COLOUR DYNAMICS IN *LEUCADENDRON*

BY

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DECLARATION

By submitting this thesis/dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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This thesis is entirely dedicated to my wife Ute Schmeisser, for her continued love and support, despite me having been a miserable sod at times while battling through this thesis. You went above and beyond the call of duty and for that I will forever be indebted.

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Summary

(Limited to 500 words)

The bright colouration of involucral leaves in *Leucadendron* is unfortunately transient in nature. Undesirable colour changes render this cut flower unmarketable, resulting in a considerable loss of profit. A deeper understanding of the mechanism leading to colour change is needed to form the framework on which future manipulation strategies can be built.

Yellow *Leucadendron* possess the ability to degreen and regreen naturally, a phenomenon linked to the controlled degradation of chlorophyll and the lesser degradation of carotenoids, which then impart the yellow colour. This colour change is directly linked to the development of the inflorescence. Involucral leaves degreen towards anthesis and are entirely yellow at full bloom. They begin to regreen again when the last florets on the cone have wilted. Deconing before flowering completely inhibits the colour change. Deconing at full bloom, results in leaves regreening sooner. Therefore the inflorescence appears to be the origin of the cue for colour change. Any factor that expedites the death of the florets, results in sooner regreening of involucral leaves. Ultra-structurally, the degreening and regreening resulted from a transdifferentiation of mature chloroplasts to gerontoplast-like plastids, which upon regreening completely redifferentiated into fully functional chloroplasts.

In the red *Leucadendron* cultivar Safari Sunset, the photosynthetic pigment degradation pattern is identical to that of yellow cultivars. However, colour expression is complexed by the presence of anthocyanins. Anthocyanin concentration was shown to be directly related to the opening of the flower head rather than to the phenological development of the inflorescence. With opening, the previously shaded inner involucral leaf surfaces are exposed to higher levels of irradiance and respond by turning red, presumably for photoprotection. Similar to yellow cultivars, any factor leading to the death of the florets before flowering, not only prevents the degreening of involucral leaves, but also prevents the opening of the flower head and therefore the associated change in anthocyanin levels.

The ecological significance of regreening was also investigated. What does a female *Leucadendron* plant stand to gain by regreening rather than discarding the involucral leaves? Regreened involucral leaves were shown not to play a significant role in providing photosynthates for the developing cone. Although the presence of regreened involucral leaves were shown to provide protection against high irradiance and radiant heating of the cone, they were not essential to ensure survival of the cone. The small floral bracts were shown to be very capable of adaptation. The most plausible reason for regreening is therefore assumed to be based on a cost-benefit relationship. As most *Leucadendron* are adapted to grow on very nutrient poor soils, the question should maybe be rephrased. Why waste valuable resources? Sclerophyllous leaves, like the involucral leaves, are costly to make and therefore reusing, rather than discarding them does seem a sensible strategy for survival.

Opsomming

(Beperk tot 500 woorde)

Leucadendron snyblomme word gekenmerk deur die helder kleure van hul omwindselblare. Die helder kleure is egter slegs vir ‘n kort periode aanwesig waarna die snyblomme onbemarkbaar word, met aansienlike verlies aan potensiële inkomste. Die ontwikkeling van manipulasies ten einde die bemarkbare periode van *Leucadendron* te verleng, berus op die verkryging van ’n dieper insig in die meganisme van kleurverandering.

Die kleurveranderinge van geel *Leucadendron* omwindselblare is te wyte aan ’n unieke vermoë tot die gereguleerde degradasie én heropbou van chlorofiele en karotenoïede onder direkte beheer van die ontwikkelende bloeiwyse. Met die aanvang van blom, lei groter proporsionele degradasie van chlorofiele tot geleidelike vergeling van omwindselblare. Die hele blomhofie verkry uiteindelik met volblom ‘n helder geel kleur. Sodra die laaste blommetjies doodgaan, neem chlorofiel- en karotenoïedsintese weer in aanvang en binnekort is die omwindselblare weer net so groen soos voor die aanvang van blom. Die geel verkleuring kan verhoed word deur die keël voor blom uit te breek. Enige faktor wat die dood van die blommetjies versnel, asook die uitbreek van keël tydens volblom, lei tot die vroeëre aanvang van vergroening. Die degradasie van plastiedpigmente hang nou saam met die differensiasie van volwasse chloroplaste tot gerontoplast-agtige plastiede wat op hul beurt weer tydens vergroening tot volkome funksionele chloroplaste herdifferensieer.

Soortgelyk aan geel *Leucadendron* kultivars, vind die veranderinge in plastiedpigmente ook plaas tydens blom van die rooi kultivar, Safari Sunset. Kleurveranderinge in ‘Safari Sunset’ is egter meer ingewikkeld vanweë die aanwesigheid van variërende konsentrasies antosianiene. Antosianienkonsentrasies en rooi kleur neem toe tydens blom vanweë die blootstelling van die beskutte adaksiale binnekante van omwindselblare aan hoë irradiasie met die oopvou van die blomhofie. Die akkumulasie van antosianiene het moontlik ’n fotobeskermende funksie. Kleurveranderinge in ‘Safari Sunset’ kan, soos in geel kultivars, voorkom word deur blom te verhoed.

Antosianiensintese word voorkom deurdat die blomhofie geslote bly en is nie direk gekoppel aan blom soos wat met plastiedpigmente die geval is nie.

Die belang van vergroening is ondersoek na aanleiding van die vraag oor wat dit ‘n vroulike *Leucadendron* baat om omwindselblare te behou na die afloop van blom? Die bydrae van foto-assimilasie deur omwindselblare tot die ontwikkeling van keëls is beperk. Alhoewel omwindselblare wel keëls teen hoë irradiasie en stralingsverhitting beskerm, is die blomskutblare in staat om aan te pas by hierdie kondisies. Die mees waarskynlike verklaring vir die behoud van die omwindselblare na blom berus moontlik op ‘n koste-voordele verwantskap. Alhoewel nie essensieel nie, is die beperkte bydrae van die omwindselblare na die afloop van blom tot die oorlewing en welstand van die keël waarskynlik genoegsaam om hul behoud te regverdig. Verskeie *Leucadendron* spesies groei in gronde wat baie arm is aan nutriënte. Sklerefiele blare, soos dié van *Leucadendron*, is vêrder duur om te vervaardig. Dit maak dus sin om hulle vir meer as een funksie te herontplooi eerder as om hulpbronne te belê in meer gespesialiseerde en minder durende blombykomstighede. Dus dui die behoud van omwindselblare dalk op ’n strategie wat gemik is op die behoud en besparing van beperkte hulpbronne.

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Overall Objective

Leucadendron are desired cut flowers on the international market due to their often brightly coloured flower heads and make up a considerable percentage of the total income (16%, SAPPEX report, 2008) generated by the export of all Fynbos products from South Africa. The bright colouration of the involucral leaves is unfortunately transient in nature. Undesirable colour changes in the involucral leaves render this product unmarketable, resulting in considerable loss of potential profit. Previous attempts to manipulate colour (unpublished data) have been unsuccessful and a deeper understanding of the mechanism leading to colour change is needed to build the framework on which future manipulation methods can be built. To achieve this goal, we studied the pigmentation patterns of yellow and red *Leucadendron* cultivars and attempted to correlate this with the phenological development of the inflorescence. This allowed us to gain a firsthand insight into the dynamics of colour expression in association with flowering, which had never been described before in *Leucadendron*. To pinpoint the origin of the cue for colour change, the inflorescences were removed before and during anthesis in both yellow and red cultivars. The effect of preventing the opening of the flower head on colour expression was also investigated. The results of these studies will be instrumental in devising commercial colour manipulating techniques and hopefully eliminate unsuccessful approaches.

The degreening and subsequent regreening of true leaves is rare in nature and appears to be a very novel process. Inherently little takes place in nature without a reason or function. Therefore the last section of this study aims to address why the female *Leucadendron* are committed to regreen their involucral leaves, irrespective of successful pollination. What does the plant stand to gain by regreening, rather than discarding these leaves? For me, the last section also highlights the pure scientific novelty of this phenomenon.

I had decided to do a short literature review on the remarkable phenomenon of regreening, but rather than regurgitating plain facts attempted to write an insightful synthesis on the regreening phenomena and the relationship to senescence, chlorophyll degradation and plastid differentiation. I

decided not to write a literature study on *Leucadendron* itself, as very recently a well-written, comprehensive review had already been published (Ben-Jaacov and Silber 2006).

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Literature Review: The regreening conundrum

1. Introduction

The yellowing of leaves and other green plant organs is considered as the tell tale sign for the occurrence of senescence (Gepstein 2004; Lim and Nam 2005), not including the yellowing caused by excessive abiotic or biotic stresses resulting in premature damage and death. As senescence is initiated, the first and most significant change at cellular level is the degradation of chlorophyll and concurrent dismantling of chloroplasts, symptomatically evident as yellowing (*et al.* 1997). Processes activated during leaf senescence, for example, are not just haphazardly run metabolic sequences, but sufficient evidence exists showing that strict genetic control underlies each process and death as a whole (Thomas and Stoddart 1980; Thomas 2002; Gepstein 2004; Lim and Nam 2005). The purpose of regulating leaf senescence is to allow for the safe breakdown of photo-dynamically active chlorophyll and therefore allow for the re-mobilisation of nutrients, such as phosphorus, potassium and nitrogen, out of the leaf to be used elsewhere in the plant (*et al.* 2002; Hörtensteiner 2006). Therefore, onset of senescence should not be viewed as solely consisting of deteriorative processes. On the contrary, it involves the activation and expression of a multiple of different genes and the *de novo* synthesis of many enzymes (Guo *et al.* 2004; Thomas *et al.* 2009).

Senescence of leaves and the associated chlorophyll degradation is a familiar occurrence that we are well aware of, as we see leaves changing into spectacular autumn colours each year. Similar degreening is observed during the seasonal ripening of many different fruit and although we talk of "fruit ripening", it is essentially a form of senescence. However, an unfamiliar phenomenon is the ability of apparently senescing plant organs, seemingly set on a path of self-destruction, to regreen naturally, which forms the focal point of this review. There are a handful of studies reporting on the natural regreening of various fruits, floral parts and non-floral accessories (Grönegress 1974; Sitte 1974; Tavares *et al.* 1998; Salopek-Sondi *et al.* 2000; Salopek-Sondi *et al.* 2002; Lino Neto *et al.* 2004; Prebeg *et al.* 2008). However, reports of the natural regreening of leaves (i.e., non-induced) seem to

be fairly rare and was rather recently thought to be non-existent (van Doorn and Woltering 2004). There are however a few studies reporting on the natural degreening and regreening of leaves (Chabot and Chabot 1975; Ikeda 1979; Koiwa *et al.* 1986). In these cases, the regreening process appears to be related to climatic changes occurring from winter to spring. The most recent addition to the literature on natural regreening of leaves, is that encountered in *Leucadendron*, where the regreening was shown to be directly linked to floral development (Schmeisser *et al.* 2010).

Time and again it is written in literature that yellowing of green plant tissues, especially the yellowing of leaves, is an external symptom of internal senescence processes (with senescence being defined as having death as its endpoint) (van Doorn and Woltering 2004). So does the ability of yellowing plant organs to regreen, especially leaves, throw a proverbial spanner into the definition of senescence?

This mini-review aims to challenge generally accepted ideas in light of the regreening phenomenon and aligning some of the concepts towards an understanding of the processes playing a role in regreening.

2. Death, Senescence & the conundrum of regreening

Death has many faces and although by definition always destructive in nature, it plays an integral part in the development of plants, as it can occur selectively on a cellular, tissue, or organ level (Gepstein 2004). It might at first appear paradoxical, referring to a process resulting in death as an “integral part of development”, when the word “development” conveys a notion of improvement rather than deterioration. However, although death is deteriorative in nature, it is often viewed as a critical process, especially on a cellular level, where it occurs according to a planned and meticulously designed programme with a defined purpose (Gepstein 2004). Consider the formation of xylem cells for example. The protoplasts of these cells die intentionally, with the defined purpose of eventually forming a conduit for the transport of water and solutes. It is this type of death, with intent and function, that is referred to when coining the term programmed cell death (PCD). The term PCD is

clearly differentiated from death as a result of excessive environmental stresses that suddenly overwhelm the plant's defence mechanism, causing a rapid, passive death, sometimes referred to as accidental death or 'murder' of cells. Although various complex definitions of PCD are given, it is best stated as a genetic programme whereby a cell actively kills itself (van Doorn and Woltering 2004).

Another, much older term used to describe the developmental processes leading to death, is senescence. In the strict sense, senescence means to grow old, but has in the biological field been defined as having death as its end point. A rather fundamental problem of definition arises in developmental biology in the use of the terms senescence and PCD. Yellowing of leaves is seen as being the symptomatic evidence of senescence occurring (which by definition is supposed to end in death) (Gan and Amasino 1997; Gepstein 2004), not including yellowing as a result of chlorosis, which is again an abiotic stress factor. However, the ability of some leaves (and other plant organs) to regreen and not end in death poses a problem with this definition. Based on the regreening ability of some plant organs, Thomas (2003) see senescence as being an ageing process that precedes PCD and when progressed past the point of no return, PCD is triggered and will result in death. The point of no return was defined as that point, when death can no longer be averted (i.e. the leaf would have lost its capacity for regreening) (Delorme *et al.* 2000; Thomas *et al.* 2003; van Doorn 2005; Thomas *et al.* 2009). Thomas has therefore defined senescence and PCD as being two separate processes, the one preceding the other and that they should not be seen as being synonymous. By this separation, the term senescence becomes redefined as being a process not necessarily ending in death, a view contested by van Doorn and Woltering (2004). Although he agrees with Thomas (2003), that yellowing leaves that are regreening are clearly not experiencing PCD, he strongly disagrees with idea of redefining the meaning of senescence. By separating these two terminologies (senescence and PCD) it implies that the older and much ingrained definition of senescence will have to be rejected. Furthermore, it has so far not been proven that all plant parts and cells (roots for example) are capable of averting death by reversal of the senescence symptoms. Therefore, by implication, cells that are not capable of reversal are not experiencing senescence, according to the definition of Thomas (2002), as they do not possess a point of no return.

Van Doorn and Woltering (2004) offer a beautiful solution to the conundrum caused by the ability of plants to regreen. A programme (such as senescence or PCD) does not need to be considered unidirectional, but rather as something that can be delayed and even reversed. Therefore in a leaf for example, if the signal for PCD has been perceived, the programme that would normally lead to death is started and the first symptoms would be the dismantling and degradation of chlorophyll (CHL), as mentioned by others (*et al.* 1997). Somewhere along the line an additional signal results in the reversal of the PCD programme, allowing regreening to occur. Therefore senescence and PCD may be seen as being synonymous (*et al.* 1997; van Doorn and Woltering 2004). Other fitting examples of regreening, to strengthen this view, is that encountered in the spathe of the arum and calla lily (*Zantedeschia aethiopica* and *Z. elliotiana*) (Grönegress 1974; Tavares *et al.* 1998), and the petals of a few orchid species (Tran *et al.* 1995; van Doorn 1997). In these cases, degreening of petals (or spathe) occurs with anthesis and only upon successful pollination does regreening occur. In the unpollinated counterparts, the petal or spathe dies and is discarded. Hence, due to a lack of signal (presumably from pollination) PCD was not reversed and allowed to run its full course.

However I pose another question. Is yellowing due to active CHL degradation always a PCD (senescence) run programme? This question is based on the very recent finding, where true leaves have shown the remarkable ability to degreen and regreen naturally in direct relation to flowering, thereby presumably aiding in pollination (Schmeisser *et al.* 2010). However, what makes this case different from other reports on regreening, is that irrespective of successful pollination, these leaves regreen and remain alive and functional for several years (at least for up to 2 years as has been observed personally). Even when the inflorescence was entirely removed, these leaves regreen, rather than abort. Therefore, is there a possibility of CHL degradation and subsequent yellowing occurring without necessarily running a senescence programme, which then needs to be reverted? Studies investigating the expression of senescence related genes of plants with the ability to regreen should shed more light on this question.

3. Chlorophyll metabolism – The unanswered question

The chlorophyll synthesis pathway is well understood and has been described in great detail since the early 1900's. The full understanding of the chlorophyll degradation pathway, however, has remained elusive for many years (Eckhardt *et al.* 2004), so far so, that CHL degradation even in the late 1980's had still been dubbed "a biological enigma" (Hendry *et al.* 1987). This is especially true for the elucidation of the multiple catabolic enzymes involved and how they are regulated. Only in the early 1990's were chlorophyll breakdown products identified in plant tissues (Matile *et al.* 1999; Hörtensteiner 2006; Harpaz-Saad *et al.* 2007), which allowed an insight into the chlorophyll degradation pathway. Recent studies further enhanced our understanding of CHL metabolism by identifying the majority of genes involved (Beale 2005). Both chlorophyll synthesis and degradation occurs throughout plant development. There is often a distinct seasonal pattern associated with chlorophyll metabolism, as seen by the greening of leaves in spring and degreening during leaf senescence in autumn. However, in both evergreens and deciduous plants a basal turnover of chlorophyll has been suggested, which may be influenced by environmental conditions such as light and temperature (Hörtensteiner 2006). The CHL metabolic pathway, enzymes involved and genes expressed have recently been reviewed by Eckhardt *et al.* (2004) and Hörtensteiner (2006) and the reader is referred to these excellent reviews on this topic.

In relation to regreening, there is really only one question regarding CHL metabolism. CHL turnover occurs in mature non-senescent tissues (even just considering leaves adapting to different light levels). After removal of the central magnesium ion of chlorophyll by magnesium chelatase (resulting in the formation of pheophorbide a), the enzyme pheophorbide a oxygenase results in the cleavage of the macrocycle, thereby producing a red coloured bilin, which is further degraded to colourless non-fluorescent chlorophyll catabolites that are sequestered in the vacuole of plant cells (Matile *et al.* 1999; Matile 2000; Eckhardt *et al.* 2004; Hörtensteiner 2006). However, according to literature, the enzyme pheophorbide a oxygenase is only expressed in senescent plant tissue (Hörtensteiner 2006). So how does basal turn-over take place in mature, green non-senescent tissues? According to Stefan

Hörtensteiner (personal communication, 2009) the simple answer is that no one knows by which mechanism CHL is turned over in green leaves. Their experiments trying to show an involvement of the pheophorbide a oxygenase pathway in CHL degradation in turnover has failed so far. The reason for posing this question, ties in with the question asked in the previous section, whether yellowing in regeening capable plants can result from alternative pathways and not necessarily be a PCD run programme. Therefore, determining the levels of pheophorbide a oxygenase in yellowing tissues of regreening capable plants, should be a suitable indicator if a PCD programme has been executed or not.

4. Plasticity of leaf Plastids

There are only a few different plastid types found in plants, their definitions sometimes ambiguous. This has been most adequately described by Pyke (1999), who states that:" plant plastids may best be described as a continuous spectrum of types and that a precise categorisation may not always be meaningful." Plastids differ among plant organs, and often within the same plant tissue depending on the specific function they perform therein, the developmental stage of the plant and environmental conditions they are exposed to. The chlorophyll containing chloroplasts are probably the most studied plant plastids, due to their central role in photosynthesis. None of the other plant plastids encountered, possess any form of photosynthetic capacity, as they lack chlorophyll.

Chromoplasts contain carotenoids and are responsible for the yellow, orange and red colour of many plant tissues, in particular that of ripening fruits, flower petals and even some roots, such as the carrot (Ljubesic *et al.* 1991). Chromoplasts, probably as a result of the *de novo* synthesis of carotenoids, have been divided into five ultra-structural categories based on the structure of carotenoid deposition e.g. globuleus, tubular etc. (Sitte 1974; Whatley 1978; Whatley and Whatley 1987). Leucoplasts are essentially colourless plastids and may be involved in lipid biosynthesis and storage (elaioplasts), protein metabolism (proteinoplasts) and are also known to partially be involved in the synthesis of some phytohormones. Another type of plant plastid, the gerontoplast, has been

defined as a plastid developing from old, mature chloroplasts as a result of leaf senescence (Sitte 1974; Matile *et al.* 1999; Zavaleta-Mancera *et al.* 1999).

Gerontoplasts contain reduced or sometimes just remnants of the former thylakoid system and large plastoglobuli. They are often reduced in size, have an electron dense stroma and show a loss of ribsomes and DNA (Nebel and Matile 1992; Matile 2000). Ultrastructurally, the gerontoplast and chromoplast are fairly similar, as even gerontoplasts still contain residual leaf carotenoids, which are responsible for the yellowing of leaves during senescence. The two plastid types however differ in the respect that chromoplasts develop from proplastids, leucoplasts or young chloroplasts and retain their capacity for biosynthesis and division. Gerontoplasts, on the other hand, develop or originate only from old senescing chloroplasts and, most importantly, have lost the capacity of biosynthesis or division (Zavaleta-Mancera *et al.* 1999). Therefore, by definition, a gerontoplast is really nothing more than an old degenerated chloroplast.

The ability of mature plastids to differentiate and redifferentiate into other functional forms seems to be accepted (Whatley 1978), although for numerous years this possibility had been contested and a unidirectional pathway proposed. Chloroplasts in young developing leaves initially originate from undifferentiated proplastids found in meristematic tissues (Pyke 1999). However, proplastids can also give rise to all other forms of plastids encountered in plants, such as amyloplasts in roots and storage tissues, chromoplasts and leucoplasts in fruit and flowers. The differentiation pathway followed is largely dependent on tissue type (Marano *et al.* 1993; Pyke 1999).

Regreening of leaves can essentially occur in two possible ways, either via the formation of mature functional chloroplasts from a pool of proplastids retained within cells or via the re-differentiation of other forms of plastids present in the cell into functional chloroplasts, possibly followed by chloroplast division (Zavaleta-Mancera *et al.* 1999; Salopek-Sondi *et al.* 2000; Salopek-Sondi and Magnus 2007). It appears that, in the majority of cases, regreening is the result of the redifferentiation and complete restoration of gerontoplast-like plastids (Grönegress 1974; Zavaleta-Mancera *et al.* 1999; Salopek-Sondi *et al.* 2000; Prebeg *et al.* 2008; Schmeisser *et al.* 2010). The term gerontoplast-like plastid is preferred to describe the plastids encountered in these leaves, until it has

been proven that a senescence programme is run in leaves capable of regreening as part of their natural development.

5. Conclusion

Regreening of plant organs, especially leaves, seemingly set on a pathway of self-destruction, is a rare occurrence in nature. The phenomenon has caused some controversy amongst biologists interested in developmental programs. However, it appears as if an amicable solution has been proposed in that PCD does not need to be viewed as being unidirectional, but should rather be viewed as a programme that can be reversed. Irrespective of the plant studied, those capable of regreening appear to follow a similar route of plastid differentiation and redifferentiation. A question that still requires further investigation is whether yellowing encountered in leaves capable of regreening is indeed a senescence run programme or whether it appears to be an alternative pathway. This should become evident from further studies on gene expression during yellowing, as well as determining the levels of pheophorbide a.

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Paper 1

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Regreening of involucral leaves of female *Leucadendron* (Proteaceae) after flowering

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Abstract. Involucral leaves of *Leucadendron* have the remarkable ability to turn yellow upon flowering and regreen naturally as the florets of the inflorescence wilt. This colour change results from degradation of chlorophyll (CHL) and to lesser degree carotenoids, resulting in the unmasking of yellow colour. CHL levels were restored upon regreening. Degreening coincided with the complete dismantling of the thylakoid system, whilst keeping the outer plastid envelope intact. Regreening resulted from the complete redifferentiation of these gerontoplast-like plastids into functional chloroplasts. The colour change was directly linked to the development of the inflorescence. Complete removal of the inflorescence before flowering prevented the colour change while removal at full bloom, when involucral leaves were yellow, resulted in significantly faster regreening. This designates the inflorescence or florets as the possible origin of the colour change trigger and suggests that the colour change is involved with attraction of pollinators. Degreening and regreening also took place in a growth chamber under continuous high light intensity. Therefore neither pollination nor the presence of roots is required for regreening. It appears that colour change in *Leucadendron* results from a well-regulated degradation and subsequent synthesis of photosynthetic pigments.

The genus *Leucadendron* consists of woody, dioecious perennials, which often display great sexual dimorphism in leaf size and floral morphology (Dekock *et al*. 1994; Rebelo *et al*. 1995; Bond and Maze 1999). In female plants, upon cessation of shoot growth, a terminal cone develops from spirally arranged floral bracts, each subtending a small yellow floret at anthesis. The distal involucral leaves

surrounding the inflorescence, often form a showy ‘flower head’ that is intensely coloured, varying from red to yellow or a combination thereof (Leonhardt and Criley 1999).

