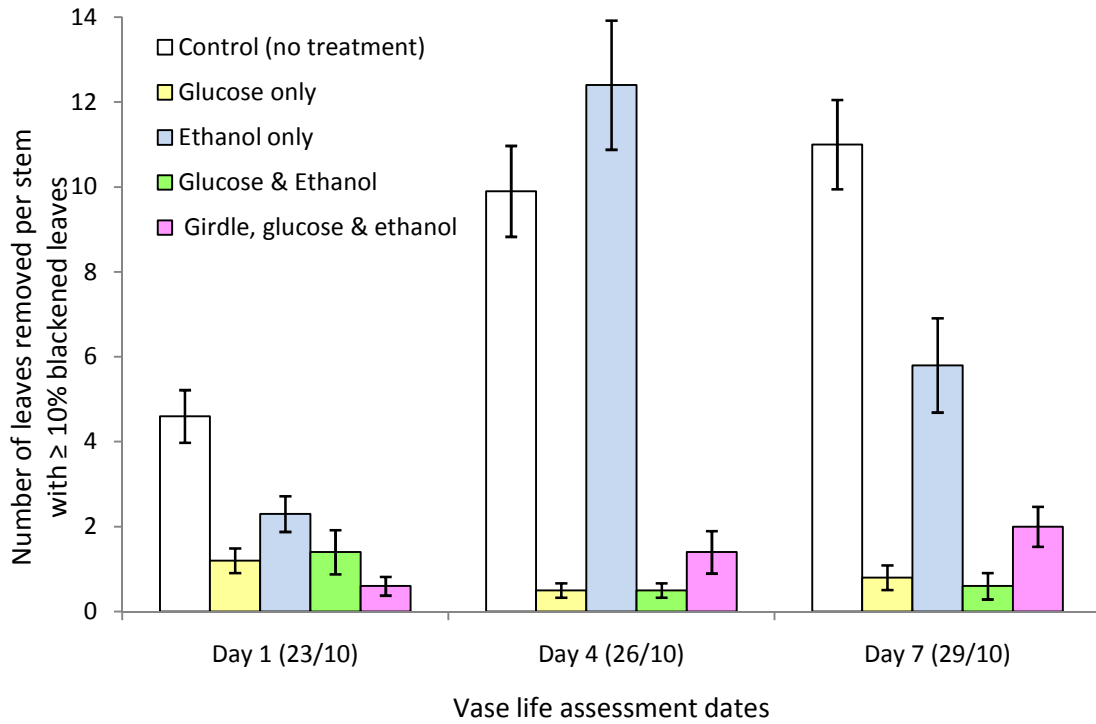


Fig. 14. Vase life assessment of *Protea* cv. *Sylvia* over a 7-day evaluation period after being subjected to various postharvest treatments prior to a cold-storage period of 3 weeks at $\pm 1^\circ\text{C}$ and $\pm 98\%$ RH.

