Flowering Physiology of Selected *Disa* Hybrids in Cultivation

By

Elri Franken

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Supervisor: Dr. E.W. Hoffman
Department of Horticultural Science
Stellenbosch University

Co-Supervisor: Prof. K.I. Theron
Department of Horticultural Science
Stellenbosch University

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Declaration

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“Disas were named after a beautiful woman.
However, like all women you may never fully understand them”.
RON MAUNDER (1999)

Summary

Disa is an unique South African orchid, currently cultivated on a limited scale. Knowledge on the timing of floral initiation is of key importance in understanding the floral biology of a new floricultural crop. Floral initiation of the Disa hybrids, 'LM0739' and 'LM0721', was determined by means of stereomicroscopy to be at the end of May and June, respectively and the duration of floral differentiation was 15-16 weeks. Floral differentiation was divided into ten stages and the vegetative phenology was studied in relation to the reproductive phenology.

It was evident that the growth strategies of tuber-forming and non-tuber forming hybrids differed. Floral abnormalities were also observed and a first report on bud abortion prior to anthesis was made. The effect of plant size and factors affecting plant size, such as the cultural practice of plant separation (separating mother plants from offsets) and higher light intensities were also studied. Furthermore, the effects of photoperiod, smoke water, the plant growth regulators MaxCel™ (6-benzyladenine (6-BA) and Promalin® (6-BA + Gibberellin G_{4+7} (GA_{4+7}) as well as potassium chlorate ($KClO_3$) on flowering of *Disa* hybrid 'LM0739' were also investigated. Plant size influenced flowering, although the critical plant size required for flowering became less obligatory as the season progressed. The separation practice showed no advantages for vegetative growth or flowering and the commercial viability of this practice should be re-evaluated. None of the growth regulators or $KClO_3$ positively affected flowering time and are not considered as viable options for future flower manipulation strategies. No short day photoperiodic effect was recorded when night break treatments, consisting of three different exposures time to night break regimes (58, 74 and 167 days), were applied.

The effect of additional lighting on the four *Disa* hybrids 'LM67' 'LM66', 'LM56' and 'LM48' were studied. On the mature leaves of light-treated plants a red/brown colouration was observed. This colouration was presumed to be induced light stress, but when quantified by photosynthesis and fluorescence recordings, no significant differences were observed between treated and control plants. Transmission electron microscope (TEM) imaging was furthermore inconclusive. The observed symptoms were suggested to possibly be an ameliorating mechanism against high light intensities by accumulating anthocyanins as screening pigments.

Photosynthesis measurements were recorded for *Disa* hybrid 'LM0739'for part of a season in which recorded maximum photosynthesis values ranged between 2 and 5 $\mu mol.m^{-1}.s^{-2}$. Non-structural carbohydrate analysis conducted on *Disa* hybrids 'LM0739' and 'LM0721' indicated that sucrose and glucose were the main soluble sugars in the plant material and that starch levels were extremely low in all samples, except the underground components of the tuber forming hybrid where up to 4.5% starch were recorded. It was furthermore recorded that the root fraction of the tuber forming hybrid ('LM0721') showed much higher levels of hydrolyzed sugars than any of the other samples. Results indicated different carbon allocation strategies for hybrids with- and without root tubers and possibly indicated glucomannan as possible storage reserve compound. These studies provide a basis for future studies with the goal to establish the successful commercial production of *Disa* as a potted plant on the international floricultural market.

Opsomming

Disa is ‘n unieke Suid-Afrikaanse orgidee wat tans slegs op ‘n beperkte skaal gekweek word. Inligting aangaande die tyd van blom inisiasie is nodig om die blom biologie van ‘n nuwe blomgewas te verstaan. Die tyd van blom inisiasie van twee *Disa* hibriede, ‘LM0739’ en ‘LM0721’ was met behulp van 'n stereomikroskoop bepaal om aan die einde van Mei en Junie respektiewelik plaas te vind, terwyl blom differensiasie tussen 15 en 16 weke geduur het. Blom differensiasie was onderskei in tien kenmerkende fases en die vegetatiewe fenologie van *Disa* was ook bestudeer met betrekking tot die reproduktiewe fenologie.

Hieruit was dit duidelik dat groei strategieë tussen hibriede wat stoorwortels vorm, en dié wat nie vorm nie verskil. Blom abnormaliteite was waargeneem en hierdie studie verskaf ook die eerste verslaggewing van blomknop-aborsie in *Disa*. Die rol van plant grootte, asook faktore soos kommersiële plant-verdeling (skeiding van ouer- en dogterplante) en hoër ligintensiteite wat plant grootte beïnvloed is bestudeer. Verder was die effek van fotoperiode, rookwater, twee groei reguleerders naamlik MaxCel™ (6-benzieladenien (6-BA) en Promalin® (6-BA + Gibberellien G_{4+7} (GA_{4+7}) asook kalium chloraat ($KClO_3$) op die blomvorming van *Disa* ‘LM0739’ getoets. Dit is vasgestel dat plantgrootte blom inisiasie beïnvloed maar dat hierdie minimum plantgrootte vereiste meer fakultatief raak later in die seisoen. Kommersiële plant-verdeling blyk om geen positiewe effek op plantgroei of blomvorming te hê nie en behoort heroorweeg te word. Verder het geen van die toegediende groeireguleerders of $KClO_3$ plantgroei of blomvorming bevoordeel nie. Geen kortdag-fotoperiodiese effek was gedokumenteer toe nagbreekbehandelings van drie verskillende tydsperiodes (58, 74 en 167 dae) toegedien was nie. Verder is die effektiwiteit van addisionele lig, om moontlik plantgrootte van vier *Disa* hibriede ‘LM67’, ‘LM66’, ‘LM56’ en ‘LM48’ te verbeter, ook getoets. Volwasse blare van plante wat aan die addisionele ligbehandeling blootgestel was, het ‘n rooi-bruin verkleuring getoon. Hierdie verkleuring was toegeskryf aan ligskade, maar met die kwantifisering van ligskade deur fotosintese en fluorosensie metings is geen verskille tussen die kontrole en lig-behandelde plante waargeneem nie. Die verkleuring word toegeskryf aan die akkumulasie van antosianiene as ‘n verdedigingssmeganisme teenoor hoë ligintensiteite.

Die fotosintetiese kapasiteit van *Disa* ‘LM0739’ was vir ‘n gedeelte van die seisoen gemonitor waartydens maksimum fotosintetiese waardes van tussen 2 en 5 $\mu mol.m^{-1}.s^{-2}$ waargeneem is. In die ontleding van nie-strukturele koolhidrate van beide bo-en ondergrondse plant materiaal van *Disa* hibriede ‘LM0721’ en ‘LM0739’ is glukose en sukrose geïdentifiseer as die twee hoof oplosbare suiker fraksies. Styselvlakke in die bogrondse materiaal van beide hibriede, asook in die ondergrondse materiaal van ‘LM0739’ was besonder laag (<2%). Die styselinhoud in die ondergrondse materiaal van hibried ‘LM0721’ was egter hoër en het tot 4.5% van die totale droë gewig bygedra. Die wortelfraksie van die stoorwortel-vormende hibried (‘LM0721’) het veral ook hoë vlakke van gehidroliseerde suikers getoon. Resultate het getoon dat hibriede met en sonder stoorwortels moontlik van verskillende koolhidraat allokasiestrategieë gebruik maak en dui ook aan dat glukomanaan moontlik gebruik word as stoorvorm . Hierdie studies verskaf ‘n basis vir toekomstige studies met die doel om die suksesvolle, kommersiële produksie van *Disa* as potplant op die internasionale ornamentele blommark te verseker.

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1. General Introduction

The Orchidaceae is the second largest of the angiosperm families, comprised of approximately 25 000 species (Linder and Kurzweil, 1999; McMurty et al., 2008). The world-wide fascination with orchids lies in their inaccessibility, the challenges of cultivation, their sometimes exclusivity and unaffordability, but ultimately in their enormous variation in colour, shape and size and long lasting floral displays. Not only has orchids captivated the attention of botanists and hobbyists alike for decades, but the commercial success of orchids is also rapidly increasing and is considered to be the fastest expanding segment of the world floriculture industry (Nash and La Croix, 2005). FloraHolland (2011) reported *Phalaenopsis* or better known by the public as "the Moth orchid" to be the top-selling indoor plant with 105 million units sold annually and a turnover of €405 million. Hereby *Phalaenopsis* topped long standing favourites like *Kalanchoe*, *Anthurium*, *Rosa* and *Chrysanthemum*, listed in descending order as top sellers for indoor plants. Another orchid reaching this list of 25 top-selling indoor plants for 2011 is the somewhat lesser known *Dendrobium* with a turnover of €18 million and five million units sold.

The positive attributes mentioned for orchids as well as a vibrant colour range and an air of elegance are also characteristic of *Disa* (Coils, 2007), an orchid genus indigenous to South Africa, harbouring immense commercial potential. The genus *Disa* consists of approximately 100 species (Crous and Duncan, 2006; Holmes, 2011), although only a small group within these species, referred to as the "Evergreen Disas", are used in hybridization programs (Holmes, 2011).

Disa is considered by many hobbyists and horticulturists as being difficult or even impossible to grow outside of its natural habitat (Venter, 2006). Still *Disa* can be amenable for cultivation as is proven by a few existing producers currently cultivating *Disa* on a small commercial scale. The striking floral characteristics of *Disa*, the limited number of successful growers as well as the short flowering season of *Disa* creates the opportunity to position *Disa* as a niche product to the most discerning floricultural markets. The successful marketing of *Disa uniflora* and its hybrids as a niche product for the international market however requires an insight into the basic phenological development of this genus over the season, as well as an understanding of the factors that control floral initiation and inflorescence development.

This study aimed to obtain a better understanding of the flowering physiology of selected *Disa*, enabling the profitable and sustainable cultivation of *Disa* hybrids. To achieve this goal a wide range of investigations were conducted, in order to acquire a broad base for studying the floral biology. These studies included reviewing all existing and traceable literature pertaining to *Disa* physiology and cultivation, but also highlighting key literature relating to the factors that influences flowering within the Orchidaceae and geophytes of the Cape Floristic region in particular. Research undertaken within this study included studies concerning the incidence of floral initiation and differentiation for two *Disa* hybrids, as linked to their vegetative phenology as well as the photosynthetic capacity and subsequent carbon allocation strategies of *Disa* throughout growth and

development. Furthermore, the effects of light, either as an increased light integral or duration (photoperiod) on plant growth and flowering was also studied. Lastly, the effect of various factors including smoke water, plant growth regulators and inorganic chemicals (potassium chlorate), were studied for their effects on timing and incidence of flowering in *Disa* hybrids.

These studies is a first report on key horticultural aspects of the floral biology of *Disa* and aim to serve as a basis for future research, ultimately to support successful commercial cultivation of *Disa* on a larger scale in South Africa, and internationally. Furthermore, due to eminent changing environmental conditions as well as under continuous urbanization in the Cape Floristic Region, some *Disa* species like *D. barbata* are considered endangered (Bytebier, 2004). One approach to conservation of *Disa* for future generations is through cultivation. Not only will successful cultivation of *Disa* hybrids enable South Africans to share this fynbos treasure with the rest of the world, but it can also be of key importance to the conservation of this indigenous genus for future generations, in the threat of a changing climate and natural habitat destruction.

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Table of Contents

The referencing style in this thesis for all papers was written according to the requirements of the Journal of American Society for Horticultural Sciences. This thesis represents a compilation of manuscripts where each chapter stands as an individual unit. Repetition between chapters that may occur was thus unavoidable.

2. Literature review: Growth and Flowering Physiology of *Disa*, with Reference to Control of Flowering in Orchidaceae and Selected Geophytes of the Cape Floristic Region

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I. INTRODUCTION

Disa uniflora, an indigenous South African orchid, is considered to be one of the most beautiful orchids world-wide. Commercial cultivation based on sound scientific principles will assist in making this unique flower accessible to South Africans and the rest of the world as a niche floricultural product.

The genus *Disa* consists of more than a 100 terrestrial species predominantly found in southern Africa (Crous and Duncan, 2006). Within this taxon group, the evergreen *Disa uniflora* displays the largest flower of all indigenous South African orchids. Its distinctive range of bright colours, triangular shaped flowers (Tibbs, 2007) and long lasting blooms (Coils, 2007) contributes greatly to the increasing popularity of this orchid as a commercially grown potted plant and/or cut flower. *Disa uniflora* and its hybrids have significant, but yet virtually untapped commercial potential as a niche product, both in the local and international floriculture market, therefore research on its cultivation has become an important priority (Reinten et al., 2011). Given more exposure, disas should become an important product in the world floriculture market (Coils, 2007).

A study of existing reports on the flowering mechanisms in Orchidaceae as well as considering the natural habitat, morphology, and growth habits of *Disa*, could possibly provide clues to the successful cultivation of *Disa* on a commercial scale. Furthermore, comparing developmental strategies of other geophytes from the Cape Floristic region such as *Gladiolus*, *Amaryllis* and *Freesia*, which has evolved with and that shares an ecological niche with *Disa*, may also contribute valuable information to the understanding of the flowering strategy of this genus.

II. TAXONOMY AND DISTRIBUTION

The family Orchidaceae with more than 25 000 species (Bytebier, 2007; Linder and Kurzweil, 1999) is the largest family of the monocotyledons (Crous and Duncan, 2006). This family is extensively represented in southern Africa with approximately 52 genera and 466 species (Linder and Kurzweil, 1999) of which most are endemic (Crous and Duncan, 2006). Orchids found in southern Africa are mostly terrestrial and are either evergreen or deciduous with, a few epiphytic species located only in the moister areas. The greatest diversity of southern African orchid species is found in the Cape Floristic Region (CFR), confined within the Western Cape province of South Africa, of which *Disa* is the largest genus (Crous and Duncan, 2006).

According to Hylander (1958), *Disa* was first described by Bergius in 1767. The genus *Disa* is classified within the subfamily Orchidoidae, tribe Disea and subtribe Disinae (Linder and Kurzweil,

1999). Of the approximately 180 *Disa* species currently documented, 100 species occur in the Mediterranean like climate of the CFR, whilst 86 species are restricted only to the CFR (Bytebier et al., 2011). Outside the CFR, in southern Africa *Disa* is also distributed in grassland areas, with isolated species also found in drier inland areas. Furthermore, *Disa* is also represented in Madagascar and Reunion, each harbouring endemic species. One species in specific, *Disa pulchella,* extends widely from tropical Africa into the Arabian Peninsula (Du Plessis and Duncan, 1989).

III. *DISA* MORPHOLOGY

A. Below ground system

Members of the Orchidaceae and thus also the genus *Disa* exhibit a common storage organ, namely a root tuber. The tubers of the Disinae consist largely of root tissue, but have a stem structure at its core, including a dormant bud (Cribb, 2003). The tubers of *Disa* is usually replaced annually (Du Plessis and Duncan, 1989) leading to a perennial mode of growth (McMurty et al., 2008). Consequently at anthesis mature plants have two such tubers, one that developed during the current growing season together with the tuber that was produced during the previous growing season (Fig. 1) (Linder and Kurzweil, 1999).

In addition to the root tuber, only a small selected number of *Disa* species such as *D. uniflora*, *D. cardinalis* and *D. tripetaloides* have thin, prostrate underground stems termed stolons. Outside of these species, stolons are rarely produced within the *Disa* group (Linder and Kurzweil, 1999). Each stolon produces a leafy plantlet at its terminal position. Subsequently, stolon formation result in the forming of dense colonies by these stoloniferous *Disa* species (Linder and Kurzweil, 1999).

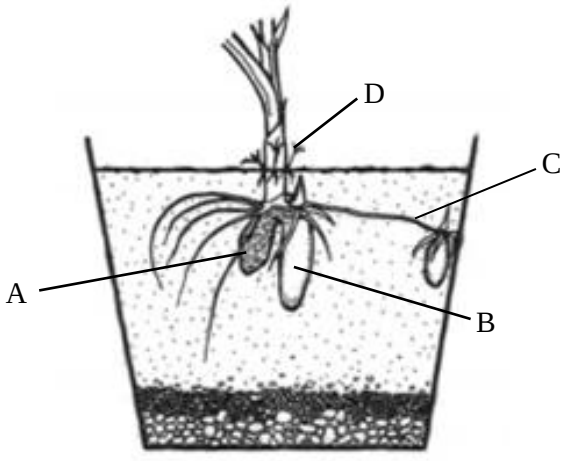


Fig. 1. The below ground system of *Disa* exhibiting stolon and tuber formation. A. An old withering tuber and roots, connected to the flowering stem. B. The new tuber displaying adventitious buds and root development. C. A stolon with a young tuber, shoot initials and roots at a distance from the main plant. D. Plantlets at the base of the existing flowering stem (Pienaar, 2005).

B. Above-ground system

1. Growth habit. Orchids display both monopodial and sympodial growth habits. However, all terrestrial orchids together with only a few isolated epiphytic orchids show sympodial growth almost exclusively (Linder and Kurzweil, 1999). Sympodial growth describes shoot growth that is terminated annually in a flowering axis, where after in the next growth season, a new vegetative shoot arises from a node at the base (below the apex) of the old shoot and resumes growth (Linder and Kurzweil, 1999).

2. *Disa* leaves. The leaves of *Disa* may appear only at the base of the stem, in an opposite arrangement or as a rosette of leaves, alternatively it may be regularly arranged along the stem (Fig. 2). In certain species the leaves merges into the bracts of the inflorescence (Du Plessis and Duncan, 1989). For *Disa uniflora* each shoot bears between five to eight linear-lanceolate leaves (Stewart and Hennessy, 1981).

Fig. 2. A photographic illustration of *Disa* hybrids in cultivation, showing bearing positions of the flowers borne on a spike as well as the basal leaf arrangement.

3. *Disa* inflorescence and floral structure as exemplified by *Disa uniflora*. The basic structure of orchid flowers is typical of monocotyledonous plants. The six perianth segments are arranged in two whorls containing three segments each, although this is a undifferentiated perigone in a strict morphological sense, the terms ‘petals’ and ‘sepals’ are commonly used in orchids (Linder and Kurzweil, 1999) and will therefore be the terminology used for the remainder of the discussion.

Flowers can be either borne solitary or be arranged in an inflorescence (Linder and Kurzweil, 1999; Stewart et al., 1982). The flowering stem of *Disa* consists of the peduncle together with the rachis, where the peduncle can be naked or bearing several leaf sheaths (Linder and Kurzweil, 1999). The peduncle is often referred to in horticultural literature as a stem. The inflorescences of the

Orchidaceae can either be a spike, corymb or raceme (Du Plessis and Duncan, 1999). An inflorescence is called a spike when the flowers are held close to the stem, each lacking a stalk (Stewart et al., 1982). The most common inflorescence found within Orchidaceae is a raceme, when each flower is borne on a short stalk (a pedicel). The inflorescences of *Disa* are understood to be a spike. *Disa* flowers, as with most other orchids, are zygomorphic, where the flower is symmetrical on one plane only. Similarly to the Iridaceae, most orchid flowers have three petaloid sepals and three petals perched above an inferior ovary (Stewart and Hennessy, 1981).

In *Disa* flowers the sepals have evolved in the most colourful part of the flower, not the petals (Fig. 3). The sepals appear on the outer perimeter of the flower and are distinguished by a dorsal or median sepal and a pair of lateral sepals. The sepals protect the other floral parts in the bud (Stewart et al., 1982). The median or dorsal sepal arranged at the top of the flower, is slightly hooded and can be elongated, forming what is called a spur. The spur may contain nectaries (Du Plessis and Duncan, 1989; Stewart et al., 1982). Similarly two of the petals are identical, and fused with the column. The third member of the petal whorl is modified to form what is called the lip or labellum. The labellum occurs at the base, in the middle of the flower and provides a landing perch for pollinating insects (McMurty et al., 2008; Stewart and Hennessy, 1981; Stewart et al., 1982). In most southern African orchids species the lip is the largest of all the tepals (Venter, 2006). The prime distinguishing feature of an orchid flower is the fusing of the sexual parts to form a unique structure, the column (McMurty et al., 2008). In the middle of the flower the male anther is united with the female stigma and style to form this unique structure (McMurty et al., 2008; Stewart et al., 1982). The column forms the stalk that bears the pollination apparatus (Stewart and Hennessy, 1981). The stigma is usually seen as a sticky surface at the base of the column. The ovary of orchid flowers is inferior and lies behind the flower (Stewart et al., 1982) and is frequently mistaken for the peduncle. The flowers of *Disa* species may range in colour including red, yellow, white, pink, blue and green. Interestingly, in addition to the red variants, a very rare yellow mutation of *Disa uniflora* is also found. Colouration is due to the two pigment groups found namely carotenoids and anthocyanins (Tatsuzawa et al., 2010).

C. Pollination strategy

The mountain pride butterfly (*Meneris tulbaghia*) is the prime pollinator of *Disa uniflora* in nature. This butterfly is attracted only by flower colour, as is perceived by the butterfly, pollinating exclusively the red *Disa uniflora* hybrids. The butterfly does not register yellow as a colour and is subsequently not attracted to yellow species or variants. Species where insect pollination is unlikely to occur, like yellow hybrids or mutations of *Disa uniflora*, the only way of propagation is by vegetative propagation. Pollination of other *Disa* species is achieved by carpenter bees, short-tongued flies, long-tongued flies, hawk moths, Anthroporid bees, settling moths, tanglewing flies, horseflies, hoverflies, muscid flies, halictid bees, spider hunting wasps and thread wasted wasps (Linder and Kurzweil, 1999).

D. Mychorrizal associations

Disa, like most orchids, needs a continued association with a mychorrizal fungus to survive. It is especially during seed germination and subsequent seedling growth that the orchids' dependence on its mychorrizal fungus is at its greatest. The evergreen *Disa* species seems to be independent of a specific mychorrizal fungus (Crous and Duncan, 2006), and this is one reason why this group is especially used in hybridization programs. In cultivation, plants are usually grown from tissue culture, and the presence of mychorrizal fungi to aid in seed germination is therefore avoided.

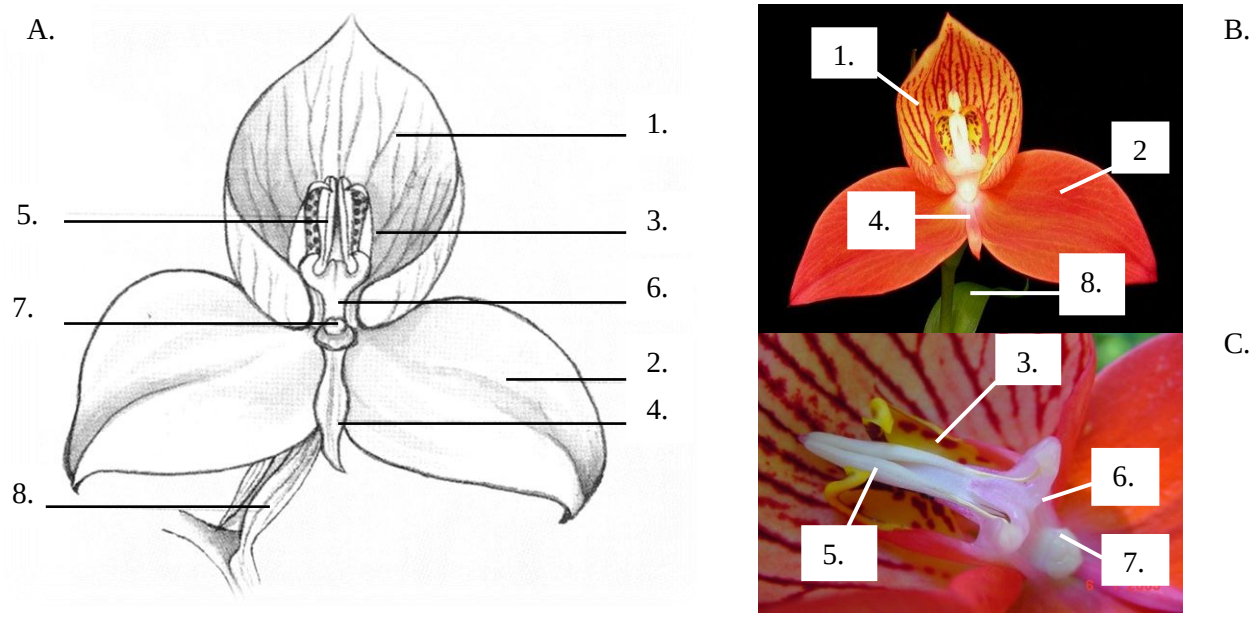


Fig. 3. A. A sketch illustration of the floral parts of *Disa uniflora* (Crous and Duncan, 2006) indicating 1. Median or Dorsal Sepal (spur), 2. Lateral Sepal, 3. Petal, fused with column 4. Lip or Labellum, 5. Pollinium containing anthers, 6. Column, 7. Stigma, 8. Bract. B and C, Photographic evidence of the corresponding floral structures of *Disa uniflora*, as illustrated in A. (Anon a, b, 2011).

IV. *DISA* SEASONAL GROWTH CYCLE

In terrestrial orchids the same bio-rhythms are manifested as found in other sympodial epiphytic or lytophytic orchids, only with most of the sequences of events hidden below ground.

In *Disa*, in contrast to epiphytic orchids, the complete plant dies after flowering, including all the roots and the old tuber of the current season. Only the rhizomes at the base of the stem below the soil surface remain alive to ensure succession. As soon as the flowered spike of the previous season has died back, plant regeneration begins by the formation of a new tuber at the end of the short rhizome or a long stolon, and a new shoot and root system arises from the newly formed tuber

(Vogelpoel, 1993) (Fig. 1). These steps give rise to a new plant when the mature tubers produce the flowering spike (Vogelpoel, 1993). This offspring is genetically identical to the parent plant and arises alongside the old inflorescence in early autumn (Pienaar, 2005).

The initial vegetative propagation phase is supported by the nutrient reserves stored in the dying parental plant (Vogelpoel, 1993). This process which occurs during January to February (SH, Southern Hemisphere) in the CFR coincides with a time of high stress in the natural environment, when the natural water table is at its lowest and temperatures are high. These stressful environmental conditions are considered to be the trigger for gene activation in the meristem of axillary buds. Cytokinin are presumably produced, which then together with their interaction with auxin and other growth factors, may play a key role in the formation of the new tuber shortly after the die back death of the mother plant (Vogelpoel, 1993).

By winter (June to August, SH), the mature *Disa* plant consists of a plump tuber, a root system of unbranched roots (often only 4 to 5 roots) and a healthy rosette of leaves (Vogelpoel, 1993). During this time growth is slow, probably due to the low day and night temperatures and long nights. In October, when different environmental conditions such as longer days, higher light intensities and rising temperatures prevail, the breaking of dormancy is triggered (Vogelpoel, 1993). Genes encoding for growth factors such as auxins and gibberellins are thought to be expressed and a period of rapid growth ensues (Vogelpoel, 1993).

The bolting phenomenon is heralded by the emergence of a flowering spike from the centre of the leaf rosette (Vogelpoel, 1993). In *D. uniflora* each plant typically carries one spike with two to four flowers. There may also be as many as eight to ten, or as few as just one flower per inflorescence. Each flower has a diameter of 8 to 12 cm (Stewart and Hennessy, 1981). Growth from bolting forth is rapid with anthesis within 8 to 10 weeks.

Within a few weeks after the emergence of the inflorescence the individual flowers start to wilt, leaves deteriorate, followed by the dying back of the stem. Concurrently the roots and tuber degenerate and turn black, while adjacent to the dying stem a rhizome connected to the new, but still immature, tuber can be found. At this stage the developing new plant consists of a tuber and small apical shoot (Fig. 4).

Studies on elucidating the possible storage metabolites found within the *Disa* tuber have not been conducted yet. In other orchid tubers, in addition to starch and sucrose, glucomannans have been prominent (Achtardjev and Koleva, 1973). Glucomannans may be found in the seeds or vegetative storage organs, where it may serve as an alternative reserve polysaccharide (Meier and Reid, 1982; Miller, 1992). It is thought that glucomannan is stored in a highly gel-like hydrated state in the vacuole of specific cells (Miller, 1992). Besides carbohydrate storage, the role of glucomannans within the plant or storage organ is still unclear.

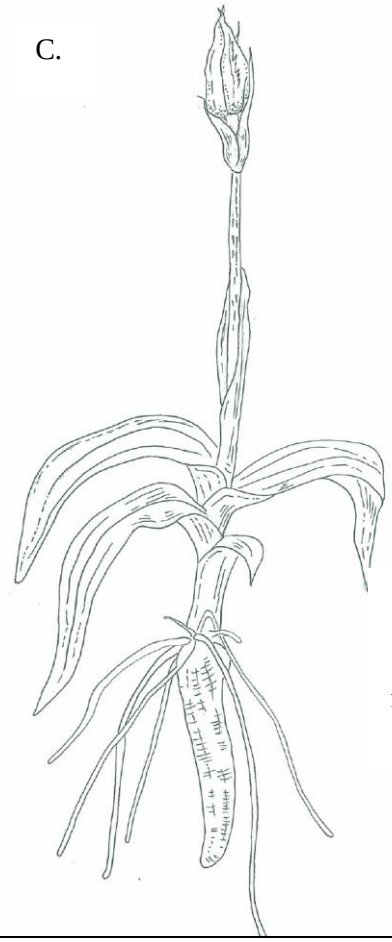
A.

Vegetative plants: Active growing or 'dormant' disas containing a plump tuber, a root system with 4-6 roots and a healthy rosette of foliage leaves.

B.

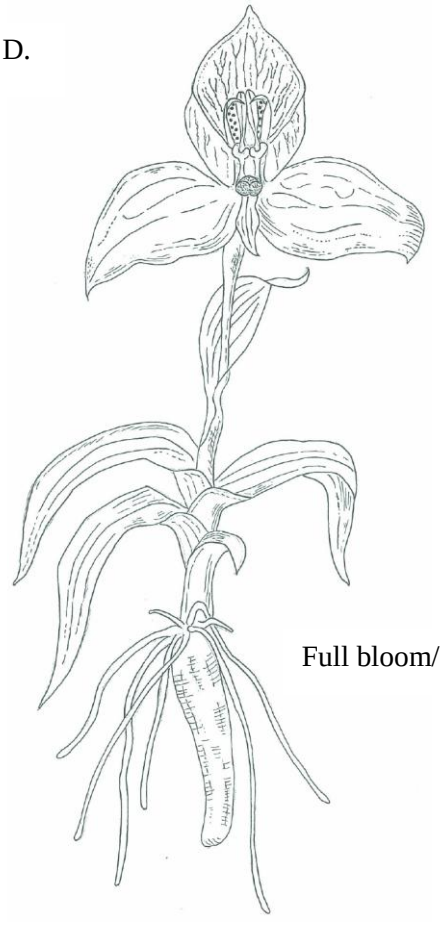
Termination of dormancy is followed by a period of rapid growth and bolting. Flowers can be seen within 8 – 10 weeks from the start of spike emergence.

C.



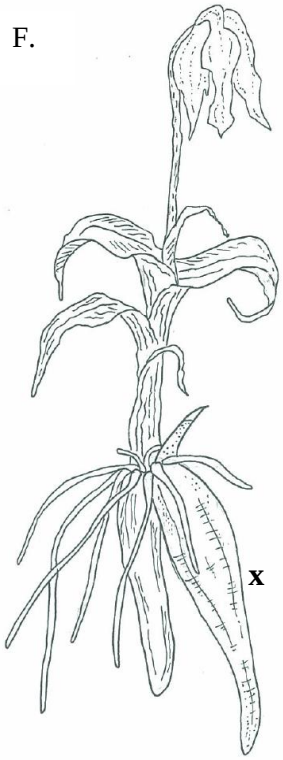
Elongating spike bearing flowering buds.

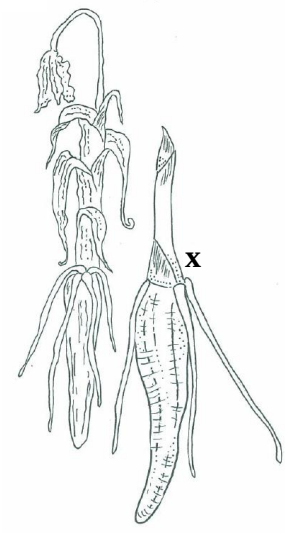
D.



Full bloom/ Anthesis

Fig. 4I. Schematic representation of the phenological events in *Disa uniflora* hybrids (A-D), from the vegetative phase through to anthesis.

<table>
<tr>
<td>E.
 
Flower senescence and wilting, followed by the deterioration and die back of leaves, stem, roots and tuber.</td>
<td>F.
 

The progeny (x) contains a near-mature, developing tuber and healthy roots, but is still attached to the withered parental plant material.</td>
</tr>
<tr>
<td>G.
 

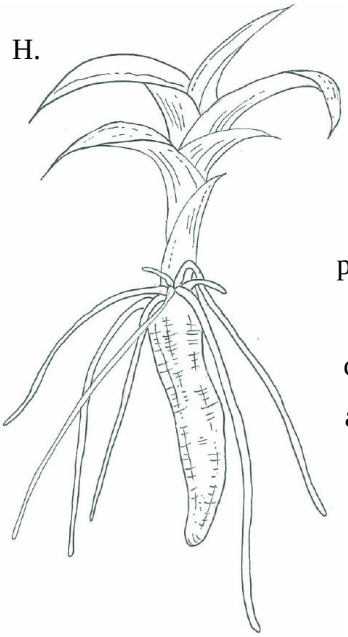
The new developing plantlet now separated from withered parental plant.</td>
<td>H.
 
Young plantlet (previously “x”), proceed to a mature vegetative plant containing a tuber, a root system with 4-6 roots and a healthy rosette of foliage leaves.</td>
</tr>
</table>

Fig. 4II. Schematic representation of the phenological events in *Disa uniflora* hybrids (F-H) showing floral senescence and die back and the regeneration of a new plantlet to maturity.

V. ECO-VARIANT GROWTH FORMS

Three basic growth variants and cycles are found in *Disa* species, separated from each other mainly based on their specific and distinctly different habitats. These growth cycles include: The evergreen cycle, winter-growth cycle and the summer growth cycle.

A. Evergreen disas

The evergreen disas include species such as *Disa aurata*, *D. cardinalis*, *D. caulescens*, *D. uniflora*, *D. marlothii*, *D. tripetaloides* and *D. uncinata* (Crous and Duncan, 2006). These species generally prefer seepage and streamside conditions and colonize perennial mountain streams alongside deep pools where water flow is not too rapid (Crous and Duncan, 2006). Evergreen disas are confined mostly to the Mediterranean climate type CFR, even at altitudes as high as 1200 m above sea level. Vegetative growth starts in late autumn or early winter, with mature tubers responsible for producing leafy shoots (Du Plessis and Duncan, 1989). Unlike the majority of *Disa* species these evergreen disas are never truly dormant. During the wet, cold winter months growth will slow down, only to increases again as temperatures rise from early spring onwards (Crous and Duncan, 2006). Flowering is expected early to late summer (November to March, SH).

B. Winter- growing (summer-dormant) disas

Species included in this group are *Disa cornuta*, *D. draconis*, *D. ferruginea*, *D. graminifolia*, *D. sagitalis*, *D. spathulata* and lastly *D. venosa.* In winter growing disas, the old tuber is replaced annually as the newly formed tuber develops throughout the winter months (Crous and Duncan, 2006). The leaf formation of winter growing disas is usually synanthous (flowers and leaves appear together). These selected disas initiate growth in early autumn, corresponding with a significant drop in night temperatures (Crous and Duncan, 2006). Flowering can be expected from mid spring to early summer, with a dormant period following through mid to late summer when average temperatures are high. New leafy shoots will only develop from the dormant tuber again in autumn, followed by the formation of new adventitious roots at the base of the leaf shoots throughout the winter months.

C. Summer- growing (winter-dormant) disas

Some of the species included in this group are *Disa crassicornis, D. nervosa*, *D. polygonoides, D. porrecta*, *D. saxicola* and *D. woodii* (Crous and Duncan, 2006). The growth cycle of this group proceeds much like its winter-growing counterpart with the exception that growth only initiates in spring (Crous and Duncan, 2006), whilst dormancy sets in during autumn and early winter (Du Plessis and Duncan, 1989). Most summer-growing disas are, like the winter-growing *Disa*, synanthous with certain species like *D. baurii* having hysteranthous leaves (leaves grow shortly after flower appearance) (Crous and Duncan, 2006). At the start of the growing season a shoot with the regenerative bud forms. As the shoot develops, adventitious roots form at the base of the stem where

the stem is attached to the tuber. The new replacement tuber develops during the period of summer growth (Crous and Duncan, 2006).

VI. COMMERCIAL HYBRIDISATION

Disa species with numerous attractive colour forms have interested plant breeders since the late nineteenth century (Du Plessis and Duncan, 1989). However, only a few *Disa* species have been used extensively in the breeding of hybrids, with *Disa uniflora*, *D. racemosa* and *D. tripetaloides* being the first *Disa* species used as hybrid parents (Holmes, 2011). Most of the commercially cultivated hybrids today are still derived from hybridization of species from the evergreen *Disa* group, as both the deciduous groups are difficult to cultivate. Species frequently used in breeding programs include the above mentioned three initial species as well as *D. cardinalis* (Holmes, 2011).

VII. SPECIES PREFERRED IN HYBRIDISATION

A. *Disa uniflora*

The flower of *Disa uniflora* is amongst the largest of terrestrial orchids of South Africa and the world (Crous and Duncan, 2006), and therefore received considerably more attention than other Cape orchids (Linder and Kurzweil, 1999). *Disa uniflora* is commonly found in the Western Cape region with its distribution extending from the Cederberg Mountains in the west, to Riviersonderend in the east, and Betty's Bay in the south (Fig. 5A). *Disa uniflora*, is also known as "The pride of Table Mountain" or the "Red disa" due to its predominant red forms (Fig. 5B), and has great horticultural potential. *D. uniflora* flowers from January to March. Flowering stems vary in length from short and robust to up to one meter tall. Distinct colour variations are found on the different mountain ranges. On Table Mountain, orange predominates in the southern parts, with a deep orange-red in the Cederberg Mountains (Crous and Duncan, 2006). Eastwards, a clear pink *D. uniflora* is found (Linder and Kurzweil, 1999).

A.

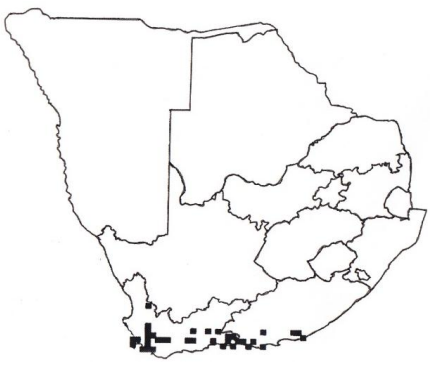
B.

Fig. 5A. The distribution map of *Disa uniflora* throughout the Western Cape Province of South Africa (Linder and Kurzweil, 1999). B. *D. uniflora* showcasing commercially important traits in large, brightly coloured flowers (Anon c, 2011).

B. *Disa racemosa*

Disa racemosa is beautiful, yet not easily observed in nature (Crous and Duncan, 2006). This *Disa* has a fairly wide distribution in marshy areas of the Western Cape as well as southern parts of the Eastern Cape, and are therefore found from Cape Town through to Grahamstown (Linder and Kurzweil, 1999) (Fig. 6A). Flowering, concentrated in November to December, exclusively occur following veld fires of the previous summer season. The plants can be tall, with flowering stems reaching lengths up to one meter (Crous and Duncan, 2006). Individual flowers are pink to pale pink with darker veins and petals having purple horizontal bars and a white to yellow lip (Fig. 6B) (Linder and Kurzweil, 1999). This species is known to flower erratically in cultivation (Du Plessis and Duncan, 1999).

A.



B.

Fig. 6A. The distribution map of *Disa racemosa* throughout the Western and Eastern Cape Provinces of South Africa (Linder and Kurzweil, 1999). B. *D. racemosa*, showing beautiful flowers which can be pink to pale pink with darker veins, with petals having purple horizontal bars and a white to yellow lip (McMaster, personal communication, 2011).

C. *Disa cardinalis*

Disa cardinalis has a restricted distribution as its occurrence is limited to the Riversdal district of the Western Cape (Fig. 7A). Populations are found, in groups or clusters resulting from their vegetative reproduction via stolons. These clusters occur alongside streams on the inland slopes of the Langeberg. Plants are slender and grow to 300 – 600 mm in height. Bright red flowers are seen from October to December (Linder and Kurzweil, 1999) (Fig. 7B). In nature *D. cardinalis* is considered to be the hardiest of all the evergreen *Disa* species. In addition to this inherent resilience, *D. cardinalis* can also introduce many other desirable attributes to breeding programmes, such as an extremely attractive flower (Du Plessis and Duncan, 1989). Surprisingly the inclusion of *D. cardinali*s in breeding programmes has not occurred to a large degree (Crous and Duncan, 2006).

A.

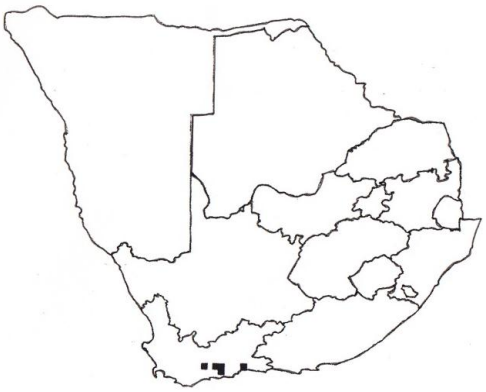
B.

Fig. 7A. The distribution map of *Disa cardinalis*, showing confinement to the Riversdal district of Western Cape Province of South Africa (Linder and Kurzweil, 1999). B. *Disa cardinalis*, showing extremely attractive red flowers (Haasbroek, personal communication, 2012).

D. *Disa tripetaloides*

Disa tripetaloides is widespread and found from the Western Cape to Southern KwaZulu-Natal, where they frequently inhabit damp mountain slopes with altitudes of up to 1000 m, as well as on the banks of perennial streams (Fig. 8A). In the Transkei region (Eastern Cape) through to KwaZulu-Natal flowers are seen from June to September, while in other areas, flowering occurs from November to January. Plants multiply by means of stolons, giving rise to clusters of plants. This extensive network of stolons may serve as protection from flooding rivers. Plants are slender and may reach a height of between 100 – 600 mm tall. Flowers are white or varying shades of pink (Fig. 8B), with a very rare yellow form found in the Transkei. Substantial variation in flower shape and size is found, which is consistent with the wide distribution of species (Linder and Kurzweil, 1999). This robust, prolific flower is probably the easiest of the evergreen species to cultivate and when used

in breeding programmes any resulting progeny will probably have an earlier flowering season (Crous and Duncan, 2006).

A. 

B. 

Fig. 8 A. The distribution map of *Disa tripetaloides* throughout the Western Cape as well as the Eastern Cape and KwaZulu–Natal Provinces (not indicated on map) of South Africa (Linder and Kurzweil, 1999). B. *D. tripetaloides*, showcasing a beautiful white flower. These flowers can also be found in varying shades of pink. (Haasbroek, personal communication, 2012).

VIII. HORTICULTURAL POTENTIAL OF *DISA* SPECIES AND HYBRIDS

Disa uniflora can be marketed as potted plants or cut-flowers (Coils, 2007). *Disa* as a floricultural crop is relatively new to the cultivated orchid market, but certainly has commercial potential. The long lasting flowers with their typical triangular shape and vibrant colours differ in appearance, from the typical orchid (Tibbs, 2007). It can therefore be introduced as a niche product in a market always demanding innovation.

Disas are grown internationally by hobbyists, but only a few nurseries have been established as commercial *Disa* enterprises (Coils, 2007) and even fewer nurseries are focussed on the cut flower market. Two of the largest nurseries trade from Taranaki, New Zealand with a combined annual production of approximately 50 000 stems (Coils, 2007).

The *Disa* has a reputation as being notoriously difficult to grow; to such an extent that Venter (2006) commented that "*Disa* should rather be left in their natural environment where the flower flourishes". In support of this view, Neels van der Linde , former floriculture nursery manager at La Motte Winery estate, currently the only commercial grower of *Disa* in South Africa stated: "There is no flower more pleasing than a *Disa*, but there is no flower as unforgiving as a *Disa*" (Van der Linde, personal communication, 2010). In addition, the few successful growers who do cultivate *Disa* on commercial scale in addition face market related challenges. A niche market for this unique product definitely exists, but the introduction of defined management systems are required to enable producers to confidently export and approach their target markets, assuring flower quality and continuity of

supply (Coils, 2007). In accordance with this statement Reinten et al. (2011) claimed that successful cultivation of South African plants does not solely rely on their unique attractiveness and aesthetic features. In order to compete internationally ornamentals need to be true to type, available in large quantities for a relatively long marketing period as well as have an acceptable vase-life. This requires sustainable propagation and cultivation practices and also effective pest and disease management (Reinten et al., 2011).

Reinten et al. (2011) included *Disa* as one of the South African genera being the subject of international interest from breeders. In a review on world marketing trends on *Disa*, Coils (2007) positively stated: "Given more exposure they cannot but take off on the world market".

IX. HORTICULTURAL CULTIVATION REQUIREMENTS

Disa requires a very well drained growing medium. Washed coarse river sand or silicon sand with a 10% peat addition is recommended (Cywes and Cywes, 1990). Combinations of sphagnum moss and perlite could also be used as well as mixtures of peat moss, vermiculite and perlite (Coils, 2007). Pienaar (2005) claimed that the growth and flowering properties of *Disa* were optimal in growth mediums of sphagnum moss and peat, but plants were negatively affected by the increased pH of peat:sand mixtures. In a study considering different growth medium for tissue culture *Disa* plantlets, when a mixture of spaghnum moss:sand in a 50:50 ratio was used plantlets showed a 45 g higher plant weight than when Funaria moss was used with sand in the same ratio (Pienaar and Combrink, 2008). Spaghnum moss also resulted in much higher water holding capacity as well as a higher ammonium content. Since disas need relatively high levels of ammonium (Pienaar and Combrink, 2007), this together with a better water supply could result in enhanced growth in the spaghnum moss mixtures, compared to Funaria moss (Pienaar and Combrink, 2008). Furthermore, clay pebbles are not a suitable growing medium for *Disa* (Pienaar, 2005). It is important that the growing medium of *Disa* should not break down when kept moist, but should be able to remain aerated, and maintain a slightly acidic pH (Coils, 2007).

Disa requires high quality water of low electrical-conductivity (EC) and with a slightly acidic pH of 5 to 5.6. Both overhead watering and hydro culture (ebb-and-flow) methods can be used, each with merits and disadvantages (Cywes and Cywes, 1990). Watering regimes and quantities can differ according to the growing medium used. If required, algae deterrents could also be added to water (Coils, 2007). Roots must never be allowed to dry out, but over watering and sogginess should be avoided at all times, as free drainage is critical. It was found that drip irrigation improved biomass accumulation compared to ebb-and -flood irrigation (Pienaar, 2005).

In a commercial nursery re-potting is practised every year, in order to promote stronger growth and to provide the new plant access to nutrients and sufficient space for root and tuber development (Coils, 2007). In South Africa re-potting is best done in autumn (Cywes and Cywes, 1990; Van der Linde, personal communication, 2010).

Nutrients can be administrated through watering tanks in a weak concentration, where the salt content of the water should not exceed 250 parts per million. In summer an EC of 0.4 $mS \cdot cm^{-1}$ is recommended, whilst in winter a lower EC of 0.2 $mS \cdot cm^{-1}$ is advisable. Higher EC levels produced desirable characteristics i.e. robust mother plants with an increased stem diameter as well as a higher root:shoot ratio (Pienaar, 2005).

Fertigation can be applied once a week with quarter to half strength in a ratio of 30N:10P:10K or 18N:18P:18K standard orchid fertilizer (eg. 'Flowering orchids',Stark Ayers (Pty) Ltd, Cape Town, South Africa). According to Pienaar and Combrink (2007) *Disa* is NH_4 tolerant, with *Disa unidiorosa* and *D. kewensis* growing best at 40 and 60% NH_4 , respectively, with the remainder of nitrogen made up by nitrate. Furthermore, in a study determining whether foliar feeding enhances plant growth under normal conditions it was found that the addition of NH_4NO_3 in the foliar spray seemed to benefit plants more than the presence of urea in the foliar spray (Pienaar, 2005). Still, despite generally accepted nutritional guidelines, practices differ widely between growers (Coils, 2007).

Disa can tolerate full sun, however shading of 40 – 50% in summer is recommended for best results (Cywes and Cywes, 1999). Two different shading levels were evaluated by Pienaar (2005), but no significant differences were found in growth between 56% and 69% shading. For disas, an environment where temperatures range between 10° and 25°C is advised. Temperature in the root zone must be kept between 5 - 15°C in winter and 10- 20°C in summer (Linder and Kurzweil, 1999). Ambient summer temperatures of above 30°C can be tolerated as long as the pots and roots are kept cool (Holmes, personal communication, 2011).

Disas are prone to fungal infections, but bacterial diseases are not seen as a problem and no viruses have been identified yet (Cywes and Cywes, 1990). Insects such as thrips, aphids, red spider, woolly aphids and gall midge have been reported to be problematic in certain areas (Cywes and Cywes, 1990).

Thus far no literature concerning postharvest physiology or handling of disas as cut flowers or potted plants exists. In a study conducted to establish the sensitivity of several orchid genera to ethylene, it was found that the response to ethylene as well as the production thereof varies greatly between the different genera. The genera tested were *Cattleya*, *Cymbidium*, *Dendrobium*, *Oncidium*, *Phapiopedilum* and *Vanda* 'Miss Joaquim' (Goh et al., 1985). *Vanda* was found to be most sensitive to ethylene, followed by the less sensitive *Cymbidium*, *Cattleya* and *Phapiopedilum*, *Dendrobium* and *Oncidium* (Goh et al., 1985). Therefore, a certain degree of ethylene sensitivity could be expected in disas. In orchids, pollination as well as emasculation (pollinia removal) accelerates ethylene production (Hew and Clifford, 1993). *Disa*, like most orchids, are perceived to have a long shelf life, as ethylene sensitivity is one of the major reasons for reduced shelf life for orchids in general,

information on the sensitivity of *Disa* to ethylene would be of key importance in the managing of the marketing chain of *Disa* as both cut-flower and a potted plant.

X. FLOWERING IN ORCHIDACEAE

When considering the size of the Orchidaceae family, not to mention the wide range of natural habitats it occupies as well as the different types of growth habits available such as epiphytic, litophytic as well as terrestrial orchids, it is safe to assume that a wide range of factors are likely to play a role in flowering of orchids. Furthermore, with great variation found between species within a genus, no generalizations can be made in terms of flowering signals.

A. Juvenility

The period of growth from seed to flowering varies between species (Goh and Arditti, 1985). The early phase of plant growth during which flowering cannot be induced by any treatment is termed juvenility. This phase is important in controlling vegetative to reproductive changes in the plant. Juvenility is seen as a way for the plant to ensure that no flowering takes place until the plant is capable of supporting the energy demands for seed production. The average time of juvenility in orchids is two to three years, but can be as long as 13 years, however most commercially important *Phalaenopsis* hybrids flowers after a 12 – 36 month period (Goh and Arditti, 1985). Following juvenility, endogenous growth factors as well as exogenous factors including temperature (vernalization and thermoperiodism), light intensity and photoperiod gives rise to floral initiation. Furthermore, combinations of these factors are often required for flowering.

B. Temperature

1. *Dendrobium*. *Dendrobium* originates from tropical and subtropical Asia, Australia as well as the Pacific islands. This is one of the largest genera within the Orchidaceae with more than 1000 species. Temperatures reported to control flowering varies greatly amongst *Dendrobium* species. *D. nobile* remained vegetative when exposed to a constant temperature of 18°C, whilst flowers were produced when plants were exposed to 13 °C, regardless of photoperiod (Goh and Arditti, 1985). On the contrary, *D. phalaenopsis* required short-day signals (nine hour daylength) as well as warmer temperatures such as 18 °C for flowering (Lopez and Runkle, 2004), as longer days at both 13 °C and 18 °C retarded flower initiation (Goh and Arditti, 1985). These results highlight the variation found in the requirements for flowering even between species within the same genus. In another *Dendrobium* cultivar 'Snowflake Red Star' flower induction was best achieved by providing 25 °C day and 10 °C night temperatures for 40 to 60 days (Lopez and Runkle, 2005).

D. crumenatum (dove or pigeon orchid) is one of the best known examples of a tropical orchid where flower induction is triggered by low temperature. Upon transition of the meristem from

vegetative to reproductive, floral buds develop up to a certain stage and then becomes dormant (Yap et al., 2008). It was noted that a sudden drop of temperature, of approximately 5°C, as is often associated with rain showers can then stimulate flower development in *D. crumenatum* (Goh and Arditti, 1985). Flowering has been recorded nine days after a rain storm (Hew and Yong, 2004). The reason for this stimulation of floral development remains uncertain, but low temperature induction and/or the hydration of flower buds are both possibilities (Hew and Yong, 2004). Furthermore, cold temperatures could also lead to the release of dormancy, leaving the non-dormant bud to react to signals. Similarly, in Florida USA, it was found that when exposed to rapid temperature changes *D. crumenatum* could be in full bloom 14 days later (Sheehan, 1983). However, chilling *D. crumenatum* plants for only eight hours at 4 °C will trigger some of the buds, but the percentage of flowering is never 100% as is obtained by natural chilling (Sheehan, 1983).

Low temperature triggers the breaking of dormancy of the buds, although the mechanism is still unknown as applications of auxin, cytokinins or gibberellins however all failed to induce further development. Apart from *D. crumenatum*, blooming following cooling has also been found in *Bromheadia alticola* and *B. finlaysoniana* as well as other *Dendrobium* and *Trixspermum* species (Goh and Arditti, 1985).

2. *Cymbidium.* Due to the large geographic distribution *Cymbidium* is often divided into groups based on their temperature tolerance. This classification according to temperature tolerances include: cool, intermediate and warm-temperature tolerant (Lopez and Runkle, 2005). In *Cymbidium*, both the duration of cold temperature exposure as well as diurnal fluctuations are important aspects to consider when studying the inductive effects of vernalization on the incidence of flower initiation. Furthermore, temperature interactions with photoperiod should also be considered.

Cymbidium cultivars are supposedly induced to flower under large diurnal fluctuations as experienced under conditions of warm days and cold nights (Lopez and Runkle, 2004). Temperate *Cymbidium* hybrids derived from the "Asian cymbidium belt" flower in response to diurnal fluctuations of 10 to 14 °C difference in day/night temperatures (Lopez and Runkle, 2005). Large flower type cymbidiums from China or the Himalayas are induced to flower by a pronounced cool period during which night temperatures should reach about 10 to 14 °C (Lopez and Runkle, 2005).

It has been reported that *Cymbidium* 'Astronaut Rajah' exposed to a photoperiod of 14 hours with 26 °C day and 12 °C night temperatures produced 5.9 inflorescences per plant on average. This average was reduced to 0.8 and 1.7 inflorescences per plant when cultivated under temperature regimes of 20/12 °C and 26/18 °C, respectively (Lopez and Runkle, 2005).

Similarly, in *Cymbidium giganteum* var. *lowianum* 'RcHb f', no inflorescences developed with day/night temperatures of 26/14 °C or 23/14 °C, whereas inflorescences developed on a limited scale at 26/10°C and 26/7°C. The greatest floral production occurred at 20/10 °C and 20/14 °C (Lopez and Runkle, 2005). Both these studies cited by Lopez and Runkle (2005) illustrate that a combination of optimum day and night temperatures are important for inflorescence production and

that flowering is not only reliant on the night temperatures alone. Contrary to *Cymbidium* 'Astronaut Rajah' which required a 14 hour photoperiod for floral initiation, Goh and Arditti (1985) claimed that photoperiod has no effect on flower initiation in *Cymbidium*. In a recent study by Kim et al. (2012) the effects of night temperature on flowering characteristics of *Cymbidium* was studied. Treatments included growing plants at night temperatures of 6, 9, 12 or 15 °C for 16 hours together with a daytime temperature of 25 °C. The lowest temperature tested (6 °C) showed the longest duration, 39 days, before flowering, whilst days to flowering when grown at 9-15 °C varied between 31-34 days. As mentioned, fluctuations between day and night temperatures are needed for flowering in *Cymbidium*, however, this study proved that too large fluctuations in temperature would delay flowering.

Flowering response to low temperature is further complicated by interactions between light and temperature. It was found that cymbidiums would flower at 21 °C and that the degree of the response to low temperature would be influenced by light intensity (Hew and Yong, 2004). Goh and Arditti (1985) claimed that high light intensity coupled with 10 to 13 °C night temperatures, a range similar to 10 to 14 °C proposed by Lopez and Runkle (2005) is required for flowering. It can therefore be concluded that according to both Hew and Yong (2004) and Goh and Arditti (1985) low temperatures coupled with high light intensity is needed for floral induction. The mechanism of low temperature induction still remains unclear, but change in endogenous cytokinins after low temperature exposure is implicated (Hew and Yong, 2004).

3. *Phalaenopsis.* Of the Orchidaceae, the genus *Phalaenopsis* is probably the best described and understood. This genus originates from tropical and sub-tropical areas of Asia and Pacific Islands. Although as expected, variation between species occurs, for most raceme elongation is triggered by exposure to relatively cooler temperatures of less than 28 °C (Lopez and Runkle, 2004). Therefore synchronization of uniform stalk lengthening can be achieved by growing plants at day and night temperatures of 25/20 °C or 20/15 °C for four to five weeks (Lopez and Runkle, 2004).

Continuous day temperatures of 25 °C for at least 12 hours were found to be effective for floral induction (bringing plants into reproductive growth), whereas at continuous high temperatures (28 °C) no flowering stalks were produced (vegetative growth continued) (Sakanishi et al., 1980). These results are confirmed by Blanchard and Runkle (2004) who reported that constant temperatures of 20, 23 or 25 °C for six weeks, and also a day/night regime of 23/17 °C led to flowering for 80% of plants from two *Phalaenopsis* hybrids. Higher, but constant temperatures of 26 °C or 29 °C or day/night regimes of 26/14 °C, 26/20 °C, 29/17°C and 29/23 °C led to no flowering over a six week period (Blanchard and Runkle, 2004).

Induced *Phalaenopsis* plants exposed to higher temperatures (above 28 °C) formed "keikei" (vegetative air plantlets) instead of floral buds or alternatively, flower buds are aborted (Sakanishi et al., 1980). "Keikei" are offsets formed at the base of the plant or next to the stem (Venter, 2006).

During conditions of devernalization, "keikei" may replace inflorescence formation (Lopez and Runkle, 2005).

Different recommended temperatures and times of exposure for floral induction are reported in the literature. Newton and Runkle (2009) claimed that certain *Phalaenopsis* and *Doritaenopsis* hybrids require 8 or 12 hours at 29 °C to completely prevent flowering. *Phalaenopsis* 'Miva Smartissimo' x 'Canberra Mosella' and *Phalaenopsis* 'Brother Pink mask' x 'Brother Success Explosion' hybrids needed eight hours, whilst *Phalaenopsis* 'Baldan's kaleidoscope Golden treasure' as well as *Doritaenopsis* 'Newberry Parfait' required 12 hours to effectively prevent flowering (Newton and Runkle, 2009). They also reported that *Phalaenopsis* required a day temperature of 26 °C or lower for three to seven weeks to flower, but that night temperatures had little or no effect on flowering (Newton and Runkle, 2009). In *Doritaenopsis* 'Newberry Parfait' and *Phalaenopsis* 'Baldan's kaleidoscope Golden treasure' flowering was completely suppressed when high temperature exposure was continuous. This confirmed work by Blanchard and Runkle (2006) that temperature during the day, but not the night controls flowering of *Phalaenopsis*. They concluded that day/night fluctuations in temperature, unlike for *Cymbidium*, are not required for flowering in two *Phalaenopsis* clones (Blanchard and Runkle, 2006), contradicting claims in early research where it was reported that both day and night temperatures affect floral induction and subsequent flowering of certain *Phalaenopsis* hybrids (Blanchard and Runkle, 2004).

Temperature controlled induction of *Phalaenopsis schillerana* has also been investigated. It was found that a drop in night temperature below 21 °C for two to three weeks was necessary for floral induction (Sanford, 1971). *Phalaenopsis amabilis* was also found to be induced by cold temperature (Sanford, 1971). Similarly to above mentioned findings flowering is prevented when grown at high temperatures (30 °C day/ 23 °C night), however this inhibition could be reversed by gibberellic acid (GA_3) application. Increases in sugar levels have been associated with GA_3 applications under warmer, non-inductive temperatures. Promotion of sucrose translocation from source leaves to the inflorescence will increase the sink activity of the inflorescence. In GA_3 treated plants an increase in sucrose synthase was also observed (Chen et al., 1994), supporting the nutrient diversion hypothesis (Hew and Yong, 2004).

Goh and Arditti (1985) claimed that *Phalaenopsis* species grown under supra-optimal temperatures for floral induction can be induced to bloom by weekly applications of 10 $mg.L^{-1}$ foliar gibberellins (GA_s) for four months. Important to note is that it was also reported that unlike other non-orchidaceous plants, GA_3 cannot replace the vernalization requirement of orchids (Hew and Yong, 2004).

4. *Cattleya.* The genus *Cattleya* is an epiphytic orchid group that grows on trees in forests and is native to Central and South America. In *Cattleya* flowering is promoted by low temperature and short days (Lopez and Runkle, 2004). Studies showed that flowering is promoted in *C. gaskelliana*, *C. mossiae* and *C. warscewcizii* through exposure to continuous short days such as a minimum of nine

hours light per day and cool day temperatures of 13 °C (Lopez and Runkle, 2004). In contrast, few plants flowered when exposed to 16 hour days at 13 °C (Lopez and Runkle, 2005). Furthermore, *C. warscewiczii* required short days and cool temperatures to promote pseudobulb development which in turn would ensure rapid flowering. Flowering was delayed and reduced by two to three months when plants were exposed to short days at 18 °C compared with similar day lengths at 13°C where pseudobulb development would occur (Lopez and Runkle, 2005). *C. schilleriana* flowered in short days irrespective of the prevailing temperatures, whilst flowering only occurred under long day conditions at temperatures below 16 °C (Lopez and Runkle, 2005). In *Cattleya* species, flowering occurs at different times throughout the year, consequently prerequisites for flowering differ between species and hybrids (Lopez and Runkle, 2005), explaining the inconsistent results found in the literature.

Thus far no cases of high temperature induced flowering have been found. It is however speculated that this method of regulation may be relevant in some Savannah orchids such as *Habenaria* and *Eulopia*, as it is a common occurrence in other bulbous plants such as *Hyacinthus orientalis* and *Tulipa gesneriana* (Sanford, 1971).

It is believed that temperature (both high and low) could be linked to moisture effects like rain or droughts as inductive conditions for orchid flowering (Sanford, 1971). An example of such a case is the orchid *Grammatophyllum rumphianum*, which requires alternating wet and dry seasons for flowering (Sanford, 1971). *Dendrobium formosum* was reported to only flower when exposed to a dry season referred to as a 'ripening season' (Sanford, 1971).

