Propagation of *Romulea* species

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Abstract

Romulea is a genus with numerous attractive and endangered species with horticultural potential. This genus in the Iridaceae has its centre of diversity in the winter-rainfall zone of South Africa. This thesis uses ecophysiological and biotechnological techniques to investigate the physiology behind the propagation of some species in this genus.

The ecophysiological techniques of soil sampling and analysis and germination physiology were used to determine the natural and *ex vitro* growth and development requirements of these plants, while biotechnological techniques are used to determine the *in vitro* growth and development requirements of these plants and to increase the rate of multiplication and development.

Soil sampling and analysis revealed that *R. monadelpha* and *R. sabulosa*, two of the most attractive species in the genus, grow in nutrient poor 1:1 mixture of clay and sandy loam soil with an N:P:K ratio of 1.000:0.017:0.189 with abundant calcium.

To investigate the physical properties of the seeds, imbibition rate, moisture content and viability of seeds were determined. The seed coat and micropylar regions were examined using scanning electron microscopy. To test for suitable stimuli for germination, the effect of temperature and light, cold and warm stratification, acid and sand paper scarification, plant growth promoting substances, deficiency of nitrogen, phosphorous and potassium, and different light spectra (phytochromes) on germination were examined. An initial germination experiment showed germination above 65% for *R. diversiformis*, *R. leipoldtii*, *R. minutiflora* and *R. flava* seeds placed at 15°C; while seeds of other species placed at 15°C all had germination percentages lower than 30%. More extensive germination experiments revealed that *R. diversiformis* and *R. rosea* seed germinate best at 10°C, *R. flava* seed germinates best when cold stratified (5°C) for 21 days and *R. monadelpha* germinates best at 15°C in the dark. Seeds of *R. diversiformis*, *R. flava*, *R. leipoldtii*, *R. minutiflora*, *R. monadelpha* and *R. sabulosa* seem to all exhibit non-deep endogenous morphophysiological dormancy while seeds of *R. camerooniana* and *R. rosea* appear to have deep endogenous morphophysiological dormancy.

The suitability of various explant types and media supplementations for culture initiation was examined for various species of *Romulea*. Both embryos and seedling hypocotyls can be used for *R. flava*, *R. leipoldtii* and *R. minutiflora in vitro* shoot culture initiation. *R. sabulosa* shoot cultures can only be initiated by using embryos as explants, because of the lack of seed germination in this species. Shoot cultures of *R. diversiformis*, *R. camerooniana* and *R. rosea* could not be initiated due to the lack of an *in vitro* explant shooting response. Shoot cultures can be initiated on media supplemented with 2.3 to 23.2 µM kinetin for all species that showed an *in vitro* response. The most suitable concentration depended on the species used. Some cultures appeared embryogenic, but this was shown not to be the case. A medium supplemented with 2.5 µM *m*TR is most suitable for *R. sabulosa* shoot multiplication. BA caused vitrification of shoots in all the experiments in which it was included and is not a suitable cytokinin for the micropropagation of these species.

The effect of various physical and chemical parameters on *in vitro* corm formation and *ex vitro* acclimatization and growth was examined. Low temperature significantly increased corm formation in *R. minutiflora* and *R. sabulosa.* A two step corm formation protocol involving placing corms at either 10 or 20°C for a few months and then transferring these cultures to 15°C should be used for *R. sabulosa*. When paclobutrazol and ABA were added to the medium on which *R. minutiflora* shoots were placed, the shoots developed corms at 25°C. This temperature totally inhibits corm formation when these growth retardants are not present. BA inhibited corm formation in *R. leipoldtii.* Corms can be commercialized as propagation units for winter-rainfall areas with minimum temperatures below 5°C during winter.

Although an incident of *in vitro* flowering was observed during these experiments, these results could not be repeated. Although none of the corms or plantlets planted *ex vitro* in the greenhouse survived, a small viability and an *ex vitro* acclimatization experiment shows that the corms produced *in vitro* are viable.

One embryo of the attractive *R. sabulosa*, produces 2.1 ± 0.7 SE shoots after 2 months; subsequently placing these shoots on a medium supplemented with 2.5 µM *m*TR for a further 2 months multiplies this value by 5.5 ± 1.3 SE. Each of these shoots can then be induced to produce a corm after 6 months. This means that 1

embryo can produce about 12 corms after 10 months or about 65 corms after 12 months (if shoots are subcultured to medium supplemented with 2.5 µM *m*TR for another 2 months). Embryo rescue can enable wider crosses within this genus. These results can be used for further horticultural development of species in this genus and their hybrids and variants.

Declarations

I Pierre André Swart, student number 207519473, hereby declare that:

- This thesis, Propagation of *Romulea* species, unless otherwise acknowledged to the contrary in the text, is the result of my own investigation, under the supervision of Professor J. van Staden and co-supervision of Doctor M.G. Kulkarni, Doctor M.W. Bairu and Professor J.F. Finnie, in the Research Centre for Plant Growth and Development, School of Biological and Conservation science, University of KwaZulu-Natal, Pietermaritzburg;
- This dissertation has not been submitted for any degrees or examination at any other university;
- This thesis does not contain data, figures or writing, unless specifically acknowledged, copied from other researchers. Where other written sources have been quoted, then
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Signed at ______________________________ on the ____________ day of

__________________________ , 2012.

____

Pierre André Swart

We declare that we have acted as supervisors for this Pierre André Swart, student number 207519473 during this PhD study entitled Propagation of *Romulea* species.

Regular consultation took place between the student and ourselves throughout the investigation. We advised to the best of our ability and approved the final document for submission to the Faculty of Science and Agriculture Higher Degrees Office for examination by the University appointed Examiners.

Professor J. van Staden
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DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis:

PUBLICATION 1:

ASCOUGH, G. D., SWART, P. A., FINNIE, J. F., and VAN STADEN, J. (2011). Micropropagation of *Romulea minutiflora*, *Sisyrinchium laxum* and *Tritonia gladiolaris* — Iridaceae with ornamental potential. South African Journal of Botany 77: 216-221.

I supplied the data on micropropagation of *Romulea minutiflora* and some editorial help. Dr. Ascough supplied all other data and did all other editing, as he is the first author. Prof. Finnie and Prof. van Staden were our co-supervisor and supervisor respectively at the time of this project.

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All the data and text of this paper I generated myself, Dr. Kulkarni, Dr. Bairu and Prof. Finnie were my co-supervisors and Prof. van Staden was my supervisor and they therefore supplied some editorial help.

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SWART, P. A., KULKARNI, M.G., FINNIE, J. F., and VAN STADEN, J. (2011). Germination physiology of four African *Romulea* species. Seed Science and Technology 39: 354-363.