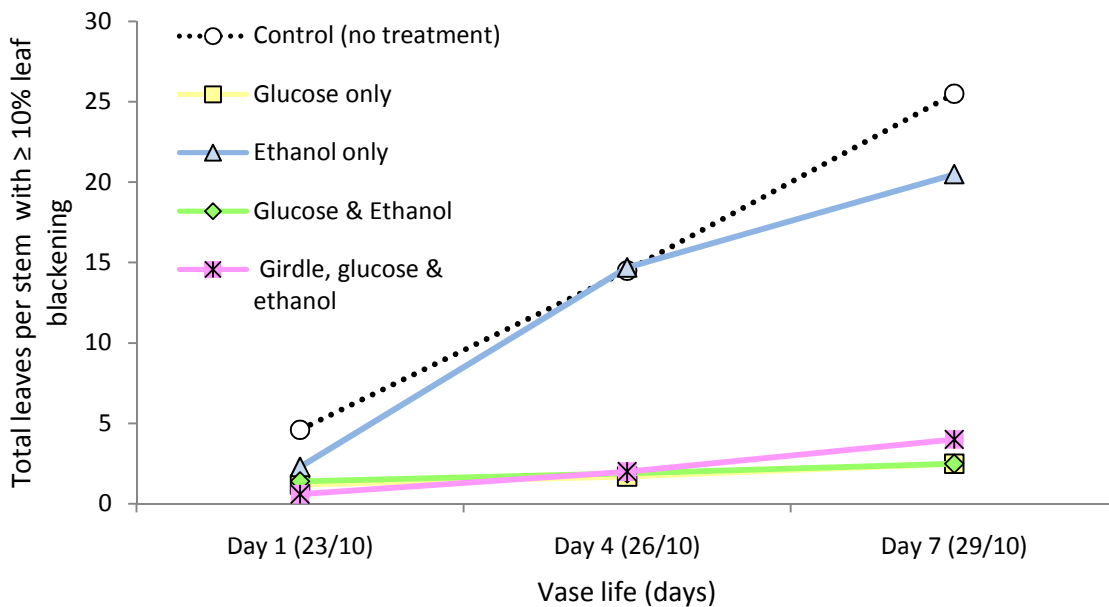


Fig. 15. Accumulative number of leaves with $\geq 10\%$ leaf blackening removed over a 7-day period during the vase life assessment of *Protea* cv. *Sylvia* after being subjected to various postharvest treatments prior to a cold-storage period of 3 weeks at $\pm 1^\circ\text{C}$ and $\pm 98\%$ RH.