C. Light

Vanda cv. Miss Joaquim was found to flower more profoundly in full sun rather than partial shade. When grown in full sunshine in south-east Asia, *Vanda* and its hybrids cultivated as cut-flowers, bloom continuously, whilst being cultivated in greenhouses in the northern temperate regions it may only flower once per year. In addition, it was found that this hybrid flowered best when exposed to a minimum of ten hours of full sunlight, as shorter times of exposure reduced the number of flowers, furthermore more flowers were produced in full sun compared to partial shade, indicating that this is a light intensity rather than a photoperiodic effect (Sheehan, 1983).

These results, as in so many cases, appear to be correlated to endogenous plant hormone levels. It was reported that auxin levels varied with exposure time to full sunlight, when auxin levels of vegetative and flowering shoots were compared, a longer exposure of *Vanda* plants to full sunlight, correlated with lower auxin levels. Interestingly, gibberellic acid levels (GAs) were unaffected by light exposure time. It was therefore concluded that auxin rather than gibberellic acid is considered a regulatory factor in flowering of *Vanda* (Sheehan, 1983).

In Hawaii the effect of shading on flowering of two orchid hybrids namely, 'Herbert Kurihara Flori' ('M-10973 Rhv.') and *Colmanara sphacetante* 'Evelyn' ('M-10878') was evaluated (Kobayashi

and Mersino, 2006). Effects on flowering however varied according to species, and thus more focussed research for individual hybrids would be required. Shade net treatments included black netting with 30% shading as control, as well as grey, blue and red netting each providing 30% shading. In this trial black and red shade cloth resulted in more inflorescences and more flowers per inflorescence and also in earlier appearance of inflorescences. Unfortunately flowering also terminated earlier than expected. Blue shade cloth caused a delayed inflorescence appearance. New inflorescence stems were produced earlier under red shade cloth, whilst under the black shade cloth no new inflorescences were formed (Kobayashi and Mersino, 2006).

The recommended light intensity for optimum growth and development of *Phalaenopsis* is between 200 and 400 $\mu mol.m^{-2}.s^{-1}$ (Lopez and Runkle, 2005) and a minimum light intensity required for in vitro flowering was established by Konow and Wang (2001). In this study 2%, 77% and 98% of *Phalaenopsis* 'Atien Kaala' seedlings flowered when grown under irradiances of 52, 82 and 240 $\mu mol.m^{-2}.s^{-1}$ respectively (Konow and Wang, 2001).

During cooling of *Phalaenopsis* a moderate light intensity is needed for flowering (Wang, 1995). Plants grown at a temperature regime of 20/15°C day/night temperature with 60 or 160 $\mu mol.m^{-2}.s^{-1}$ produced flowering stalks (racemes) at 34 and 28 days respectively. Plants grown at 0 or 8 $\mu mol.m^{-2}.s^{-1}$ with the same temperatures of 20/15°C produced no flowering stalks during the trial duration of six weeks (Wang, 1995).

In a study by Leite et al. (2008), management of the light spectrum by coloured shade nets was found to improve commercial characteristics in *Phalaenopsis* plants. Red shade nets lead to precocity in all cultivars with one exception. Leaf area was found to be larger under blue shade nets, and plants grown under blue shade nets also had an enhanced green colour and were glossier (Leite et al., 2008).

D. Photoperiodic response

Photoperiod has an effect on various orchid species, although not all genera, e.g. *Phalaenopsis,* are responsive (Lopez and Runkle, 2004). Plants, including orchids respond to day length broadly in three ways: day neutral plants (DNP), short day plants (SDP) and long day plants (LDP) (Dole and Wilkins, 2005). Tropical plants are believed to be more sensitive to smaller differences in day length than their temperate region counter parts (Hew and Yong, 2004).

1. **Day neutral plants (DNP).** Orchid hybrids such as *Vanda* 'Miss Joaquim', *Aranda* 'Wendy Scott', *Aranda* 'Deborah', *Arachnis* 'Maggie Oei' and several *Dendrobium* hybrids are all tropical orchids considered to be insensitive to day length, thus classified as DNP (Goh and Arditti, 1985). Similarly, day length studies that were conducted on *Dendrobium* 'Jacquelyn Thomas' and *Dendrobium* 'Lady Hay', both flowering all year round, lead to the conclusion that these plants are probably insensitive to day length (Sheehan, 1983).

2. Short day plants (SDP). In a few cases, it was found that the raceme emergence as well as raceme length is promoted by short days in *Phalaenopsis*, while long days promoted vegetative growth and development of "keikeis" (Lopez and Runkle, 2005). Furthermore, Goh and Arditti (1985) found that long days stimulated the growth of vegetative aerial shoots. However, this effect of photoperiod was ascribed to the length of exposure to cooler temperatures at night rather than the shortening of the days itself (Lopez and Runkle, 2005; Sakanashi et al., 1980).

In temperate zones it was found that short days with low temperatures stimulated flowering in some species of *Cattleya*, *Dendrobium* and a few species of *Phalaenopsis* (Sheehan, 1983), however the majority of the *Phalaenopsis* genus is not considered photosensitive. In the genus *Cattleya*, flowering occurred when plants were exposed to continuous photoperiods of nine hours at 13 °C. Few plants flowered when plants were exposed to 16 hours of light per day at 13 °C (Lopez and Runkle, 2004). Remarkably this genus contains both LDP as well as SDP flowering types (Lopez and Runkle, 2004). Short day species are *C. labiata*, *C. mossiae* and *C.trianae*, whilst *C. dowinia*, *C. intermedia* and *C. granulose* are considered by some to be long day species (Batchelor, n.d.).

Cattleya hybrids grown in Hawaii under continuous long days for two years, produced double the number of pseudobulbs compared to plants grown continuously under short- or normal day lengths (Sheehan, 1983). When considering flowering success it was found that on average nine plants flowered under short days, compared to 0.6 (one plant flowered) under continuous long days (Sheehan, 1983).

Temperature is known to modify photoperiodic plant responses. For the short day *Cattleya* species it was found that exposure of plants to higher night temperatures such as 20 °C prevented flowering under short day conditions, but did not have any effect on plants grown under long day conditions (Sanford, 1971). Similar effects were found in *Phalaenopsis amabilis* when grown under short days at night temperature of 20 °C. It was reported that *P. amabilis* flowered at various times during the year when cultivated under the short day conditions, but when grown under long days and 20 °C night temperature it may only flower once per year (Sanford, 1971).

Dendrobium inflorescence development and consequential flowering could be accelerated by placing plants for six weeks under nine hour day lengths, at 18 °C. The same effect was found at 13 °C, although development was slowed by the cooler temperature (Lopez and Runkle, 2005).

The influences of a night interruption of four hours (10:00PM – 02:00AM) on *Cymbidium* at both low light intensities (3-7 $\mu mol.m^{-1}.s^{-2}$) of the night break as well as high light intensities (120 $\mu mol.m^{-1}.s^{-2}$) increased the number of flowers as well as decreased the days to anthesis when compared to the control groups receiving no night interruption (Kim et al., 2012).

E. Hormonal control and decapitation

Orchid branching can proceed in two basic ways, either monopodial or sympodial (Fig. 9). In sympodial orchids growth is limited as the apical meristem terminates in a flower or inflorescence;

therefore growth by means of axillary shoots can only continue by the formation of laterally located axillary buds. For monopodial orchids, growth is unlimited and continuous at the apex (Hew and Yong, 2004). Examples of monopodial orchids are *Aranda* and *Vanda*, whilst the sympodial orchid group contain amongst others *Cattleya* and *Dendrobium*.

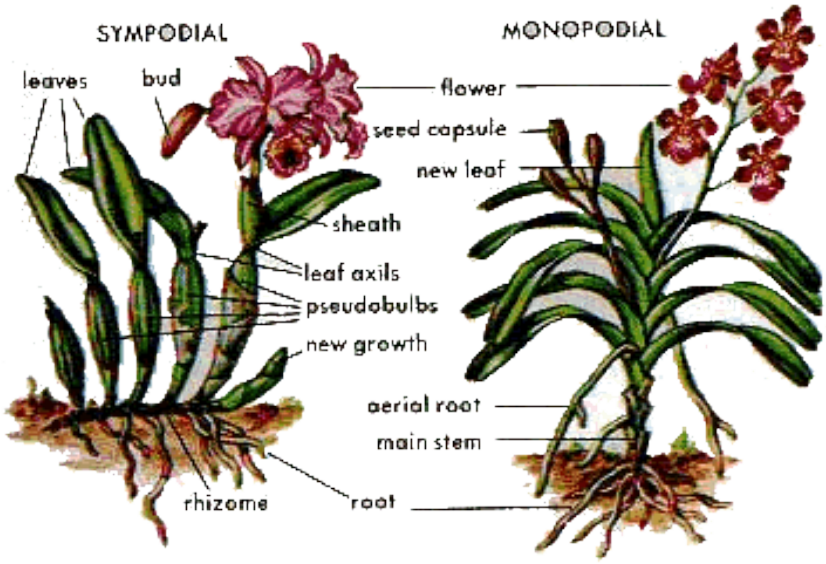


Fig. 9. Illustration of the growth habits of sympodial and monopodial orchids showing growth continuation by means of lateral located axillary buds or unlimited apical growth respectively (Anon d, 2011).

1. Monopodial orchids. *a. Decapitation.* The phenomenon of a flowering gradient seems to be wide spread in monopodial orchids and is exhibited by many *Aranda* hybrids. Decapitation of the shoot apex leads to the formation of axillary shoots. The nature of these axillary shoots (reproductive or vegetative) corresponds to its position on the stem axis (Hew and Yong, 2004). Buds further down the axis develop into vegetative shoots, with those closest to the apex developing into inflorescences. Flowering capacity is therefore the greatest at the shoot apex and diminishes basipetally along the stem axis, therefore representing a floral gradient (Goh, 1975).

The flowering capacity along the gradient was found to differ widely in *Aranda* hybrids. Plants that were decapitated at various levels between leaves 6 to 30 below the apex, showed a dramatic decrease in the number of flowering spikes compared to decapitation between nodes 10 to 15 (Sheehan, 1983). Decapitation at the 15th or 16th node produced an inflorescence in *Aranda* 'Meiling' and in *Aranda* 'Lucy Laycock', whereas decapitation at the 18th to 20th node produced an inflorescence in *Aranda* 'Hilda Galstan' and *Aranda* 'Nancy'. Similarly decapitation at the 25th node produced an inflorescence in *Aranda* 'Deborah'. Decapitation is currently not practiced commercially by orchid cut flower growers.

b. Flowering gradient. Differences in flowering gradient can either be genetic, due to nutritional differences or hormonal in nature (Hew and Yong, 2004). Auxin amongst others is suggested to play an important role (Goh, 1977), as the flowering buds can be released from apical dominance by the application of anti-auxins such as tridobenzoic acid (TIBA) and maleic hydrazide (MH) at concentrations of 10^{-3}M and 10^{-4}M, respectively, although only a low percentage of the initiated flowers developed to maturity (Goh, 1977). The effect of growth retardants such as daminozide (B-nine), 2-chloroethyltrimethyl ammonium chloride (CCC/Cycocel) and succinic acid 2,2-dimethylhydrazide (B995) on flower initiation was also studied (Hew and Yong, 2004). CCC (10^{-3}M and 10^{-4}M) stimulated the initiation of multiple flowers, with a third developing to maturity, whilst B995 showed a similar number of initiated flowers, with even fewer flowers developing to maturity (Goh, 1977). No initiation of flowers was found after applications of IAA (10^{-3}M and 10^{-4}M), GA_3 (10^{-4}M and 10^{-5}M) or abscisic acid ABA (10^{-6}M to 10^{-4}M) (Goh, 1977). By comparison, the synthetic cytokinin 6-benzylaminopurine (BAP) at 10^{-3}M or a double application of 10^{-3}M was greatly successful in releasing apical dominance, with the majority of the initiated flowers developing to maturity (Goh, 1975). BAP was the only application thoroughly successful in induction as well as the development of the inflorescences to anthesis. In addition, the effect of synthetic cytokinin was found to be enhanced by GA_3 (Goh, 1977).

c. Root originated hormones. Root tips of aerial monopodial orchids are known to constitute an important source of endogenous cytokinin, ABA and auxin. Higher levels of cytokinin were found in root tips of flowering *Aranda* 'Noorah Alsagoff' plants than in root tips of non-flowering plants (Zhang et al., 1995), whilst a correlation between spike formation and the number of roots present were reported.

Concluding from the above studies, cytokinin, either synthetic as in the study by Goh (1977) or endogenous from the roots (Zhang et al., 1995) may play an important role in flower induction of monopodial orchids.

2. Sympodial orchids. *a. Cytokynin: Benzyladenine (BA).* Similar effects were found in monopodial orchids as in sympodial orchids with BA, especially in regard to the control of flowering. The application of exogenous cytokinin induced flowering of sympodial orchids such as *Dendrobium* hybrids, where BA (10^{-3}M and 10^{-4}M) stimulated flower induction in mature pseudobulbs of *D.* 'Lady Hochoy' and *D.* 'Buddy Shepler' x *D.* 'Peggy Shaw' (Goh and Yang, 1978). An application of GA_3 (10^{-3}M and 10^{-4}M) alone was unsuccessful in inducing flowers, but in combination with BA it slightly enhanced the BA effect. Consequently it was also found that IAA (10^{-5}M) suppresses the promotive effect of BA (Goh and Yang, 1978).

Flowering did not occur while vegetative growth occurred, but only after the apex became quiescent. The actively growing vegetative apices inhibited the development of axillary buds (Goh, 1977). This illustrates the role of apical dominance in sympodial orchids, highlighting the role of endogenous hormones in flower induction.

BA was found to promote flowering in *Doritaenopsis* and *Phalaenopsis* orchid clones (Blanchard and Runkle, 2008), by hastening the occurrence of a visible inflorescence by 3-9 days, and by increasing the number of inflorescences by 1 to 3.5 and the number of flowers per inflorescence by 3-8 (Blanchard and Runkle, 2008). This suggests that cytokinin, may at least partially regulate inflorescence induction. Still, although inductive, BA could not substitute for the necessary inductive low temperature (Blanchard and Runkle, 2008).

The number of inflorescences of *Dendrobium* 'Jaquelyn Thomas' were significantly increased from 0.5 inflorescences per control plant to 8.9 inflorescences per treated plant by injecting 100mM BA into one-year-old leaved stems (Lopez and Runkle, 2005). Similarly, when 10 or 100 mM BA were injected into the vegetative buds of two-year-old leafless plants, the number of inflorescences per plant increased from 0.2 to 4.0 or 6.3 inflorescences, respectively. However, the higher concentration of 100 mM BA led to abnormalities in inflorescences and reduced inflorescence length, although the addition of 1000 mM GA_3 to the solution counteracted this effect. Once again, similarly to Goh and Yang (1978), the promoting effect of GA_3 on BA treatments, with regard to flowering in orchids was reported.

It is well documented that both short days and low temperatures can affect the endogenous levels of growth regulators (Goh, 1977). Consequently flowering responses to these environmental conditions are only secondary due to the changing levels of endogenous hormones. A strong correlation is seen between increases in cytokinin levels following a period of cold exposure, and the flower induction of orchids such as *Dendrobium* (Hew and Yong, 2004).

b. Cytokinin: Auxin ratio. The correlation between light intensity and endogenous auxin levels have also been investigated. Endogenous levels of auxin was found to be lower in *Vanda* 'Miss Joaquim' plants grown under high irradiance and higher in plants grown under low irradiance (Hew and Yong, 2004). Furthermore, it was observed that under high light intensity more flowers were produced (Hew and Yong, 2004). The level of cytokinin was not reported in this trial, as the cytokinin/auxin ratio is considered more important than the absolute concentration of auxin (Hew and Yong, 2004).

Campos and Kerbauy (2004) discussed the importance of cytokinin and auxin in flower initiation, but emphasized that although important, it may not necessarily be the only contributing factors to flowering. Cloned *Dendrobium* 'Second love' plants were exposed to a 12 hour photoperiod at 25 °C and 10 °C over a period of 30 days. On monitoring the levels of cytokinin and IAA, both were found to increase after 15 days of treatment, whilst the levels of ABA decreased throughout the treatment period. The photoperiodic and temperature treatment accelerated inflorescence development and hormones were suggested to play a role in the signal transduction pathway of thermo-periodic flowering.

c. Gibberellic acid (GA_3). In studying the role of GA_3 on flower induction in orchids, four

year old non-flowering *Paphiopedilum* ('Macabre' x *P. glanduliferum*) plants were treated either with GA_3, BA, or a combination of the first two treatments or a combination of BA and 1- naphthalene acetic acid (NAA) (Miguel et al., 2008). The ten GA_3 treated plants all flowered and produced an average of 3.8 inflorescences per plant. However, the BA or BA/NAA treated plants produced no inflorescences. Interestingly, when in combination with other growth regulators, BA seemed to reduce the effectiveness of GA_3 as only six (of ten) plants flowered and produced an average of 3.2 inflorescences within the GA_3/BA combination treatment (Miguel et al., 2008).

Similarly, an experiment was conducted to promote flowering of *Miltoniopsis* orchids by treatment of GA_3 (2.5 or 5 mM) and N^{6}-benzyladenine (BA) (25 or 50 mM), alone or in combination. This orchid is normally induced by cool temperatures and a short photoperiod (Matsumoto, 2006). BA treatments promoted the growth of new vegetative shoots and decreased the number of flowering plants compared to the control group. These effects were reduced by GA_3 when BA and GA_3 were used in combination, but results were still negative in comparison to untreated plants. GA_3 hastened inflorescence emergence by between 10.9 to 48.7 days, depending on the hybrid used. In 'Eileen' the number of inflorescence per plant increased by 0.8 with the GA_3 treatment (Matsumoto, 2006).

d. Potassium chlorate (KClO3). In addition to, or replacing the requirement of cool temperatures for inflorescence induction, it was found that certain chemicals can substitute for vernalization and can induce flowering for some orchid species and sub-tropical fruits. These chemical treatments included potassium chlorate ($KClO_3$), salicylic acid and gibberellic acid (Li et al., 2006). A study with $KClO_3$ on the non-photoperiodic, but temperature sensitive longan tree, has resulted in flower induction (Li et al., 2006). This resulted in the evaluation of $KClO_3$ on the non-photoperiodic, but temperature sensitive *Phalaenopsis*. In longan trees, $KClO_3$ resulted in higher cytokinin and lower GA_3, followed by the subsequent induction of flower differentiation and morphogenesis (Manochai, et al. 2005). Considering the positive effects found with BA applications in both sympodial orchids (Goh and Yang, 1978; Zhang et al., 1995) and in monopodial orchids by Goh (1975), further research into the efficacy of $KClO_3$ to induce flower initiation seems justified. The efficiency of $KClO_3$ when evaluated on *Phalaenopsis* was not as expected. Various levels of $KClO_3$ applications (0-16 $mmol.L^{-1}$) differentially delayed time to visible raceme emergence and raceme length was also decreased. However $KClO_3$, at concentrations of 4 and 8 $mmol.L^{-1}$ decreased the days to anthesis of the first flower by 13 and 24 days respectively and increased the number of viable buds by 16% (Li et al., 2006).

F. Nutrition

The common horticultural practice to reduce nutrient levels (especially nitrogen) to encourage flowering does not apply to orchids, as nutrient levels do not fluctuate drastically from one season to another in their natural environment, but flowers are still induced (Lopez and Runkle, 2004). A possible explanation for the observed occasional floral induction of orchids following reduced

nutrient supply is that the plants grown under such severe conditions are stressed to the extent where they resort to flowering as a survival mechanism. This, however, is not an approach that is advisable or sustainable in commercial cultivation. Another method described by hobbyist is to induce flowering by the addition of Epsom salts (magnesium sulphate). Magnesium forms an integrated component of chlorophyll and therefore plays a central role in photosynthesis. There is however no scientific proof that these techniques are effective in inducing flowering in a large range of species (Lopez and Runkle, 2004). Nevertheless, it is well known that the N:K ratio in a plant plays a major role within flowering, where a ratio of 2:1 or less is generally promotive of flowering (Malan, 2013).

In vitro flowering of *Cymbidium niveomarginatum*, a plant that requires four to seven years before flowering, has been achieved on a growth medium of restricted nitrogen, enriched phosphorus and cytokinin (6-benzylaminopurine), together with root excision as a cultural practice. This technique however has limited commercial potential (Lopez and Runkle, 2005).

G. Orchid flowering time manipulations

Current manipulation of *Cattleya* to flower twice a year in order to meet the market demand in June and over the Christmas period is based on photoperiod manipulation (Fig. 10). Steps to follow (Northern hemisphere (NH) or Southern hemisphere (SH)) as described by Sheehan (1983) are listed below.

1. Create 16 hour day length from 5 June to 10 October (125 days) by the addition of lights, and 27°C day- and 17°C night temperature.
2. Flowers should be harvested by 15 December, 65 days after the removal of additional lights.
3. Lights are turned on again from 15 December to 13 April, over a 105 day period.
4. Provide shading from 13 April to 5 June to create an eight hour day for 60 days, at day temperatures of between 27 °C and 29 °C, and night temperatures of 17°C.
5. Resulting flowers will then be cut by 5 June, where after steps 1 to 5 is repeated year after year.

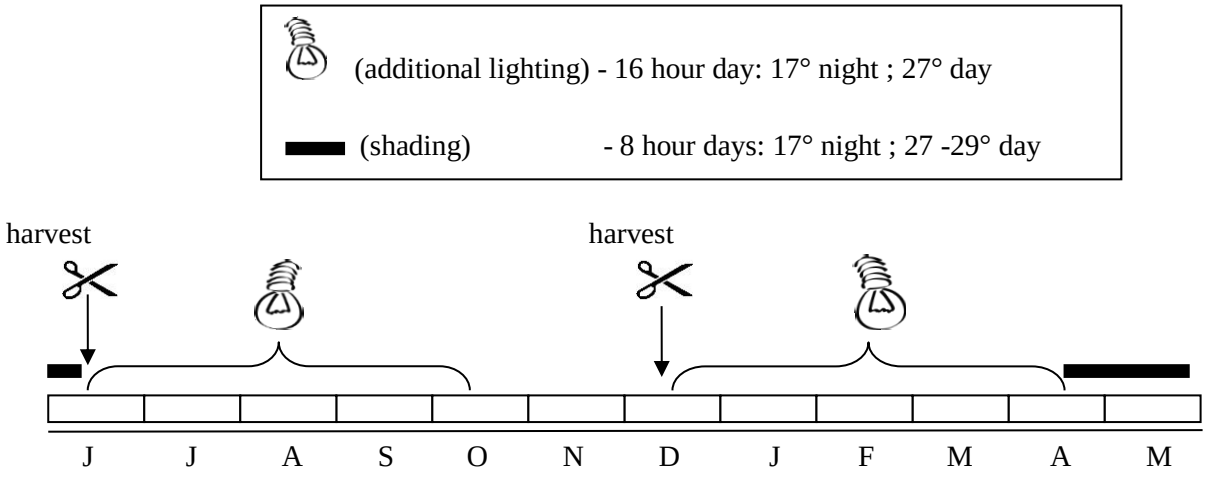


Fig. 10. Schematic representation of flowering manipulation model of *Cattleya* as accounted in Sheehan (1983).

No manipulating of flowering time has yet been researched or applied for the genus *Disa* (Van der Linde, personal communication, 2010). When considering the growth habitat of *Disa*, photoperiodism together with a minimum plant size may play a role in flower induction. The natural habitat and environmental growing conditions together with the short flowering window in summer and the rapid spike emergence in spring, suggests a short day photoperiod requirement for *Disa* and warrants further research.

XI. DISA, A GEOPHYTE OF THE CAPE FLORISTIC REGION

High plant diversity is characteristic of Mediterranean climates. This is especially noted in the high number of geophytes recorded in the CFR of South Africa (Proches et al., 2006). Geophytes are defined as all plants with a perennial organ which fulfils a storage function (Proches et al., 2006), or plants that survive not only as seeds, but also as specialized underground storage organs (Theron and De Hertogh, 2001).

Many of these geophytic species are of horticultural importance due to their synthetic appeal and are being cultivated worldwide. The largest horticultural important geophytic families within the CFR include: Iridaceae with 767 species, followed by Hyacinthaceae with 285 species and Orchidaceae with 230 species, as well as the Amaryllidaceae with 147 species (Proches et al., 2006). Almost half of the species richness in the Cape floral kingdom is accounted for by only 33 Cape floral clades, with *Disa*, being one of these (Bytebier et al., 2011).

Disas are considered well adapted to a fire prone environment along with many other Fynbos geophytes (Keely, 1993). Some *Disa* species even may be obligate fire dependant for flowering in the following season. One such example is *D. racemosa* (Crous and Duncan, 2006). The mechanism or triggers for this phenomenon is not yet understood (Bytebier et al., 2011). Another, well known fire

dependant species is *Cyranthus ventricosus* or the Fire lily. Within days after a veld fire, these geophytes are induced to flower. Interestingly, in *Cyranthus* flowering can be induced at any time of year, but is completely linked to fire (Keely, 1993). Furthermore, flowering is stimulated by smoke, but exogenous ethylene, a gaseous component of smoke was not successful in stimulating flowering alone (Keely, 1993). The active component that has recently been identified for stimulating flower and also stimulating seed germination is smoke-derived butenolides (Light et al., 2008).

As *Disa* has evolved alongside other geophytes, which has adapted to the fire-prone ecology and Mediterranean climate of the CFR, insight into the mechanisms that control flowering of these geophytes, sharing this natural niche with *Disa*, may provide some understanding of the floral initiation processes still unknown in *Disa* (C.A. Pauw, personal communication, 2011).

A. *Gladiolus*

Gladiolus is classified as a geophyte having a turnicated corm as the geophilic organ, where the corms are replaced annually (Halevy, 1985). Flowering in *Gladiolus* is autonomous, always following the differentiation of a definite number of leaves (eight to ten) (Shillo and Halevy, 1976a). Apart from this, some of the main driving factors affecting flowering in *Gladiolus* are photoperiod, light intensity and temperature.

1. Light intensity. *Gladiolus* has been described as one of the plants being most efficient in the utilization of available light, although the failure to flower in winter can mainly be attributed to low light intensities found during this season (Shillo and Halevy, 1976a). The effect of light intensity on *Gladiolus* seems to be cumulative, as shading for longer periods caused a greater reduction in flowering than shading for shorter periods. Furthermore, the reduction of flowering increased as light intensity decreased (Shillo and Halevy, 1976a). Cultivars differ in their sensitivity to low light intensities. In the more sensitive cultivar ‘San Souci’ exposure to low light intensities reduced the flowering percentage as well as the number of flowers per spike. In a less sensitive cultivar ‘Spic and Span’, only the number of flowers per spike was affected. The most sensitive developmental stage is between the fifth leave stage and spike emergence (Shillo and Halevy, 1976a).

2. Photoperiod. The decrease in the number of daylight hours in winter caused a reduction in the flowering capacity of the plants. This effect differed in severity according to the sensitivity of different cultivars (Shillo and Halevy, 1976b). A reduction in flowering capacity was three fold, firstly a reduction in the number of secondary inflorescences was seen, secondly the number of flowers per spike was decreased, and lastly the flowering percentage decreased. In addition, the sensitivity to short days (SD) differed with different developmental stages and plants were found to be most sensitive during the period of the appearance of the second leaf to spike emergence.

It is a possibility that the effects found in reducing the number of daylight hours could be a secondary effect due to a reduction in total irradiance, rather than a photoperiodic effect (Shillo and Halevy, 1976b). This was also implicated in a report by Halevy (1985), as in winter-grown *Gladiolus*

the failure to flower or the reduction of the number of flowers per spike is a common phenomenon. It was found that a reduction in light intensity as well as SD induced flower blasting (Halevy, 1985).

Flower development was further correlated with the light integral during the time of inflorescence development. It is hypothesized that the main factor causing flowering in summer, is the daily high solar energy flux, which is double in summer compared to winter (Shillo and Halevy, 1976c). Alternatively, winter with its marginal light intensities and lower temperatures, typically cause flower blasting. Therefore, although SD was found to advance flowering, the flowering percentage, number of flowers per spike as well as spike length were negatively affected (Halevy, 1985). Similarly, applications of night breaks delayed flowering by up to three weeks, but improved flower quality in increasing the number of secondary inflorescences and the number of flowers per spike. Furthermore, the flowering percentage rose and the stem length of the flowers were also greater (Shillo and Halevy, 1976b). In some cultivars long days (LD) did not affect flowering time, although flowering percentage and flower quality were promoted. This indicates a photoperiodic effect of flower development, independent from light integral affects (Halevy, 1985).

Gladiolus has two competing sinks during development, the new daughter corms and the inflorescence (Halevy, 1985). Distribution of assimilates between these sinks are directly affected by photoperiod in that the flower sink is promoted in LD, whilst the daughter corm sink is promoted during SD. Increasing the competition for assimilates, may induce flower blasting in photosynthate limiting times (Halevy, 1985). In greenhouses, supplementary lighting has been used to promote flowering in winter flowering *Gladiolus* cultivars. By using additional low light intensity lighting plant density could be tripled and flowering was improved, with the best effects obtained by supplying additional lighting throughout the night.

3. Temperature. Two stages of *Gladiolus* development were particularly sensitive to low temperature, shortly after planting and during the seven leaf phase. Low temperature during the first phase retarded growth while during the latter phase a lower number of flowers per spike were produced (Shillo and Halevy, 1973c).

Considering the role of temperature on flowering, the greatest effect of temperature was found on the number of days to flowering, by influencing growth and development. Chilling of plants during the day inhibited stalk elongation (Shillo and Halevy, 1976c). Summer-grown gladioli bloom within 60 to 80 days, whilst winter-grown gladioli required 120 to 140 days from sprouting before flowering occurs. Conditions of low night temperatures (1 to 4 °C) and low light intensities induced the blasting of entire inflorescences. However, night chilling with high irradiance did not reduce flowering.

Gladiolus has been known to withstand temperatures of up to 50 °C, provided humidity and soil moisture are high. Since flower induction occurs after sprouting, temperatures applied to the corms have limited influence on flowering (Shillo and Halevy, 1976c). More recent studies however indicate the importance of cold treatment on corms to accelerate the breaking of dormancy (Gonzales

et al., 1998). In *Gladiolus tritis* cold storage of three or six weeks at 5°C and a relative humidity of 90% advanced flowering by 20 and 11 days respectively. Furthermore, the length of the flowering period was increased by 35 days when stored for six weeks. Quality parameters of the flowers were not affected by corm cold storage, with the only difference the increased flowering stem lengths when corms were stored for six weeks (Gonzales et al., 1998).

B. *Freesia*

Freesia x hybridia was developed from species native to the Western Cape of South Africa. The geophytic organ of the *Freesia* is a turnicated corm of several internodes. The presence of the corm classifies *Freesia* as a geophyte. Freesia, are commercially grown from seed or corms. Similar factors that affect flowering of *Gladiolus* can also be seen prevalent in *Freesia* hybrids.

1. Temperature. Complete differentiation of the *Freesia* inflorescence requires 6 to 9 weeks at temperatures ranging from 12 °C to 15 °C but induction can be assumed to be irreversible after four weeks at 13 °C. Although temperatures ranging from 12 °C to 15 °C are considered optimum, induction can occur between 5 °C to 20 °C (Gilbertson-Ferriss, 1985). When flower induction is interrupted by high temperatures (above 16 °C) abnormal inflorescence development occurred, such as that a flower may developed some distance below others on the spike.

After initiation, higher temperatures decrease the time to anthesis, while lower temperatures in the 10 °C to 12 °C range will advance days to flowering (Gilbertson-Ferriss, 1985). Decreased flower numbers per inflorescence, inflorescence stem length and number of lateral inflorescences may also occur at temperatures above 16 °C. Berghoef et al. (1986) planted three cultivars, 'Blue Heaven', 'Ballerina' and 'Aurora', at temperatures ranging between 9 and 25 °C. All cultivars showed flower initiation at five weeks after planting in the temperature range of 9 °C to 15 °C. At higher temperatures floral initiation was delayed in the different cultivars to varying degrees. Flower initiation in 'Blue Heaven' was delayed when planted and grown at 16 °C, 'Ballerina' at 17 °C and 'Aurora' at the highest temperature of 18 °C (Berghoef et al., 1986). Furthermore, at higher temperatures which delayed flowering, more leaves developed (15-20), compared to the number of leaves (10-12) when planted at lower temperatures. Commercially *Freesia* corms are forced at temperatures between 12 °C to 15 °C to ensure rapid flower initiation and development as well as good quality flowers. When grown under eight hours at 13 °C, irrespective of dark or light, and 16 hours of non-inductive temperature of 24 °C, *Freesia* will flower and better quality is produced at the constant temperature range of 12 °C to 15 °C (Gilbertson-Ferriss, 1985). Berghoef and Zevenbergen (1990) found that timing of floral initiation depends on the average temperature of the air and the soil. Flowering was delayed at higher average temperatures. This delay was ascribed to lower assimilate levels in leaves at higher temperatures, compared to plants grown at lower temperatures. The plant leaves displayed a considerably higher soluble carbohydrate level at 13 °C than at 21 °C. At a lower carbohydrate level (21°C), the apex continued to form leaves, driven by higher soil and air

temperatures which enhanced leaf growth and subsequently reduced the available carbohydrates (Berghoef and Zevenbergen, 1990).

Flowering of *Freesia* hybrids are furthermore also influenced by pre-plant corm storage temperature and duration. Wulster and Gianfagna (1991) found that corms stored at 5 °C for four weeks flowered 20 days earlier than non-stored, control corms. In addition, cold storage of corms also decreased both the plant and flower height (Wulster and Gianfagna, 1991). Three *Freesia* cultivars were grown at greenhouse temperatures of 10, 15 and 20°C, respectively. The warmest greenhouse temperature resulted in the tallest plants in all three cultivars. Furthermore, supporting the findings of Gilbertson-Ferris (1985), the number of days to flowering decreased as the greenhouse temperatures increased (Wulster et al., 1989).

2. Photoperiod. The response of *Freesia* to photoperiod has been found to be inconsistent. Freesias seem to be less responsive to photoperiod than to temperature (Gilbertson-Ferriss, 1985). Flower induction may be stimulated by SD, whilst flower development is enhanced in LD. SD increases the number of flowers, the length of the inflorescence and the number of lateral inflorescences, with LD producing the exact opposite effect. However, LD was observed to hasten the time to anthesis by up to two weeks (Gilbertson-Ferriss, 1985). The promoting effects of both LD and SD are considered less pronounced as temperature increases, suggesting that temperature are more influential than photoperiod and that high temperatures could mask any photoperiodic effects (Gilbertson-Ferriss, 1985).

3. Growth regulators. Corms of *Freesia* was found to be non-responsive to hormone treatments as the quality and rate of flowering could not be successfully altered by using vacuum infusion or soaking freshly harvested *Freesia* corms in gibberellins, BA or IAA or combinations of the above mentioned (Gilbertson-Ferriss, 1985). Ancymidol, as active ingredient of certain growth retardants delayed flowering by nine days when applied as a soil drench of 3 mg per pot (Wulster and Gianfagna, 1991). Wulster et al. (1989) found similar results, but only found the delay at higher growing temperatures. Furthermore, Ancymidol™ decreased the leaf and flower height. When applied as a soil drench, Wulster and Gianfagna (1991) found a reduction of 50% in plant and flower height of *Freesia*. The reduction of plant and flower height was less pronounced at higher greenhouse temperatures (Wulster et al., 1989).

XII. CONCLUSION

Orchids are some of the most beautiful ornamentals available to the floricultural market. Therefore the ability to manipulate flowering time for more effective commercial production is important for successful and sustainable marketing in an environment constantly demanding novel floriculture products. When taken into account the size of the Orchidaceae and the limited availability of literature, especially concerning the factors which control flower induction and flower development, it is evident that further research is required. Existing research based mainly on

environmental factors affecting the flowering processes within the Orchidaceae, shows great variation between the various genera within the family, and even within species, making any generalizations or extrapolations impossible.

Judging by the consumer demand and the healthy market prospects as well as the availability of the well-studied *Phalaenopsis*, this could only indicate the untapped potential for other less well-known and documented taxa within the Orchidaceae. The South African *Disa* and its hybrids are one such example. *Disa*, one of the most beautiful orchids worldwide, is currently being cultivated commercially only on a limited scale. However, a greater understanding of the mechanisms that controls flowering in this genus, would allow far greater access to local and international markets.

A good starting point for research into the flowering system of a specific orchid is to study the origin and habitat native to that species. Firstly, the study of other genera or species within Orchidaceae showed limited similarities with *Disa*, as *Disa* is a terrestrial, sympodial orchid, also classified as a geophyte, acclimated to the Mediterranean type climate of the Cape Floristic Region of South Africa. Studying the flowering mechanisms of geophytes, having evolved with *Disa* and sharing an ecological niche, could therefore be another approach to unravel the flowering mechanism. However, the wide variety of geophytes found in the CFR, together with their adaptations to specific niches complicate the search for a useful model to compare, to the cultivation and flowering model of *Disa*. The amount of information that can be accumulated through observation of growth cycles in nature, and also from hobbyists, should not be underestimated.

Scientific experimentation based on the experience of growers could give a better understanding of *Disa* and in the development of this genus to its full horticultural economic potential.

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Paper 1: Florogenesis and Physiological Disorders of Greenhouse Cultivated *Disa* Hybrids (Orchidaceae)

ABSTRACT. The indigenous South African orchid *Disa* has significant horticultural appeal. The lack of relevant literature and science-based information on *Disa* prevents the cultivation of this orchid and it realising its full economic potential. Floral initiation and differentiation of *Disa* hybrids 'LM0739' and 'LM0721' was studied in cultivation using light and scanning electron microscopy. Floral initiation was observed between May and June for *Disa* 'LM0739' and 'LM0721' respectively and the duration of floral differentiation was 15-16 weeks. The differentiation of *Disa* hybrid flowers was divided into ten characteristic stages. Vegetative phenology of the above mentioned hybrids was studied in relation to the reproductive phenology by recording various growth parameters such as plant height (mm), leaf width (mm), number of unfolded leaves, number of floral primordia and dry weight of above-and below ground components. From transplant through to anthesis the growth strategy of the tuber-producing hybrid 'LM0721' differed from the non-tuber forming hybrid 'LM0739'. Floral abnormalities were observed in these cultivated *Disa* hybrids. A first report on the abortion of the apical meristem as well as the two youngest developing flowers of the inflorescence in *Disa* was documented. Understanding the phenology of a plant in cultivation and especially elucidating the exact time of floral initiation can be invaluable in the management and manipulation of flowering regimes. Therefore, this study provides a strong basis for future studies, with the common goal to establish the successful commercial production of *Disa* as a potted plant.

ADDITIONAL INDEX WORDS. *Disa uniflora*, terrestrial orchid, geophyte, inflorescence initiation, floral differentiation, bud abortion, floral abnormalities

The indigenous terrestrial orchid genus *Disa* consist of more than a 100 species, predominantly found in southern Africa (Crous and Duncan, 2006). A group within this genus, often referred to as "Evergreen disas" are used by breeders, mainly due to its large and showy inflorescences (Holmes, 2011). Incidentally, these evergreen disas also lends itself best to cultivation and commercialization and consequently, all the *Disa* species known to be used in hybridisation programs are derived from this group (Holmes, 2011). Evergreen disas include species such as *Disa aurata, D. cardinalis, D. caulescens, D. uniflora, D. marlothi, D. tripetaloides* and *D. uncinata* (Crous and Duncan, 2006), with *D. uniflora, D. cardinalis, D. racemosa* and *D. tripetaloides* most prominently used in hybridisation programs (Holmes, 2011). *D. uniflora,* the most renowned of this group, also commonly referred to as the 'Pride of Table Mountain' or 'Red *Disa*', has the largest flower of all indigenous South African orchids and is considered to be one of the most beautiful

orchids worldwide. Adding to this a distinctive range of colours and unique triangular shaped flowers, it is evident why this species harbours commercial potential (Tibbs, 2007).

The basic structure of an orchid flower reflects morphological characteristics of monocotyledonous plants. The flowering stem of *Disa* consists of the peduncle together with the rachis, where the peduncle can be naked or bearing several leaf sheaths (Linder and Kurzweil, 1999). The peduncle is often referred to in horticultural literature as a stem. The most common inflorescence type found within Orchidaceae is a raceme, where each flower is borne on a short stalk (pedicel). Such an inflorescence is mostly present in *Disa*. The flowers of *Disa* are zygomorphic, where the flower is symmetrical on one plane only (Linder and Kurzweil, 1999; McMurty et al., 2008; Schelpe, 1966; Stewart et al., 1982). The perianth is arranged in two whorls containing three segments each. Although the floral structure is classified as a perigone in a strict morphological and botanical sense, the terms 'petals' and 'sepals' are more commonly used in orchid literature (Linder and Kurzweil, 1999).

The first whorl of an orchid flower consists of one dorsal and two petaloid lateral sepals (Xu et al., 2006). The sepals protect the other floral parts in the bud (Stewart et al., 1982). The median or dorsal sepal, arranged at the dorsal side of the flower, is slightly hooded and can be elongated, forming what is called a spur. The spur may contain nectaries (Du Plessis and Duncan, 1989; Stewart et al., 1982). In *Disa*, the inner perianth whorl is referred to as the petals, and is smaller than the sepals, contrary to other orchids like *Dendrobium* where the petals are broader than the sepals (Xu et al., 2006). The petals of *Disa* are enclosed by the median sepal (Linder and Kurzweil, 1999). Similarly to *Dendrobium* in particular (Xu et al., 2006) and most orchids in general (Linder and Kurzweil, 1999), the median petal of *Disa* is modified into a lip/labellum which differs in size and shape compared to the other two petals. The lip which aids in cross-pollination, could be spurred and may contain nectar, and therefore acts as attractant and landing platform for pollinators (Pridgeon, 1999). Though, as the dorsal sepal of *Disa* is spurred, the lip does not exhibit this adaptation.

An interesting feature of orchid flowers is the phenomenon of resupination where the ovary is twisted by 180°C on the pedicel to achieve its position (Linder and Kurzweil, 1999; Stewart et al., 1982). Most southern African orchids are resupinated, implicating the lip is the lowermost part of the inner perianth lobes. In the developing bud, the lip usually faces away from the axis, whilst the column faces upwards. However, at anthesis, the lip is lowermost, and this is achieved by resupination. Resupination occurs to position the lip, as landing platform for the pollinator, in the correct lowermost position. The ovary of orchid flowers is inferior and lies below the attachment of the other floral parts. The position of the ovary is also described as being borne on a pedicel, with the floral parts borne on the apex of the ovary (Pridgeon, 1999). The ovary is green, slender and can be indistinguishable from the pedicel at time of flowering (Stewart et al., 1982).

The prime distinguishing feature of an orchid flower is the fusing of the sexual parts to form a unique and central structure, the column (Linder and Kurzweil, 1999; McMurty et al., 2008). The

column, made up by the male anther, female stigma and style, forms the stalk that bears the pollination apparatus (Stewart and Hennessy, 1981). The anther on top of the column always has two thecae (pollen or anther sacs) which contains the pollinia (Linder and Kurzweil, 1999), a term that refers to a condition where the pollen is united to form firm masses. The pollinia have a viscous stalk termed the caudicle which attaches the pollinia to a sticky gland, the viscidium. This sticky viscidium adheres to a visiting pollinator, and when the pollinator leaves the flower the attached pollinarium (pollinia and caudicle) is transported to the stigma of the next flower to be visited by the pollinator. The stigma is usually seen as a sticky surface at the base of the column (Linder and Kurzweil, 1999; Stewart et al., 1982). Between the anther and stigma, a sterile strip, flap or bulge of tissue, called the rostellum can be seen. The tip of the rostellum supports and cradles the viscidium (Stewart et al., 1982).

Orchids display both monopodial and sympodial growth habits, with terrestrial orchids showing almost exclusively sympodial growth (Linder and Kurzweil, 1999). All orchids found in the Western Cape Province are terrestrial and therefore presumably sympodial. These orchids are adapted to a wide range of habitats within the Mediterranean-type climate characteristic of the Cape Floristic region (CFR) of South Africa.

In nature, mature *Disa* plants have two root tubers at anthesis, one that develops during the current growing season together with the remaining tuber from the previous growing season, which supports the flowering spike (Linder and Kurzweil, 1999). In *Disa,* in contrast to epiphytic orchids, the above-ground plant material completely dies back after flowering and a new shoot and root system is initiated from the current season's tuber. The progeny is genetically identical to the parent plant and arises alongside the old spike in early autumn (Vogelpoel, 1993). Unlike the majority of *Disa* species, the cultivated *Disa* hybrids derived from the evergreen *Disa* group are never truly dormant. During the cold winter months growth slows down, but vegetative growth continues and accelerates when temperature increases from early spring onwards (Crous and Duncan, 2006). Flowering commences in spring (October) and, depending on the species or hybrid, may continue up to mid-and late summer (March) (Crous and Duncan, 2006).

The lack of relevant literature and science-based information on *Disa* prevents the cultivation of this orchid to its full economic potential. Although the growth cycles of *Disa* in the wild were described by Du Plessis and Duncan (1989), the specific phenological stages and its synchronisation of plants in cultivation, have not received attention.

In cultivation, a clear understanding of how the vegetative phase precedes the reproductive phases, as well as the optimum environmental requirements for these stages, is needed to construct manipulation strategies for flowering and harvest predictions. Bio-mass allocation patterns between above and below ground organs during the 'dormant' and 'bolting' phases can provide a better understanding of the nutritional or environmental requirements of the *Disa* in cultivation.

An in-depth understanding of the various phenological stages during cultivation can aid in risk assessments and management when unfavourable external conditions may occur during certain production stages. For example, it is known that *Phalaenopsis* will form vegetative air plantlets called 'keikei', or even more severely, flowers will be aborted with temperatures above 28 °C directly after floral initiation (Sakanishi et al. 1980). Similarly, in roses, "bullhead syndrome" can be induced by low night temperatures, inappropriate cultural practices or certain hormone levels prevailing during the early differentiation stages of the flower, just prior to the differentiation of the anthers and stamens (Horridge and Cockshull, 1974). "Bullhead syndrome" can be prevented by growth regulator application on flower buds, but only when done at a diameter of not more than 4 to 6 mm. This illustrates the advantage of understanding and linking phenological responses to production based interventions. Currently, our understandings of the mechanisms driving growth and flowering in *Disa* are insufficient to assist growers in predicting the impact of unfavourable external conditions or cultural practices on production outcomes.

The aim of this research is to describe the growth and development phases of two *Disa* hybrids in cultivation, in order to construct a phenological diagram that will accurately document the time of floral initiation, the differentiation and the progression of the consecutive stages of floral development for the construction of vegetative and reproductive growth curves. Floral abnormalities and physiological disorders observed will also be reported.

Materials and Methods

PLANT MATERIAL. All plant material was obtained from La Motte Winery Estate in Franschhoek (33°52'49.9"S; 19°4'28.3"O), the only commercial *Disa* producer in South Africa. Hybrids used were 'LM0739', 'LM0721' and 'LM61'. Hybrid 'LM0739' has large red flowers, and spike emergence occurs at the beginning of October and anthesis in November. 'LM0721', with smaller orange flowers reaches anthesis approximately one month later than 'LM0739'. For studying the incidence and timing of bud abortion another hybrid 'LM61' was used. This is an early flowering (July - October) hybrid with bright red flowers, similar in size to that of 'LM0739'. Parent plants used in the hybridization programs for all the above mentioned hybrids are however unknown.

EXPERIMENTAL SITE. Franschhoek has a mild Mediterranean-type climate with cool, wet winters and hot, dry summers. Long-term data indicate average and absolute maximum summer temperatures to be 29 and 37.5 °C respectively and average and absolute minimums to be 16 and 8 °C, respectively. In winter, temperature maximums (average and absolute) are 19.3 and 30.5 °C, with minimum average and absolute temperatures of 7 and 0 °C, respectively (Anon, 2012). Annual rainfall of between 520 and 800 mm occurs, with highest precipitation in mid-winter (June).

The active growing vegetative phase for plants used in this study began in Oct. – Nov. 2010 with the transplanting of young plantlets from tissue culture into 9 x12 cm plastic pots (Fig. 1). During this growth stage plants were cultivated in a shade house under 80% black and white shade

netting on one meter high raised benches (Fig. 2A). In April 2011 all plants were separated and re-potted to one plantlet per pot. Newly re-potted plants were then moved to a polycarbonate greenhouse facility (Fig. 2B) for the consecutive dormant and reproductive phases, where plants were cultivated in a hydroponic 'ebb-and-flow' tray system. Hybrid 'LM61' was kept in the shade house for the entire duration of its development, and was only moved to the greenhouse at anthesis.

NUTRITION, WATER AND GROWTH MEDIUM. The growth medium used was a mixture of sphagnum moss and polystyrene pellets in a 50:50 $^{V}/_{V}$ ratio with coarse pebbles at the base of the pots, to facilitate drainage. Standard commercial nutrition was provided through the hydroponic tray system (ebb-and-flow method), whilst additional watering was applied by means of a manually operated overhead system. The water source was obtained from a mountain spring which had a general EC of 14 $\mu S.cm^{-1}$.

TEMPERATURE AND GROWING CONDITIONS. Temperatures and humidity were recorded, using LogTag® temperature and humidity loggers (Model: HAX-8, LogTag Recorders, Northcote, Auckland, New Zealand). Temperatures recorded in the greenhouse and shade house are presented in Fig. 3. Relative humidity as recorded in the greenhouse is presented in Table 1. Light levels inside the greenhouse and shade house were recorded with a light meter (Li-250, LI-COR, Lincoln, Nebraska, USA). Light levels of 158.95 ± 6.03 $\mu mol \cdot m^{2} \cdot s^{-1}$ (12 Dec., 11:00) and 364.4 ± 28.15 $\mu mol \cdot m^{2} \cdot s^{-}1$ (18 Apr., 12:00) were recorded inside the greenhouse with the alumni-net closed and opened, respectively. In the shade house light levels were 170.13 ± 14.94 $\mu mol \cdot m^{2} \cdot s^{-1}$ (18 Apr. 12:00) and 134.71.4 ± 4.5 $\mu mol \cdot m^{2} \cdot s^{-1}$ (13 Sept. 10:30) at bench level. All measurements were conducted on clear days.

TREATMENT AND EXPERIMENTAL DESIGN. Ten plants of hybrid 'LM0739' and eight plants of 'LM0721' were dissected biweekly from 31 Mar. to 14 Oct. 2011. Dissections were conducted until the flowering buds were visible to the naked eye and both hybrids showed complete floral differentiation of the floral whorls. The typical sized plants selected for both hybrids showed a plant height of ± 30 mm and a crown width of ± 70 mm. All the plants were selected at the start of the trial and allocated to a dissection treatment, according to a complete randomised design.

To study the timing and incidence of bud abortion, eight hybrid 'LM67' plants of similar size, but with varying stem lengths, and various phases of bud developments and/or flower opening were selected during June 2012.

DATA RECORDED. *Growth parameters.* Growth parameters was selected by which growth could be monitored without using destructive sampling with the aim to identify a fast and reliable method to determine plant growth commercially. In addition, dissected plant material was used to determine fresh and dry weights. The height of each plant was recorded from the base of the stem, just above the root attachment, to the tip of the newest unfolding leaf enclosing the apical meristem (Fig. 4A). The leaf base diameter (Fig. 4B) together with the width of the five principle leaves (when five or more leaves were present) and number of leaves were also recorded. As the basal leaves died

off, the 5 largest and more distal leaves were measured. Leaf width was measured at the widest part of the leaf lamina (Fig. 4C) The roots were separated from the above-ground plant parts and the leaves were then spirally removed from the plant, proceeding from the outer, oldest leaf to the youngest leaf at the most inner basal position. The more fibrous and reclining leaf base (± 1.5 – 2 cm) was removed to standardise measurements (Fig. 5). The below-ground components that were weighed consisted of roots as well as possible root tubers (Fig. 6). Total leaf area (cm^2) of each plant was measured using a portable area meter (Li-3000A, Li-COR, Lincoln, Nebraska, USA). The separated harvested roots and leaves were freeze-dried, weighed and subsequently ground to a powder using liquid nitrogen for non-structural carbohydrate analysis (Paper 2).

Floral initiation and differentiation. Each plant was dissected to the meristem using a stereo microscope equipped with x160 magnification (eyepiece x20) (Leica S6D, Leica DFC295 (camera) and KL200 LED, Leica Microsystems Inc., Illinoise, USA). Firstly, the number of leaves on each plant were removed consecutively and recorded up to the smallest unfolding leaf visible to the naked eye, where after the leaf primordia surrounding the apical meristem were removed microscopically. The state of the meristem whether vegetative or reproductive, was subsequently determined and recorded. Plants that were damaged during dissection or died beforehand were recorded as "Discarded".

Furthermore, when floral initiation was determined, the floral differentiation stages were also described and photographed by means of a stereo microscope. The differentiation stages of the reproductive axillary meristems were subsequently allocated according to predetermined classification criteria. Dissected meristems were then stored in FAA (formaldehyde, acetic acid – 50% ethanol, 1:1:18 by volume). Morphological changes taking place during bud development were further observed using a Leo® 1430VP Scanning Electron Microscope at Stellenbosch University (LEO Microscopy Ltd., Cambridge, England). Prior to imaging the dissected axillary meristems were mounted on a stub with double sided carbon tape to ensure surface electrical conductivity. The SEM photographs were either taken with a secondary electron detector (SED/SE) or by using a variable pressure secondary electron detector (VPSED/VPSE) with magnification ranging between x30 and x250 and a working distance (WD) ranging between 9 and 27 mm, depending on the development phase and subsequent size of the sample. For use in SE mode the samples were ethanol-dehydrated, critical point dried and then sputter coated with a thin layer of gold using a gold ion coater (Edwards S150A Sputter Coater). Beam condition (accelerating voltage) for the SE mode during surface analysis was 7 kV. Using the VPSE mode, samples were taken directly from FAA and mounted on the stub, using double sided carbon tape (no drying or sputter-coating) and viewed with beam condition (accelerating voltage) of 20 kV. The best representation of each differentiation phase was selected from either hybrid 'LM0739' or 'LM0721' as the various developmental phases were similar for both hybrids. SEM images were used to determine the timing of the differentiation of the different flowering whorls until the flowering bud was clearly visible with the naked eye. At flower anthesis,

further morphological studies were conducted to describe structures not visible during dissections or any differentiation which occurred after the conclusion of dissection.

Physiological abnormalities. The possible occurrence and timing of flower bud abortion in *Disa* hybrids under greenhouse cultivation were also investigated in June 2012 by dissecting eight plants of hybrid 'LM61' as hybrids 'LM0739' and 'LM0721' were not available at La Motte nursery during the 2012 season. Plants of similar size, with varying spike lengths and at different stages of floral maturity (bud stage - anthesis) were selected. Each plant of hybrid 'LM61' was dissected to the apical meristem using the same stereo microscope described for the floral initiation and differentiation studies on 'LM 0739' and 'LM0721'. Stem length (mm), crown width (mm), number of unfolded leaves, number of opened flowers, number of visible buds and the number of aborted flowers for each plant were recorded. Furthermore, flowering of a randomly selected population (n=170) of *Disa* hybrid 'LM0739' plants were monitored. Abnormalities typically found within the population is presented and discussed.

STATISTICAL ANALYSIS. In graphs presenting seasonal growth, trend lines were fitted using SAS 9.2 (SAS Institute Inc., Cary, USA). *P* and R^2 values are shown.

Results

FLORAL INITIATION AND DIFFERENTIATION. The various phenological phases of the developing apical meristem and later the differentiation of the oldest flower of the inflorescence of *Disa* hybrids were divided into ten stages. The stages were named according to the structure(s) differentiated first within that stage.

The vegetative phase was observed as a domed apical meristem (ap) together with some visible leaf primordia, and was referred to as stage "V" (Fig. 7). Fig. 8a provides a diagrammatic illustration (Crous and Duncan, 2006) of a *Disa* flowerat anthesis which was used for the verification of floral parts and stages were named accordingly. Floral initiation was first detected in stage "I" as a protruding structure (i) lateral to the apical meristem (Fig. 8b). The lateral sepals (ls), were the first floral parts to differentiate from the protruding structure (i) and later developed into a more triangular structure (Fig. 8c), this stage is then referred to as "LS". At the next stage (Fig. 8d) the lateral sepals were observed to be more distinct and defined. Already at this stage, described as stage "PI" the differentiation of the petaloid initials (pi) was visible (Fig. 8d and e). The petaloid initials eventually gave rise to the petaloid whorl containing three petals (p) of which one petal were destined to form the lip (l). The next stage, "MS" showed the differentiation of the median sepal (ms), as this sepal together with the lateral sepals (formed in "LS", Fig. 8c), completed the differentiation of the sepal whorl (Fig. 8f and 9a). During the subsequent "late MS" stage, the median sepal elongated further, enclosing the reproductive whorls for the first time (not shown). Dissections from this stage and onwards required the median sepal to be removed/opened in order to view the enclosed, developing reproductive structures. The following distinguishable phase "PO" showed the differentiation of the

pollinia (po) (Fig. 9b and c). The pollinia differentiated from the 'dome-like' structure (pp – pre-pollinium), already visible in phase "PI" and "MS" between the developing petals (p) (Fig. 8e and f). The stage just prior to the last developmental stage is named as stage "C" during which the column (c), a structure unique to the Orchidaceae differentiated. This structure was seen below the pollinia (po) and above the lip (l) (Fig. 9d). In the final developmental stage "ST" the further differentiation of the column (c) could be seen, together with the differentiation of the stigma (st) (Fig. 9e and f), which conclude floral differentiation.

Resupination of the *Disa* flower was demonstrated where the lip in the differentiating flowers was in an uppermost position of the flower (Fig. 8f), whilst in flowers at anthesis (Fig. 8a) the lip was borne on the lowermost side of the flower. Resupination occurred after flower differentiation, but before anthesis. The ovary of *Disa* flowers is situated below the examined floral parts and the exact timing of the differentiation of the ovary could not be determined, but the differentiation is assumed to occur simultaneously or soon after differentiation of the stigma. Further morphological examination at anthesis revealed and confirmed the position of the ovary (with seeds) as being below the floral parts, above the pedicel (Fig. 10). The approximate time of the differentiation of the spur could not be observed during dissection. The differentiation of the spur was observed macroscopically in developing flower buds. The consecutive growth of the spur with bud age is shown in Fig. 11. Upon longitudinal dissection of the spur, a sticky clear fluid was detected within (presumably nectar), although no nectaries were observed within the spur. At anthesis the column had further differentiated into the rostellum (a flap of sterile tissue) below the pollinia. The pollinia displayed anther sacs, caudicles (pollinia stems) and viscidiums supported by the rostellum (Fig. 12). The white coloured anther sac (an; Fig. 10) enclosed the bright yellow pollinarium (pollinia with its caudicle) (Fig. 10 and 12).

The percentage (%) floral meristems representing each of the floral developmental stages, from "V" to "ST", throughout the period of dissections are shown in Fig. 13 and Fig. 14 for hybrid 'LM0739' and 'LM0721', respectively. Dissection of the potted *Disa* plant meristems revealed that the timing of floral initiation differed by approximately one month (4 weeks) between the respective hybrids. Floral initiation of hybrid 'LM0739' was first recorded on 25 May (Fig. 15), whilst initiation for hybrid 'LM0721' was first recorded on 23 June (Fig. 16). Dissections of plants were concluded towards middle October, when flower buds were visible with the naked eye and with the differentiation of the floral whorls completed by that time. Floral initiation ("I") for hybrid 'LM0739' was first recorded by 25 May (Fig. 13 and 15) and floral differentiation was concluded by 9 Sept. when the first flower reached the "ST" stage (Fig. 13). Therefore, the duration of floral differentiation for hybrid 'LM0739' was calculated as 107 days, approximately 15 weeks. In hybrid 'LM0721' initiation was observed by 23 June (Fig. 14 and 16), and floral differentiation of the floral whorls was completed on 14 Oct. (Fig. 14). The duration of floral differentiation was therefore calculated as 113 days (16 weeks). The time of floral initiation for the two respective hybrids differed by

approximately four weeks, whilst floral differentiation was found to take approximately a week longer for hybrid 'LM0721' than for hybrid 'LM0739'.

PLANT GROWTH PARAMETERS. A Julian day conversion table (Table 2) is provided for conversion of Julian days to calendar dates.

Plant height (mm) of both hybrids 'LM0739' and 'LM0721' remained constant throughout autumn and winter, only to increase exponentially from spring (day 252) onwards (Fig. 17). The leaf width (mm) of both hybrids 'LM0739' and 'LM0721' was consistently between 16 and 20 mm throughout the first period of measurement up to the end of July, day 120 to 210 (Fig. 18). From day 157 onwards the two hybrids showed different trends in leaf width of the principal leaves of the leaf whorl as measured per dissection date until the end of recordings in November (day 330). In hybrid 'LM0739' leaf width did not differ significantly over time and remained stable between 15 to 20 mm through autumn up to early summer. In contrast, in 'LM0721', a significant linear decrease in leaf width as measured on the 5 principles leaves occurred where the more recent distal leaf widths adjusted to approximately 12 mm by the end of November (330).

Both hybrids 'LM0739' and 'LM0721' showed no significant trend over time in the leaf base diameter (mm) of the dissected plants as the leaf base diameter for both hybrids stayed within a 6 mm range throughout the entire monitoring period (Fig. 19). For hybrid 'LM0739' an average leaf base diameter of 11.75 ± 0.47 was recorded throughout the season, whilst 'LM0721' showed a similar value of 11.58 ± 0.52 mm.

The total leaf area (cm^2) of both *Disa* hybrids showed a significant cubic increase over the recorded time frame (Fig. 20). The initial total leaf area per plant was between 30 and 50 cm^2, with final values for total leaf area per plant recorded between 70 and 80 cm^2, by the end of October (day 299).

The number of photosynthetically active unfolded leaves for the two hybrids displayed an opposite trend throughout the season (Fig. 21). Hybrid 'LM0739' did not vary significantly over time at the 5% confidence level as this hybrid showed a constant number of unfolded leaves of between 6 and 8, throughout the season, as older basal leaves died off and more recent, distal leaves unfolded. In contrast, hybrid 'LM0721' displayed a significant, linear increase in leaf numbers over the season, reaching a maximum number of leaves of 10-11, in spring.

The number of leaf primordia removed from the apical meristem of dissected plants followed the same significant quadratic trend for hybrid 'LM0739' and 'LM0721' from late summer to late spring (Fig. 22). Leaf primordia in both hybrids increased throughout late summer up to early autumn and winter (day 128 to 203), where after a rapid decline in number of leaf primordia were recorded. Similarly to the number of unfolded leaves (Fig. 21), a consistently higher number of leaf primordia were recorded in 'LM0721' plants compared to that of 'LM0739'.

The total leaf dry weight per plant of both hybrids increased linearly over the season (Fig. 23). In hybrid 'LM0739' total dry leaf weight was consistently higher than in hybrid 'LM0721' (Fig.