Involucral leaf colour however is transient in nature, as these leaves possess the ability to change colour from green to bright yellow, followed by a phase of rapid, natural regreening. This colour change is most striking in yellow species, but a distinct yellowing and regreening is also evident in red *Leucadendron*. Petals of many angiosperms change colour in relation to their reproductive development, commonly linked to the start of the pollination phase. The colour change itself aids in the attraction of pollinators by directly influencing their foraging behaviour (Weiss 1991; Neeman and Nesher 1995; O'Neill 1997). Even non-floral accessories such as sepals and bracts often possess the ability to change colour and form part of the floral advertisement and are in some plants solely responsible for the display of colour (Tavares *et al.* 1998; Salopek-Sondi *et al.* 2000; Xu SuXia *et al.* 2009). However, it is very uncommon for leaves to change colour to aid pollination and especially to then regreen again into a functional leaf (van Doorn and Woltering 2004). Only a few studies reporting on the natural degreening and regreening of leaves have been encountered so far (Chabot and Chabot 1975; Ikeda 1979; Koiwa *et al.* 1986). In these cases, the regreening process appears to be related to climatic changes occurring from winter to spring. Considering the vast plant diversity, natural regreening of plant organs appears to be relatively uncommon and has only been reported to occur in a few fruits, such as oranges (Coggins Jr. and Lewis 1962; Mayfield and Huff 1986), pumpkins (Devide and Ljubesic 1974), cucumber (Prebeg *et al.* 2008), and floral structures, such as sepals of *Helleborus niger*, *H. foetidus* (Salopek-Sondi *et al.* 2002; Herrera 2005) and the Golden-saxifrage (*Chrysosplenium alternifolium*) (Sitte 1974), the spathe of the arum and calla lily (*Zantedeschia aethiopica and Z. elliotiana*) (Grönegress 1974; Tavares *et al.* 1998), and the petals of a few orchid species (Tran *et al.* 1995; Doorn 1997).

Regreening can essentially occur in two ways, either via the formation of new chloroplasts from a pool of proplastids retained within the cell or the reconstruction of the degreened chloroplasts as encountered in *Euonymus* (Ikeda 1979) and *Buxus* (Koiwa *et al.* 1986). Ultra-structural studies have been used successfully to determine which of the two mentioned scenarios is responsible for the

observed regreening of *H. niger* (Salopek-Sondi *et al.* 2000), cucumber (Prebeg *et al.* 2008) and the induced regreening of tobacco leaves (Zavaleta-Mancera *et al.* 1999).

Although we are by no means the first to have observed the remarkable colour change, this study appears to be the first report on the colour dynamics of *Leucadendron.* The investigation aims at relating the observed degreening and regreening phenomenon to changes in pigment content, ultra-structural changes of the chloroplast, and most importantly to specific phenological stadia of the inflorescence. To determine the commonality of the observed change in seasonal pigmentation within the genus, photosynthetic pigment levels were determined for a pure yellow hybrid, a cultivated yellow selection of *L. salignum*, as well as for a wild species population of *L. laureolum*. Although this does not establish a strong case for comparative biology, it does serve to indicate that the colour change is not confined to a single species of the genus. Strong evidence is given to show that pollination is not a pre-requisite for colour change. Furthermore, the possible origin of the signal for colour change was investigated.

MATERIALS AND METHODS

Terminology

Terms used to describe parts of the flower head in Leucadendron seem to vary amongst authors, despite using similar literary references. Depending on the author, the colourful elements of the Leucadendron flower head have been referred to as petal-like bracts and involucral bracts, as well as involucral leaves (Ben-Jaacov *et al*. 1986; Robyn and Littlejohn 2003; Pharmawati *et al*. 2005). To create some form of consistency in our publications, the terms set out by Rebelo *et al*. (1995) and as used by Hemborg and Bond (2005) will be employed (Fig. 1). The unit formed by involucral leaves and the inflorescence is called the flower head. Involucral bracts are brown and inconspicuous as they die and shrivel up, but remain attached at the base of the inflorescence. Floral bracts are green and initially cover the small developing floret. These bracts eventually die and form the dry, woody cone. The involucral leaves (often erroneously called involucral bracts) are the colourful elements of the showy flower head, displaying the observed colour changes subsequently discussed.

Plant material

Female shoots of the cultivar Goldstrike (*L. salignum* x *L. laureolum*), harvested in 2004 and 2006, were obtained from Arnelia Farm in Hopefield (Western Cape, South Africa, 33º02'S, 18º19'E). A selection of wild *L. salignum* (female), grown non-commercially, was also obtained from Arnelia Farm in 2004. Female *L. laureolum* shoots were sampled during 2006 from a wild population growing on the mountain slopes of the Eikenhof farm in the Elgin area of the Western Cape (34º08'S, 19º02'E). In 2007, 'Goldstrike' shoots were obtained from the commercial protea farm Vredekloof situated in Paarl (Western Cape, South Africa, 33º02'S, 18º19'E). All sample regions are characterised by a Mediterranean-type climate, with flowering times of 'Goldstrike' starting in August, *L. salignum* in early June and that of *L. laureolum* in August.

Sampling procedure and statistical layout

To determine seasonal pigmentation patterns as well as the effect of deconing on these patterns, weekly sampling of shoots took place from 27 July until 19 October for 'Goldstrike' during the 2004 season. Sampling commenced well after cessation of shoot growth, but prior to anthesis and continued until all florets on the inflorescence had wilted. A week prior to the start of a trial, all shoots required were randomly selected and tagged based on visual assessment of the apical cone to ensure phenological uniformity. At the start of the sampling period, randomly selected shoots from the pool of pre-tagged shoots were deconed by pinching out the entire inflorescence. The random picking of non-deconed shoots and deconed shoots occurred weekly. Sampling of the species selection *(L. salignum*) and the wild growing *L. laureolum* was done on three dates only, representing the main stages of inflorescence development (preanthesis, anthesis and postanthesis). Some *L. salignum* shoots were deconed at full bloom and harvested 19 days later, when a significant colour difference was evident between deconed and non-deconed shoots. For all trials, shoots were harvested at random (complete random design), divided into 5 repetitions with 4 shoots per replicate. For 'Goldstrike' and *L. salignum*, 8 to 9 involucral leaves (counting from youngest to oldest) were removed per shoot, frozen in liquid nitrogen, freeze-dried, milled and stored at -80ºC prior to pigment analysis. Involucral leaves of *L. laureolum* were not collected for pigment analysis and only colour measurements were taken.

Pigment extraction and colour analysis

Photosynthetic pigments were extracted by adding 10 ml cold aqueous acetone (80%) to 500 mg freeze-dried sample. Extraction took place for 1 hour in the dark at 4 ºC whilst stirring. After centrifugation for 10 min at 12000 X *g*, the supernatant was decanted into a vial. For a fast re-extract, 5 ml acetone was added to the pellet and vortexed for 5 seconds. The re-extract was centrifuged for 10 min at 12000 X *g*, after which the supernatant of the re-extract was pooled together with the first extract. The supernatant was filtered through a 0.45 μm filter (Millex-HV, Millipore Corporation, Milford, MA) and analysed spectrophotometrically using a Cary 50 Spectrophotometer Series (Varian, Mulgrave, Australia). Absorbance of carotenoids (A_{470}) and chlorophyll (A_{663} and A_{647}) were measured and concentrations calculated according to Lichtenthaler (1987). When presented, leaf colour measurements were taken using a chromameter (Nr-3000; Nippon Denshoku, Tokyo, Japan) and expressed in terms of hue angle, chroma and lightness. The colorimetric co-ordinates L*, C* and H are directly based on light reflectance of an illuminated surface. The reflected light is measured and defined as a specific colour within a colour space that correlates with human perception of lightness, saturation and hue. Although reflection may give an indication of colour, L*, C* and H values used to express colour change (change in lightness, saturation and tint) are more intuitively understood and visualised by readers. Lightness values are proportional to the amount of light reflected from an illuminated surface, with 0 indicating the total absorption of the illuminating light and a value of 100, a 100% reflection thereof. Increasing pigment concentrations generally decrease lightness values, due to an increase in the total light absorbed. Hue angle values are indicative of a definite colour or tint of colour. A hue angle of 90 º is considered to be yellow, with colour changing from yellow towards yellowish-green to bluish-green as hue angles increase towards 180 º. Chroma is a measurement of saturation of vividness of colour. Both chroma and hue angles are calculated from the tristimulus CIELab colour scale (for a more detailed explanations on colour measurement the reader is referred to (Mcguire 1992; Gonnet 1998; Gonnet 1999; Gonnet 2001). Colour measurements were taken of three involucral leaves (leaf number 4, 5 and 6, counting from youngest to oldest) per flower head for all shoots in a replicate. One reading was taken in the mid-section and always on the morphological outside face of the vertically orientated leaf.

Transmission electron microscopy

Five 'Goldstrike' shoots were harvested before flowering (green involucral leaves), at full bloom (yellow involucral leaves) and again after the involucral leaves had regreened. Involucral leaf number five, counting from youngest to oldest was removed from each shoot. Green leaves lower down on the shoot (leaf number 19 or 20), that do not undergo any colour change during flowering, were also collected at the same time. From here onwards these will be referred to as regular leaves. Using a standard stereo microscope, leaf segments of 1–2 mm^2 were cut from each leaf in the middle of the leaf left of the midrib. Leaf segments were fixed for 4 h in 5% (v/v) glutaraldehyde in a 0.075M phosphate buffer, pH 7.4, containing 2% formaldehyde and 0.5% caffeine, at room temperature (22ºC). The first 30 min of fixation was conducted under partial vacuum, as normal fixation at ambient pressure only resulted in poor infiltration of the fixative. Further fixation (same fixative) took place overnight at 4°C. Segments were washed with the phosphate buffer and post-fixed in 1% osmium tetroxide (in the same buffer) for 3 hours. After washing the segments with distilled water, they were dehydrated in a series of ethanol solutions (30, 50, 70, 80 and 90%) for 5 min each. The final dehydration step was done in 100% ethanol (twice for 20 min). Ethanol was then replaced by 100% acetone (twice for 20 min each). Half the acetone was replaced with Spurr's epoxy resin (Wirsam, Johannesburg, South Africa) and left overnight while on a shaker to prevent solidification of the resin. The resin concentration was increased (2:1, resin: acetone) and after 8 h to 100% resin for a further 2 days and then to be hardened off in an oven at 60ºC for 24 h. Thin sections (90-100nm) were cut on a Reichert Ultracut-S ultra-microtome using glass knives, with sections being picked up on 200µm mesh square copper grids. Sections were stained with uranyl acetate and lead citrate as described by Reynolds (1963). Specimens were visualized with a *LEO* 912 transmission electron microscope equipped with a CCD camera.

Colour change in growth chamber

During 2006, 'Goldstrike' shoots were harvested when the tip of the fused perianth of the most basal flowers was just visible at the rim of the floral bracts. Five two-shoot replications were cut to a length of about 30 cm, placed in glass jars containing tap water. Three involucral leaves were marked (leaf number 4, 5 and 6) and leaf colour measured as explained. Jars were then randomly placed in a growth chamber held at 22 ºC ± 2 ºC. Shoots were exposed to a photosynthetic photon flux of

800-900 µmol·m^{-2}·s^{-1} provided by two 400-W high-pressure sodium lights (SON-T; Osram MgBh, Munich, Germany) situated on top of the growth chamber. An acrylic (Perspex) sheet of 5 mm thickness separated the shoots from lights, preventing direct heating of shoots and aiding temperature control within the chamber. Water was refilled every few days as required. Each week, the bottom 0.5 cm of each stem was removed to ensure efficient water uptake. Colour of marked involucral leaves was measured weekly and shoots assessed for signs of senescence.

Phenological development

Five 'Goldstrike' shoots of each main developmental phase (non-flowering, flowering and after floral death) of the inflorescence were sampled during the 2007 season. Floral cones were removed and dissected by hand to reveal the small developing flowers. Macroscopic studies of florets were conducted using a Wild M400 Photomacroscope (Wild-Heerbrugg, Switzerland) equipped with a Zeiss Axiocam digital camera (Carl Zeiss, Germany). Colour measurements were taken of the corresponding involucral leaves (leaf number 4, 5 and 6).

Data analysis

The data were analysed using the General Linear Models (GLM) procedures of the SAS program (SAS release 9.1, SAS Inst., Cary, NC).

RESULTS

Phenological development in relation to colour changes

The colour development period investigated, in relation to inflorescence development, can be divided into 4 phases marked by 3 main events. Phenological development of the female *Leucadendron* inflorescence did not seem to differ markedly among cultivars and the yellow cultivar Goldstrike was chosen as a suitable representative. During early reproductive development (Phase A, Fig. 2 A1-4), each floral bract covers and hides a young, developing flower (floret) in its axil. The floral stigma has not expanded yet and is still partially enclosed in tissue. The ovary is small and subtended by developing needle-shaped nectaries. During Phase A, the involucral leaves are green (L=62; H=109). The first main event is the 'emergence of the floret'. This marks the start of Phase B (Fig. 2 B1-4). Prior

to anthesis, the fused perianth of the older basal florets emerge above the rim of the floral bract. By now, the perianth has turned bright yellow and is visible against the light green background of the cone. The stigma appears slightly more expanded, but little change in ovary shape and size occurred. Nectary morphology changed, as they became more needle-shaped with distinct yellowing of the tips. The involucral leaves often appeared of a slightly lighter green colour with a haze of yellow than during Phase A, as indicated by the increase in lightness and decrease in hue values (L=66; H=105). The second main event is 'anthesis', which occurs approximately 1 to 2 weeks after the emergence of the first yellow florets. At anthesis, the perianth splits open, with each perianth segment bearing the anthers curling backwards to expose a fully expanded stigma. This marks the onset of the pollination phase (Phase C**,** Fig. 2 C1-4) as the stigma is receptive for pollen. An exudate covering the papillae, as reported by Robyn and Littlejohn (2003), was not observed. Phase C is characterised by a rapid yellowing and increase in lightness of the involucral leaves (L=77; H=87). The start of Phase D occurs with the death of florets, which is associated with a rapid, natural re-greening of involucral leaves (L=67; H=107). With floral death, the ovary slowly changes into a characteristic seed shape, with a small ovule inside (Fig. 2D1-4).

Seasonal pigment fluctuations

During early floral development (Phase A), when involucral leaves are green, chlorophyll (CHL) and carotenoid (CAR) levels were high in 'Goldstrike', with initial concentrations of about 303 $\mu g \cdot g^{-1}$ and 98 $\mu g \cdot g^{-1}$ dry weight, respectively (Fig. 3). Upon protrusion of the florets (Phase B), there was a gradual decrease in CHL and CAR during the first week, with CHL levels dropping rapidly (52%) within the week just after anthesis (Phase C). The net pigment degradation was 65% for CHL and 59% for CAR, reaching the lowest level of 107 $\mu g \cdot g^{-1}$ and 40 $\mu g \cdot g^{-1}$ respectively, resulting in a CHL:CAR ratio of 2.7, a drop from an initial ratio of 3.1. At this point, the flower heads were of a light yellow colour. With floral death (Phase D), marking the end of the pollination period, a rapid synthesis of both CHL and CAR occurred, but the increase was more gradual relative to the earlier steep degradation rate encountered after anthesis. CHL and CAR concentrations increased between 30-70 $\mu g \cdot g^{-1}$ weekly for about 4 weeks, resulting in the complete regreening of involucral leaves. Both pigment levels returned to similar concentrations encountered before anthesis. The yellow species selections of *L. salignum* and the wild species *L. laureolum* showed precisely the same colour change pattern as

'Goldstrike', again being linked to the main phenological development stages of the inflorescence. In *L. salignum*, about 75% CHL was degraded by anthesis and only 54% of the CAR, resulting in a CHL:CAR ratio of 1.9, a decrease from an initial ratio of 3.5 (Fig. 4). After floral death (regreening phase), both CHL and CAR returned to levels encountered before flowering. The yellowing of *L. laureolum* is evident from the 12º decrease in hue angle from 104º (non-flowering) to 91º upon flowering (Fig**.** 5). The involucral leaves turned a bright yellow colour as indicated by the concurrent increase in lightness from 72 to 82. Upon floral death, and the associated regreening, the hue angle increased back again to 103º.

Ultra structural changes

Green involucral leaves before anthesis contained chloroplasts that were ultrastructurally similar to those encountered in regular leaves lower down on the stem (Fig. 6 A-B). Involucral leaves did however contain fewer chloroplasts per cell than regular leaves. Both leaf types contained chloroplasts with normal, well-developed thylakoid systems, with distinct granal stacks. Both had electron-dense stroma, sometimes containing a few small plastoglobules. Most conspicuous were the excessively large starch granules observed in both leaf types. The starch granules of regular leaves, although being of similar size to those observed in involucral leaves, were far more numerous. Commonly three granules of various sizes were visible within a single chloroplast. Chloroplasts of involucral leaves also sometimes contained more than one starch granule, but less frequently. At anthesis, when involucral leaves had turned yellow, most chloroplasts had dedifferentiated into gerontoplast-like plastids of irregular shapes with varying degrees of ultrastructure degeneration. The term "gerontoplast-like plastids" is proposed for yellow involucral leaves, as strictly speaking the term gerontoplast is reserved for plastid development in senescing leaves (Sitte 1974; Thomas 1997). In *Leucadendron*, however, the yellowing does not appear to be senescence-related as regreening is the rule rather than the exception. In general, plastids of yellow involucral leaves had loosely arranged, disorganised thylakoid systems, deteriorated granal stacks and a stroma of low electron density (Fig. 6 C-D). Many gerontoplast-like plastids contained swollen thylakoids with relatively large plastoglobules. However, in all samples investigated, a considerable range of chloroplasts ultrastructures were encountered, varying in the degree of thylakoid degeneration as well as in size and number of plastoglobules present. Even some completely intact chloroplasts with seemingly

functional thylakoid systems were encountered in yellow involucral leaves. As involucral leaves regreened, the gerontoplast-like plastids redifferentiated into functional chloroplasts of the usual ultrastructure as observed before degreening (Fig. 6 E-F). Plastoglobules of variable sizes were observed in the redifferentiating chloroplasts, but they tended to be smaller and more numerous than in the plastids of yellow involucral leaves. As encountered in yellow involucral leaves, there was again a considerable range of chloroplast ultrastructures present, depending on the degree of reconstitution they had undergone. However, fully redifferentiated chloroplasts seemed to be predominant. Completely redifferentiated chloroplasts tended to contain smaller plastoglobules, but not always and a clear pattern was difficult to discern. The plastids of regular leaves lower down on a shoot, yellowing due to normal senescence, showed distinct thylakoid degeneration with large plastoglobules being present. During the more advanced stages of senescence, many of the plastids had ruptured or partly disintegrated double membrane envelopes (Fig. 7A). This was not at all observed in yellow involucral leaves. Plastids of regular leaves in an advanced stage of senescence had completely disintegrated, leaving behind remnants of large starch granules (Fig. 7B).

Effect of deconing

The complete removal of the inflorescence well before anthesis inhibited the colour change of involucral leaves in 'Goldstrike' (Fig. 8). Deconing before flowering prevented the severe degradation of CHL as well as the lesser degradation of CAR, which in turn prevented the unmasking of yellow colour and the involucral leaves remained green. Similar results were obtained or observed by the deconing of *L. salignum*, *L. laureolum*, *L. microcephalum* and other cultivars such as Laurel Yellow (*L. laureolum x L. discolor*), Chameleon (*L. salignum x L. eucalyptifolium*) and Inca Gold (*L. salignum x L. laureolum*) (data not presented). The deconing of *L. salignum* at full bloom, when involucral leaves are yellow, resulted in a significantly faster regreening. Nineteen days after deconing, the control shoots were still yellow, whereas the deconed shoot had already regreened significantly with CHL and CAR levels almost double that of the control (Fig. 9 A-B).

Colour change in excised shoot

The degreening and regreening phenomenon observed under field conditions also took place in harvested 'Goldstrike' shoots placed into a growth chamber under continuous high light intensity and

set at 22 ºC (Fig. 10). Similar to the described field observations, as shoots flowered in the growth chamber, there was a significant decrease in hue (indication of yellowing) with the concurrent increase in lightness.

Discussion

The seasonal colour change in *Leucadendron* results from an apparently well-regulated degradation and subsequent synthesis of photosynthetic pigments. The almost complete degradation of CHL unmasks the presence of the CAR, which are degraded to a lesser extent and become the pigment imparting the bright yellow colour in yellow *Leucadendron* cultivars and species. The subsequent synthesis of CHL upon floral death to levels encountered before degreening, results in the complete regreening of involucral leaves. The colour intensity differences between pure yellow flower heads result from varying degrees of CHL and CAR degradation, giving specific pigment ratios leading to differences in colour expression. In 'Goldstrike' 65% and in *L. salignum* 75% of the initial CHL was degraded by anthesis resulting in CHL:CAR ratios of 2.7 and 1.9, respectively. The difference in ratios accounts for *L. salignum* having a more intense yellow colour than 'Goldstrike', which has a more bland yellow appearance. The reason that 'Goldstrike' shows a yellow colour, although the CHL:CAR ratio is close to the original 3.1, is best explained by Bougeur's Law, whereby the influence of a minor pigment on perceived colour becomes exponentially greater as concentration of both major and minor pigments decrease (see Biran (1974) for further explanation). The developmental pattern in photosynthetic pigmentation and the underlying mechanism seems to be common to *Leucadendron* showing a distinct yellowing of their involucral leaves, irrespective of dealing with a wild species, clonal species selection or cultivar.

Along with CHL and CAR degradation, there was the concurrent dismantling of the chloroplasts' inner structure whilst maintaining the integrity of the outer envelope attaining a gerontoplast-like appearance. Evidence indicates that the regreening of involucral leaves results from the reconstruction of gerontoplast-like plastids to fully functional chloroplasts. This notion is supported by the fact that regreened chloroplasts retained features of the gerontoplast-like plastids. No dividing chloroplasts or pool of proplastids was encountered in yellow or regreened leaves. Furthermore, the

plastid numbers per cell showed no significant increase between yellow and regreened leaves (data not presented), providing further evidence that the chloroplasts of regreened leaves unlikely originated from proplastids, but rather from the redifferentiation of partially dismantled plastids. The ontogeny of plastids in yellow involucral leaves compared well with the natural regreening phenomenon encountered in the green hellebore (Salopek-Sondi *et al*. 2000), but ultrastructurally even more so to the induced regreening of tobacco leaves (Zavaleta-Mancera *et al*. 1999). Unlike senescing regular leaves, gerontoplast-like plastids of yellow involucral leaves never deteriorated so far as to lose the integrity of the outer envelope. Regreening of involucral leaves is the rule and forms an integral part of the normal flowering cycle of *Leucadendron*. It would therefore appear logical that the yellowing of involucral leaves is not the result of a senescence-related sequence. This view is strengthened by the fact that involucral leaves of some species can remain green and alive for almost a year after regreening (personal observation). However, a senescence-related degreening can so far not be ruled out, as it might be the case of redifferentiation of gerontoplasts before the point of no return (van Doorn and Woltering 2004).

Compelling evidence was gathered showing that colour development in *Leucadendron* is developmentally regulated and linked directly to the phenology of the inflorescence, but not linked to successful pollination and subsequent seed development. The first noticeable colour change occurs when the fused perianth of the most basal florets appears above the rim of the floral bracts, which coincides with a distinct change in the appearance of the nectaries in becoming more needle-shaped and yellow in appearance. This might be a sign of maturation and gaining the potential for nectar production, but there is no other literature to support this notion. The yellowing of involucral leaves intensifies towards flowering as the plant enters the pollination phase. Rapid regreening of involucral leaves seems to occur only when the last of the most apical florets on the cone are wilting. These developmental changes were noted for all *Leucadendron* investigated and are so closely related to the colour change, that the developmental stage of the inflorescence of individual shoots in the field can be closely estimated by externally assessing their colour from a distance. The removal of the inflorescence before flowering prevented the drastic degradation of CHL and CAR and involucral leaves remained green. This again indicates that the developmental regulation underlies pigment

degradation, but furthermore designates the inflorescence or florets as being the most likely origin of the trigger for colour change.

Natural regreening as encountered in the sepals of Helleborus niger (Salopek-Sondi and Magnus 2007) and the floral spathe of Zantedeschia elliottiana (Grönegress 1974) and Z. aethiopica (Tavares et al. 1998) is dependent on critical events like pollination or the presence of developing seeds. In these species, the floral appendages capable of regreening wilt if the flower remains unpollinated and, in comparison to the pollinated counterpart, show no signs of regreening (Grönegress 1974; Tavares et al. 1998). In contrast, the sepals of unpollinated or depistillated H. niger flowers have the same life-span as pollinated ones, but tend not to regreen significantly. Active fruit development is required in H. niger for regreening of sepals to occur and removal of developing fruit arrests the regreening process (Salopek-Sondi *et al*. 2002; Tarkowski *et al*. 2006). Leucadendron differ in that pollination or subsequent seed development does not seem to be a prerequisite for regreening of involucral leaves. It appears that flowering is the critical step to determine initial CHL degradation and floral death for the subsequent CHL synthesis during the regreening process. The fact that female Leucadendron shoots, harvested well before flowering, are able to complete the entire colour change (green to yellow and back to green) in a growth chamber where there is no possibility of pollination, indicates that a signal from events such as pollination or seed development is not required for regreening. Self-pollination can be ruled out, as all Leucadendron are dioecious with male organs on the female inflorescence being sterile (Rebelo *et al.* 1995).