All the data and text of this paper I generated myself, Dr. Kulkarni and Prof. Finnie were my co-supervisors and Prof. van Staden was my supervisor and they therefore supplied some editorial help.

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List of Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
2-iP	N6(2-isopentenyl)-adenine
ABA	Abscisic acid
BA	6-Benzyl-aminopurine
Butenolide	3-methyl-2*H*-furo[2,3-*c*]pyran-2-one
$CaCl_2$	Calcium chloride
$CaCO_3$	Calcium carbonate
GA	Gibberellic acid
HCl	Hydrochloric acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butryric acid
Kinetin	6-Furfurylaminopurine
KNO_3	Potassium nitrate
KOH	Potassium hydroxide
Me*m*T	6-(3-methoxybenzylamino)purine
Me*m*TR	6-(3-methoxybenzylamino)-9-b-D-ribofuranosylpurine
MGT	Mean germination time
MPa	Mega Pascal
MS	Murashige and Skoog
*m*T	6-(3-hydroxybenzylamino)purine
*m*TR	6-(3-hydroxybenzylamino)-9-b-D-ribofuranosylpurine
NAA	Naphthaleneacetic acid
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NaOH	Sodium hydroxide
nm	nanometer
PGR	Plant Growth Regulator
Thidiazuron	N-phenyl-N-1,2,3,-thidiazol-5-ylurea
TMS	Table Mountain Sandstone
TTC	2,3,5-triphenyltetrazolium chloride
Zeatin	Trans-6-(4-hydroxyl-3-methylbut-2enyl) aminopurine
µM	Micromole
µm	Micrometer

Namakwaland

Die wêreld lê oop en kaal in die snikhete son
hittegolwe bewe op die horison
dit maak skimme op die grens
die skaap soek koelte onder pens.
The world lies open and naked in the hot sun
heat waves quiver on the horizon
it makes shimmers on the border
the sheep searches for shade under paunch

Die koggelmander skarrel stywebeen
oor skroeiende granietklip heen.
Geen koeltebome hier – die aarde is plat –
Skilpad soek maar bossie, ander graaf maar gat
The lizard scurries stiff-legged
over scorching granite rock
No shade trees here – the earth is flat –
Tortoise looks for a small bush, others dig a hole

Die klippe lê asof soos kaiings uitgebraai
maar onder – deur die sandjies toegewaai –
lê fyne saad gesaai,
van moederplant al lank gespeen
wagtend – op die reën.
The rocks lie as if they were pieces of roasted crackling
But underneath – covered by windblown sand –
fine seeds are sown
from parent plant weaned for long
in waiting - for the rain.

Die vlakhaas spits sy oor
hy het die donderweer gehoor
skilpad loer ook uit sy dop.
Wildsbok lig en draai sy kop –
sy neusgat vleuel soos hy die reënlug snuif
daar gaan ñ rilling deur sy lyf
sy stert staan kuif.
The rabbit lifts his ear
he has heard the thunder
tortoise also peaks out his shell
The wild buck lifts and turns his head -
his nose flares as he smells the rain-filled air
a shiver goes through his body
his tail stands rigid

Die donderweerswolke maak ñ donker sluier
en die voorwind dwarrel en kuier
hier en daar, van wie weet waar
kielie ñ graspol en ritsel ñ blaar.
Die eerste druppels val met sware plof
in die dorre verpoeierde stof –
dit maak ñ wasem op die klip
en laat die sandtjies dans en wip.
Water drup van die klip se rand
in die dorstige rooi-rooi sand.
The thunderclouds make a dark slur
and the fore-wind tornadoes and visits
here and there, from who knows where
a grass is tickling and a leaf scurrying.
The first drops fall with heavily, explosively
in the dull powdered dust -
it makes a haze on the rock
and makes the sand particles dance and whip
Water drips from the rock’s edge
in the thirsty red-red sand.

Dit lyk kompleet of die wêreld wil sing
oor die genade wat weer uitkoms bring,
want gou is die wêreld met blomme verfraai
wat jubel van kleur as die wind daaroor waai
It appears truly that the world wants to sing
about mercifulness that again brings salvation,
because instantaneously the world is bedazzled with flowers
that rejoice with colour as wind blows over them

Het iemand met ñ towerstaf
ongesiens hier deurgedraf?
Lap-lap lê dit aanmekaar
ñ mooi gesig voorwaar!
Gousblom en vergeet-my-nietjie
met blou en pers so bietjie-bietjie.
Did someone with a magic wand
run through here unseen?
Patch-patch it lies continuously
a pretty face for sure!
Arctotis hirsuta* and *Anchusa capensis
with blue and purple little-little

Slanguinjtjie weet nie wat haar noop
maar stoot haar blommetjie skaam-skaam oop
sy mag mos ook in die vreugte deel
met skuterwit en spikkelgeel.
***Morea serpentina* does not know what and where**
but pushes open her flower shy-shy
she may also have her share of the happiness

Geel en goud, bankvas aanmekaar
met blouselblou, ñ bietjie hier, ñ bietjie daar.
Yellow and gold, tightly packed together
with blossom-blue, a little here, a little there

ñ Wuiwend blommeparadys
om sekerlik die Heer te prys.
A lush flower paradise
to surely praise the Lord

As die mens dan ook so sy dankbaarheid betoon,

sal Hy sekerlik ook nader aan ons woon.

If man then also can show his gratitude like this,

then He will surely also live closer to us

A poem by my late father, Pierre André Swart Senior; to whom this thesis is dedicated (English translation in grey bolded text).

1 Introduction

1.1 PROPAGATION OF *ROMULEA* SPECIES FOR HORTICULTURAL AND CONSERVATION PURPOSES

Romulea is a genus with many species of potential horticultural value. The fast growth, attractive growth forms, regular flowering and diverse flower variation with many aesthetically pleasing colours, makes species of this genus prime candidates for commercialisation as miniature potted plants and cut flowers (MANNING & GOLDBLATT, 1996; 1997; NIEDERWIESER *et al.*, 2002).

The Iridaceae is one of the most horticulturally important families of monocotyledons. Most of the cultivated ornamental species indigenous to South Africa have come from this family (COETZEE *et al.*, 1999; NIEDERWIESER *et al.*, 2002; REINTEN & COETZEE, 2002). The two genera of Iridaceae most in demand by the world market as floricultural crops are *Gladiolus* and *Freesia* (COETZEE *et al.*, 1999). The production of cut flowers of *Gladiolus* and *Freesia* is a million dollar industry in many parts of the world (GOLDBLATT, 1991). These two genera are placed in the same tribe, Ixieae, as the genus *Romulea* (GOLDBLATT, 1990).