23). When considering the total dry weight of the underground components, for hybrid 'LM0721' it was consistently higher than for 'LM0739' (Fig. 24), an opposite trend to that described for the above ground organs (Fig. 23). A significant linear increase in the total dry weight of the underground components for 'LM0721' was recorded, whereas this parameter did not differ significantly over time for 'LM0739' (Fig. 24). Dissections also established that only one of the hybrids, namely, 'LM0721', produced a root tuber. No root tuber formation was observed throughout the season for hybrid 'LM0739' (Fig. 6).

The shoot:root ratio of both hybrids showed a slow, but significant linear increase over time (Fig. 25), thus indicating a greater investment of dry matter in above-ground structures towards spring, relative to the below-ground components.

PHYSIOLOGICAL DISORDERS.

Abortion. Flower bud abortion was suspected as the number of floral initials (five to six) seen while dissecting hybrid 'LM0739' and 'LM0721'did not correspond with the number of visible flower buds and flowers (one to three) at anthesis (data not shown). An early flowering (May – June) cultivar ('LM61') was dissected to determine the incidence as well as the time of bud abortion (Table 3). As all dissected plants of hybrid 'LM61' showed abortion irrespective of the crown width and spike length, time of abortion was assumed to take place before bolting of the inflorescence. Bud abortion of the one or two youngest developing flowers (Fig. 26) as well as the abortion of the apical meristem was observed with each dissection. Aborted floral parts were distinguished by the translucent and/or brown colour of the plant tissue, compared to the light green appearance of living plant tissue (Fig. 26). Neither the exact differentiation stage of the aborted flower buds nor the exact time of abortion relative to the reproductive phenology could be determined.

Abnormalities. Flower abnormalities were observed within hybrid 'LM0739' (Fig. 27). These abnormalities included deviations of colour, petal, sepal and sexual floral parts and were especially prevalent early in the flowering season, with approximately 50% of plants observed showing abnormalities early in the season. Abnormalities were however less prevalent (approximately 5%) as the season continued. Colour was observed to be streaky and faded (Fig. 27 A-F). Sepals were either duplicated (Fig. 27A), absent (Fig. 27B), or deformed (Fig. 27C). Furthermore, petals and sexual floral parts were deformed (Fig. 27A-F). Sexual floral parts were also duplicated (Fig. 27D) and lips were regularly missing (Fig. 27B) even in otherwise well-developed flowers.

Discussion

FLORAL INITIATION AND DIFFERENTIATION. Knowledge of the time of floral initiation is important for any future flower manipulation strategies to be successful. The developmental strategy of related hybrids, but with different flowering times (as seen for hybrid 'LM0739' and 'LM0721'), may vary in the time of floral initiation, in the rate of floral differentiation or may be a combination of both. The timing of floral initiation as determined by dissection differed for the two hybrids in

question by approximately four weeks (one month). In the hybrid 'LM0721', floral initiation was determined to be by approximately 25 June, whilst the hybrid 'LM0739', showed the first signs of floral initiation on 25 May. The duration of floral differentiation of the hybrid 'LM0721' however required only an additional week (16 weeks) compared to that required for hybrid 'LM0739' (15 weeks). The intervals between subsequent differentiation stages were similar between hybrids and required approximately two weeks to complete for most stages, except for the interval between the differentiation of the column ("C") and stigma ("ST"), where approximately four weeks (02 Aug. – 09 Sep.) and five weeks (09 Sep. – 14 Oct.) were required, respectively for hybrid 'LM0739' and 'LM0721' to complete. Hybrid 'LM0721' flowers generally approximately one month later than hybrid 'LM0739' (Van der Linde, personal communication, 2011). Therefore it can be assumed that floral differentiation of the hybrids is similar, and that differences in flowering times can mainly be ascribed to the month difference in time of initiation.

The period of floral differentiation for *Disa* is longer than reported for other orchids, where the differentiation of *Vanda* 'Arachnis' inflorescences usually require approximately eight weeks (Goh and Arditti, 1985). *Vanda* is described as a tropical orchid, preferring day temperatures between 21 and 35 °C (McConnell and Cruz, 1996). The additional heat unit requirement of this orchid most likely underpins the much faster differentiation rate compared to *Disa*. Furthermore, differentiation of the geophyte *Lilium* was completed within a similar period to *Vanda*, approximately 1.5 – 2 months (six to eight weeks) (De Hertogh and Le Nard, 1993). Advancement of flowering time could therefore possibly be achieved by increasing temperatures in the greenhouse during winter to accelerate floral development. This may however affect the display life of the inflorescence as temperature affects not only the rate of flower initiation and differentiation, but also the quality of the flowers (De Hertogh and Le Nard, 1993). However, geophytes which required a much longer period for floral differentiation than reported for *Disa* have been documented. For example, *Nerine bowdenii* and *N. sarniensis* required a 26 or 18 month period from floral initiation to anthesis, respectively, whilst *Amaryllis belladonna* required 12-13 months to complete differentiation (Theron and Jacobs, 1994). Therefore, the period of floral differentiation for *Disa* is therefore more closely related to orchids than to these Cape Flora geophytes.

For many crops, including orchids and geophytes, a pre-floral (just prior to initiation) doming of the vegetative apical meristem is common (De Hertogh and Le Nard, 1993; Hew and Yong, 2004). Such an occurrence was not clearly observed in this study for either of the dissected *Disa* hybrids. A slight increase in dome size that occurred immediate prior the reproductive switch, signalling the onset of floral initiation is likely, but the general domed appearance of the meristem throughout all dissections carefully concealed detection of the phenomenon.

The morphology of the *Disa* flower was further documented at anthesis to include any structures or changes not documented during dissections. Interestingly, with the exception of Schelpe (1966) who indicated a viscidium and caudicle in a line sketch of a '*Disa*-like' orchid, no scientific

literature could be sourced that specifically report a rostellum, anther sac, viscidium or caudicle for *Disa*, although it is clearly distinguishable when inspecting the flower. The structures were thus identified by means of comparison to various other orchids in literature where these structures are widely documented (Linder and Kurzweil, 1999; McMurty et al., 2008; Stewart et al., 1982).

Resupination is common in most orchids. Resupination implicates that the lip is at the lowermost part of the inner perianth lobes. This position results from the torsion of the developing flower bud through 180°, which is necessary as initially the lip is in an uppermost position. The lip needs to acquire a lowermost position in order to perform its role as landing platform for pollinators. The point of torsion is the ovary and pedicel which consequently appears twisted as a result (Linder and Kurzweil, 1999). In *Dendrobium*, the process of resupination can be seen by following the position of the spur on flowers of different maturities along the axes of an inflorescence (Hew and Yong, 2004). In *Disa*, following the position of the lip throughout the differentiation and development of the flower is clear confirmation of resupination.

Considering the observed time of floral initiation for the *Disa* hybrids 'LM0739' and 'LM0721' of late autumn to early winter, speculations about possible flowering signals can be made. Possible floral induction signals preceding or coinciding with the time of initiation can be a short day photoperiodic response or a response to the decline in temperatures from autumn to winter. The involvement of both these responses has been well documented within the Orchidaceae as well as for other geophytes. Low temperature induction has been recorded for *Cymbidium* and *Phalaenopsis* hybrids (Hew and Yong, 2004), whilst various geophytes are known to require vernalization to enable bud initiation (De Hertogh and Le Nard, 1993). Therefore, a drop in temperatures (especially night temperatures) or an unknown period of vernalization may be required as induction for floral initiation in *Disa*. However, certain geophytes initiate flowers independently from environmental control. In *Amaryllis* and *Tritleia*, no temperature or photoperiodic specific responses is needed, as flowering in these genera is considered to be autonomous (De Hertogh and le Nard, 1993). Therefore, other factors, such a possible minimum plant size or threshold reserve capacity, rather than temperature or photoperiod may be required for floral initiation.

VEGETATIVE PHENOLOGY. Considering the various parameters recorded to study the vegetative phenology of the respective *Disa* hybrids, the exponential increase in plant height for both hybrids 'LM0739' and 'LM0721' from late winter (day 252) onwards, was the most prominent indicator of a change in phenological status (Fig. 17). This bolting phase commenced on approximately 09 Sept. 2011 for both hybrids, between three and four months, after the observed initiation for hybrids 'LM0739' and 'LM0721' respectively. This bolting phase (increasing plant height) visually signalled the onset of the reproductive stage.

Firstly considering the tuber forming hybrid, 'LM0721', an increase in number of leaves was seen throughout the season, together with an increase in total leaf area as well as total dry leaf- and root weight for this hybrid (Fig. 20, 21, 23 and 24). However, despite the higher number of unfolded

leaves and thus leaf primordia recorded for ‘LM0721’ compared to that of ‘LM0739’, ‘LM0721’ consistently showed a lower total leaf dry weight and shoot to root ratio than that of the non-tuberous ‘LM0739’. The cost of the production and sustained growth of the root tuber in ‘LM0721’ is subsequently reflected in the lower total investment in above-ground structures compared to that of ‘LM0739’.

The significant decline in leaf width for ‘LM0721’ recorded over the season is difficult to explain (Fig. 18). A reduction in leaf size can be ascribed to the presence of high temperatures and high light intensities during leaf formation and extension (Friend et al., 1962). As a decrease in mature leaf width was almost consistently recorded from early April and onwards, these particular leaves could possibly have differentiated in January to March, a time when both temperature and light intensity were peaking. However, the decrease in leaf width did not negatively influence total leaf area, as the increase in the number of unfolded leaves, as well as sufficient extension of the leaves (personal observation) compensated for the reduction in leaf width.

‘LM0739’, without the need to support an energy-requiring, developing root tuber, did not show a significant change in unfolded leaf number or width or an increase in total root dry weight. However, a significant increase in both leaf area and total leaf dry weight, similar to ‘LM0721’, was still recorded and could possibly be ascribed to leaf extension. Leaf length was not measured throughout the season, as it was not regarded as a practical and accurate estimation of growth to be used by producers. The relatively stable leaf width recorded in hybrid ‘LM0739’ throughout the season, may indicate ‘LM0739’ to be genetically more tolerant towards high temperatures and high light intensities, therefore not adjusting leaf width in response to unfavourable conditions during leaf formation, as was observed for ‘LM0721’.

Both hybrids, irrespective of their strategy to optimise leaf area towards anthesis, were able to support a flowering spike. In addition, the greater investment of ‘LM0739’ in photosynthetic capacity may be one of the underlying causes for initiating inflorescences approximately four weeks earlier than ‘LM0721’. The presence of a root tuber and a higher total root dry weight in ‘LM0721’ did not offer an advantage in terms of an earlier flowering time, as ‘LM0721’ flowered approximately a month later than ‘LM0739’.

The significant increase in the dry weight of below-ground components of hybrid ‘LM0721’ could be ascribed to the sustained development of the tuber throughout the season, although this was not quantified. The non-tuberous hybrid ‘LM0739’ did not exhibit a similar significant increase in total dry weight of underground components over time. The below ground dry weight for both hybrids was however consistently higher than that of the total dry leaf weight, showing greater investment in the below ground organs rather than above-ground organs. This strategy is characteristic for geophytes, adapted to a fire-prone, Mediterranean ecological system. Such storage organs facilitate a fast re-growth of above ground plant parts in the case of damage, either by fire, trampling, wind or herbivory (Belsky et al., 1993).

Due to the presence of the root tuber in 'LM0721' the dry root weight of 'LM0721' was consistently higher than that of 'LM0739' throughout the season. The absence of tubers in 'LM0739', which are considered to be characteristic in *Disa* as a member of the Orchidaceae, is not clear. Cultivation practices such as the use of growth stimulants may cause a re-allocation of reserves to above-ground growth at the expense of tuber formation (Mellett, F.D., personal communication, 2012). However, in tuber formation of potato the main factors affecting tuber formation is nitrogen levels (Aksenov et al., 2012; Jackson, 1999), light (high light promoted tuber formation), temperature (high temperature inhibits tuber formation), photoperiod (SD promotes tuber formation) (Jackson, 1999), sufficient carbohydrates (Aksenov et al., 2012) and high sucrose content which promoted tuber formation (Jackson, 1999). Concerning plant growth regulators the main hormones affecting tuber initiation and formation are gibberilic acid (GA_s) which inhibits tuber formation and abscisic acid (ABA) which promotes tuber formation (Aksenov et al., 2012; Jackson, 1999; Xu et al., 1998). However, this does not explain why certain hybrids, such as 'LM0721' subject to the same horticultural practices persistently produced tubers in cultivation, while others do not. In addition, as the parentage within the hybridisation program used for hybrid 'LM0739' and 'LM0721' is unknown, differences observed in growth strategies could be genetic.

The leaf base diameter did not differ significantly over time for either of the hybrids studied. The leaf base recordings varied inconsistently within a small margin throughout the season as new leaves unfolded and older leaves abscised from the outer perimeter. Leaf base diameter, though useful in most crop growth studies, is considered an inappropriate growth estimate of growth and above-ground plant size in *Disa*. Above-ground growth was most effectively recorded by the destructive measurements of dry leaf weight and total leaf area, for both hybrids.

Leaf primordia removed from dissected plants, for both hybrids, decreased from early winter onwards, coinciding with the time of floral initiation determined for both hybrids (Fig. 23). Hybrid 'LM0739' showed a decrease in number of leaf primordia removed from May (day 157) onwards, whilst this hybrid showed the first onset of floral initiation on 25 May (Fig. 13 and 15). Similarly, hybrid 'LM0721' initiated flowers by approximately 25 June, whilst leaf primordia started a decline by 23 June (day 174). The data on the decline of recorded leaf primordia production thus positively confirmed the observed timing of the switch from the vegetative to the reproductive phase (Fig. 14 and 16) as the meristem would have stopped producing leaf primordia upon initiation, and the number of primordia would decrease as they developed to unfolded bracts.

The growth of both hybrids increased throughout winter as defined by plant height, total leaf area and total leaf dry weight. This trend is consistent with literature claiming that a true state of dormancy is never achieved for *Disa* and that growth continues, though at a slow pace, throughout winter (Crous and Duncan, 2006).

PHYSIOLOGICAL DISORDERS. *Bud abortion.* Hybrid 'LM61' was used to investigate the occurrence of bud abortion (Table 3). As *Disa* is classified as an orchid exhibiting a sympodial

growth habit, the apical meristem is destined to terminate in a flower or inflorescence, whilst vegetative growth continued through the formation of lateral axillary buds on the stem (Hew and Yong, 2004). In *Disa*, the inflorescence initiates laterally to the apical meristem. During this study of hybrid 'LM61', the apical meristem was observed to consistently abort together with the youngest one to two developing axillary flowers (Table 3). Repeated observations of dissected meristems of hybrids 'LM0721' and 'LM0739', in which the number of floral initials visible (five to six) were compared to the flowers that eventually reached anthesis (one to three), strongly suggested that flower abortion may be common in *Disa* hybrids.

Flower abortion has been widely reported in the Orchidaceae. Goh and Arditti (1985) reported that initiated flower buds may not always develop to maturity and bloom whilst, Hew and Yong (2004) reported that it is not known whether this phenomenon is of a genetic, physiological or pathological origin. In *Aranda* cv. 'Deborah' 30% of the initiated buds failed to develop and was subsequently aborted (Goh and Arditti, 1985). This relatively high value of mortality is comparable to the percentage of aborted flowers we found in *Disa* (20 – 30%). For *Aranda* cv. 'Deborah' a minimum bud size of 2 mm was required to ensure progression to maturity (Goh and Arditti, 1985). A similar minimum developmental stage or bud size as pre-requisite for development to maturity could not be determined for *Disa*, neither could the exact phenological stage of abortion be established.

Bud abortion is furthermore also widely reported in many commercially cultivated geophytes such as *Alstroemeria*, *Dahlia*, *Freesia*, *Gladiolus*, *Amaryllis*, *Iris*, *Lilium*, *Muscari* and *Tulip* (De Hertogh and Le Nard, 1993). In *Iris*, the reproductive meristem has up to five floral primordia, although only one or two reach anthesis, a number also comparable to *Disa*. In these geophytes, this phenomenon is classified as a physiological disorder and can be ascribed to various factors, including high temperatures, low light intensities, root damage (mechanical or due to high salinity), shortage of storage reserves and exposure to ethylene (De Hertogh and Le Nard, 1993). Considering the cultivation conditions of our experimental *Disa* plants at high temperatures or suboptimal light intensities as recorded in the greenhouse (Fig. 2B), these factors could also possibly be responsible for the observed bud abortion. The cultural practice of separating and re-potting plants to one plant per pot in April, could also conceivably cause mechanical root damage and induce stress, which could contribute to bud abortion. However, whether abortion is a natural process in *Disa*, a result of hybridization or whether it is due to suboptimal environmental and nutritional conditions, is not clear and requires further studies.

The developing inflorescence of *Disa* is concealed and enclosed by mature leaf bases until stem elongation, making non-destructive inspection impossible. Observations from this study suggest flower abortion in *Disa* occurs in the period preceding stem elongation as plants within various stages of stem elongation, even at the first protrusion of the buds above the foliage, already displayed flower abortion. Similarly to *Gladiolus* where flower abortion affects the youngest flowers at the tip of the

inflorescence (Shillo and Halevy, 1976), the youngest two to three of the developing flowers and the apical meristem were aborted in *Disa.* As bud abortion was only studied in a single, early flowering hybrid (July) it is unknown to what extend the presumable early initiation of this hybrid during the high temperatures of summer may be the cause of bud abortion.

Abnormalities. Personal observations made indicated that a high number of the oldest flowers of an inflorescence that opened showed abnormalities, especially concerning colour development as well as fusing and symmetry of the outer floral whorl parts (Fig. 27). This was particularly the case for 'LM0739'. Frequently, flowers were observed to have incomplete sepal or petal arrangements or several cases were observed where additional floral parts were produced. Similar abnormalities, with regards to multiple column and lip formations, were also documented for *Phalaenopsis* (van der Knaap et al., 2005). In this tropical orchid, these abnormalities were ascribed to genetic mutations and stressful conditions such as extreme climatic conditions, root stress and a harsh winter environment. Supporting the link of abnormalities due to unfavourable temperature regimes (rather than genetic mutations) is the observation that the remaining flowers on the 'LM0739' inflorescence which reached anthesis later (the younger flowers on a spike) or inflorescences of hybrids with a later flowering time, did not show these abnormalities; neither did any flowers of hybrid 'LM0721', suggesting that initiation closer to July may be more optimal to support normal flower differentiation.

Floral abnormalities concerning the formation of additional floral organs or omission of some whorls or elements (similar to *Disa* and *Phalaenopsis*) also occur in the genus *Iris* (De Hertogh and Le Nard, 1993). In *Iris*, these abnormalities are ascribed to anoxia or a drop in temperature below 0 °C during floral differentiation. Conditions of such low temperatures or anoxia were not experienced during the cultivation of *Disa* 'LM0739'. In addition, colour deviations such as pale coloured *Iris* flowers (also observed in *Disa*) was ascribed to high relative humidity coupled with insufficient air circulation. This could possibly also be the case with *Disa* 'LM0739' as an average high relative humidity of above 80% was mostly recorded within the greenhouse (Table 1). The pigments mainly responsible for the colour development in *Disa* has been determined to be carotenoids and anthocyanins (Tatsusawa et al., 2010). High temperatures, similar to that recorded within the cultivation of *Disa* are often known to cause a faded petal colour in flowers containing theses pigments. Furthermore, low light intensities (also recorded in cultivation of *Disa*) are also known to decrease flower colour in many plants (Ben-Tal and King, 1997).

The occurrence of physiological disorders in *Disa* emphasises the importance of a clear understanding of the various consecutive floral differentiation stages, together with the sensitivity of these hybrids to external climatic factors during these respective stages in cultivation and its possible link to physiological disorders.

Conclusion

Flowering manipulation strategies require knowledge of both the time of flower initiation, as well as the factors that control initiation. In this paper the time of floral initiation for two commercial *Disa* hybrids were elucidated. Initiation was found to be a month apart, at the end of May and June for hybrids 'LM0739' and 'LM0721' respectively. This research therefore provides a basis for further studies on manipulation of flowering time. Furthermore, the flower development stages for the two *Disa* hybrids were also documented and proceeded identically between hybrids concerning the observed stages as well as timing, and are therefore proposed to be representative of floral differentiation in *Disa*. The difference in flowering time seen between the hybrids is therefore mainly due to timing of floral initiation rather than varying differentiation times between hybrids.

The timing of the differentiation stages are of key importance in understanding the underlying mechanism responsible for floral abnormalities and can aid risk assessment and harvest predictions. Early initiated flowers appears to be especially linked to floral abnormalities under conditions where floral differentiation can be expected to occur at an accelerated rate compared to later in the season. The phenomenon of resupination within *Disa* was also reported for the first time within this study. If the floral induction factors of *Disa* hybrids is known the timing of initiation can be scheduled to avoid periods of risk where environmental conditions may be conducive to floral abnormalities. The initiation of *Disa* hybrids, coinciding with the onset of winter, leads to speculation of possible low temperature or short day photoperiod induction. Other endogenous factors like hormones or critical plant size might also be responsible for floral induction.

Various growth parameters were used to report on growth of the above and below-ground components. The formation of a root tuber by *Disa* hybrids 'LM0721' seemed to affect the growth strategy of the affected hybrid. Still, it can be concluded that total leaf area and total dry leaf weight for both hybrids increased, indicating growth and optimisation of the photosynthetic capacity of the plant, in order to support a flowering spike. Plant height as defined by the elongation of the spikes was unmistakeably indicative of flowering, whilst it was established that leaf base diameter is not a reliable indicator of growth.

This study provided the first report on bud abortion within this genus and, as in many commercial geophytes, this might be an important physiological disorder affecting marketability and profitability of potted *Disa* hybrids. In the *Disa* hybrid studied, abortion of the one to three youngest developing flowers together with the apical meristem was seen. It is not clear whether this is common for of all *Disa* hybrids, or what the exact cause of the abortion is. The minimum threshold size or phase for flowers to ensure development would proceed to maturity could not be identified; neither could the timing of abortion in relation to plant development be established.

Considerable progress has been made into the understanding of the growth and flowering of *Disa* hybrids in cultivation. However, more extensive research is needed before the development of commercially sound and viable techniques to control flowering in cultivated *Disa* hybrids can be

achieved. This study provides a strong basis for future studies, with the main purpose to establish the successful commercial production of *Disa* hybrids.

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Table 1. Relative humidity (RH; %) recorded (average, minimum and maximum) in the greenhouse of La Motte Winery estate, Franschhoek, as prevalent during the developmental phases of *Disa* hybrids 'LM0739' and 'LM0721' from Mar. 2011 to Jan. 2012.

	Relative Humidity (%)										
	Months (2011 -2012)										
	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec	Jan
Average	86.9	81.5	88.2	92.4	81.4	80.7	82.6	79.2	74.9	69.0	73.3
Maximum	99.6	96.3	97.5	99.8	94.3	95.6	98.6	98.1	95.3	85.9	96.5
Minimum	53.5	50.5	65.4	71.6	56.2	51.0	49.4	44.6	40.9	41.0	32.0

Table 2. A table converting Julian days to dates (2011), for the days on which growth measurements were recorded for *Disa* hybrids in 'LM0739' and 'LM0721'.

Julian days	**Date (2011)**
82	23-Mar
102	12-Apr
124	04-May
129	09-May
145	25-May
157	06-Jun
174	23-Jun
189	08-Jul
203	22-Jul
214	02-Aug
229	17-Aug
252	09-Sep
257	14-Sep
272	29-Sep
287	14-Oct
299	26-Oct
313	09-Nov

Table 3. Vegetative and reproductive characteristics of *Disa* hybrid 'LM61' (n=8) relating to the incidence and timing of bud abortion.

Spike length (mm)	Crown width (mm)	Number of leaves	Flowers (anthesis)	Developing flower buds	Aborted flowers
129.15	138.01	10	1	2	2
137.67	133.09	7	1	2	2
131.93	146.43	13	1	2	1
152.90	137.39	10	1	2	1
127.35	152.40	9	1	1	2
95.81	115.88	8	1	2	2
81.33	81.37	11	1	1	2
115.21	92.20	-[z]	1	2	1

[z] - Represents a missing value.

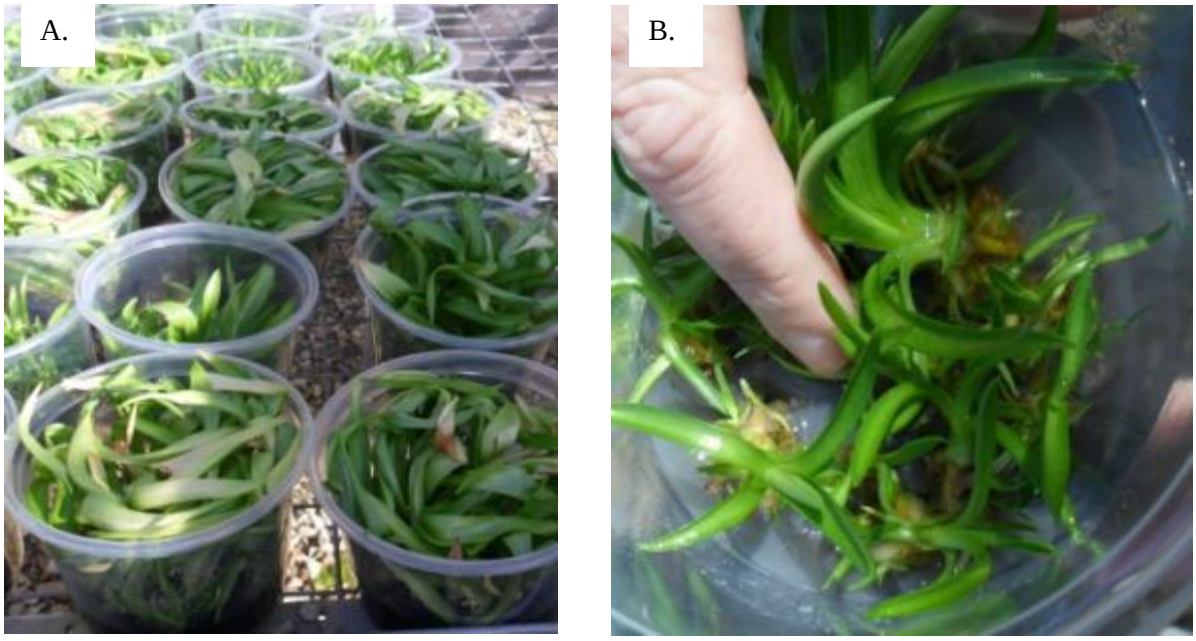
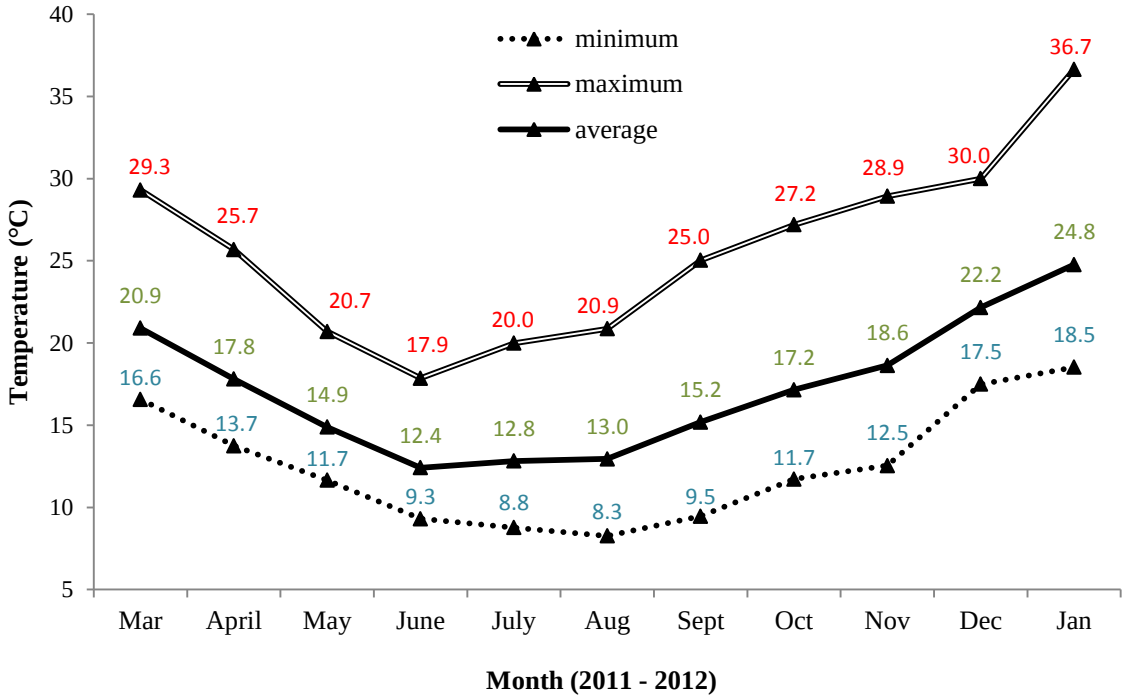


Fig. 1. A. and B. *Disa* hybrid 'LM0739' is shown as tissue cultured plants, just prior to being transplanted into pots, Oct. – Nov. 2010. The tissue culture plants ready to be planted, consisted of 4-5 leaves, were approximately 3 cm in height and had a well-developed root system.

Fig. 2. The cultivation areas of the commercial *Disa* hybrids 'LM0739'and 'LM0721' with A. The shade house, where the potted *Disa* plants were grown during the vegetative phase of development, from tissue culture until re-potting (Oct. 2010 – Apr. 2011). *Disa* potted plants were kept on raised benches under a misting system for cooling. B. The greenhouse where the *Disa* plants were relocated after re-potting, and cultivated throughout the dormant and reproductive phase, until anthesis (Apr. 2011 – Dec. 2011). The benches of the 'ebb-and- flow' hydroponic system, as well as misters and wind tunnels used for cooling, can be seen. The greenhouse was also equipped with mechanical alumni-net, which was left open during the winter months.

A.



B.

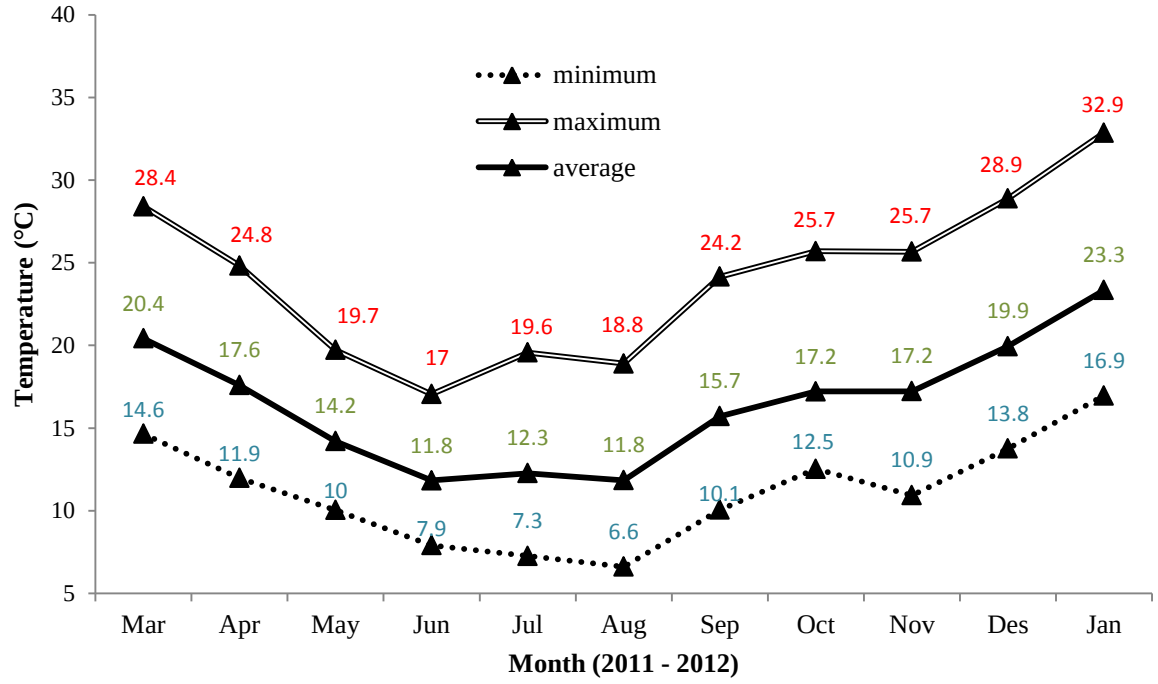


Fig. 3. A. Temperatures (°C) recorded (average, minimum and maximum) in the greenhouse at La Motte Winery estate, Franschhoek, as prevalent during the developmental stages of *Disa* hybrids 'LM0739' and 'LM0721' from Mar. 2011 to Jan. 2012. B. Temperatures (°C) recorded (average, minimum and maximum) in the shade house at La Motte Winery estate, Franschhoek as prevalent during the developmental stages of *Disa* hybrids 'LM0739' and 'LM0721' from Mar. 2011 to Jan. 2012. Hybrid 'LM61' was grown in the shade house for the full duration until anthesis.

Fig. 4. A vegetative plant of *Disa* hybrid 'LM0739', indicating the positions at which the growth parameters of (A) plant height (mm), (B) leaf base diameter (mm) and (C) leaf width were measured.

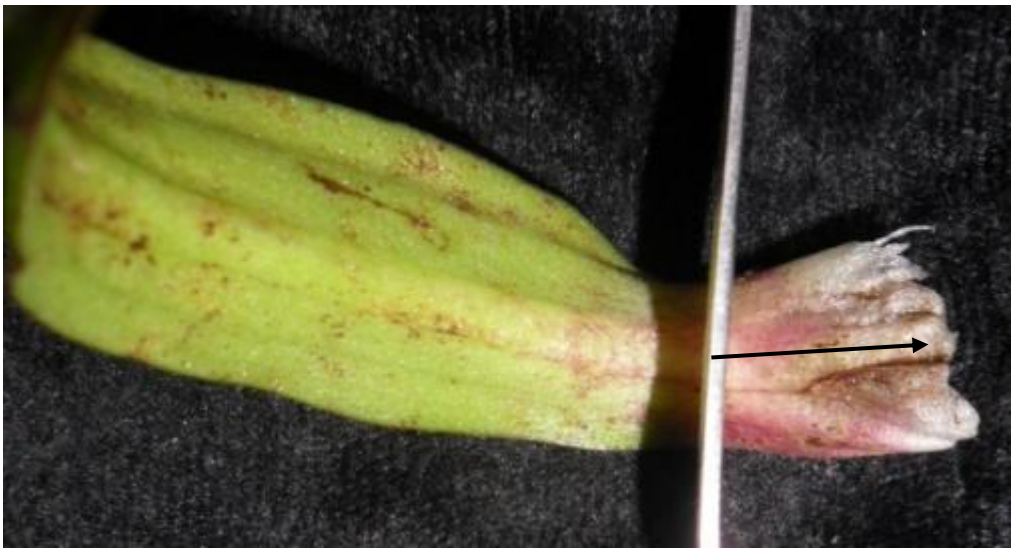


Fig. 5. A leaf of *Disa* hybrid 'LM0739' is shown to illustrate where the fibrous part of the leaf base was removed each time to standardise and facilitate leaf measurements.

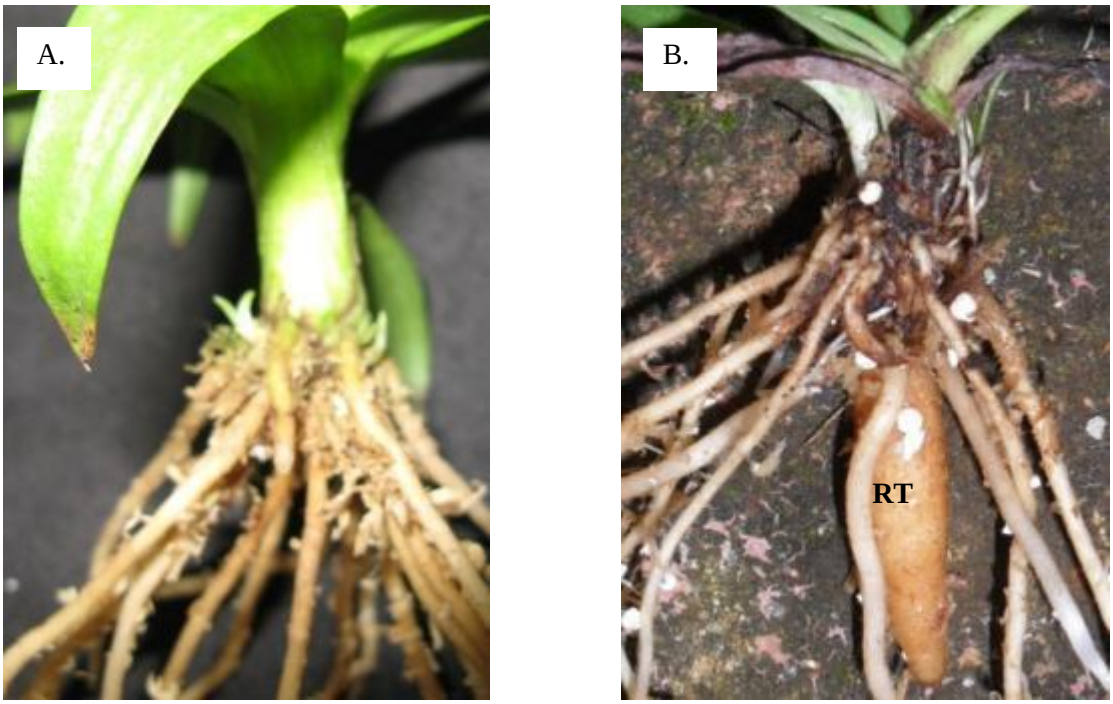


Fig. 6. A. *Disa* 'LM0739' showing well-developed roots, but no root tuber as part of the underground structures, as is characteristic of Orchidaceae. B. *Disa* hybrid 'LM0721' showing a well-developed root tuber (RT) as was seen throughout the growing season.

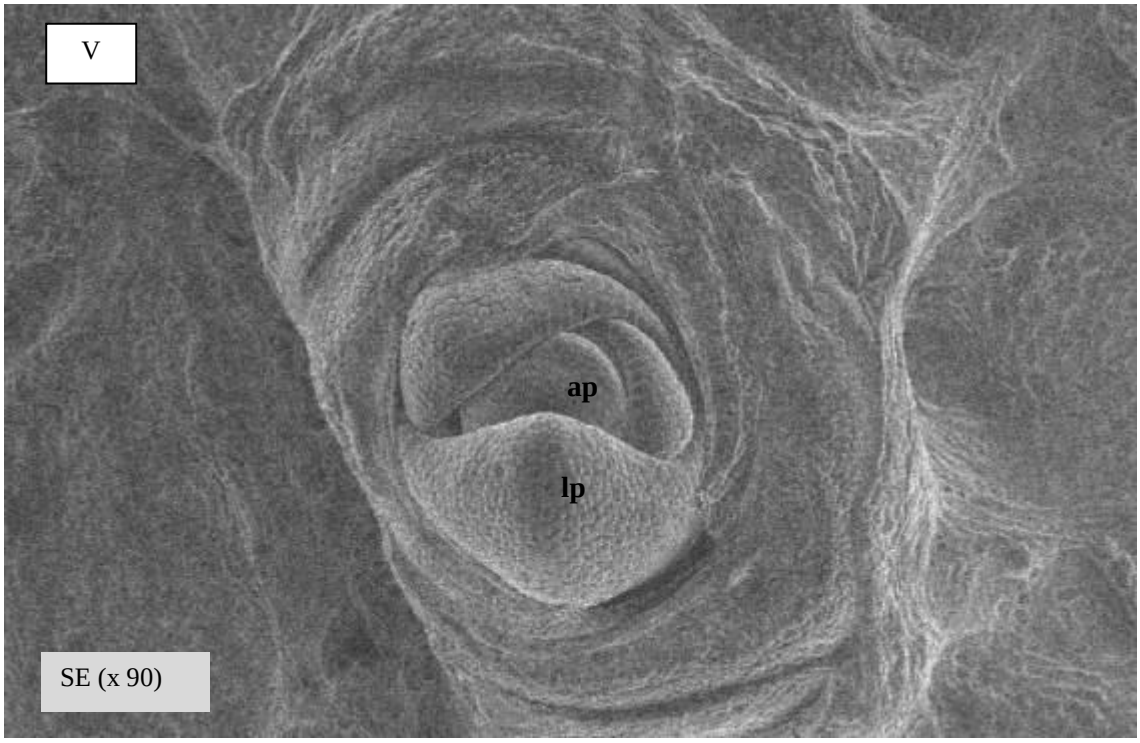


Fig. 7. A vegetative apical meristem of a potted *Disa* hybrid 'LM0739' plant, consisting of a relatively domed vegetative apex (ap) and protruding leaf primordia (lp), as dissected on 12 Apr. 2011 and photographed with a scanning electron microscope (SEM) using a secondary electron detector (SE).

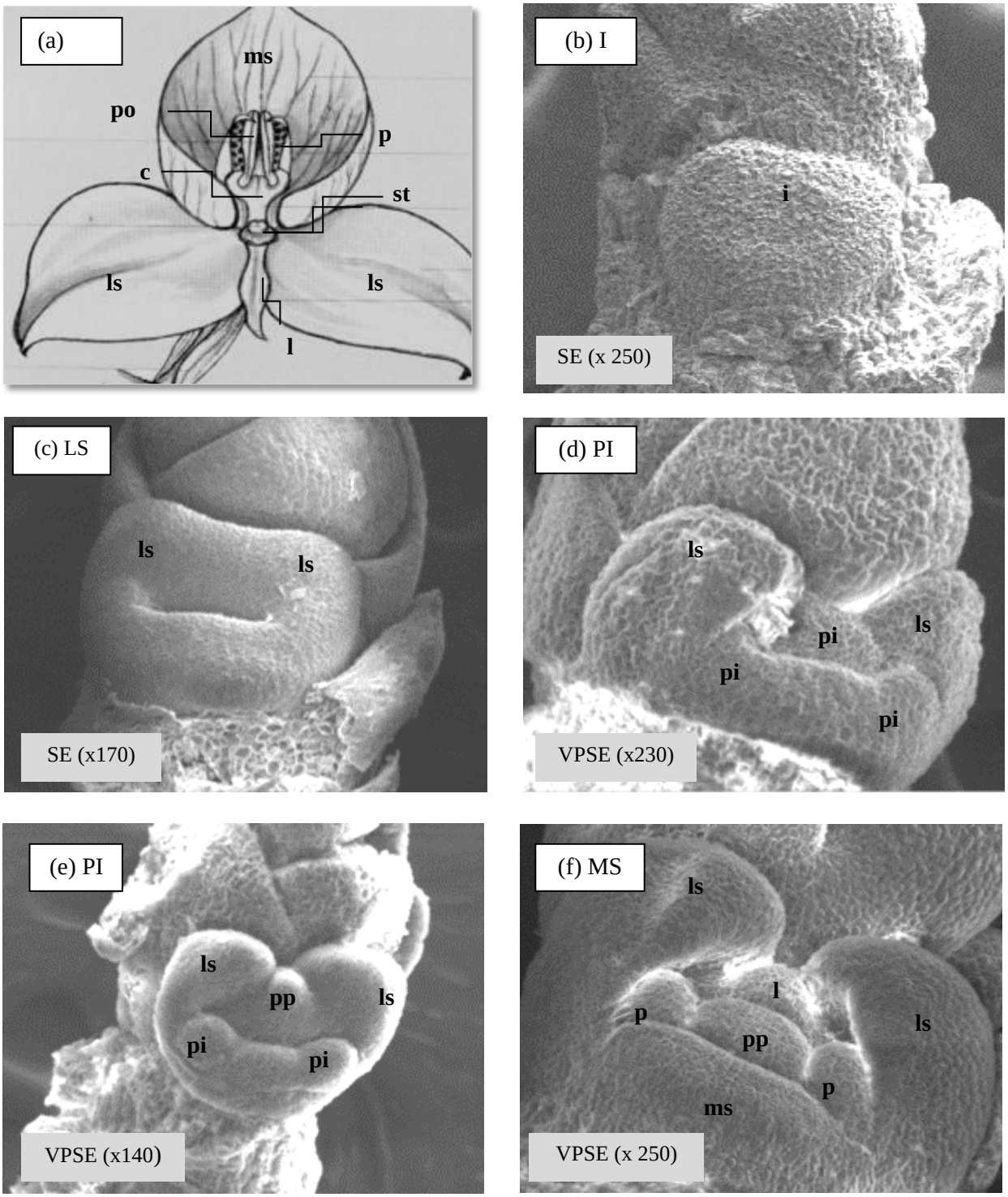


Fig. 8. Development of a flower from an axillary meristem of cultivated *Disa* hybrids, commencing with the first reproductive stage "I" up to stage "MS" a). A diagrammatic representation of a fully differentiated *Disa* flower showing various floral parts: ls (lateral sepals), ms (median sepal), p (petals), l (lip), po (pollinia), c (column) and st (stigma) b). "I" - Floral initiation, the first stages of flower development visible as a protruding structure (i) below the apical meristem. c). "LS" - Lateral sepal (ls) development. d). and e). "PI" - More advanced lateral sepals (ls) together with the appearance and development of petaloid initials (pi). f). "MS" - Differentiation of the median sepal (ms) together with the petaloid initials now clearly distinguishable as three petals, 1 lip (l) and 2 petals (p). The pre-pollinium structure (pp) is also visible at this stage. The detector (SE - secondary electron detector or; VPSE – variable pressure secondary electron detector) and magnification used is indicated on the each photograph as applicable.

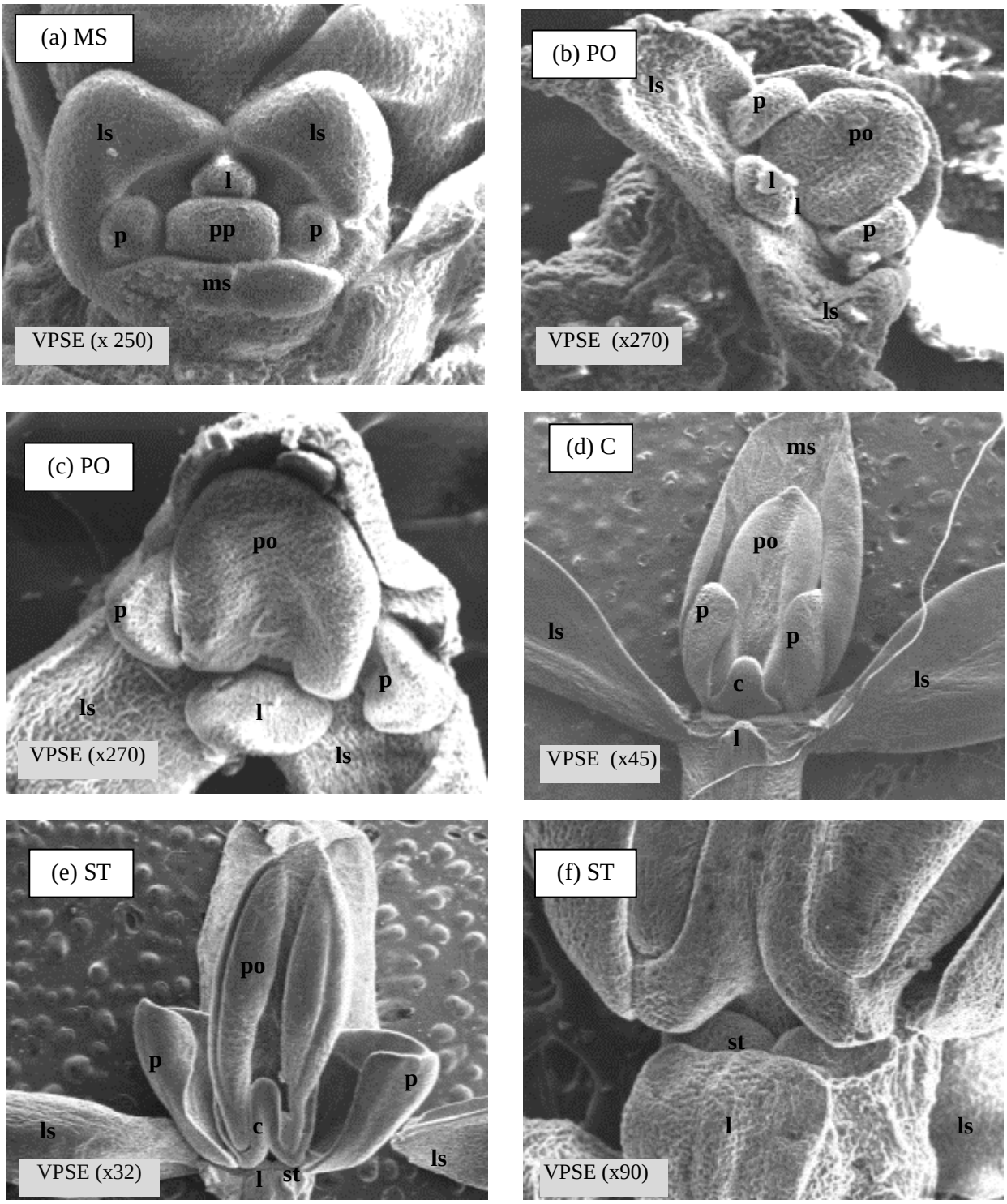


Fig. 9. The development of the flower from an axillary meristem of cultivated *Disa* hybrids from stage "MS" to the end of floral differentiation at stage "ST". The median (ms) and lateral sepals (ls) is seen to surround the reproductive whorls. a). "MS" - Further development of the median sepal together with the differentiation of the petaloid initials now clearly distinguishable as the three petals, 1 lip (l) and 2 petals (p). The pre-pollinium (pp) is also visible at this stage. b). and c). "PO" - The development of the pollinia (po). d). First visible signs of the developing column (c). e). and f). "ST" - The final phase of floral differentiation, the development of the stigma (st). A variable pressure secondary electron detector (VPSE) was used, magnification are indicated on each photograph.

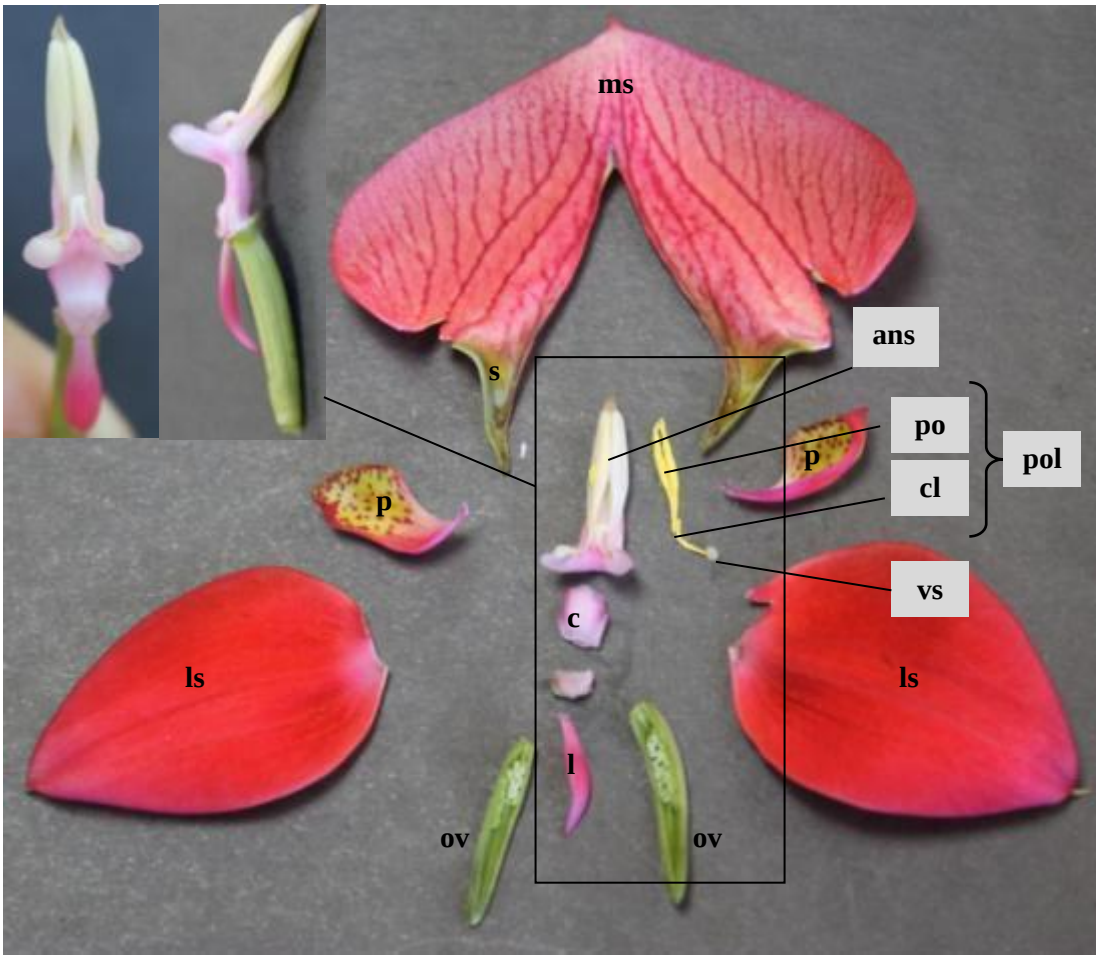


Fig. 10. A *Disa* flower (hybrid 'LM61') dissected to display the various differentiated floral parts complete at anthesis. The floral parts include the lateral sepals (ls), median sepal (ms) elongated into a spur (s), the petals (p) and lip (l). The column (c) and the anthers, consisting of the pollinarium (pol) enclosed in an anther sac (ans) as well as the ovary (ov) which was dissected longitudinally to reveal enclosed seeds, are presented. The pollinia (po) together with the caudicle (cl; pollinia stem) is referred to as the pollinarium (pol). The caudicle (cl) attach the pollinia (po) to a sticky gland, the viscidium (vs). The fused reproductive parts together with the lip and ovary are shown in the inserted blocks.

Fig. 11. Flower buds of *Disa* (hybrid 'LM61') displaying the consecutive development of the spur (s) (from left to right) on the outer side of the median sepal (ms). The elongation of the spur is observed with progressive maturity of the floral buds.

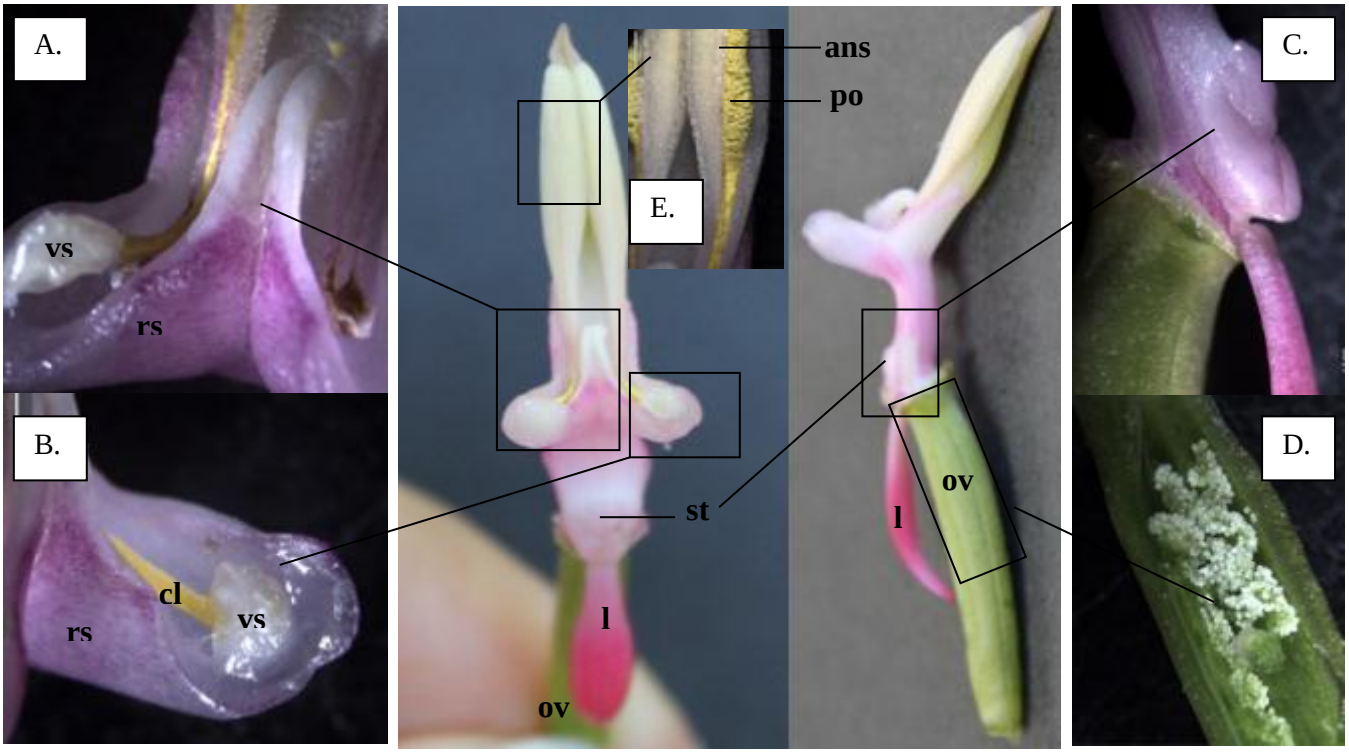
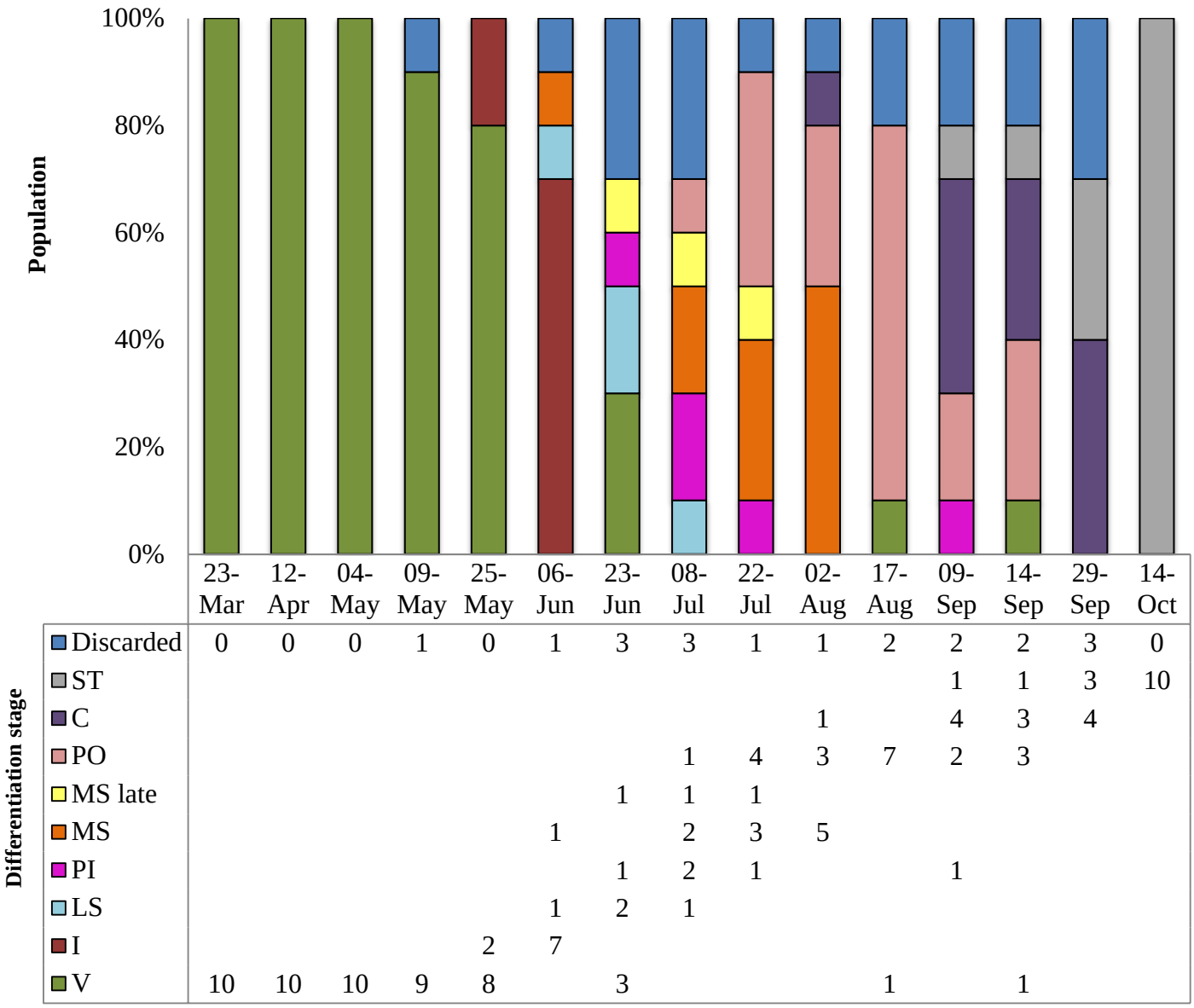


Fig. 12. The fused sexual parts, together with the positions of the lip (l) and ovary (ov) of a *Disa* (hybrid 'LM61') flower. A. Showing the rostellum (rs), a sterile flap of tissue, supporting the viscidium (vs; sticky, viscid gland) attached by the caudicle (yellow pollinia stem) to the pollinia. B. The viscidium (vs) and caudicle (cl). C. Side view of the stigma (st), as seen below the rostellum at the lowermost part of the column, with the visible ovary, at an inferior position. D. Dissection of the ovary longitudinally revealed minute seeds. E. The anther sac, white (ans) enclosing the yellow pollinia (po).



Differentiation stage	23-Mar	12-Apr	04-May	09-May	25-May	06-Jun	23-Jun	08-Jul	22-Jul	02-Aug	17-Aug	09-Sep	14-Sep	29-Sep	14-Oct
Discarded	0	0	0	1	0	1	3	3	1	1	2	2	2	3	0
ST												1	1	3	10
C										1		4	3	4	
PO								1	4	3	7	2	3		
MS late							1	1	1						
MS						1		2	3	5					
PI							1	2	1			1			
LS						1	2	1							
I					2	7									
V	10	10	10	9	8		3				1		1		

Fig. 13. Number and percentage (%) of dissected *Disa* hybrid 'LM 0739' (n=10) plants in each of the specific stages of flower differentiation at each dissection date during 2011. The stages are named according to the floral part(s) visible within that stage. "V" (vegetative), "I" (initiation), "LS" (lateral sepals), "PI" (petaloid initials), "MS" (median sepals), "PO" (pollinia), "C" (column) and "ST" (stigma). "Discarded" in the legend refers to plants that had died since selection or were damaged during dissection.