It is estimated, that about 89% of *Leucadendron* are insect pollinated (Williams 1972) with beetles outweighing the other pollinator guilds such as wasps, flies and bees (Hattingh and Giliomee 1989; Rebelo *et al.* 1995). The fact that Leucadendron have well developed nectaries (Rao 1967) and are capable of producing nectar (Littlejohn and Robyn 2000), suggest that the most logical function of colour change in involucral leaves is to aid in pollination. The true flowers of *Leucadendron* are often small and in some instances remain partially or even completely hidden by involucral leaves as evident in *L. salignum* (Fig. 9). It appears that the involucral leaves in *Leucadendron* have adopted a floral function to attract pollinators through yellowing and then regreening when pollination is no longer possible. In total, ten wind pollinated *Leucadendron* have been recorded so far, based on their

inability to produce nectar, being odourless and the female cones having large stigmas to capture sufficient pollen from the air (Rebelo *et al*. 1995). The wind-pollinated species still display yellow florets, but generally do not show an elaborate colour change in their involucral leaves at anthesis, giving credence to the idea that the colour change itself is an adaptation to aid pollination. Interestingly, only one of the ten wind-pollinated species (*L. olens*) yellows significantly at anthesis, which might indicate a transitional state between wind and insect pollination or a mixed pollination system as the inflorescence is also scented in this species.

Working with cultivars, there is always an inherent danger of miss-interpretation, due to artificial selection or possible introgression. However, the following reasons should support the feasibility of using cultivars to conduct studies on understanding the mechanisms of colour change. Cultivars are often more easily accessible and their clonal nature considerably reduce variability. The cultivars used all have a distinct species parentage, without backcrossing of interspecific hybrids having occurred. Therefore, introgression can be ruled out. Furthermore, *Leucadendron* are important cut flowers in various countries due to the colourful flower head formed by the involucral leaves. From a horticultural perspective, the regreening of the flower head is detrimental to the profitability of the product. If anything, artificial selection would have been aimed at out-breeding the regreening phenomenon.

Colour change in the involucral leaves of *Leucadendron*, resulting from the regulated degradation and synthesis of CHL and CAR, is directly linked to the development of the inflorescence. This is common to species and cultivars alike. Any factor (biotic or abiotic) advancing, delaying, or even preventing flower development has direct bearing on the colour change. The regreening of leaves is a rare phenomenon in nature, making *Leucadendron* a novel model for studies of leaf senescence and its reversal if it is shown to be senescence-related degreening, as well as for ultrastructural investigations on the plasticity of plastid differentiation.

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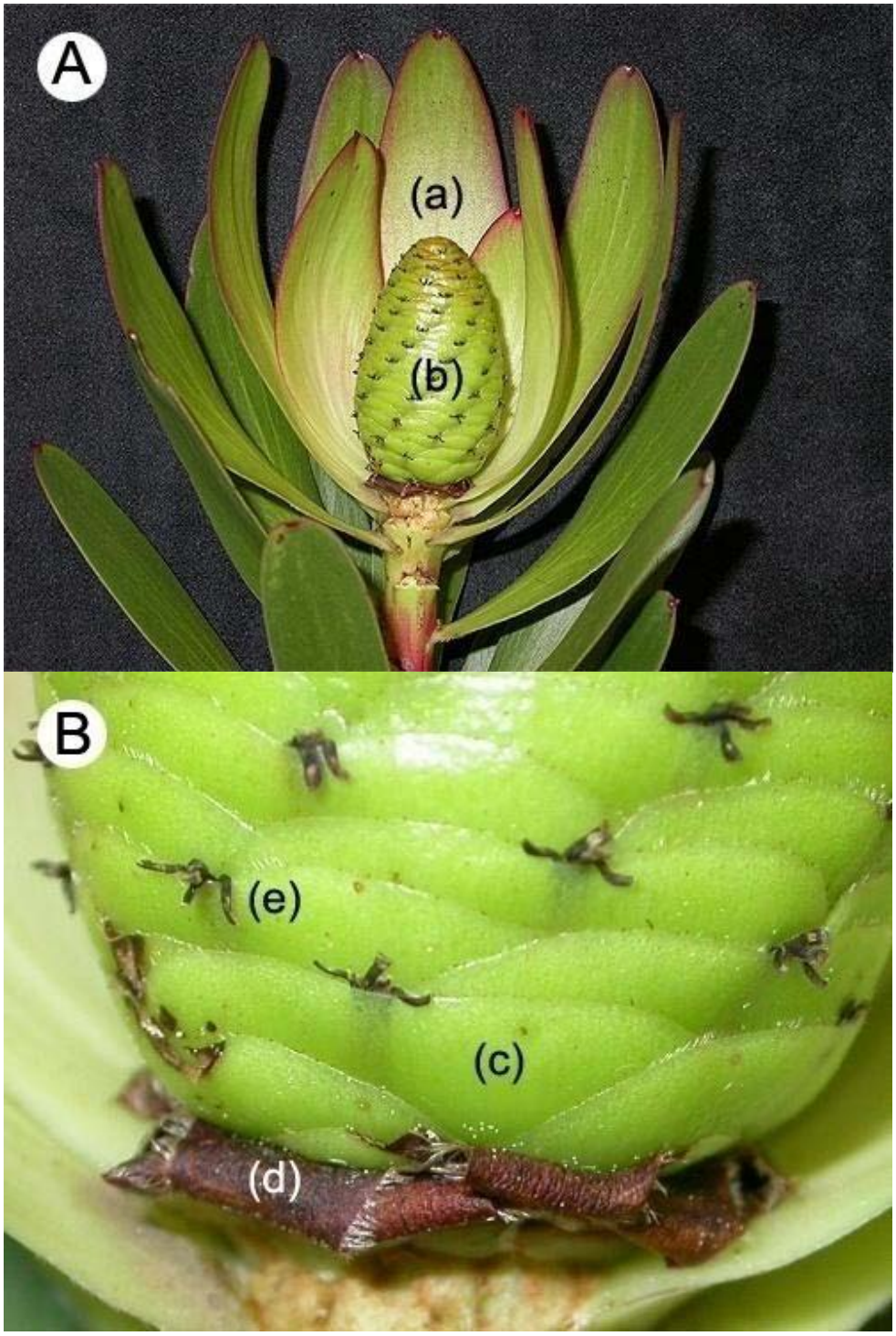

Fig. 1. A typical *Leucadendron* flower head with letters denoting the morphological terms as set out by Rebelo *et al.* (1995) and as used by Hemborg & Bond (2005). A. The 'flower head' is a unit consisting of (a) involucral leaves or display leaves (often brightly coloured) and (b) the inflorescence/cone. B. The floral bracts (c) in females are generally large and fleshy partly covering the small florets. The floral bracts eventually form the woody cone. Involucral bracts (d) die and shrivel up as the season progresses, but remain attached to the base of the inflorescence. Wilted floret (e) above the rim of the floral bract.

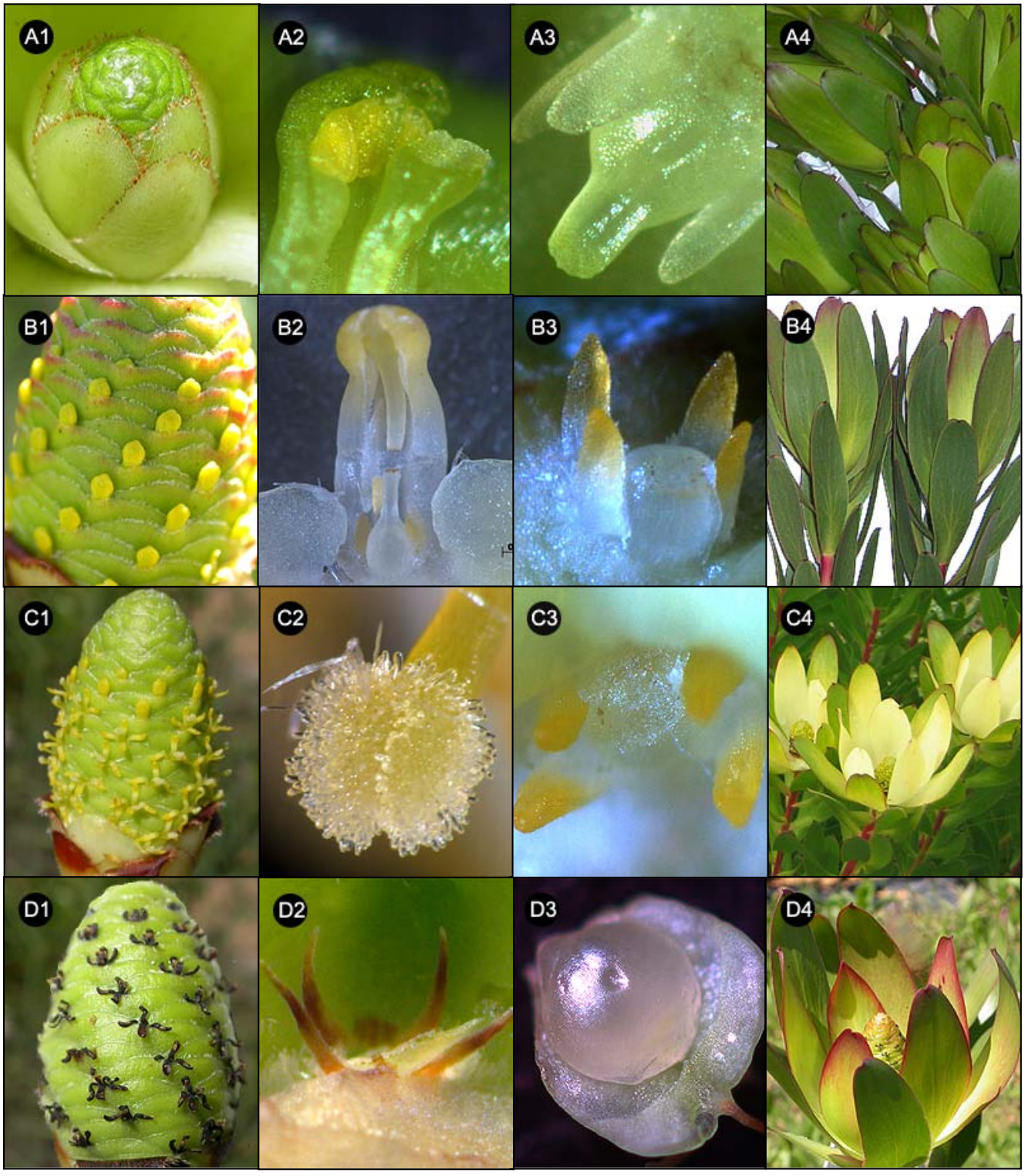

Fig. 2. Important developmental phases of the female *Leucadendron* inflorescence and florets in relation to pigment fluctuations observed during a season. Phase A: Young inflorescence with floral bracts pushing through involucral bracts (A1). In the axil of each floral bract, a young developing floret can be found. The stigma is not fully developed (A2) connected to a small ovary subtended by four immature needle-shape nectaries (A3). The flower head is green (A4). Phase B: Protrusion of florets (still a fused perianth) over the rim of floral bracts (B1). Stigma shows a yellow colouration and is slightly more expanded (B2). The nectaries appear larger and more mature with yellow tips (B3). Shoots appear of a slightly lighter green colour. Phase C: Perianth has split open, with sterile anthers curled backwards to expose the fully expanded stigma. This marks the start of the pollination phase (C1). The stigma soon becomes receptive (C2) with no obvious change in the nectaries (C3). The flower head turns a bright yellow. Phase D: Floral death, marking the end of the pollination period (D1). Nectaries become wilted (D2) and the ovary gradually changes into the characteristic seed shape with (D3) showing a small developing ovule lying on the integument. The flower-head has re-greened substantially (D4).

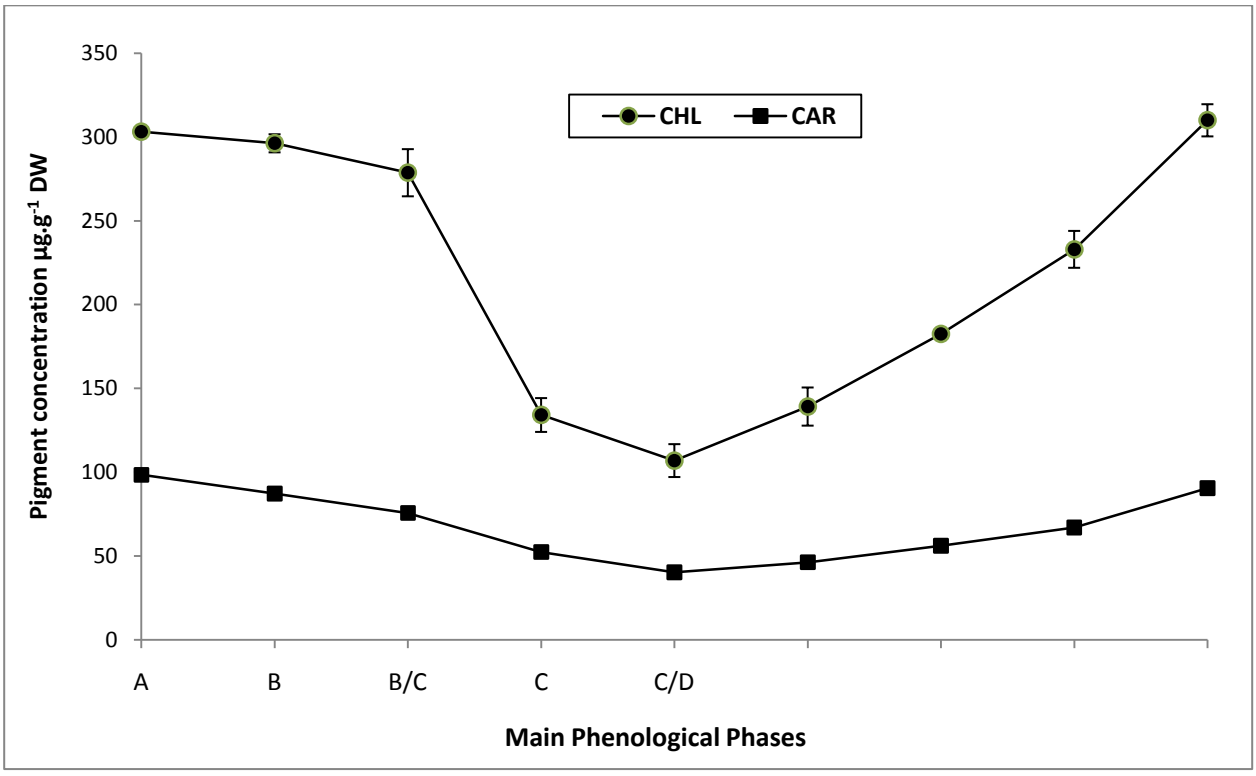

Fig. 3. Seasonal fluctuation patterns of photosynthetic pigments (CHL and CAR) in the involucral leaves of the yellow *Leucadendron* cultivar 'Goldstrike' in relation to the main developmental phases of the inflorescence. Phases: A. Early preanthesis. B. Advanced preanthesis characterised by fused perianth protruding above floral bract. C. Anthesis. D. Death of florets marks beginning of postanthesis phase. The B/C and C/D notation represents a phase change, when sample collected contained some shoots of the next phase. Bars represent ±SE (n=5), but may be hidden by marker if standard error is small.

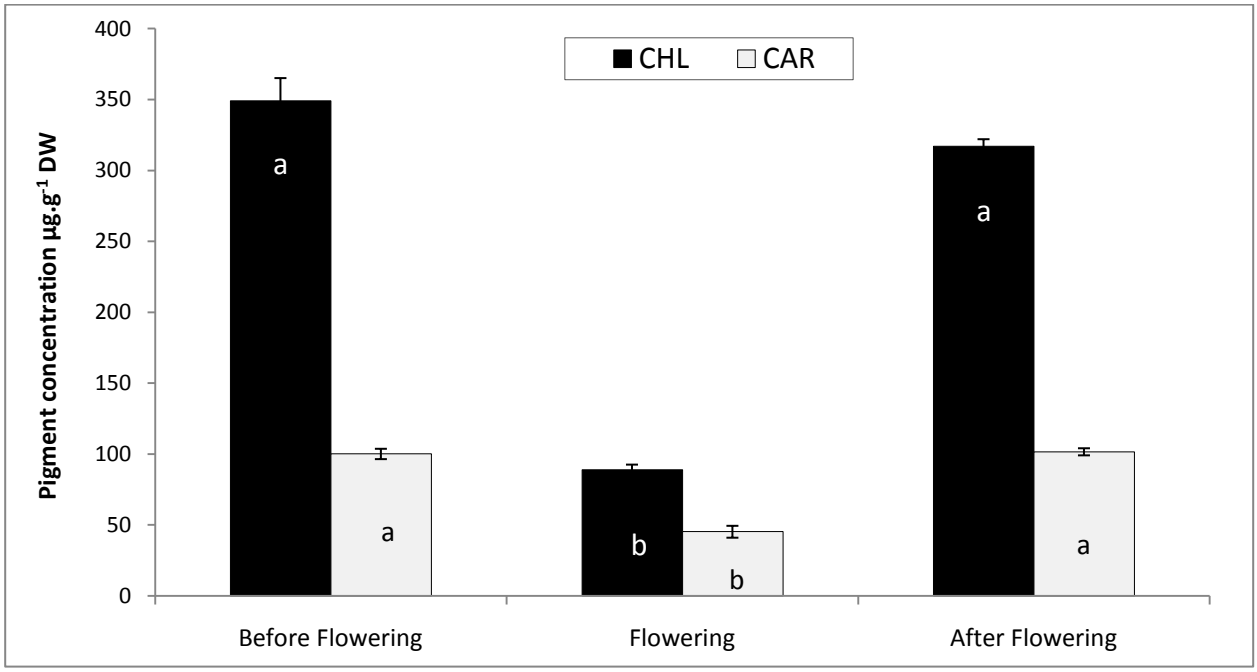

Fig. 4. Chlorophyll (CHL) and carotenoid (CAR) fluctuations in involucral leaves of *L. salignum* in relation to inflorescence development. Mean separation of a pigment class between the three developmental stages was done by least significant difference (5%) following significant F test with $p \leq 0.001$. Bars represent ±SE (n=5).

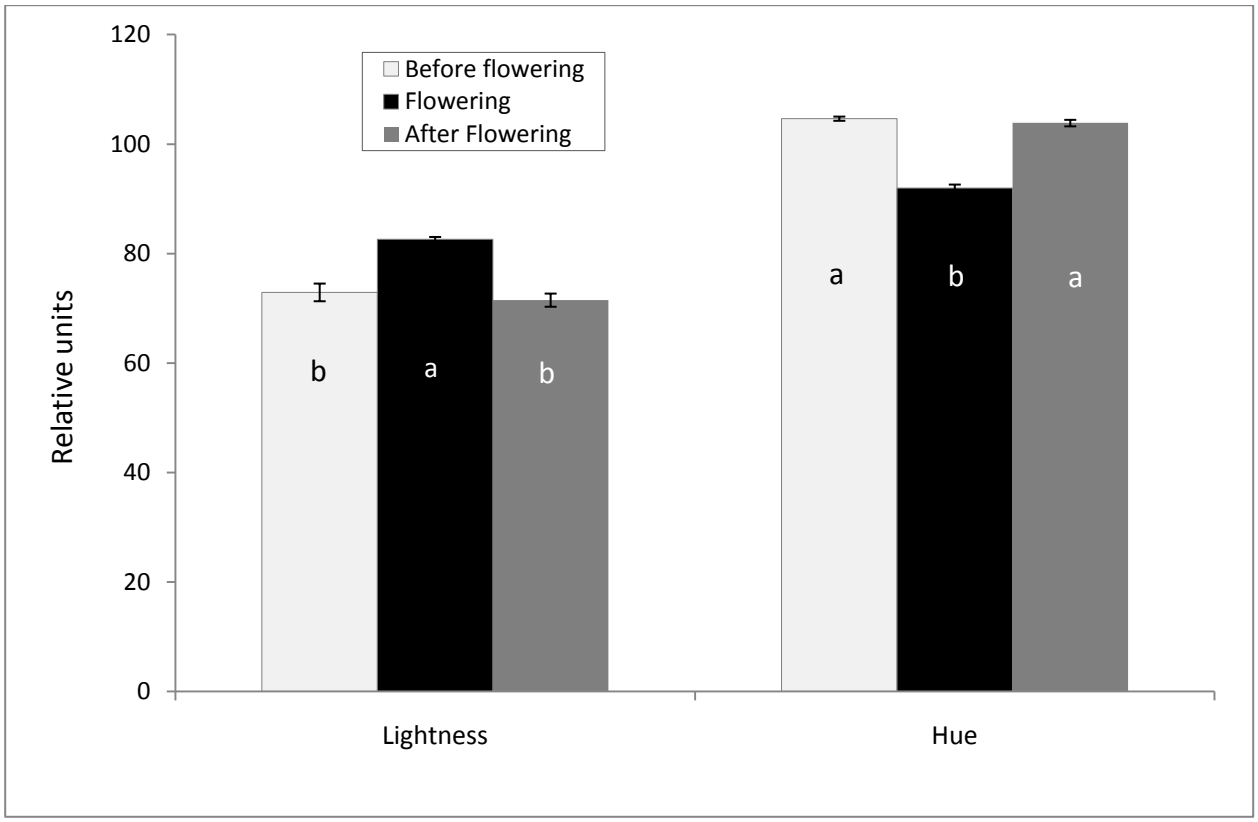

Fig. 5. Colour changes in the involucral leaves of *L. laureolum* (wild population) in relation to inflorescence development. Colour expressed as hue angle (0º = red; 90º= yellow; 180º = green), lightness (0-100). Decrease in hue angle denotes an increase in yellowness, while an increase in L value denotes that the colour becomes lighter. Mean separation of a colour measurement was done by least significant difference (5%) following significant F test with $p \leq 0.001$. Bars represent ±SE (n=15).

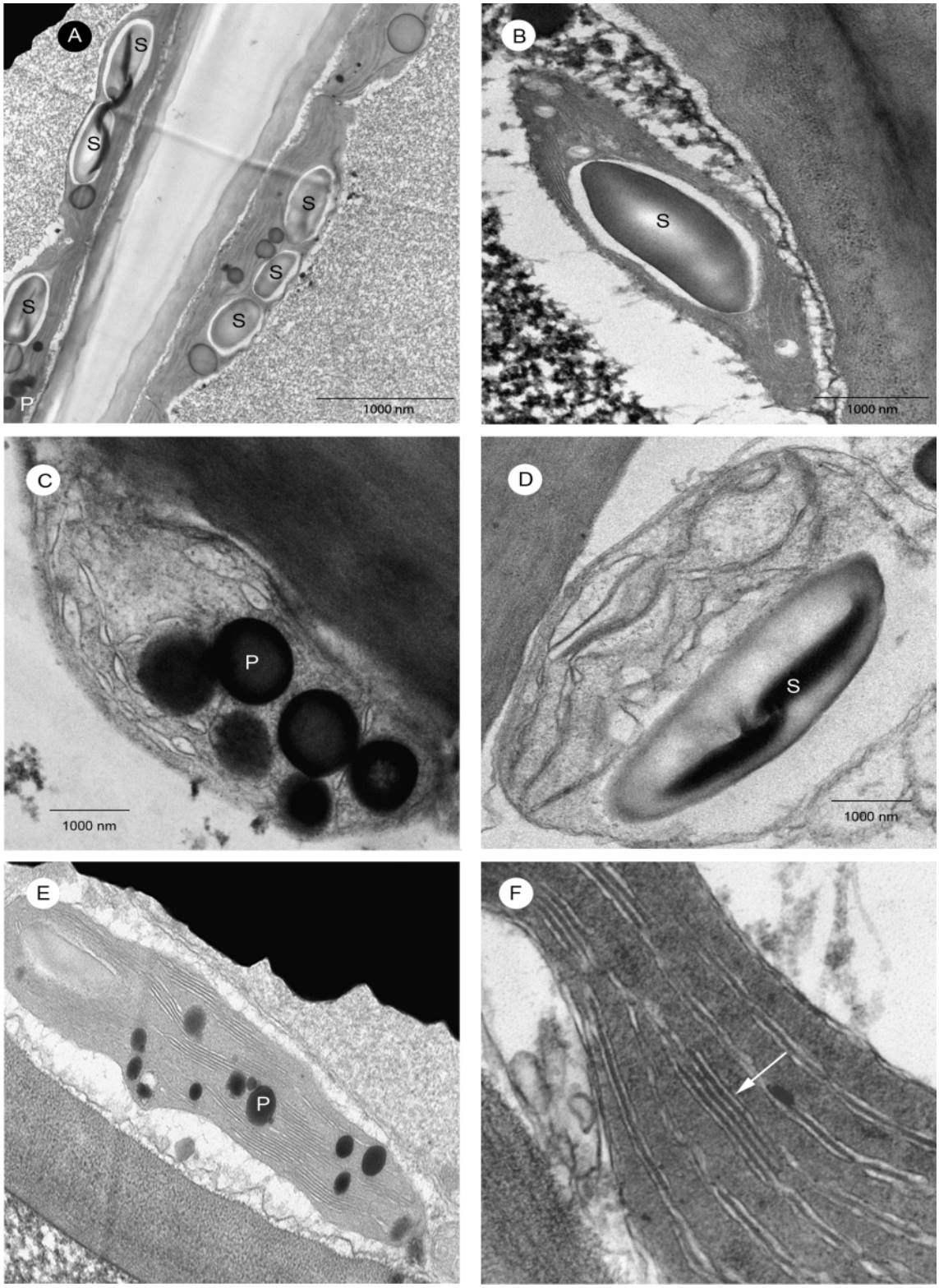

Fig. 6. Chloroplasts encountered in leaves and involucral leaves of *Leucadendron* at the three main phases of inflorescence development (see results section). Phase A (before anthesis) – (A) Chloroplasts of mature, green leaves lower down on the stem with well defined thylakoid systems and several large starch granules (S). (B) Chloroplasts of green involucral leaves with well defined thylakoid systems and generally showing one predominant starch granule. Phase C (anthesis) – (C & D) Plastids of yellow involucral leaves with deteriorated thylakoid systems and large plastoglobules (P). Plastoglobule size and numbers varied considerably. Phase D (floral death) – (E & F) After complete regreening of involucral leaves, chloroplasts had completely restored thylakoid systems with well defined granal stack (arrow). Again the presence of plastoglobules in terms of size and number varied considerably.