The name *Romulea* was borrowed from the city of Rome, in vicinity of which the genus was first described by Maratti in a small taxonomic study published in 1772. He proposed that this species was distinct from *Crocus*, *Colchicum*, *Sisyrinchium*, *Bulbocodium* and *Ixia* (DE VOS, 1972).

According to MANNING & GOLDBLATT (2001) there are approximately 90 species of *Romulea*. These species are found in sub-Saharan Africa, the Mediterranean basin, the Canary Islands, the Azores, and southern Europe (DE VOS, 1972; MANNING & GOLDBLATT, 2001). This attractive genus of the Iridaceae has its centre of diversity in the winter-rainfall zone of South Africa where 73 species are now recognized (MANNING & GOLDBLATT, 2001). Viewing the flowering plants in this area is an important tourist attraction. It attracts international tourists including world renowned botanists and nature lovers. Many South Africans also travel across the country each year to immerse themselves in the beauty of this floral spectacle,

where a great number of species of *Romulea* can be seen. Within the summer-rainfall zone of southern Africa, the species is restricted to upland and montane habitats (MANNING & GOLDBLATT, 2001). Species belonging to *Romulea* are deciduous perennial geophytes and the tunicated corms of these plants enable them to survive the dry season (DE VOS, 1972; MANNING & GOLDBLATT, 2001). At the start of the growing season, a group of adventitious roots are first formed near the base of the corm, after which the uppermost axillary bud develops into an inflorescence stem (DE VOS, 1972).

According to HILTON-TAYLOR (1996) there are 18 rare, 10 vulnerable and 2 extinct species in the genus *Romulea*. RAIMONDO *et al* (2009) however lists this genus as having only 4 rare, 4 near threatened, 23 vulnerable, 7 endangered and 3 critically endangered species. In their book, RAIMONDO *et al* (2009) displays a photograph of the vulnerable *Romulea sabulosa* on the cover. This species was used in this study. Despite the fragile conservation status of many species in this genus, the area which hosts its centre of diversity is also under threat from climate change. The longer periods and higher intensity of drought in the Cape Floral Region is likely to have a large negative impact on the endemic flora (WEST, 2009).

This study will form the groundwork for the commercialisation and conservation of this genus, as there has been no extensive work done on its ecophysiology and propagation.

Micropropagation is an important tool for ornamental plant culture and breeding, which has been applied to almost all commercial geophytes (ZIV, 1997). It enables high propagation rates, which is especially useful for the commercialisation of new species (LILIEN-KIPNIS & KOCHBA, 1987; PIERIK, 1997). In many instances it has also been shown that micropropagation can play a vital role in plant conservation, especially when combined with methods such as cryopreservation (WOCHOCK, 1981; SARASAN *et al.*, 2006; SHIBLI *et al.*, 2006; WITHERS, 2008).

1.2 AIMS AND HYPOTHESES

The general aim of this study was to investigate the conditions that promote growth and development in a number of *Romulea* species both *ex vitro* and *in vitro* to aid its commercialisation and conservation. In more detail, the aims of this study were:

- To investigate the environmental factors that influence the development and growth of *R. sabulosa* and *R. leipoldtii* in their natural habitat and to replicate these conditions for *ex vitro* growth;

- To investigate the germination physiology of *R. camerooniana*, *R. diversiformis*, *R. flava*, *R. leipoldtii*, *R. minutiflora*, *R. monadelpha*, *R. rosea* and *R. sabulosa* and to improve seed germination in some of these species that show low germination;

- To obtain suitable protocols to initiate cultures for the micropropagation of *R. diversiformis, R. flava*, *R. leipoldtii*, *R. monadelpha*, *R. minutiflora* and *R. sabulosa;*

- To establish suitable protocols for shoot multiplication for *R. leipoldtii*, *R. minutiflora* and *R. sabulosa;*

- To investigate whether embryogenesis readily occurs in the presence of 2,4-D;

- To establish protocols for *in vitro* corm production and *ex vitro* corm germination for *R. leipoldtii*, *R. minutiflora* and *R. sabulosa;*

- To establish an *in vitro* flowering protocol for *R. minutiflora; and*

- To promote the commercialisation of *Romulea* species

It was expected that there will be a correlation between geographical distribution and suitable *ex vitro* and *in vitro* stimuli. The suitable culture conditions were expected to be similar to that of *Crocus* species due to their close relationship with this genus.

The subgenera *Romulea* and *Spatulanthus* were expected to have differentially suitable conditions for *ex vitro* and *in vitro* development and growth.

1.3 GENERAL OVERVIEW OF THESIS CONTENT

Chapter 2 is a review of literature available on aspects relative to this study. It firstly covers the distribution, morphology, life-cycle, habitat and conservation status of species in the genus *Romulea*. It then discusses other studies performed on ecophysiology and propagation of this genus with descriptions of phylogeny and taxonomy. It further reviews the ecophysiological techniques of soil sampling and analysis. A review of seed physiology and techniques applicable to this study is included in this chapter. It also gives a review on micropropagation in general, discusses some *in vitro* techniques applicable to the study, placing emphasis on explant selection, culture initiation and multiplication, embryogenesis, *in vitro* corm formation, *in vitro* flowering and *ex vitro* acclimatization. A summary of the micropropagation of species in the family Iridaceae is included.

In **Chapter 3** the habitat of some *Romulea* species is investigated further through ecophysiological techniques of soil sampling and analysis.

Chapter 4 is an examination of the germination physiology of some *Romulea* species. This was done firstly by examining the physical properties and viability of the seeds, and then investigating the effect of an array of physical and chemical stimuli on germination. The physical properties of the seeds; imbibition rate, moisture content and viability of seeds were determined. The seed coat and micropylar regions were examined using scanning electron microscopy. To test for suitable stimuli for germination, the effect of temperature and light, cold and warm stratification, acid and sand paper scarification, plant growth promoting substances, deficiency of nitrogen, phosphorous and potassium, and different light spectra (phytochromes) on germination were examined.

Chapter 5 is an examination of the suitability of various explant types and media supplementations for culture initiation. Two explant types were used; seedling organs and embryos. It also investigates the effect of various physical and chemical

parameters on shoot multiplication and describes some cultures that appeared embryogenic.

Chapter 6 is a report on the effect of various physical and chemical parameters on *in vitro* corm formation and *ex vitro* acclimatization and growth. It includes a description of an incident of *in vitro* flowering and some experiments conducted in an attempt to replicate these conditions and further stimulate *in vitro* flowering.