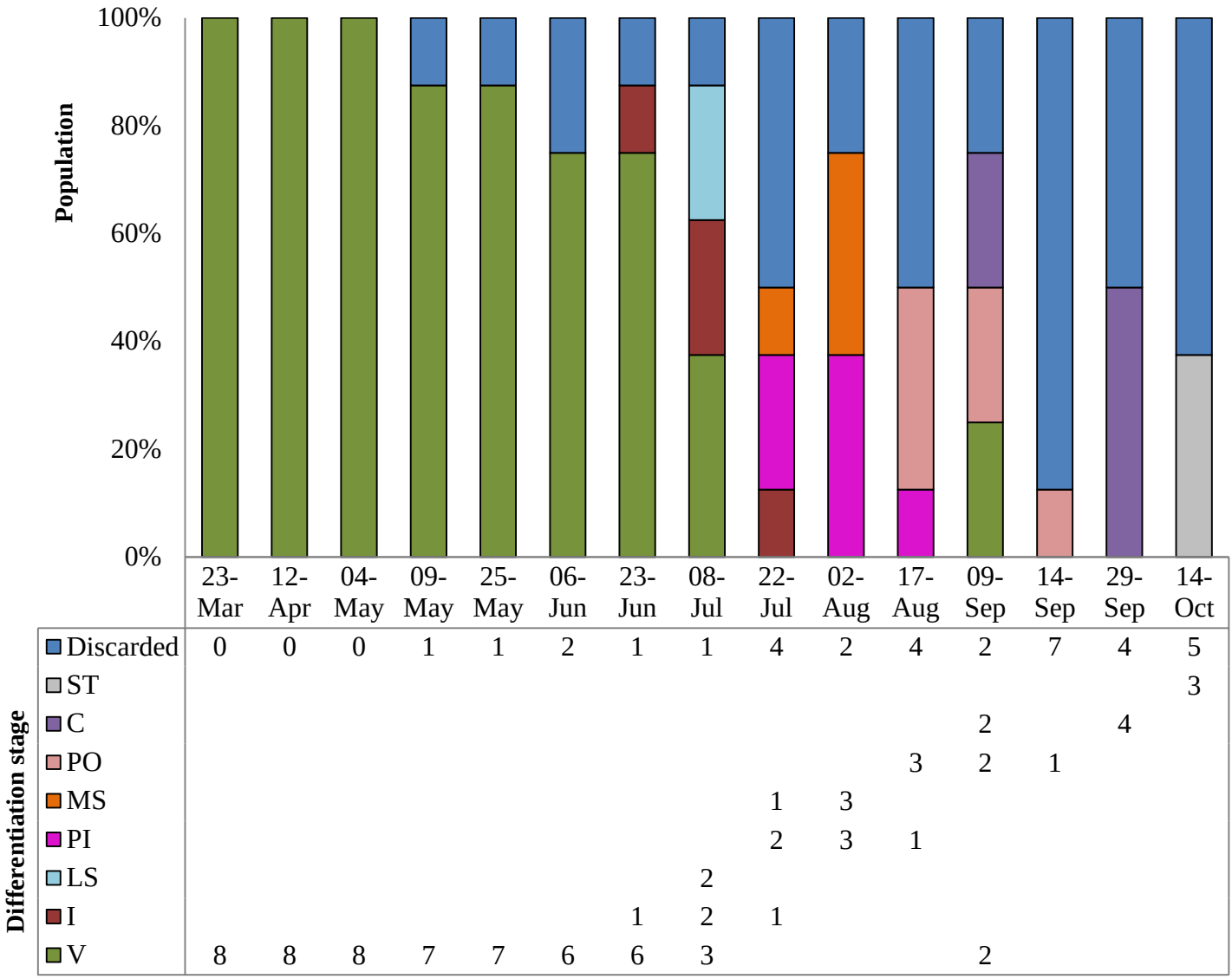


Fig. 14. Number and percentage (%) of dissected *Disa* hybrid 'LM 0721' (n=8) plants in each of the specific stages of flower differentiation at each dissection date during 2011. The stages are named according to the floral part(s) visible within that stage. "V" (vegetative), "I" (initiation), "LS" (lateral sepals), "PI" (petaloid initials), "MS" (median sepals), "PO" (pollinia), "C" (column) and "ST" (stigma). "Discarded" in the legend refers to plants that had died since selection or were damaged during dissection.

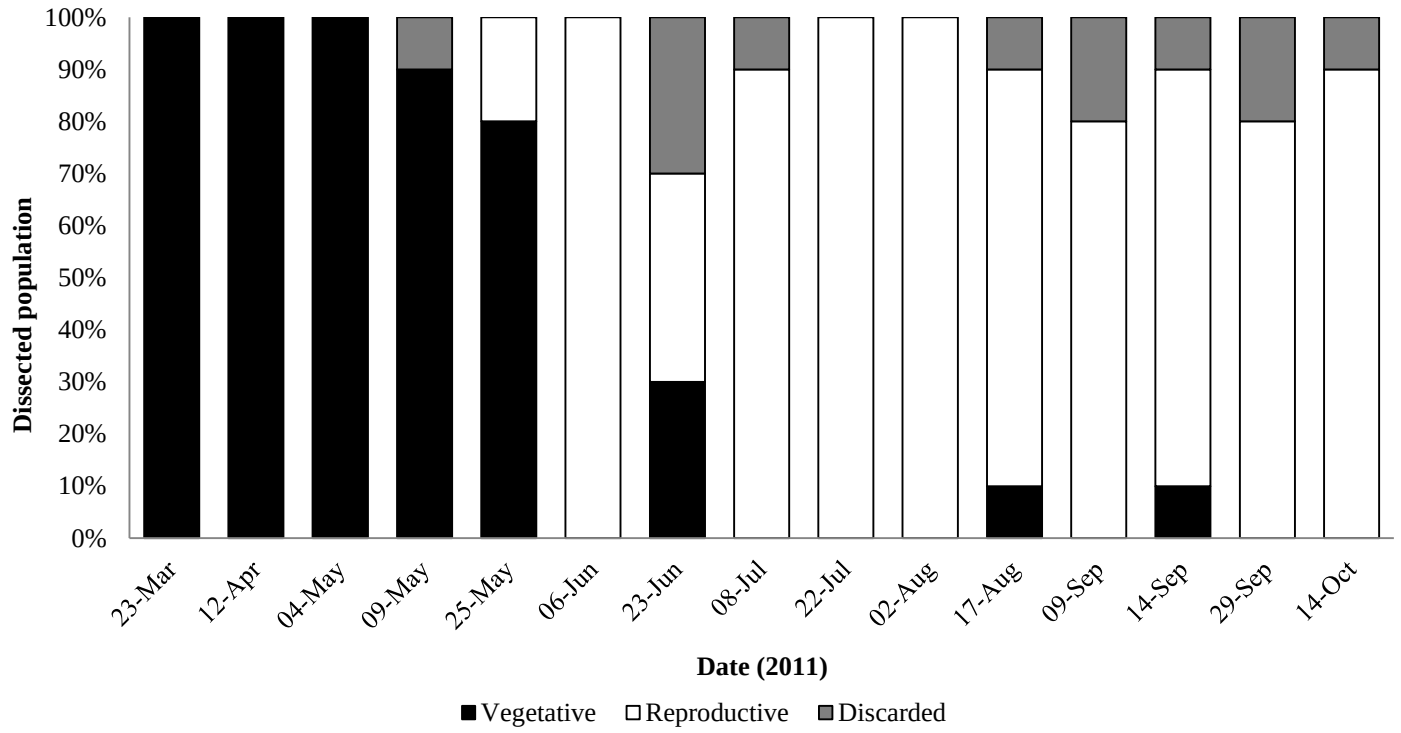


Fig. 15. The meristem status as revealed for potted plants of the *Disa* hybrid 'LM0739' (n=10), when dissected biweekly from 23 Mar. – 14 Oct. 2011. "Discarded" in the legend refers to plants that had died since selection or were damaged during dissection.

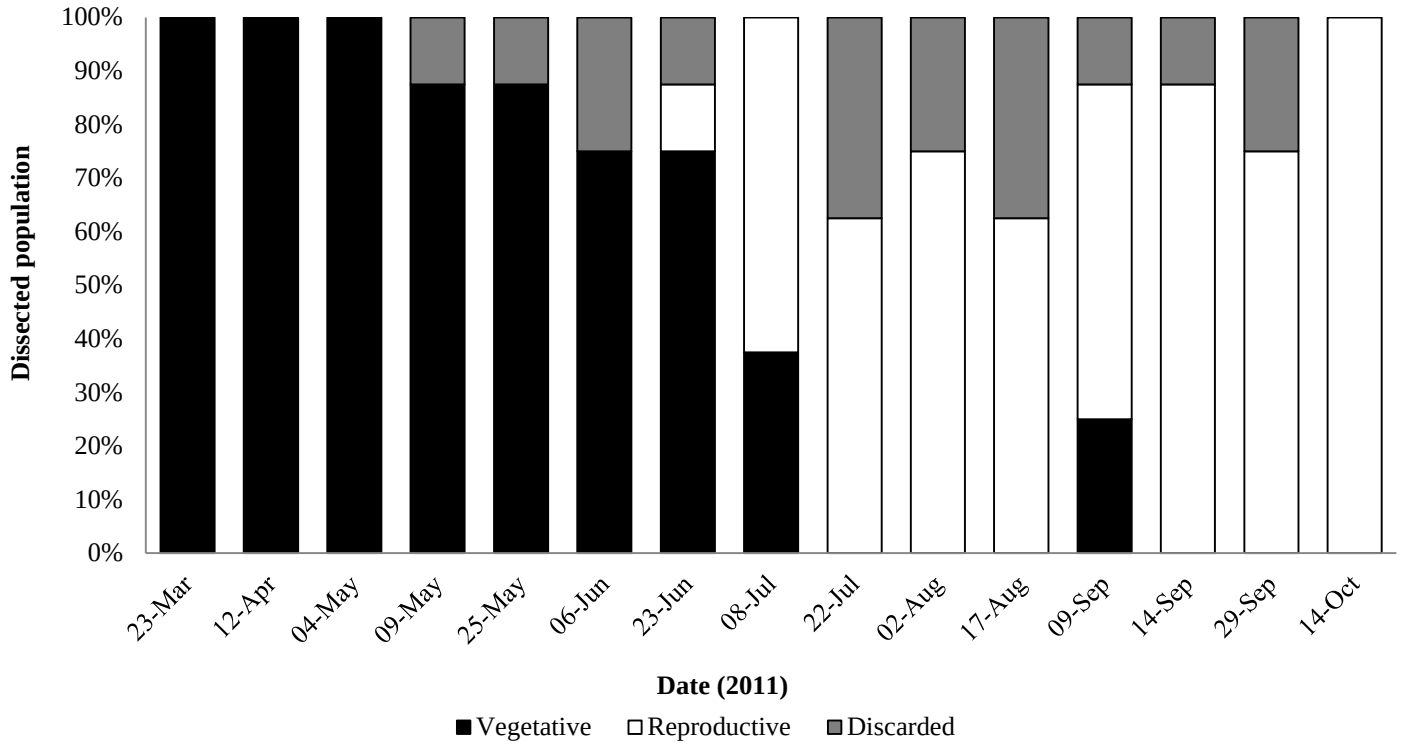
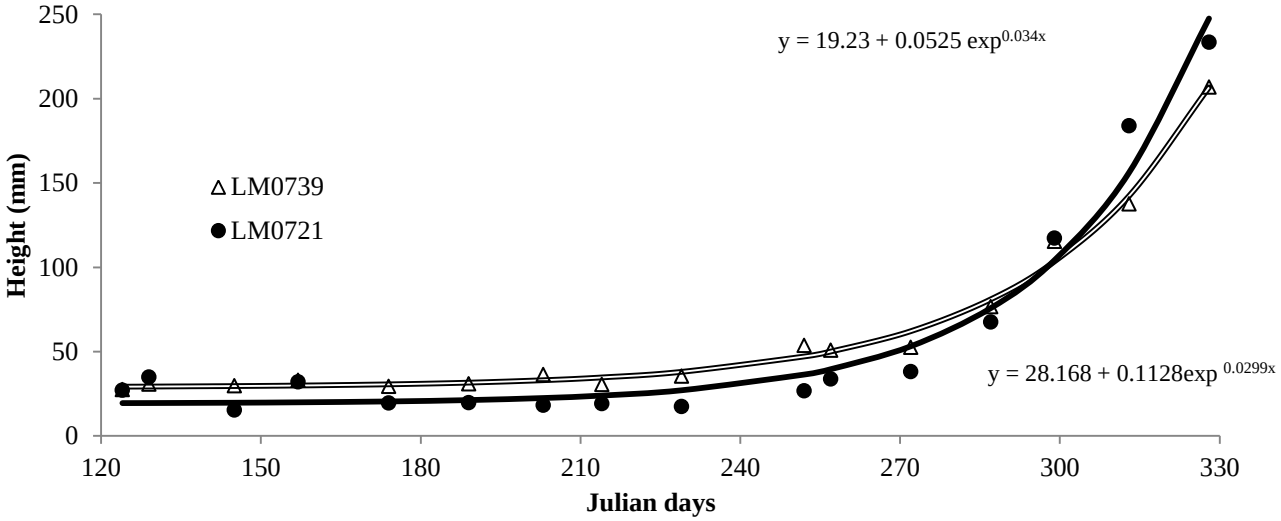
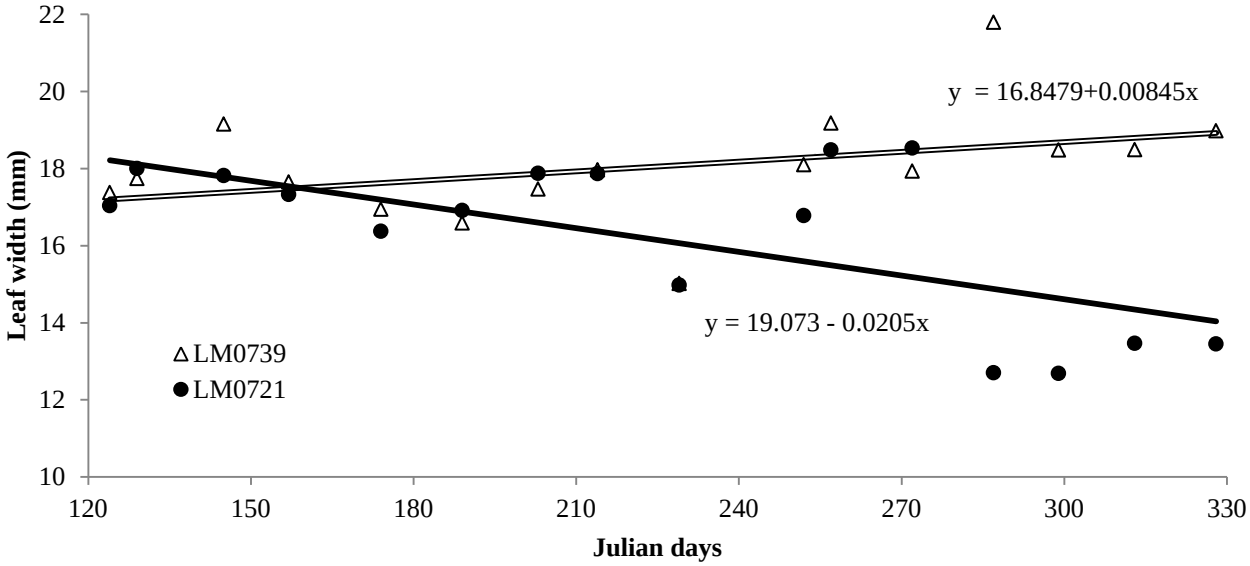


Fig. 16. The meristem status as revealed for potted plants of the *Disa* hybrid 'LM0721' (n=8), when dissected biweekly from 23 Mar. – 14 Oct. 2011. "Discarded" in the legend refers to plants that had died since selection or were damaged during dissection.



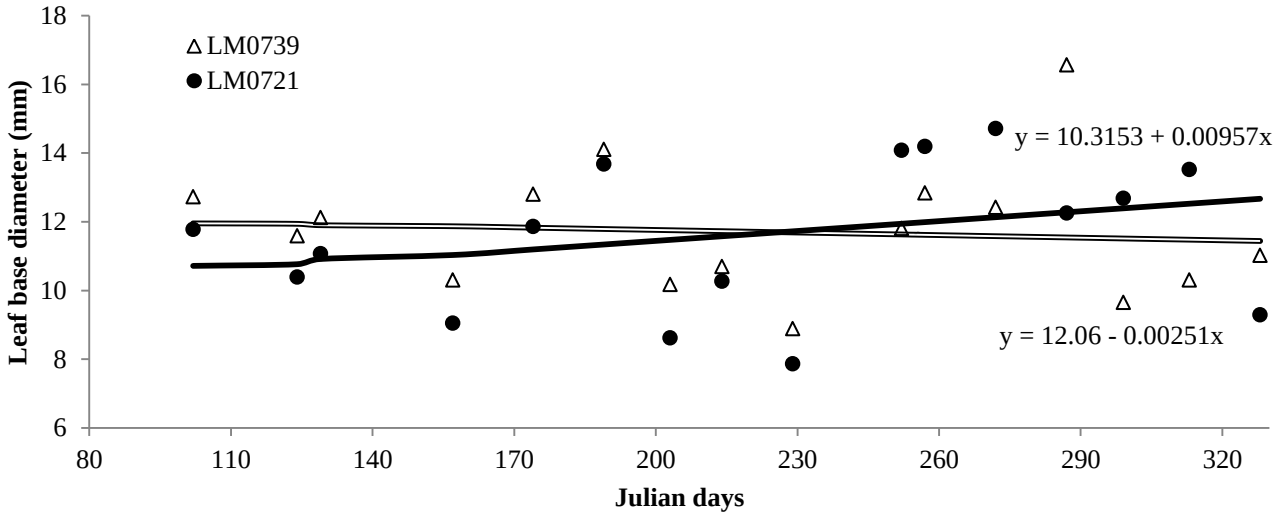
	R^2	*P* - value	Significance
LM0739	0.99519	<0.0001	***[z]
LM0721	0.98297	<0.0001	***

Fig. 17. The mean plant height (mm) of potted *Disa* hybrids 'LM0739' (n=10) and 'LM0721' (n=8) measured biweekly from 4 May – 24 Nov. 2011. Dates are displayed as Julian days. Height was measured from the leaf base to the tip of the newest unfolding leaf. The R^2 and p-values of the trend lines at 5% significance are presented.
[z]* Indicates the level of significance.



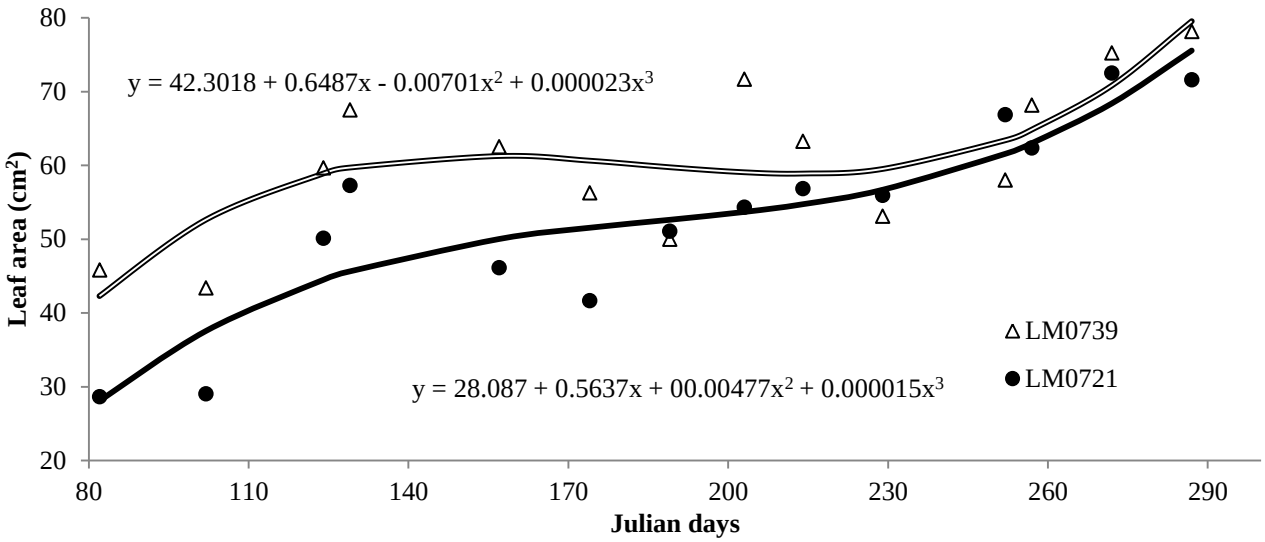
	R^2	*P* - value	Significance
LM0739	0.39902	0.1258	NS[z]
LM0721	0.64856	0.0066	**[y]

Fig. 18. The mean leaf width (mm) of potted *Disa* hybrids 'LM0739' (n=10) and 'LM0721' (n=8) measured biweekly from 4 May – 24 Nov. 2011. Dates are presented as Julian days. Leaf width was measured at the widest section of the lamina of the 5 principle leaves. The R^2 and p-values of the trend lines at 5% significance are presented.
[z]NS. Indicates no significant difference at the 5% confidence level. [y]*Indicates the level of significance.



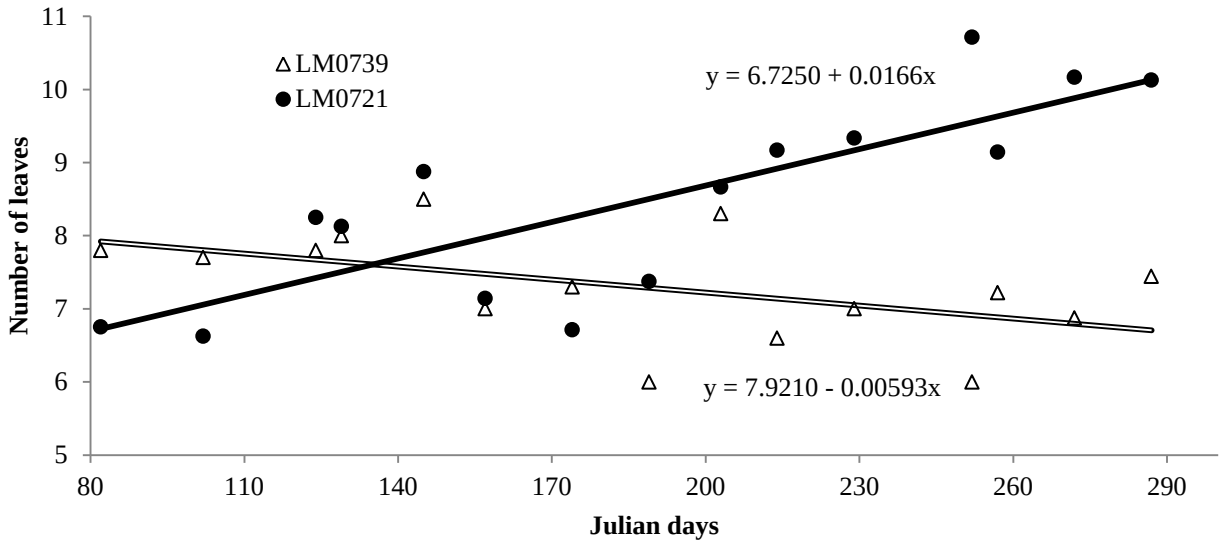
	R^2	*P* - value	Significance
LM0739	0.05801	0.8310	NS[z]
LM0721	0.29480	0.2677	NS

Fig. 19. The mean leaf base diameter (mm) of potted *Disa* hybrids 'LM0739' (n=10) and 'LM0721' (n=8) measured biweekly from 4 May – 24 Nov. 2011. Dates are presented as Julian days. Leaf base was measured at the plant base, just above the root attachments. The R^2 and p-values of the trend lines at 5% significance are presented. [z]NS. Indicates no significant difference at the 5% level.



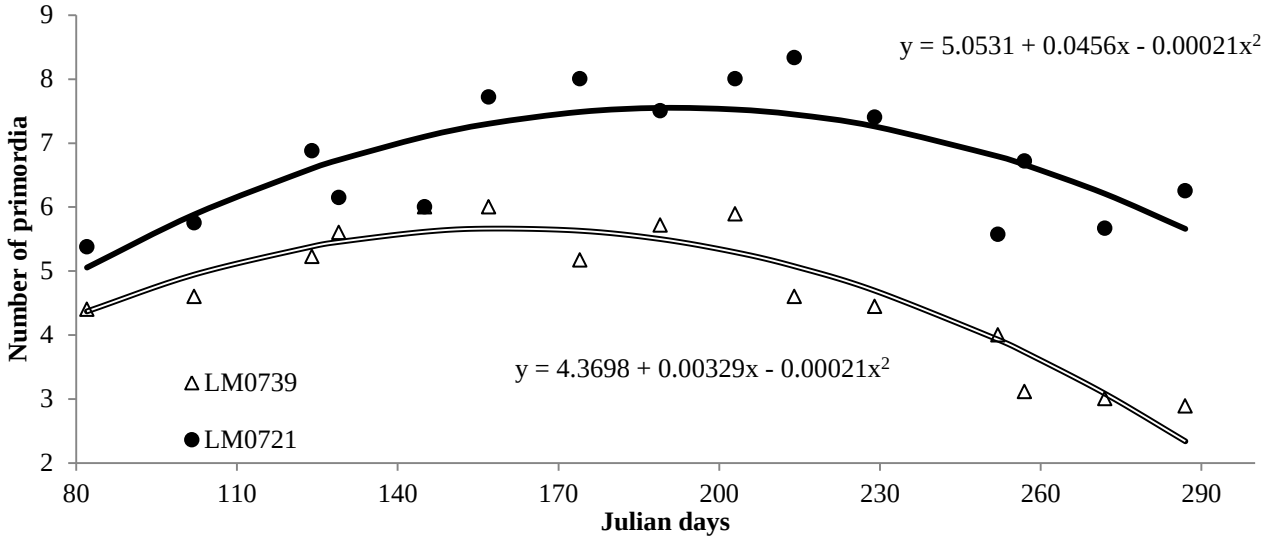
	R^2	*P* - value	Significance
LM0739	0.78865	0.0008	***[z]
LM0721	0.90781	<0.0001	***

Fig. 20. The mean leaf area (cm^2) of potted *Disa* hybrids 'LM0739' (n=10) and 'LM0721' (n=8) measured biweekly from 23 Mar. to 14 Oct. 2011. Dates are presented as Julian days. The R^2 and p-values of the trend lines at 5% significance are presented. [z]* Indicates the level of significance.



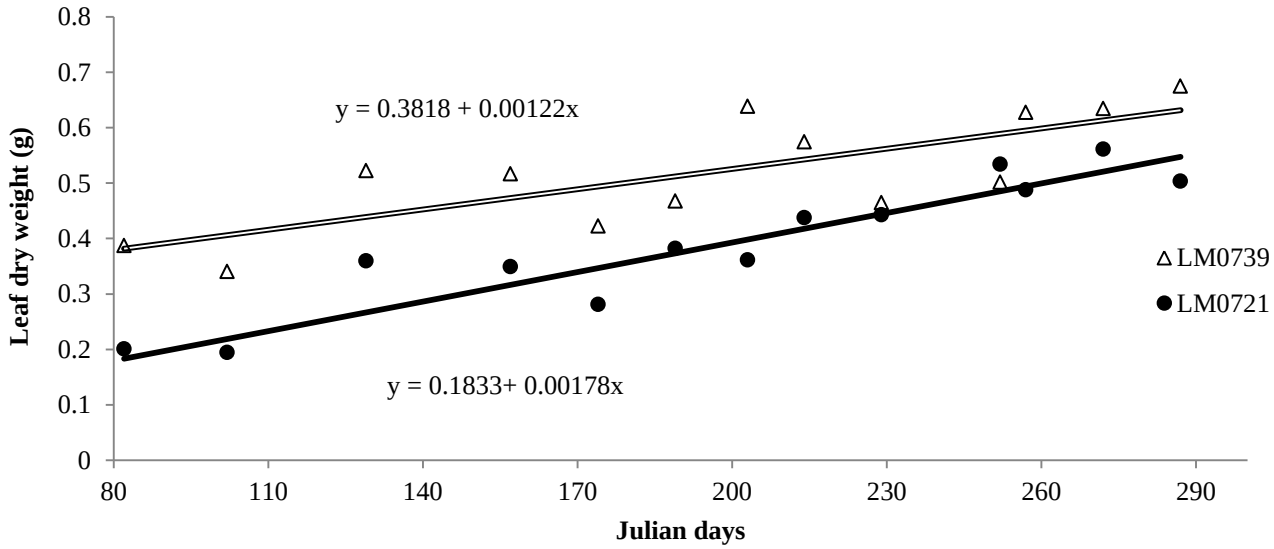
	R^2	*P* - value	Significance
LM0739	0.49086	0.0632	NS^z
LM0721	0.81177	0.000	$***^y$

Fig. 21. The mean number of leaves of potted *Disa* hybrids 'LM0739' (n=10) and 'LM0721' (n=8) measured biweekly from 23 Mar. – 14 Oct. 2011. Dates are presented as Julian days. The R^2 and p-values of the trend lines at 5% significance are presented. ^{z}NS. Indicates no significant difference at the 5% confidence level. ^{y}* Indicates the level of significance.



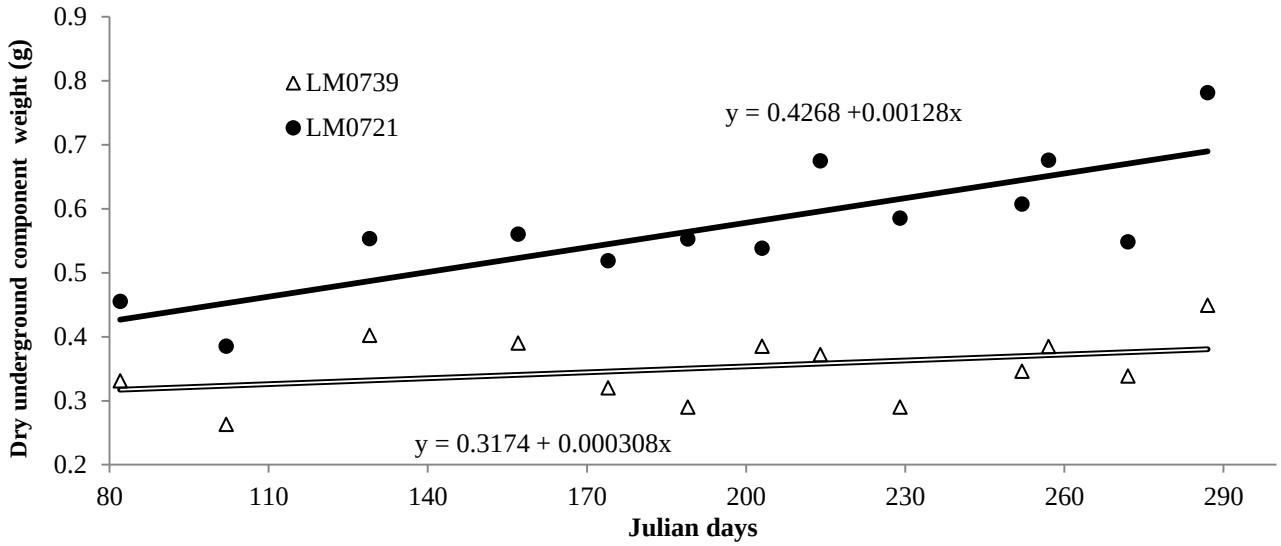
	R^2	*P* - value	Significance
LM0739	0.93639	<0.0001	$***^z$
LM0721	0.78970	<0.0001	***

Fig. 22. The mean number of leaf primordia of dissected *Disa* hybrids 'LM0739' (n=10) and 'LM0721' (n=8) measured biweekly from 23 Mar. – 14 Oct. 2011. Dates are presented as Julian days. The R^2 and p-values of the trend lines at 5% significance are presented. ^{z}* Indicates the level of significance.



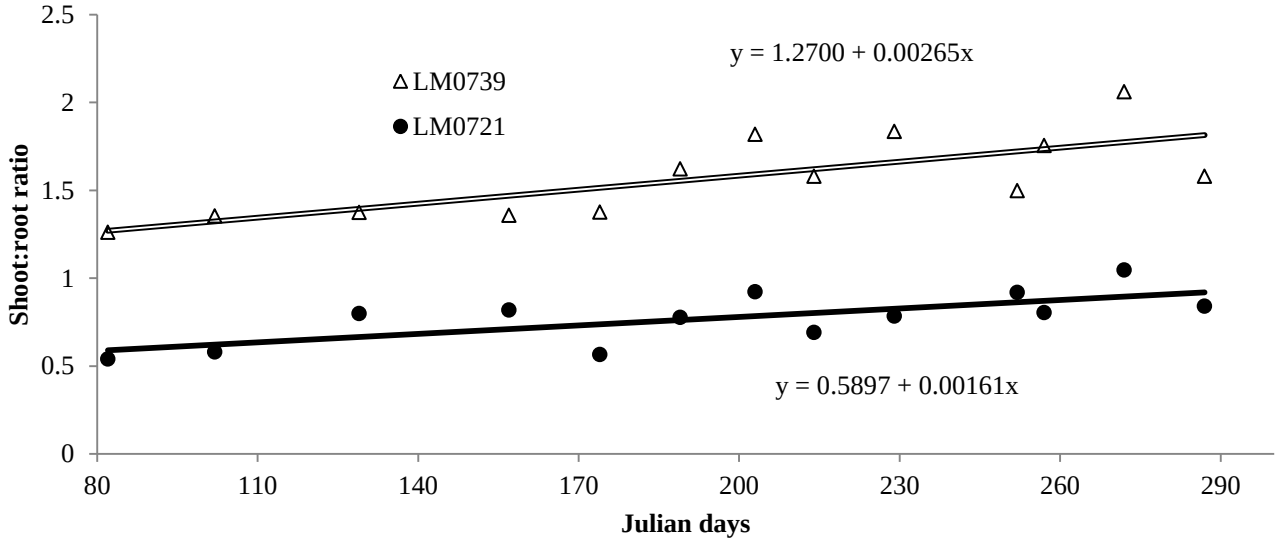
	R^2	*P* - value	Significance
LM0739	0.33056	0.0002	***[z]
LM0721	0.63979	<0.0001	***

Fig. 23. The total mean dry weight (g) of leaves per plant of potted *Disa* hybrids 'LM0739' (n=10) and 'LM0721' (n=8) measured biweekly from 23 Mar. – 14 Oct. 2011. Dates are presented as Julian days. The R^2 and p-values are of the trend lines at 5% significance are presented. [z] * Indicates the level of significance.



	R^2	*P* - value	Significance
LM0739	0.13215	0.1451	NS[z]
LM0721	0.26780	0.0107	*[y]

Fig. 24. The total mean dry weight (g) of underground components per plant of potted *Disa* hybrids 'LM0739' (n=10) and 'LM0721' (n=8) measured biweekly from 23 Mar. – 14 Oct. 2011. Dates are presented as Julian days. The R^2 and p-values of the trend lines at 5% significance are presented. [z]NS. Indicates no significant difference at the 5% confidence level. [y]*Indicates the level of significance.



	R^2	*P* - value	Significance
LM0739	0.27556	0.0018	**[z]
LM0721	0.33266	0.0013	**

Fig. 25. The shoot:root ratio of potted *Disa* hybrids 'LM0739' (n=10) and 'LM0721' (n=8) measured biweekly from 23 Mar. - 29 Sept. 2011. The R^2 and P-values of the trend lines at 5% significance are presented. [z]* Indicates the level of significance.

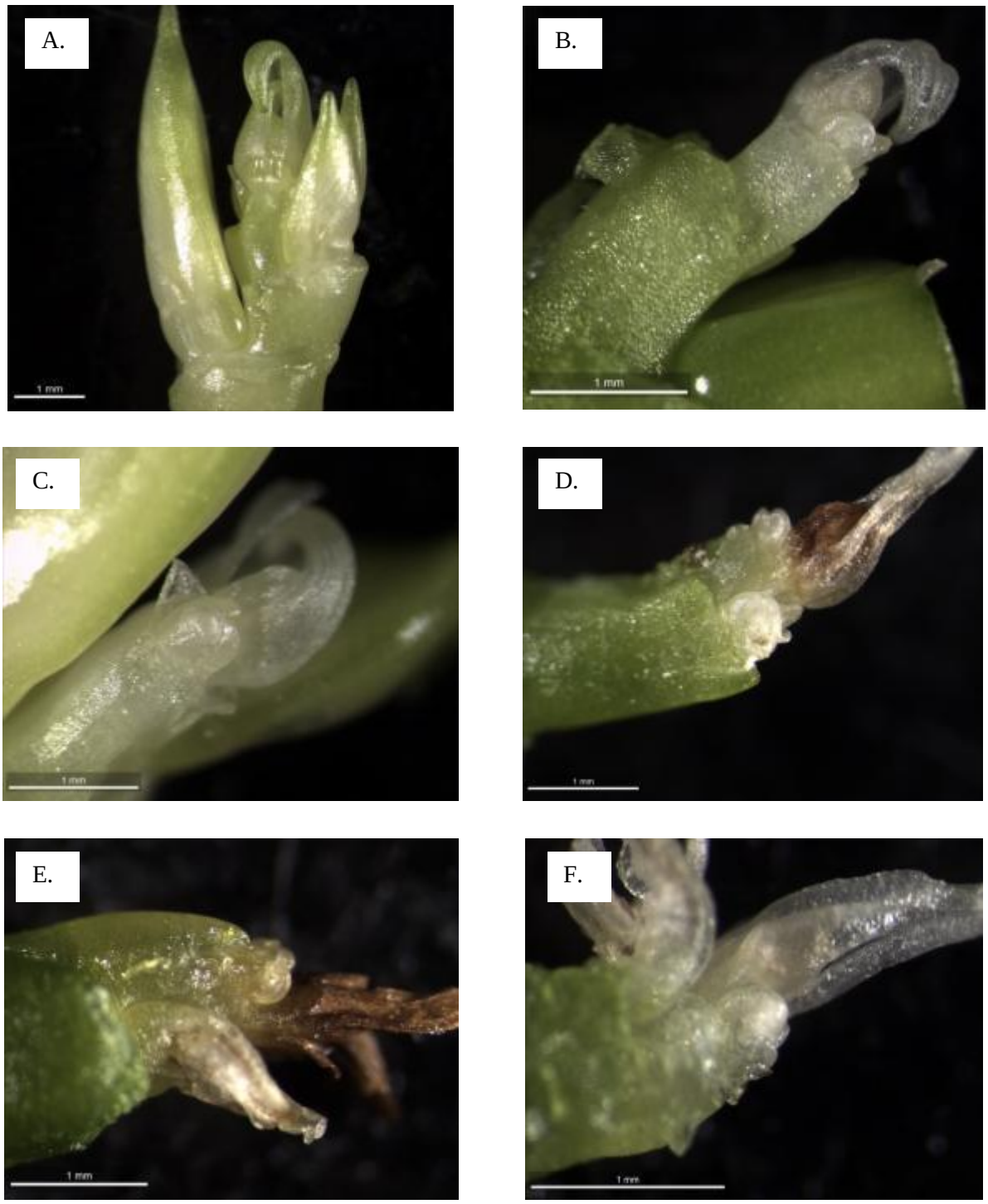


Fig. 26. A. An example of a healthy terminal spike of *Disa* hybrid 'LM0739' showing developing flowers here the youngest 1-2 flowers have not been aborted. B-E. Flowering meristems of hybrid 'LM61' with aborted flower buds identified by a translucent and brown colouration of the tissue.

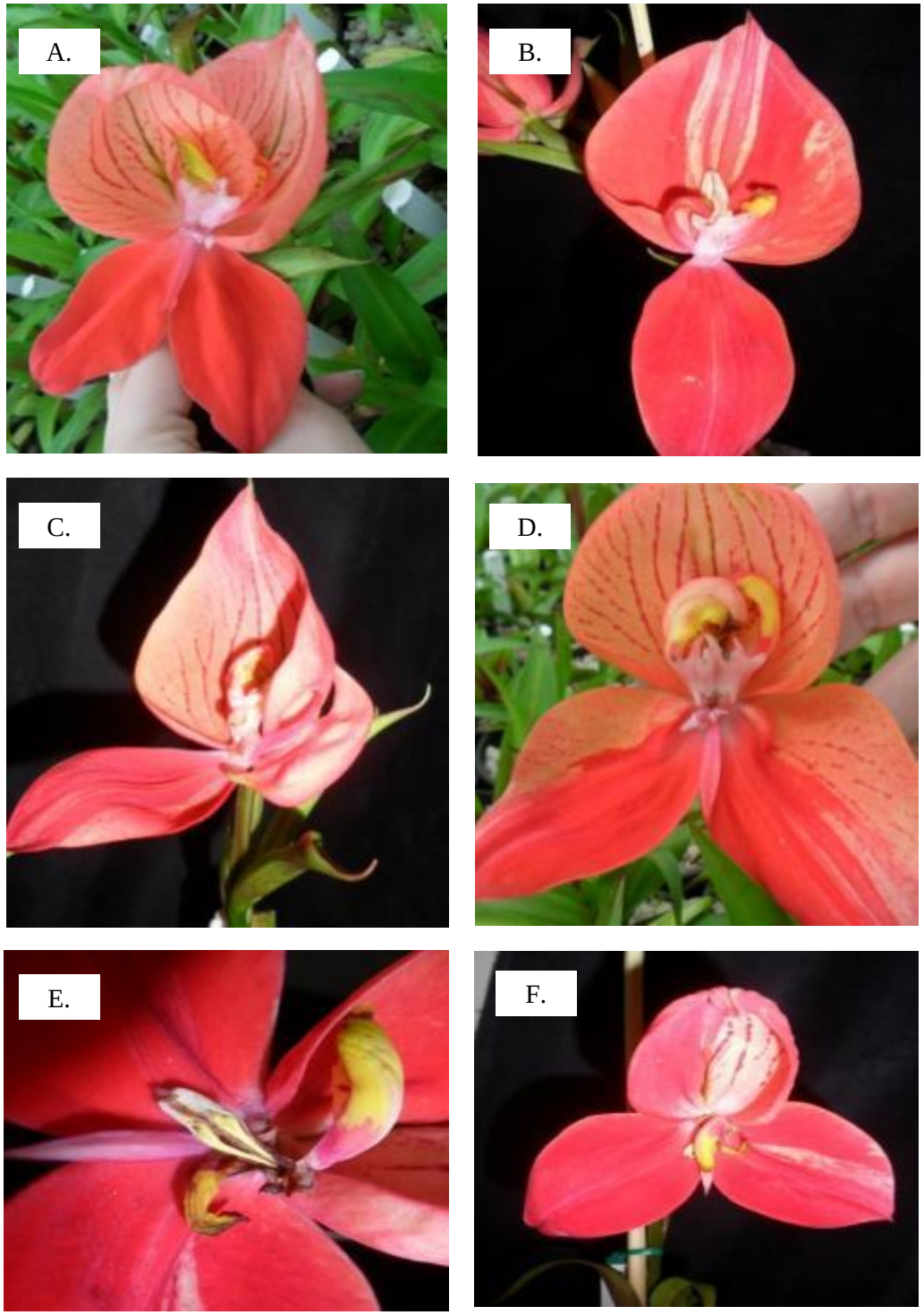


Fig. 27. *Disa* hybrid 'LM0739' flowers showing abnormalities concerning colour expression and the morphology of the petal, sepal and sexual floral parts. Abnormalities were found to be especially prevalent early in the flowering season. Colour deviations were observed to be streaky and faded (A - F). Sepals were either duplicated (A), absent (B) or deformed (C). Deformed petals and sexual floral parts were frequently observed (A - F). Sexual floral parts were found duplicated (D) with the lips regularly missing (B) even in otherwise well-formed flowers.

Paper 2: Photoperiod, Photosynthesis and Carbon Allocation Strategies for Selected *Disa* Hybrids in Cultivation

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ABSTRACT. **The indigenous South African orchid *Disa* shows potential for being a niche floricultural crop, however, the lack of scientific research regarding cultivation practices hampers the realization of the potential of *Disa*. The role of light, as pertaining to photoperiod effects, photosynthesis and carbon allocation strategies were studied in *Disa* hybrids in cultivation, in order to determine baseline values for this newly established floricultural crop.**

Firstly, photoperiodic effects were considered by subjecting plants to four night break treatments namely a control (no night break) and then 58, 74 and 167 days of night break cycles respectively where a night break cycle consisted of sixteen hours direct exposure to a 100W incandescent bulb daily from 04:00PM to 08:00AM. The vegetative growth of plants was monitored using plant height (mm), crown width (mm) and number of unfolded leaves as growth parameters. Flowering was documented as date and percentage of spike emergence, anthesis and senescence respectively. No photoperiodic effect was however recorded for the *Disa* hybrid 'LM0739' studied.

In a next trial additional lighting was supplied to four *Disa* hybrids 'LM56', 'LM66', 'LM67' and 'LM48' in the form of a Papillon day light source which was switched on from 06:00AM to 06:00PM daily. A red/brown colouration which was observed on the unfolded leaves and subsequently suspected to be the consequence of light stress, was further examined by means of transmission electron microscope images (TEM) as well as photosynthesis and fluorescence recordings. No significant differences were however observed between light-treated (mature and newly formed leaves) and control plants regarding any of the quantifying measures, whilst TEM imaging was considered unsuccessful and inconclusive. The observed pigmentation was suggested to be ascribed to anthocyanin formation, as part of an adaptive, protective mechanism against higher light intensities.

In a third trial, photosynthetic measurements of A_{max} (maximum rate of light-saturated net CO_2 assimilation), A_{sat} (maximum rate of light- and CO_2-saturated net CO_2 assimilation) and R_d (dark respiration rate), were recorded for the *Disa* hybrid 'LM0739' from Mar. to Sept. 2011. Carbon assimilation rates similar to that of other Cape Floristic Region (CFR) species and orchids (3–12 $\mu mol.m^{-2}.s^{-1}$) were found. Carbon allocation strategies were studied by means of non-structural carbohydrate analyses of above-and below ground material of two *Disa* hybrids, 'LM0739' and 'LM0721'. Sucrose and glucose were the main soluble sugars extracted within the ethanol fraction. Starch levels were found to be extremely low in plant tissue of both hybrids, except in the underground components of the tuber-forming hybrid 'LM0721'. The hydrolysis of the polymers extracted in the water fraction confirmed these compounds to be at

least partially of a polysaccharide nature, possibly glucomannans. The root fraction of hybrid 'LM0721' showed much higher levels of the hydrolyzed sugars when compared to that of 'LM0739'. *Disa* hybrids containing root tubers is thus proposed to employ to different carbon utilization strategies compared to non-tuber forming hybrids. These studies form a first report on light, photosynthesis and carbon allocation strategies for *Disa* hybrids in cultivation.

ADDITIONAL INDEX WORDS: irradiance, short days, day continuation, carbohydrate reserves, glucomannans.

High plant diversity is characteristic of Mediterranean climates and one such climatic region, especially rich in geophytes, is the Cape Floristic Region (CFR) of South Africa (Proches et al., 2006). Almost half of the species richness in the Cape floral kingdom is accounted for by only 33 Cape floral clades, with *Disa* being one of these clades (Bytebier et al., 2010). Geophytes are defined as all plants that not only survive as seeds, but also by means of specialized underground storage organs (Theron and De Hertogh, 2001) or plants with perrenation organs which fulfils a storage function (Proches et al., 2006). *Disa*, as a member of the Orchidaceae, is also considered a geophyte due to the presence of a characteristic root tuber which is replaced annually (Du Plessis and Duncan, 1989). A tuber from the current growing season as well as another tuber from the previous growing season is present in plants at anthesis (Linder and Kurzweil, 1999). Re-growth (new shoot and roots systems) are enabled and supported by the new root tuber and occurs as soon as the old flowering spike have died back (Vogelpoel, 1993).

Disa, as an indigenous South African orchid shows considerable commercial potential, although various factors currently limit the cultivation of this exceptional orchid. An understanding of the possible flowering signal in *Disa*, together with a known timing of floral initiation is important requirements for the development of future flower manipulation strategies. When this established timing of floral initiation is correlated with changes in phenology it could shed light on the nature of the flowering signal. Results from Paper 1 reported floral initiation for both the studied *Disa* hybrids to occur at the end of May and June, respectively. As initiation coincide with the onset of winter (southern hemisphere), a consistent drop in temperature, the shortening of days (photoperiod) or possibly a combination of the two as the flowering signal in natural habitats is argued.

A short day flowering response is widely reported within the Orchidaceae (Goh and Arditti, 1985; Lopez and Runkle, 2004; Lopez and Runkle 2005) as well as in other CFR fynbos genera such as *Leucadendron* (Hettasch and Jacobs, 2006) and therefore a possible short day flowering response in *Disa* warrants research.

Carbohydrates derived directly from photosynthesis account for 90% of the dry weight in crops and subsequently light irradiance is a key factor for maximal photosynthesis (Guo et al., 2012). Light not only affects plant growth strategies as a signal for floral induction due to the duration of

exposure to light (photoperiod), but light is also essential for all growth, photosynthesis and carbon allocation strategies. In cultivation, knowledge on the optimum light intensity required for orchid growth is important, as the emergence of an inflorescence and the quality of the flowers are highly correlated with high levels of carbohydrates stored in orchid leaves (Guo et al., 2012).

The growth cycle of *Disa* has been described in its natural habitat (Crous and Duncan, 2006). This information, together with shifts in photosynthetic capacity throughout the season, can provide growers with a valuable indication on the carbon usage and storage capacity of *Disa*. Knowledge of the phenology of any plant in cultivation is important to obtain a clear understanding of the carbon partitioning and the bio-mass allocation patterns between plant parts. This information is of critical importance in the decision to implement certain cultural practices such as the time and feasibility of re-potting during the cultivation of *Disa* hybrids and thus forms part of baseline information when studying a new horticultural crop.

In general, little is known about the photo-assimilation strategies of CFR plants, including *Disa* (Van der Heyden and Lewis, 1989). Earlier studies report the photosynthetic capacity of CFR plants to be mostly low, with maximum CO_2 assimilation rates of 16 fynbos species recorded to range between 2.8–12.7 $\mu mol.m^{-2}.s^{-1}$ (Von Willert et al., 1989). A more recent study by Bezuidenhout (2010) studying the photosynthetic characteristic of *Protea* cv. 'Pink Ice' species under elevated temperatures, as is expected with global warming, recorded similar maximum CO_2 assimilation rates of between 3 and 12.5 $\mu mol.m^{-2}.s^{-1}$. The photosynthetic capacity of fynbos can be limited by nitrogen availability (Stock et al., 1992), which is mostly unavailable in the nutrient leached sandy soils of the CFR. The age of leaves were also found to influence the photosynthetic capacity in Proteaceae (Von Willert et al., 1989). As *Disa* is also endemic to the CFR a low photosynthetic capacity is therefore expected for this genus.

In orchids, studies indicate that thick-leaved orchids such as *Aranda* species and *Phalaenopsis* (Guo et al., 2012) primarily show the characteristic gas exchange as is seen typically for crassulacean acid metabolism (CAM) plants, while thin-leaved orchids such as *Arundina graminifolia* and *Oncidium* mainly photosynthesize via the C_3 photosynthetic pathway (Goh et al. 1983). Orchids do, however, also have non-foliar green organs such as pseudo-bulbs, stems, flowers and roots that can also contribute, to varying degrees, to the carbon balance of the plant. In general, it was found that the photosynthetic activity from non-foliar organs is not sufficient to sustain growth and is mostly utilised within the organ itself and not exported to other sinks (Hew and Yong, 2004). However, this is not the case in shoot-less orchids where roots form more than half of the total biomass and therefore plays a major part in carbon fixation. In *Chiloschista usneoides* this carbon fixation alternative is referred to as "astomatal CAM" (Cockburn et al. 1985). Roots of the well-known orchid genus, *Phalaenopsis*, are thick and succulent and some of the aerial roots contain chlorophyll and are thus able to photosynthesize (Hou et al., 2011). Furthermore, stems of many monopodial orchids are green and can contribute positively to the carbon assimilation of the plant, whilst stomata found on orchid

flowers are non-functional and therefore unlikely to be under the same diurnal stomatal control as the leaves. However, green flowers found on certain *Cymbidium* species can photosynthesize. Signs of C_4 photosynthesis have been found in orchids such as *Cymbidium* and *Arundina graminifolia*, although uncertainty and contrasting results prevents concluding evidence on this phenomenon (Hew and Yong, 2004). However, no photosynthetic data have been recorded for *Disa* and the preferred pathway of carbon fixation, seasonal trends in photosynthesis, and all the organs involved in photosynthate assimilation, carbon allocation patterns and the preferred storage metabolites have never been studied.

The characteristic strategy of geophytes is to evade unfavourable environmental conditions by means of a resting bud attached to an underground storage organ (Orthen, 2001). For geophytes species, such as *Disa*, incidences of environmental stresses, typical of the CFR, can be high temperatures experienced during long, dry summer months (Orthen, 2001). Consequently the lifecycle of CFR geophytes are synchronised to coincide with favourable environmental conditions. Enabling such a growth strategy is the large amount of reserve materials contained in an underground organ (Orthen, 2001; Orthen and Wehrmeyer, 2004), as these storage reserves allow the initiation of new growth following seasonal dormancy. Underground storage organs can contain a variety of reserve carbohydrates; the most common are sucrose and starch together with non-starch reserve polysaccharides like fructans and glucomannans (Miller, 1992; Orthen, 2001; Ranwala and Miller, 2008).

During certain physiological stages, which coincide with periods of rapid photosynthesis, plant cells accumulate reserve polysaccharides. These reserve compounds are stored (in roots, underground storage organs and even leaves) and are temporarily withdrawn from cellular metabolism. Polysaccharides are later catabolised to carbohydrate monomers which can re-enter the general metabolism (Meier and Reid, 1982). Non-starch reserve polysaccharides are found in plant organs such as seeds, roots, rhizomes, tubers, bulbs and shoot axes, but not so frequently in leaves (Meier and Reid, 1982). These reserve metabolites can occur alongside starch or as the sole reserve polymer in a given plant organ (Meier and Reid, 1982). Glucomannan has been extracted from Orchidaceae species like *Orchis moria* (Watanabe et al., 1991), *Satyrium coriifolium* and *S. carneum* (Mabusela et al., 1989), whilst pure mannan has been also been extracted from the pseudobulbs of the thin leaved orchid *Oncidium* (Wang et al., 2006). A type of polysaccharide has also been extracted from the leaves, stems and roots of orchid species from the genus *Dendrobium*, although the nature of the polysaccharide was not specified (Lu et al., 2007). Glucomannan has further been reported as a reserve carbohydrate in other major geophyte families like Araceae, Amaryllidaceae, Liliaceae and Iridaceae (Achtardjiev and Koleva, 1973). However, little is known about the carbohydrate metabolism in geophytes and especially concerning carbohydrate partitioning together with the preferred form of carbohydrate reserves (Miller, 1992).

Carbon allocation, partitioning and sufficient utilisation of reserves are essential for plant development and subsequent flowering. Understanding the carbon allocation and partitioning in relation to optimum photosynthetic conditions throughout the season, and also the plant's photosynthetic response to changing environmental factors such as higher irradiance, can lead to better management strategies for the successful cultivation of *Disa*.

The aims of this study were firstly to examine the effects of light on *Disa* growth and flowering by studying two possible light strategies and its effects, namely the duration of light exposure in the form of short day photoperiod as well as higher light irradiance. Secondly, to study the photosynthetic capacity of *Disa* hybrids throughout the active growing-, dormant- and bolting phases, and lastly, to describe and monitor carbon allocation and partitioning throughout the growth cycle in selected *Disa* hybrids.

Materials and Methods

PLANT MATERIAL. All plant material was obtained from La Motte Winery Estate in Franschhoek (33°52'49.9"S; 19°4'28.3"O), the only commercial *Disa* producer in South Africa. The material selection included hybrid 'LM0739' for the short day photoperiod and photosynthesis trials as well as hybrid 'LM0721' together with 'LM0739' when reporting on the non-structural carbohydrates and carbon allocation strategies of *Disa*. For studying the effect of additional lighting on the growth and flowering of *Disa* hybrids in cultivation, hybrids 'LM56', 'LM66', 'LM67' and 'LM48' were used. Parent plants used in the hybridization programs for all the above mentioned hybrids are unknown.

EXPERIMENTAL SITE. The same experimental site was used as described in Paper 1. In Apr. 2011 all plants were separated and re-potted to one plant per pot. Newly re-potted plants of hybrid 'LM0739' and 'LM0721' were then transferred to a polycarbonate greenhouse facility for the consecutive dormant and reproductive phases. However, in 2012 after plant separation, hybrids 'LM56', 'LM66', 'LM67' and 'LM48' were kept in the shade house for the entire duration of its development, and was only transferred to the greenhouse at anthesis. For details on the greenhouse and shade house structures refer to Paper 1.

NUTRITION, WATER AND GROWTH MEDIUM. The nutrition, water and growth medium used for cultivation of plants were similar as described in Paper 1.

TEMPERATURE AND GROWING CONDITIONS. Temperatures and growing conditions as recorded within the shade- and greenhouses were reported within Paper 1.

PHOTOPERIOD. Sixty, medium to large sized plants (crown widths of between 119.4 ± 3.2 and 158.6 ± 6.1 mm) of the *Disa* hybrid 'LM0739' were selected and randomly allocated to four treatments. These treatments consisted of the control, and plants that were exposed to a night break regime for either 58, 74 or 167 days, with 15 replications per treatment. All treatment groups were placed under a 100W, 240V incandescent bulb, with the exception of the control group, which

received no night interruption treatment. Growing conditions were otherwise similar for all experimental plants. A curtain constructed of black plastic, which hung from the ceiling down to the level of the growth tables, was erected to contain the light only to the allocated experimental area (Fig. 1). This ensured that the rest of the greenhouse was not affected by the night interrupting regime. The light levels were recorded at night, with a light meter (Li-250, LI-COR, Lincoln, Nebraska, USA), approximately ± 1m below the light source, in concentric circles at 13 positions. The average values of three recordings at each of the 13 positions ranged between 1.4 and 3.4 $\mu mol.m^{2}.s^{-1}$ (n=39).

The light was set to a timer which allowed for illumination from 04:00PM to 08:00AM, providing a night break (or day continuation) of 16 h and ensuring that the plants received no natural darkness or short nights. The light was first switched on 22 Mar. 2011 when all the treatments, with the exception of the control, were placed directly under the light to receive an effective photoperiod of 24 h. The first group of plants was removed on 17 May 2011, after 58 days of the night break regime. The second treatment was removed on 13 Jun. 2011, after 74 days, whilst the last group was removed on 12 Sept. 2011, after 167 days of the night break regime respectively. After removal of the treated plants from the night break regime all plants were placed under normal growing conditions of the greenhouse as described in Paper 1.

After baseline data were obtained in March 2011, growth as described by plant height (mm), crown width (mm) and number of unfolded leaves were recorded at six week intervals. Height measurement was taken from the base of the plant to the tip of the newest unfolding leaf, whilst the crown width was measured form the tip of one unfolded mature leaf to the tip of the opposite mature unfolded leaf. All measurements were recorded with a 15 or 30 cm digital calliper and digital logger (Mitutoyo, Mitutoyo Corporation, Kawasaki, Japan). Together with the date of spike emergence, the date on which the first flower of the inflorescence opened (anthesis) and flower senescence were also recorded to construct a harvest distribution. Differences seen in percentage of anthesis and senescence were ascribed to the experimental loss of the flowering plants before senescence could be documented.

ADDITIONAL LIGHTING. Four hybrids, 'LM56', 'LM67', 'LM66' and 'LM48' were chosen to study the effects of additional lighting (higher daily irradiance) on *Disa* in cultivation. Fifteen plants of each hybrid were selected randomly in June 2012 for both the control and treatment groups. Plants selected were of medium to large size with crown widths of between 119.4 ± 3.2 and 158.6 ± 6.1 mm. The treatment entailed placing the selected plants under an additional light source, namely a D-Papillon 315W, 230V Daylight fixture, with an Agro 315W 930 grow lamp (Lights interaction, Achtseweg, Eindhoven, Netherlands), mounted approximately 60 cm above plant level and switched on daily from 06:00AM to 06:00PM. This light regime was followed from June until Sept. 2011, after which recordings and observations were documented. The light intensities measured under the D-Papillon lamp at plant level reached 359 $\mu mol.m^{-2}.s^{-1}$, whilst the light intensities measured on plant

level of the control group reached up to 279 μmol.m^{-2}.s^{-1}, where both readings were measured on a clear morning, at approximately 12:00 AM.

No growth data were recorded for the treated or control plants within this trial. Visually observed differences between the control and treatment group which was exposed to the additional light was documented and quantified by means of Transmission Electron Microscopy (TEM), photosynthetic readings and fluorescence measurements. The hybrid with the broadest leaf characteristics, hybrid 'LM56', was selected for photosynthetic recordings, whilst another hybrid, 'LM67', was selected to represent the other three hybrids during the fluorescence recordings. Photosynthesis recordings were obtained using an IRGA (LI -6400XT Portable Photosynthesis system, LI-COR®, Lincoln, Nebraska, USA), whilst fluorescence measurements were made by means of a pulse modulated fluorimeter (FMS2, Hansatech Instruments Ltd., Norfolk, UK). Mineral leaf analyses of the leaves from control plants and both mature and newly formed leaves of light- treated plants were conducted by Bemlab (Van der Berg Crescent, Gant's Centre, Strand, South Africa).

Photosynthesis recordings (IRGA). For photosynthetic measurements three hybrid 'LM56' plants were used for both the treated and control plant groups respectively. In the light-treated plants, two leaves per plant were selected where one leaf was represented by a mature leaf which showed a typical dark red colouration and necrotic lesions, whilst the other leaf was a newly unfolded green leaf (visible after approximately two months of light treatment) formed after plants presumably adapted to the higher light environment. Photosynthesis recordings, similar to that described for the trial where additional lighting were supplied, were conducted at a photosynthetic photon flux (*PPF*) of 800 μmol.m^2.s^{-1} and a CO_2 flow rate set at 150 μmol.s^{-1}.

Fluorimeter. A single leaf from each of three plants of hybrid 'LM67' of the control group, together with two leaves (one newly formed adapted leaf and one mature light-damaged leaf) from each of three light-treated plants were dark-adapted with leaf clips for one hour before maximum quantum efficiency (Fv/Fm) measurements were taken. This Fv/Fm value represents the maximal quantum yield or current photochemical efficiency of Photosystem II (PSII), usually ranging between 0.75 and 0.85 for non-stressed plants (Guo et al., 2012).