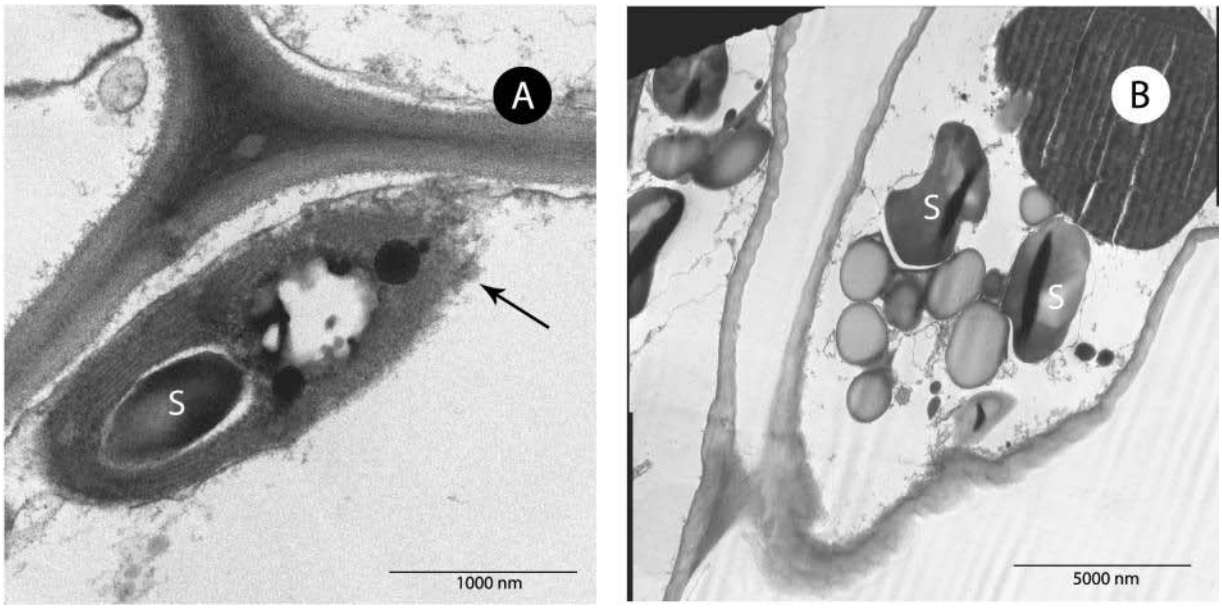

Fig. 7. Chloroplast ultra-structure encountered in regular leaves lower down on the shoot at an early stage and a late stage of senescence. (A) The plastids of yellowish regular leaves at a more advanced stage of senescence showed, besides highly degenerated thylakoid systems, the rupturing and disintegration of the chloroplast envelope (arrow). (B) At an advanced stage of senescence, the double membrane envelope of gerontoplasts had completely disintegrated, leaving behind large starch granules (S).

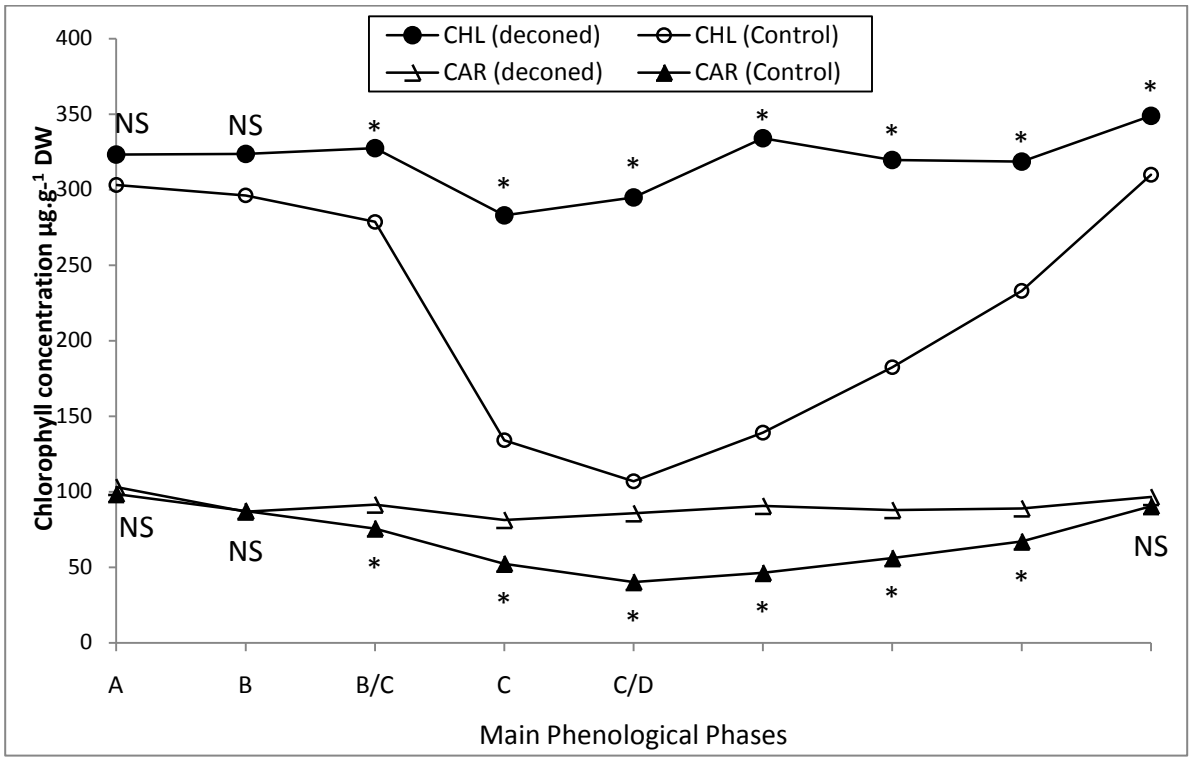

Fig. 8. The effect of deconing 'Goldstrike' shoots before flowering on the chlorophyll (CHL) and carotenoid (CAR) concentration in involucral leaves. The apical inflorescence was completely removed early in the season, well before any significant floral development. Phases: A. Early preanthesis. B. Advanced preanthesis characterised by fused perianth protruding above floral bract. C. Anthesis. D. Death of florets marks beginning of postanthesis phase. The B/C and C/D notation represents a phase change, when sample collected contained some shoots of the next phase. NS = non significant between treatments; * = significant difference ($p<0.05$) between means of treatments within a pigment class at each measurement point.

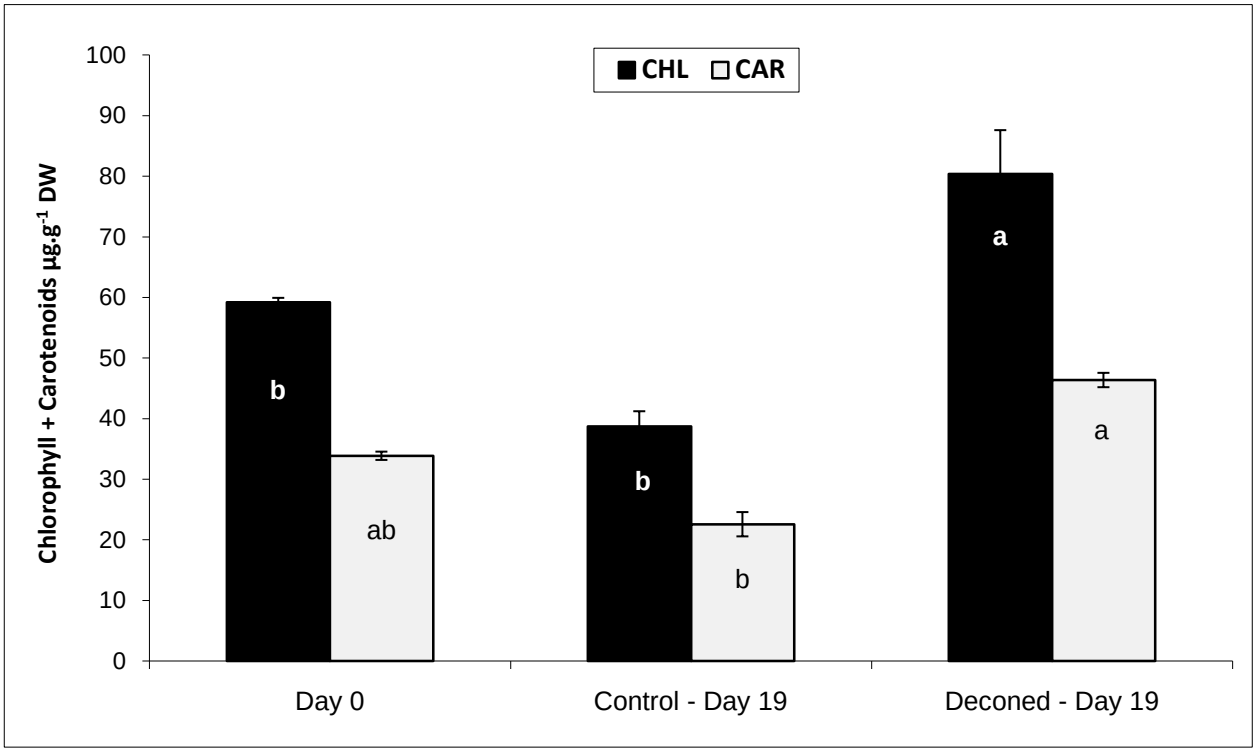

Fig. 9. A. The effect of deconing *L. salignum* shoots at full bloom on the chlorophyll (CHL) and carotenoid (CAR) concentration in the involucral leaves. "Day 0" denotes the pigment concentration on the day deconing occurred. Control and deconed shoots were harvested about 19 days later. Mean separation of a pigment class between treatments was done by least significant difference (5%) following significant F test with $P \leq 0.01$. Bars represent ±SE (n=5). **B.** The colour difference between *L. salignum* shoots deconed at full bloom (right) and the non-deconed control (left) 19 days after deconing.

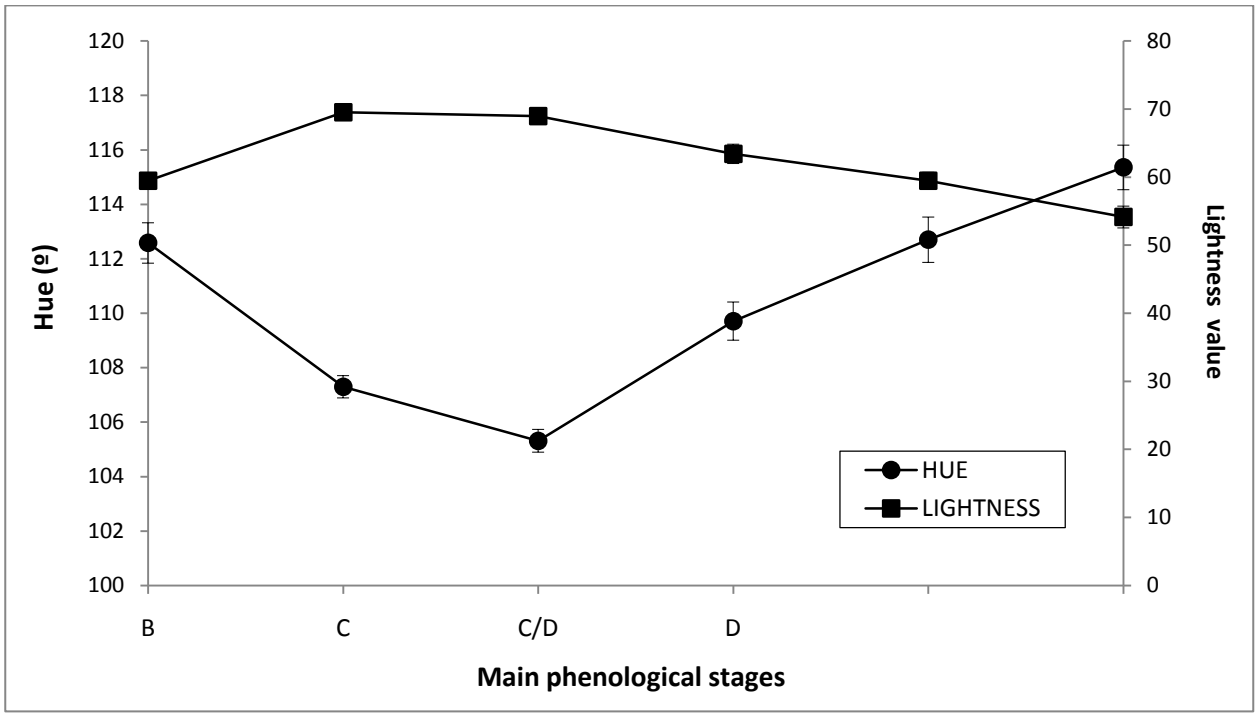

Fig. 10. Degreening and regreening of harvested 'Goldstrike' shoots in a growth chamber under continuous light (800-900 µmol·m^{-2}·s^{-1}) and set at 22 ºC. Shoots were harvested when involucral leaves were green and the fused perianth barely visible at the rim of the floral bracts. Phases: B. Fused perianth barely visible above floral bract. C. Flowering. D. Death of florets. The C/D notation represents a phase change, when sample collected contained shoots of both phases. Bars represent ±SE (n=5), but may be hidden by marker if standard error is small. Decrease in hue angle denotes an increase in yellowness (0º = red; 90º= yellow; 180º = green), while an increase in L value denotes that the colour becomes lighter.

Paper II: Dynamics of foliar anthocyanins in involucral leaves of the *Leucadendron* 'Safari Sunset' (Proteaceae)

M. Schmeisser, G. Jacobs, W.J. Steyn

Introduction

Leucadendron are sought after cut flowers on international markets due to their brightly coloured flower heads, ranging from bright yellow to red and mixtures thereof (Leonhardt and Criley 1999). The red cultivar Safari Sunset (*L. salignum x L. laureolum*) is, world-wide, one of the most widely grown commercial cultivar, making up the majority of exported stems reaching the European market (Littlejohn and Robyn 2000; Robyn and Littlejohn 2001). The long vase life, long shoot length, ease of packing and exceptionally large yields makes 'Safari Sunset' such a desirable cultivar (Barth *et al.* 1996; Leonhardt and Criley 1999).

The prime harvesting period of yellow *Leucadendron* cultivars as yellow cut flowers is relatively short, about two to three weeks, due to a rapid natural regreening of the flower heads (Schmeisser *et al.* 2010). As the involucral leaves of flower heads begin to turn from yellow to green, they no longer meet the export quality standard and are often downgraded on international markets or not accepted at all, resulting in a considerable loss of profit. Similarly, the marketing period of red cultivars, such as Safari Sunset, is shortened due to undesirable colour changes in the involucral leaves of the flower head. In South Africa, 'Safari Sunset' is harvested a few weeks after cessation of shoot growth, as shoots with dark red to maroon flower heads. As the inflorescence develops, the red colour of the involucral leaves fades and flower heads attain an unattractive muddy-red colour, making them unmarketable. The fading of red colour during this phase of development was addressed in a previous study (Schmeisser 2002). Fortunately another positive colour change occurs later in the season, rendering shoots marketable once again. Towards anthesis, the flower heads open up completely and display involucral leaves that are no longer a muddy-red, but instead mixtures of bright yellow and red and as a result shoots can be marketed as "Safari - Rainbow Colours". Were it not for this additional colour change, 'Safari Sunset' could only be exported, from South Africa, for about the first three month of the year. After the "Rainbow Colours" stage, the flower head closes completely and

flower heads once again attain the unmarketable muddy-red colouration. The yellowing and subsequent regreening of yellow Leucadendron was shown to result from a well regulated degradation and subsequent synthesis of photosynthetic pigments, directly linked to the development of the inflorescence (Schmeisser *et al*. 2010). Yellowing of involucral leaves was due to the rapid degradation of chlorophyll and to a lesser degree carotenoids, resulting in an unmasking of yellow colour. As the colour change is so tightly linked to flowering in yellow cultivars, a pollination function was proposed (Schmeisser *et al.* 2010). The colour dynamics of red Leucadendron appears to be complexed by the presence of fluctuating levels of anthocyanins, which may partly obscure the underlying changes of photosynthetic pigments.

It would be beneficial to be able to extend the marketable period of 'Safari Sunset' by delaying or even preventing the initial colour change from maroon red to muddy red or alternatively extending the time the shoots exist as "Rainbow Colours". 'Safari Sunset', like many other cut flowers, are sold on the vividness of their red colour and therefore attempts to increase redness during the muddy red phases could also be taken into consideration. Preliminary trials to increase redness were unsuccessful and therefore further basic studies to understand the colour dynamics of red *Leucadendron* were required. In order to devise successful methods to extend the marketable period of red *Leucadendron* cultivars, by manipulating the pigmentation pattern, it is necessary to understand how the dynamics of anthocyanins relates to that of the photosynthetic pigments and how these pigments together are inter-twined with the phenological development of the inflorescence.

Materials and Methods

Plant material: 'Safari Sunset' shoots, harvested in 2004, and 2005, were obtained from Arnelia Farm in Hopefield (Western Cape, South Africa, 33º02'S, 18º19'E). The sample region is characterised by a Mediterranean-type climate, with flowering times of 'Safari Sunset' starting end of June to early July, which in the southern hemisphere is mid-winter.

Sampling and statistical layout: Weekly sampling of 'Safari Sunset' commenced on 8 June and continued to 7 September in 2004 and again from 1 June to 7 September in 2005. In both years, sampling of 'Safari Sunset' commenced well after cessation of shoot growth during the muddy red phase, but prior to anthesis and continued until all florets on the cone had wilted. Flowering started between 22 and 29 June for 'Safari Sunset' for 2004 and 2005. A week prior to the start of sampling, all shoots required were randomly selected and tagged based on visual assessment of the apical cone to ensure phenological uniformity. At the same time, in 2004 and 2005, randomly selected shoots from the pool of pre-tagged shoots were deconed by pinching out the entire inflorescence and designated as deconed before flowering. In addition, in 2005, the flower heads of some of the pre-tagged shoots were prevented from opening by spiralling a 25 cm long pipe cleaner with soft bristles around the flower head. The weekly random picking of non-deconed control shoots, deconed shoots and shoots with tied flower heads (2005 only) commenced a week later. Later in the season of 2005, randomly selected shoots from the pool of pre-tagged shoots were deconed at full bloom and the treatment designated as deconed at anthesis. During all years, the random picking of twenty tagged shoots occurred weekly, divided into 5 repetitions with 4 shoots per replicate. Eight to nine involucral leaves (counting from youngest to oldest) were removed, frozen in liquid nitrogen, freeze-dried, milled and stored at -80ºC prior to pigment analysis.

Pigment extraction and analysis: Simple spectrophotometry was used to quantify chlorophyll (CHL) and carotenoids (CAR) of 'Safari Sunset' as described in Paper I. For anthocyanin quantification, 10 ml cold 3M HCL:H_2O:MeOH (1:3:16, by vol.) was added to 250 mg sample. Extraction took place for 1 hour in the dark at 4 ºC whilst stirring. After centrifugation for 10 min at 12000 X *g*, the supernatant was decanted into a vial. For a fast re-extract, 5 ml of the respective solvents were again added to the left over pellet and vortexed for 5 seconds. The re-extract was centrifuged for 10 min at 12000 X *g*, after which the supernatant of the re-extract was pooled together with the first extract, resulting in a final volume of 15 ml. After centrifugation for 10 min at 12000 X *g*, the supernatant was filtered through a 0.45µm filter (Millex-HV, Millipore Corporation, Milford, MA) and analysed spectrophotometrically using a Cary 50 Spectrophotometer Series (Varian, Mulgrave, Australia). Anthocyanin levels were measured at A_{530} and A_{653} and calculated as A_{530} - 0.24 A_{653} to compensate for the small overlap of absorbance of chlorophyll in the A_{530} region (Murray and Hackett 1991).

Microscopy: Macroscopic studies of leaf cross sections were conducted using a Wild M400 Photomacroscope (Wild-Heerbrugg, Switzerland) equipped with a Zeiss Axiocam digital camera (Carl Zeiss, Germany). Involucral leaves were taken from flower heads that had been flowering for a while already and both leaf surfaces were red. Cross sections were done by hand, using a sharp razor blade.

Digital imaging: Flower opening was measured during 2005 by taking digital images of the flower heads from above, using a high resolution Nikon DXM1200 digital camera with a 0.63x relay lens (Innovative solutions (IMP) Scientific and Petitions (Pty) Ltd, Johannesburg, South Africa), mounted on top of a standardised photographic cabinet. Lighting within the cabinet was provided by a microlite fluorescent ring light for epi-illumination (Innovative solutions (IMP) Scientific and Petitions (Pty) Ltd, Johannesburg, South Africa). A piece of measuring tape was included in the flower head photographs to provide a scale for the correct calibration of the analysis software, Image Pro-Plus 4.5 (Innovative solutions (IMP) Scientific and Petitions (Pty) Ltd, Johannesburg, South Africa). Flower head opening was determined by measuring the average distance between involucral leaves three, four and five using the same software.

Results

Anthocyanins accumulated in the vacuoles of epidermal, hypodermal as well as mesophyll cells of mature involucral leaves of 'Safari Sunset'. The leaf cross sections, as seen in as seen in Fig. 1, were made from involucral leaves of flower heads that had been flowering for a while already and therefore both leaf surface were red (see photograph of Phase D in Fig. 3). Mesophyll cells to the depth of the vascular bundles were coloured red in many leaves and no distinct difference between the upper and lower surfaces of the horizontally orientated involucral leaves were noted (n=10). The seasonal pigmentation period under investigation was divided into four phases, as defined during the phenological study of 'Goldstrike' (Schmeisser *et al.* 2010). The development of the 'Safari Sunset' inflorescence is identical to 'Goldstrike', only differing in the time of anthesis, which is about a month earlier for 'Safari Sunset'. Phase A is defined as being the early reproductive phase when floral bracts still cover and hide the tiny developing florets. Phase B is marked by the appearance of the fused

perianth above the rim of the floral bract (preanthesis). Phase C is defined as anthesis and Phase D occurs with the wilting (death) of the small florets (postanthesis).

Seasonal pigmentation pattern

During early reproductive development (Phase A), CHL and CAR levels were high, at concentrations of 246 $\mu g \cdot g^{-1}$ and 80 $\mu g \cdot g^{-1}$ respectively in 2004 and 267$\mu g \cdot g^{-1}$ and 72 $\mu g \cdot g^{-1}$ in 2005 (Fig. 2). The high photosynthetic pigment concentration coincided with relatively high concentrations of anthocyanins (1110 $\mu g \cdot g^{-1}$ in 2004 and 1197 $\mu g \cdot g^{-1}$ in 2005, Fig. 2), resulting in a muddy red-coloured flower head (Fig. 3, Phase A). During Phase A, the flower heads were completely closed, with the outermost leaves touching in the centre. As the fused perianth of florets began to appear above the rim of the floral bracts (Phase B), the flower head started opening (Fig. 4) and CHL and CAR showed signs of decreasing gradually, with the most rapid loss of both pigments coinciding with the start of anthesis, marking the start of Phase C (Fig. 2). The net degradation of photosynthetic pigments was 77% and 54% for CHL and CAR respectively during 2004 and about 74% of CHL and 50% of CAR in 2005. This resulted in a significant drop in CHL:CAR ratios from roughly 3.1 to about 1.5 in both years. Contrary to photosynthetic pigments, the anthocyanin concentration increased significantly during this period (Fig. 2), by as much as 41% in 2004 and 30% in 2005. The pattern of anthocyanin synthesis did differ between 2004 and 2005, in that the initial response to flowering, as well as the highest anthocyanin peak occurred later in 2004. At the start of anthesis, the flower head is wide open, displaying bright red and yellow colours (Fig. 3, Phase C). At this point, the cut flowers are often referred to as “Safari - Rainbow Colours”. There is a vast difference in colouration of the involucral leaves from the inside to the outside leaves, as well as the inside facing (later upward facing) and corresponding outside facing (later downward facing) leaf surface, seemingly dependent on the amount of light exposure (Fig. 3). During Phase A, the innermost involucral leaves are fairly green, with the inside facing surface being greener than the corresponding outside facing, light-exposed surface, which was redder. Distinct green patches are evident on the outside facing surfaces where leaves must have overlapped or caused distinct shading. As the flower heads open towards anthesis, the previously shaded interior surfaces become exposed to light and turn red. During the early stage of anthesis, the innermost involucral leaves are still mostly yellow, with varying degrees of redness, but by the time the flowering period comes to an end, the entire inner surface of involucral leaves has turned red and the

difference between the inside surface and outside surface becomes less apparent (Fig. 3, Phase D). As the florets wilt, the flower head gradually closes again. A few weeks later the head is once again completely closed and very similar in appearance to the flower heads seen at the start of the flowering period (Fig. 3, Late Phase D).