In **Chapter 7** the attributes of various *Romulea* species is considered and their suitability for commercialization is discussed.

2 Literature review

Leef van daad en woord dan so gepas,
Dat jy nooit wens dat môre gister was

Live of word and deed then in such a becoming way,
That you never shall wish tomorrow was yesterday

Pierre André Swart Senior

2.1 MORPHOLOGY, DISTRIBUTION AND HABITAT

There are approximately 90 species of *Romulea* (MANNING & GOLDBLATT, 2001). These species are mainly confined to sub-Saharan Africa and the Mediterranean (DE VOS, 1972; MANNING & GOLDBLATT, 2001). Twelve to 15 species occur in the Mediterranean basin, Canary Islands, the Azores, and southern Europe (MANNING & GOLDBLATT, 2001). The remaining species occur in sub-Saharan African, which includes the Arabian Peninsula and Socotra (MANNING & GOLDBLATT, 2001). It is reported that 3 species occur in tropical Africa and 2 are endemic in East Africa and the Peninsula (MANNING & GOLDBLATT, 2001). The genus has its centre of diversity in the winter-rainfall zone of southern Africa; here 73 species are now recognized (MANNING & GOLDBLATT, 2001). The distribution of 7 species used in the propagation experiments are shown in Figure 2.1. Within the summer-rainfall zone of southern Africa the species are restricted to upland and montane habitats (MANNING & GOLDBLATT, 2001). Those within the winter rainfall part of southern Africa occur from sea level to high altitudes, being especially common in medium to high altitudes (MANNING & GOLDBLATT, 2001). Winter-rainfall species generally flower during the spring (August to September, with a few in May and June) and summer-rainfall species flower from September to February (MANNING & GOLDBLATT, 2001) as shown in Figures 2.2 to 2.4.

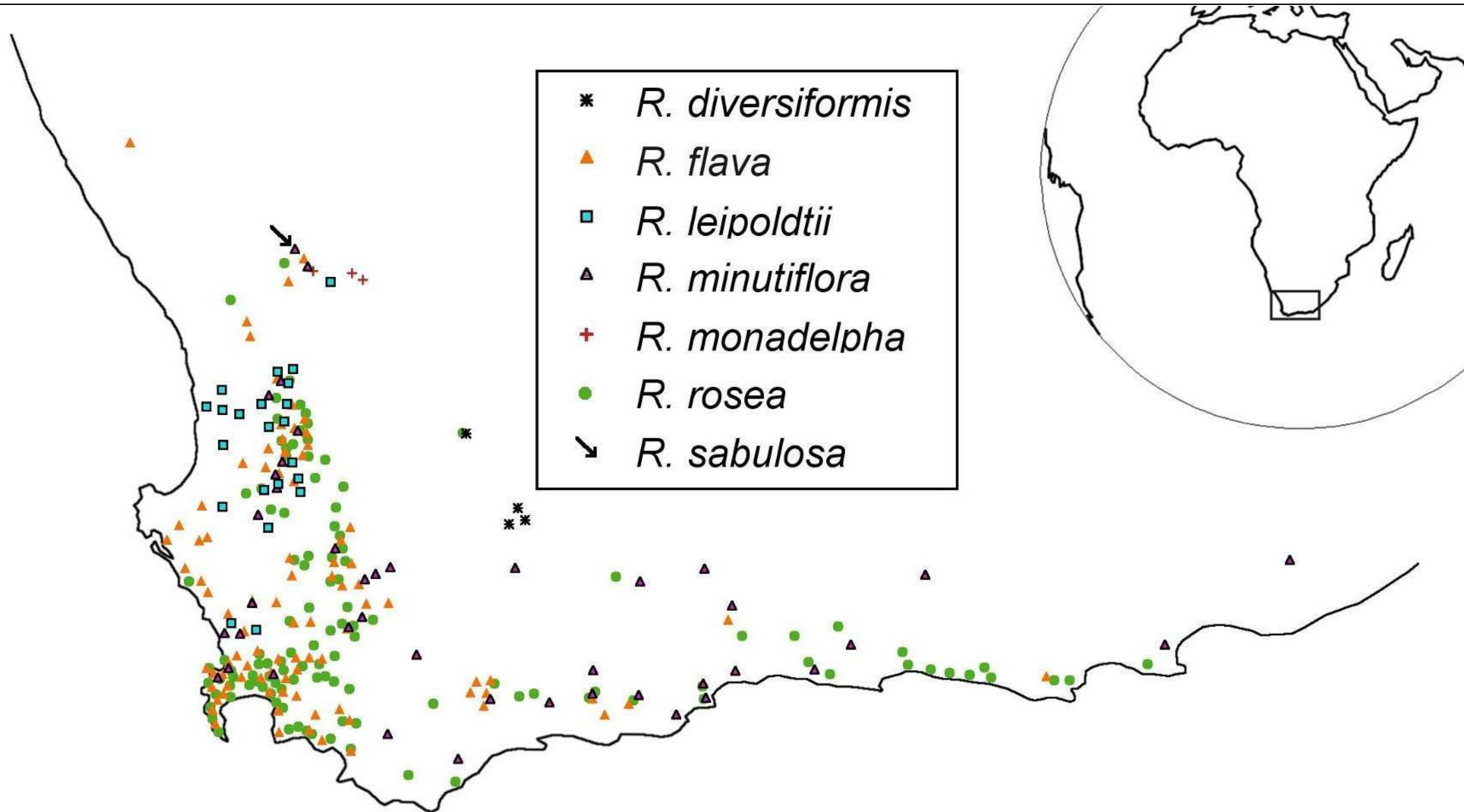

Figure 2.1: Map showing the distribution of seven of the species used in propagation experiments. The inset of the globe in the top right corner indicates the location of this map on the African continent with a rectangle. Modified from DE VOS (1972; 1983).