TEM. For inspection by means of TEM, a representative leaf of the control group, as well as one non-adapted mature (red coloured) and one newly formed and adapted (green) leaf from the treated plants, were prepared for examination of the chloroplasts. Sample preparation was done by the method of Schmeisser (2010) as described for studying the visualization of chloroplasts of *Leucadendron* plants. Leaf segments of 5 mm^2 were cut from leaf centre, next to the midrib for each leaf. Leaf segments were fixed for 4 h in 5% ($^v/_v$) glutaraldehyde in a 0.075M phosphate buffer, pH 7.4, containing 2% formaldehyde, at room temperature (22 ºC). Further fixation at 4 °C, took place until processing of the samples approximately two months later. For the next stage, segments were washed once, for 5 min with the phosphate buffer and transferred to an 1% osmium tetroxide (Apollo Scientific, S-02600-BA), in the same buffer, for 3 h. After washing the segments with distilled water,

they were dehydrated in an ethanol series (30%, 50%, 70%, 80% and 90%) for 10 min each (Leica EM TP, sample processor). The final dehydration step was performed in 100% ethanol, twice for 20 min. Subsequently, ethanol was then replaced by 100% acetone where samples was immersed for an additional 20 min, repeated twice, where after half the acetone was replaced with Spurr's epoxy resin (Wirsam, TLS032 D), with samples left overnight on a shaker to prevent solidification of the resin. The resin concentration was increased after 8 h to 100% resin for a further 2 days at room temperature set at approximately 20 °C, where after samples were transferred to 60 ºC for 24 h. Thin sections (70-100 nm) were then cut on a Leica EM UC7 ultra-microtome using glass knife, with sections being placed on 200 µm mesh square copper grids. Imaging of the samples was accomplished using a JEOL 1200-EX II Transmission Electron Microscope at Stellenbosch University. Transmission Electron images were taken using conditions of 120 kV, Objective Aperture setting of 150 microns and a spot size of three. Samples were imaged with digital imaging acquisition using an Orius™ SC 200 CCD Camera with Gatan Microscopic software with largest resolution of 1376 x 1032 and exposure time of 2 s.

PHOTOSYNTHESIS. Eight, large (crown width of 158.6 ± 6.1 mm) plants of *Disa* hybrid 'LM0739' with sufficient leaf width to fit the 6 cm^2 of the leaf chamber of the Infra red gas analyzer (IRGA) (LI -6400XT Portable Photosynthesis system, LI-COR®, Lincoln, Nebraska, USA) were preselected on 24 Mar. 2011 for consecutive photosynthetic recordings throughout the season until 30 Sept. 2011. Recordings of the eight plants were taken in random order onsite at La Motte Winery Estate, with approximate three-weekly intervals between recordings. A light curve (CO_2 assimilation vs. photosynthetic photon flux (*PPF*)) which was constructed in Mar. 2011 for hybrid 'LM0739' prior to the start of the photosynthetic recordings on 24 Mar. 2011, determined the optimum light levels required for photosynthesis readings concerning this *Disa* hybrid to be approximately 800 $\mu mol.m^2.s^{-1}$ (Fig. 2).

Recordings were consistently taken in the mornings between 09:00AM and 12:00AM. The CO_2 flow rate set for photosynthetic measurements using the IRGA were 150 $\mu mol.s^{-1}$. Three photosynthetic measurements were taken for each of the eight plants: A_{max} (maximum rate of light-saturated net CO_2 assimilation; $\mu mol.m^{-2}.s^{-1}$), A_{sat} (maximum rate of light- and CO_2-saturated net CO_2 assimilation; $\mu mol.m^{-2}.s^{-1}$) and R_d (dark respiration rate, $\mu mol.m^{-2}.s^{-1}$). Furthermore, A_{max} was measured at ambient CO_2 levels of 380 µmol $CO_2.mol^{-1}$ and *PPF* of 800 $\mu mol.m^2.s^{-1}$. A_{sat} was measured at CO_2 levels of 1200 µmol $CO_2.mol^{-1}$ and *PPF* of 800 $\mu mol.m^2.s^{-1}$, whilst R_d was measured at ambient CO_2 of 380 µmol $CO_2.mol^{-1}$ and *PPF* of zero.

NON-STRUCTURAL CARBOHYDRATE ANALYSES. Starch and sugar analyses were conducted on the freeze-dried plant material of the dissected plants from the floral initiation and differentiation trials in Paper 1. Both the above- and underground components of five plants per dissection week of hybrids 'LM0739' and 'LM0721' each were analyzed. The data are presented as weeks of the year,

but the respective dates are also provided. At harvest the plant materials were freeze-dried and stored at -80 °C until carbohydrate analysis was conducted. A 100 ± 0.05 mg of each sample was used for the carbohydrate analyses.

The analyses of the non-structural carbohydrates were done through the extraction of three different fractions of the samples. Firstly, an aqueous 80% ethanol solution was used for extraction of the soluble sugar fraction, which is considered to be readily available to the plant. As polysaccharides have been shown to be present as storage reserve in both geophytes and orchids (Wozniewski et al., 1991), the pellets were then re-extracted with water (2nd fraction) to obtain possible polymers that would have been insoluble in 80% ethanol. Lastly, a starch fraction was extracted from the pellet.

Ethanol fraction (soluble sugars). The samples were weighed into marked kimax tubes and 4 ml of 80% aqueous ethanol was added to the tubes. The kimax tubes were capped, vortexed and placed on an 80 °C heating block for 30 min. After removal from the heating block the samples were centrifuged for 3 min at a relative centrifugal force (rcf) of 1800 g_n and 4°C. The supernatants were decanted into clearly marked 20 ml glass vials. This process was repeated and the supernatants were pooled, resulting in approximately 8 ml of the supernatants in the 20 ml glass vials. Another 4 ml of 80% ethanol was added to the kimax tubes containing the pellets and the kimax tubes were again capped, vortexed and placed on an 80 °C heating block, this time for 15 min. After removal from the heating block the samples were centrifuged (3 min at 1800 g_n; 4 °C), decanted and pooled with the previous supernatants. The process was repeated once more to yield approximately 16 ml of the 80% ethanol extract in each of the glass vials. The residues or pellets were now assumed to contain no soluble sugars and were retained for the further extractions of possible polysaccharides and starch. The total volume of the ethanol extracts were then placed into a SC210A Speed vac® Plus (Thermo Savant) for evaporation. Once dry, the samples were reconstituted with 1 ml of deionised water and were placed on the shaker at 400 revolutions per minute (rpm) for 15 min. The reconstituted samples were then transferred to a clean set of 2 ml eppendorf tubes and centrifuged (15 min at 20 000 g_n; 4 °C) after which the resulting supernatants were decanted to a clean set of eppendorf tubes.

C18 column cartridges, containing preparative C18 bulk packaging material (high performance polymeric solid phase extraction (SDE) products) from Phenomenex® (Promolab Pty. Ltd. T/A Separation, Randburg, Gauteng, SA) were firstly conditioned with methanol and then equilibrated with deionised water. This was done with the aid of a VacMaster® Sample processing station (International Sorbent Technology Ltd., Hengoed, UK). After the conditioning process, clean, clearly marked 20 ml glass vials were placed beneath the C18 cartridges and 200 µL of the supernatants were transferred onto the C18 cartridge. The samples were allowed to move freely through the column into the 20 ml glass vials, without using vacuum. The cartridges were then rinsed three times with 1.5 ml of deionised water, facilitated by low vacuum levels. The filtered and rinsed fraction samples were once again dried using the Speed Vac® Plus and reconstituted with 1 ml deionised water. The extracts were poured into disposable syringes and filtered through 0.45 µm

filters directly into high performance liquid chromatography (HPLC) vials. The sugars were separated and quantified using an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany) equipped with ChemStation software, a G1322A degasser, G1311A Quarternary pump, G1316A Column Thermostat, G1329A thermostatted autosampler and a G1362A Refractive Index Detector. A Resex™ RCM – Monosaccharide (300 x 7.8 mm) column (Phenomenex® product nr: 00H-0130-KO) with a Rezex™ RCM Monosaccharide (50 x 7.8mm) pre-column (Phenomenex® product nr: 03B-0130-KO) was used at 80 °C. The sugars were eluted with deionised water and a flow rate of 0.5 $ml.min^{-1}$ was maintained throughout the analysis. The sugars were quantified against external standards of the individual sugars that were being analyzed.

Water fraction. Deionised water (6 ml) were added to the kimax tubes containing the residues/pellets (from the 80% ethanol extraction), which were then capped, vortexed and placed on a heating block at 80 °C for 23 h. The samples were removed from the heating block and centrifuged for 3 min at 1800 g_n and 4 °C. The supernatants were then decanted into 50 ml Falcon centrifuge tubes. This extraction process was repeated another two times. The above process was repeated for a similar third extraction, but with the exception that only an additional 2 ml of deionised water was added and only 30 min extraction time in the heating block was allowed. The resulting supernatants were pooled with the previous supernatants stored in the 50 ml Falcon tubes. The total volume of the water extracts now resulted in approximately 20 ml per sample, whilst the pellets were retained to use for the starch extractions. The water extracts were transferred into disposable syringes and filtered through 0.45 µm filters. The water extracts had a "jelly-like", "mucous-like", or "mucilage-like" consistency, especially evident in the underground component samples of hybrid 'LM0721'. The mucilage-like nature of the water extract confirmed the necessity of including the water extraction step in the analyses to study the inclusion of possible polymers (polysaccharides) responsible for this gelatinized fraction. Extraction processes such as filtering and pipetting was complicated due to this "mucous-like" consistency. Before being hydrolyzed the water fractions were first analyzed using the same HPLC system, column and conditions as described for the soluble sugars obtained in the 80% ethanol fractions, to verify that there were no soluble sugars left in the water fractions before hydrolization.

Next, the water fractions were hydrolyzed to liberate any sugars from the polysaccharides assumed to be present. The water extract (5 ml), containing polymers, were evaporated using the Speed Vac® Plus. The polymers were then hydrolyzed with 0.5 ml of 2 M trifluoroacetic acid for 5 hours at 100 °C. The hydrolysates were dried using the Speed Vac® Plus and the resulting residues were dissolved in 1 ml deionised water and filtered directly into HPLC vials. The sugars that were released during hydrolysis were separated and quantified using the same HPLC system, column and conditions as described for analyses of the free sugars in the 80% ethanol fraction. The sugars were quantified against external standards for the individual sugars that were being analyzed. The sugars

that were released by the hydrolization process confirmed that some of the polymers within the samples are at least partially polysaccharide in nature.

Starch. For the extraction of starch 2 ml of 5 mM acetate buffer (pH=4.8) was added to the kimax tubes containing the pellets (residue from the 80% ethanol and water extractions). The tubes were placed on a heating block (100 °C) for one hour after which the tubes and heating block were cooled down to 60 °C and removed from the heating block when 60 °C was reached. At removal from the heating block, 2 ml of the enzyme amyloglucosidase (AMG) solution, 7 U/ml in 5 mM Acetate Buffer (pH=4.8), were added to the samples and the tubes were capped, vortexed and placed on a heating block for 14 h (overnight) at 60 °C. The heat of the heating block was increased from 60 °C to 100 °C, and the tubes were kept on the heating block at the higher temperature for 10 min to deactivate the enzymes. The tubes were removed and centrifuged for 5 min at 1800 g_n and 4 °C. The supernatants were decanted into new, clearly marked 15 ml Falcon tubes. Deionised water (3ml) were added to the kimax tubes containing the pellets and the mixture were vortexed and centrifuged for 5 min at 1800 g_n and 4 °C. The supernatants were decanted into the same 15 ml Falcon tubes. This process was repeated with another 3 ml of deionised water. All the supernatants were pooled (totalling approximately 10 ml) and mixed thoroughly. The extracts were filtered directly into HPLC vials using 0.45 µm filters. The starch was quantified from the amount of glucose liberated during the enzymatic hydrolysis, which was determined by using the same HPLC system and conditions as mentioned before, but using a Transgenomic® (Transgenomic Ltd., 40 Watt Road, Hillington Park, Glasgow, UK) ICS SEP ICE-ION-300 column at 20 °C. The column was eluted with a filtered 2 mM H_2SO_4 solution at a flow rate of 0.5 ml. min^{-1} throughout the analysis.

STATISTICAL ANALYSIS. Growth parameters were analyzed by repeated analyses of variance (RANOVA) using Statistica 9.0 (Stasoft, Inc., Tulsa, Oklahoma, USA). Furthermore, on the last date of measurements (30 Sept.) and for the carbohydrate analysis, a comparison of means was done by means of a one-way ANOVA, using Fishers' LSD posthoc separation test in Statistica 10.0 (Stasoft (SA) Inc., Tulsa, Oklahoma, USA). Flowering parameters were analyzed using M-L Chi square analyses in Statistica 10.0 (Stasoft (SA) Inc., Tulsa, Oklahoma, USA). The polynomial trendline on the constructed light curve was fitted using Microsoft Excel 2007 (Microsoft Corporation, One Microsoft Way, Redmond, USA).

Results

PHOTOPERIOD. No significant time and treatment interaction was observed for the growth parameters of number of unfolded leaves (Fig. 3), crown width (Fig. 4) and plant height (Fig. 5) respectively. However, a significant change over time was observed for all growth parameters (Fig. 3-5). No significant differences were seen between treatments for any of the growth parameters just prior to spike emergence on 30 Sept., after a six month period (Table 1). No significant differences were observed for the percentages of spike emergence (P = 0.968), anthesis (P = 0.415) or senescence

(P = 0.415) (Table 2). Furthermore similar timing of spike emergence, anthesis and senescence were also observed for all the treatments and control (Table 2).

ADDITIONAL LIGHTING. Personal observations of the plants exposed to the additional D-Papillon day light source reported an initial dark red colouration on all the leaves which led to the assumption that light damage occurred (Fig. 6; ML). However, new emerging leaves that developed later after a period of exposure to the higher light intensities were observed to be visually healthy and green, not exhibiting any of the presumed stress signs as detected in the older leaves (Fig. 6; YL). To evaluate the pigmented leaves for possible light stress, mature, red coloured leaves as well as new, healthy emerging leaves were prepared for inspection under a TEM microscope, to observe possible disintegration of the chloroplasts within the older, symptom-showing leaves. The TEM results was however inconclusive as no imaging could be obtained and therefore no observations or quantification of possible light damage could be made.

No significant difference in photosynthesis and fluorescence (Table 3) with P -values of 0.612 and 0.349 respectively, was however obtained between either the mature red, and younger green leaves of light-treated plants or leaves of the control plants as indication of light stress.

Mineral analysis of the leaves from control plants and that from both mature and newly formed leaves of light- treated plants were conducted to establish whether the observed symptoms could not be ascribed to nutrient deficiencies. Results (data not shown) indicated no significantly differences between the treated and control plants, with regard to either macro- or micronutrient levels.

PHOTOSYNTHESIS. The photosynthetic rate declined from autumn through to winter (Apr. – June 2011) when expressed both as A_{max} and A_{sat} (Fig. 7A and B). These rates remained relatively stable from July to Sept. 2011, but would be expected to increase again towards summer (data not recorded). The rates of respiration recorded for *Disa* hybrid 'LM0739' stayed relatively stable with no clear polynomial trend observed throughout the season (Fig. 7C).

CARBOHYDRATE ANALYSES. For *Disa* hybrids 'LM0739' and 'LM0721', both in the leaf and root components, fructose, glucose, galactose and sucrose were identified as the four soluble sugars present, although the respective concentrations differed between hybrids and plant organs (Fig. 8-9). Two additional, but unidentified sucrose-equivalent sugars were also detected in root and leaf samples of both hybrids, but as their contribution to the total non-structural carbohydrate pool were considered not significant, no further reference to them will be made. In Fig. 8 – 9 the total soluble sugars (the total of the fructose, glucose, galactose and sucrose fractions) together with the starch fraction for the above-and below-ground components for both hybrids are shown respectively.

Starch. The contribution of starch to the total non-structural carbohydrate pool for 'LM0739' leaves (Fig. 8A) were negligible (<0.09% of total dry weight) and did not differ significantly throughout the season (P = 0.336). The corresponding contribution of starch in the leaves of hybrid

'LM0721' (Fig. 8B) was even smaller than recorded for hybrid 'LM0739' at <0.05% of total dry weight. However in this case the fraction did differ significantly throughout the season (P = 0.01).

Starch in the roots of hybrid 'LM0739' was below 0.4% of the total dry weight from April to August, where after an increase in leaf starch above 0.7% was observed during early September with a maximum of 2% dry weight reached by mid-September (Fig. 9A). Significant differences in root starch of 'LM0739' over the season were consequently observed (P <0.001). Starch in the roots of hybrid 'LM0721' (Fig. 9B) made a considerably larger contribution to the total non-structural carbohydrate pool, by contributing up to 4.5% of the total fraction, although significant differences between the starch fractions over time were not observed.

Soluble sugars in ethanol extraction. Glucose constituted the largest fraction of the ethanol–soluble, extractable sugars within the leaves of hybrid 'LM0739' by contributing approximately 10-16% of the total dry weight, with sucrose being the second largest fraction by contributing 1.5-3% of the total dry weight respectively (Fig. 8A). However, only sucrose levels differed significantly throughout the season (P <0.001). For the leaves of hybrid 'LM0721' (Fig. 8B) the sucrose and glucose fractions were comparable, although glucose contributed slightly more to the total non-structural carbohydrate pool throughout the season at 2.5 – 4% of the total dry weight. The sucrose, but not the glucose fraction, differed significantly throughout the season (P <0.001).

In the leaves of hybrid 'LM0739' and 'LM0721' the contribution of both fructose and galactose was small, each contributing < 0.5% of the total dry weight. The percentages galactose differed significantly over the season with P = 0.016 and P = 0.043 for hybrids 'LM0739' and 'LM0721' respectively. Only in hybrid 'LM0721' did the low percentage contribution of fructose differ significantly over the season (P <0.001).

For the underground components of both hybrids 'LM0739' and 'LM0721' sucrose contributed in the greatest proportion of the total dry weight with 6-12% and 3.5-7.2%, respectively. Sucrose was followed by glucose and fructose contributing 3-5% and 1.5-2.3% each for hybrids 'LM0739' and 'LM0721', respectively. In the roots, for both hybrids, the galactose fractions made the smallest contribution to the total dry weight at <0.5% and <0.3% for hybrid 'LM0739' and 'LM0721', respectively. For hybrid 'LM0739' all three major sugar fractions differed significantly over the season. In hybrid 'LM0721' only the galactose fraction differed significantly over time (P <0.001).

Combined soluble-sugar and starch fraction. The total combined soluble sugar and starch fractions differed throughout the season for the leaves (P = 0.031) and roots (P = 0.051) of hybrid 'LM0739' and for the leaves (P = 0.055) of 'LM0721', whilst it did not differ (P = 0.605) for the roots of the latter cultivar.

Hydrolysable fraction. The hydrolization of the polymers within the extracted water fraction yielded four main sugars namely arabinose, glucose, galactose and mannose (Fig. 10 and 11). The retention time on the chromatograph (set for conditions which favoured the separation of glucose,

fructose and sucrose) of galactose and mannose were very close to one another, to such an extent that a merged peak was frequently recorded. As integration of these two individual sugars proved problematic and inaccurate these sugars are presented as a combined fraction, referred to as "Galactose-Mannose" ("GalMan").

The total hydrolyzed sugars contributed a relatively small percentage of the total dry weight for leaves (<2.4%) and roots (<2.0%) of hybrid 'LM0739' as well as for the leaves of 'LM0721' (<1.4%). However, for the roots of hybrid 'LM0721' the total hydrolyzed sugars of the underground components contributed 6-10% of the total dry weight. The total hydrolyzed sugars for the leaves of both hybrids differed significantly throughout the season ($P < 0.001$) (Fig. 10). For hybrid 'LM0739' (Fig. 10A) each of the three individual sugar fractions differed significantly over the season compared to hybrid 'LM0721' (Fig. 10B) where only the galactose-mannose fraction differed significantly over time ($P < 0.001$) in the leaves.

Within the underground components of both hybrids 'LM0739' and 'LM0721' neither the total hydrolyzed sugar percentages or any of the sugar fractions, when considered separately, differed significantly over the season (Fig. 11). The galactose-mannose fraction extracted from the roots of hybrid 'LM0739'contributed most significantly to the total hydrolyzed sugars at 0.6-1.1%, whilst the contribution from the arabinose and glucose fractions were relatively small (0.2 – 0.6%). The roots of hybrid 'LM0721' showed the most significant total hydrolyzed sugar fraction, between the respective hybrids and above or below-ground components analyzed. Within the total hydrolysable fraction of both 'LM0739' and 'LM0721', the principal contribution was made by the galactose-mannose fraction at 3-5% of the total dry weight, whilst glucose also contributed significantly at 2-3% of the total dry weight. The arabinose fraction made a lesser contribution (0.1 -0.5%), compared to the other two fractions.

Discussion

PHOTOPERIOD. The control plants that received natural short days (during autumn and winter) did not show any significant differences either in growth or flowering compared to the treated groups which received various periods of day-continuation. Day continuation did not inhibited flowering in the treated plants, as was reported in *Leucadendron* species (Hettasch and Jacobs, 2006). In contrast to many CFR species, daylength does not seem to influence flowering in commercially cultivated *Disa* hybrids. The possibility of using night breaks or day continuation regimes for flowering time manipulations are therefore currently eliminated. *Disa* is thus either indifferent to photoperiod, a phenomenon common in various orchids, including the commercially important *Phalaenopsis* (Lopez and Runkle, 2004). Alternatively, experimental plants may not have received sufficient cycles of day continuation to inhibit flowering, as at the time the study was conducted, the exact timing of floral initiation was still unknown. However, from results in Paper 1, it is now known that the hybrid 'LM0739' which was also used for the photoperiod study experienced floral initiation

at the end of May. The first group of night break-treated plants was removed from day continuation on 17 May 2011, after receiving 58 days of night breaks, whilst plants subjected to the second treatment group was removed from the night break regime on 13 June 2011, after receiving 74 days of day continuation. Both treatments presumably provided sufficient day continuation before the natural time of flower initiation at the end of May. Furthermore, the light intensity of the breaks as supplied wass comparable to that used to successfully inhibit flowering in Leucadendron plants (Hettasch and Jacobs, 2006).

In cases where orchids such as *Phalaenopsis* are found to be indifferent to day light duration, a drop in temperature is required to induce flowering. A drop in temperature, as found with the onset of autumn and early winter, coincides with the natural time of floral initiation in *Disa* and thus warrants further investigation.

ADDITIONAL LIGHTING. Considering another possible light effect on *Disa* hybrids, that of the exposure of hybrids to an additional light source to increase the total light integral, was studied. The use of higher light intensities could possibly provide the producer with larger plants with a higher propensity to flower, if supplied from a very early stage of plant growth. However, personal observations revealed possible light damage and stress symptoms to light-treated plants. Yet, none of the means by which the damage or stress was quantified showed any significant differences between the treated plants presenting these symptoms and the corresponding control plants.

It is known that plants can cope with excess light in numerous ways, including leaf movement and the production of ‘sunscreen’ pigments such as carotenoids and anthocyanins (Sherwin and Farrant, 1998). Anthocyanins, the red pigment in plants, can affect the profiles of light absorbed by a leaf and have been documented to accumulate in vacuoles of plants in response to light and temperature stress (Neill and Gould, 2003). The involved mechanism are suggested to be by masking chlorophyll and/or act as filters preventing excess light absorption by the leaf (Sherwin and Farrant, 1998). The red colouration on the leaves of the light treated *Disa* plants observed in this study can possibly be ascribed to the accumulation of anthocyanin, as a defence mechanism in response to higher light intensities. The use of anthocyanin as defence mechanism was also seen in the resurrection plants *Craterostigma wilmsii* and *Xerophyta viscose* when exposed to high light and dehydration. Both species showed physical (leaf curling for avoidance) and chemical changes in response to these stress conditions. Together with curling of leaves the leaf tissue turned purple/brown in colour and this morphological change coincided with a three-fold increase in the anthocyanin concentration, and a 30% decrease in the chlorophyll content (Sherwin and Farrant, 1998). None of the stress quantifying measures used in this study showed any significant differences between the red/brown coloured leaves of the treated plants and the normal leaves of the control plants, suggesting that no light damage occurred to the photosynthetic apparatus of the light-treated *Disa* plants. Therefore the observed symptoms could possibly be ascribed to a defence mechanism rather than to light damage or stress. However, the state of the chloroplasts as well as the anthocyanin

content of leaves would have to be recorded in order to serve as evidence in support of this hypothesis.

Adaptations of the new developing leaves to the higher light intensities demonstrated the resilience of *Disa* regarding changing environmental conditions (Fig. 6). *Disa* is said to be able to tolerate high light intensities if not in conjunction with too high temperatures. Furthermore, the fact that *Disa* naturally occurs within high light intensities, together with the construction of a light curve which showed that *Disa* are capable of optimal photosynthesis at relatively high light intensities confirmed that *Disa* can successfully accommodate elevated light intensities. For future studies it is suggested that the effect of additional lighting on plant growth and flowering, when increased gradually from the tissue culture stage, be evaluated.

Although *Disa* is considered to be well adapted to high light intensities, the defence response by accumulating anthocyanins was presumably necessary as the plants have been grown at relatively low light intensities throughout cultivation. Recent studies investigated the use of monochromatic LED diodes emitting red, blue, yellow or green light on *Oncidium* orchid protocorm-like bodies and subsequent growth of plantlets (Liu et al., 2011). Exposure of these plants to the red light spectrum enhanced proliferation and the carbohydrate content of protocorm-like bodies as well as subsequent plantlet lengths. A combination of red and blue LEDs resulted in higher energy efficiency as well as higher dry-weight and enzyme activity in plantlets (Liu et al., 2011). The effect of LEDs on tissue culture plantlets of *Disa* hybrids in cultivation, either before or after transplant could possibly lead to stronger healthier plants and warrants future research.

PHOTOSYNTHESIS. Photosynthesis values generally decreased during the autumn and winter months as would be expected under conditions of lower temperatures and light intensity. Reynoso et al. (2000) divided the seasonal diurnal pattern of eight Proteaceae species (*Banksia speciosa*, *Telopia speciosissima*, *Protea compacta*, *P. cynaroides*, *P. macrocephala*, *P. longifolia*, *P. neriifolia* and *P. repens*) into three groups. Species from the first group (*B. speciosa* and *P. macrocephala*) showed a decline in assimilation rate during summer and winter with an increase in spring and autumn, whereas species from the second group (*P. compacta* and *T. speciosissima*) showed a decline in the assimilation rate in winter and an increase in summer and autumn, whilst the last group (*P. cynaroides*) showed a small fluctuating assimilation rate throughout the year. The recorded seasonal photosynthetic data of *Disa* in this study may react similar to species belonging to the second group, but data was not collected further into summer and therefore does not allow for direct comparisons. Although only partial seasonal data was recorded it is speculated that the assimilation pattern of *Disa* would increase during spring into summer and decrease during autumn towards winter. This however requires verification through further experimentation.

The CO_2 assimilation values for *Disa* were considered relatively low, especially compared to other floricultural crops like cut roses. For two *Rosa* species the net photosynthetic rate at saturating irradiances were recorded to be 16.8 $\mu mol.m^{-1}.s.^{-2}$ for *Rosa rugosa* and 18.7 $\mu mol.m^{-1}.s.^{-2}$ for *R.*

bracteata respectively (Ueda et al., 2000). Yet, when comparing the photo-assimilation rate of *Disa* with that of other CFR species and orchids, it is not uncharacteristically low. Von Willert et al. (1989) reported maximum photosynthetic assimilation values of between 2.8 and 12.7 $\mu mol.m^{-1}.s.^{-1}$ for sixteen fynbos species, whilst Reynoso et al. (2000) reported a minimum of 1.4 $\mu mol.m^{-1}.s.^{-1}$ for *T. speciosissima* and a maximum of only 8.8 $\mu mol.m^{-1}.s.^{-1}$ in *B. speciosa*. During a diurnal cycle the maximum net CO_2 assimilation recorded for *Phalaenopsis amabilis* was 8 $\mu mol.m^{-1}.s.^{-1}$ (Guo et al., 2012). The light levels for optimal photosynthesis for *Disa* at 800 $\mu mol.m^{-1}.s.^{-1}$ as determined by construction of a light curve was much higher than the light levels of 200 $\mu mol.m^{-1}.s.^{-1}$ determined for maximum photosynthesis of *Phalaenopsis* (Guo et al., 2012). Again, when compared to studies on other CFR species, light saturation of photosynthesis for sixteen plant species was not achieved until 2000 $\mu mol.m^{-1}.s.^{-1}$ was reached. In a study by Van der Heyden and Lewis (1990) similar photon flux densities was used for measuring photosynthesis in *Erica plukenetii* at 800 and in *P. laurifolia* and *Thamnochortus lucens* at 1000 $\mu mol.m^{-1}.s.^{-1}$ respectively, again supporting similar results obtained for *Disa* in this study.

Phalaenopsis and various other orchids have been known to utilize either the CAM or C_4 photosynthetic pathways in addition to the C_3 photosynthesis (Guo et al., 2012; Hew and Yong, 2004). Contrasting this, in the sixteen fynbos species studied by Von Willert et al. (1989), only evidence for the C_3 photosynthetic pathway were found. It is presumed that *Disa* uses the C_3 photosynthetic pathway, although it was not established in this study. The photosynthetic capacity and photon flux at which *Disa* light saturates suggests that with regards to light utilisation *Disa* is closer related to the CFR fynbos species with which it share an ecological niche than to other epiphytic orchids such as *Phalaenopsis*.

CARBOHYDRATE ANALYSES. Root tubers are considered as taxonomically important morphological characteristic of orchids in general, although in some *Disa* hybrids like 'LM0739' in cultivation, a root tuber was curiously absent, with the reason therefore unknown. However, hybrid 'LM0721' did form a conspicuous root tuber throughout the season. This tuber was combined with the non-tuberous roots in the carbohydrate analysis of the underground-components.

Despite the importance of storage carbohydrate metabolism in ornamental geophytes and their economic significance, there is limited information concerning their identity and distribution among species (Ranwala and Miller, 2008). The main plant carbohydrates found within geophytes are soluble sugars (sucrose, glucose, fructose, hexoses and oligosaccharides), starch, glucomannan and fructans (Miller, 1992). Substantial literature concerning starch metabolism in plants all identify starch as a major plant storage carbohydrate, which is nearly ubiquitous in the plant kingdom (Miller, 1992; De Hertogh and Le Nard, 1993). Starch has been found to be the main reserve polysaccharide for some floricultural important South African geophytes like *Haemanthus pubescence* (Amaryllidaceae), *Sparaxis grandiflora* (Iridaceae) (Orthen, 2001) and *Nerine bowdenii* (Theron and Jacobs, 1996). Therefore, the very low percentage of starch, in all the samples, except for the

underground component samples of hybrid 'LM0721' were unexpected as it clearly disregard starch as main storage carbohydrate for *Disa* hybrids in cultivation.

Fructans are the most common alternative to starch as a reserve carbohydrate in plants (Ranwala and Miller, 2008). It has been estimated that fructans occurs as the principle carbohydrate in approximately 36 000 species, which is about 15% of angiosperm species world-wide (Hendry, 1987; Hendry, 1993). Geophytic plant species are frequently divided into three groups when considering the polysaccharide accumulated within the storage organs. These are geophytes storing starch, or fructans or a combination of both starch and fructans (Orthen, 2001). However since no fructose and/or sucrose fractions, the main constituents of fructans (Brocklebank and Hendry, 1989), was released upon hydrolization of the *Disa* polymer extracts, it is unlikely that fructans play a major role as a reserve metabolite within *Disa* hybrids. Consequently the absence of significant quantities of starch and fructans as reported within this study would not allow for *Disa* hybrids to be classified in any of the above-mentioned groups.

The extraction and identification of hydrolyzed sugars from the water fractions confirmed the polymers obtained from the *Disa* plant tissue to be fully, or at least partially of a polysaccharide nature. The percentages of these hydrolyzed sugars were especially high within the underground components of hybrid 'LM0721', the hybrid which exhibited a root tuber. This higher incidence of polysaccharides associated with the root tuber of hybrid 'LM0721' strongly suggests that this more readily available form of sugars may be an alternative to starch and/or fructans, although the specific advantageous that this holds for *Disa* is uncertain and speculative.

The presences of the glucomannan in geophytic organs are reported, amongst others within the families Orchidaceae, Liliaceae, Amarylidaceae, Iridaceae and Araceae (Wozniewski et al., 1990), where they may occur in seeds or vegetative storage organs such as roots, tubers and bulbs, serving as an alternative reserve polysaccharide (Miller, 1992). Glucomannan is composed of β-1,4 linked D-mannose and D-glucose monomers (Alonso-Sande et al., 2009). The ratio of glucose:mannose may vary substantially between origins. Konjac glucomannan as extracted from *Amarphophallus konjac* has a molar ratio of 1.6:1 whilst glucomannans extracted from orchid tubers and Scotch pine have ratios of 3.6:1 and 2.1:1, respectively (Alonso-Sande et al., 2009).

In *Aloe arborescence*, hydrolyses of the polysaccharide glucomannan gave fractions of mannose, glucose, arabinose and galactose (Wozniewski et al., 1990). As glucomannans are commonly reported within the Orchidaceae as well as other geophytic genera, together with results from this study which reported that the hydrolyses of the polymer fraction to release arabinose, glucose, galactose and mannose fractions, the same components that was obtained with the hydrolization of the glucomannan in *Aloe*, it is concluded that glucomannan as a polysaccharide is also present within *Disa* hybrids, especially so within tubers. However, the exact determination of the glucose:mannose ratio of the glucomannans presumably present within *Disa* did not fall within the scope of this study.

In vegetative storage organs it is thought that glucomannan is stored in a highly gel-like hydrated state in the vacuole of specific cells (Miller, 1992). Besides serving as a carbohydrate reserve, the role of glucomannans in geophytes is unclear, although, due to its colloidal nature, a role in water relations was suggested (Meier and Reid, 1982; Miller, 1992). In its hydrated state rapid hydrolysis of glucomannans could occur, resulting in adjustments of water potential within cells (Miller, 1992). It is also suggested that glucomannans enhances the water holding capacity of bulb scales (Miller, 1992). In both *Disa* hybrids the hydrolyzed sugar fraction within root tissue remained fairly constant throughout the season, further supporting the role as osmolyte of the glucomannan within the underground storage tissues of *Disa*.

A clear difference was observed between the carbohydrate compositions of the root components of the two studied hybrids. The presence of the root tuber in hybrid 'LM0721' most likely were responsible for these observed differences. Ultimately the presence of a root tuber might also be responsible for different carbon utilization strategies between hybrids. Higher percentages of the freely available sugars such as sucrose, fructose and glucose were present within the roots of hybrid 'LM0739' compared to the roots of hybrid 'LM0721'. However, root samples of hybrid 'LM0721' had higher starch as well as much higher concentrations of hydrolyzed sugars, providing evidence for the preferred use of polysaccharides (starch and possibly glucomannans) as reserve carbohydrates within the roots and tuber of hybrid 'LM0721'. In addition, when considering the leaves of both hybrids, 'LM0739' showed a much higher availability of soluble free sugars compared to that recorded in the leaves of 'LM0721'.

Hybrid 'LM0739' which exhibits low starch levels, low or no fructans as well as apparently a low polysaccharide content are therefore presumably required to depend on soluble sugars supplied by current photosynthesis. The higher leaf area, leaf dry weight and shoot to root ratio (Paper 1) recorded in hybrid 'LM0739' compared to that for hybrid 'LM0721', provides this hybrid with a greater above-ground capacity to provide sufficient photosynthesis and subsequently photosynthates (soluble-sugars) to maintain growth. A similar strategy was also observed within *Protea* where mainly sugars derived from current photosynthesis, were utilised for growth (Gerber et al., 2001). However, the possible presence of other polymers and polysaccharides not elucidated within this study might provide additional reserve storage in *Disa*.

Hybrid 'LM0721' clearly invests more resources to below-ground organs in producing a root tuber to maintain a stable pool of reserve polysaccharides than investing in above-ground plant parts, which supply freely available sugars for current growth and maintenance. The strategy of this tuber-forming hybrid makes it ecologically possible to evade periods of unfavourable conditions. When considering the Mediterranean habitat of *Disa*, such a growth strategy would require a sufficient supply of reserve materials to enable sprouting upon re-growth (Du Plessis and Duncan 1989; Orthen, 2001), a strategy which would not be feasible for hybrid 'LM0739', having limited reserve carbohydrates. The strategy of hybrid 'LM0721', possibly utilizing different polysaccharides in the

form of glucomannans, is similar than found in other geophytes like *Galanthus nivalis*, which utilizes polysaccharides for carbon and as a supply of energy for re-growth and flowering (Orthen and Wehrmeyer, 2004).

Since the hybridization of parent-plants used for hybrids within this study is unknown, differences in strategies could also be ascribed to genetic variances with a possibility that the inherent growth strategy of producing a root tuber, might have been lost in *Disa* hybrid 'LM0739' due to cross-hybridisation. Furthermore, certain cultivation practices and applications of growth regulators may affect the growth strategies of specific hybrids, although then it is not clear why not all hybrids in cultivation respond similarly towards these cultivation systems. For future studies it is strongly recommended to analyze he root tuber separately from the roots, in order to clearly distinguish whether certain carbon allocation patterns could be ascribed to inherent genetic differences or due to the presence of the root tuber. The evident differences in carbon utilization strategies between the tuber-forming and non-tuber forming hybrids form a basis for future studies in order to obtain a better understanding on the robustness of *Disa* storage strategies to support growth and flowering.

Conclusion

A short day photoperiodic response was not observed for *Disa* hybrids in cultivation, although this flowering signal is widely documented within the Orchidaceae as well as in various other CFR species. The determined time of floral initiation of *Disa* hybrids coincides with the onset of autumn and winter a time when a drop in temperatures, especially night temperatures, and shortening of days occur. However, since a short day signal is apparently not required for floral initiation in *Disa*, future studies on the effects of vernalization or diurnal cycles on flowering of *Disa* is recommended.

When the effects of a higher light intensity by providing an additional light source were studied for its efficacy to advance flowering, *Disa* hybrids displayed plasticity to high light stress by accumulating anthocyanins as possible screening protection against photo damage. These pigmented leaves that developed under the higher light conditions did not register measurable stress levels and testifies towards the resilience of *Disa* towards changing environmental conditions. Studies on additional light sources (possibly LEDs) for *Disa* plants grown from tissue culture is recommended to possibly enable more plants to reach the threshold size for flowering. *Disa* hybrids harbours low CO_2 assimilation rates, likely due to their adaptation to sub-optimal light conditions in cultivation, and its inherent CFR geophyte adaptation to nutrient poor soils. The photosynthetic capacity of *Disa* was found to be more closely related to the CFR species than to epiphytic orchids.

Sucrose and glucose made the largest contribution to the non-structural carbohydrate compounds present in above-and underground plant organs of *Disa* hybrids. The tuber-forming hybrid, 'LM0721', showed much higher hydrolyzed sugar and starch content within the underground components than the non-tuber forming hybrid 'LM0739'. These findings indicate different strategies

of carbon utilization between tuber and non-tuber forming hybrids. Furthermore, this forms the first report on the possible presence of glucomannans within *Disa*.

Results from this study provide some insight into the response of *Disa* in cultivation to light, either to photoperiod or intensity, as well as produced the first reports on the photosynthetic capacity and carbon allocation patterns within *Disa*, aspects which are of key-importance in understanding the floral biology of *Disa* as a new floricultural crop.

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Table 1. The vegetative growth parameters of *Disa* hybrid 'LM0739' as number of unfolded leaves, crown width (mm) and plant height (mm) after exposure to night break treatments over a period of 58, 74 and 167 days respectively (n=15). In control plants night break treatments were omitted. Data was recorded on 30 Sept. 2011.

Photoperiod	**Days of night break regime**	**Unfolded leaves**	**Crown width (mm)**	**Height (mm)**
(n=15)	Control	6.89 NS[z]	153.90 NS	48.81 NS
	58	5.50	144.97	42.50
	74	6.40	162.81	46.62
	167	6.92	181.90	53.36
ANOVA	F	2.153	2.627	0.859
	P	0.110	0.065	0.471

[z]NS. Indicates no significant difference at the 5% confidence level.

Table 2. The distribution of flowering patterns of *Disa* hybrid 'LM0739' plants exposed to various night break regimes that consisted of the treatments control (no night break regime), 58, 74 and 167 days of night break regime respectively. Flowering was monitored using spike emergence, anthesis and senescence as parameters. Measurements were taken from 21 Oct. 2011 (first signs of spike emergence) until 19 Jan. 2012. Data are presented as percentages (%) of the monitored population.

				Monitoring dates (2011 - 2012)						
Photoperiod (night break regime)	**n**	**Total (%)**	**Date ± SE**	**21-Oct. - 27 Oct.**	**03-Nov. - 08 Nov.**	**18-Nov. – 23 Nov.**	**02-Dec. – 09 Dec.**	**12-Dec. – 20 Dec.**	**30 Dec. - 04-Jan.**	**09 Jan. – 19-Jan.**
Experimental plants	**15**									
Control (no treatment)										
Died prior to spike emergence	6	40	N/A[z]							
Non flowering	2	13	N/A							
Spike emergence	7	47	22 Nov. ± 4.6		14.2	42.9	42.9			
Anthesis	5	33	02 Jan. ± 2.0						80	20
Senescence	5	33	15 Jan. ± 2.4							100
58 days of night break										
Died prior to spike emergence	5	33	N/A							
Non flowering	4	27	N/A							
Spike emergence	6	40	22 Nov. ± 4.6		16.7	50	33.3			
Anthesis	3[y]	20	30 Dec. ± .0.0						100	
Senescence	2[y]	13	14 Jan. ± 7.1							100
74 days of night break										
Died prior to spike emergence	6	40	N/A							
Non flowering	4	27	N/A							
Spike emergence	5	33	26 Nov. ± 6.7		20	20	40	20		
Anthesis	2	13	04 Jan. ± .7.1						50	50
Senescence	2	13	14 Jan. ± 7.1							100
167 days of night break										
Died prior to spike emergence	6	40	N/A							
Non flowering	3	20	N/A							
Spike emergence	6	40	22 Nov. ± 6.0		33.3	16.7	50			
Anthesis	1	7	[x]						100	
Senescence	1	7	[x]							

M-L Chi square

	M-L Chi square	***P*-value**	**Significance**
Spike emergence	0.558	0.968	NS[z]
Anthesis	3.934	0.415	NS
Senescence	3.934	0.415	NS

[z] N/A indicates not applicable.

[y] Differences seen in number of anthesis and senescence can be ascribed due to a loss of plant material before senescence could be documented.

[x] Insufficient number of replicates to calculate an average or Standard Error of the Mean.

[w] NS. Indicates no significant difference at the 5% confidence level.

Table 3. The photosynthetic capacity (CO_2 assimilation) of *Disa* hybrid 'LM56' plants and fluorescence of *Disa* hybrid 'LM67' plants as recorded in Nov. 2012 after four months' exposure to an additional D-Papillon day light, daily from 08:00AM to 06:00PM at light levels of up to 359 µmol.m^{-2}.s^{-1}, compared to control plants that was grown under normal growing conditions and light intensity of up to 279 µmol.m^{-2}.s^{-1}. A "young leaf" refers to a newly formed green leaf that developed during the approximately three months of exposure to additional lighting, a period throughout which adaptation has presumably taken place. A "mature leaf" refers to a pigmented leaf that developed prior to the additional lighting and was therefore presumably light stressed when exposed to the additional lighting.

Treatment		**CO_2 assimilation (µmol·m^{-1}·s^{-2})**	**Fluorescence (Fv/Fm)** [z]
Control		1.71 ± 0.13	0.77 ± 0.02
Light treated: young leaf		2.04 ± 0.95	0.78 ± 0.01
Light treated: mature leaf		1.37 ± 0.12	0.75 ± 0.03
ANOVA	F	0.532	1.261
	P	0.612	0.349
	Significance	NS[X]	NS

[z] 'Fv/Fm' value < 0.5 indicates a stressed plant whilst 0.75-0.85 indicates no stress.
[x]NS. Indicates no significant difference at the 5% confidence level.

Fig. 1. The construction of a black curtain is shown as was used during a night break regime on *Disa* hybrid 'LM0739' to exclude light from the control group and the commercial population.

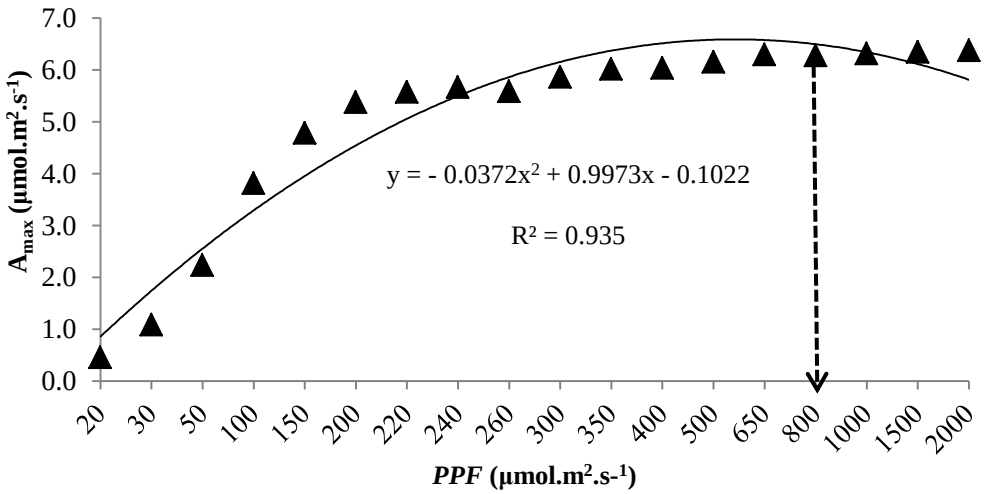
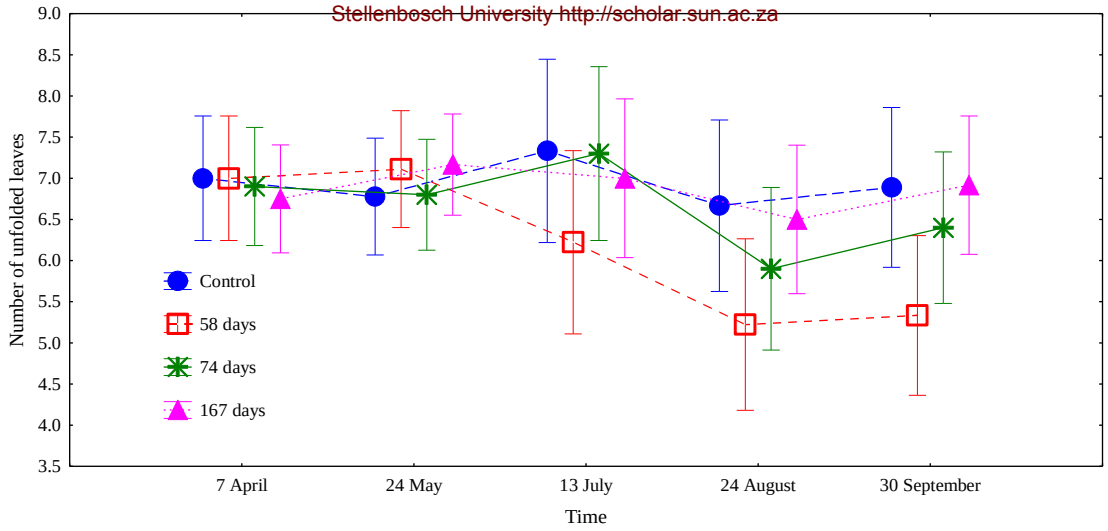
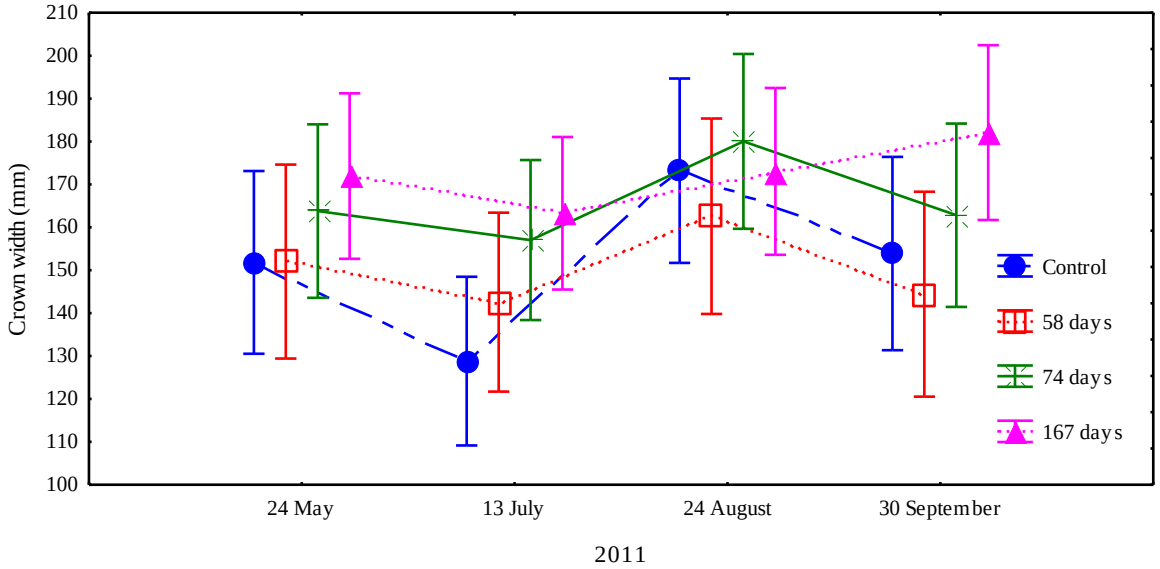


Fig. 2. A light saturation curve (CO_2 assimilation vs. photosynthetic photon flux (*PPF*)) as constructed for *Disa* hybrid 'LM0739' in order to determine the maximum rate of light-saturated net CO_2 assimilation. The curve was constructed in Mar. 2011, within a laboratory environment at room temperature (22 ˚C).



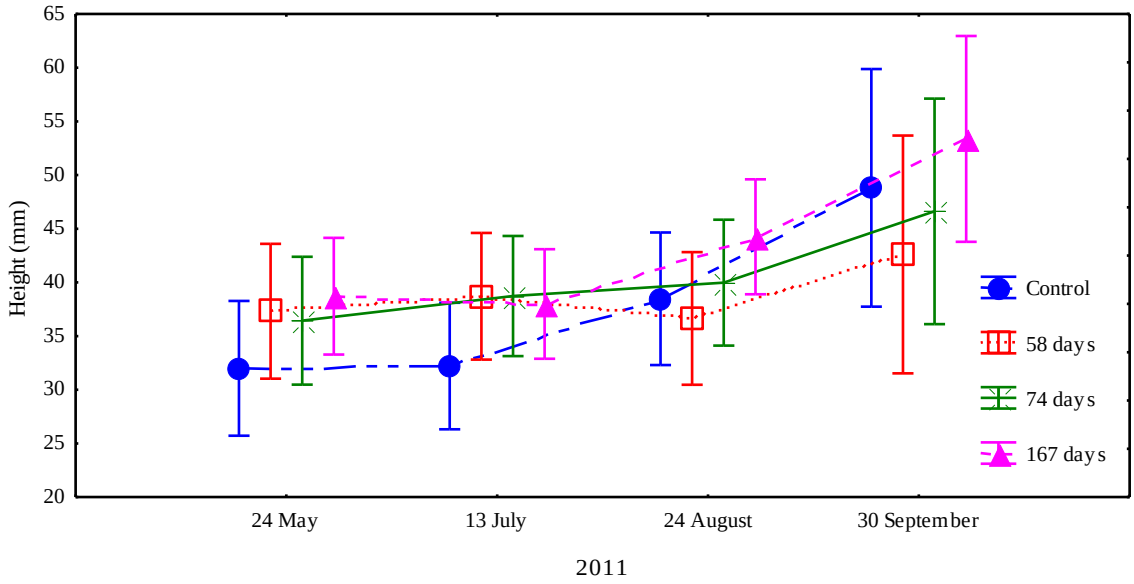
RANOVA	F-value	*P* -value	Significance
Treatment	1.500	0.231	NS[z]
Time	4.498	0.002	**[y]
Treatment*Time	1.158	0.319	NS

Fig. 3. Growth response curves of *Disa* hybrid 'LM0739' over a six month period using number of unfolded leaves as growth parameter (n=15) after plants were exposed to various night break treatments namely: Control (no night break regime), 58, 74 and 167 days of night break regime, respectively. Vertical error bars indicate the Standard Error (SE) of the Mean for each data point. [z]NS. Indicates no significant difference at the 5% confidence level. [y]* Indicates the level of significance.



RANOVA	F-value	*P* - value	Significance
Treatment	1.486	0.236	NS[z]
Time	11.870	<0.001	***[y]
Treatment*Time	1.829	0.072	NS

Fig. 4. Growth response curves of *Disa* hybrid 'LM0739' over a six month period using crown width (mm) as growth parameter (n=15) after plants were exposed to various night break treatments namely: Control (no night break regime), 58, 74 and 167 days of night break regime, respectively. Vertical error bars indicate the Standard Error (SE) of the Mean for each data point. [z]NS. Indicates no significant difference at the 5% confidence level. [y]* Indicates the level of significance.



RANOVA	F-value	*P* - value	Significance
Treatment	0.8587	0.471	NS[z]
Time	16.8844	0.000	***[y]
Treatment* Time	1.1315	0.347	NS

Fig. 5. Growth response curves of *Disa* hybrid 'LM0739' over a six month period using plant height (mm) as growth parameter (n=15) after plants were exposed to various night break treatments namely: Control (no night break regime), 58, 74and 167 days of night break regime, respectively. Vertical error bars indicate the Standard Error (SE) of the Mean for each data point. [z]NS. Indicates no significant difference at the 5% confidence level. [y]* Indicates the level of significance.

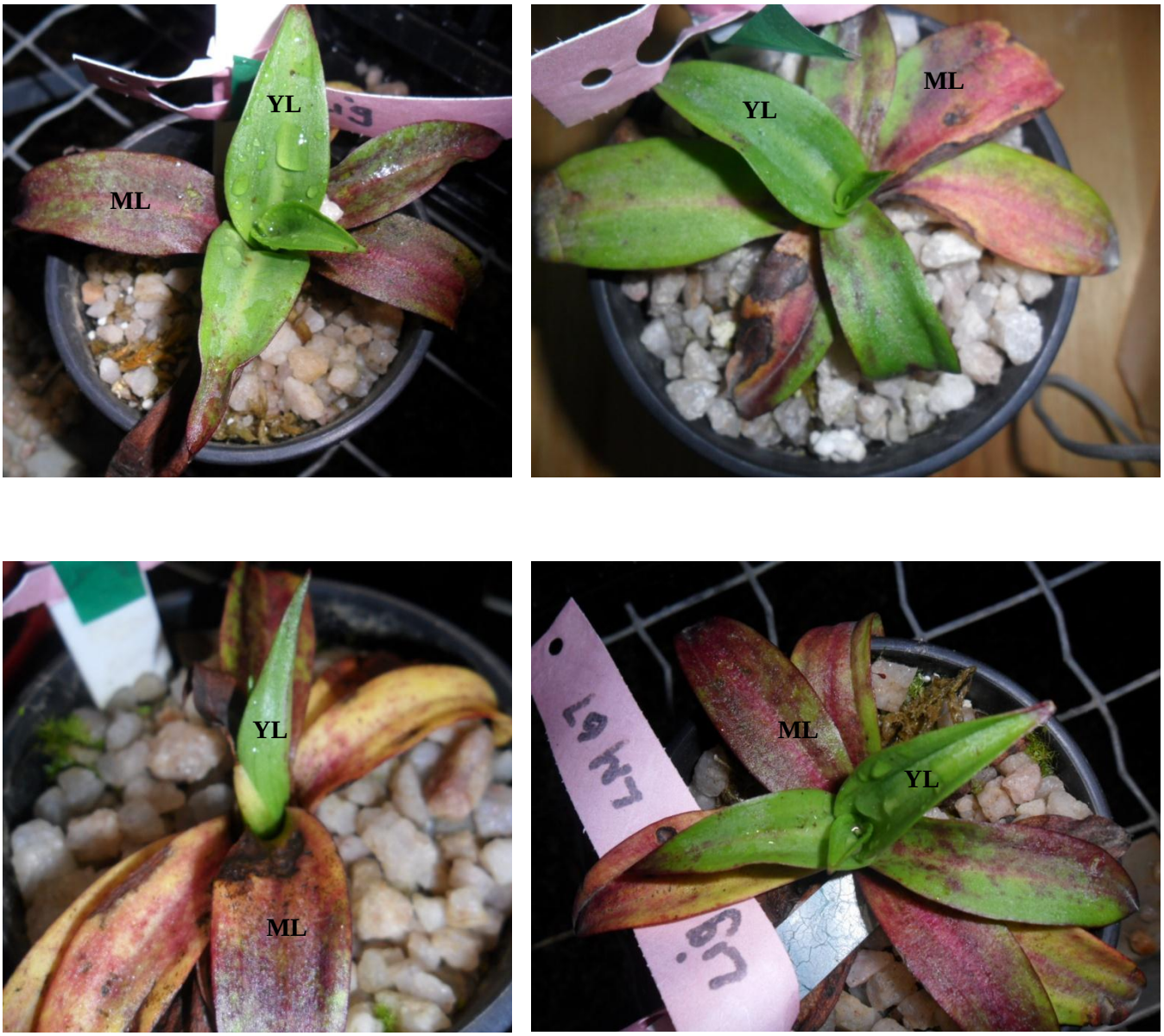
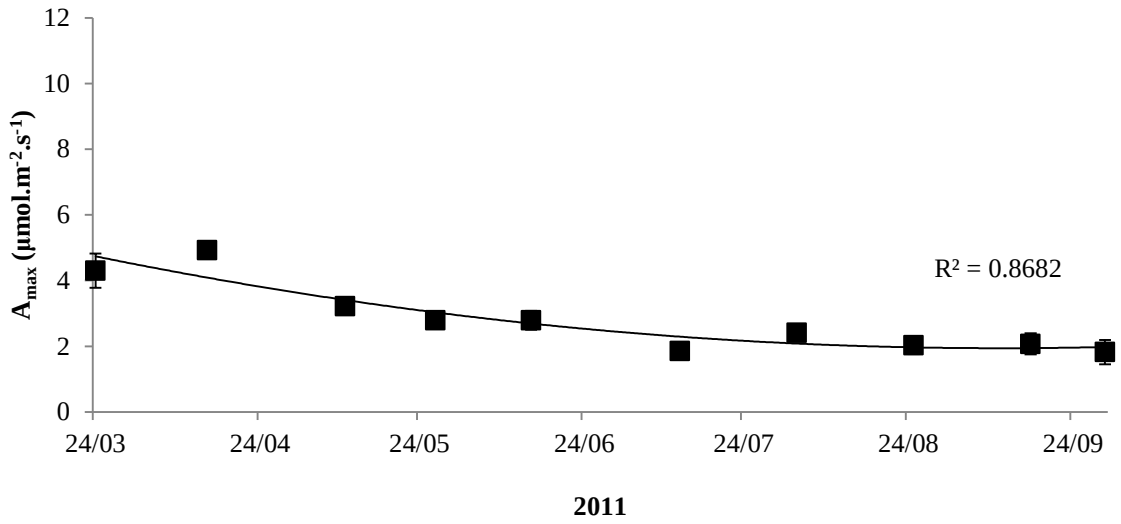
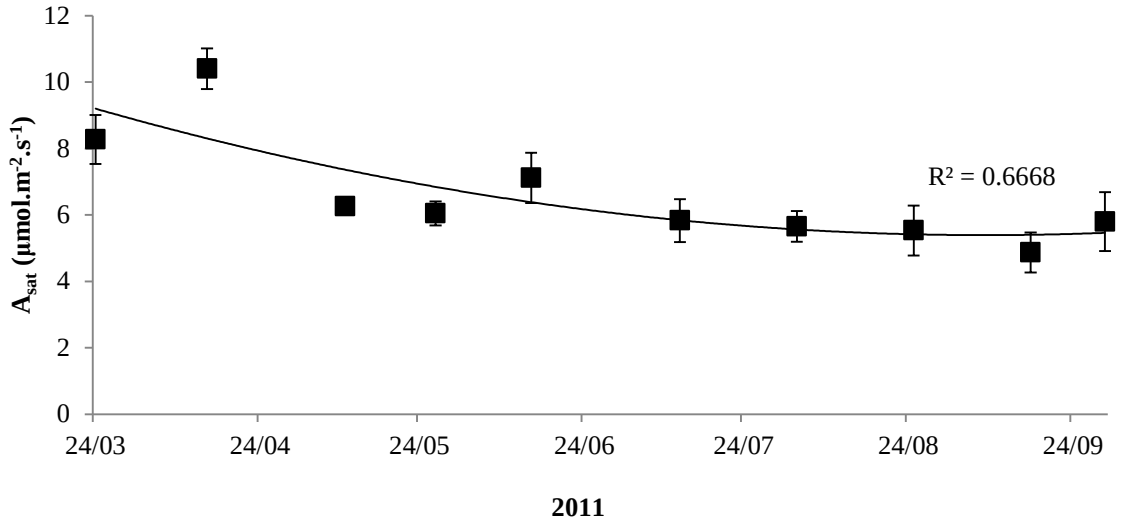


Fig. 6. Plants of *Disa* hybrid 'LM67' showing the development of a red to purple pigmentation on mature leaves (ML) under the exposure of additional light as supplied by a D-Papillon 315W, 230V Daylight fixture, with an Agro 315W 930 grow lamp (Lights interaction, Achtseweg, Eindhoven, Netherlands) mounted approximately 60 cm above plant level. Plants were exposed to this additional light daily from 06:00AM to 06:00PM from June until Sept. 2011. Light levels recorded beneath the additional light treatment on a clear day at 12:00AM were between 113 and 359 $\mu mol.m^{-2}.s^{-1}$. Healthy, younger leaves (YL) which developed later the additional light regime, presumably after adaptation to the higher light environment has taken place, are also shown (YL).

A.



B.



C.