Effect of preventing the flower head from opening

Keeping the flower head physically closed during the season prevented the increase of anthocyanins generally associated with the start of the flowering phase (Fig. 4). There appears to be a good correlation between the opening and closing of the flower head and the changing anthocyanin concentration. Flowering of shoots that were kept closed appeared to be normal, although in some shoots a little delayed. In general, the pattern of CHL and CAR degradation was very similar between closed heads and control shoots that opened naturally. There was a significant initial increase of both CHL and CAR a week after winding the pipe cleaners around the flower heads, pointing to some initial adjustments of photosynthetic pigments. This might be due to lower light conditions within the flower heads, as the artificially closed heads tended to be more tightly closed than the control shoots. Both treatments reached similar low levels of ±70 $\mu g \cdot g^{-1}$ CHL and ±40 $\mu g \cdot g^{-1}$ CAR at the height of anthesis. The forced closing treatment did differ significantly from the control in that the CHL, as well as CAR levels never quite recovered to the levels encountered at the start of measurement, remaining significantly lower than the control.

Effect of deconing the flower head

The complete removal of the inflorescence (deconing) well before anthesis inhibited the colour change of involucral leaves in both years (Fig. 5 & Fig. 6), as it prevented the severe degradation of CHL, the lesser degradation of CAR, as well as the synthesis of anthocyanins. Furthermore, deconing before anthesis (DBA) prevented the opening of the flower head. When shoots were deconed at anthesis (DAA), shoots appeared to regreen faster than control shoots, while also maintaining a prolonged higher level of anthocyanins (Fig. 7). CAR showed a weak significant treatment-time interaction ($P=0.0483$), whereas CHL showed an almost significant interaction ($P = 0.0535$). Anthocyanins increased significantly about 14 days after deconing ($P = 0.0216$) and then remained consistently higher than the control until the last week of sampling. CHL and CAR showed a very

delayed reaction to deconing, relative to control shoots, in that a change in pigmentation only occurred after about 28 days (Fig. 7). The CHL:CAR ratio which differed significantly (P = 0.0155) 28 days after deconing, changed from 2.31 to 2.9 and 2.39 to 2.5 for deconed and control shoots respectively (data not shown).

Discussion

The cut flower 'Safari Sunset' is marketable at two distinct phases during a season. A few weeks after cessation of shoot growth, they are sold as dark red to maroon cut flowers and again at anthesis when flower heads have turned into mixtures of bright yellow and red. The yellowing part of the 'Safari Sunset' colour change appears to be very similar to changes observed in yellow *Leucadendron*, resulting from a well-regulated degradation and subsequent synthesis of photosynthetic pigments (Schmeisser *et al.* 2010). However, the colour dynamics is not as clear cut as changing from green to yellow and back, as the whole colour change is complexed by the presence of varying levels of anthocyanins.

The general colour change of the flower heads of 'Safari Sunset' from just prior to anthesis (preanthesis) to anthesis and postanthesis is evident from Fig. 3. Also very noticeable in Figure 3 is the concurrent opening and closing of the entire flower head as the inflorescence proceeds through its developmental stages. The significance of this opening phenomenon in terms of colour will be discussed below. Although the intensity of red colour is directly related to anthocyanin concentration, the expression of final leaf colour as perceived by a human observer is based on complex interactions between the presence or absence of a pigment class, the types present within each class, their relative concentrations and the blending effect of all types of pigment classes present (Brouillard 1983; Lancaster *et al.* 1994; Lancaster *et al.* 1997). In chrysanthemums, for example, the presence of anthocyanins together with carotenoids was reported to modify the colour from orange-red to bronze (Teynor *et al.* 1989). Colour and/or the vividness of a specific colour may change, as the ratios of relevant pigments change.

The underlying pigmentation pattern in terms of the levels of CHL and CAR is identical to that encountered in yellow *Leucadendron* (Schmeisser *et al.* 2010), in that CHL is almost completely

degraded and to a lesser degree the carotenoids. The first sign of gradual photosynthetic pigment degradation occurs when the fused floral perianth appears above the rim of the floral bract and intensifies towards anthesis. 'Safari Sunset' degrades up to 77% of its chlorophyll and 54% of its carotenoids towards anthesis, resulting in a CHL:CAR ratio of about 1.5 (Fig. 2). Were it not for the presence of anthocyanins, 'Safari Sunset' would sport a far more intense yellow flower head than the actual yellow cultivar 'Goldstrike', which has a CHL:CAR ratio of 2.7 (Schmeisser *et al.* 2010). With floral death, CHL and CAR levels are again restored to levels encountered before anthesis. The notion that the photosynthetic pigmentation pattern in 'Safari Sunset' also underlies strict developmental regulation is strengthened by the following facts. The main colour changes happen at very distinct stages of inflorescence development such as the protrusion of the florets, subsequent flowering and death of florets. The degradation pattern and its link to specific developmental phases is identical each year and an identical pattern is encountered in all yellow cultivars, where it was shown that the colour change was indeed developmentally regulated (Schmeisser *et al.* 2010). Furthermore, the removal of the inflorescence before anthesis completely prevented CHL and CAR degradation (Fig. 5) designating the cone as the origin of the cue for colour change.

The possible controlling factor accounting for the anthocyanin pattern is more difficult to discern. At first glance it does appear that anthocyanins levels are also developmentally regulated, simply responding to the phenological development of the inflorescence (Fig. 2). Anthocyanins increase significantly with the onset of anthesis and the fact that deconing before anthesis completely inhibits anthocyanin synthesis does point toward this fact (Fig. 2, Fig. 6). However, when considering the other results obtained, anthocyanin dynamics does not seem to be quite as simple. Regreening of involucral leaves of most *Leucadendron* is the rule, rather than the exception. The degradation of CHL, however, is an inherently dangerous process which requires meticulous regulation and protective mechanisms to prevent damage to leaf tissue (Hörtensteiner 2006), and ensure successful regreening. Amongst the myriad of functions that have been ascribed to anthocyanins, photoprotection appears to be one function that is generally accepted (Steyn *et al.* 2002; Gould *et al.* 2010; Zhang *et al.* 2010), at least in the sense that if anthocyanins are present, they will act as a light screen, attenuating light levels striking the photosynthetic system. In 'Safari Sunset' therefore, due to location of anthocyanins in the upper and lower tissue layers (Fig. 1), it can be assumed that they will provide a form of

photoprotection. Whether the anthocyanins were primarily synthesised to perform this function is a different matter. 'Safari Sunset' is a hybrid of *L. salignum* and *L. laureolum* and was selected for its red colouration and it is inherently dangerous to ascribe an ecological function to a human selected trait, such as colour. Although red cultivars have essentially been selected for their enhanced red colour, the reddening of leaves during the regreening phase also occurs in some yellow cultivars during the regreening phase (Fig. 8). Keeping the flower head artificially closed prevented anthocyanin synthesis toward anthesis (Fig. 4A). The closed flower heads flowered normally (personal observation, M. Schmeisser), as is also evident from the characteristic CHL and CAR degradation pattern towards anthesis (Fig. 4B). The fact that the CHL degradation of closed flower heads was more or less identical to control shoots and that there was no concurrent anthocyanin synthesis weakens the idea of anthocyanins being developmentally regulated. A different hypothesis is therefore proposed. Previously shaded plant tissue that is subsequently exposed to high irradiance has been shown to synthesise anthocyanins to provide photoprotection and reduce photo-oxidative stress in these tissues (Ma and Cheng 2004). Therefore in 'Safari Sunset', as the flower head opens towards anthesis and the involucral leaves become more horizontally orientated, previously shaded leaf surfaces are exposed to high light intensities and become photosensitised, therefore requiring additional photoprotection. In response to this, anthocyanins are synthesised in the upper layers of the newly exposed leaf surface, resulting in the drastic increase of their total concentration. The photographs in Figure 3 depict this quite clearly. The inside surfaces of involucral leaves become more uniformly red as the flower head opens up towards anthesis (Phase A to Phase D). The time between Phase C and Phase D as seen in these photographs is roughly 10 days, explaining the more intense and more uniform red colouration as leaves had been exposed to light stress for longer. At a much later stage after flowering, when the flower head has completely closed up again (Fig. 3, Late Phase D), anthocyanin levels decreased to levels encountered before flowering. At this stage, photoprotection is no longer required, as inner facing leaf surfaces are once again shaded (Fig. 2). The initially outside facing leaf surfaces (and later downward facing surfaces as the flower head opens) are consistently red through all phases of development, although they vary in intensity of red colour, depending on the changing CHL content. The observed anthocyanin pattern in 'Safari Sunset' is therefore presumed to be linked to the opening of the flower head or more precisely to the amount of photo-oxidative stress experienced by the involucral leaves, rather than directly on the development of the

inflorescence. This would explain why additional anthocyanin synthesis does not occur when flower heads are kept closed, as light stress would not be experienced. Similarly, disbudding before anthesis completely prevents opening of the flower head and therefore anthocyanin synthesis is also not required. CHL and CAR degradation was also prevented by deconing before anthesis, unlike when only keeping the flower head artificially closed, as both are developmentally regulated and the signal for colour change had been removed by deconing (Fig. 5). The new hypothesis also explains why the anthocyanin pattern may be variable between years, as the pattern can most probably be ascribed to environmental conditions (mostly light and temperature) that exist during opening of the flower head. Lower temperatures along with high light intensities are known to increase photo-stress in leaves (Steyn *et al.* 2002) and would therefore increase anthocyanin synthesis.

Deconing of shoots at full bloom (DAA) resulted in rapid regreening of the pure yellow cultivar *L. salignum* (Schmeisser *et al.* 2010). Similarly, deconing 'Safari Sunset' shoots at anthesis resulted in the faster regreening of the flower head and prolonged elevated levels of anthocyanins, relative to the control. There was an almost greater increase in CHL (P= 0.0535), a definite significant change in CAR combined with the significant increase of anthocyanins (about 20% higher than control shoots) (Fig. 7A-C). The significantly higher CHL:CAR ratio of deconed shoots further supports the notion that shoots definitely regreened faster than non-deconed shoots, although visually more difficult to discern when compared to yellow cultivars. Interestingly, the colour difference only really became apparent after about 28 days, which is somewhat longer than noted for *L. salignum*, where the main colour difference was recorded after 19 days (Schmeisser *et al.* 2010). Whether the presence of the high anthocyanin levels has any effect on the regreening process is uncertain. However, the response pattern of photosynthetic pigments to the complete removal of the inflorescence is identical to that encountered in yellow *Leucadendron* (Schmeisser *et al.* 2010) and again designates the inflorescence as the origin of the cue for colour change. More importantly, however, in terms of red colour development is the response of anthocyanins to deconing at anthesis. The raised levels of anthocyanins appeared to be consistently higher than control shoots for more than 20 days. The economic feasibility of deconing shoots at anthesis requires further investigation in terms of additional labour cost and possible damage caused by deconing. The response is, however, worth keeping in mind as an opportunity to possibly enhance red colour in red *Leucadendron* cultivars.

From a horticultural perspective, this study has significant value as it helps in defining the correct strategy to manipulate colour in red *Leucadendron* cultivars. Colour manipulation techniques can be approached from several angles, based on the results of this study, each with a different aim in mind. *Leucadendron* are short-day plants and flowering time can be shifted by subjecting plants to artificial long-day conditions using incandescent lamps (Hettasch and Jacobs 2006). Therefore, flowering of 'Safari Sunset' can theoretically be shifted, at least for growers in the southern hemisphere, to the more lucrative marketing window of October, November or December. Whether such a large shift is possible requires further investigation. Fortunately the change in photosynthetic pigmentation and opening of the flower head (which determines the increase in red colouration) is directly linked to flowering and therefore the colour change will still take place even if the flowering period is shifted. The effect of the generally warmer climate during that time of year on red colour development will have to be evaluated, as higher ambient temperatures often cause a decrease in anthocyanin concentrations. A different approach is needed if the aim is to intensify red colour expressed by the flower head, by actively promoting anthocyanin synthesis and possibly promoting chlorophyll degradation, which will make the flower head appear a more bright red. To produce a more intense red flower head, the manipulation techniques should be aimed at increasing photo-oxidative stress experienced by the involucral leaves, but during the stage when the flower head begins to open, as anthocyanin synthesis is directly linked to opening of the flower head. Attempts to manipulate red colour when the flower head is still closed would be futile or at best, less effective. Manipulations could also be aimed at prolonging the characteristic of a specific phase, such as the "Rainbow colour" product. Strategies should be geared towards prolonging the flowering period in order to delay the regreening of the involucral leaves as well as the closing of the flower head.

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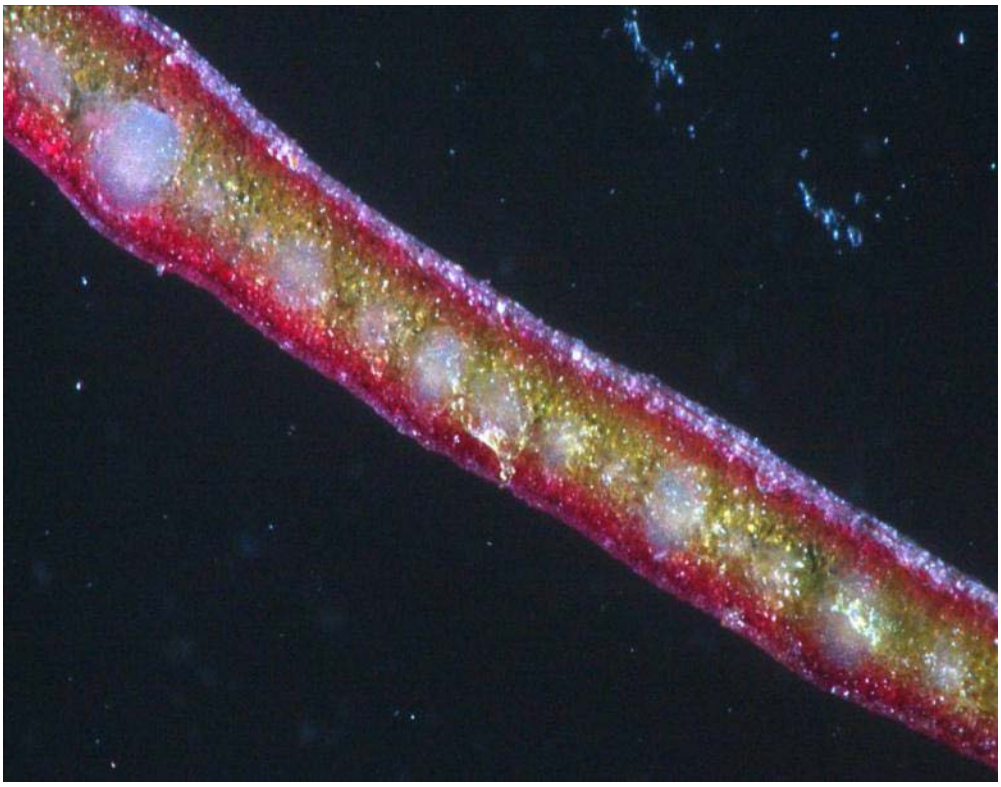


Fig. 1. Cross-section of a ‘Safari Sunset’ involucral leaf showing the location of anthocyanin containing tissue. The photograph was taken a few weeks after all florets on the inflorescence had wilted and the entire flower head was red (see photograph of Phase D in **Fig. 3**). No significant difference was noted between the inside and outside surfaces of the horizontally orientated involucral leaves (n=10).

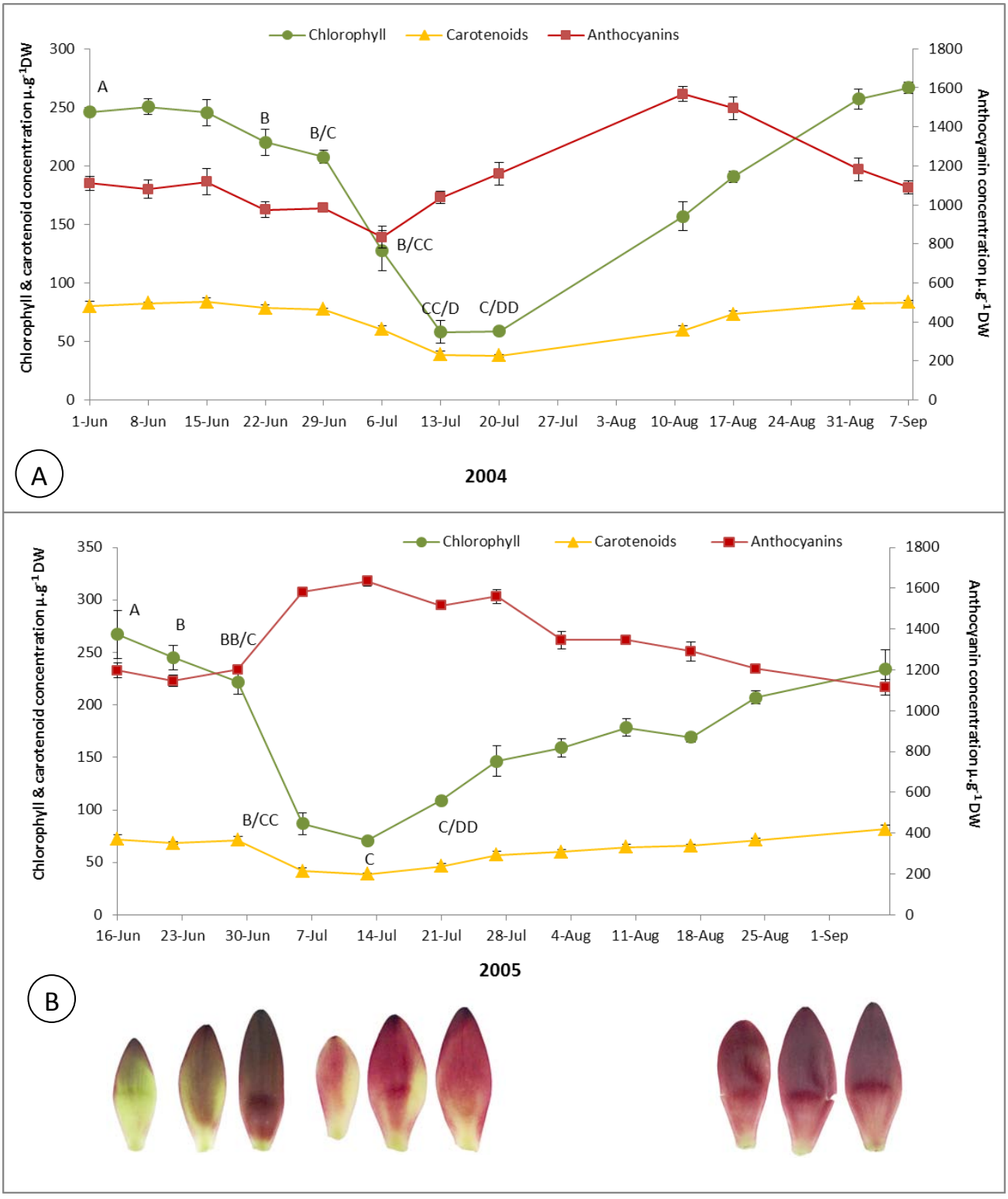


Fig. 2. Seasonal pigmentation patterns of involucral leaves of 'Safari Sunset' in relation to main developmental phases of the inflorescence during 2004 (A) and 2005 (B). Phases: A. Non flowering inflorescence. B. Fused perianth protruding above floral bract. C. Flowering D. Death of florets. The BB/C, B/CC notation for example represents phase changes, when sample collected contained shoots of two phases. The double letter as in B/CC for example, indicates that the sample collected had more flowering shoot than shoots with protruding floral sacks. Bars represent ±SE (n=5). Photograph of the involucral leaves serves to indicate the general colour change in relation to changes in pigmentation.

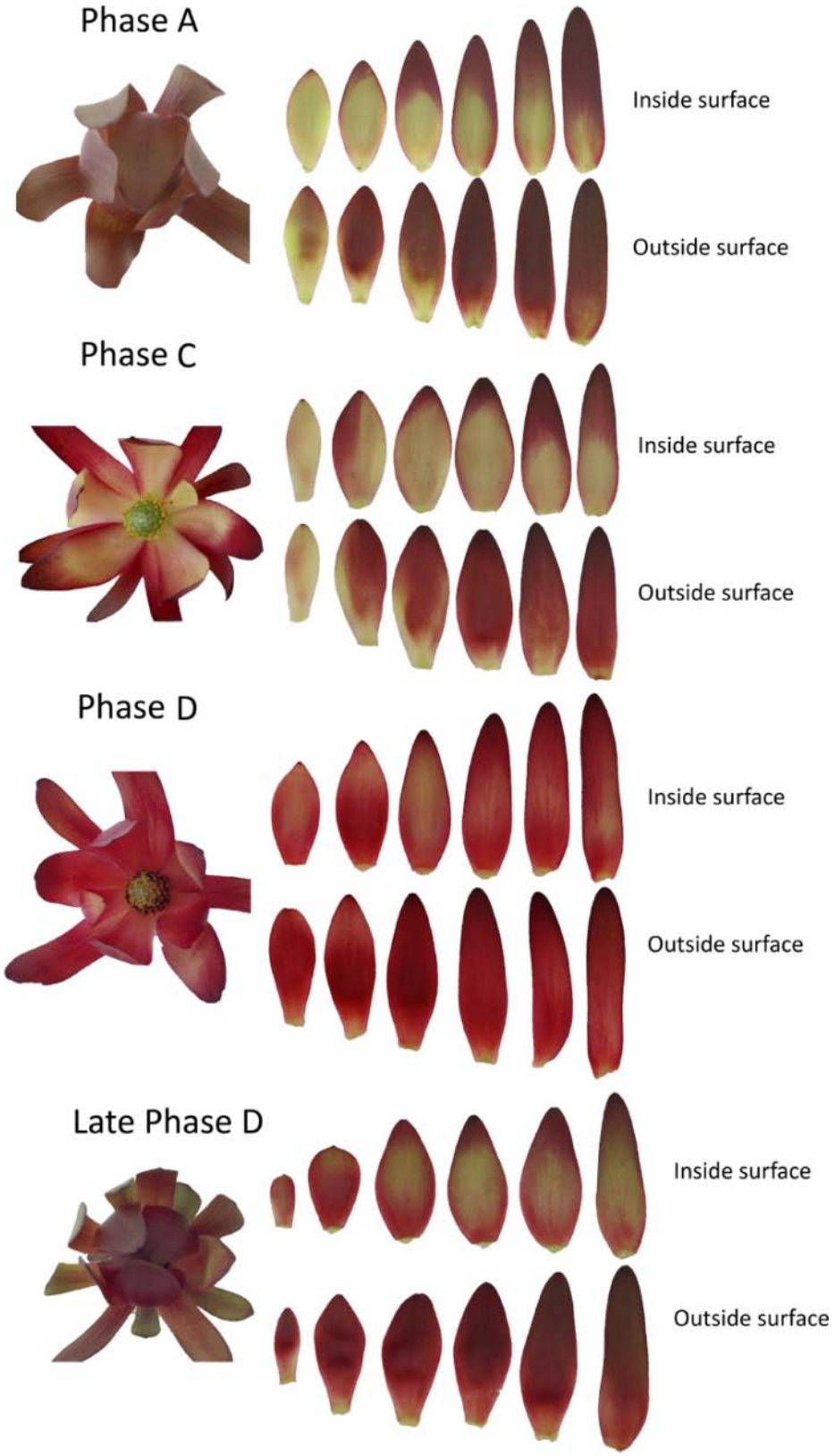
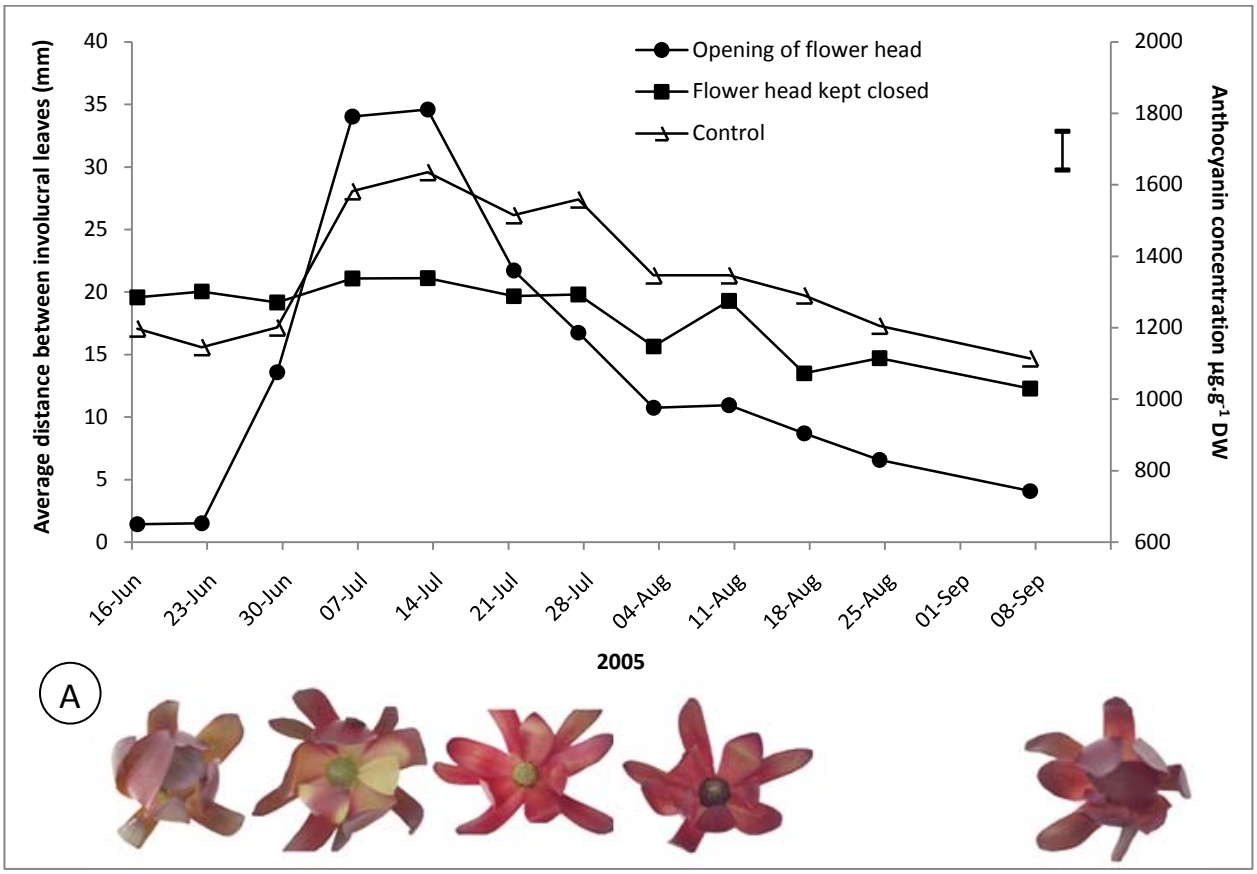


Fig. 3. Colour changes of the flower head, individual involucral leaves and their respective inner and outer facing surfaces throughout a flowering season. Phase A – Early reproductive development, before any visual signs of flowering. Phase C – Early anthesis. Phase D – Death of the florets and the gradual closure of the flower head. Late Phase D – Sample taken a few weeks after the start of floral death. The flower head is completely closed again and similar in appearance as at the start of the reproductive period. Phase B, when the fused perianth just appears above the floral bract, was not included here as a significant colour change was not yet apparent in the photographs.