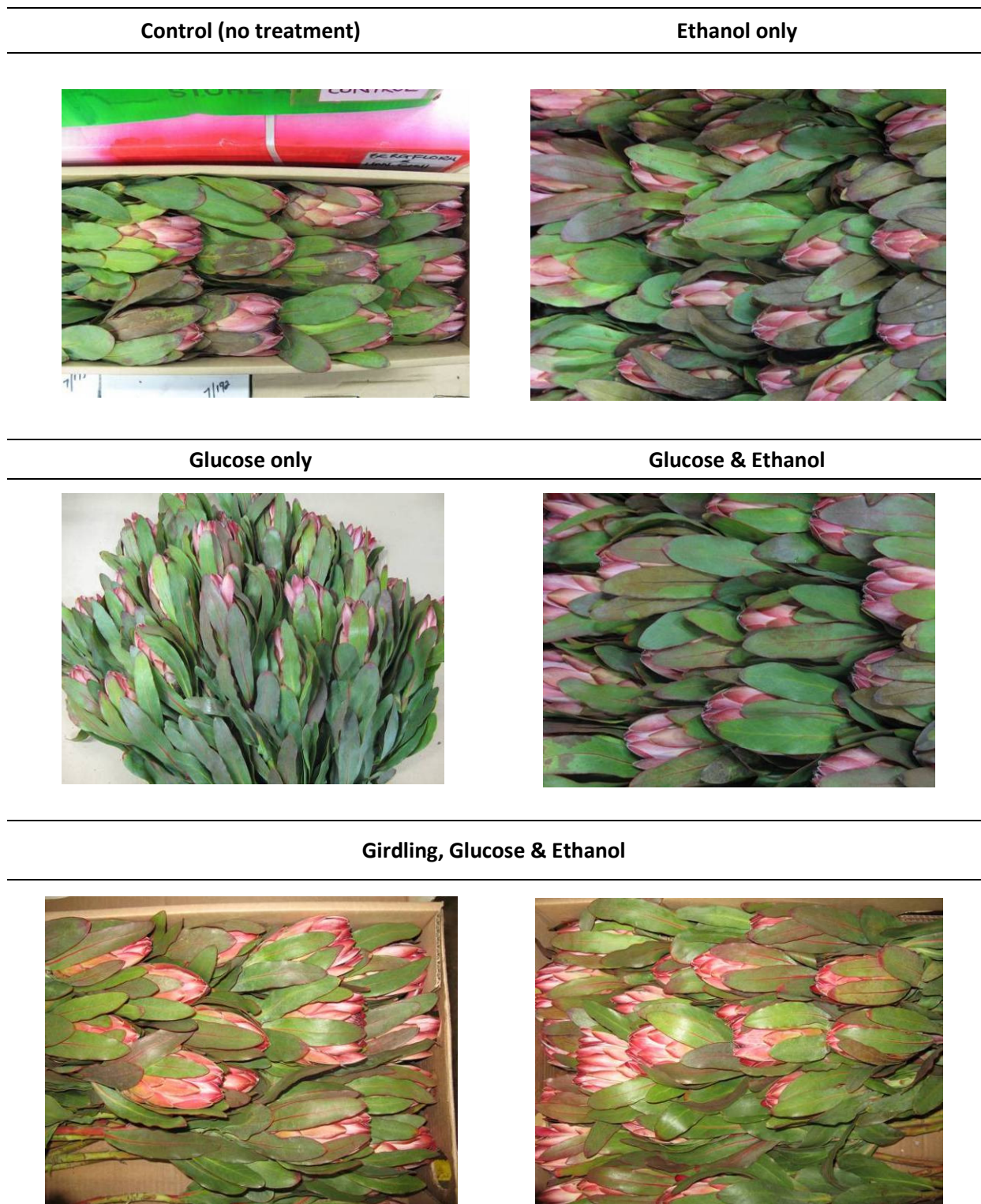


Fig. 16. Photographic evidence of *Protea* cv. Sylvia stems on arrival at Oudendijk Imports, the Netherlands. Stems were harvested in week 40 of 2009, where after the stems were subjected to various postharvest treatments and a cold-storage period of 3 weeks at $\pm 1^{\circ}\text{C}$ and $\pm 98\%$ RH.

***Protea cv. Sylvia* (control) on day 4 of assessment**



***Protea cv. Sylvia* treated with glucose and ethanol on day 4 of assessment**



Fig. 17. Photographic evidence of *Protea cv. Sylvia* stems on day 4 of vase life assessment at Oudendjik Imports, the Netherlands. Stems were harvested in week 40 of 2009, where after the stems were subjected to various postharvest treatments, of which the control (no treatment) and the combined ethanol ($3\text{g}\cdot\text{kg}^{-1}$ fresh weight) and 5.7% glucose treatment are presented above.

Protea cv. Sylvia vase life assessment day 7

Control 'Sylvia' stems that received no postharvest treatment



'Sylvia' stems treated with glucose, showing significant control of leaf blackening (left) and high quality flowers opening (right)

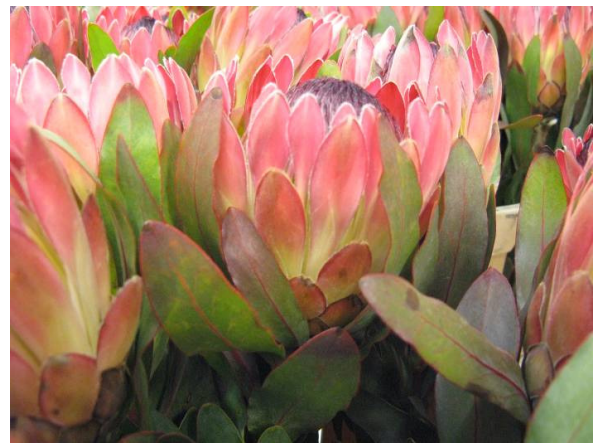


Fig. 18. Photographic evidence of *Protea cv. Sylvia* stems on day 7 of vase life assessment at Oudendijk Imports, the Netherlands. Stems were harvested in week 40 of 2009, where after the stems were subjected to various postharvest treatments, of which the control (no treatment) and the 5.7% glucose treatment are presented above.

'Sylvia' stems treated with ethanol, showing advanced leaf blackening (left) and dehydration of the flowers (right)



'Sylvia' stems treated with a combination of glucose and ethanol



Fig. 19. Photographic evidence of *Protea* cv. Sylvia stems on day 7 of vase life assessment at Oudendijk Imports, the Netherlands. Stems were harvested in week 40 of 2009, where after the stems were subjected to various postharvest treatments, of which the ethanol ($3\text{g}\cdot\text{kg}^{-1}$ fresh weight) and the combined ethanol and 5.7% glucose treatment are presented above.

'Sylvia' stems treated with a girdling, glucose and ethanol



Fig. 20. Photographic evidence of *Protea* cv. Sylvia stems on day 7 of vase life assessment at Oudendijk Imports, the Netherlands. Stems were harvested in week 40 of 2009, where after the stems were subjected to various postharvest treatments, of which the combined girdling, ethanol ($3\text{g}\cdot\text{kg}^{-1}$ fresh weight) and 5.7% glucose treatment are presented above.