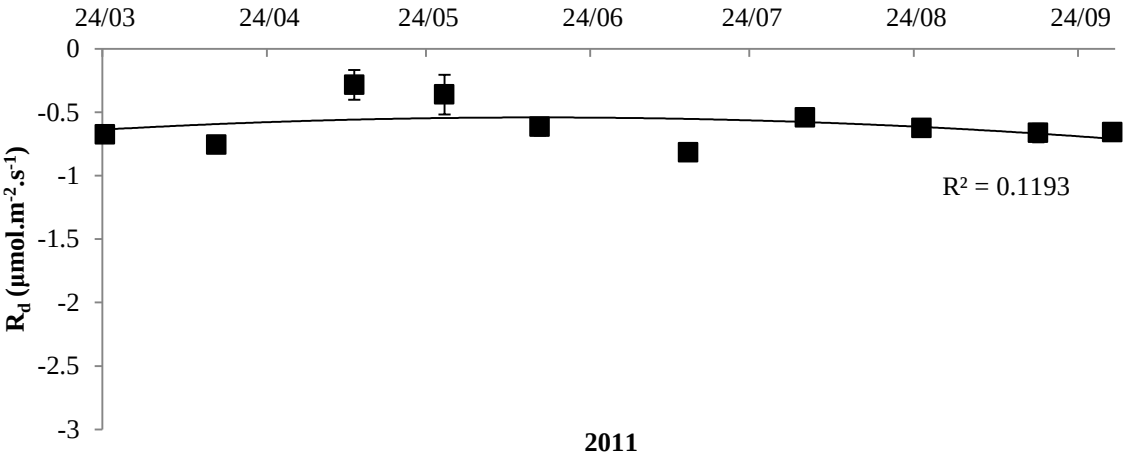
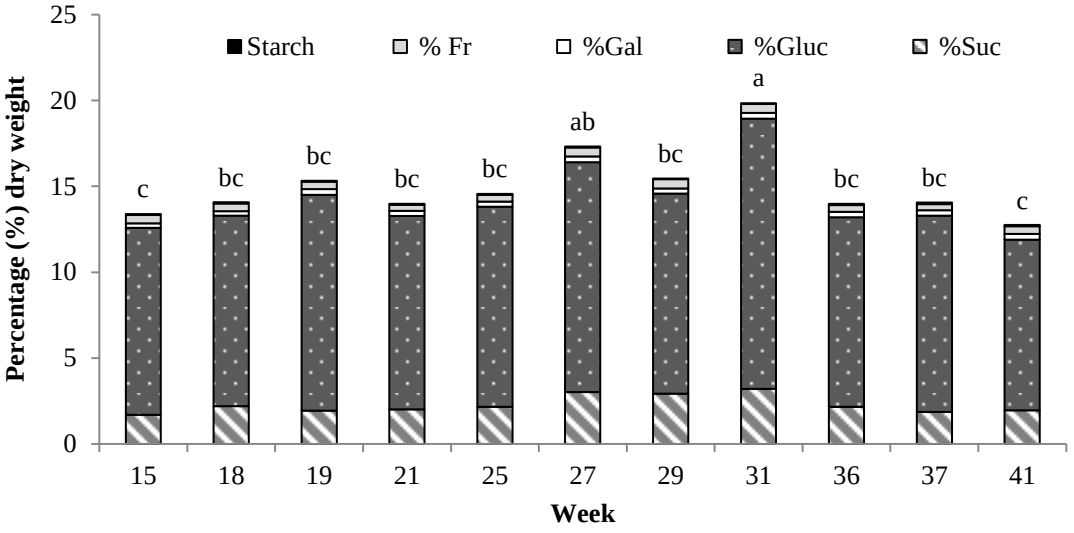


Fig. 7. Spot measurements of gas exchange taken from Mar. 2011 to Sept. 2011 on mature leaves of *Disa* hybrid 'LM0739'. A. Maximum rate of light-saturated net CO_2 assimilation (A_{max}). B. Maximum rate of light-and CO_2 saturated and net CO_2 assimilation (A_{sat}). C. Dark respiration rate (R_d). Data are represented as mean values ± SE.

A.

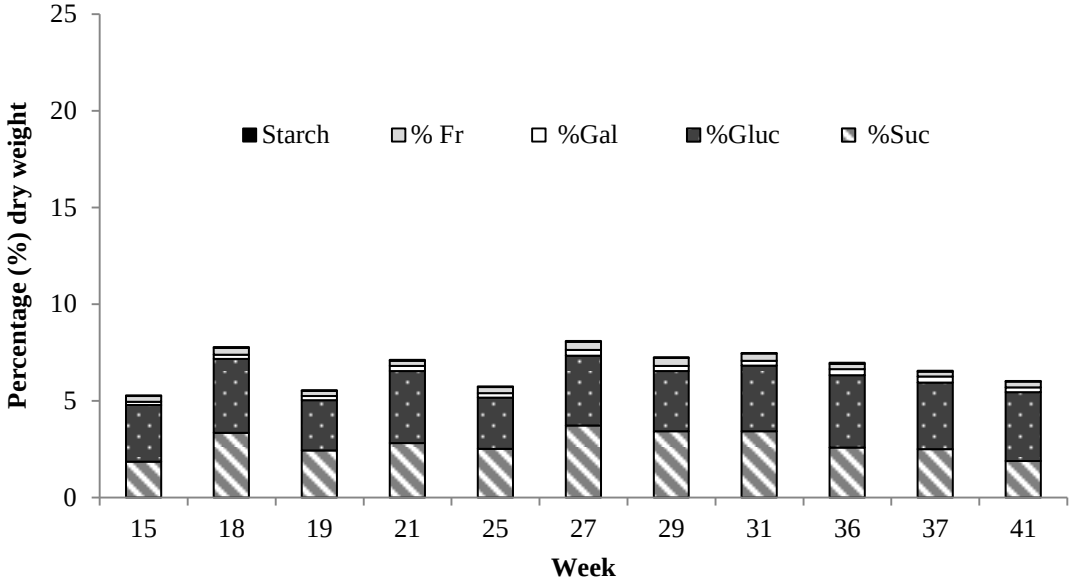
Week	Date
15	12-Apr
18	04-May
19	09-May
21	25-May
25	23-Jun
27	07-Jul
29	22-Jul
31	02-Aug
36	09-Sep
37	14-Sep
41	14-Oct



'LM0739' Leaves	F-value	*P*-value	Significance
Total sugars and starch	2.257	0.031	*[z]
Starch	1.170	0.336	NS[y]
Sucrose	3.838	<0.001	***
Glucose	1.956	0.063	NS
Galactose	2.539	0.016	*
Fructose	1.965	0.061	NS

B.

Week	Date
15	12-Apr
18	04-May
19	09-May
21	25-May
25	23-Jun
27	07-Jul
29	22-Jul
31	02-Aug
36	09-Sep
37	14-Sep
41	14-Oct

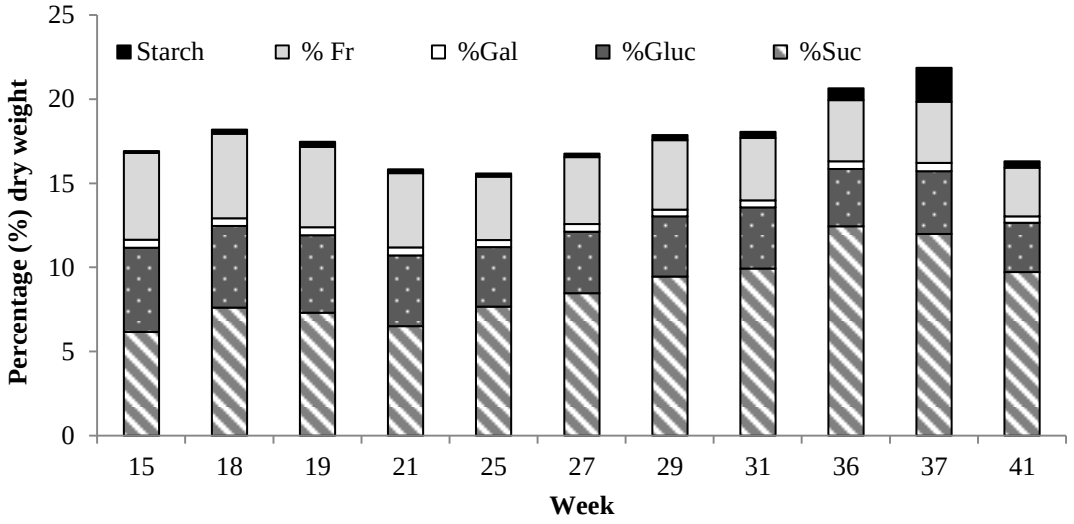


'LM0721' Leaves	F-value	*P*-value	Significance
Total sugars and starch	2.024	0.055	NS
Starch	2.772	0.01	*
Sucrose	14.659	<0.001	***
Glucose	0.606	0.800	NS
Galactose	2.134	0.043	*
Fructose	5.804	<0.001	***

Fig. 8. The non-structural carbohydrate fractions of leaves of A. *Disa* hybrid 'LM0739' and B. *Disa* hybrid 'LM0721' as recorded throughout the season after transplant from tissue culture on 12 Apr. 2011 (Week 15), to just prior to anthesis, around 14 Oct. 2011 (week 41). Different letters denote significant differences between means of weeks throughout the season. [z]* Indicates the level of significance. [y]NS. Indicates no significant differences at the 5% confidence level.

A.

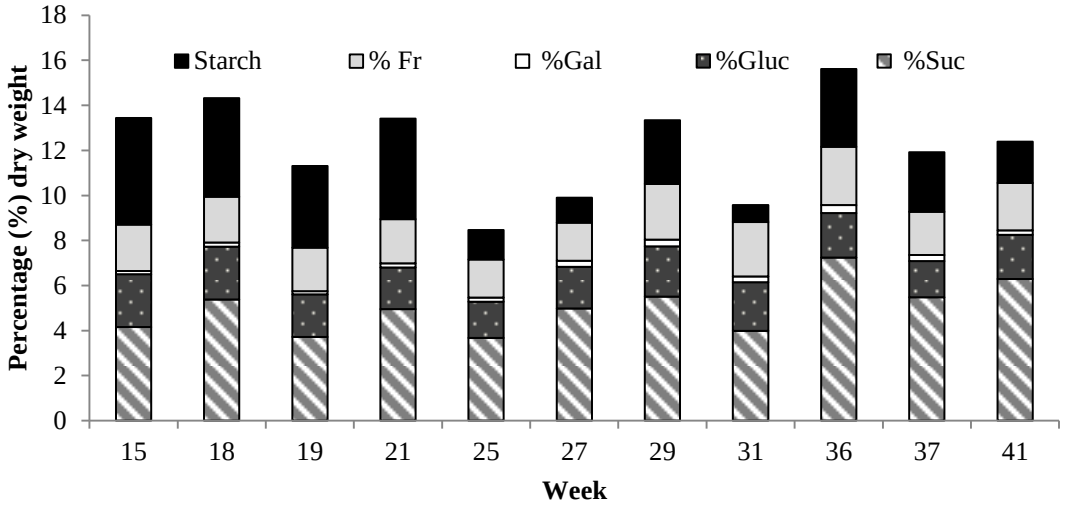
Week	Date
15	12-Apr
18	04-May
19	09-May
21	25-May
25	23-Jun
27	07-Jul
29	22-Jul
31	02-Aug
36	09-Sep
37	14-Sep
41	14-Oct



'LM0739' Roots	F-value	*P*-value	Significance
Total sugars and starch	2.046	0.051	NS[z]
Starch	6.182	<0.001	*** [y]
Sucrose	3.481	0.002	**
Glucose	4.378	<0.001	***
Galactose	0.652	0.762	NS
Fructose	4.530	<0.001	***

B.

Week	Date
15	12-Apr
18	04-May
19	09-May
21	25-May
25	23-Jun
27	07-Jul
29	22-Jul
31	02-Aug
36	09-Sep
37	14-Sep
41	14-Oct

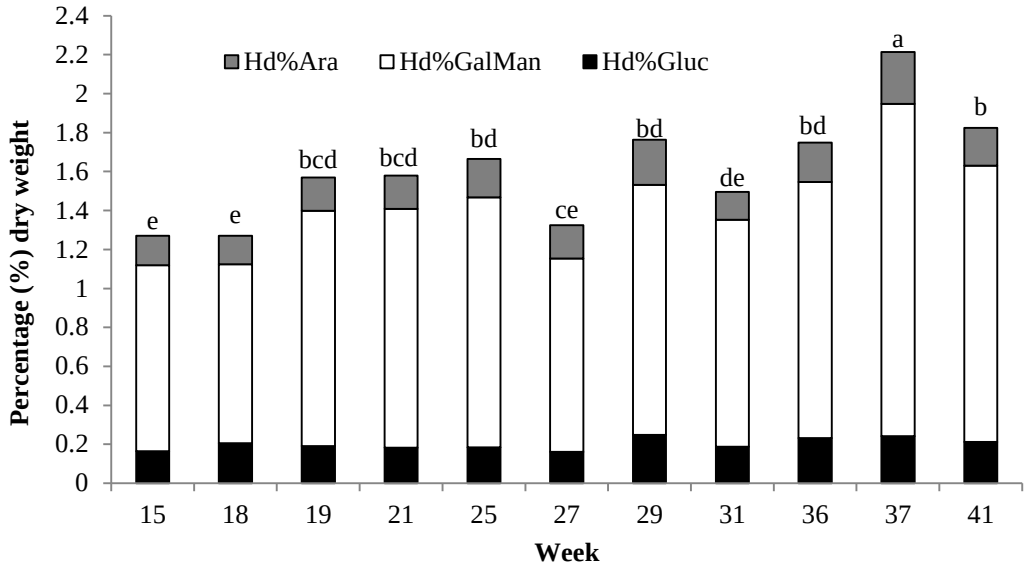


'LM0721' Roots	F-value	*P*-value	Significance
Total sugars and starch	0.825	0.605	NS
Starch	0.711	0.712	NS
Sucrose	1.470	0.163	NS
Glucose	0.535	0.861	NS
Galactose	5.114	<0.001	***
Fructose	0.215	0.994	NS

Fig. 9. The non-structural carbohydrate fractions of roots of A. *Disa* hybrid 'LM0739' and B. *Disa* hybrid 'LM0721' as recorded throughout the season after transplant from tissue culture on 12 Apr. 2011 (Week to just prior to anthesis , around 14 Oct. 2011 (week 41). Different letters denote significant differences between means of weeks throughout the season. [z]NS. Indicates no significant difference at the 5% confidence level. [y]* Indicates the level of significance.

A.

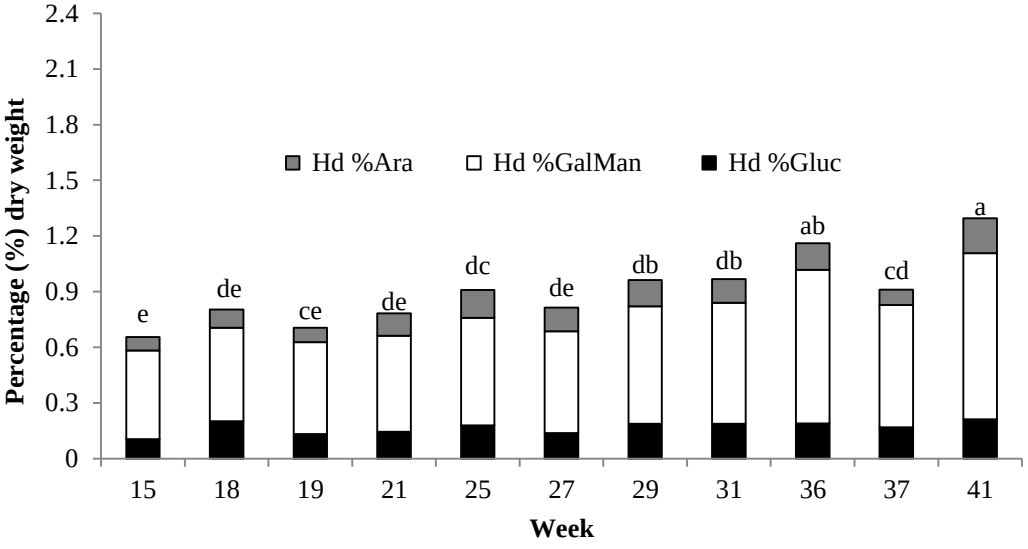
Week	Date
15	12-Apr
18	04-May
19	09-May
21	25-May
25	23-Jun
27	07-Jul
29	22-Jul
31	02-Aug
36	09-Sep
37	14-Sep
41	14-Oct



'LM0739' Leaves	F-value	*P*-value	Significance
Total hydrolyzed sugars	7.525	<0.001	***[z]
Glucose	3.706	0.001	**
Galactose and Mannose (GalMan)	8.092	<0.001	***
Arabinose	3.419	0.002	**

B.

Week	Date
15	12-Apr
18	04-May
19	09-May
21	25-May
25	23-Jun
27	07-Jul
29	22-Jul
31	02-Aug
36	09-Sep
37	14-Sep
41	14-Oct

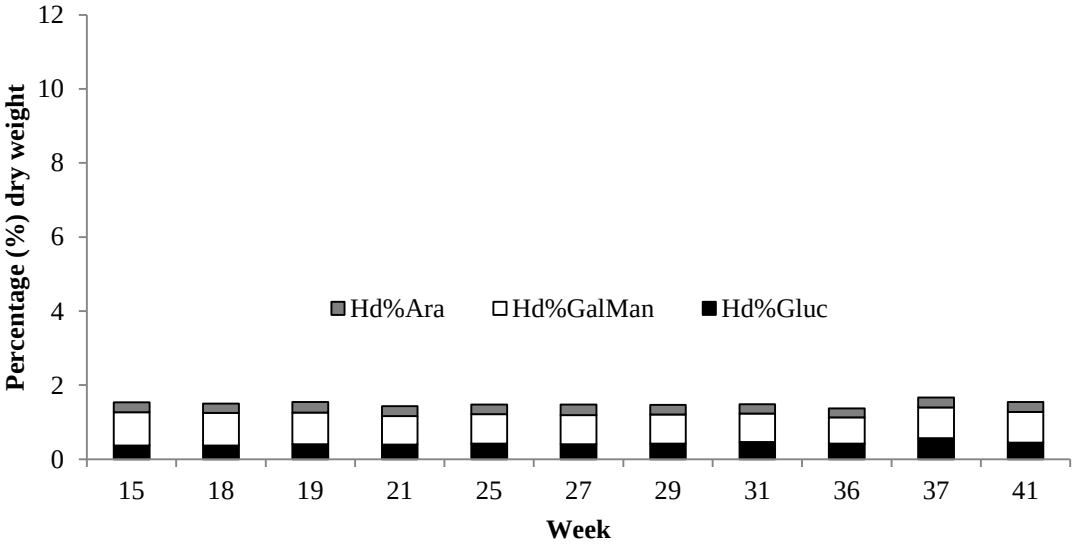


'LM0721' Leaves	F-value	*P*-value	Significance
Total hydrolyzed sugars	6.009	<0.001	***
Glucose	1.410	0.211	NS[y]
Galactose and Mannose (GalMan)	7.876	<0.001	***
Arabinose	2.968	0.007	NS

Fig. 10. The hydrolyzed sugar fractions of leaves of A. *Disa* hybrid 'LM0739' and B. *Disa* hybrid 'LM0721' as recorded throughout the season after transplant from tissue culture on 12 Apr. 2011 (Week 15), to just prior to anthesis, around mid Oct. 2011 (week 41). Different letters denote significant differences between means of weeks throughout the season. [z]* Indicates the level of significance. [y]NS. Indicates no significant difference at the 5% confidence level.

A.

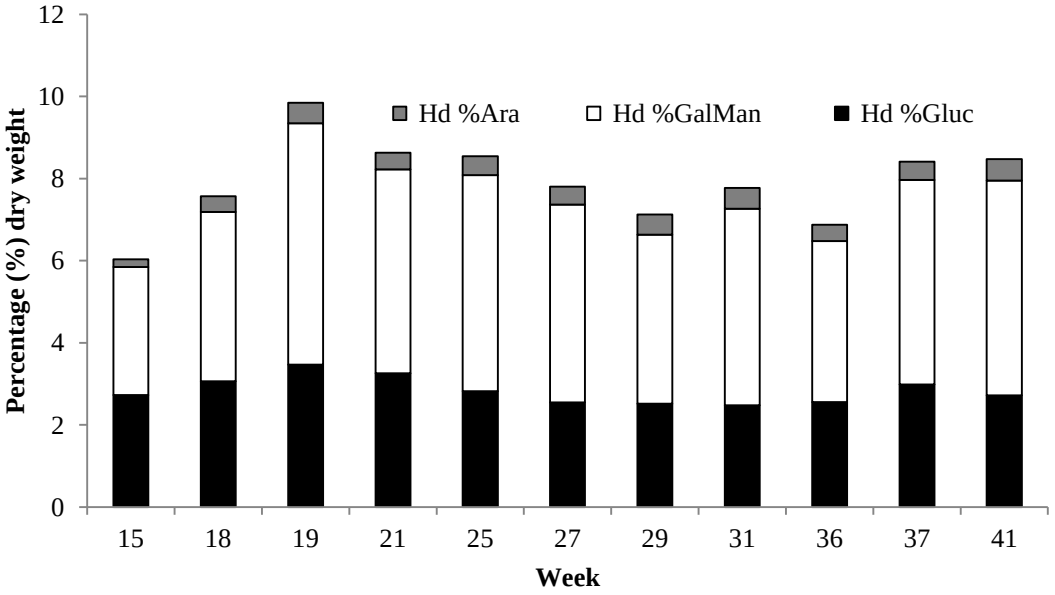
Week	Date
15	12-Apr
18	04-May
19	09-May
21	25-May
25	23-Jun
27	07-Jul
29	22-Jul
31	02-Aug
36	09-Sep
37	14-Sep
41	14-Oct



'LM0739' Roots	F-value	*P* -value	Significance
Total hydrolyzed sugars	0.608	0.799	NS
Glucose	1.689	0.114	NS
Galactose and Mannose (GalMan)	1.371	0.225	NS
Arabinose	0.572	0.828	NS

B.

Week	Date
15	12-Apr
18	04-May
19	09-May
21	25-May
25	23-Jun
27	07-Jul
29	22-Jul
31	02-Aug
36	09-Sep
37	14-Sep
41	14-Oct



'LM0721' Roots	F-value	*P* -value	Significance
Total hydrolyzed sugars	0.156	0.999	NS
Glucose	0.216	0.994	NS
Galactose and Mannose (GalMan)	0.255	0.989	NS
Arabinose	0.890	0.545	NS

Fig. 11. The hydrolyzed sugar fractions of roots of A. *Disa* hybrid 'LM0739' and B. *Disa* hybrid 'LM0721' as recorded throughout the season after transplant from tissue culture on 12 Apr. 2011 (Week 15), to just prior to anthesis, around mid Oct. 2011 (week 41). [y]NS. Indicates no significant difference at the 5% confidence level.

Paper 3: Elucidating the Flowering Signal in *Disa* Hybrids: Endogenous Control through Plant Size as a Possible Mechanism

ABSTRACT. The indigenous South African orchid *Disa* is cultivated on a limited scale. Research is required to elucidate the possible flowering signal to enable flower manipulation strategies and successful marketing. The effect of plant size and factors possibly affecting plant size, such as the cultural practice of re-potting and separating plants, were investigated in this study. *Disa* hybrid plants (n = 30) were selected into three distinct size categories, "Small" (18.1 ± 1.5 mm), "Medium" (26.7 ± 1.1 mm) and "Large" (50.23 ± 2.1 mm). The vegetative growth of the size groups were monitored over a six month period using plant height (mm), crown width (mm) and number of unfolded leaves as growth parameters. Flowering was documented as date and percentage of spike emergence, anthesis and senescence, respectively. In addition, the association between plant size and floral initiation was investigated by means of stereo microscopy. Number of unfolded leaves and crown width emerged as possible parameters that could be used in predicting the propensity of plants to flower. Furthermore, a floral gradient was observed where the critical size required for flowering became more facultative and less obligatory as the season progressed. The interaction between plant size and external factors such as temperature and light intensity should be further researched before plant size can be used as a reliable indicator in flowering predictions. In a next experiment, the current cultivation practice where clumped plants within a pot are separated to single plants per pot was also investigated to determine the effect of this practice on plant growth and flowering. The separation of plants showed no advantages for vegetative growth or flowering, therefore the commercial viability of this practice should be re-evaluated.

ADDITIONAL INDEX WORDS: *Disa uniflora*, leaf area, number of leaves, Orchidaceae, orchids

In *Phalaenopsis*, and other commercial orchids, the ability to manipulate flowering time in response to environmental conditions has been the key to successful and sustainable marketing. The indigenous, terrestrial orchid genus *Disa* consists of more than a 100 species, predominantly found in southern Africa (Crous and Duncan, 2006). The "Evergreen disas", a group within the genus *Disa*, is renowned for its large and showy flowers and is especially popular to breeders. All of the known *Disa* hybrids are derived from species belonging to this group; not only due to the striking inflorescences, but also their relative ease in cultivation (Holmes, 2011). *D. uniflora*, *D. cardinalis*, *D. racemosa* and *D. tripetaloides* are the evergreen species most prominently used in hybridisation (Holmes, 2011). *D. uniflora*, also commonly referred to as the 'Pride of Table Mountain' or 'Red *Disa*' is the most renowned species of this group and has the largest flower of all indigenous South

African orchids. However, basic understanding of the requirements for cultivation, especially with regard to the nature of the flowering signal of *Disa*, is still lacking. The absence of scientifically based research is limiting the commercial success of *Disa* hybrids in cultivation.

A wide range of factors are known to influence flowering in floricultural crops. For plants adapted to the Cape Floristic region triggers for flowering includes photoperiod (Halevy, 1985; Hettasch and Jacobs, 2006; Shillo and Halevy, 1976a); temperature (Gilbertson-Ferriss, 1985; Shillo and Halevy, 1976b), light (Halevy, 1985; Shillo and Halevy, 1976c; Wang, 1995) and fire or smoke (Brown and van Staden, 1997; Keely, 1993). The active component in smoke has been identified as butenolides (Light et al., 2009).

In orchids an even more extensive range of factors influence flowering. This is not surprising when considering the size of the Orchidaceae family, the various growth habits and the variation in natural habitats. Factors that influence flowering of orchids include juvenility, photoperiod, light, low temperatures (vernalization) and diurnal fluctuations (Goh and Arditti, 1985; Hew and Yong, 2004; Lopez and Runkle, 2004; Lopez and Runkle, 2005), alternating wet and dry seasons (Sanford, 1971), but also plant growth regulators (Blanchard and Runkle, 2008; Goh and Arditti, 1985; Matsumoto, 2006; Miguel et al., 2008) and chemicals such as potassium chlorate (Li et al., 2006). The influence of these factors can be seen alone or in combination, and the reaction differs widely between and within genera within the Orchidaceae. The factors affecting flowering in terrestrial, polycarpic orchids are mainly the climate, plant size/age, the flowering history (whether the plant had flowered in the previous season(s) (Pfeifer et al., 2006) together with herbivore damage and cost of reproduction (Jacquemyn et al., 2010).

In commercial geophytic genera such as *Amaryllis*, *Anigozanthos*, *Hyacinthis*, *Iris*, *Liatris*, *Lilium*, *Nerine*, *Polianthis*, *Ranunculus*, *Tulipa* and *Zantedeschia* a critical minimum size of the geophytic organ, was found necessary to ensure floral initiation (De Hertogh and Le Nard, 1993). A certain plant size threshold that is required as prerequisite for flowering has also been demonstrated in monocarpic orchids (Jacquemyn et al., 2010). This relationship becomes more complicated in polycarpic plants as conditions in one year may affect flowering of the subsequent season. This may be particularly true for terrestrial orchids occurring in their natural habitat, as most species have below-ground perrenating structures, where stored resources of the current year are used for floral development and sustaining of the flowering process of the following season (Jacquemyn et al., 2010). In *Disa*, root tubers are replaced annually (Crous and Duncan, 2006; Du Plessis and Duncan, 1989). Immediately prior to flowering mature plants have two tubers, one from the current growing season and one from the previous growing season (Linder and Kurzweil, 1999). The new tuber gives rise to the progeny (new shoots and roots), and will later support the flowering spike (Vogelpoel, 1993).

Jacquemyn et al. (2010) and Pfeifer et al. (2006) conducted research on factors affecting flowering on natural occurring populations of terrestrial orchids. Within a natural habitat, the

irregular flowering of orchids has been reported as an intrinsically triggered and unpredictable phenomenon (Pfeifer et al., 2006). Under cultivation, certain factors like flowering history and climate can either be controlled or are irrelevant. For instance, plants are usually cultivated within a temperature controlled greenhouse and are grown from tissue culture annually, thereby excluding the effects of flowering history. Still, certain prerequisites for floral initiation such as critical plant size remain relevant in cultivation. When a critical plant size can be determined, only plants of this threshold size could be selected for cultivation. Harvest predictions could then be based on plant size and lastly measures to increase plant size, using additional light or nutrition could be applied, enabling more plants to reach this critical size and subsequently increase profitability. Size-dependant flowering, referring to the size of a geophytic organ or plant size as measured by leaf area or number of leaves in orchids, has been widely documented for orchids, geophytes and other biennial and perennial plants (De Hertogh and Le Nard, 1993; Pfeifer et al., 2006). For *Disa*, this information is not available.

No manipulation of flowering time is used commercially for the genus *Disa* (Van der Linde, personal communication, 2010). Terrestrial orchids, similar to geophytic plants, rely on a developmentally competent meristem to perceive environmental or endogenous signals, essential for the transition from a vegetative to a reproductive phase. For tuberous terrestrial orchids plant size, together with factors affecting plant size (light, photosynthesis and carbon allocation), appears to be the critical factor which will determine whether a plant will flower within a given year (Jacquemyn et al., 2010; Pfeifer et al., 2006).

Vegetative propagation of *Disa* plants often leads to multiple plants developing in a pot. This clumped growth is also described for *Disa* growing in its natural habitat (Crous and Duncan, 2006) and seems to be inherent to *Disa*, as plants grown from tissue culture also readily form these clumps. During cultivation, to avoid competition, plants are separated and re-potted (Haasbroek, personal communication, 2010-2012). However, the impact on plant growth and flowering has not been established. Currently separation and re-potting is done in fall before the onset of a presumed dormancy.

The aim of this chapter is to study the relationship between plant size and the incidence of flowering. The first hypothesis proposes that a minimum plant size is required for flower initiation. The second hypothesis states that separation and re-potting influences plant growth and flowering negatively and that this cultivation practice should be omitted.

Materials and Methods

PLANT MATERIAL. All plant material was obtained from La Motte Winery Estate in Franschhoek (33°52’49.9”S; 19°4’28.3”O), the only commercial *Disa* producer in South Africa. The material selection included hybrid ‘LM0739’ for the plant size and separation trials with ‘LM56’ and ‘LM67’ used for the study correlating floral initiation and plant weight through dissections. In hybrid

'LM0739' spike emergence occurred at the beginning of October, with anthesis in November. Hybrids 'LM56' and 'LM67' are generally considered early flowering (July-October) hybrids, approximately similar in size to 'LM0739', with crown widths between 119.4 ± 3.2 and 158.6 ± 6.1 mm. Parent plants used in the hybridization programs for all the above mentioned hybrids are unknown.

EXPERIMENTAL SITE. The same experimental site as described in Paper 1 was used. Newly re-potted plants of hybrid 'LM0739' together with the 'non-separated' treatment plants were then moved to a polycarbonate greenhouse facility for the consecutive dormant and reproductive phases, where plants were cultivated in a hydroponic tray system. Hybrids 'LM56' and 'LM67' were kept in the shade house for the entire duration of development, and was only moved to the greenhouse at anthesis. For detail on the green- and shade houses refer to Paper 1.

NUTRITION, WATER AND GROWTH MEDIUM. The nutrition, water and growth medium used for cultivation of plants used within this study were similar as described in Paper 1.

TEMPERATURE AND GROWING CONDITIONS. Temperatures and growing conditions as recorded within the shade house and greenhouses were reported in Paper 1.

PLANT SIZE TRIAL. Three size categories of vegetative *Disa* hybrid 'LM0739' plants were selected from the commercial population at the end of Apr. 2011, at the time of re-potting. Each plant size category group consisted of 30 plants. The first group consisted of small plants with height and crown width measurements of approximately 18.1 ± 1.5 mm and 76.7 ± 3.3 mm, respectively. The "Small" size category is considered non-viable and would have been discarded commercially. The second group consisted of medium sized plants with height and crown width measurements of 26.1 ± 1.1 mm and 119.4 ± 3.2 mm, respectively. This group is used commercially as some may flower during the next spring. The last group consisted of large, commercially viable plants. Plants in this group were approximately 50.2 ± 2.1 mm in height with a crown width of 158.6 ± 6.1 mm. A further 158 randomly selected plants were monitored from the commercial population of hybrid 'LM0739' which served as a control treatment for the plant size trial. The representative plants within the population were tagged at the approximate time of spike emergence and flowering data was subsequently collected and described.

SEPARATED VS. NON-SEPARATED PLANTS. Separation or re-potting entails the separation of mother plants from daughter plantlets growing in the same pot, followed by re-potting, with only one plant per pot. To study the impact of this cultural practice on vegetative growth and flowering of *Disa* hybrid 'LM0739', 50 potted plants were randomly selected during vegetative growth. The plants randomly selected for use within this trial could be best compared in size with the "Medium" plant size category. These plants were allocated to two treatment groups of 25 pots each, namely "Separated" (n = 74 plants from 25 pots) and "Non-separated" (n = 50 plants from 25 pots). In the "Separated"' group plants were divided on 28 Mar. 2011 and each plant was transplanted into a

separate pot. The plant height after separation was 21.4 ± 1.1 mm. The "Non-separated" group were left to grow undisturbed with numerous plants per pot.

DATA RECORDED. After baseline measurements were obtained, growth as described by plant height (mm), the crown width (mm) and number of unfolded leaves, of all three plant size category groups as well as for the "Separated" and "Non-separated" plant groups were recorded at six week intervals. Height was measured from the base of the plant to the tip of the newest unfolding leaf (Paper 1), whilst the crown width was measured form the tip of one unfolded mature leaf to the tip of the opposite mature unfolded leaf (Fig. 1). All measurements were made using a 15 or 30 cm digital calliper and logger (Mitutoyo, Mitutoyo Corporation, Kawasaki, Japan).

The flowering was first recorded on 8 Oct. 2011 and continued until 19 Jan. 2012. Together with the date of spike emergence, the date on which the first flower of the inflorescence opened (anthesis) and senesced were also recorded. Differences seen in percentage anthesis and senescence can be ascribed to the experimental loss of the flowering plants before senescence could be documented.

DISSECTIONS. Dissections were conducted at two week intervals from 28 Mar. to 4 Jun. 2012 in order to establish when floral initiation occurred in plants of different sizes. Eight and ten plants of hybrid 'LM67' and 'LM56' were selected respectively for each dissection date. Plants were selected to include a comprehensive range of plant sizes at each dissection date. Plants were dissected to the meristem using a stereo microscope equipped with x160 magnification (eyepiece x20) (Leica S6D, Leica DFC295 (camera) and KL200 LED, Leica Microsystems Inc., Illinoise, (USA). At dissection, each plant was cleaned and weighed, where after the number of leaf primordia together with the number of unfolded leaves and the meristem status were determined. Data concerning the number of unfolded leaves and the number of leaf primordia are presented separately as counting the number of unfolded leaves are seen as a practical technique for producers to use when estimating the propensity of a plant to flower. Time is presented as Julian days.

STATISTICAL ANALYSIS. The change in all growth measurements (unfolded leaf number, height and crown width) over time was analyzed by Repeated Analysis of Variance (RANOVA), using Statistica 9.0 (Stasoft, Inc., Tulsa, Oklahoma, USA). On the last date of measurements the comparison of means was done by means of a one-way ANOVA, using Fishers' LSD posthoc separation test (Statistica 9.0, Stasoft (SA) Inc., Tulsa, Oklahoma, USA). Discriminate analyses were conducted using XLStat 2011 (Addinsoft, New York, USA) and the ANOVA's for the dissection trial were done using SAS 9.2 (SAS Institute Inc., Cary, USA). M-L Chi square analyses were also conducted to determine significant differences between flowering parameters (Statistica 10.0, Stasoft (SA) Inc., Tulsa, Oklahoma, USA).

Results

PLANT SIZE CATEGORIES. No significant interaction occurred between the size categories and time for number of unfolded leaves and crown width, respectively (Fig. 2 and 3). A significant

difference was found between treatments and over time ($P < 0.001$) for both growth parameters where the "Large" size group showed, on average, a constant higher number of unfolded leaves and crown width, than the "Medium" and "Small" categories. The plant height showed a significant interaction between size categories and time ($P < 0.001$) as the height of "Large" plants increased significantly more from the end of August onwards in comparison with the other two size categories (Fig. 4). At the last observation date prior to flowering (30 Sept. 2011), the three size treatment groups differed significantly from each other with respect to all three growth parameters (number of unfolded leaves, crown width and plant height) (Table 1).

Table 2 shows the flowering data as collected for the three plant size categories compared to that of the commercial control group. The total percentage of spike emergence (93%) and anthesis (63%) in the "Large" plant size category was higher compared to both the commercial control and "Medium" sized groups with 70% and 50% for spike emergence and 29% and 23% for anthesis percentages, respectively. Percentages spike emergence, anthesis and senescence all differed significantly between treatments with *P*-values of <0.001, $P = 0.002$ and $P = 0.003$, respectively. No spike emergence was observed in any plants within the "Small" size category. Plants of the "Large" treatment group displayed the onset of spike emergence on the same date than that of the control plants. The time span over which spike emergence occurred within the "Large" plants category was however more condensed, resulting in one month earlier average spike emergence for "Large" plants. Spike emergence in the "Medium" size category first occurred approximately two weeks later than in the commercial control. Anthesis of the "Large" size category plants extended over an eight week period, while the distribution in the "Medium" plant size category was the most condensed of all the treatment groups and was completed within a month. The "Large" plants flowered two to six weeks earlier (anthesis on 29 Nov. ± 2.6) compared to both the commercial control group (16 Jan. ± 14.0) and the "Medium" sized plants (12 Dec. ± 3.1). The date of senescence of the first flower in "Large" plants was synchronized with that of the other treatment groups, but similar to anthesis, was more condensed than that of the commercial control group (Table. 2). In the "Medium" size category the first flower senesced a week later than in the "Large" or commercial control plant groups.

SEPARATION VS. NON-SEPARATION. No significant interaction between the separation treatment and time ($P = 0.130$) occurred in number of unfolded leaves (Fig. 5). Separating plants did not influence the number of unfolded leaves, but a significant difference in unfolded leaf number was found over time ($P <0.001$). Non-separated plants showed an increase in number of unfolded leaves from April to July, followed by a decrease thereafter to September, whereas the number of leaves in plants that were separated remained fairly constant throughout fall, winter and early spring. The crown width did not show a significant interaction between the treatments and time ($P = 0.784$) or between treatments ($P = 0.062$) or time ($P = 0.495$) (Fig. 6). No significant interaction between treatments and time ($P = 0.556$) existed for plant height (Fig. 7). However, in both treatments, a significant increase in plant height occurred towards 30 Sept. ($P < 0.001$). When growth parameters

were compared between treatments after a six month period following separation, no statistical differences were obtained between number of unfolded leaves, crown width and plant height (Table 3).

When the flowering patterns of the separated and non-separated plants were compared, the timing of spike emergence, anthesis and senescence were similar between the two groups. Separated plants showed a higher spike emergence (36%) and anthesis (19%) percentages compared to the non-separated group where spike emergence was 24% and anthesis 6%, respectively, however none of these differences were significant at the 5% confidence level (Table 4).

DISSECTIONS. When data describing growth by means of the number of unfolded leaves, plant weight and number of leaf primordia were submitted to a discriminate analysis (DA) for both hybrids 'LM56' and 'LM67' (Fig. 8 and 9), distinct separation of the vegetative and reproductive meristems were not detected on the observational charts, despite the ability of Factor 1 and 2 to explain approximately 90% and 93%, in total, of the variance for hybrids 'LM56' and 'LM67', respectively (Fig. 8A and 9A). Factor 1, for both hybrids, was partially successful to distinguish between the vegetative and reproductive meristems from left to right, explaining 62% and 84% of the variance for hybrids 'LM56' and 'LM67', respectively. The DA model could only predict 41.9% and 44.0% of the samples correctly into the correct day and developmental phase for hybrids 'LM56' and 'LM67' respectively (Table 5 and 6). Comparing the variable and observation charts for both hybrid 'LM56' and 'LM67', plant weight and number of unfolded leaves were positively associated with the "Reproductive" (R) groups (Fig. 8 and 9).

The plant weight of vegetative and reproductive plants of the hybrid 'LM56' showed a significant interaction with time (P = 0.039) (Fig. 10A). In hybrid 'LM67' this interaction was however not significant (Fig. 10B). Floral initiation occurred much later in 'LM67' than in hybrid 'LM56', despite its more robust vegetative growth compared to that of 'LM56' where vegetative plants had a weight of below six grams throughout the monitoring period (Fig. 10). The reproductive 'LM56' plants weighed more than the comparative vegetative plants, especially earlier in the season. A similar trend was observed for 'LM67', with vegetative plants consistently showing weights of below 15 grams (Fig. 10B), while reproductive plants weighed more by at least 5 gram. The number of unfolded leaves did not show an interaction between treatment and time for either of the two hybrids (Fig. 11). However, for both hybrids a significant difference was seen in the number of unfolded leaves for the two developmental categories with P-values of 0.035 and <0.001 for 'LM56' and 'LM67', respectively. Reproductive plants of the hybrid 'LM56' (Fig. 11A) had more leaves (9.9 ± 0.4) than the vegetative plants (8.5 ± 0.5), especially earlier in the season. A similar trend was seen in 'LM67' where reproductive plants had more leaves (9.5 ± 1.0) in comparison to the vegetative plants (6.1 ± 0.4) (Fig. 11B).

The number of leaf primordia removed showed a significant phenological phase and time interaction for hybrids 'LM56' and 'LM67' with *P*-values of 0.039 and 0.035, respectively (Fig. 12). These values were inconsistent between hybrids, over time and between the phenological classes.

Discussion

PLANT SIZE. *Disa* hybrids in cultivation must reach a threshold size before floral initiation could occurr. According to the dissection study, this critical size becomes a less obligatory requirement as the season progresses. This size requirement is also found in other floricultural crops (Schmid et al., 1995; Willems and Dorland, 2000). The term 'plant size' is generally used in literature, but more specifically refers to leaf area, rosette size or to the size of the underground storage organ. Plant size provides an indication of sufficient leaf area or reserve status to sustain photosynthetic capacity required to support the flowering process. Furthermore, plant size or plant age should also be clearly distinguished. Although the two concepts are closely related, plant size or leaf area can be manipulated by external conditions to reach critical sizes, whilst plant age is time dependant. Generally, plant size is more important in determining the phenological development than age (Gross, 1981).

In the Orchidaceae, size dependant flowering has mostly been studied in natural occurring populations of terrestrial orchids (Jacquemyn et al., 2010). However, in commercial *Phalaenopsis* nurseries, a plant is considered viable and mature (able to support a flowering spike) when a leaf span (similar to 'crown width' measured in this study) of 25 cm is reached (Van der Knaap et al., 2005).

Size dependant flowering in geophytes, where the size of the tuber, rhizome or corm is the determining factor, is also widely documented. Minimum geophytic storage organ sizes have been reported for *Amaryllis*, *Anigozanthos*, *Hyacinthis*, *Iris*, *Liatris*, *Lilium*, *Nerine*, *Polianthis*, *Ranunculus*, *Tulipa* and *Zantedeschia* (De Hertogh and Le Nard, 1993). Large bulbs would be likely to produce thicker, larger and more vigorous shoots with more leaves and subsequently more flowers than smaller bulbs (De Hertogh and Le Nard, 1993). The geophyte organ size extends its influence wider than only the ability to flower. In *Lilium* and *Ranunculus* earlier flowering was reported for plants with larger geophytic organs compared to flowering times for smaller organs. Frequently, a minimum weight or circumference of a geophytic organ is reported below which flower initiation will not occur. Increases in a storage organ above a certain maximum threshold level would not favour stem growth or flowering any more than for plants having a geophytic organ of a threshold size. However, the size of the underground storage components do not always influence flowering as is claimed to be the case in *Dahlia*. Tuberous root sizes with weights of 80 and 120 g, respectively were reported to show equal growth potential (De Hertogh and Le Nard, 1993), although the threshold size was not specified in this study. Evergreen commercial *Disa* hybrids are not directly comparable to other terrestrial orchids in natural occurring populations, neither to epiphytic orchids in cultivation or to other geophytes typical of the Cape Flora, but seems to follow the same requirement for a threshold plant size/weight which allows floral initiation

A rapid and significant increase in plant height was the only growth parameter measured which predicted the probability of flowering reliably in *Disa*, as the sudden increase in plant height (bolting) was a sign that floral initiation had occurred. Plants in the "Large" plant size category which were significantly higher on 30 Sept. than the other two plant size categories (Table 1; Fig. 4) also had significantly more spikes emerging and flowering than the control and "Medium" plants (Table 2). Gross (1981) found similar results in the four biennial plant species *Verbascum thapsus*, *Oenothera biennis*, *Daucus carota* and *Tragopogon dubius*, concluding that in general, for all four species, a minimum plant size must be reached before a plant is capable of flowering. Also, contrasting to what is claimed for the size of geophytic organs, an increase in plant size above this critical size will significantly increase in the probability to flower.

For this study, the total inability of the "Small" plants size category to flower and the lower percentage of flowering recorded for the "Medium" plant size category clearly confirmed the importance of plant size in floral initiation of *Disa*. In these size categories, lower leaf numbers and smaller crown leaf widths may implicate lower photosynthetic capacity and thus available assimilates to support flowering. Interestingly, significant differences between percentages of spike emergence and anthesis were recorded. The reason why many (21% - 26%) (data not shown) of the plants that differentiated a flowering spike with buds did not reach anthesis of at least one flower is unclear and warrants further research, especially considering the link to low carbohydrate reserve status (Table 2).

The commercial control and "Medium" plant size category showed similar low anthesis percentages of 29% and 23% respectively. This percentage however more than doubled within the "Large" plant size category, reaching 63% anthesis, supporting the hypothesis that sufficient carbohydrates (high leaf number and leaf area) are needed to support flowering to anthesis. Smaller plants may have enough carbohydrates to support spike elongation, but were not able to support flower opening, in contrast to the "Large" plant size category plants in which stored carbohydrates could support the whole flowering process. From the flowering time distribution of the "Medium" group it was clear that both spike emergence and flower opening occurred later for this group than for the "Large" group (Table 2). In the commercial control group the window for both flower opening and spike emergence were wider. This could probably be ascribed to the range of plant sizes that was represented within this random selection of plants. The larger size plants within the population group would then be responsible for the early spike emergences and anthesis, whilst the medium and smaller sized plants would contribute to the later spike emergences and anthesis. Thus to achieve a longer flowering period the inclusion of more than one size category of plants would be beneficial.

The lack of flowering of plants in the "Small" plant size category supports the cultural practice of discarding small plants as this group is unlikely to flower during the current season as all plants died either during the trial or at the end of the season. This practice is also used for commercial *Phalaenopsis* plants as small plants show a disadvantage from the start, and would require an additional four months' growth to reach a flowering size. If not discarded, these plants are to be

cultivated separately, although they are unlikely to reach the same potential as the larger plants (Van der Knaap et al., 2005). However, unlike for small *Phalaenopsis* plants which will only flower later than its larger counterparts, a low probability exist in *Disa* for these "Small" plant size category plants to achieve later flowering, or even flowering in the following season. All the plants in this "Small" plant size category died at the end of the season, even though no flowering occurred. *Disa* in their natural habitat has access to storage carbohydrates from a root tuber which was produced during the previous growth season, together with assimilates produced by current photosynthesis to support flowering (Du Plessis and Duncan, 1989). However hybrids grown from tissue culture only rely on photosynthesis of the current growing season, as tuber formation appears to be fairly uncommon (personal observation) for *Disa* propagated through this technique. It might thus be possible for smaller plants to flower in nature, provided a storage organ of sufficient size is present. Thus, currently, the only flower manipulation strategies possible through plant size selection is to advance flowering and increase productivity by selecting large plants with a crown width and height of approximately 160 mm and 50 mm, respectively, and to extend the flowering period by including medium sized plants with crown width and height of approximately 120 mm and 25 mm respectively. In *Disa*, leaf span, or crown width could possibly be used in future studies to predict flowering propensity. Future studies should focus on developing methods by which plant size can be enlarged or optimized, although an optimum size would have to be considered hybrid specifically, as great variation occurs between *Disa* hybrids.

The confusion matrix for hybrid 'LM56' and 'LM67' had a total of 42% and 44% correctly predicted sample groups respectively. Miss-classified samples were randomly distributed in the matrixes (Table 5 and 6). However, when comparing the observations and variable charts, plants dissected with reproductive meristems were associated with a higher number of leaves and plant weight for both hybrids (Fig. 8 and 9). In 'LM56' the mean plant weight for plants with reproductive meristems decreased over time, displaying a floral gradient where plant size became increasingly less important as the season progressed and plants have possibly been exposed to several cycles of the inductive signal(s). Although floral initiation in hybrid 'LM67' during the monitoring period occurred much later, the same trend could be seen where the reproductive plants exhibited a higher plant weight earlier in the season than later in the season. Recordings indicated that a minimum unfolded leaf number is necessary for flowering in *Disa*. The threshold number of leaves would be hybrid specific, but may also depend on the presence and number of floral inductive cycles. Larger reproductive plants of 'LM56' and 'LM0739' displayed consistently eight or more leaves (Table 1; Fig. 11). Plants with a lower number of unfolded leaves could possibly also initiate inflorescences, but would probably require more induction cycles. In addition, spike emergence may occur in these smaller plants with a lower number of leaves, but the flowering process may not be fully supported through to anthesis. The "Medium" plant size category of 'LM0739' which had a lower spike

emergence and flowering percentage than the "Large" plant size category, had between five and six leaves (Table 2 and 3). The "Small" plants that did not flower had fewer than 5 leaves.

Therefore, five leaves are considered as a threshold minimum required for flowering of *Disa* hybrids in cultivation. This is similar to the number of leaves Pfeifer et al. (2006) found necessary for flowering in terrestrial orchids like *Himantoglossum hircinum.* Since *Disa* plants seem to maintain a relatively constant leaf number throughout the season, selection of plants early in the season could be a practical tool to identify viable plants.

SEPARATION VS. NON-SEPARATION. No previous scientific studies have been conducted to determine the effect of plant separation on *Disa*. The assumption is that plant separation, by eliminating competition, would promote plant size and therefore flowering. We found no significant differences between the "Separated" and "Non-separated" plants growth, spike emergence and anthesis (Fig. 5-7; Table 4). Disturbance of the plants through the separation practice therefore cancelled the advantages of eliminating competition even though no separation induced stress symptoms were visible. Still, mechanical damage to the roots due to separation of plants, when it is performed at a sensitive time just prior to floral initiation, could possibly result in physiological disorders such as bud abortion, as was reported in Paper 1. Separation of the plants may be justified only if the market specifies only one flowering spike per pot. The separation of *Disa* appears to be similar to the separation of the keikeis of orchids like *Cymbidium* and *Phalaenopsis*, although these practices are also not done commercially. Based on results differences found concerning flowering between the "Separated" and "Non-separated" is not substantial enough to validate the extra time and labour costs the separation process entails.

The separation and re-potting of plants (orchids, *Agapanthus* or *Clivia*) are used as a means of vegetative propagation and to increase the size of plant collections (Tibbs, 2007). Divisions of *Clivia*, *Agapanthus* and certain orchids (*Cymbidium*) are done by removing new plantlets from the mother plant (Duncan, 2002; Duncan, 2008; Tibbs, 2007). In *Clivia* and *Agapanthus*, offspring should be removed by tugging. If these plantlets cannot be removed in this manner it is considered not ready to be separated from the mother plant. Newly produced plantlets can also be severed from the mother plant with a sharp object, but then it is critical to ensure that enough root material is included in the cutting process. The main difference between these examples and *Disa* is that these plants use rhizomatous rootstocks, so that even when the new offspring is ready to be divided from the mother plant, a cut through the rhizome is needed for complete separation. The rhizomatous rootstock itself could also be divided by cutting, still ensuring that every sectioned segment of rhizome would have sufficient foliage and roots (Duncan, 2002; Duncan, 2008; Tibbs, 2007). The only time considered to divide evergreen *Agapanthus* species is after the end of their flowering period in March (Southern hemisphere) (Duncan, 2002). Clivias however, in warmer areas like Kwa-Zulu Natal and the Lowveld, can be divided at any time of the year. In colder areas like the Western Cape, spring and early summer is recommended as the best time to divide summer growing *Clivia* species, whilst

winter-growing species (which seldom produce offsets) are best divided in autumn (Duncan, 2008). Through personal observation of *Disa*, although not mentioned anywhere in literature, the separation of *Disa* is best compared to the separation of the keikeis of orchids like *Cymbidium* and *Phalaenopsis*, although these practices are not reported for commercial production. Keikei are the adventitious growths produced on the mother plants (keikei meaning 'little child' in Hawaii) and can be easily separated when the keikei has developed a few strong roots (Tibbs, 2007). In *Agapanthus* and *Clivia* it is reported that the separated plantlet will only flower after a two year vegetative growth period. Personal observation of the size of the separated *Disa* plantlets after 6 months predicts this might also be the case in *Disa*. Therefore, in a commercial production system where new tissue culture plants are acquired annually, the separated plant material will evidently be of no use to the producer. Considering this, together with the results from this study indicating that the separation practice does not harbour significant advantages for the plant in growth or flowering, it is clear that on a commercial level, the cultural practice of plant separation is not justified. Furthermore, the possibility that the plant separation practice could lead to bud abortions (Paper 1), the costs involved in the separation practice and ultimately a claim by Crous and Duncan (2006) that *Disa* plants are gregorious of nature and enjoy being clumped to a minimum of three plants in a six centimetre pot confirms the notion that the separation practice is not commercially viable.

In general, genetic differences between hybrids makes the extrapolation of results obtained for one hybrid to related hybrids is not recommended as was shown for critical minimum sizes of bulbs between tulip cultivars (De Hertogh and Le Nard, 1993). The role of the root tuber size to control and support flowering as well as a possible propagation technique of tuber planting to replace tissue culture should be investigated for the commercial cultivation of *Disa*. Furthermore, it is important to note that many external factors may firstly affect plant size, and secondly after a certain plant size is reached, additional factors might still be required for floral induction (Willems and Dorland, 2000). Studies on *Dahlia* demonstrated how different nutritional treatments could affect plant height, number of branches per plant, flowering time and number of flowers per plant (Ahmed et al., 2004). Taking cognisance of results from this study that plant size affects flowering in commercially cultivated *Disa* hybrids, future studies could investigate the effect of temperature, light and nutrition on plant size and whether this could modify threshold plant size values for flowering.

Conclusion

Plant size, as well as external conditions such as available light and prevailing temperature, can play a role in the propensity of a plant to flower. In this study it was clearly demonstrated that larger plants (whether measured by plant weight, plant height, and number of unfolded leaves or crown width) had an advanced flowering time together with a higher spike emergence and the ability to support the individual flowers of an inflorescence until anthesis. "Medium" plant size category plants with lower plant weight, number of leaves or crown width are also capable of initiating

inflorescences and achieving spike emergence and anthesis, but to a lesser extent and later than larger sized plants. The inclusion of medium sized plants in a commercial population should be carefully considered in terms of the expenses involved compared to the expected harvest. A homogenous population of large sized plants would increase anthesis percentage, but will also lead to a more restricted flowering season compared to a population that include both large and medium sized plants. Cultivating small plants (a crown width of 76.7 ± 3.3 mm or below) will not produce inflorescences and should therefore be discarded. The hypothesis that a critical plant size is needed for flowering is therefore not rejected and increasing commercial plant size by providing additional light intensity or by adjusting fertilisation programs could also be studied as management strategies to promote flowering in cultivation.

The hypothesis proposing that plant separation negatively influences plant size is rejected, as no differences, negative or positive, were found between separated and non-separated plants and the necessity of plant separation as commercial practice can be questioned as no significant differences was found in the growth and flowering parameters between the separated and non-separated plants. However, separation of plants will result in a single flowering spike per pot, which may be preferred by the market. To ultimately be able to manipulate flowering time and percentage by using plant size, approaches of increasing the number of plants in a population that reach optimum size, together with studies to elucidate the flowering signal, are required before *Disa* can achieve its full economic potential.

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Table 1. The growth response of *Disa* hybrid 'LM0739' in three size classes, "Small" (18.1 ± 1.5 mm; n = 22); "Medium" (26.7 ± 1.1 mm; n= 29) and "Large" (50.2 ± 2.1 mm; n = 28), using number of unfolded leaves, crown width (mm) and height (mm) as growth parameters. Measurements as recorded on 30 Sept. 2011 are presented. This date was just prior to spike emergence after plants had been monitored over a 6 month period. The average heights ± SE of each plant size category (mm) are presented in brackets next to the size category.

Treatment		**Number of unfolded leaves**	**Crown width (mm)**	**Height (mm)**
Small (18.1 ± 1.5 mm)		4.09 c[z]	85.31 c	31.04 c
Medium (26.1 ± 1.1 mm)		5.52 b	127.33 b	45.19 b
Large (50.2 ± 2.1 mm)		7.79 a	160.37 a	115.25 a
ANOVA	F	39.42	91.78	161.74
	P	<0.001	< 0.001	<0.001

[z] Different letters denote a significant difference for Means at the 5% confidence level.

Table 2. Distribution of flowering patterns of *Disa* hybrid 'LM0739' between 8 Oct. 2011 and 19 Jan. 2012 in three plant size categories, "Small" (18.1 ± 1.5 mm), "Medium" (26.7 ±1.1 mm) and "Large" (50.2 ± 2.1 mm), also including a commercial control group. Flowering response was monitored using spike emergence, anthesis and senescence as parameters. Data are presented as a percentage (%) of the monitored population.

				Monitoring dates (2011 - 2012)							
Treatment:	**n**	**Total (%)**	**Date ± SE**	**08-Oct. - 13 Oct.**	**21-Oct. - 27 Oct.**	**03-Nov. - 08 Nov.**	**18-Nov. – 23 Nov.**	**02-Dec. – 09 Dec.**	**12-Dec. – 20 Dec.**	**30 Dec. - 04-Jan.**	**09 Jan. – 19-Jan.**
Commercial control	**158**										
Non flowering	47	30	N/A[z]								
Spike emergence	111	70	08 Nov ± 1.8	13.5	27.0	19.8	26.1	1.8	11.7		
Anthesis	45[y]	29	16 Jan ± 14.0				4.4	33.3	37.8	17.7	6.7
Senescence	39[y]	25	29 Jan ± 15.6					2.6	23.1	33.3	41
Experimental plants	**30**										
Large	**28**										
Died prior to spike emergence	2	7	N/A								
Non flowering	0	0	N/A								
Spike emergence	28	93	10 Oct ± 0.9	82.1	17.9						
Anthesis	19	63	29 Nov ± 2.6			10.5	21.1	63.2	5.3		
Senescence	18	60	21 Dec ± 5.7					33.3	22.2	33.3	11.1
Medium	**29**										
Died prior to spike emergence	1	3	N/A								
Non flowering	14	47	N/A								
Spike emergence	15	50	20 Nov ± 4.9		20.0	6.7	33.3	40.0			
Anthesis	7	23	12 Dec ± 3.1					57.1	42.9		
Senescence	6	23	04 Jan ± 5.7						16.7	33.3	50
Small	**22**										
Died prior to spike emergence	8	27	N/A								
Non flowering	22	73	N/A								
Spike emergence	0	0	-[x]	-	-	-	-	-	-	-	-
Anthesis	0	0	-	-	-	-	-	-	-	-	-
Senescence	0	0	-	-	-	-	-	-	-	-	-

M-L Chi square			
	M-L Chi square	***P*-value**	**Significance**
Spike emergence	15.24	<0.001	***[w]
Anthesis	10.08	0.002	***
Senescence	8.80	0.003	***

[z] N/A. indicates not applicable.

[y] Differences seen in number of anthesis and senescence can be ascribed due to a loss of plant material before senescence could be documented.

[x]- Indicates no data collected as no flowering incidences occurred.

[w]* Indicates the level of significance.

Table 3. The growth response of *Disa* hybrid 'LM0739' as determined by number of unfolded leaves, crown width (mm) and plant height (mm) where plants have either been separated as a cultural practice and were referred to as "Separated" (n = 74 plants from 25 pots) or were left undisturbed as a "Non-separated" treatment (n = 50 plants from 25 pots). Measurements were taken on 30 Sept. 2011, the last date of growth parameter data recording, just prior to spike emergence and 6 months after separation.

Treatment		**Number of unfolded leaves**	**Crown width (mm)**	**Height (mm)**
Non-separated		5.64 NS[z]	111.83 NS	39.11 NS
Separated		5.52	118.84	35.47 NS
ANOVA	F	0.573	1.099	0.760
	P	0.451	0.297	0.385

[z] NS. Indicates no significant difference at the 5% confidence level.

Table 4. Distribution of flowering patterns of *Disa* hybrid 'LM0739' between 8 Oct. 2011 and 19 Jan. 2012 where plants had been separated ("Separated"; n = 74 plants from 25 pots) or were left undisturbed (non-separated; n = 50 plants from 25 pots). Flowering response was monitored using spike emergence, anthesis and senescence as parameters. Data are presented as percentages (%) of the population.

				Monitoring date (2011 - 2012)							
Treatment	**n**	**Total (%)**	**Date ± SE**	**08-Oct. - 13 Oct.**	**21-Oct. - 27 Oct.**	**03-Nov. - 08 Nov.**	**18-Nov. – 23 Nov.**	**02-Dec. – 09 Dec.**	**12-Dec. – 20 Dec.**	**30 Dec. - 04-Jan.**	**09 Jan. - 19-Jan.**
Experimental plants	**74**										
Separated											
Died prior to spike emergence	11	15	NA[z]								
Non flowering	36	49	NA								
Spike emergence	27	36	19 Nov. ± 3.9	3.7	18.5	11.1	29.6	18.5	14.8	3.7	
Anthesis	14[y]	19	12Dec. ± 3.0				7.1	57.1	14.3	21.4	
Senescence	11[y]	15	30 Dec. ± 3.2					9.1	9.1	45.5	36.4
Non-separated	**50**										
Died prior to spike emergence	18	36	NA								
Non flowering	20	40	NA								
Spike emergence	12	24	16 Nov. ± 7.9	25.0		16.7	8.3	41.6	8.3		
Anthesis	3	6	17 Dec. ± 10.0					33.3	33.3	33.3	
Senescence	3	6	26 Dec. ± 4.1						33.3	66.6	

M-L Chi square

	M-L Chi square	***P* - value**	**Significance**
Spike emergence	0.253	0.615	NS[x]
Anthesis	2.606	0.107	NS
Senescence	1.178	0.278	NS

[z] NA. Indicates not applicable.

[y] Differences seen in number of anthesis and senescence can be ascribed due to a loss of plant material before senescence could be documented.

[x] NS. Indicates no significant difference at the 5% confidence level.