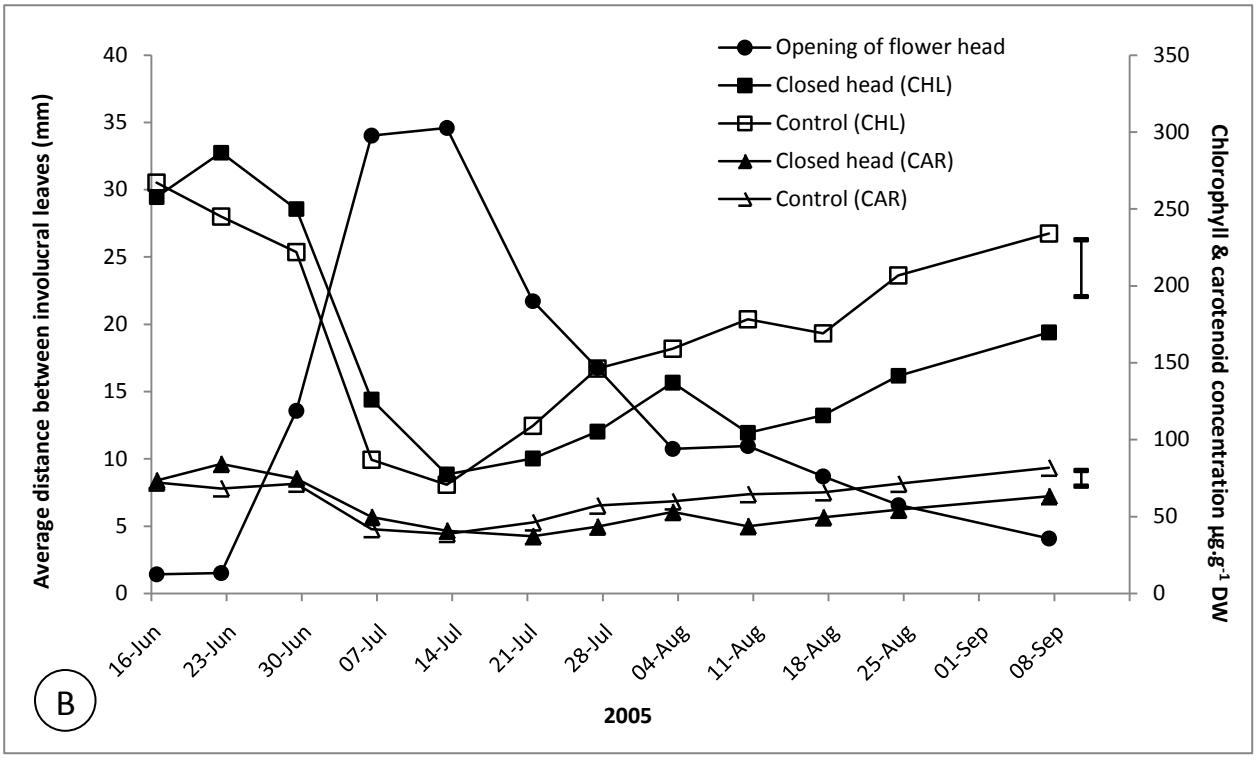


Fig. 4. Seasonal photosynthetic pigmentation pattern of involucral leaves of 'Safari Sunset' in relation to the natural opening of the flower head and the effect of artificially keeping the flower head closed. A. Changes in anthocyanin content B. Change in chlorophyll (CHL) and carotenoid (CAR) content. The photographs serve to indicate the various stages of normal flower head opening. (The vertical bars indicate means separated by LSD 5% for $P_{date*treatment} < 0.05$.). Arrow indicates anthesis.

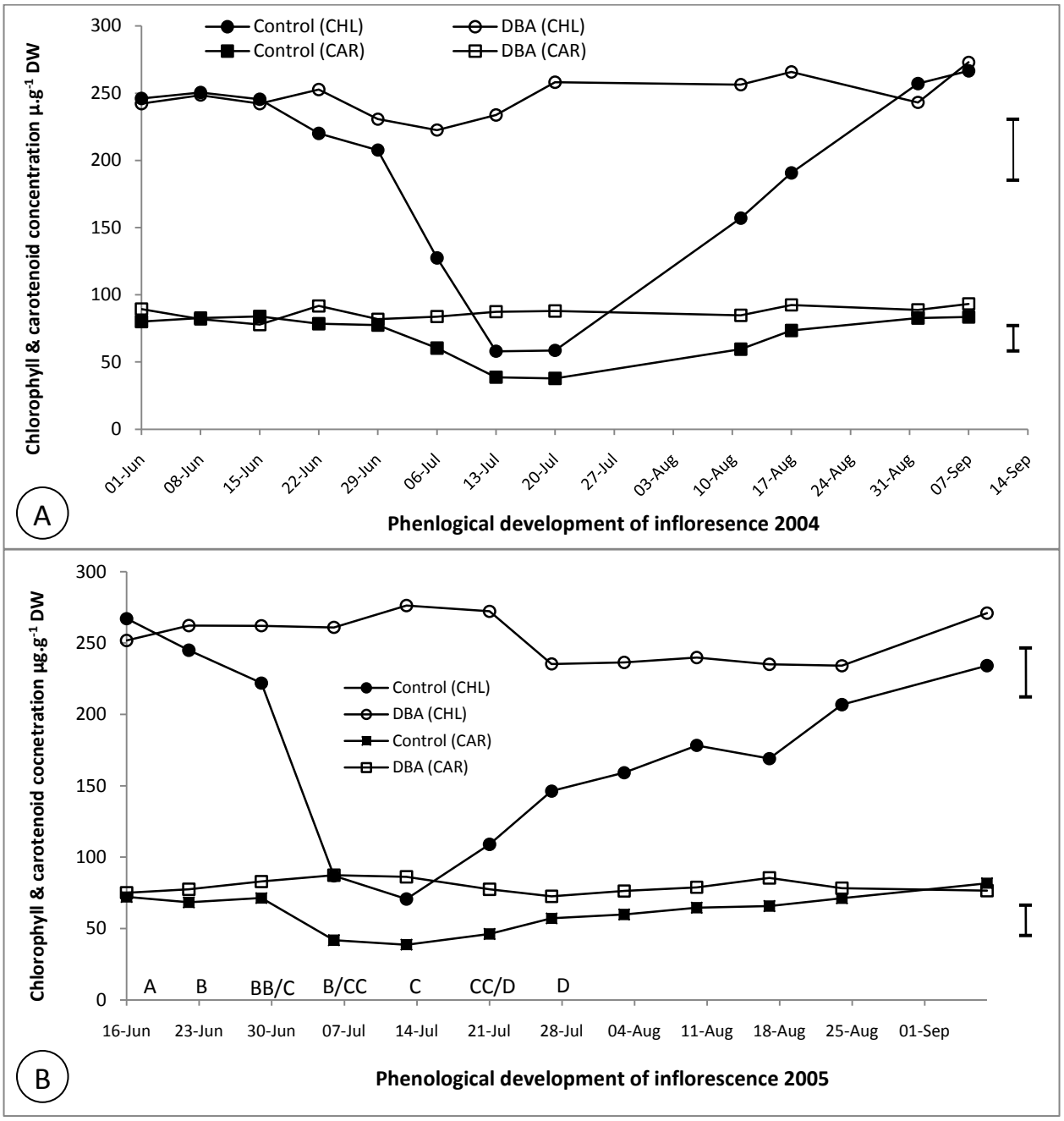


Fig. 5. Effect of deconing shoots before anthesis (DBA) on the chlorophyll (CHL) and carotenoid (CAR) concentration of involucral leaves of 'Safari Sunset' during 2004 (A) and 2005 (B). Phases: A. Non flowering inflorescence. B. Fused perianth protruding above floral bract. C. Flowering D. Death of florets. The BB/C, B/CC notation for example represents phase changes, when sample collected contained shoots of two phases. The double letter as in B/CC for example, indicates that the sample collected had more flowering shoot than shoots with protruding floral sacks. (The vertical bars indicate means separated by LSD 5% for $P_{date*treatment} < 0.05$.)

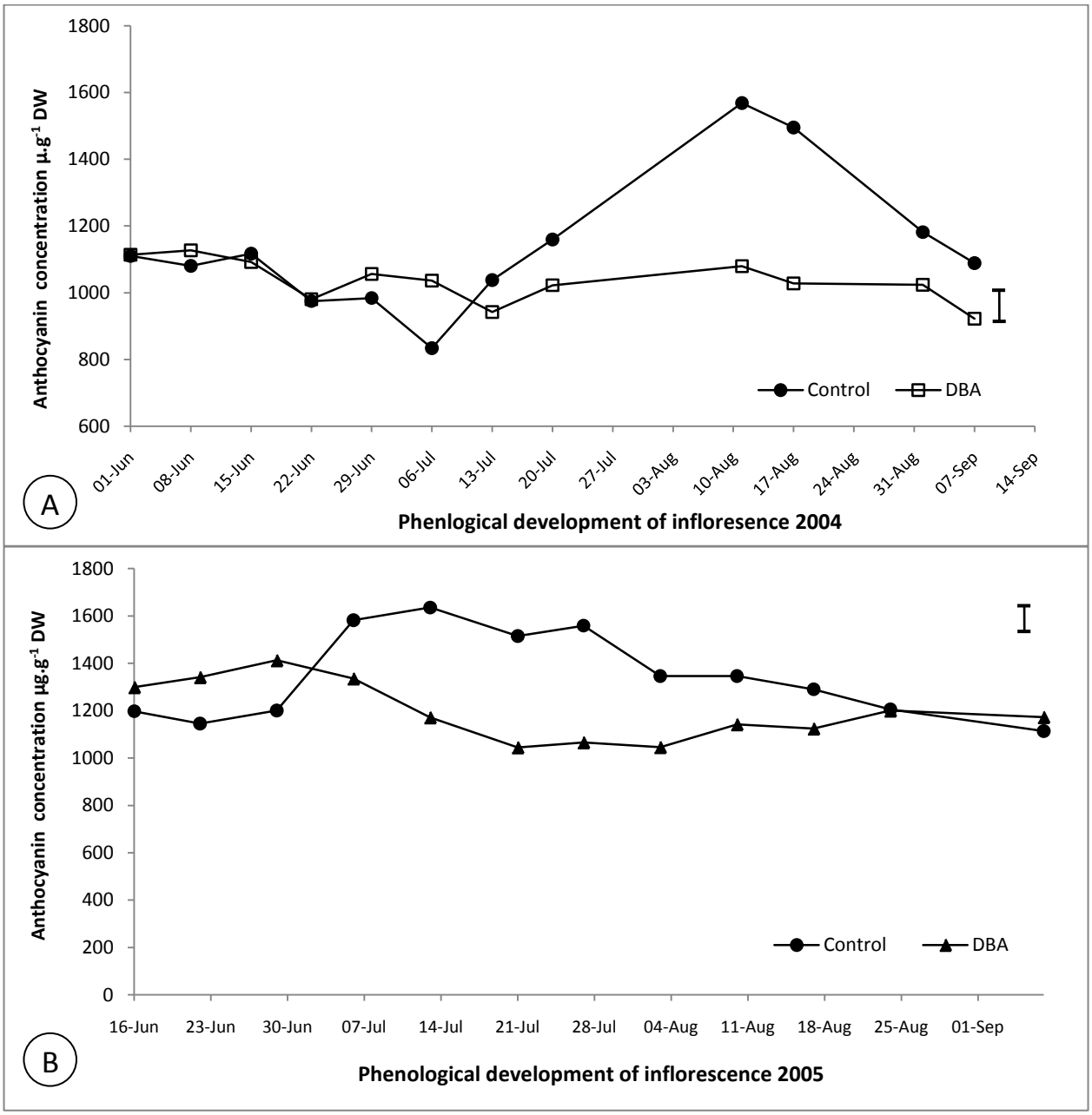


Fig. 6. Effect of deconing shoots before anthesis (DBA) on the anthocyanin concentration of involucral leaves of 'Safari Sunset' during 2004 (A) and 2005 (B). Phases: A. Non flowering inflorescence. B. Fused perianth protruding above floral bract. C. Flowering D. Death of florets. The BB/C, B/CC notation for example represents phase changes, when sample collected contained shoots of two phases. The double letter as in B/CC for example, indicates that the sample collected had more flowering shoot than shoots with protruding floral sacks. (The vertical bars indicate means separated by LSD 5% for $P_{date*treatment} < 0.05$.)

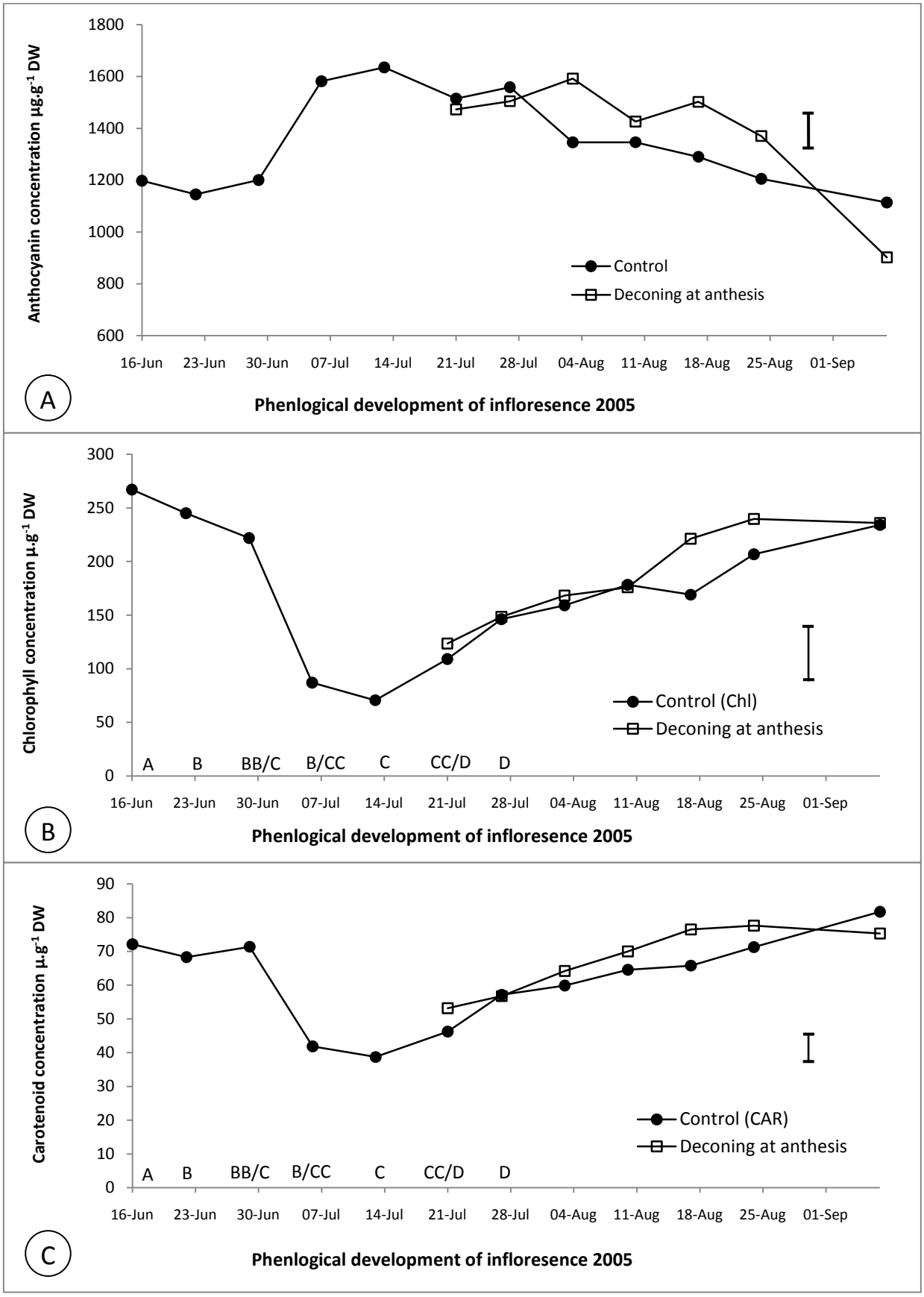


Fig. 7. Effect of deconing shoots at anthesis on the (A) anthocyanin (B)chlorophyll (CHL) and (C) carotenoid (CAR) concentration of involucral leaves of 'Safari Sunset' during 2005. Phases: A. Non flowering inflorescence. B. Fused perianth protruding above floral bract. C. Flowering D. Death of florets. The BB/C, B/CC notation for example represents phase changes, when sample collected contained shoots of two phases. The double letter as in B/CC for example, indicates that the sample collected had more flowering shoot than shoots with protruding floral sacks. (The vertical bars indicate means separated by LSD 5% for $P_{date*treatment} < 0.05$.)

Fig. 8. Anthocyanin synthesis during the regreening involucral leaves of the yellow *Leucadendron* cultivar 'Inca Gold'

Paper III: Economy in function: regreening in female *Leucadendron* (Proteaceae)

Introduction

Natural regreening of yellow plant organs is a rare phenomenon in nature, considering the vast species diversity on our planet. So far it has only been reported to occur in a few flower and floral accessory structures (Grönegress 1974; Sitte 1974; Tran *et al.* 1995; van Doorn 1997; Tavares *et al.* 1998; Salopek-Sondi *et al.* 2002), some fruit (Coggins Jr. and Lewis 1962; Devide and Ljubesic 1974; Mayfield and Huff 1986; Prebeg *et al.* 2008) and contrary to belief (van Doorn 2005) even in leaves (Ikeda 1979; Koiwa *et al.* 1986). The natural regreening of the leaves of *Euonymus* and *Buxus* as reported by Ikeda (1979) and Koiwa *et al.* (1986) is related to the seasonal shift from winter to summer. In *Leucadendron*, the involucral leaves of flower heads turn yellow and then regreen naturally, but unlike *Euonymus* and *Buxus*, this colour change is directly linked to the development of the inflorescence, rather than directly to environmental conditions. The degreening of involucral leaves, due to its close link to floral development, is thought to play a significant role in the pollination syndrome (Schmeisser *et al.* 2010).

Of all the studies dealing with the natural regreening of plant structures, only a few address the significance of the regreening phenomenon, in terms of what the plant stands to gain by prolonging the longevity of these structures. Although mature green leaves are generally the main source of photosynthates, green floral structures (both sterile and fertile) are reported to significantly contribute to carbon fixation (Aschan *et al.* 2005). Therefore a possible function of regreening would be to contribute to the net carbon gain of the plant, especially during fruit development. The regreened sepals of *Helleborus niger* were shown to provide a significant amount of photosynthates during initial fruit development in spring, when mature leaves were not yet present (Salopek-Sondi *et al.* 2000; Aschan *et al.* 2005; Herrera 2005). Indeed, in many of the species mentioned, the perianth, spathe or sepals do not regreen unless successful pollination and subsequent fruit development takes place (Grönegress 1974; Sitte 1974; Salopek-Sondi *et al.* 2002). *Leucadendron* differ in this respect, as involucral leaves will regreen irrespective of successful pollination. Regreening of involucral leaves

appears to be the rule and occurs as a response to the senescence of the small florets. Any factor that results in the expedited death of the florets will cause the involucral leaves to regreen sooner (Schmeisser *et al.* 2010). It is uncertain to what extent the involucral leaves contribute to the carbon budget of the plant. If the primary function of leaf regreening in *Leucadendron* is not photosynthesis to support the developing inflorescence, as was shown in the other cases of regreening, then an alternative reason for regreening must exist.

The question of what a female *Leucadendron* plant stands to gain by regreening its involucral leaves is the focal point of this study. A few possible hypotheses as to why regreening occurs in *Leucadendron* are postulated and evaluated.

Materials and Methods

Plant material: Female shoots of the cultivars Goldstrike, Safari Sunset, Rosette, Laurel Yellow and Inca Gold were obtained from the commercial protea farm Vredekloof situated in Paarl (Western Cape, South Africa, 33º02'S, 18º19'E) during the seasons of 2006, 2007 and 2008. The sample region is characterised by a Mediterranean-type climate with flowering time of 'Safari Sunset' (*L. laureolum x L. salignum*) and 'Inca Gold' (*L. laureolum x L. salignum*) starting in July, and that of 'Goldstrike' (*L. salignum* x *L. laureolum*), 'Rosette' (*L. laureolum x L elimense*) and 'Laurel Yellow' (*L. laureolum* x *L. discolor*) in late August. Cultivars were used in this study for the same reasons discussed in Schmeisser *et al.* (2010).

The development period has been divided into three distinct phases (the reader is referred to Paper I and Paper II for a more detailed descriptions and photographs of each phase). As the *Leucadendron* inflorescence is distinctly cone shaped, the term inflorescence and cone will be used synonymously:

I. *Preanthesis* - when florets are not yet visible above the rim of the floral bracts and involucral leaves are green in yellow cultivars ('Goldstrike', 'Inca Gold' and 'Laurel Yellow') and dark maroon in the red cultivar Safari Sunset.

II. *Anthesis* – when the inflorescence is flowering. Involcural leaves are yellow in yellow cultivars and a mixture of yellow and red in 'Safari Sunset'.
III. *Postanthesis* – all florets on the cone have wilted and involucral leaves of yellow cultivars have regreened as well as in the red cultivar 'Safari Sunset' resulting in a dark maroon flower head.

Photosynthetic measurements: Leaf gas exchange parameters (net assimilation rate and stomatal conductance) were measured of 'Laurel Yellow', using an open gas exchange system with independent CO_2 control (Li-6400; LI-COR Inc, Lincoln, NE, USA). Measurements were done on attached involucral leaves (n=20), at the three main phases of cone development (preanthesis, anthesis and postanthesis). Photosynthetic activity of the regular leaves lower down on the shoot (between leaf number 20 and 25) was measured only during the postanthesis phase to allow for relative comparison of photosynthetic rates between regular and involucral leaves. Measurements were taken at a constant flow rate of 300 $\mu mol.s^{-1}$. CO_2 levels were maintained at 380 $\mu mol.mol^{-1}$, temperature at 24 ºC and photosynthetic photon flux (PPF) at 1500 $\mu mol.m^{-2}.s^{-1}$, which was at light saturating level for this cultivar. The leaf vapour pressure deficit was maintained between 1.5 and 2 kPa to prevent stomatal closure. To determine whether there was any form of photosynthetic activity in yellow involucral leaves at anthesis, the flow rate was reduced to 100 $\mu mol.s^{-1}$. The measurements taken at this low flow rate were not included in the statistical analysis, as strictly speaking only measurements at the same flow rate should be compared.