Figure 2.2: Life cycle of *Romulea sabulosa*, a species endemic to the winter-rainfall area of South Africa (Modified from ASCOUGH (2008); DE VOS (1972); and photographs taken by Dr. John C. Manning)

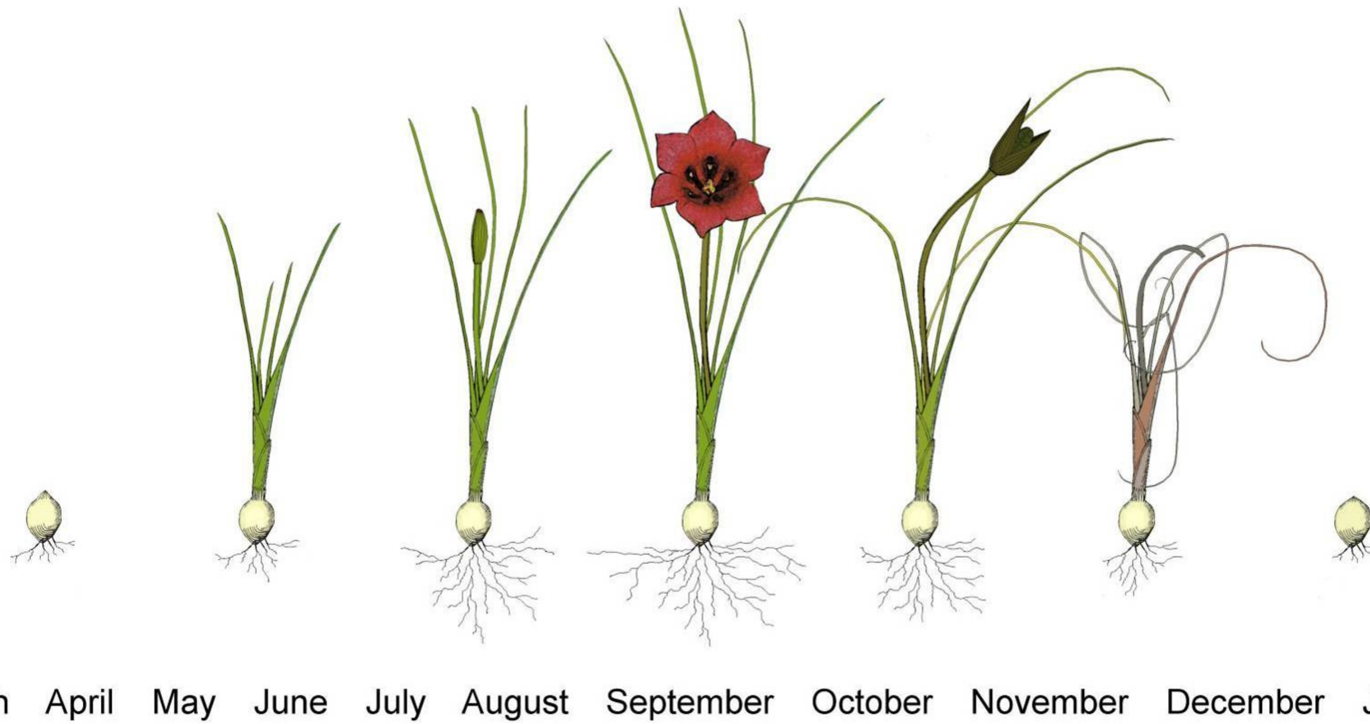

Figure 2.3: Life cycle of *Romulea monadelpha*, another species endemic to the winter-rainfall area of South Africa (Modified from ASCOUGH (2008); DE VOS (1972); and photographs taken by Dr. John C. Manning).

Figure 2.4: Life cycle of *Romulea camerooniana*, a species occurring in summer-rainfall regions of Africa (Modified from ASCOUGH (2008); DE VOS (1972) and photographs taken by Dr. John C. Manning).

Species belonging to *Romulea* are deciduous perennial geophytes (DE VOS, 1972; MANNING & GOLDBLATT, 2001).The tunicated corms of these plants enable them to survive the dry season (DE VOS, 1972). At the start of the growing season a group of adventitious roots are first formed near the base of the corm after which the top axillary bud develops into a inflorescent stem (DE VOS, 1972). During growth the corm gradually shrinks and a new corm is formed, which remains dormant until the next season (DE VOS, 1972).

Most of the species used in this study generally occur in seasonally moist or inundated open sandy or clay flats (MANNING & GOLDBLATT, 2001). The genus *Romulea* is not as substrate-specific as many other southern African Iridaceae and in *Romulea* sp. true edaphic endemics are rare (MANNING & GOLDBLATT, 2001). These edaphic endemics include most of the species endemic to the western Karoo, which is found only in fine-grained doleritic clay soil and *R. barkerae*, which is restricted to the coastal limestone deposits of the Saldanha district (MANNING & GOLDBLATT, 2001).

The corms of species belonging to *Romulea* are described by as globose, bell-shaped or asymmetrical and woody (MANNING & GOLDBLATT, 2001). The corm consists of a few swollen, basal internodes of the axis covered by the tunics, which consists of persistent leaf bases (DE VOS, 1972). DE VOS (1972) noted that the adventitious roots of species belonging to this genus originate from a basal ridge or basal point on the corm in the form of a row or cluster that represents the ventral side of the rhizome from which the distinguishing corm of the species in the family Iridaceae evolved (DE VOS, 1972). When the plant is too high in the ground a contractile root may develop from the basal scar (DE VOS, 1972). This root is thicker than other adventitious roots (DE VOS, 1972). The new corm is most commonly obliquely attached to the old corm via the basal scar (DE VOS, 1972). This basal scar is not quite basal and is actually situated towards one side (DE VOS, 1972). A new corm develops when a basal internode of the axis develops into leafy shoots (DE VOS, 1972). The newly formed corms are still partially enclosed in the old corm tunics after formation (DE VOS, 1972).

The plants are short stemmed or stemless. The flowering stems of *Romulea* species are also usually reduced and often subterranean. The flowers are each borne singly on a branch or peduncle (MANNING & GOLDBLATT, 2001). The leaves of *Romulea* are linear to filiform, with most species having two grooves on each surface. When the leaf is examined anatomically, it consists of a wide central rib separated from the smaller marginal ribs by wide to narrow longitudinal grooves. The stomata are located in these longitudinal grooves (MANNING & GOLDBLATT, 2001).

The flowers of most species are very similar except for pigmentation, which is exceptionally variable. The colour array includes uniformly yellow to white, pink, orange, apricot, red, magenta, lilac and purple, with the cup usually being yellow (DE VOS, 1972; MANNING & GOLDBLATT, 2001). Dark markings commonly appear below the rim of the cup. The perianth is cup-shaped with a short perianth tube. The flower has six tepals, which are cupped below and spreads horizontally above. The floral cup includes the stamens which are adjoining and coherent. The style divides into three distinct style arms above mid-anther level (MANNING & GOLDBLATT, 2001). The flowers are short lived and not suitable for picking (MANNING & GOLDBLATT, 2001).

2.2 SPECIES SPECIFIC MORPHOLOGY AND DISTRIBUTION

According to MANNING & GOLDBLATT (2001) and DE VOS (1970a) the corm and its tunics appear to provide the most useful characteristics for identifying different species within the genus. In most species the corm develops a sharp lateral or basal ridge through intercalary growth of the tunics. The margins of the tunics along this fold consist of fine fibrils, forming a fibrous fringe (MANNING & GOLDBLATT, 2001). MANNING & GOLDBLATT (2001) classifies these species as belonging to the subgenus *Romulea*.