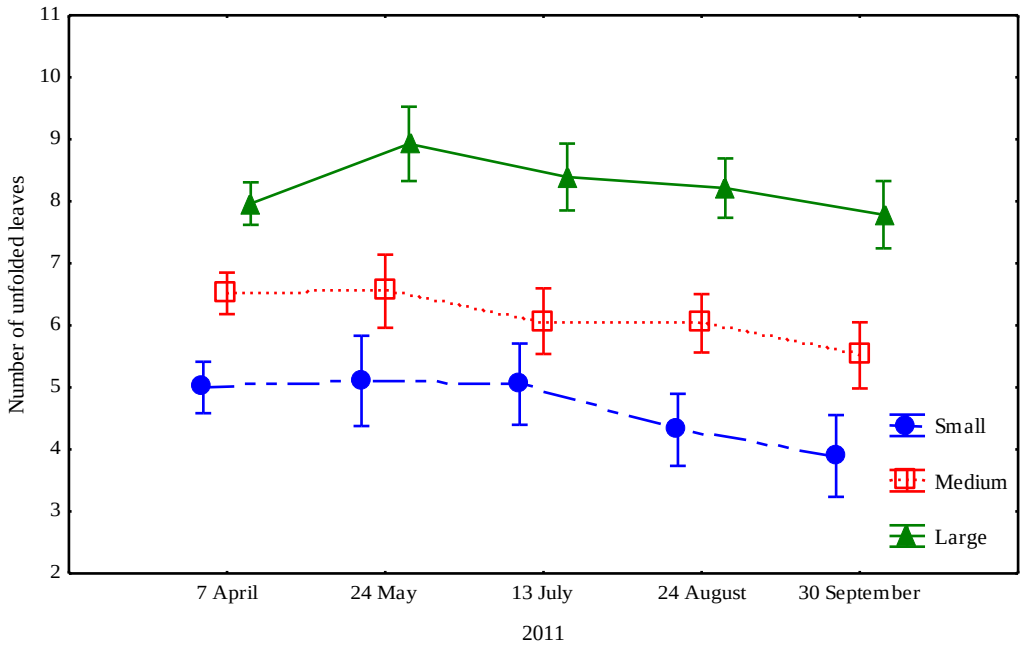
Table 5. A confusion matrix indicating the percentage (%) of correctly predicted meristems for *Disa* hybrid 'LM56' according to the two classes namely "Vegetative"(V) and "Reproductive" (R) on each dissection date, using plant weight, number of unfolded leaves and number of leaf primordia as parameters. The numbers following the symbols indicate the day of dissection, with the first dissection date at day 0.

from \ to	**R0**	**R13**	**R26**	**R40**	**R53**	**R67**	**R79**	**V0**	**V13**	**V26**	**V40**	**V53**	**V79**	**Total**	**% correct**
R0	1	0	0	0	1	0	0	0	0	1	0	0	0	3	33.3
R13	0	2	0	0	0	0	0	0	0	0	0	0	0	2	100.0
R26	0	0	1	0	1	1	0	0	0	0	0	0	0	3	33.3
R40	0	0	0	1	1	4	0	0	0	1	0	0	0	7	14.3
R53	0	0	1	0	4	0	0	0	1	0	0	0	0	6	66.7
R67	1	0	0	0	1	3	1	0	2	1	0	0	0	9	33.3
R79	0	0	1	0	0	0	5	0	1	0	0	0	0	7	71.4
V0	0	0	0	0	0	0	0	2	2	0	0	0	0	4	50.0
V13	0	0	0	1	0	1	0	1	4	0	0	0	0	7	57.1
V26	0	0	0	1	1	1	0	0	1	2	0	0	0	6	33.3
V40	0	0	0	0	0	0	0	0	2	0	1	0	0	3	33.3
V53	0	0	0	0	0	0	0	0	2	0	1	0	0	3	0.0
V79	0	0	0	0	0	0	2	0	0	0	0	0	0	2	0.0
Total	2	2	3	3	9	10	8	3	15	5	2	0	0	62	41.9

Table 6. A confusion matrix indicating the percentage (%) of correctly predicted meristems for *Disa* hybrid 'LM67' according to the two classes namely "Vegetative" (V) and "Reproductive" (R) on each dissection date, using plant weight, number of unfolded leaves and number of leaf primordia as parameters. The numbers following the symbols indicate the day of dissection, with the first dissection date at day 0.

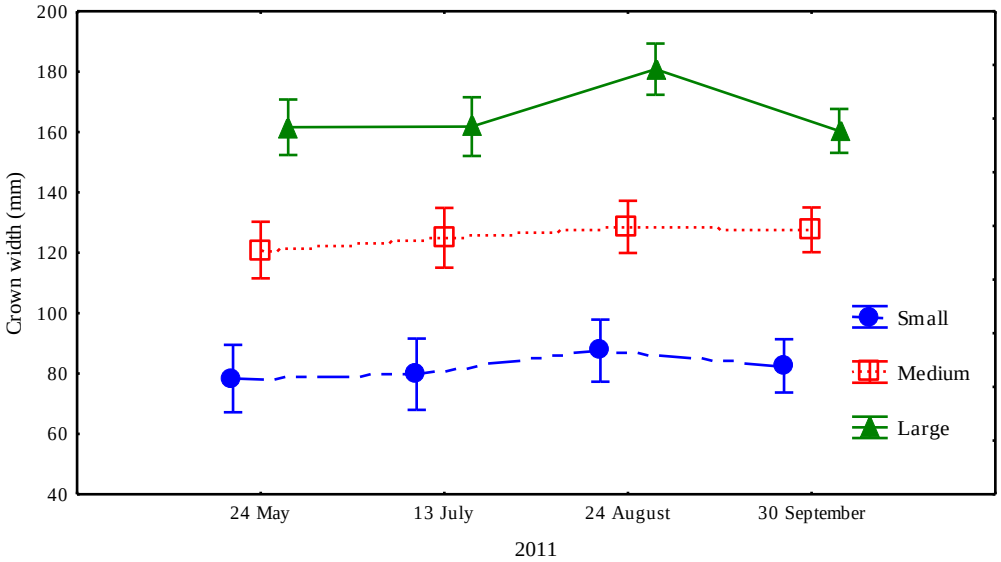
from \ to	**R53**	**R67**	**R79**	**V0**	**V13**	**V26**	**V40**	**V53**	**V67**	**Total**	**% correct**
R53	2	0	0	0	0	0	0	0	0	2	100.0
R67	0	0	1	1	0	2	0	0	0	4	0.0
R79	0	0	6	0	0	1	0	0	0	7	85.7
V0	0	0	0	8	0	0	0	0	0	8	100.0
V13	0	0	0	3	0	1	2	0	1	7	0.0
V26	0	0	3	0	1	3	1	0	0	8	37.5
V40	0	0	1	2	0	2	2	0	0	7	28.6
V53	0	0	1	1	0	0	1	0	0	3	0.0
V67	0	0	1	0	0	0	2	0	1	4	25.0
Total	2	0	13	15	1	9	8	0	2	50	44.0

Fig. 1. A vegetative plant of *Disa* hybrid ‘LM0739’ indicating the position at which the growth parameter of crown width (mm) was taken.



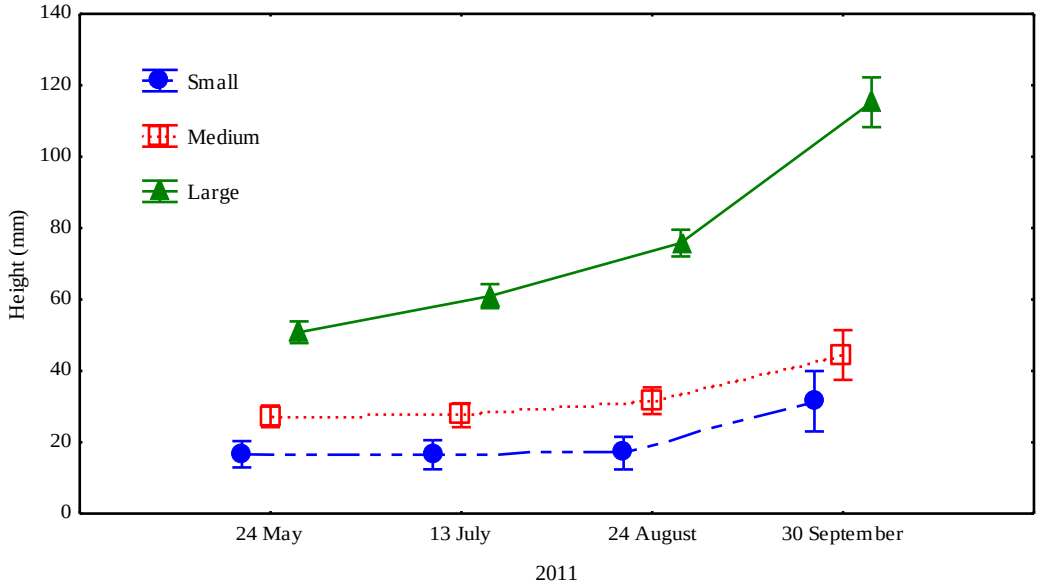
RANOVA	F-value	*P* - value	Significance
Treatment	82.721	<0.001	***[z]
Time	11.728	<0.001	***
Treatment *Time	1.485	0.162	NS[y]

Fig. 2. Plant size growth responses curves of *Disa* hybrid 'LM0739' in the various size categories of "Small" (18.14 ± 1.53 mm), "Medium" (26.68 ± 1.1 mm) and "Large" (50.22 ± 2.07 mm) over a six month period using number of unfolded leaves as growth parameter (n=30). Vertical error bars indicate the Standard Error (SE) of the Mean for each data point. [z]* Indicates the level of significance. [y]NS. Indicates no significant difference at the 5% confidence level.



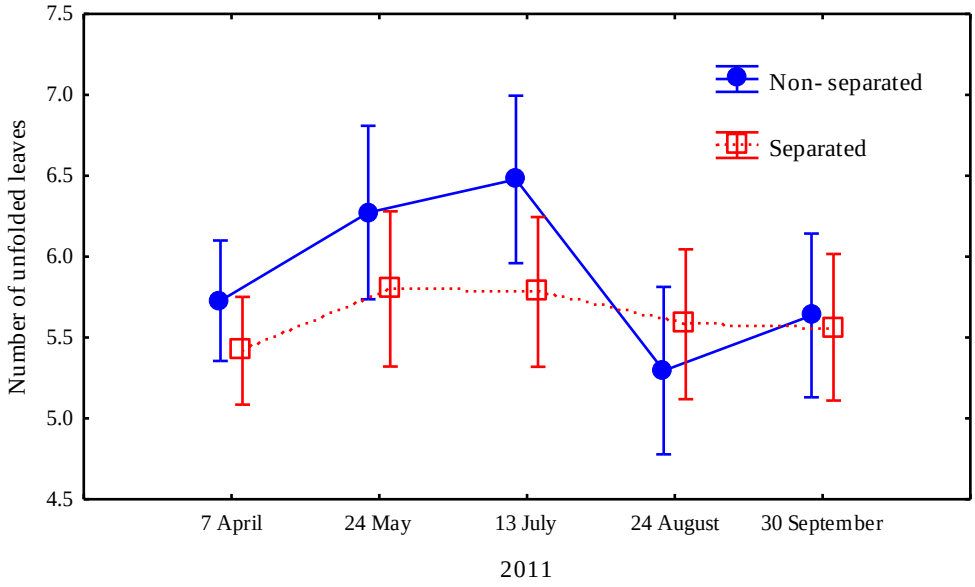
RANOVA	F-value	*P* - value	Significance
Treatment	3480.509	<0.001	***[z]
Time	126.228	<0.001	***
Treatment *Time	1.836	0.093	NS[y]

Fig. 3. Plant size growth responses of *Disa* hybrid 'LM0739' in the various size categories of "Small" (18.14 ± 1.53 mm), "Medium" (26.68 ± 1.1 mm) and "Large" (50.22 ± 2.07 mm) over a six month period using crown width (mm) as growth parameter (n=30). Vertical error bars indicate the Standard Error (SE) of the Mean for each data point. [z]* Indicates the level of significance. [y]NS. Indicates no significant difference at the 5% confidence level.



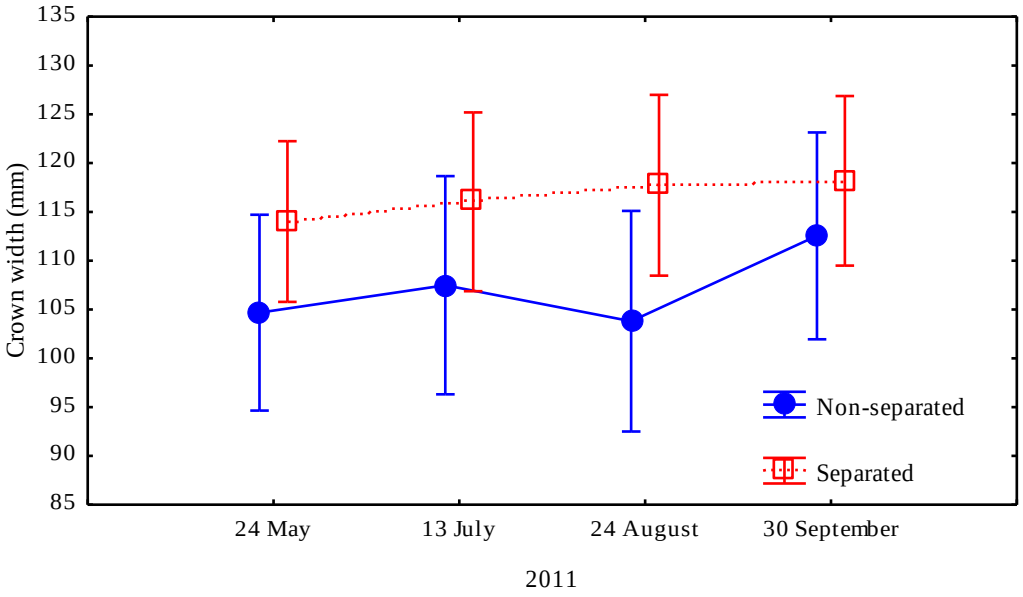
RANOVA	F-value	*P*- value	Significance
Treatment	212.492	<0.001	***[z]
Time	239.257	<0.001	***
Treatment *Time	60.343	<0.001	***

Fig. 4. Plant size growth responses of *Disa* hybrid 'LM0739' for the size categories "Small" (18.14 ± 1.53 mm), "Medium" (26.68 ± 1.1 mm) and "Large" (50.22 ± 2.07 mm) over a six month period using plant height (mm) as growth parameter (n=30). Vertical error bars indicate the Standard Error (SE) of the Mean for each data point. [z]* Indicates the level of significance.



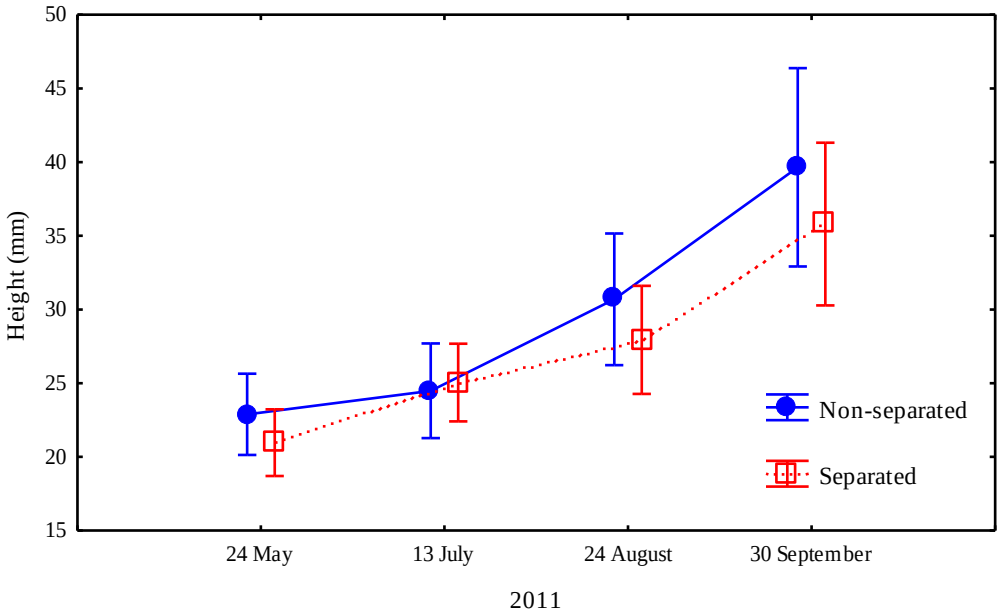
RANOVA	F-value	*P* - value	Significance
Treatment	1.339	0.250	NS[z]
Time	4.735	<0.001	***[y]
Treatment *Time	1.793	0.130	NS

Fig. 5. Plant size growth responses of *Disa* hybrid 'LM0739' for treatments "Separated" and "Non-separated", using number of leaves of the two groups as growth parameter. "Non-separated" plants have not been repotted in autumn as conventional cultural practices depicts. Vertical error bars indicate the Standard Error of the Mean for each data point. [z] NS. Indicates no significant difference at the 5% confidence level. [y]* Indicates the level of significance.



RANOVA	F-value	*P* - value	Significance
Treatment	3.550	0.062	NS[z]
Time	0.800	0.495	NS
Treatment *Time	0.357	0.784	NS

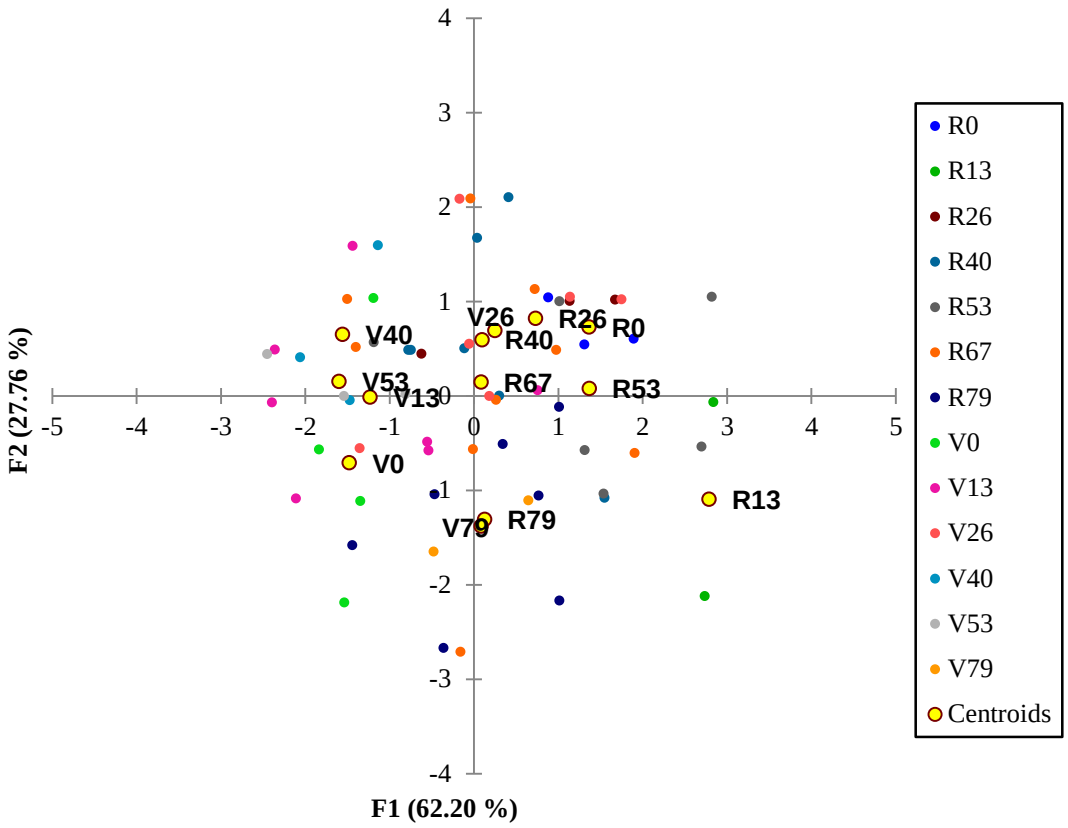
Fig. 6. Plant size growth responses of *Disa* hybrid 'LM0739' for treatments "Separated" and "Non-separated" using crown width (mm) as growth parameter. "Non-separated" plants have not been repotted in autumn as conventional cultural practices depicts. Vertical error bars indicate the Standard Error (SE) of the Mean for each data point. [z] NS. Indicates no significant difference at the 5% confidence level.



RANOVA	F-value	*P* - value	Significance
Treatment	0.7874	0.377	NS[z]
Time	37.6085	<0.001	***[y]
Treatment *Time	0.6942	0.556	NS

Fig. 7. Plant size growth responses of *Disa* hybrid 'LM0739' for treatments "Separated" and "Non-separated" using plant height (mm) of the two groups as growth parameter. "Non-separated" plants have not been repotted as conventional cultural practices depicts. Vertical error bars indicate the Standard Error (SE) of the Mean for each data point. [z]NS. Indicates no significant difference at the 5% confidence level. [y]* Indicates the level of significance.

A.



B.

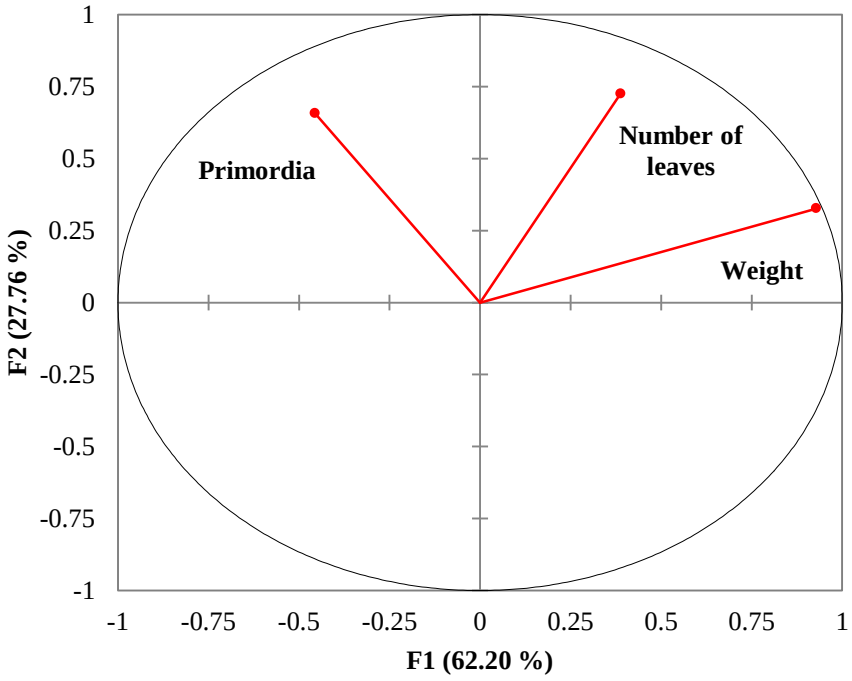
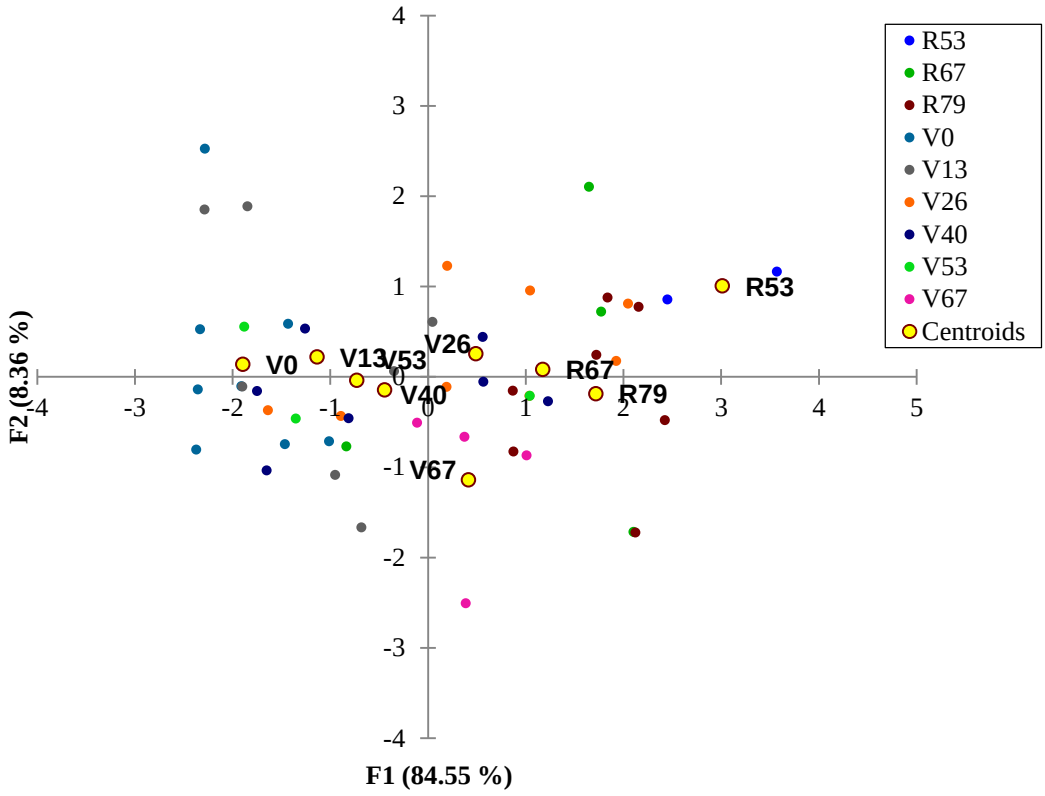


Fig. 8. A. Observations chart and B. Variables chart for the "Vegetative" and "Reproductive" phases of *Disa* hybrid 'LM56', using number of unfolded leaves, plant weight and number of leaf primordia as variables in a discriminant analysis (DA). The symbols "V" and "R" represent a vegetative and reproductive phenological state, whereas the number following the symbols indicates the day of dissections with the first dissection at day 0. The percentage (%) of variance described by each factor is indicated.

A.



B.

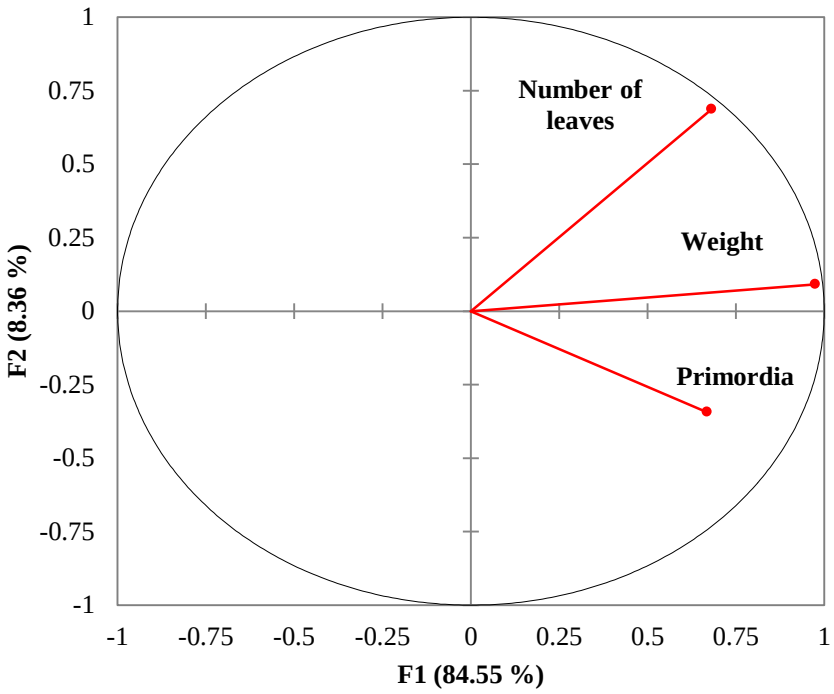
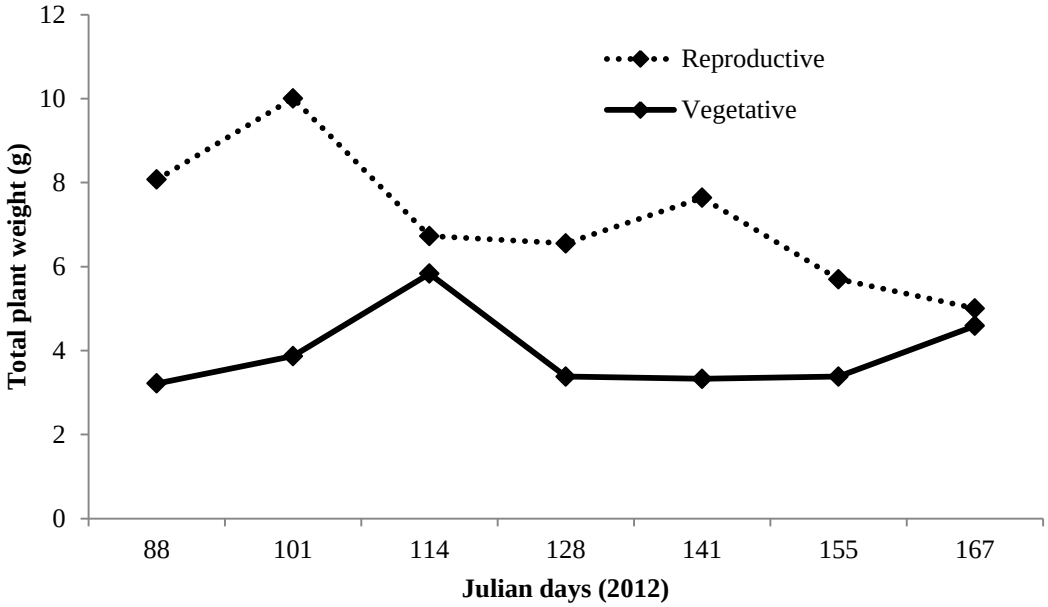


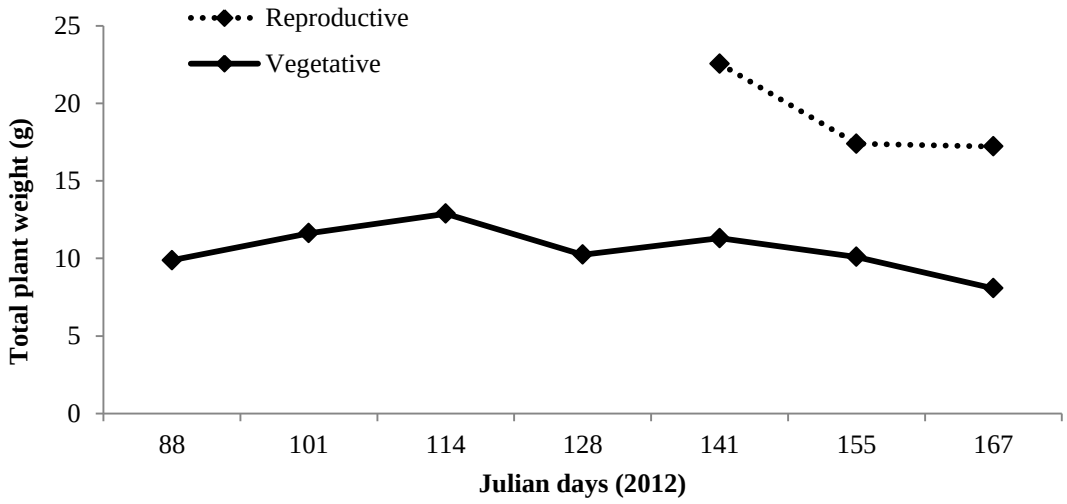
Fig. 9. A. Observations chart and B. Variables chart for the "Vegetative" and "Reproductive" phases of *Disa* hybrid 'LM67' using number of unfolded leaves, plant weight and number of leaf primordia as variables in a discriminant analysis (DA). The symbols "V" and "R" represent a vegetative and reproductive phenological state, whereas the number following the symbols indicates the day of dissections with the first dissection at day 0. The percentage (%) of variance described by each factor is indicated.

A.



ANOVA	F-value	*P* - value	Significance
Treatment	4.45	0.040	*[z]
Time	1.70	0.139	NS[y]
Treatment*Time	2.42	0.039	*

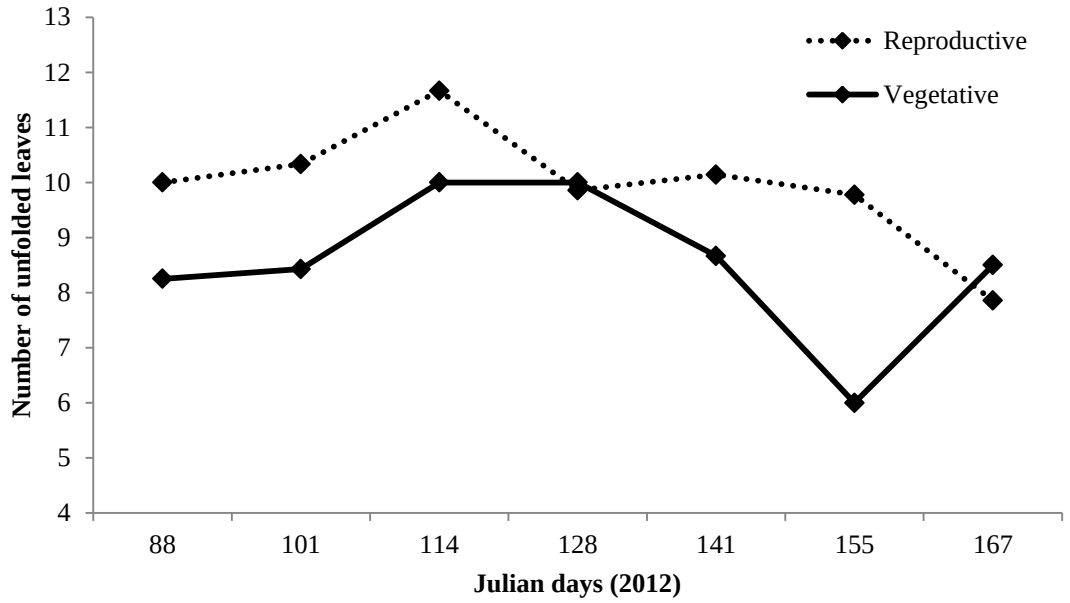
B.



ANOVA	F-value	*P* - value	Significance
Treatment	24.58	<0.001	***
Time	0.77	0.595	NS
Treatment*Time	0.29	0.750	NS

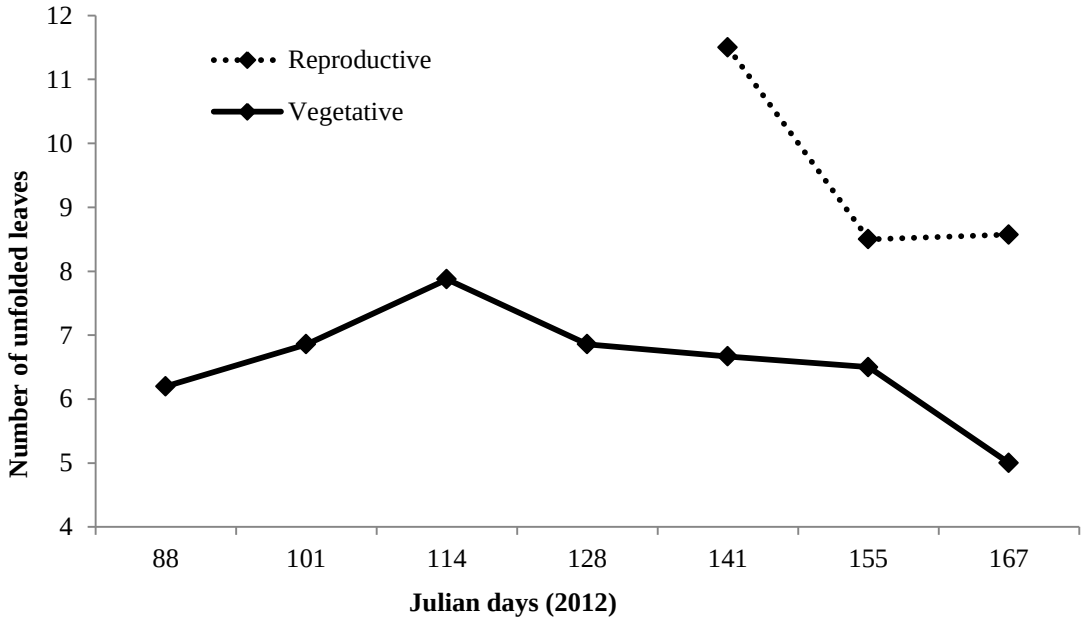
Fig. 10. The mean plant weight (g) of A. plants of *Disa* hybrid 'LM56' (n=8) and B. hybrid 'LM67' (n=8) as dissected biweekly from 29 Mar. – 16 Jun. 2012. Dates are indicated as Julian days. [z]* Indicates the level of significance. [y]NS Indicates no significant difference at the 5% confidence level.

A.



ANOVA	F-value	*P* - value	Significance
Treatment	4.68	0.035	*[z]
Time	3.55	0.005	**
Treatment* Time	1.34	0.256	NS[y]

B.

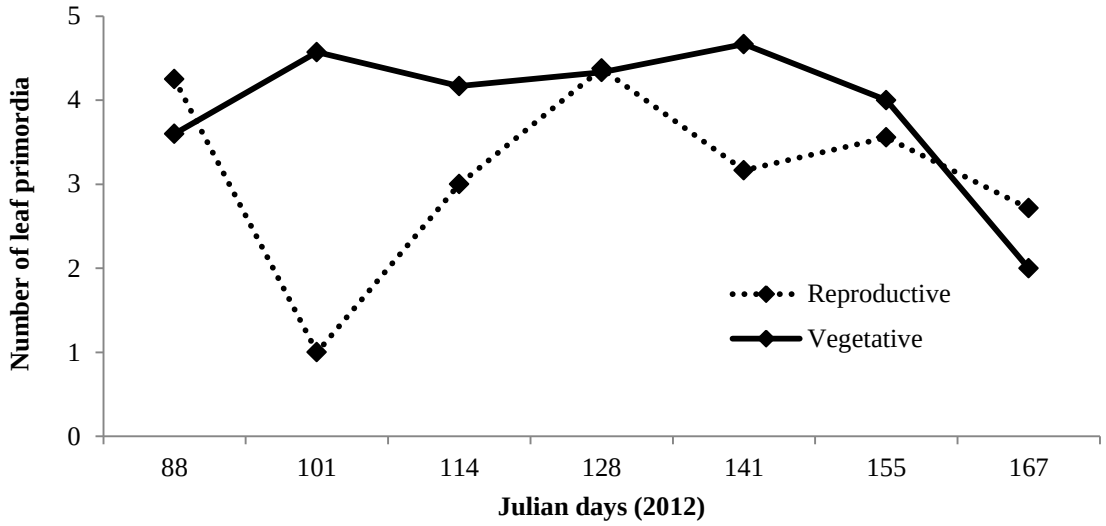


ANOVA	F-value	*P*-value	Significance
Treatment	20.61	<0.001	***
Time	1.82	0.118	NS
Treatment *Time	1.35	0.271	NS

Fig. 11. The mean number of unfolded leaves of A. plants of *Disa* hybrid 'LM56' (n=8) and B. hybrid 'LM67'(n=8) as dissected biweekly from 29 Mar. – 16 Jun. 2012. Dates are presented as Julian days.

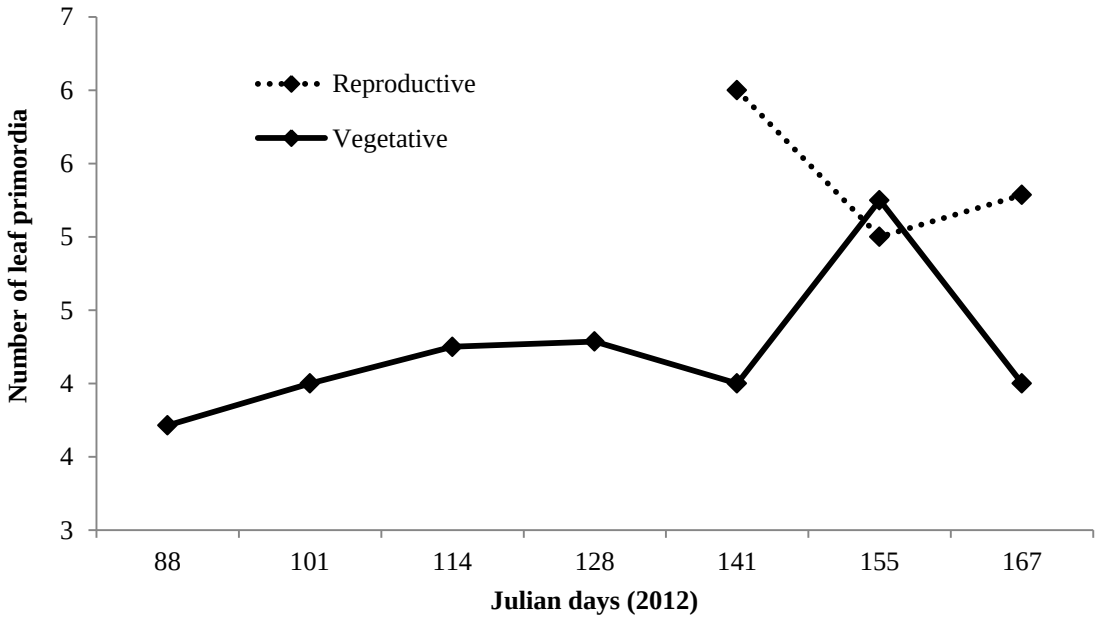
[z]* Indicates the level of significance. [y]NS. Indicates no significant difference at the 5% confidence level.

A.



ANOVA	F-value	*P*-value	Significance
Treatment	4.45	0.039	*[z]
Time	1.70	0.139	NS[y]
Treatment*Time	2.42	0.039	*

B.



ANOVA	F-value	*P* - value	Significance
Treatment	21.36	<0.001	***[z]
Time	1.12	0.369	NS[y]
Treatment*Time	3.66	0.035	*

Fig. 12. The mean number of leaf primordia of A. plants of *Disa* hybrid 'LM56' (n=8) and B. hybrid 'LM67' (n=8) as dissected biweekly from 29 Mar. – 16 Jun 2012. Dates are indicated as Julian days.

[z]* Indicates the level of significance. [y]NS. Indicates no significant difference at the 5% confidence level.

Paper 4: Growth Regulators and Potassium Chlorate to Induce Floral Initiation in Commercial *Disa* Hybrids.

ABSTRACT. A greater understanding of the flowering signal of the South African indigenous orchid genus *Disa* is needed to enable flower manipulation strategies and increase profitability. The efficacy of various factors known to affect flowering in orchids, as well as that of geophytes of the Cape floristic region, were evaluated for its ability to induce flowering in *Disa*. *Disa* hybrid plants (n = 20) were treated with a dilution series of smoke water and distilled water, ranging from 0, 1:10 000, 1:5000, 1:2000, 1:1000 and 1:500. The efficacy of the plant growth regulators MaxCel™ (6-benzyladenine (6-BA), 1.9% active ingredient $^{w}/_{w}$) (n = 20) and Promalin® (6-BA + Gibberellin G_{4+7} (GA_{4+7}), 1.8% active ingredient $^{w}/_{w}$, 1:1 ratio) (n = 20) to induce flowering in *Disa* were investigated by applying a concentration range of 0, 200, 400 and 600 mg.L^{-1} MaxCel™ and 0, 25, 50 and 100 mg.L^{-1} Promalin®, respectively. Lastly the role of potassium chlorate ($KClO_3$) (n=15) on flowering of *Disa* was studied by applying a $KClO_3$ concentration range of 0, 2, 4, 8 and 16 mmol.L^{-1}. The vegetative growth of the experimental plants were monitored over a six month period, using plant height (mm), crown width (mm) and number of unfolded leaves as growth parameters. Flowering was documented as date and percentage of spike emergence, anthesis and senescence respectively. None of the smoke water, growth regulators or $KClO_3$ treatments was able to induce flowering or advance flowering time. To the contrary, MaxCel™ and $KClO_3$ at the higher concentration levels impacted negatively on plant growth. Lower concentrations of the smoke water treatment appeared to have some beneficial effects on flowering and warrants further evaluation.

ADDITIONAL INDEX WORDS: Orchidaceae, terrestrial orchid, flowering signal

Disa uniflora is an indigenous South African orchid with unexploited commercial potential as *Disa* hybrids are currently cultivated commercially on a limited scale only (Coils, 2007). Scientific research is required to develop management systems which would enable the commercial viability of this orchid as a potted plant on the international market. One of the main factors contributing to successful cultivation and marketing of floriculture crops is a clear understanding of the nature of their flowering signal and the availability of flower manipulation strategies, based on the knowledge of these floral inducive signals. The different growth habits and wide range of natural habitats that orchids occupy world-wide allows for a variety of factors responsible for floral induction and initiation within the Orchidaceae. Acknowledging that evergreen Disas are temperate, terrestrial orchids, flower manipulation strategies commonly used in commercial tropical or sub-tropical orchids, might not be applicable to *Disa*. In *Disa* hybrids, 'LM0739' and 'LM0721' the timing of floral initiation was determined to be between the end of May and June, respectively (Paper 1). Floral

initiation in late autumn to early winter possibly implicates inductive conditions to include lower day- and night temperatures or a short day photoperiodic response. In a pilot study *Disa* hybrids did not respond to a short day signal (Paper 2). However, considering the flowering signals already established in orchids as well as inductive conditions known to affect geophytes of the Cape Floristic Region (CFR), additional clues may be provided to unravel the nature of the flowering signal for *Disa*.

SMOKE WATER. The genus *Disa* is considered to be well adapted to a fire prone environment along with many other Fynbos geophytes (Keely, 1993; Brown and Van Staden, 1997). Some *Disa* species may even be obligate fire dependant to secure flowering in the following season e.g. *D. racemosa* (Crous and Duncan, 2006). Another well-known fire dependant species of the CFR is *Cyranthus ventricosus*, the Fire lily, where these geophytes' flower development is stimulated at any time of the year within days after a veld fire (Keely, 1993).

Smoke treatments, either airborne or as aqueous extract have been studied for various commercial applications such as the release of dormancy in *Freesia* corms when exposed to smoke generated from smouldering plant material (Uyemura and Imanishi, 1983). In addition, smoke water has also been considered useful in promoting flowering of certain geophytes, as is shown in *Watsonia* where a 70% increase in flowering spike production was seen upon a single smoke water application (1:500) (Light et al., 2007). The inclusion of a 10% smoke-saturated-water (SSW) treatment enhanced the embryonic potential of *Geranium* hypocotyls. As SSW treatment also accelerated embryo development, a growth regulatory role was suggested for smoke (Senaratna et al., 1999). Smoke derived extracts have also been proved to stimulate root initiation and development (Taylor and Van Staden, 1996). However, probably the best known effect of smoke water is the enhancing of seed germination as this phenomenon has been observed in many families typical of fire prone environments such as the Proteaceae, Asteraceae, Ericaceae and Restionaceae (Brown, 1993). The promotive effect of smoke, derived from burnt vegetation, on seed germination was also evident when seeds from Western Australian species that were deemed difficult (even impossible) to germinate responded positively to smoke treatments (Dixon et al., 1995). However, the mechanism(s) or triggers for these smoke or fire dependant responses are not yet fully understood (Bytebier et al., 2011), but the active component has been identified as smoke-derived butenolides (Light et al., 2009).

PLANT GROWTH REGULATORS. Plant growth regulators have been used successfully to modify flowering of many orchid species. Responses are however strongly dependant on the orchid species and therefore extrapolation to other orchid species is not recommended. 6-Benzyladenine (6-BA) promotes flowering in both *Phalaenopsis* and *Doritaenopsis* orchids (Blanchard and Runkle, 2008). Three applications of each treatment were made at 2 hours, 7 days and 14 days after plants were moved into a greenhouse where conditions were inductive for flowering. When foliar application in a concentration range of 100, 200 and 400 $mg.L^{-1}$ was used, the higher concentrations of 200 and 400 $mg.L^{-1}$ produced inflorescences three to nine days earlier. In addition, these

treatments produced more inflorescences per plant as well as more flowers per inflorescence (Blanchard and Runkle, 2008). However, when gibberellin$_{4+7}$ (GA_{4+7}) was used in combination with 6-BA on *Phalaenopsi*s and *Doritaenopsis*, none of the beneficial effects reported for 6-BA alone were observed (Blanchard and Runkle, 2008). Matsumoto (2006) studied the effects of 6-BA and gibberellic acid (GA_3) separately as well as in combination on the flowering of *Miltoniopsis* orchids. Applications were made as soil drenches eight months after the plants have been transplanted and plants had 2-3 pseudobulbs. 6-BA alone and in combination with GA_3 produced fewer inflorescences than the control plants, while GA_3 alone hastened inflorescence emergence by at least 10 days, an advancement of flowering up to 48 days were reported for hybrid 'Rouge Akatsuka'. GA_3 also increased the number of inflorescences per plant (Matsumoto, 2006). In four-year-old *Paphiopedilum* ('Macabre' x *glanduliflerum*) orchids with three to four matured growths (fans), GA_3 induced flowering in 100% of the experimental plants in which no flowering had occurred previously over a four year period. However, 6-BA alone, or in combination with GA_3 did not induce inflorescences (Miguel et al., 2008). The different positive responses observed in other orchid genera in response to plant growth regulators, especially the advancement of flowering, the increase in the number of inflorescences per plant or the number of flowers per inflorescence, warranted a pilot-study to evaluate the efficacy of 6-BA alone and in combination with GA_{4+7} on the flowering of commercial *Disa* hybrids.

POTASSIUM CHLORATE. Some inorganic compounds can be used as an alternative to low temperature floral induction in some orchids and subtropical fruit trees (Manochai et al., 2005). One such chemical is potassium chlorate ($KClO_3$) which has been successfully used in Longan? plants to completely overcome the requirement for cool temperature induction (Manochai et al., 2005). $KClO_3$ also stimulated floral transition in spinach plants (Li et al., 2006). $KClO_3$ is reported to promote flower differentiation by increasing cytokinin-like substances whilst decreasing gibberellin-like substances (Wangsin and Pankasemuk, 2005). These mechanisms and also an increase in ethylene biosynthesis possibly mimic the effects of low temperature induction (Li et al., 2006). Li et al. (2006) further reported that $KClO_3$ at appropriate concentrations advanced the flowering of *Phalaenopsis* orchid and also increased the number of floral buds per spike. However, flower size was negatively affected.

The aim of this study was therefore an attempt to establish a possible inductive signal for flowering in *Disa* hybrids by conducting pilot-trials with treatments that have previously been found to promote flowering in either orchids or geophytes. These treatments include smoke water, 6-BA and GA_{4+7} and $KClO_3$. The likely inclusion of fire obligatory *Disa* species within the breeding program led to the addition of a range of smoke water concentrations as treatments. Furthermore, various reports on the efficacy of external growth regulator applications to induce flowering motivated the evaluation of MaxCel™ (BA), and Promalin® (BA:GA_{4+7}; 1:1) for its role in flowering of *Disa* hybrids. Lastly applications of $KClO_3$ which were found to be effective in modifying the

flowering biology of *Phalaenopsis*, a non-photoperiodic, but low temperature sensitive plant, justified a similar investigation on *Disa* hybrids.

Materials and Methods

PLANT MATERIAL. All plant material was obtained from La Motte Winery Estate in Franschhoek (33°52'49.9"S; 19°4'28.3"O), the only commercial *Disa* producer in South Africa. Hybrid 'LM0739' used in this trial has large red flowers at anthesis, with spike emergence at the beginning of October and anthesis during November. Parent plants used in the hybridization programs for 'LM0739' are unknown.

EXPERIMENTAL SITE. For a detailed description of the experimental site refer to Paper 1.

NUTRITION, WATER AND GROWTH MEDIUM. The nutrition, water and growth medium used for cultivation of plants were similar as described in Paper 1.

TEMPERATURE AND GROWING CONDITIONS. Temperatures and growing conditions as recorded within the shade- and greenhouses were reported in Paper 1.

TREATMENT AND EXPERIMENTAL DESIGN. *Smoke water*. For this trial, 120 *Disa* hybrid 'LM0739' plants were selected and grouped in a complete randomised design. The trial comprised of 6 treatments with 20 plants per treatment. The treatments included a control group and a series of smoke water (SW) dilutions that ranged between: 0 (distilled water), 1:10 000, 1:5000, 1:2000, 1:1000 and 1:500. The smoke water dilutions were applied by hand as a soil drench to each individual pot. The volume smoke water to ensure optimum exposure was determined to be 40 ml per plant, administered as 20 ml on opposite sides of the plant in the pot. Furthermore, four re-applications (n=20) of all treatments were done approximately one week apart on 28 Mar., 6 Apr., 13 Apr. and 20 Apr. 2011.

The smoke water concentrate used was prepared by Kirstenbosch Botanical Gardens (Rhodes Drive, Newlands, Cape Town, South Africa). As the biological activity of the active component in the smoke water sample was unknown, seed germination bio-assays were conducted. The bio-assay using the same dilution series was performed on both 'Grand Rapid' lettuce seeds as well as on fynbos *Buchu* seeds. The bio-assays were done by placing 25 each of *Buchu* and 'Grand Rapid' lettuce seeds into separate petri dishes on Whatman filter paper, with three repetitions for each seed type and treatment (25 seeds x 3 petri-dishes x 6 treatments). Smoke water dilutions from the same batch as used for *Disa* applications was applied to keep seeds moist throughout the experiment. The bio-assay was performed in darkness at room temperature (± 25°C), with only a safe green light switched on during seed inspections. 'Grand Rapid' lettuce seeds are known not to sprout in darkness, unless in the presence of smoke water, which serves as a substitute for light. *Buchu* seeds were included as a second bio-assay as the presence of smoke water is known to significantly increase germination of fynbos species.

Plant growth regulators. For the evaluation of floral initiation following plant growth regulator applications, 160 *Disa* hybrid 'LM0739' plants were selected in a complete randomised design. Different concentration ranges of both MaxCel™ (6-benzyladenine (6-BA), 1.9% active ingredient $^{w}/_{w}$; Valent BioSciences Corporation, Philagro South Africa, Pty. Ltd., Somerset West, South Africa) and Promalin® (6-BA:Gibberellins 4+7 (G_{4+7}), 1.8% active ingredient $^{w}/_{w}$, 1:1 ratio; Valent BioSciences Corporation, Philagro South Africa, Pty. Ltd., Somerset West, South Africa) were applied respectively. MaxCel™ concentration treatments consisted of 0, 200, 400 and 600 $mg.L^{-1}$, whereas Promalin® concentrations ranged between 0, 25, 50 and 100 $mg.L^{-1}$. All growth regulator treatments were prepared with the surfactant ABG-7044 (Valent BioSciences Corporation, Philagro South Africa, Pty. Ltd., Somerset West, South Africa) at 0.5 $ml.L^{-1}$. Treatments were applied once on 6 Apr. 2011. Each treatment comprised of 20 plants and the treatments were applied until run-off with a paint brush, to ensure adequate penetration to the apical meristem. The control treatment groups of both MaxCel™ and Promalin® were treated with distilled water.

Potassium chlorate. For this trial the efficacy of $KClO_3$ on the floral initiation of *Disa* hybrid 'LM0739' was evaluated on 75 plants which were selected in a complete randomised design. The experimental design comprised of 5 treatments with 15 plants per treatment. The treatments included a concentration range of $KClO_3$ (Sigma-Aldrich Pty. Ltd. Johannesburg, South Africa; Catalogue number: 255572) at 0, 2, 4, 8 and 16 $mmol.L^{-1}$. Treatments were applied once on 6 Apr. 2011. The $KClO_3$ dilutions were applied by hand as a soil drench to each individual pot. The optimum volume to ensure proper exposure was determined as 40 ml per plant, applied as 20 ml on opposite sides of the plant in the pot.

DATA RECORDED. In all three trials, after baseline measurements were obtained, growth as described by plant height (mm), crown width (mm) and number of unfolded leaves were recorded at six week intervals. Height was measured from the base of the plant to the tip of the newest unfolding leaf (Paper 1), while the crown width was measured form the tip of one unfolded mature leaf to the tip of the opposite mature unfolded leaf (Paper 3). All measurements were made using a 15 or 30 cm digital calliper and logger (Mitutoyo, Mitutoyo Corporation, Kawasaki, Japan).

The flowering data were first recorded on 8 Oct. 2011 and continued until 26 Jan. 2012. Together with the date of spike emergence, the date on which the first flower in the inflorescence opened (anthesis) and senesced were also recorded. Differences seen in percentage anthesis and senescence can be ascribed to the experimental loss of the flowering plants before senescence could be documented.

STATISTICAL ANALYSIS. Vegetative growth parameters, number of unfolded leaves, plant height (mm) and crown width (mm) over time was analyzed by means of Repeated Analysis of Variance (RANOVA), using Statistica 9.0 (Stasoft, Inc., Tulsa, Oklahoma, USA). In addition, on the last date of measurements (30 Sept.), just prior to spike emergence, the comparison of means was done by one-way ANOVA, using Fishers' LSD posthoc separation test using Statistica 10.0 (Stasoft

(SA) Inc., Tulsa, Oklahoma, USA). M-L Chi square analyses were also conducted to determine significant differences between flowering parameters with Statistica 10.0 (Stasoft (SA) Inc., Tulsa, Oklahoma, USA).

Results

SMOKE WATER. The results for both bio-assays were negative as 'Grand Rapid' lettuce seed germination occurred in all treatments, whilst no germination resulted with the *Buchu* seeds (data not shown). Number of unfolded leaves as indicator of growth showed a significant interaction between treatment and time ($P = 0.037$) (Fig. 1). All treatments showed a slight initial increase in leaf number from April to May/June, except for plants treated with the 1:1000 dilutions which showed a decline in unfolded leaf number directly after treatment. From May/June to August all treatments showed a decline in number of unfolded leaves, after which only plants treated with 1:1000 and 1:10 000 smoke water dilutions, respectively showed a slight increase towards spring.

Plant crown width and plant height as growth parameters showed no significant interaction between treatments and time ($P = 0.072$ and 0.119, respectively) (Fig. 2 and 3). Significant increases were found in crown width and height over time ($P < 0.001$), but not between treatments ($P = 0.484$ and 0.438, respectively) (Fig. 2 and 3). On 30 Sept., the last day of growth measurements just prior to spike emergence, no significant differences were seen between treatments for number of unfolded leaves ($P = 0.226$), crown width ($P = 0.459$) or plant height ($P = 0.202$) (Table 1).

No significant differences were found between the various smoke water treatments for spike emergence ($P = 0.403$), anthesis ($P = 0.753$) or senescence ($P = 0.753$). In addition, no real advancement of flowering time was observed for the smoke water treated plants when compared to the control plants (Table 2).

PLANT GROWTH REGULATORS. *MaxCel™.* For plants treated with MaxCel™ the growth parameter number of unfolded leaves showed a significant interaction between treatment and time ($P < 0.001$) (Fig. 4). The number of leaves of plants treated with MaxCel™ at 600 $mg.L^{-1}$ (MaxCel 600) was negatively affected as a severe drop in number of leaves was observed within 6 weeks after treatment. MaxCel™ 400 and Maxcel™ 200 followed a similar decline in number of unfolded leaves, but only from May onwards. When the number of unfolded leaves was compared on 30 Sept., the last date of measurements prior to spike emergence, the control group had a significantly higher number of unfolded leaves compared to plants treated with MaxCel™ (Table 3).

Crown width (mm) of plants treated with MaxCel™ showed a significant interaction between treatments and time ($P < 0.001$) (Fig. 5). MaxCel™ applied at 400 and 600 $mg.L^{-1}$ resulted in a sharp decrease in crown width from July to September. On 30 Sept. control plants had a significantly wider crown width, compared to plants that received MaxCel™. On this day plants treated with the two highest MaxCel™ concentrations had the lowest mean crown widths (Table 3).

Similar to the number of unfolded leaves and crown width, plant height as an estimation of growth, also showed a significant interaction between treatments and time ($P < 0.001$) (Fig. 6). All treatments showed an increase in plant height from April to September/October. Plants treated with MaxCel™ reached a maximum value already between May and July/August, whereas control plants continued to increase in height until Sept. On 30 Sept. no significant differences were found for plant height between the treatments, just prior to anthesis (Table 3).

The highest spike emergence percentage (60%) as well as the highest flowering percentage (15%) occurred in the control group (Table 4). No flowering occurred in plants treated with MaxCel™ at 400 mg.L^{-1}, whilst only one spike emerged and developed to anthesis in plants treated with 600 mg.L^{-1} MaxCel™. Plants treated with MaxCel™ at 200 mg.L^{-1} showed a low spike emergence of 25% with only 5% of spikes eventually reaching anthesis (Table 4). However, both anthesis and senescence percentages did not differ significantly between treatments and the control (Table 4).

Promalin®. Plants treated with Promalin® showed no significant interaction between the treatments with time ($P = 0.665$) in the number of unfolded leaves (Fig. 7). The number of unfolded leaves decreased significantly over time ($P < 0.001$), but no differences were found between treatments ($P = 0.600$). On 30 Sept., just prior to spike emergence the treatment groups did not differ significantly from each other in terms of number of unfolded leaves (Table 5).

No significant interaction between Promalin® treatments and time was found in either crown width or plant height (Fig. 8, $P = 0.070$; Fig. 9, $P = 0.099$). Significant differences were however found in both parameters over time ($P < 0.001$) as well as between treatments ($P < 0.001$) (Fig. 8 and 9). Crown width increased consistently over time for all treatments, whereas plant height increased over time for all treatments, from May to June/July onwards (Fig. 9). Plants treated with Promalin® at 50 and 100 mg.L^{-1} showed a consistently higher plant height throughout the trial as well as on 30 Sept. (Table 5; Fig. 9) compared to the other two treatments. Significant differences occurred between treatments in crown width on 30 Sept. The highest crown width was found in plants treated with 100 mg.L^{-1} Promalin® and differed significantly from the control and plants treated with 50 mg.L^{-1} Promalin® (Table 5).

Differences in percentage spike emergence, anthesis and senescence between treatments were non-significant. Furthermore, no real differences were observed in the time of anthesis between the treatments, except for plants treated with 50 mg.L^{-1} Promalin®, where anthesis occurred a week later than in other treatments (Table 6).

POTASSIUM CHLORATE. Significant interactions between treatment and time were observed in the number of unfolded leaves, crown width and height when plants were treated with $KClO_3$ ($P < 0.001$) (Fig. 10, 11 and 12). All $KClO_3$ treated plants showed a decline in number of leaves, except for the control plants where leaf number remained constant between 7 and 8 leaves throughout the monitoring period. Unfolded leaf number recorded on 30 Sept. showed that plants treated with 8

mmol.L^{-1} $KClO_3$ had significantly fewer leaves than the control and plants treated with 4 mmol.L^{-1} (Table 7).

The highest concentration of $KClO_3$ at 8 mmol.L^{-1} had a negative or toxic effect on crown width, showing a sharp decline from between 140 and 160 mm in July, to 80 mm in September/October, whereas crown width all the other treatments remained relatively constant at 140 and 160 mm throughout the monitoring period. On 30 Sept. plants treated with 8 mmol.L^{-1} $KClO_3$ had a much lower mean crown width than all the other treatments (Table 7).

The height of all the treated plants, except those treated with the highest $KClO_3$ concentration at 8 mmol.L^{-1} increased over time. Measurements on 30 Sept. showed that the control plants differed significantly in height from plants treated with $KClO_3$ at 4 and 8 mmol.L^{-1} (Table 7). All plants treated with $KClO_3$ at 16 mmol.L^{-1} died before any measurements could be recorded.

Total spike emergence and flowering of 67% and 53%, respectively were obtained in control plants (Table 8). Six percent (%) spike emergence was found in plants treated with 8 mmol.L^{-1} $KClO_3$, while plants treated with $KClO_3$ at 2 mmol.L^{-1} had 47% spike emergence and 33% flowering. Although spike emergence of plants that received a 4 mmol.L^{-1} $KClO_3$ application was fairly similar to that of plants treated with 2 mmol.L^{-1} $KClO_3$, the corresponding flowering percentage was much lower. The time of spike emergence, anthesis and senescence seemed to differ between the control plants and the plants treated with $KClO_3$. Spike emergence was recorded much later for the 4 and 8 mmol.L^{-1} $KClO_3$ treated plants on 21 Dec. and 01 Dec., respectively compared to the control plants where spike emergence was recorded on 03 Nov. Anthesis of the 2 mmol.L^{-1} $KClO_3$ treated group was also delayed by one week (Table 8).