Photosynthetic contribution of involucral leaves: To determine the importance of involucral leaves for inflorescence development in terms of dry weight and size, different types of leaves were removed just after the last florets on the 'Goldstrike' inflorescence began to wilt (07 August 2006). The four treatments consisted of: the removal of only involucral leaves (ten leaves, counting from the youngest to the oldest); the removal of only regular leaves on the stem; the removal of all leaves and control shoots which were left intact. All leaves were removed with scissors to prevent damage to the cone axis or stem. The trial was laid out in a randomised complete block design, with 8 blocks and two shoots per plot. When the trial ended on 07 October 2006, all shoots were harvested and brought to the laboratory for further measurements. Cone size (length and diameter) was measured and fresh

mass determined. Cones were freeze-dried, weighed, milled and stored in a -80ºC freezer awaiting further analysis.

Carbohydrate analysis

Reducing sugars: Carbohydrates were extracted overnight (16 hours) from 200 mg freeze-dried involucral leaf tissue by adding 5 ml of a methanol, chloroform and water (MCW) solution (12:5:3). The extracts were centrifuged at 4000 X *g* for 10 minutes (20± 1ºC) and the supernatant decanted into a new set of polycarbonate tubes. One ml of MCW was again added to the residue pellet and vortexed for 5 seconds. The re-extract was centrifuged for 10 min at 4000 X *g*, after which the supernatant of the re-extract was pooled together with the first extract, resulting in a final volume of 6 ml. The remaining residue was stored at 4 ºC for further starch analysis. One ml of chloroform was added to the pooled extracts now containing the reducing sugars and shaken vigorously before adding 1 ml of de-ionised water and shaken again. The pooled extract was centrifuged for 10 min at 4000 X *g*, to aid the separation of layers. The aqueous top-layer was pipetted into glass vials and rotary evaporated on a SC 210 A Speed Vac Plus (Thermo Savant) on low heat for 80 min and a further 90 min without heat. The dried extracts were re-dissolved in 5 ml de-ionised water on a shaker. C18 cartridges packed with C18 (55-105 µm, Bulk Packing Material, Waters, Milford, USA), that had been conditioned with 100% pure methanol the previous night, were washed four times with 3 ml de-ionised water. Cartridges were then loaded with 1 ml sample, which under vacuum moved through the column into respective glass vials. The cartridges were washed four times with 2 ml de-ionised water into the same glass vials. The extracts were filtered through 0.45 µm filters (Millex-HV, Millipore Corporation, Milford, MA, USA) into reverse-phase high performance liquid chromatography (HPLC) vials. Sugars were separated and quantified using an HPLC system (HP 1100; Agilent Technologies, Palo Alto, CA) equipped with a refractive index detector (GA 1362A, Agilent Technologies). Sugar separation was achieved by eluting the extracts with a 4.5 mM H_2SO_4 solution at a constant flow rate of 0.4 $ml.min^{-1}$ through a Transgenomic, IC Sep, Ice-Ion-300 column held at 30ºC. Individual sugars were quantified against external standards (Sigma, St. Louis, USA).

Starch: The stored residue from the reducing sugar analysis was washed with 2 ml of 20% methanol, vortexed and centrifuged for 10 minutes at 4000 X *g*. The supernatant was discarded and the residue washed three times with 5 ml de-ionised water, again being vortexed and centrifuged at 4000 X g, discarding the supernatant after each wash. After discarding the last supernatant, a further 4.5 ml de-ionised water was added to the residue, along with 0.5 ml of an internal sorbitol standard (5 $g.L^{-1}$). Residues were placed on a heating block at 100 ºC for 2 hours. After cooling, 100 µl of a 2.5 mM potassium hydrogen succinate buffer (pH 4.6) was added to each residue along with 0.05 g of polyvinylpolypyrrolidone (PVPP, P-6755, Sigma, St. Louis, USA) and shaken for 15 minutes (300 rpm). Then 200 µL of a buffered amyloglucosidae enzyme solution (20 $\mu g.ml^{-1}$ in 0.6 mM potassium succinate, *Aspergillus niger*, Fluka) was added and mixed gently. Tubes were placed on a heating block (56 ºC) overnight to allow for enzymatic hydrolysis of starch. After 17 hours, tubes were place in boiling water for 5 min to denaturise the enzyme and then centrifuged at $3000g_n$ for 10 minutes. Supernatant was pipetted off in to clean 10ml flasks. Another 2ml of deionised water was added to the residue to ensure complete extraction of starch breakdown products. After centrifugation at $3000g_n$ for 10 minutes this supernatant pipetted off and added to first collected supernatant. This process was repeated gain to ensure that all glucose has been extracted from the residue. The supernatants were filtered through 0.45 µm filter (Millex-HV, Millipore Corporation, Milford, MA) into HPLC vials and analysed by HPLC using the identical machine and settings as described in the analysis of the reducing sugars.

Protection of inflorescence: To determine whether involucral leaves provide some degree of photoprotection, the involucral leaves were removed in 'Safari Sunset', 'Inca Gold' and 'Rosette' exposing the entire inflorescence and control shoots were left intact. These cultivars were chosen, because they differ in the degree their flower head opens up during anthesis. The 'Safari Sunset' flower head is entirely closed during early inflorescence development, but opens up completely towards anthesis. After the small florets have wilted, the flower head closes again as the involucral leaves regreen. In 'Inca Gold', the flower head never opens during any time of inflorescence development nor anthesis. 'Rosette' on the other hand, is characterised by having a completely open flower head at all stages of development, never showing signs of opening or closing. To determine the degree of photoinhibition between exposed and non-exposed inflorescences, chlorophyll

fluorescence was measured using a pulse modulated fluorimeter (FMS2, Hansatech Instruments Ltd., Norfolk, UK). The trial was laid out in complete randomised block design with 15 blocks and 1 shoot per plot. Shoots of 'Safari Sunset' and 'Inca Gold' were harvested monthly starting on 31 August 2007 and ending 30 January 2008. 'Rosette' shoots were also collected monthly, but the trial commenced later (20 September 2007), as the shoots only finished flowering by end of September. Before harvesting the shoot, the north facing side (sun-facing side) of the cone shaped inflorescence was marked. In the laboratory the inflorescences were removed and cut longitudinally in half. The half of the cone that had been marked as the north-facing side was immediately floated on water in a petridish, with the marked side facing upward. After allowing the cones to dark adapt for 2 hours, maximum quantum efficiency (Fv/Fm) measurements were taken. Cones were randomly placed in a growth cabinet held at 17 ºC (±2ºC) and subjected to a high PPF of 900-1000 $\mu mol.m^{-2}.s^{-1}$ for 16 hours. Irradiance was provided by two 400-W high-pressure sodium lights (SON-T; Osram MgBh, Munich, Germany) situated on top of the growth chamber. An acrylic (Perspex) sheet of 5 mm thickness separated the shoots from lights, preventing direct heating of shoots and aiding temperature control within the chamber. The water in the petridish was replenished as needed, to prevent drying out of the cone. After the 16 hours of high light exposure, cones were dark adapted for 2 hours and Fv/Fm was measured. Cones were placed back into a dark growth chamber, held at 23 ºC (±2ºC) and allowed to recover overnight for 16 hours, after which Fv/Fm measurements of the cones were taken again.

Colour measurements: When presented, leaf colour measurements were taken using a chromameter (Nr-3000; Nippon Denshoku, Tokyo, Japan) and expressed in terms of hue angle (H). For a short expose on why H values were used to express colour, rather than reflection measurements, the reader is referred to Schmeisser *et al.* (2010).

Pigment extraction and analysis:

Simple spectrophotometry was used to quantify chlorophyll (CHL), carotenoids (CAR) and anthocyanins (ANT) as described in Schmeisser *et al*. (2010) and Paper II.

Data analysis

The data were analysed using the General Linear Models (GLM) procedures of the SAS program (SAS release 9.1, SAS Inst., Cary, NC).

Results

Photosynthesis: There was a drastic decrease of photosynthesis (from about 4 $\mu mol.m^{-2}.s^{-1}$ to essentially zero) as involucral leaves changed from green (preanthesis) to yellow at anthesis (Table 1). Stomatal conductance followed a similar pattern to that of the photosynthetic rate, decreasing from 0.09 mol $H_2O.m^{-2}.s^{-1}$ before anthesis to 0.01 mol $H_2O.m^{-2}.s^{-1}$ at anthesis. Upon regreening, the photosynthetic rate, as well as stomatal conductance of involucral leaves was restored to levels comparable to rates before anthesis. In comparison, the photosynthetic rate and stomatal conductance of regular leaves, was significantly higher, about 11 $\mu mol.m^{-2}.s^{-1}$ and 0.3 mol $H_2O.m^{-2}.s^{-1}$ respectively. These values are comparable to rates found in other studies on *Leucadendron* at nutrient poor and elevated nutrient levels (Midgley *et al.* 1999; Mohammadian *et al.* 2007). Therefore, the photosynthetic rate of green and regreened involucral leaves before and after anthesis is less than half the rate encountered in regular leaves. Slight photosynthetic activity was detected in yellow involucral leaves of about 0.8 $\mu mol.m^{-2}.s^{-1}$, but only if the flow rate was reduced to 120 $\mu mol.s^{-1}$.

Cone size and carbohydrate content: This part consisted of four treatments differing in the types of leaves that had been removed. Besides the control shoots that were left intact, the treatments consisted of the removal of only involucral leaves, removing all regular stem leaves but leaving involucral leaves and the removal of all leaves. Removing only the involucral leaves had no significant effect on the size (length and diameter) or dry weight of the inflorescence relative to control shoots (Table 2). This is especially evident from the percentage increase in size relative to cone measurements taken at the start of the trial. With complete leaf removal, the cone was 13 mm shorter than the cones of the control shoots and only showed a relative size increase of 30% compared to the roughly 45% of control shoots. Interestingly, there was also a significant difference in

cone size between the treatment of removing only regular stem leaves and the treatment of removing all leaves. When all the leaves had been removed, the cones were smaller in size (~6mm in both length and diameter) and had 9% and 6% lower increase in length and size respectively. Despite the difference in cone size between the two treatments, there was no difference in dry weight. Dry weight was however significantly lower in comparison to the control (29% for stem leaves removed and 44% lower for all leaves removed). In terms of total carbohydrate content per cone (the summation of starch, glucose, fructose and sucrose), there was no significant difference between control shoots, the treatment where only involucral leaves had been removed and the treatment where only regular leaves had been removed. When all leaves were removed, the total carbohydrate content per cone was 42% lower than control shoots. In terms of total carbohydrate concentration per cone, the treatment where all regular leaves had been removed from the stem contained the highest carbohydrate concentration of 31.7 $mg.g^{-1}$, which differed significantly from control shoots, as well as from the treatment where only involucral leaves were removed. The treatment where all leaves had been removed differed significantly from all other treatments in having the lowest carbohydrate levels in terms of both content and concentration.

Cone protection: In all three cultivars, namely Safari Sunset, Inca Gold and Rosette, the data showed a significant increase in photoinhibition experienced by the inflorescences in the field, as the season progressed, as well as a significant effect of removing involucral leaves. Exposed cones of all three cultivars showed slightly reduced Fv/Fm values (0.76 to 0.79), evident of sun adaptation in comparison to Fv/Fm values of 0.82 to 0.84 of control cones. The response to 16 hours of severe light stress, expressed as % photoinhibition, differed between the cultivars. The cones of 'Safari Sunset' control shoots, when subjected high irradiance, showed a high percentage of photoinhibition (around 45%) throughout the sampling period (Fig.1A). Fv/Fm values of around 0.45 were measured in control cones, indicating chronic photoinhibition if not photodamage. It appears more likely that photodamage has occurred, as control cones hardly showed any signs of recovery after 16 hours of dark adaptation (Fig. 1B). Exposed inflorescences of 'Safari Sunset' were significantly less inhibited (~54% less) by the light stress treatment throughout the season. In the second month, there was even an improvement in coping with light stress. Although the ability to recover from the light stress treatment decreased in both control and exposed cones as the season progressed, the exposed

inflorescences had a higher ability to recover than the enclosed inflorescences. In comparison, the inflorescence of 'Inca Gold' control shoots became increasingly photoinhibited throughout the season when subjected to high light stress. Photoinhibition increased from 34% in the beginning to 55% at the end of the sampling period (Fig. 2A). The exposed inflorescences of 'Inca Gold' seemed to cope significantly better with the light stress, throughout the entire season (15% inhibition at the start of the season increasing only to 43% inhibition at the end), as well as the recovery thereof (Fig. 2B). Although in 'Rosette' the % photoinhibition caused by light stress increased throughout the season, it was relatively low (12 to 20% photoinhibition), and there was no difference between treatments (Fig. 3A). The inflorescences of 'Rosette' recovered well overnight, with the amount of residual photoinhibition averaging around 3.5% during the first month and around 7.5% during the second month of sampling (Fig. 3B).

There was no significant difference in the cone colour of 'Rosette' (data not presented). As cones are never enclosed, they already sported a dark red colour (Hue angle of 30) at the start of the trial. In both 'Safari Sunset' and 'Inca Gold', the exposed cones were redder than the control cones (Fig. 4 and Fig. 5). Interestingly, there appeared to be distinct loss of red colour in exposed 'Inca Gold' cones. By the last sampling date, the floral bracts of the 'Rosette' inflorescence had become completely brown. So much so, that neither fluorescence nor colour could be measured after two months. A distinct browning was also recorded on the exposed cones of 'Inca Gold' and 'Safari Sunset', but little to none in the covered control cones (Fig. 6). Closer inspection of floral bracts revealed that it was just the epidermal to hypodermal layers that had become necrotic and that below that the tissue was still green and alive, similar to that encountered in *Hakea* and *Pinus* (Midgley and Enright 2000). A distinction was made between browning floral bracts or the necrosis of these bracts. Necrosis was recorded when the floral bracts was dead and dry and had, as a consequence of drying out, opened up. No significant difference was recorded in terms of cones that were necrotic, whether for treatment or cultivar.

Discussion

There are several plausible reasons as to why the involucral leaves of *Leucadendron* should regreen. First of all, they could significantly contribute to the carbon budget of the plant during inflorescence development, by directly supplying the developing inflorescence with photosynthates. This appears to be the most common function associated with regreening of floral and floral accessory structures (Salopek-Sondi *et al.* 2000; Aschan *et al.* 2005). The spathe in *Zantedeschia* aborts rapidly and never regreens if the inflorescence is not pollinated (Grönegress 1974; Tavares *et al.* 1998). Although the life-span of sepals in unpollinated flowers of *Helleborus niger* is reported to be the same as that of pollinated counter parts, they never regreen (Salopek-Sondi *et al.* 2002). In insect pollinated *Leucadendron*, regreening appears to be the rule and will occur irrespective of successful pollination and reproductive development. The 50% lower photosynthetic rates of involucral leaves relative to regular leaves lower down on the stem (Table 1) and the fact that the removal of involucral leaves did not significantly alter the size or dry weight of the inflorescence (Table 2), does not point towards regreening playing a major role in improving the carbon budget of the plant during floral development.

The difference in the total carbohydrates content per inflorescence, where total carbohydrates refers to the sum of starch, glucose, fructose and sucrose, is more difficult to explain. Removing involucral leaves did not appear to affect the development of the inflorescence at all, as evident from the similar size, weight, and total carbohydrate and total carbohydrate concentration per cone. Removing all the regular leaves on the stem did however result in smaller cones. The fact that the total carbohydrate content is not significantly lower relative to the control, point towards an alteration in the partitioning of the available carbohydrates. It appears as if the carbohydrates are preferentially allocated to the non-structural carbohydrate pool, rather than being invested in growth of the cone. The significantly higher total non-structural carbohydrate concentration in the cones of this treatment (31.7 $mg.g^{-1}$ relative to 25.4 $mg.g^{-1}$ for the control) results from the smaller cone size, whilst maintaining a high carbohydrate content. When all leaves are removed, the cone size as well as carbohydrate levels are significantly reduced. There was a 57% decrease in total carbohydrate content relative to the control and despite the significantly smaller cone size, there was even a 25% drop in total carbohydrate concentration. Therefore it appears that *Leucadendron* shoots are not able translocate carbohydrates

from other, more richly supplied plant parts. The fact that the treatment where the regular leaves have been removed from the stem is able to maintain its total carbohydrate content relative to the treatment where all leaves have been removed, does point towards the involucral leaves supplying the inflorescence with photosynthates. It appears as if involucral leaves possess the ability to compensate for the loss of regular leaves to a small extent. Compensation is commonly reported in plants in response to excessive herbivory (Dyer *et al.* 1991; Meyer 1998; Thomson *et al.* 2003). To satisfactorily answer the degree of compensation, further empirical data on gas exchange parameters of involucral leaves will have to be obtained. However, the focus should remain on the fact that the removal of involucral leaves did not significantly affect the non structural carbohydrate status of the cone. The question is, if regreening does not seem essential to support the developing inflorescence, what other functions of regreening exist? In 'Safari Sunset' and 'Inca Gold' the involucral leaves are cup-shaped around the inflorescence and overlap each other to a large degree, resulting in considerable in between leaf shading. This is not ideal, but does appear as if some kind of protective function might be involved.

Leucadendron are considered to be weakly serotinous in that they retain their seeds within the cone for one to three years depending on the species (Midgley and Enright 2000). From personal observation, it was noticed that when the floral bracts of *Leucadendron* become necrotic and dry, they tend to split apart, thereby exposing the seeds for dispersal. Therefore it stands to reason that the floral bracts need to remain alive and sufficiently supplied with water in order to have successful serotiny of at least a few years. The *Leucadendron* flower head of many species and cultivars open up towards anthesis, entirely exposing the inflorescence. This may be an advantage during pollination (Schmeisser *et al.* 2010), but it may be considered a disadvantage to expose them to environmental extremities after anthesis. Flowering of most species occurs in mid to late winter and therefore the floral bracts of the cone will be exposed to higher irradiance and elevated radiant heating experienced during the hot and dry summer following anthesis. The degree to which the flower head opens differs significantly between the different species and cultivars. Some cultivars, such as Safari Sunset open their flower heads completely while others not at all, like Inca Gold for example. In species where the flower head does open with anthesis (not 'Rosette'), it was shown that it closes again with regreening to once again entirely enclose the inflorescence (Schmeisser *et al.* 2010).

Therefore it does seem plausible that keeping the involucral leaves alive and enclosing the cone could provide protection against high irradiance and radiant heating. However the current data does not support the notion that the protection offered by the regreened involucral leaves is the primary function of regreening. The cones did not seem perturbed by being exposed to higher irradiance and temperatures relative to enclosed cones under natural conditions and were shown to adapt to higher light levels by reddening of the floral bracts. The exposed shoots were far less susceptible to photoinhibition caused the light stress and appeared to recover more easily, indicating a high degree of adaptation to high irradiances. It was observed, that the floral bracts of exposed cones (also the continuously exposed cones of 'Rosette') showed strong browning of the epidermal and subepidermal layers, so much so that one last date of sampling fluorimeter readings became senseless (between 88% of sun-facing surface). No browning was encountered on cones of 'Safari Sunset' and 'Inca Gold' that were enclosed by the involucral leaves. The surface temperature of exposed cones, at an ambient temperature of 34 ºC, was on average between 6 to 8 ºC higher than ambient and between 3 to 5 ºC higher than enclosed cones. It appears that the browning encountered in exposed *Leucadendron* cones likens the bract browning reported to occur in waratahs (*Telopea* spp.) in that the bracts exposed to full sunlight showed significantly more browning (McConchie *et al.* 2005; Martyn *et al.* 2007; Martyn *et al.* 2008). The term sunburn browning is advocated to describe observed cone browning. This is term used to describe sunburn in apples as a result of high fruit temperatures in the presence of light (Schrader *et al.* 2008). However, the main fact debunking the entire idea that involucral leaves regreen to protect the cones, is that there was no significant difference in the amount of necrotic cones encountered between the exposed and non-exposed 'Safari Sunset', 'Inca Gold' or 'Rosette' inflorescences, especially since cones of 'Rosette' are exposed at all times.

Maybe the question should rather be phrased differently. Does the female *Leucadendron* have anything to gain by not regreening and discarding its involucral leaves after anthesis? The answer to that appears to be a definite no, as it really only stands to lose by discarding its involucral leaves. *Leucadendron* generally grow on nutrient poor soils (Midgley *et al.* 1999) and therefore display the characteristic leaf traits generally associated with poor nutrient conditions i.e. having thick, sclerophyllous leaves with a high dry mass per leaf area and a long individual leaf lifespan (Wright and

Westoby 2003). By discarding the involucral leaves, the female *Leucadendron* would lose a leaf that had a large construction cost.

The answer to why the female *Leucadendron* regreen their involucral leaves is most likely related to a cost-benefit calculation rather than directly to photosynthetic output or protection alone. Retaining expensive building material and nutrients in a nutrient poor environment appears to be the most sensible adaptation for survival. The real novelty lies in the degreening of involucral leaves to aid in pollination (Schmeisser *et al.* 2010), which appears like a transient snaphot of leaves evolving into petals.

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Table 1. Gas exchange parameters (net assimilation rate and stomatal conductance) measured on attached involucral leaves (n=20) of 'Laurel Yellow', during the three main phases of inflorescence development. Gas exchange parameters for regular leaves lower down on the shoot were only measured at the postanthesis phase and have been included as a comparison.

Leaf type	*Phase*	*Photosynthesis ($\mu mol\ CO_2.m^{-2}.s^{-1}$)*	*Conductance ($mol\ H_2O.m^{-2}.s^{-1}$)*
Involucral	Preanthesis	4.28 b*	0.0967 b
	Anthesis	~ 0 c	0.0113 c
	Postanthesis	4.82 b	0.0864 b
Regular	Postanthesis	11.36 a	0.262 a
(P < 0.05)		< 0.0001	< 0.0001

* Values within a column followed by the same letters are not significantly different (means separated by LSD 5%).

Table 2. Impact of leaf removal on the size, dry weight and carbohydrate content of the inflorescence of the cultivar Goldstrike. Besides control shoots where no leaves were removed, the treatments consisted of removing only the involucral leaves, removing the regular stem leaves and leaving behind only the involucral leaves, as well as removing all leaves.

Leaf type removed	*Dry weight/cone (g)*	*Length (mm)*	*Diameter (mm)*	*Total carbohydrates / cone [y]*	
				Content (mg)	*Conc. ($mg.g^{-1}$)*
Control	5.7 a	50.9 a (44%[z])	29.9 a (46%)	141.14 a	25.4 b
Involucral	5.5 a	49.6 a (44%)	28.8 a (44%)	127.1 a	23.1 b
Stem leaves	4.0 b	43.2 b (35%)	25.2 b (36%)	124.0 a	31.7 a
All leaves	3.2 b	37.8 c (26%)	22.7 c (30%)	60.3 b	18.8 c
(P < 0.05)	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

* Values within a column followed by the same letters are not significantly different (means separated by LSD 5%)

[Y] Total carbohydrate content = starch + glucose + fructose + sucrose

[z] Values in brackets indicate the percentage increase relative to measurements taken at the start of the trial.

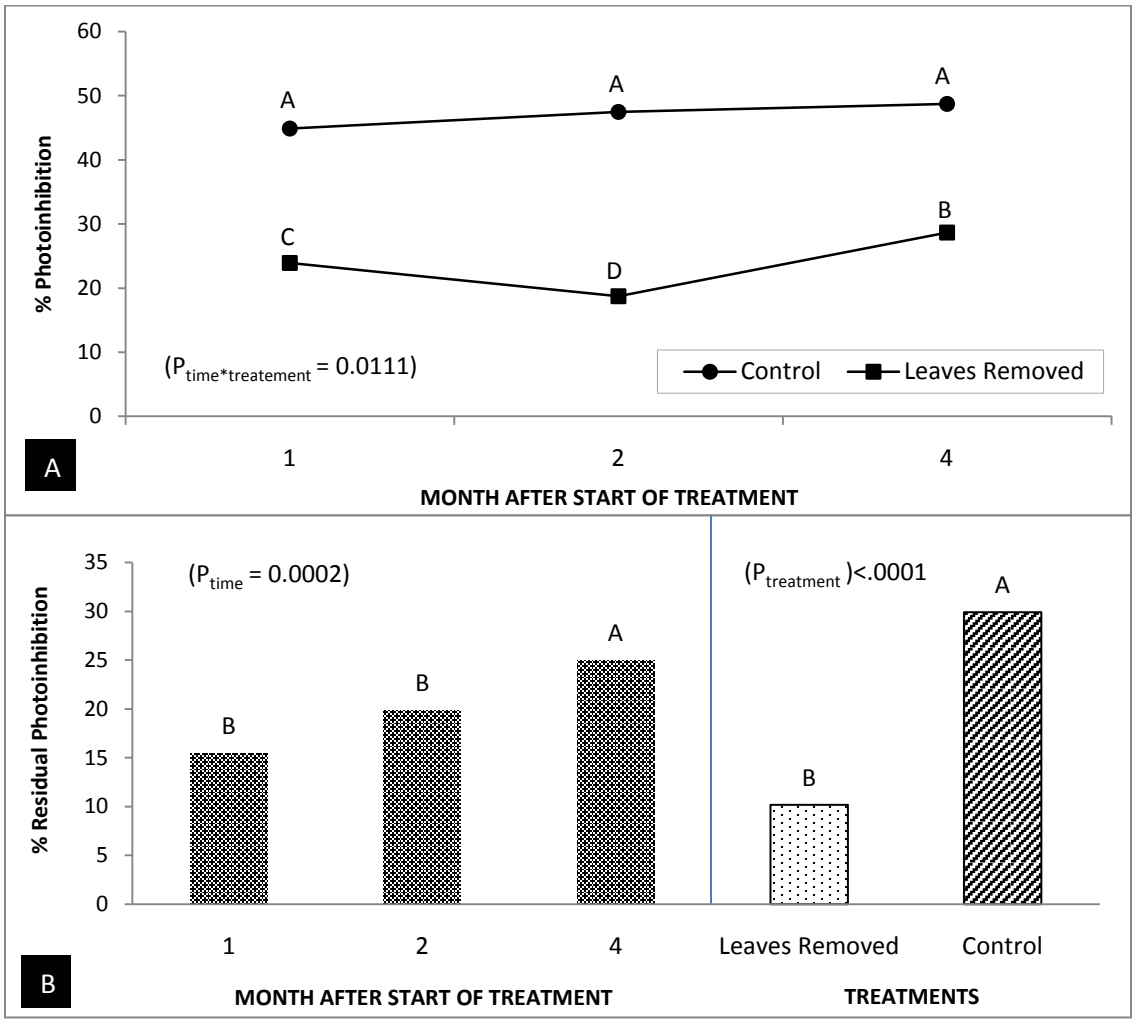

Fig. 1 The effect of removing the involucral leaves on the photostress experienced by the exposed floral bracts of the 'Safari Sunset' inflorescence. A. % photoinhibition of cones, after having been subjected to 16 hour light stress (900-1000 $\mu mol.m^{-2}.s^{-1}$) in a growth chamber held at 17ºC ± 2 ºC B. Residual photoinhibition after cones were allowed to recover for 24 hours in the dark. Columns with the same letters are not significantly different (means separated by LSD 5%).