The corms of some other species have a rounded or pointed base and lack a basal ridge. In this case the tunics split into several well-defined acuminate teeth that do not have a fibrous appearance (MANNING & GOLDBLATT, 2001). MANNING &

GOLDBLATT (2001) classifies these species as belonging to the subgenus Spatalanthus. Manning and Goldblatt (2001) goes on to divide the genus into sections based on corm morphology, this is not discussed.

Research was done on the character of flowers and growth form of a number of *Romulea* species found in South Africa. Descriptive localities, habitats, identifying features and subgenus classification of these species used in propagation experiments are also discussed.

2.2.1 *Romulea amoena*

The flowers of *R. amoena* are deep rose-pink to carmine-red with purple-black blotches (DE VOS, 1983; MANNING & GOLDBLATT, 1997; MANNING & GOLDBLATT, 2001). These blotches are sometimes also replaced by stripes around the cream or yellow cup. The tepals are elliptic to oblanceolate (MANNING & GOLDBLATT, 2001). The bracts are green or sometimes reddish (DE VOS, 1983). The outer bracts have narrow or inconspicuous membranous margins while the inner bracts have wide and colourless or brown-streaked margins. It has 1 to 2 flowers which can be seen in August (DE VOS, 1972; MANNING & GOLDBLATT, 1997). Plants of *R. amoena* are between 50 and 300 mm in height (DE VOS, 1983; MANNING & GOLDBLATT, 2001). Its stem is subterranean or reaches 100 mm above the ground. These plants have 3 to 4 leaves that are usually all basal in origin (MANNING & GOLDBLATT, 2001).

R. amoena occurs in sandy soils and is mostly found in rocky places. It is indigenous to the Bokkeveld mountains south of Nieuwoudtville (MANNING & GOLDBLATT, 2001). MANNING & GOLDBLATT (2001) has placed this species in the subgenus *Romulea*.

2.2.2 *Romulea autumnalis*

The flowers are pink or magenta-pink to white with a yellow to orange cup. The tepals are elliptic and the fruiting peduncles are erect. The bracts are green or greenish with the outer bracts having narrow membranous margins while the inner bracts have wide colourless margins (DE VOS, 1983; MANNING & GOLDBLATT, 2001). *R. autumnalis* flowers from April to July during which the plant develops 1 to 3

flowers (DE VOS, 1972; MANNING & GOLDBLATT, 2001). The plants of this species grow 150 to 350 mm in height with subterranean stems. The plant has 3 to 4 leaves which are basal and thread like or filiform to compressed cylindrically (DE VOS, 1983; MANNING & GOLDBLATT, 2001).

This species is found in Eastern Cape from Grahamstown towards Kariga where it occurs on grassy flats or mountain slopes (DE VOS, 1983; MANNING & GOLDBLATT, 2001). It is closely allied with *R. camerooniana*, but can be distinguished from *R. camerooniana* by its short stamens and style which do not reach the middle of the perianth, as opposed the stamens and styles of *R. camerooniana,* which do reach the floral cup (MANNING & GOLDBLATT, 2001). This means that the stamens of *R. autumnalis* are included in the floral cup (MANNING & GOLDBLATT, 2001). MANNING & GOLDBLATT (2001) places this species in the subgenus *Romulea*.

2.2.3 *Romulea camerooniana*

The flowers are magenta or pink to white and the cup is yellow. The tepals are elliptic (MANNING & GOLDBLATT, 2001). The outer bracts have narrow or inconspicuous membranous margins. The inner bracts also have narrow and colourless membranous margins (MANNING & GOLDBLATT, 2001). *R. camerooniana* mostly flowers from December to April (BURROWS & WILLIS, 2005). The plants are normally 80 to 200 mm in height with a stem which is subterranean. There are 2 to 6 filiform leaves per plant which are all basal (MANNING & GOLDBLATT, 2001).

R. camerooniana occurs in rocky or grassy highlands. In these habitats their distribution extends from the Drakensberg of the Eastern Cape, South Africa to Kenya, Sudan and Southern Ethiopia. Outlying populations also occur in the Cameroon in west Africa (MANNING & GOLDBLATT, 2001). MANNING & GOLDBLATT (2001) places this species in the subgenus *Romulea*.

2.2.4 *Romulea citrina*

The flowers are lemon-yellow and unscented with tepals that are elliptic and between 20 and 32 mm long (DE VOS, 1983; MANNING & GOLDBLATT, 2001). The fruiting peduncles are at first curved and later suberect. The outer bracts have narrow

membranous margins while the inner bracts are slightly shorter with broad, brown streaked, membranous margins (DE VOS, 1983; MANNING & GOLDBLATT, 2001). *R. citrina* flowers from August to September with 1 to 4 flowers (DE VOS, 1972; MANNING & GOLDBLATT, 2001). These plants reach a height of 80 to 350 mm, and sometimes reach up to 450 mm. The stem is subterranean or reaches 20 mm above the ground. The plant has 3 to 4 filiform leaves of which the lower two are basal (DE VOS, 1983; MANNING & GOLDBLATT, 2001). The leaves are narrowly grooved with 4 grooves. They are compressed cylindrically and curve outward (MANNING & GOLDBLATT, 2001).

This species occurs in wet sites with sandy or stony ground in Namaqualand where it is common in the Kamiesberg area (DE VOS, 1983; MANNING & GOLDBLATT, 2001). It also occurs at lower elevations and is found around Grootvlei, west of Kamieskroon (MANNING & GOLDBLATT, 2001). MANNING & GOLDBLATT (2001) places this species in the subgenus *Romulea*.

2.2.5 *Romulea cruciata*

Flowers are magenta-pink to lilac-pink in colour. There are dark blotches around a dark or golden yellow to orange cup (DE VOS, 1983; MANNING & GOLDBLATT, 1996; MANNING & GOLDBLATT, 2001). The flower is unscented with elliptic to oblanceolate tepals. The fruiting peduncles remain erect or spread slightly. The bracts are greenish or purplish red with the outer bracts having narrow inconspicuous membranous margins and the inner bracts submembranous which are commonly brown-flecked (MANNING & GOLDBLATT, 2001). Its flowering period is between July and September during which a plant may have 2 to 4 flowers (DE VOS, 1972; MANNING & GOLDBLATT, 2001). These plants are 150 to 400 mm in height with a totally subterranean stem (DE VOS, 1972; MANNING & GOLDBLATT, 2001). The plant has 2 to 8 filiform leaves which are all basal (MANNING & GOLDBLATT, 2001).