Discussion

SMOKE WATER. Firstly, the lack of response obtained from the conducted smoke water bio-assays impedes the interpretation of the smoke water results. The reason(s) for the inability of viable *Buchu* seeds to germinate is not clear and questions the bioactivity of the smoke water extract. Similarly, the very high percentage of germination recorded for the 'Grand Rapid' lettuce seeds, even for the control seeds, which germinated in the absence of light or smoke water, is difficult to explain and nullified the use of 'Grand Rapid' seeds as a bio-assay for smoke water activity. This problem was also experienced by other researchers (Hills, personal communication, 2012). The general growth vigour of the smoke water treated plants as well as high spike emergence and anthesis percentages recorded within this study suggests a possible positive effect of smoke water on *Disa* growth and flowering although the negative bio-assay results impedes further discussion of the smoke water results. Nevertheless, before smoke water as a treatment to enhance flowering in *Disa* is dismissed as ineffective, it is suggested that such a trial should be repeated. In a re-assessment trial, a positive bio-assay should be obtained first, using freshly prepared smoke water dilutions. Such a trial should also

include more replications per treatment together with various combinations of single as well as multiple application times.

PLANT GROWTH REGULATORS. *MaxCel*™. The number of leaves of a plant is important, as it directly determines the photosynthetic capacity of a plant, and ultimately whether a plant will be able to support a flowering spike. All MaxCel™ treatments negatively influenced the number of unfolded leaves compared to the control.

Similarly to number of unfolded leaves, crown width, which also implicates photosynthetic capacity through leaf area, was also negatively influenced by all the MaxCel™ treatments. Similarly the non-treated MaxCel™ control plants were the highest. Therefore, when considering only the vegetative growth parameters, MaxCel™ treatments clearly had a negative to toxic effect on plant growth. This detrimental effect impacted on flowering as significantly fewer spikes emerged. However, even in the control, a low flowering percentage of only 15% was recorded, which did not differ significantly from that of the cytokynin treated plants. Considering both the negative effects seen on the vegetative growth as well as on flowering incidences, treatments at the tested concentration levels with MaxCel™ is currently not recommended for flower manipulation strategies at the concentrations tested. However, further tests to establish the efficacy of much lower concentrations such as 25, 50, 100 and 150 $mg.L^{-1}$ MaxCel™ to manipulate flowering is recommended.

Promalin®. In contrast to the results found with MaxCel™, Promalin® exerted more positive effects on vegetative plant growth in treated plants with regards to leaf crown width and plant height. However, no significant differences in spike emergence or anthesis were recorded between treatments. Furthermore, no advancement of flowering time was seen in the Promalin® treated plants. No advancements of flowering time or higher flowering percentages were documented for any of the plant growth regulator treated plants, compared to the control. The plant growth regulators MaxCel™ and Promalin®, at the concentrations tested, did not display any potential for use on commercial *Disa* hybrids to improve flowering. Considering the positive effects found with the application of GA_3 alone on the advancement of flowering in *Miltoniopsis* orchids (Matsumoto, 2006) and the promotion of floral initiation in non-flowering *Paphiopedilum* orchids (Miguel et al., 2008), the effects of GA_3 on *Disa* hybrids warrants further research.

POTASSIUM CHLORATE. Vegetative growth was mostly negatively affected by the application of $KClO_3$. Plants treated with $KClO_3$ at 8 $mmol.L^{-1}$ showed clear symptoms of toxicity with regards to all the parameters measured and virtually no flowering occurred with this treatment. Furthermore, all the plants treated with the highest concentration of $KClO_3$ at 16 $mmol.L^{-1}$ died shortly after application. These effects might be ascribed to the fact that *Disa* is known to be intolerant to saline environments (Crous and Duncan, 2006). Although the $KClO_3$ treatments at lower concentrations of 2 and 4 $mmol.L^{-1}$ might not be toxic, the lower spike emergence and flowering percentages recorded for these two treatments compared to the control, also with no real difference in time of flowering

suggests that $KClO_3$, even at low concentrations, is not a viable option for future manipulation strategies of flowering time in *Disa* hybrids.

Conclusion

Results from these pilot studies showed that neither smoke water, MaxCel™, Promalin®, or $KClO_3$ at the concentrations tested currently provides any indication of a possible flowering signal or can be used as viable methods to manipulate flowering time. The uncertainty regarding the activity of the smoke water solution used in this study underpins the recommendation that such a study should be repeated using confirmed biological active smoke water. Although the flowering signal for *Disa* was not elucidated through these studies, the results obtained serve as a reference and baseline to pursue future studies using lower concentration levels of the evaluated compounds or evaluate other known elicitors of flowering, yet untested on *Disa*.

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Table 1. Vegetative growth of *Disa* hybrid 'LM0739' plants determined by the parameters number of unfolded leaves, crown width (mm) and plant height (mm) after treatment with a range of smoke water dilutions ranging from a control (smoke water omitted), 1:10 000; 1:5000; 1:2000; 1:1000 and 1:500 respectively. Measurements were taken on 30 Sept. 2011 on the last day of growth measurements, just prior to spike emergence, after plants have been exposed to treatments for a 6 month period.

Smoke water	**Treatment**	**Number of unfolded leaves**	**Crown width (mm)**	**Height (mm)**
(n=20)	0	6.65 NS[z]	149.06 NS	46.78 NS
	1:10 000	7.24	144.28	56.54
	1:5000	6.47	147.71	55.89
	1:2000	6.06	141.37	49.97
	1:1000	6.12	135.95	47.27
	1:500	6.29	137.57	44.59
ANOVA	F	1.413	0.940	1.4822
	P	0.226	0.459	0.202

[z] NS. Indicates no significant differences for mean values at the 5 % confidence level.

Table 2. The distribution of flowering patterns of *Disa* hybrid 'LM0739' plants after soil drenches with smoke water diluted in a concentration range of 0 (control), 1:10 000; 1:5000; 1:2000; 1:1000 and 1:500. Flowering was monitored from 21 Oct. 2011 (first signs of spike emergence) until 19 Jan. 2012 using spike emergence, anthesis and senescence as indicators. Data are presented as percentages (%) of the monitored population.

				Monitoring dates (2011 - 2012)						
Treatment: Smoke water	**n**	**Total (%)**	**Date ± SE**	**21-Oct - 27 Oct**	**03 Nov - 08 Nov**	**18 Nov - 23 Nov**	**02 Dec - 09 Dec**	**12-Dec - 20 Dec**	**30 Dec - 04-Jan**	**09 Jan - 19-Jan**
Experimental plants	**20**									
0 (Control)										
Died prior to spike emergence	3	15	N/A[z]							
Non flowering	4	20	N/A							
Spike emergence	13	65	21 Nov. ± 4.3	7.7	15.4	46.2	15.4	15.4	0	0
Anthesis	6	30	22 Dec. ± 3.1					66.7	33.3	
Senescence	6	30	01 Jan. ± 4.5					16.7	66.7	16.7
1:10 000										
Died prior to spike emergence	3	15	N/A							
Non flowering	0	0	N/A							
Spike emergence	17	85	19 Nov. ± 2.7	5.9	11.8	52.9	29.4			
Anthesis	9	45	21 Dec. ± 2.6					77.8	22.2	
Senescence	9	45	05 Jan. ± 0.9						66.7	33.3
1:5000										
Died prior to spike emergence	3	15	N/A							
Non flowering	3	15	N/A							
Spike emergence	14	70	14 Nov. ± 4.0	28.6		50.0	21.4			
Anthesis	8	40	19 Dec. ± 3.7				25	37.5	37.5	
Senescence	8	40	02 Jan. ± 4.4					25	50	25
1:2000										
Died prior to spike emergence	2	10	N/A							
Non flowering	3	15	N/A							
Spike emergence	15	75	15 Nov. ± 3.9	20	20	33.3	26.7			
Anthesis	6	30	24 Dec. ± 5.0					50	33.3	16.7
Senescence	6	30	31 Dec. ± 3.0					33.3	50	16.7
1:1000										
Died prior to spike emergence	4	20	N/A							
Non flowering	5	25	N/A							
Spike emergence	11	55	12 Nov. ± 3.4	9.1	54.5	27.3	9.1			
Anthesis	8	40	18 Dec. ± 5.3			12.5		50	37.5	
Senescence	8	40	07 Jan. ± 1.3						87.5	12.5
1:500										
Died prior to spike emergence	4	20	N/A							
Non flowering	3	15	N/A							
Spike emergence	13	65	20 Nov. ± 4.2	7.7	23.1	38.5	7.7			
Anthesis	5	25	24 Dec. ± 5.4				60	20	20	
Senescence	5	25	03 Jan. ± 5.4				20	60	20	

M-L Chi square

	M-L Chi square	***P* -value**	**Significance**
Spike emergence	5.105	0.403	NS[y]
Anthesis	2.658	0.753	NS
Senescence	2.658	0.753	NS

[z] N/A. Indicates not applicable.

[y] NS. Indicates no significant difference at the 5% confidence level.

Table 3. The vegetative growth of *Disa* hybrid 'LM0739' determined by the parameters number of unfolded leaves, crown width (mm) and plant height (mm) after treatment with a concentration range of MaxCel™ (Benzyladenine (BA), 1.9% active ingredient $^{w}/_{w}$) applications at 0, 200, 400 and 600 mg.L^{-1}. Measurements were taken on 30 Sept. 2011, just prior to spike emergence, after plants have been exposed to the MaxCel™ treatments for a 6 month period.

MaxCel™	**Treatment (mg. L^{-1})**	**Number of unfolded leaves**	**Crown width (mm)**	**Height (mm)**
(n=20)	0	6.69 a[z]	142.41 a	46.52 NS[y]
	200	5.41 b	111.78 b	37.40
	400	5.39 b	74.34 c	32.31
	600	5.07 b	83.35 c	37.40
ANOVA	F	2.882	13.655	1.644
	P	0.044	<0.001	0.189

[z] Different letters indicate a significant difference for means at the 5% confidence level.
[y] NS. Indicates no significant differences for mean values at the 5 % confidence level.

Table 4. The distribution of flowering patterns of *Disa* hybrid 'LM0739' plants following foliar treatments with MaxCel™ (Benzyladenine (BA), 1.9% active ingredient $^{w}/_{w}$) in a concentration range of 0, 200, 400 and 600 mg.L^{-1}. Flowering was monitored from 21 Oct. 2011 (first signs of spike emergence) until 19 Jan. 2012 using spike emergence, anthesis and senescence as indicators. Data are presented as percentages (%) of the monitored population.

				Monitoring dates (2011 - 2012)						
Treatment: MaxCel™	**n**	**Total (%)**	**Date ± SE**	**21-Oct. – 27 Oct.**	**03-Nov. - 08 Nov.**	**18-Nov. - 23 Nov.**	**02-Dec. - 09 Dec.**	**12-Dec. - 20 Dec.**	**30 Dec. - 04-Jan.**	**09 Jan. - 19-Jan.**
Experimental plants	**20**									
0 (Control)										
Died prior to spike emergence	3	15	N/A[z]							
Non flowering	5	25	N/A							
Spike emergence	12	60	22 Nov. ± 5.0	16.7		41.7	25	16.7		
Anthesis	3[y]	15	16 Dec. ± 7.0						33.3	66.7
Senescence	2[y]	10	19 Jan. [x]							100
200 mg.L^{-1}										
Died prior to spike emergence	3	15	N/A							
Non flowering	12	60	N/A							
Spike emergence	5	25	18 Nov. ± 5.0	20	20	20	40			
Anthesis	1	5	09 Dec. [x]				100			
Senescence	1	5	04 Jan. [x]							100
400 mg.L^{-1}										
Died prior to spike emergence	7	35	-[w]	-	-	-	-	-	-	-
Non flowering	13	65	-	-	-	-	-	-	-	-
Spike emergence	0	0	-	-	-	-	-	-	-	-
Anthesis	0	0	-	-	-	-	-	-	-	-
Senescence	0	0	-	-	-	-	-	-	-	-
600 mg.L^{-1}										
Died prior to spike emergence	5	25	N/A							
Non flowering	14	70	N/A							
Spike emergence	1	5	21 Oct. [x]	100						
Anthesis	1	5	12 Dec. [x]					100		
Senescence	1	5	04 Jan. [x]						100	

M-L Chi square

	M-L Chi square	***P* - value**	**Significance**
Spike emergence	27.952	<0.001	***[v]
Anthesis	4.617	0.202	NS[U]
Senescence	2.878	0.411	NS

[z] N/A. Indicates not applicable.

[y] Differences seen in number of anthesis and senescence can be ascribed due to a loss of plant material before documented senescence.

[x] Insufficient number of replicates to calculate a Standard Error of the Mean.

[w] -. Indicates no data collected as no flowering incidences occurred.

[v] * Indicates the level of significance.

[u] NS. Indicates no significant differences at the 5% confidence level.

Table 5. The vegetative growth of *Disa* hybrid 'LM0739' determined by the parameters of number of unfolded leaves, crown width (mm) and plant height (mm). Treatments consisted of a concentration range of Promalin® (BA:Gibberellic acid (GA_{4+7}), 1.8% active ingredient $^{w}/_{w,}$ 1:1) at 0, 25, 50 and 100mg.L^{-1} respectively. Measurements were taken on 30 Sept. 2011, just prior to spike emergence, after plants have been exposed to the Promalin® treatments for a 6 month period.

Promalin®	**Treatment (mg.L^{-1})**	**Number of unfolded leaves**		**Crown width (mm)**		**Height (mm)**	
(n=20)	0	6.41	NS[z]	129.61	c[y]	47.33	b
	25	6.13		181.97	ab	53.40	b
	50	6.65		158.59	bc	81.10	a
	100	6.07		201.78	a	81.01	a
ANOVA	F	0.458		5.176		6.415	
	P	0.712		0.003		0.008	

[z]NS. Indicates no significant difference for mean values at the 5% confidence level.
[y]Different letters indicate a significant difference for means at the 5% confidence level.

Table 6. The distribution of flowering patterns of *Disa* hybrid 'LM0739' plants following foliar treatments with Promalin® (BA:Gibberellic acid (GA_{4+7}), 1.8% active ingredient $^{w}/_{w}$, 1:1) in a concentration range of 0, 25, 50 and 100 $mg.L^{-1}$. Flowering was monitored using spike emergence, anthesis and senescence as indicators. Measurements were taken from 21 Oct. 2011 (first signs of spike emergence) until 19 Jan. 2012. Data is presented as percentages (%) of the monitored population.

				Monitoring dates (2011 - 2012)						
Treatment: Promalin®	**n**	**Total (%)**	**Date ± SE**	**21-Oct. - 27 Oct.**	**03-Nov. - 08 Nov.**	**18-Nov. - 23 Nov.**	**02-Dec. - 09 Dec.**	**12-Dec. - 20 Dec.**	**30 Dec. - 04-Jan.**	**09 Jan. - 19-Jan.**
Experimental plants	**20**									
0 (Control)										
Died prior to spike emergence	3	15	N/A[z]							
Non flowering	8	40	N/A							
Spike emergence	9	45	23 Nov. ± 6.1		33.3	33.3	11.1	22.2		
Anthesis	3	15	30 Dec. ± 0.0						100.0	
Senescence	3	15	04 Jan. ± 7.1							100.0
25 $mg.L^{-1}$										
Died prior to raceme emergence	4	20	N/A							
Non flowering	12	60	N/A							
Raceme emergence	4	20	23 Nov. ± 6.1			75.0	25.0			
Anthesis	2	10	30 Dec. ± 0.0						100.0	
Senescence	2	10	04 Jan. ± 7.1						50.0	50.0
50 $mg.L^{-1}$										
Died prior to raceme emergence	3	15	N/A							
Non flowering	13	65	N/A							
Spike emergence	4	20	24 Nov. ± 4.8			33.3	11.1	55.6		
Anthesis	2	10	05 Jan. ± 5.8						33.3	33.3
Senescence	2	10	14 Jan. ± 6.1						66.7	66.7
100 $mg.L^{-1}$										
Died prior to spike emergence	5	25	N/A							
Non flowering	12	70	N/A							
Spike emergence	1	5	4 Dec. ± 10.7			50.0		50.0		
Anthesis	1	5	30 Dec.[y]						100.0	
Senescence	1	5	04 Jan.[y]						100.0	

M-L Chi square

	M-L Chi square	***P* - value**	**Significance**
Spike emergence	5.810	0.121	NS[x]
Anthesis	1.513	0.679	NS
Senescence	1.513	0.679	NS

[z] N/A. Indicates not applicable.

[y] Insufficient number of replicates to calculate a Standard Error of the Mean.

[x] NS. Indicates no significant differences at the 5% confidence level.

Table 7. The vegetative growth of *Disa* hybrid 'LM0739' determined by the parameters number of unfolded leaves, crown width (mm) and plant height (mm) after treatment with a concentration range of $KClO_3$ at 0, 2, 4 and 8 $mg.L^{-1}$. Measurements were taken on 30 Sept. 2011, just prior to spike emergence, after plants have been exposed to treatments for a 6 month period.

$KClO_3$	**Treatment ($mmol.L^{-1}$)**	**Number unfolded of leaves**		**Crown width (mm)**		**Height (mm)**	
(n = 15)	0	7.25	a[z]	143.57	a	61.59	a
	2	6.25	ab	123.92	a	50.04	ab
	4	6.55	a	146.17	a	37.51	bc
	8	4.91	b	80.01	b	31.66	c
ANOVA	F	12.862		8.807		6.169	
	P	<0.001		<0.001		0.0014	

[z] Different letters indicate a significant difference for means at the 5% confidence level.

Table 8. The distribution of flowering patterns of *Disa* hybrid 'LM0739' plants after soil drenches with a concentration range of $KClO_3$ at of 0, 2 4, 8 and 16 mmol.L^{-1}. Flowering was monitored from 08 Oct. 2011 (first signs of spike emergence) until 04 Jan. 2012, using spike emergence, anthesis and senescence, as indicators. Data are presented as percentages (%) of the monitored population.

				Monitoring dates (2011 - 2012)						
Treatment: $KClO_3$	**n**	**Total (%)**	**Date ± SE**	**08 Oct - 13 Oct**	**21-Oct - 27 Oct**	**03 Nov - 08 Nov**	**18 Nov - 23 Nov**	**02 Dec - 09 Dec**	**12-Dec - 20 Dec**	**30 Dec - 04-Jan**
Experimental plants	**15**									
0 mmol. L^{-1}										
Died prior to spike emergence	4	27	N/A[z]							
Non flowering	1	6	N/A							
Spike emergence	10	67	03 Nov. ± 5.2		50.0	30.0	10.0		10.0	
Anthesis	8	53	13 Dec. ± 2.5					37.5	62.5	
Senescence	8	53	21 Dec. ± 2.2						75	25
2 mmol. L^{-1}										
Died prior to spike emergence	3	20	N/A							
Non flowering	5	33	N/A							
Spike emergence	7	47	31 Oct. ± 4.8	57.1	28.6	14.3				
Anthesis	5[y]	33	13 Dec. ± 3.0					60	40	
Senescence	1[y]	6	30 Dec. ± 0.0							100
4 mmol. L^{-1}										
Died prior to spike emergence	4	27	N/A							
Non flowering	3	20	N/A							
Spike emergence	8	53	21 Dec. ± 10.9				37.5	62.5		
Anthesis	1	6	20 Dec. ± [x]						100	
Senescence	0	0	-[w]	-	-	-	-	-	-	-
8 mmol. L^{-1}										
Died prior to spike emergence	7	47	N/A							
Non flowering	7	47	N/A							
Spike emergence	1	6	01 Dec. ± 4.1			100				
Anthesis	0	0	-	-	-	-	-	-	-	-
Senescence	0	0	-	-	-	-	-	-	-	-
16 mmol. L^{-1}										
Died prior to spike emergence	15	100	-	-	-	-	-	-	-	-
Non flowering	0	0	-	-	-	-	-	-	-	-
Spike emergence	0	0	-	-	-	-	-	-	-	-
Anthesis	0	0	-	-	-	-	-	-	-	-
Senescence	0	0	-	-	-	-	-	-	-	-

M-L Chi square

	M-L Chi square	***P* - value**	**Significance**
Spike emergence	28.905	<0.001	***[v]
Anthesis	26.794	<0.001	***
Senescence	26.963	<0.001	***

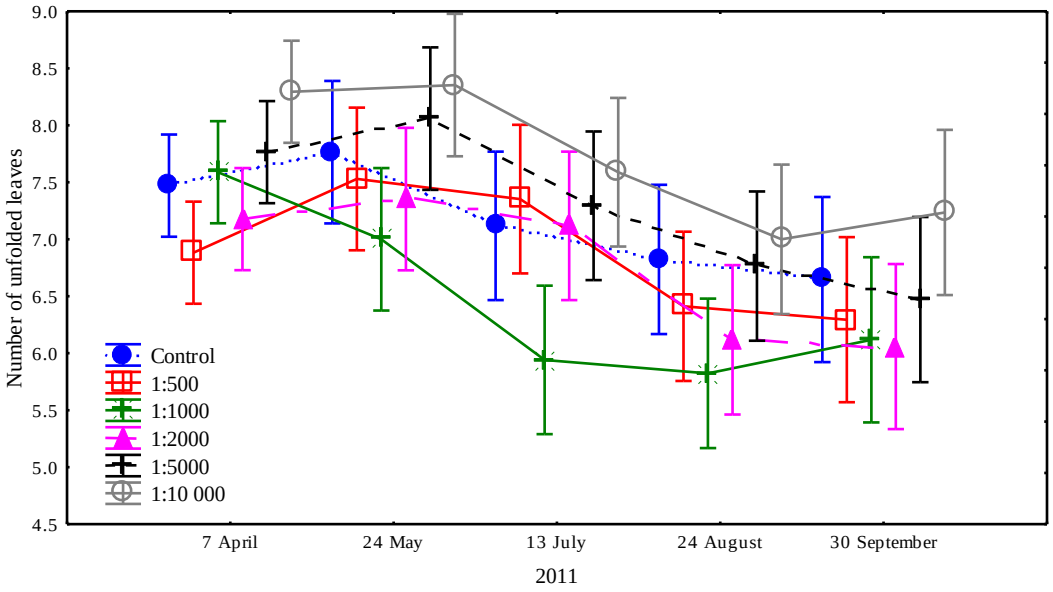
[z] N/A. Indicates not applicable.

[y] Differences seen in number of anthesis and senescence can be ascribed due to a loss of plant material before documented senescence.

[x] Insufficient number of replicates to calculate a Standard Error of the Mean.

[w]- Indicates no data collected as no flowering incidences occurred.

[v]* Indicates the level of significance.



RANOVA	F-value	*P* - value	Significance
Treatment	2.878	0.018	*[z]
Time	40.045	<0.001	***
Treatment*Time	1.662	0.037	*

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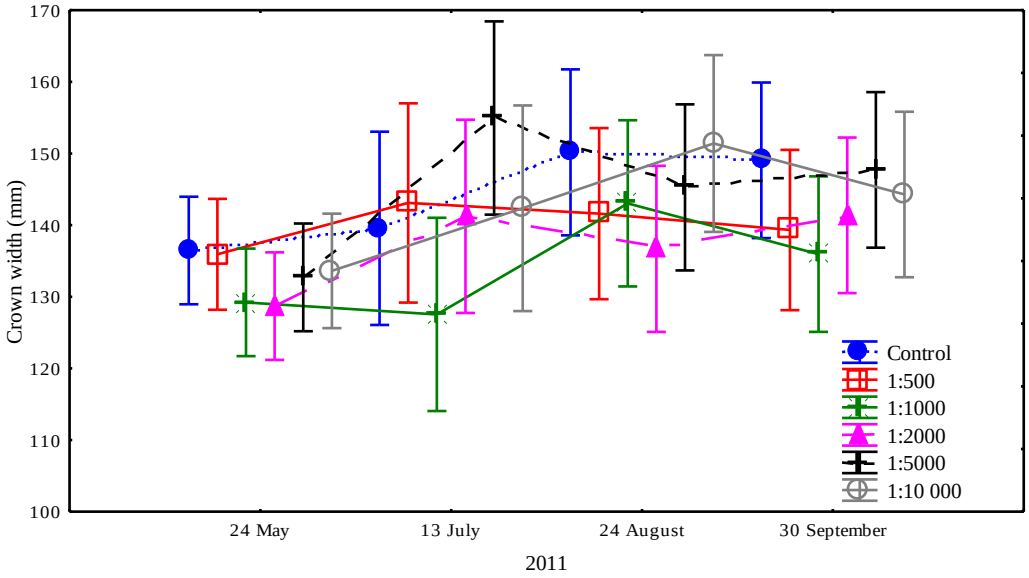
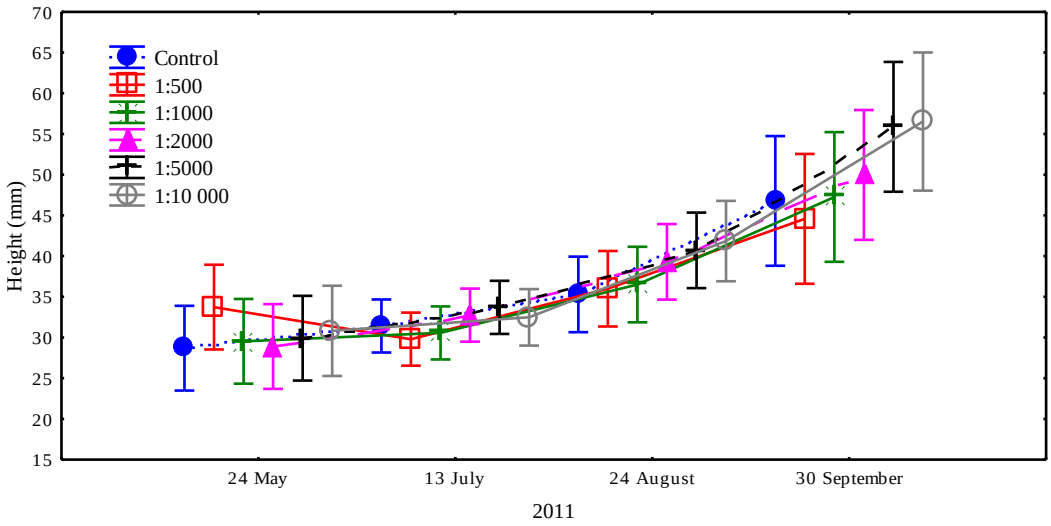
Fig. 1. Growth responses of *Disa* hybrid 'LM0739' over a six month period using number of unfolded leaves (n=20) as growth parameter after plants were treated with a smoke water dilution range of 0; 1:10 000; 1:5000; 1:2000; 1:1000 and 1:500 on 28 Mar., 6 Apr., 13 Apr. and 20 Apr. 2011 respectively. Vertical error bars indicate the Standard Error of the Mean for each data point. [z]* Indicates the level of significance.



RANOVA	F-value	*P* - value	Significance
Treatment	0.901	0.484	NS[z]
Time	11.777	<0.001	***[y]
Treatment* Time	1.605	0.072	NS

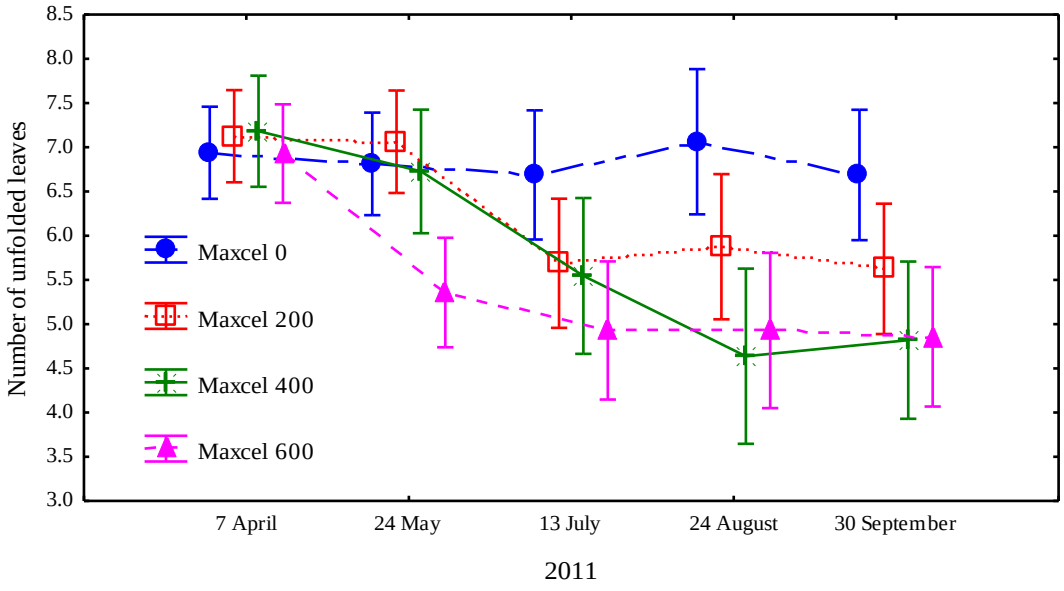
Fig. 2. Growth responses of *Disa* hybrid 'LM0739' over a six month period using crown width (mm) (n=20) as growth parameter after plants were treated with a smoke water dilution range of 0; 1:10 000; 1:5000; 1:2000; 1:1000 and 1:500 on 28 Mar., 6 Apr., 13 Apr. and 20 Apr. 2011 respectively. Vertical error bars indicate the Standard Error of the Mean for each data point. [z]NS. Indicates no significant difference at the 5% confidence. [y]* Indicates the level of significance.



RANOVA	F-value	*P* - value	Significance
Treatment	0.974	0.438	NS[z]
Time	113.268	<0.001	***[y]
Treatment*Time	1.462	0.119	NS

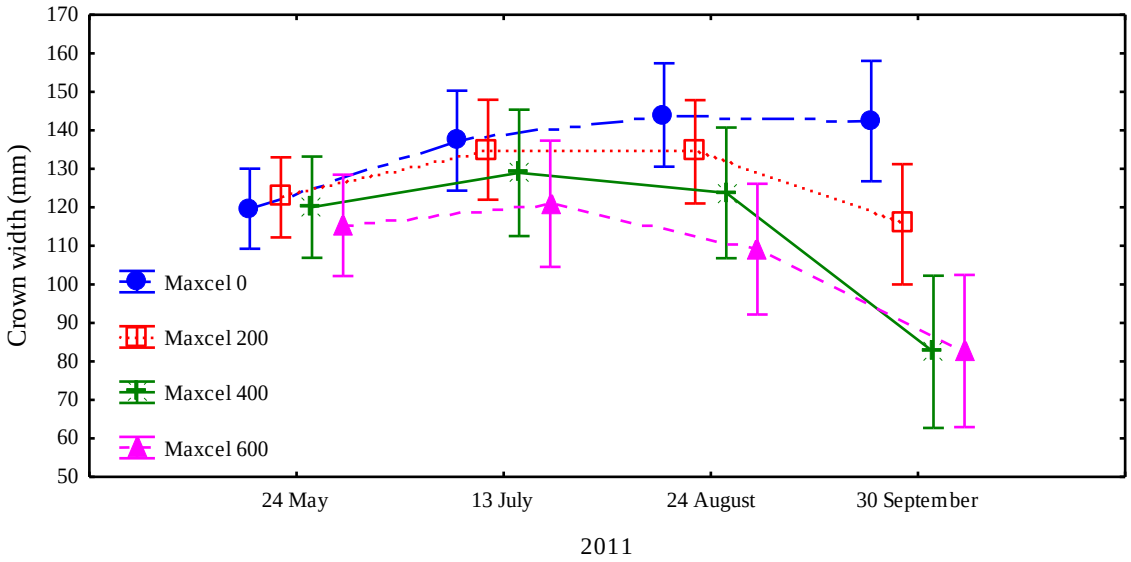
Fig. 3. Growth responses of *Disa* hybrid 'LM0739' over a six month period using plant height (mm) (n=20) as growth parameter after plants were treated with a smoke water dilution range of 0; 1:10 000; 1:5000; 1:2000; 1:1000 and 1:500 on 28 Mar., 6 Apr., 13 Apr. and 20 Apr. 2011 respectively. Vertical error bars indicate the Standard Error of the Mean for each data point. [z]NS. Indicates no significant differences at the 5% confidence.

[y]* Indicates the level of significance.



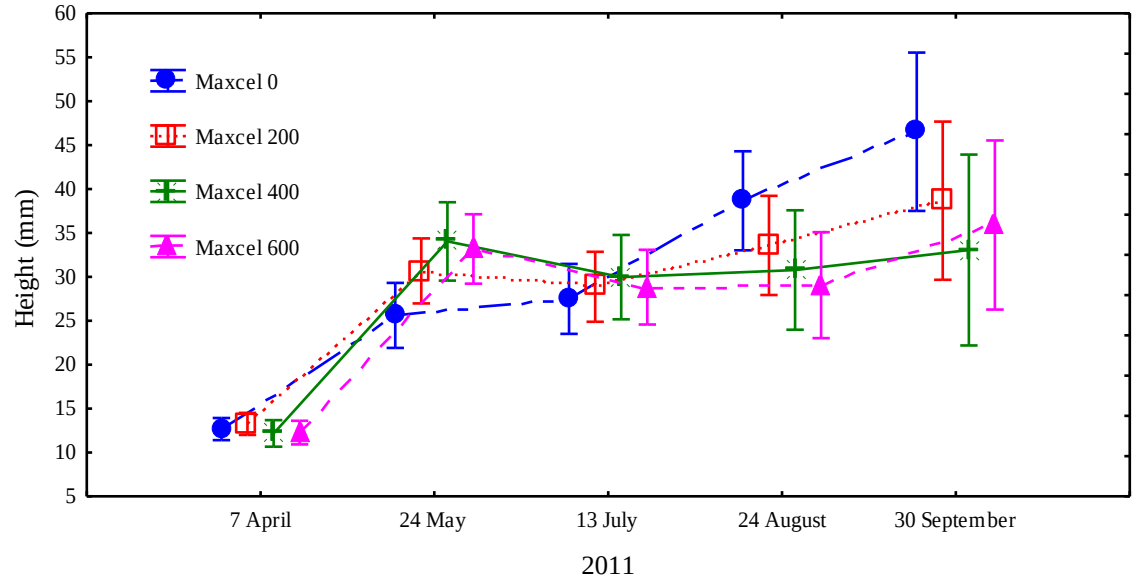
RANOVA	F-value	*P* - value	Significance
Treatment	5.677	0.002	***[z]
Time	22.742	<0.001	***
Treatment*Time	3.734	<0.001	***

Fig. 4. Growth responses of *Disa* hybrid 'LM0739' plants over a six month period using number of unfolded leaves (n=20) as growth parameter after plants were treated with a concentration range of MaxCel™ (Benzyladenine (BA), 1.9% active ingredient w/w) at 0, 200, 400 and 600 mg.L^{-1} respectively. Vertical error bars indicate the Standard Error of the Mean for each data point. [z]* Indicates level of significance.



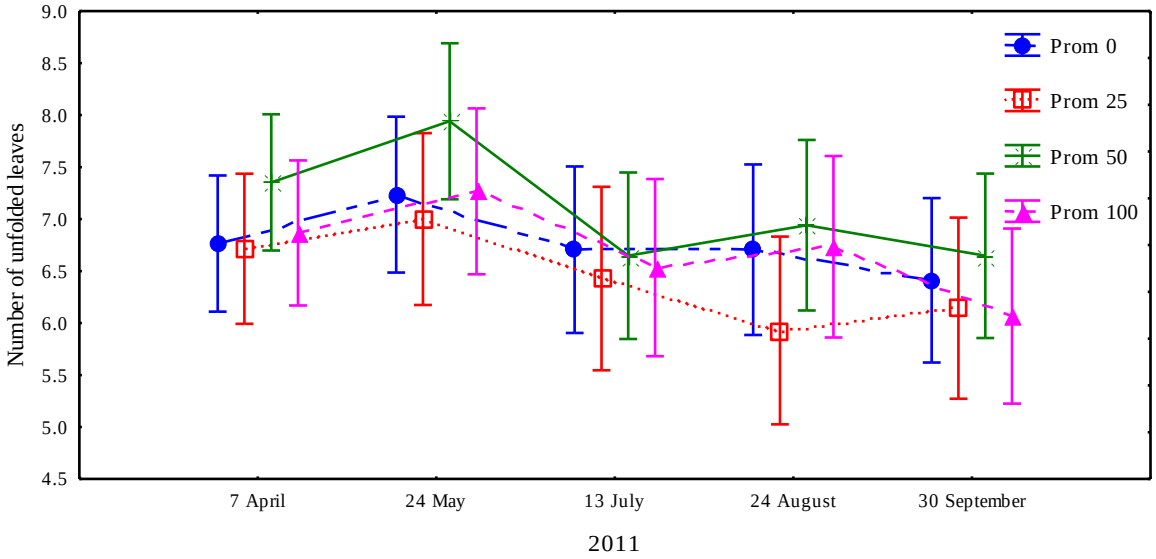
RANOVA	F-value	*P* - value	Significance
Treatment	4.463	0.008	***[z]
Time	20.365	<0.001	***
Treatment*Time	6.529	<0.001	***

Fig. 5. Growth responses of *Disa* hybrid 'LM0739' plants over a six month period using crown width (mm) (n=20) as growth parameter after plants were treated with a concentration range of MaxCel™ (Benzyladenine (BA), 1.9% active ingredient w/w) at 0, 200, 400 and 600 mg.L^{-1} respectively. Vertical error bars indicate the Standard Error of the Mean for each data point. [z]* Indicates level of significance.



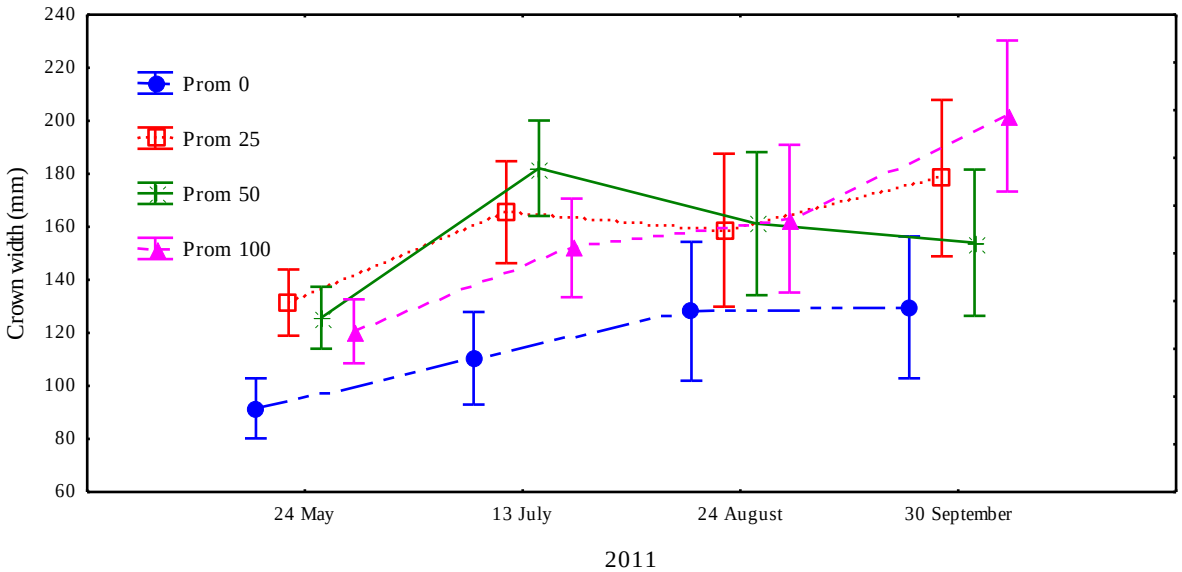
RANOVA	F-value	*P* - value	Significance
Treatment	0.2819	0.838	NS[y]
Time	78.0187	<0.001	***[z]
Treatment*Time	3.2326	<0.001	***

Fig. 6. Growth responses of *Disa* hybrid 'LM0739' plants over a six month period using plant height (mm) (n=20) as growth parameter after plants were treated with a concentration range MaxCel™ (Benzyladenine (BA), 1.9% active ingredient w/w) at 0, 200, 400 and 600 mg.L^{-1} respectively. Vertical error bars indicate the Standard Error of the mean for each data point. [z]* Indicates level of significance. [y]NS. Indicates no significant difference at the 5% confidence level.



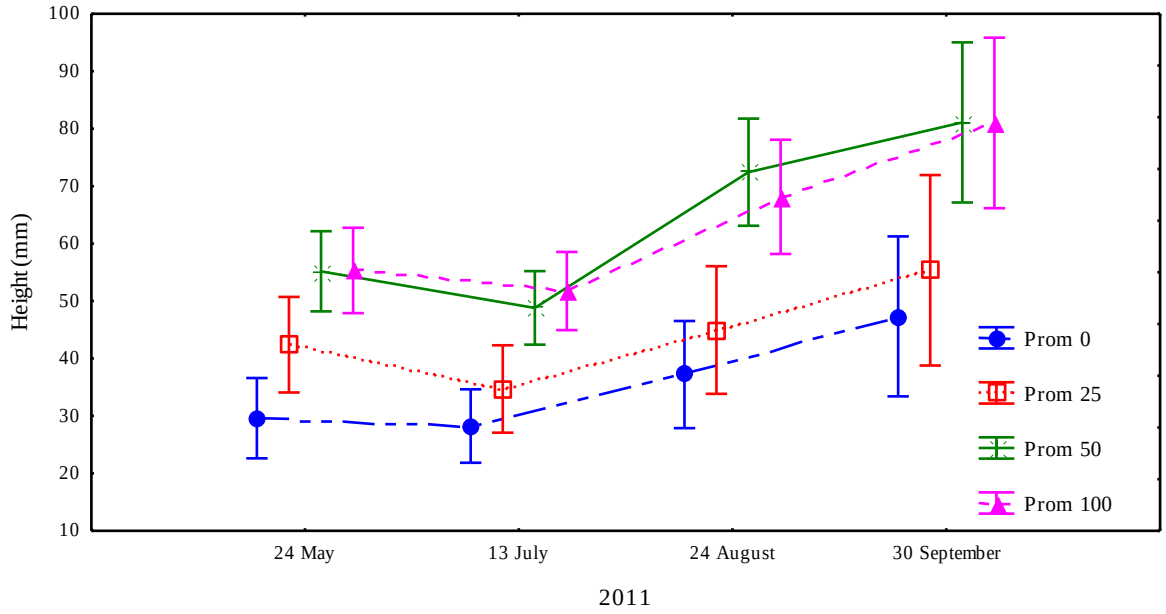
RANOVA	F-value	*P* - value	Significance
Treatment	0.628	0.600	NS[z]
Time	12.940	<0.001	***[y]
Treatment*Time	0.786	0.665	NS

Fig. 7. Growth responses over a six month period of *Disa* hybrid 'LM0739' plants using number of unfolded leaves (n=20) as growth parameter following treatment with Promalin® (Benzyladenine (BA):Gibberellic acid (GA_{4+7}); 1.8% active ingredient $^{w}/_{w}$, 1:1) of 0, 25, 50 and 100 mg.L^{-1} respectively. Vertical error bars indicate the Standard Error of the Mean for each data point. [z]NS. Indicates no significant difference at the 5% confidence levels. [y]* Indicates level of significance.



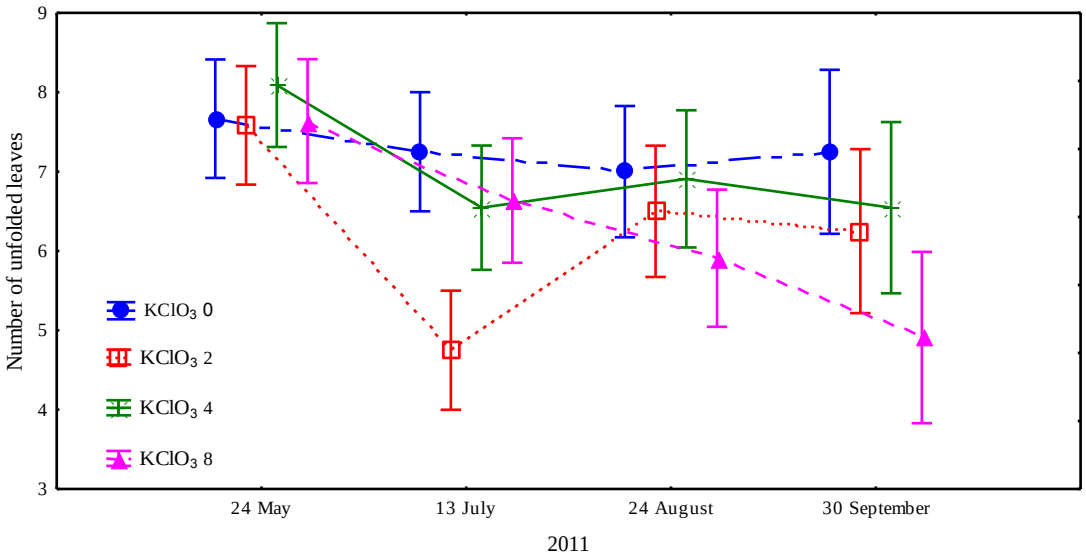
RANOVA	F-value	*P* - value	Significance
Treatment	0.365	<0.001	***[z]
Time	2.136	<0.001	***[y]
Treatment*Time	2.065	0.070	NS

Fig. 8. Growth responses over a six month period of *Disa* hybrid 'LM0739' plants using crown width (mm) (n=20) as growth parameter following treatment with Promalin® (Benzyladenine (BA):Gibberellic acid (GA_{4+7}); 1.8% active ingredient $^{w}/_{w}$, 1:1) at 0, 25, 50 and 100 mg.L^{-1} respectively. Vertical error bars indicate the Standard Error of the Mean for each data point. [z]NS. Indicates no significant difference at the 5% confidence level. [y]* Indicates level of significance.



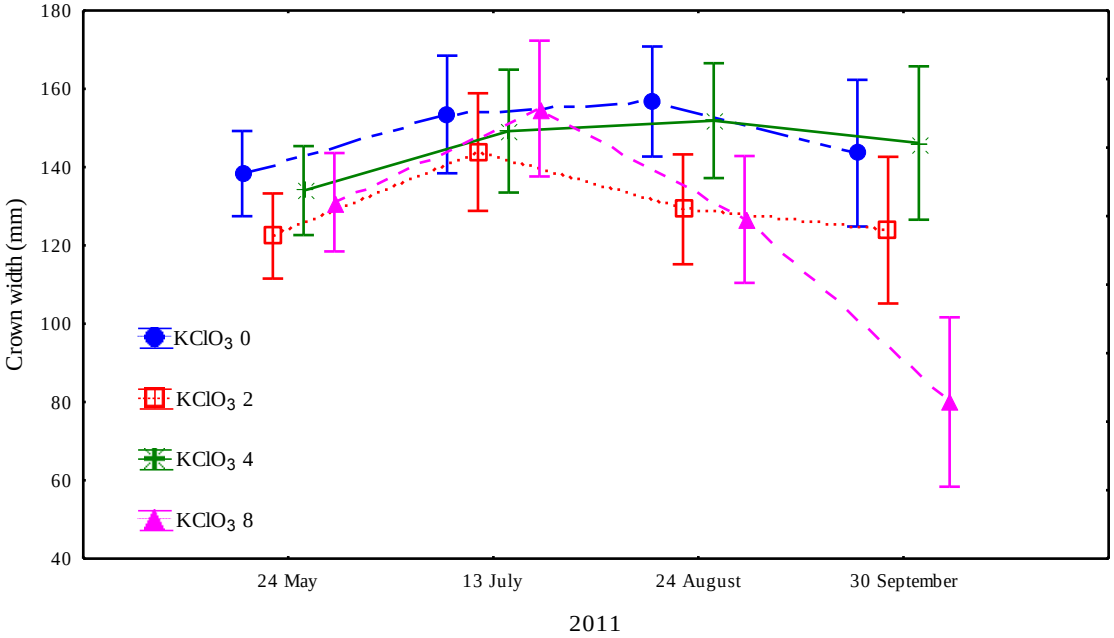
RANOVA	F-value	*P* - value	Significance
Treatment	11.4763	<0.001	***[z]
Time	56.0991	<0.001	***
Treatment*Time	1.6742	0.0985	NS[y]

Fig. 9. Growth responses over a six month period of *Disa* hybrid 'LM0739' plants using plant height (mm) (n=20) as growth parameter following treatment with Promalin® (Benzyladenine (BA): Gibberellic acid (GA_{4+7}); 1.8% active ingredient $^{w}/_{w}$, 1:1) at of 0, 25, 50 and 100 $mg.L^{-1}$ respectively. Vertical error bars indicate the Standard Error of the mean for each data point. [z]NS. Indicates no significant difference at the 5% confidence levels. [y]* Indicates level of significance.



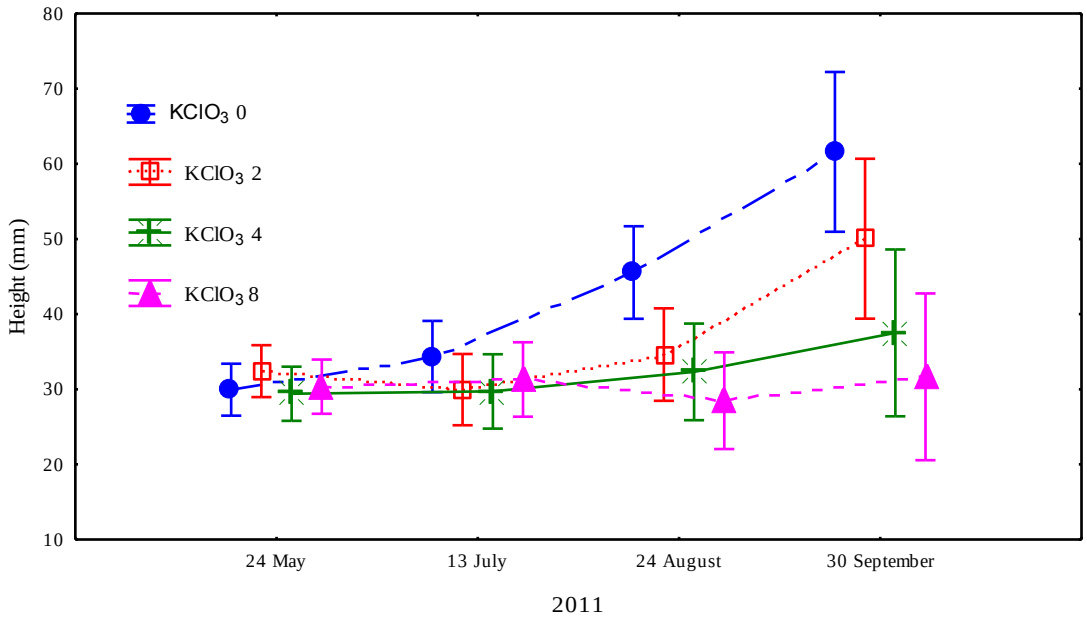
RANOVA	F-value	*P* - value	Significance
Treatment	2.232	0.098	NS[z]
Time	24.581	<0.001	***[y]
Treatment*Time	5.574	<0.001	***

Fig. 10. Growth responses over a six month period of *Disa* hybrid 'LM0739'plants using number of unfolded leaves (n=15) as growth parameter following treatment with $KClO_3$ at 0, 2, 4 and 8 $mmol.L^{-1}$ respectively. Vertical error bars indicate the Standard Error of the Mean for each data point. [z]NS. Indicates no significant difference at the 5% confidence level. [y]* Indicates level of significance.



RANOVA	F-value	*P* - value	Significance
Treatment	3.762	0.0181	**[z]
Time	17.672	<0.001	***
Treatment*Time	6.372	<0.001	***

Fig. 11. Growth responses over a six month period of *Disa* hybrid 'LM0739'plants using crown width (mm) (n=15) as growth parameter following treatment with $KClO_3$ at 0, 2, 4 and 8 mmol.L^{-1} respectively. Vertical error bars indicate the Standard Error of the Mean for each data point. [z]* Indicates level of significance.



RANOVA	F-value	*P* - value	Significance
Treatment	3.6949	0.0190	*[z]
Time	38.5341	<0.001	***
Treatment*Time	8.2229	<0.001	***

Fig. 12. Growth responses over a six month period of *Disa* hybrid 'LM0739'plants using plant height (mm) (n=15) as growth parameter following treatment with $KClO_3$ at 0, 2, 4 and 8 mmol.L^{-1} respectively. Vertical error bars indicate the Standard Error of the Mean for each data point. [z]* Indicates level of significance.

7. General Conclusion

The floriculture retail is ever seeking new exciting products to stimulate the market. Competitive production of established floricultural crops today are largely based on cutting edge technology, to such an extent, that the trial-and-error approach generally adopted by hobbyists and small scale growers cannot be relied upon to deliver high quality products of new floricultural crops sustainably to a global cut-flower market. Therefore, for the advancement of such new crops scientifically based research to develop viable cultivation techniques is of the utmost importance. The latter statement is extremely relevant with regard to the indigenous South African orchid genus *Disa*, harbouring undoubtedly commercial potential, but for which the current commercial success is greatly hampered by the lack of science based research on the flowering physiology and cultivation methodology. The aim of this study was therefore to provide commercial *Disa* growers with baseline information which would primarily aid in our understanding of the flowering physiology of *Disa*, but which simultaneously could also be implemented to improve current commercial cultivation practices, enabling these producers to compete on the international market.

Firstly, when considering the cultivation of floriculture crops, the ability to control flowering time in order to coincide with periods of high market demand is of critical importance. Control of flowering time can only be achieved if firstly the timing of floral initiation and secondly the flowering signal is known. To accomplish this milestone can be challenging, especially because a wide range of factors or even combinations thereof should be considered as possibilities for a flowering signal.

Within this study the floral initiation times of two commercial *Disa* hybrids were determined to be towards the end of May and June respectively. The floral differentiation for the two hybrids studied progressed at a comparable rate and ten characteristic stages of floral differentiation was identified, described and labelled for *Disa*. Floral abnormalities were observed at anthesis for a concerning a large percentage of the population. The occurrence of floral defects could possibly be linked to sub-optimal environmental conditions during the time of differentiation of specific floral parts. The knowledge of the differentiation stages of *Disa*, together with research facts on optimum environmental conditions during each of these stages could aid both in harvest predictions and in ensuring optimum greenhouse conditions prevail during the floral differentiation period to eliminate floral abnormalities which will result non-marketable flowers.

This study also documents the first report on bud abortion of *Disa* flowers, prior to anthesis. Whether this is a natural occurring phenomenon, also seen in nature, or whether it is due to sub-optimal cultivation conditions, is unknown. However, more research on this phenomenon is warranted, as bud abortion is a common physiological disorder within many floricultural crops which is managed strictly to ascertain more flowers per inflorescence and an increased appeal and marketability of the product.

Various trials to elucidate the flowering signal were conducted in this study, based on similar inductive signals reported in literature to govern flowering of either orchids or geophytes, both in their natural habitat or in a commercial production system. Possible elicitors of flowering were chosen for their reported positive effects on floral initiation, but also for their ease of application within cultivation. The flowering factors studied included photoperiod (short days), plant size, plant growth regulators, smoke water and chemical applications ($KClO_3$).

Except for plant size, none of the above listed flowering factors were effective in altering flowering incidences in *Disa* hybrids, as evaluated at the reported times and concentrations. The exact timing of floral initiation was not known at the time these studies were conducted. However, the timing of floral initiation as determined within in Paper 1 revealed to coincide with the onset of autumn and winter, under natural shortening of days and declining temperatures. A short day photoperiod response was considered within this study, but was found ineffective to delay flowering. Subsequently cycles of vernalization, or a general decline in night temperatures is proposed as a subject for future studies to track the elusive inductive signal.

The vigour and healthy appearance visually observed with all smoke water treated plants, as well as the overall high flowering incidences warrants further research on smoke water, especially when using weaker dilutions than applied within this study and also including a range application times in the evaluation. Even if smoke water treatments proves ineffective to alter flowering it should be evaluated for its perceived positive effects on plant growth as an inexpensive way of improving overall plant quality.

Certain cultural practices like discarding small plants at the beginning of the season led to studies on the effects of plant size on flowering. It was concluded that a minimum plant size is required for flowering and that larger plants not only showed higher spike emergence and anthesis percentages than medium and small sized plants, but flowering incidences were also recorded to occur earlier within the flowering season. Although the cultivation of only large plants in a population will considerably enhance the productivity, the flowering season will also be condensed significantly. Therefore, for commercial producers the inclusion of medium sized plants is recommended as these plants showed acceptable percentages of flowering whilst flowering occurred later than for large plants, effectively lengthening the flowering period of the total population.

No flowering incidences were recorded for the smallest plant size category, and as all the small plants within the study died during the duration of the trial, the commercial practice of discarding these small plants are supported. Furthermore, through dissections, it was shown that the plant weight as well as the number of leaves of plants can be linked to the phenological phase of the plants. However, the requirement for a larger plant size as a threshold requirement to flower apparently becomes less obligatory as the season progressed. Since *Disa* hybrids harbours a relatively stable number of leaves throughout the season, leaf number at the beginning of the season could be used as a useful tool to predict the flowering capacity of the commercial population, and can be used

as a screening tool to remove plants from the commercial population that, based on these studies, do not harbour the propensity to flower within the current season. In doing so, no resources are wasted in cultivating plants unlikely to flower within a reasonable span of time. Although it was proven that an optimum plant size is an underlying requirement for flowering, it is suspected that an additional external regulatory mechanism should be active to trigger flowering. Thus, reaching a sufficient plant size for *Disa* alone is not sufficient to induce flowering.

Together with plant size, the commercial practice of plant separation and re-potting of plantlets to one plant per pot, and the effect it has on plant growth and flowering were also studied. The current hypothesis that plant separation and re-potting will ultimately reduce competition within pots linked this study to the study of plant size as it is was assumed that growing plants separately in a pot will reduce competition and ultimately produce larger plants with a higher propensity to flower. However, no differences were found between separated and non-separated plants regarding any of the growth parameters or flowering incidences. This separation process is labour intensive and should be reconsidered based on the results found, as these expenses are not justified unless markets specifically demand one plant per pot for marketing purposes or for visual appeal.

Plant separation did not seem to affect plant size. However, in addition to studying photoperiod, the effect of higher light intensities for enhance vegetative growth were also evaluated. It was hypothesized that plants grown in higher light will ultimately reach an optimum flowering size earlier in the season, and therefore have the ability to produce a spike earlier than plants grown under low lighting conditions. Furthermore, the availability of light can also be directly linked to the photosynthetic capacity of a plant as well as to the carbon allocation and utilization strategies. Plants treated daily with an additional light source showed a visual reddening of leaves. This colouration was initially ascribed to light stress as induced by the sudden increased in light intensities, but after none of the techniques used to quantify the light stress showed any significant differences between the control plants, not exhibiting these symptoms and the treated plants with the colouration, it was concluded that the red colouration could possibly be ascribed to an ameliorating and adaptive strategy of *Disa* by accumulating anthocyanins as screening pigments to avoid light stress. Further studies are required to test this hypothesis. Furthermore, new leaves that became visible after approximately two months of light treatment, did not exhibit these colouration symptoms. It is therefore proposed that the *Disa* plants have adapted to the higher light intensities and produced new leaves now adapted to the additional light, again pointing to the resilience of *Disa* to potentially unfavourable environmental conditions. Since it was evident from photosynthetic studies that *Disa* is capable of utilizing moderate to high light intensities, it is suggested that further studies on the effects of additional lighting on *Disa* hybrids should be continued. The effect of LED lights specifically, and exposing *Disa* plantlets from an early stage to this type of lighting, warrants research. Closely correlated to light intensity is the photosynthetic capacity of plants. The photosynthetic capacity of *Disa* plants were measured throughout a part of the season and although the general photosynthetic capacity of

Disa was considered to be low in comparison to other conventional floricultural crops, when compared to other CFR species which have evolved with *Disa*, the recorded photosynthetic assimilation was not seen as uncharacteristically low. Higher light intensities will allow for a higher rate of photosynthate assimilation which in return will allow plants to optimally support growth and enable the support a flowering spike through to anthesis. Within the plant size study it was furthermore also evident that larger plants with a higher leaf area and leaf number, and consequently a higher photosynthetic capacity was better able to support the flowering process supporting the use of additional light within plant cultivation.

Considering the studies on the carbon utilisation strategies of two *Disa* hybrids, both the above- and below ground components of two *Disa* hybrids were studied. Interestingly, one of the studied hybrids did not produce a root tuber, as is said to be characteristic of orchids and thus also *Disa*. The absence of the tuber within this hybrid is difficult to explain. However it does seem to affect the growth strategy of the hybrid, especially concerning carbon allocation and partitioning.

Three non-structural carbohydrate fractions were extracted from the plant tissue: a soluble sugar fraction extracted with an aqueous ethanol solution, a polymer fraction extracted with water only and a starch fraction digested and extracted from the pellet. Starch is considered to be the main storage carbohydrate in many plant species. However, very low starch levels recorded within this study disregarded this polymer as reserve metabolite for *Disa*. Only the tuber forming hybrid expressed slightly higher starch levels within the underground components containing the root tuber. The soluble sugar fractions, which are considered to provide readily available resources for growth and maintenance processes within the plant, consisted of four main sugars namely glucose, sucrose, galactose and fructose, with sucrose and glucose being most abundant within the plant tissue. Within the extracted polymer fraction, four sugars became available upon hydrolization namely: galactose, glucose, mannose and arabinose. The presence of these sugars confirmed that the polymers within *Disa* plant tissue are fully or at least partly of a polysaccharide nature. Glucomannans are widely reported to be present within the storage organs of orchids and geophytes. This study presented some evidence that glucomannan may also present within the plant tissue of *Disa* hybrids, especially within the underground components of the tuber forming hybrid, which showed significantly higher concentrations of the hydrolyzed sugars than any of the other sample types.

However, the non-tuber forming hybrid showed higher soluble sugars levels within both its above- and below ground material when compared to the tuber forming hybrid. These findings suggest that tuber forming *Disa* hybrids might use different carbohydrate utilisation strategies than the non-tuber forming *Disa* hybrids and also put forward that the non-tuber forming hybrid is mostly reliant upon soluble sugars as is supplied by current photosynthesis. Further support for this statement lies in the higher leaf area, above-ground plant weight and a higher shoot:root ratio for the non-tuber forming hybrid in comparison to the tuber forming hybrid. A greater investment of energy in above ground plant organs will able the supply sufficient photosynthesis to maintain growth and

flowering. Alternatively, the tuber-forming hybrid is suggested to allocate more reserves to underground storage organs to form storage reserves, presumably in the form of glucomannan and starch.

The current research possibilities on *Disa* as a fascinating indigenous South African orchid, are seemingly endless. It is firmly believed when the research from this and future studies are used in creating sound commercial practices, *Disa* has the potential of reaching the same commercial success than currently experienced by other commercially available orchids. Furthermore, sustainable cultivation practices of *Disa* will aid in conservation of this fynbos treasure for future generations in uncertain times and an ever changing natural environment.