***Protea* cv. Sylvia**

Second consignment on 'Mol Cullinan' (harvested in week 41 of 2009): For 'Sylvia' in the second consignment, the stems that received a combination of girdling, glucose and ethanol had the most saleable stems, followed by the glucose treatment (Fig. 21). Stems that received no treatment (control) had the highest rejection rate (all leaves removed) due to leaf blackening, followed by the stems treated with either ethanol alone or in combination with glucose (Fig. 21). However, the benefit that any of the postharvest treatment may have had up onto arrival, had dissipated by day 3 of vase life when all stems, irrespective of treatment, rated a score 1 ("bad, consumer would consider discarding the product") on the scoring index.

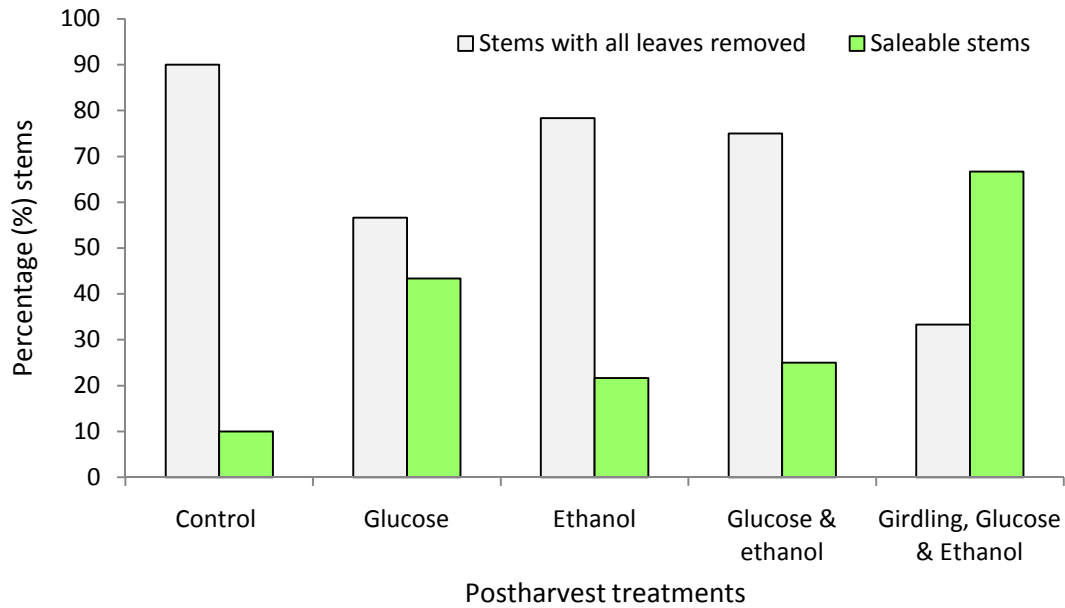


Fig. 21. Percentage of *Protea* cv. *Sylvia* stems that were considered saleable or with all leaves removed due to leaf blackening on arrival (5 November 2009, day 1 assessment) at Oudendijk Imports, the Netherlands. Stems were harvested in week 41 of 2009, where after it was subjected to various postharvest treatments and a cold-storage period of 3 weeks at $\pm 1^{\circ}\text{C}$ and $\pm 98\%$ RH.