R. cruciata is most common in the south western Cape from Nieuwoudtville to Riversdale (MANNING & GOLDBLATT, 1996; MANNING & GOLDBLATT, 2001). Its distribution spreads from the Bokkeveld Mountains of the Northern Cape Province in the north to the Gourits river in the Western Cape Province in the east where it is

often found on clay or granitic soils in renosterveld (MANNING & GOLDBLATT, 2001). On the 7th of September 2006 *R. cruciata* was observed in renosterveld outside Malmesbury by a group from Naturetrek (PONTING, 2006). MANNING & GOLDBLATT (2001) place this species in the subgenus *Spatalanthus*.

2.2.6 *Romulea diversiformis*

The flowers are buttercup-yellow and unscented (DE VOS, 1983; MANNING & GOLDBLATT, 1997; MANNING & GOLDBLATT, 2001). Tepals are obovate with the inner tepals broader than the outer tepals (MANNING & GOLDBLATT, 1997; MANNING & GOLDBLATT, 2001). The fruiting peduncles are bent (MANNING & GOLDBLATT, 2001). Bracts are green to greenish (DE VOS, 1983). The outer bracts have narrow white membranous margins and apices while the inner bracts have wider membranous margins. *R. diversiformis* flowers (1 or more flower per plant) from August to September (DE VOS, 1972; MANNING & GOLDBLATT, 1997). These plants are 80 to 200 mm in height and are classed as stemless geophytes. It has 6 to 10 filiform leaves which are all basal (DE VOS, 1983; MANNING & GOLDBLATT, 2001).

The plants occur in moist or waterlogged dolerite and clay in the Western Karoo and Roggeveld of South Africa (MANNING & GOLDBLATT, 1997; MANNING & GOLDBLATT, 2001). MANNING & GOLDBLATT (2001) place this species in the subgenus *Spatalanthus*.

2.2.7 *Romulea flava*

Flowers are white or yellow and rarely flowers are also blue, blue-violet or pinkish (DE VOS, 1983; MANNING & GOLDBLATT, 1996; MANNING & GOLDBLATT, 2001). The flowers have a yellow cup (MANNING & GOLDBLATT, 1996; MANNING & GOLDBLATT, 2001). The white flowers of *R. flava* are usually scented (MANNING & GOLDBLATT, 2001). The tepals are oblanceolate and the outer tepals are uniformly green on the abaxial side. The fruiting peduncles are recurved and later erect (MANNING & GOLDBLATT, 2001). Outer bracts have narrow or inconspicuous membranous margins while inner bracts are submembranous or membranous and are often brown streaked (DE VOS, 1983; MANNING & GOLDBLATT, 2001). These plants flower from June to September and each plant generally has 1 to 4 flowers

(DE VOS, 1972; MANNING & GOLDBLATT, 1996; MANNING & GOLDBLATT, 2001). They are 50 to 550 mm in height with a stem that is subterranean or reaches 300 mm above the ground (DE VOS, 1983; MANNING & GOLDBLATT, 2001). The plant has 3 to 4 leaves of which one is a basal leaf. Leaves are narrowly or widely grooved with 4 grooves and are sometimes minutely ciliate or filiform (DE VOS, 1983; MANNING & GOLDBLATT, 2001).

R. flava populations are widespread in the southern African winter-rainfall zone (MANNING & GOLDBLATT, 2001). These plants grow in sandy or clay soils from Namaqualand in the north to Humansdorp in the southeast where it occurs in fynbos and renosterveld (MANNING & GOLDBLATT, 2001). MANNING & GOLDBLATT (2001) places this species in the subgenus *Romulea*.

2.2.8 *Romulea leipoldtii*

Flowers are white to cream with a yellow cup and sweetly scented (DE VOS, 1983; MANNING & GOLDBLATT, 2001). Tepals are elliptic and 18 to 35 mm long. Filaments are 5 to 8 mm long and anthers are 5 to 8 mm long. Fruiting peduncles are bent and later erect (MANNING & GOLDBLATT, 2001). Outer bracts are green with inconspicuous membranous margins. Inner bracts have colourless or brown speckled membranous margins (DE VOS, 1983; MANNING & GOLDBLATT, 2001). *R. leipoldtii* flowers from September to October (DE VOS, 1972; MANNING & GOLDBLATT, 2001). These plants usually have 4 to 6 flowers or more and are 100 to 300 mm in height, with some plants reaching up to 600 mm (DE VOS, 1972; MANNING & GOLDBLATT, 2001). The stem reaches 50 to 350 mm above ground (DE VOS, 1983; MANNING & GOLDBLATT, 2001). The plant has 4 to 6 leaves of which the lower 2 are basal. These leaves are grooved narrowly with 4 grooves (MANNING & GOLDBLATT, 2001).

R. leipoldtii occurs from the Bokkeveld Mountains in the Northern Cape Province in the north to Klipheuwel near Malmesbury in Western Cape Province in the south where it is found growing in damp sandy soil (DE VOS, 1983; MANNING & GOLDBLATT, 2001). This species is closely allied with *R. tabularis* (MANNING & GOLDBLATT, 2001). The main difference is the larger, bicoloured, cream to white

flowers of *R. leipoldtii* which has a dark yellow to orange centre (MANNING & GOLDBLATT, 2001). MANNING & GOLDBLATT (2001) place this species in the subgenus *Romulea*.

2.2.9 *Romulea minutiflora*

The small flower of this species is pale mauve with a yellowish cup. The tepals are 4 to 9 mm long and elliptic (MANNING & GOLDBLATT, 2001). The fruiting peduncles of this species are curved and later erect. The outer bracts have narrow margins which are frequently brown-spotted. Inner bracts can be observed to also have these brown-spotted margins (sometimes submembranous) (MANNING & GOLDBLATT, 2001). Flowering occurs from July to September (DE VOS, 1972; MANNING & GOLDBLATT, 2001). These plants have been observed to have 1 to 4 flowers and to reach 60 to 200 mm in height (DE VOS, 1972; MANNING & GOLDBLATT, 2001). There are several basal leaves present. These measure between 0.5 and 1.5 mm in diameter and are narrowly 4-grooved (MANNING & GOLDBLATT, 2001).

R. minutiflora is abundant throughout the South African winter-rainfall region. Its range extends from the Bokkeveld Mountains in the west to Grahamstown in the east (MANNING & GOLDBLATT, 2001). This species has been introduced into Australia (DE VOS, 1972). *R. minutiflora* is closely allied to *R. sinispinosensis*. Both these species have corms with a spade-shaped basal ridge (MANNING & GOLDBLATT, 2001). *R. minutiflora* is however easily identifiable by its very small pale mauve or pink flowers of which the largest flower observed had tepals measuring a mere 9 mm in length (MANNING & GOLDBLATT, 2001). The tepals of *R. sinispinosensis* are white and between 10 and 12 mm in length (MANNING & GOLDBLATT, 2001). MANNING & GOLDBLATT (2001) place this species in the subgenus *Romulea*.