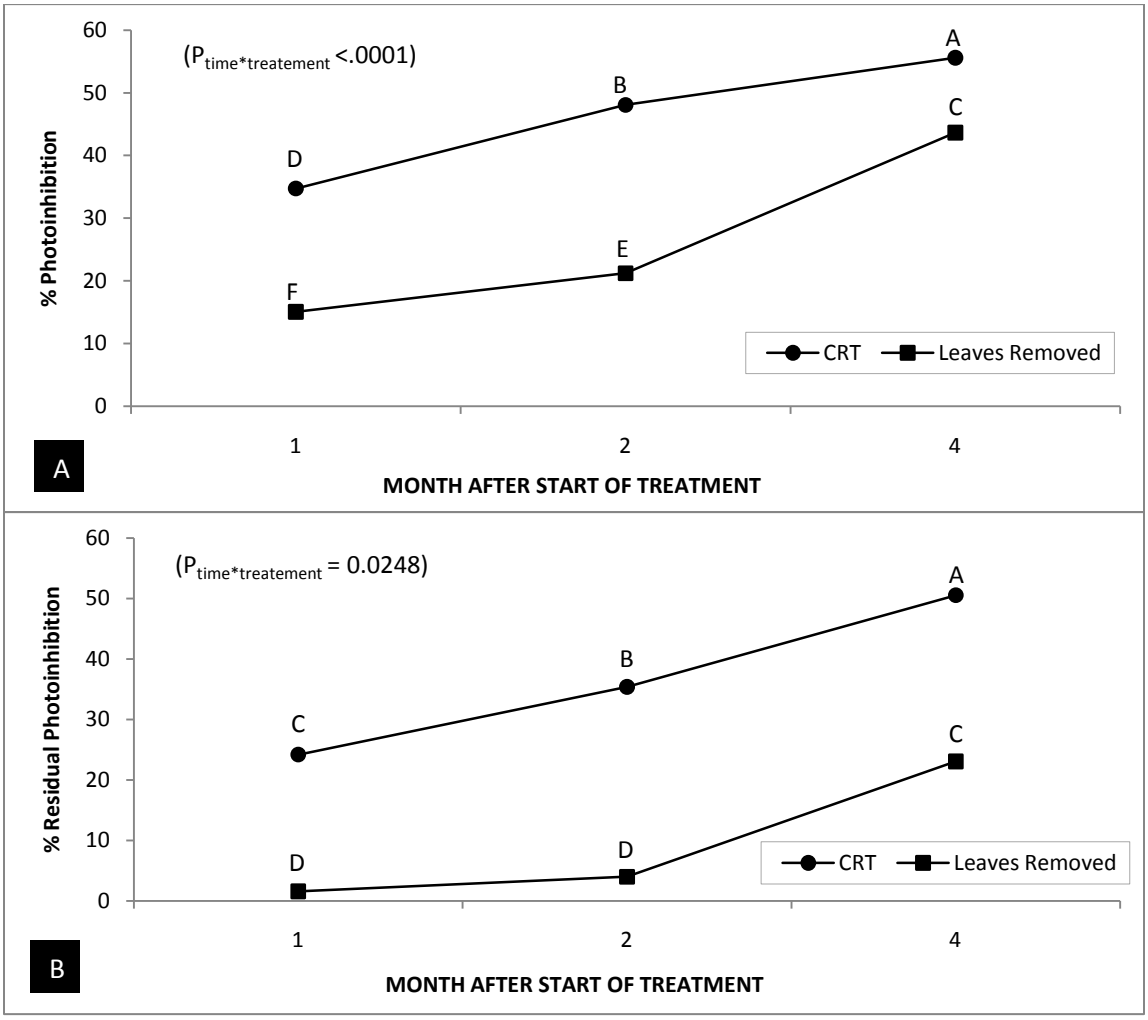

Fig. 2. The effect of removing the involucral leaves on the photostress experienced by the exposed floral bracts of the 'Inca Gold' inflorescence. A. % photoinhibition of cones, after having been subjected to 16 hour light stress (1100-1200 $\mu mol.m^{-2}.s^{-1}$) in a growth chamber held at 17ºC ± 2 ºC B. Residual photoinhibition after cones were allowed to recover for 16 hours in the dark. Data points with the same letter within a treatment and between treatments are not significantly different (means separated by LSD 5%).

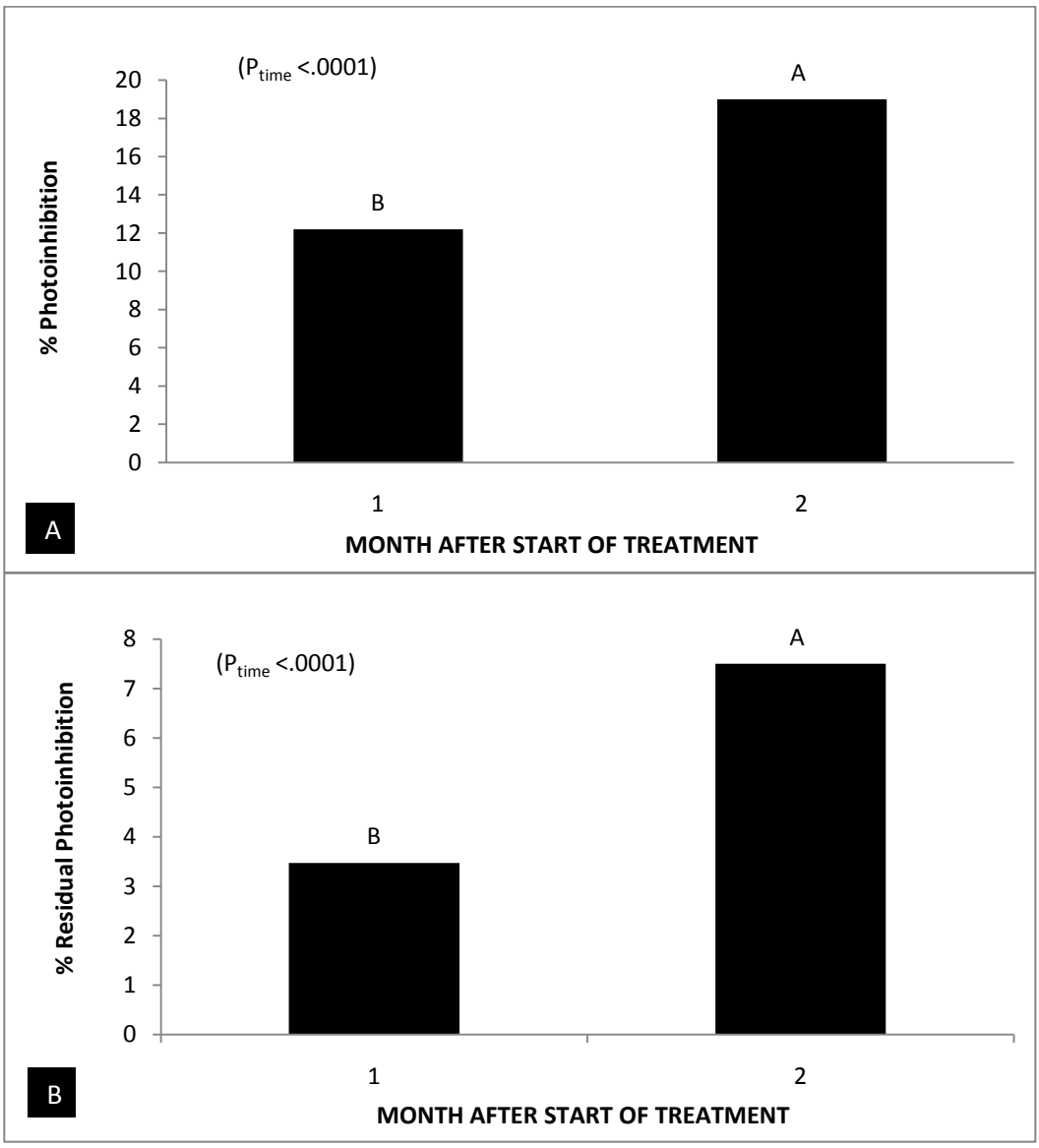

Fig.3 The effect of removing the involucral leaves on the photostress experienced by the exposed floral bracts of the 'Rosette' inflorescence. A. % photoinhibition of cones, after having been subjected to 16 hour light stress (900-1000 µmol.$m^{-2}.s^{-1}$) in a growth chamber held at 17ºC ± 2 ºC B. Residual photoinhibition after cones were allowed to recover for 24 hours in the dark. Columns with the same letters are not significantly different (means separated by LSD 5%).

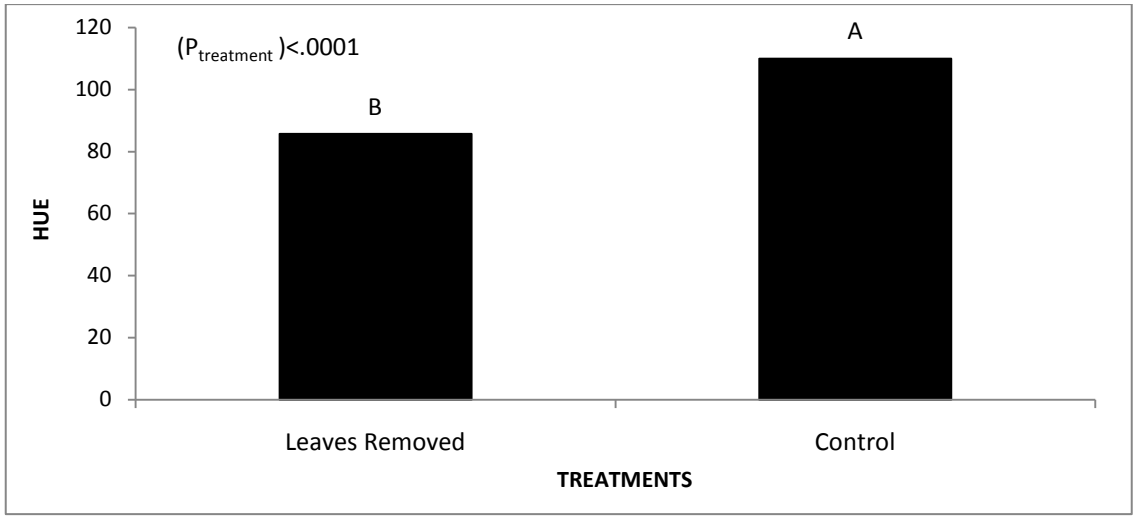

Fig. 4. The effect of removing involucral leaves on the colour of floral bracts of the 'Safari Sunset'. Columns with the same letters are not significantly different (means separated by LSD 5%)

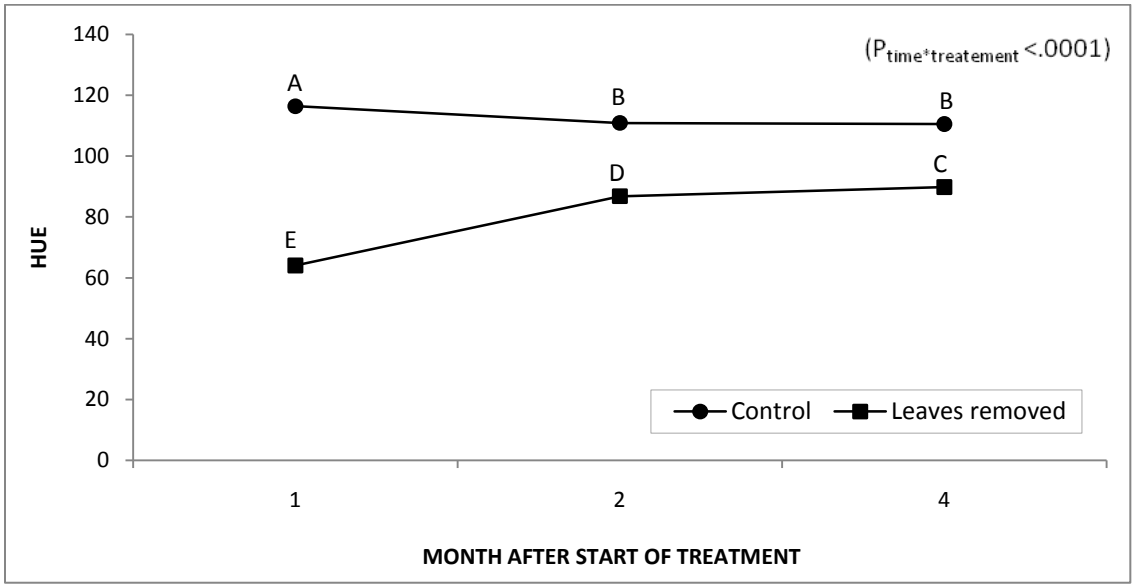

Fig. 5. The effect of removing involucral leaves on the colour of floral bracts of the 'Inca Gold'. Data points with the same letter within a treatment and between treatments are not significantly different (means separated by LSD 5%)

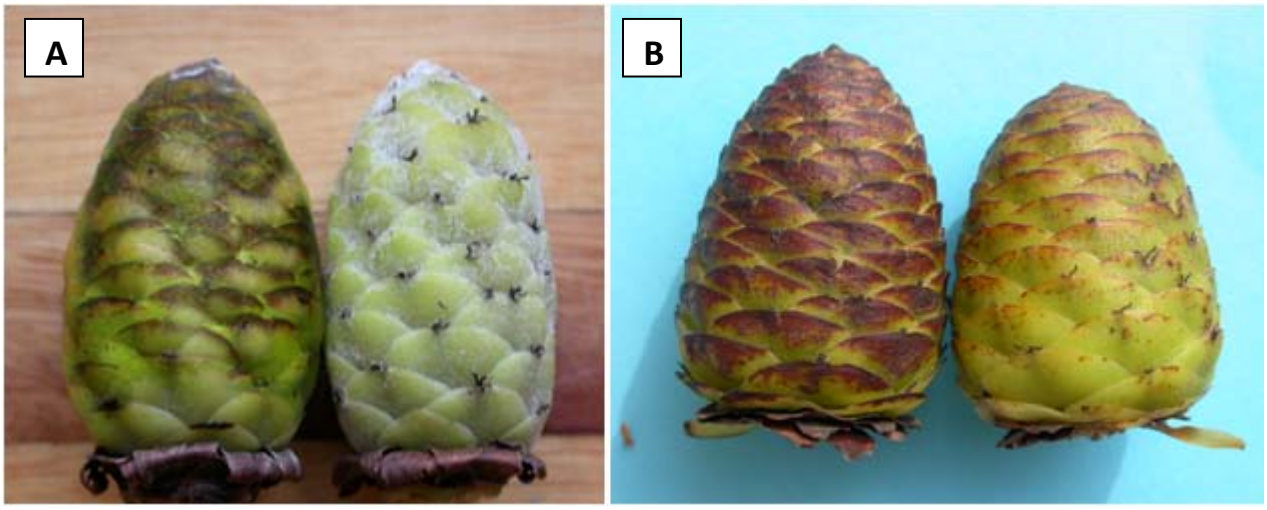

Fig. 6 Examples of browning in A. 'Inca Gold' cones. This depicts the more extreme differences between the control (right) and the treatment where the involucral leaves had been removed (left) B. Cones of 'Rosette'. Cones of 'Rosette' are always exposed, but there is a significant difference between the sun-facing side (left) and the more shaded back(right).

General Discussion and Conclusions

Leucadendron are sought after cut flowers on the international market, due to their colourful flower heads, ranging from bright yellow to red or mixtures thereof. However, it appears that the vibrant colouration of the flower heads is at the same time its economic Achilles heel. Unfavourable colour changes in both yellow and red *Leucadendron* considerably shorten the marketability and lower the potential profitability of these cut flowers. The current study on the colour development of *Leucadendron* aims at providing the cornerstone towards an understanding of the colour expression dynamics of *Leucadendron*. It should form the foundation on which the development of colour manipulation techniques can be based.

Pigmentation patterns: Colour development in *Leucadendron* is transient in nature as the colour of involucral leaves changes throughout the season in direct relation to the development of the inflorescence. Yellow cultivars change from green (preanthesis) to yellow at anthesis, as a result of the well-regulated degradation of the plastid pigments chlorophyll (CHL) and carotenoids (CAR). CAR are degraded to a lesser degree than CHL and therefore impart yellow colour to the leaves. The vividness of yellow colour (a quality parameter for cut flowers) appears to be related to the CHL:CAR ratio. After florets have wilted (postanthesis), the same involucral leaves regreen rapidly and are restored again into fully functional leaves. The degradation pattern of plastid pigments was identical in the red *Leucadendron* cultivar Safari Sunset. However, although the change of plastid pigments might be identical to that encountered in yellow *Leucadendron*, the final colour expressed is not, as the colour dynamics is complexed by the presence of red anthocyanins. The expression of final colour is correlated to the relative ratios of all three pigments present. Anthocyanins increased significantly with flowering and decreased again after anthesis, just inversely to CHL and CAR. During the first year of my PhD, I had therefore concluded that the change in anthocyanins, just like the photosynthetic pigments, was also directly linked and regulated by the development of the inflorescence. Indeed, all data appeared to support this notion and would have remained the final conclusion, were it not for the addition of a simple trial in the subsequent year. Along with flowering, the flower head of 'Safari Sunset' opens up completely, exposing the previously shaded inner surfaces of involucral leaves to

high irradiation and radiant heating, possibly causing photo-oxidative stress. To ameliorate this photo-oxidative stress the plant responded by increasing anthocyanin levels, to presumably provide photoprotection. When the flower is kept closed artificially, there is no increase in anthocyanins, despite normal flowering, as further indicated by the typical degradation pattern of photosynthetic pigments. Therefore anthocyanin synthesis is directly related to the opening of the flower head rather than inflorescence development. However, anthocyanin pigmentation is indirectly linked to flowering, as deconing before anthesis completely prevented the opening of the flower head and therefore also the changes in anthocyanin concentration. It was once said that: "It is a good morning exercise for a research scientist to discard his pet hypothesis every day before breakfast." (Konrad Lorenz, 1966). I had just not expected to discard my very first hypothesis regarding red colour development so soon, which in itself made this PhD very rewarding. I attempted to show that anthocyanins provide photoprotection to involucral leaves of 'Safari Sunset', by measuring the maximum quantum efficiency of PSII (Fv/Fm ratio) as flower heads were opening and subjected to high light stress and those that were not. Unfortunately, due to the very low CHL, I was unable to obtain reliable data. This is something I would like to revisit in the near future, by adapting the methodology further.

Colour manipulation: Despite my understanding of the seasonal pigmentation patterns and the correlation between flowering, opening of the flower head and colour development, effective manipulation of colour has remained elusive. It is easy to prevent colour changes from occurring, by deconing the flower head before anthesis. However, for the yellow cultivars, this is hardly an option, as the cut flower will then remain as the less profitable green product. The only approach to extending the yellow period of individual shoots is to delay death of the florets. To obtain more vibrant yellow flower heads, efforts should be geared towards enhancing chlorophyll degradation (maybe in combination with attempts to enhance carotenoids synthesis before flowering) to lower the relative ratio of CHL:CAR even further. This approach can even be used to improve colour intensity in the red cultivar Safari Sunset, as lower CHL levels will result in a more vivid yellow and red mixture at anthesis (Rainbow colour product). Important, when evaluating any technique used to modify pigment concentrations, a vase life study should be included to make certain it is no affected by the treatment. *Leucadendron* proved relatively unresponsive to the various colour manipulation techniques they were subjected to. The majority of treatments applied, such as lower temperature

treatments, sugar pulsing, higher irradiation treatments, hormonal treatments showed no significant visual response and were therefore not pursued further. It is inherently difficult to separate a direct effect on colour from an effect on flowering. Attempt to enhance red colour of 'Safari Sunset' by means of increasing anthocyanin synthesis will most likely be unsuccessful as it requires the opening of the flower head.

The last section addressed the significance and novelty of the regreening phenomenon encountered in the involucral leaves of *Leucadendron*. Regreening of yellowing plant organs is rare in nature and even less known to occur in true leaves. It was exciting to study a plant where true leaves have adopted a similar function to petals, by degreening at anthesis to aid in pollination. They differ from petals in that these leaves regreened and are long lived structures. Based on our data, the regreening does not occur to support or protect the developing inflorescence, but is rather related to a cost-benefit relationship. *Leucadendron* grow on very nutrient poor soils and rather than discarding a sclerophyllous leaf, it appears far more cost effective to reuse these expensive investments.

Insights – post scriptum: I would like to do follow-up trials on the function of the regreening of involucral leaves. Although it is apparent why cultivars are suitable for these kinds of studies, it might be important to evaluate the effect of removing involucral leaves in wild *Leucadendron* populations, under natural field conditions, where water and nutrients are not supplemented. Water stress, as often experience by *Leucadendron* growing on the sandy soils, could lead to an increase in the prevalence of sunburn necrosis, which would point to a definite protective role of involucral leaves during stressed conditions. Our data shows that involucral leaves are not needed for protection when *Leucadendron* grow under non-stressed conditions. The study would also benefit from including seed viability testing to see whether the plant gains in survival fitness.

A future endeavour would be to include male plants as part of the study. The involucral leaves of male plants have been observed to undergo the same degreening process as encountered in female *Leucadendron*. From personal observation, I have seen that they also regreen, but it is not certain to what extend as their lifespan appears to be abruptly ended after regreening.

This study highlighted the importance of including objective colour measurements as part of any trial dealing with colour and the manipulation thereof. Unfortunately, this was something I had initially deemed as unnecessary work and only realised later, the utmost significance of these measurements. Indeed the numerical expression of colour, in hind sight, appears to be of great importance, especially when three pigment classes are involved. Expression of final colour as perceived by a human observer is based on complex interactions between the presence or absence of a pigment class, the types present within each class, their relative concentrations and the blending effect of all types of pigment classes present. This does not even take into consideration the numerous other factors influencing colour expression such as the presence of co-pigments, and the chemical milieu in which all these chromophores co-exist. Due to this complexity, pigmentation concentrations as determined in a laboratory, although very useful to include in a study, convey no information on what colour the specific plant part under investigation expresses. The classic example is that of 'Safari Sunset', where seemingly high levels of anthocyanins by no means resulted in a marketable red flower head, but rather an unmarketable red-bronze, due to the concurrent presence of high CHL levels.

Future considerations:

This study has opened several interesting avenues for further study, which may roughly considered as those with horticultural merit in terms of colour manipulation techniques and those with a stronger pure scientific bend, laying the future foundations for horticultural studies dealing with colour change or the manipulation thereof.

It seems unlikely, that hormones do not play a role in the colour development of *Leucadendron.* Trials conducted with numerous growth promoters and retardants and respective inhibitors, at three different concentration levels, did not lead to any significant results (data not presented). Therefore I would suggest a combinational approach of measuring hormone changes in the cones throughout the reproductive development period, in conjunction with differential gene expression in involucral leaves. The latter will say a lot about transcriptional factors (and possible signals) and together with changes hormone levels in the cone should give a better indication of which hormones are likely candidates. Based on that, the hormone issue can be revisited, but with a more purposeful approach.

Since potted *Leucadendron* plants have become more easily available, they will be very suitable for further controlled studies on the effect of climate, especially temperature and light, on the expression of anthocyanins in red cultivars.