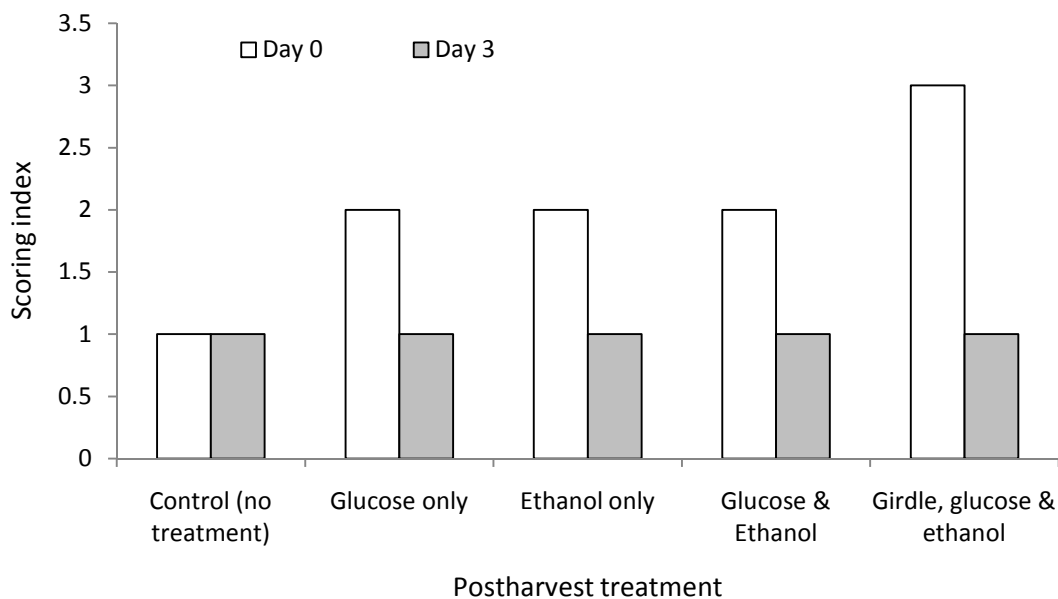


Fig. 22. Scoring index (0=very bad, consumer discarded product already; 1=bad, consumer would consider discarding the product; 2=average, product is saleable; 3= sufficient, product certainly saleable; 4=good, just one small quality remark; 5= very good, no quality remarks on stems) of *Protea* cv. *Sylvia* on day 0 (at arrival) and day 3 of vase life assessment. Stems were harvested in week 41 of 2009, where after it was subjected to various postharvest treatments and a cold-storage period of 3 weeks at $\pm 1^{\circ}\text{C}$ and $\pm 98\%$ RH.

Protea cv. *Sylvia* assessment on arrival

Control 'Sylvia' stems that received no postharvest treatment



'Sylvia' stems treated with glucose



Fig. 23. Photographic evidence of *Protea* cv. *Sylvia* stems on arrival at Oudendijk Imports, the Netherlands. Stems were harvested in week 41 of 2009, where after the stems were subjected to various postharvest treatments, of which the control (no treatment) and the 5.7% glucose treatment are presented above.

'Sylvia' stems treated with ethanol



'Sylvia' stems treated with a combination of glucose and ethanol



Fig. 24. Photographic evidence of *Protea* cv. Sylvia stems on arrival at Oudendijk Imports, the Netherlands. Stems were harvested in week 41 of 2009, where after the stems were subjected to various postharvest treatments, of which the ethanol ($3\text{g}\cdot\text{kg}^{-1}$ fresh weight) and the combined ethanol and 5.7% glucose treatment are presented above.

'Sylvia' stems treated with girdling, glucose and ethanol



Fig. 25. Photographic evidence of *Protea* cv. *Sylvia* stems on arrival at Oudendijk Imports, the Netherlands. Stems were harvested in week 41 of 2009, where after the stems were subjected to various postharvest treatments, of which the combined girdling, ethanol (3g.kg^{-1} fresh weight) and 5.7% glucose treatment are presented above.

4. References:

- Cannon, A., McConchie, R., 2001. Controlling leaf blackening in *Protea*. A report for the Rural Industries Research and Development Corporation, RIRDC publication No. 01/098, Project No. US-89A, Australia.
- Crick, S.G., McConchie, R., 1999. Ethanol vapour reduces leaf blackening in cut flower *Protea* 'Pink Ice' stems. *Postharvest Biol. and Technol.* 17, 227-231.
- Jones, R.B., McConchie, R., Van Doorn, W.G., Reid, M.S., 1995. Leaf blackening in cut *Protea* flowers. *Hortic. Rev.* 17, 173 – 201.
- Stephens, I.A., Holcroft, D.M., Jacobs, G., 2000. Low temperatures and girdling extend vase life of 'Sylvia' proteas. *Acta Hortic.* 545: 205-214.
- Stephens, I.A., Holcroft, D.M., Jacobs, G., 2001. Glucose prevents leaf blackening in 'Sylvia' proteas. *Postharvest Biol. Technol.* 23: 237-240.
- Stephens, I.A., Meyer, C., Holcroft, D.M., Jacobs, G., 2005. Carbohydrates and postharvest leaf blackening of Proteas. *J. Hortic. Sci.* 40(1), 181 – 184.
- Van Doorn, W.G. 2001. Leaf blackening: Recent developments. *Acta Hortic.* 545: 197-204.