2.2.10 *Romulea monadelpha*

This attractive unscented species has large dark red flowers with black blotches at the edge of its creamy cup. The tepals of this species is 25 to 40 mm long and obovate-cuneate, resulting in an almost bell-shaped flower (DE VOS, 1970b). Its anther filaments are 3 to 4 mm long and oblong, adnate or fused into a column. The fruiting peduncles are curved. The upper portion of outer bracts is commonly one keeled and the inner bracts are two keeled. The outer and inner bracts both have

brown membranous margins. Flowering is observed from August to September. The plants have 1 to 4 flowers (DE VOS, 1970b). DE VOS (1972) mentions that this is one of the most beautiful romuleas. The plants are 150 to 300 mm high with a subterranean stem. The plant has 3-5 filiform (1 to 2 mm in diameter) basal leaves which have 4 grooves (MANNING & GOLDBLATT, 2001).

The habitat of *R. monadelpha* is restricted to the Northern Cape Province of South Africa. Here it occurs on dolerite clay in an area that starts in the proximity of Nieuwoudtville, extends south to the top of the Gannaga Pass near Middelpos and stretches along the Bokkeveld and Roggeveld Escarpments in the western Karoo (MANNING & GOLDBLATT, 2001). The flowers of the Gannaga Pass population has salmon pink flowers with large silvery grey and black markings around the cup (MANNING & GOLDBLATT, 2001).

It is interesting to note that a plant collector brought a few corms of *R. monadelpha* to England in 1825. Two of the corms germinated, developed and flowered in Colvill's Nursery soon after planting. Unfortunately the locality of collection was not known and the only knowledge that existed for 139 years was drawings made during this discovery (DE VOS, 1970b). It appears that no herbarium specimens were made in this study.

Then in 1964 on a search for *R. sabulosa* in the area of Calvinia, a species that was so similar to *R. sabulosa was found*, that it was mistaken for *R. sabulosa* (DE VOS, 1970b). After being cultivated (no details on growth conditions given) in the Stellenbosch Nursery it was however noted that these had a fused filament column, a distinguishing feature of *R. monadelpha* (DE VOS, 1970b).

In a more recent collection by MANNING & GOLDBLATT (2001) it was noted that it is more usual for the filaments to be merely adnate. They suggest identifying *R. monadelpha* by its short black filaments which are oblong, unlike the usually pale green slender, tapering filaments of *R. sabulosa*. These two species can also be distinguished by their fruiting peduncles (MANNING & GOLDBLATT, 2001). *R. monadelpha* typically has stout and semiterete peduncles with conspicuously

flattened upper sides which are curved to the fruit. The fruiting peduncles of *R. sabulosa*, on the other hand, are commonly more slender and rounded in section and remain suberect in fruit. The peduncles of the latter species also remain suberect during fruiting. Apart from these morphological differences *R. sabulosa* is also restricted to light sandy clay soils near Nieuwoudtville whereas *R. monadelpha* is found on heavy, dolerite clay in several localities along the Bokkeveld and Roggeveld escarpments (MANNING & GOLDBLATT, 2001). MANNING & GOLDBLATT (2001) places this species in the subgenus *Spatalanthus.*

2.2.11 *Romulea pearsonii*

The flowers are lemon-yellow with elliptic tepals and the fruiting peduncles are suberect (DE VOS, 1983; MANNING & GOLDBLATT, 2001). Both the outer and the inner bracts are green. The outer bracts are firm and closely veined with narrow brown streaked membranous margins and an apex (DE VOS, 1983; MANNING & GOLDBLATT, 2001). The inner bracts have broad brown streaked membranous margins (MANNING & GOLDBLATT, 2001). The plant flowers in August to September with 1 to 3 flowers (DE VOS, 1972; MANNING & GOLDBLATT, 2001). Plants of this species have a height of 100 to 250 mm (DE VOS, 1983; MANNING & GOLDBLATT, 2001). The stem is completely subterranean or reaches 30 mm above ground (DE VOS, 1983; MANNING & GOLDBLATT, 2001). The plant has 3 to 4 filiform leaves of which 2 are basal (MANNING & GOLDBLATT, 2001).

R. pearsonii is restricted to higher elevations in central Namaqualand (MANNING & GOLDBLATT, 2001). Here it occurs from Grootvlei and the main Kamiesberg range and grows in sandy and granitic slopes and flats (MANNING & GOLDBLATT, 2001). MANNING & GOLDBLATT (2001) places this species in the subgenus *Romulea*.

2.2.12 *Romulea rosea*

The flowers are pink to magenta or white with a purplish zone around the yellow cup and are occasionally scented. The tepals are elliptic to oblanceolate and 4 to 6 mm long. The outer bracts have narrow membranous margins whereas the inner bracts have wide brownish membranous margins (MANNING & GOLDBLATT, 2001). *R. rosea* flowers from July to October and the plants have several flowers (DE VOS, 1972; MANNING & GOLDBLATT, 2001). These plants reach 150 to 600 mm from the

ground and have a subterranean stem (MANNING & GOLDBLATT, 2001). *R. rosea* has 3 to 6 leaves which are all basal.

R. rosea is common and many varieties and forms are found (DE VOS, 1972). It occurs in a variety of habitats which include stony clay flats and slopes (MANNING & GOLDBLATT, 2001). It is found throughout the Cape region from the Bokkeveld range to Port Elizabeth.

This species is a known invasive in other parts of the world, including Australia, New Zealand and USA (EDDY & SMITH, 1975; CROSSMAN *et al*., 2008; VAN KLEUNEN *et al*., 2008; FLEMING *et al*., 2009). Here it is regarded as a weed and numerous strategies can be found to eradicate this species due to its moderate toxicity to sheep and goats (EDDY & SMITH, 1975; SIMMONDS *et al*., 2000). MANNING & GOLDBLATT (2001) places this species in the subgenus *Spatalanthus*.

2.2.13 *Romulea sabulosa*

The flowers are currant or glossy red and rarely pink with black blotches at the edge of the creamy or greyish green cup (DE VOS, 1983; MANNING & GOLDBLATT, 2001). The flowers are unscented with tepals that are obovate-cuneate. The fruiting peduncles are suberect (MANNING & GOLDBLATT, 2001). The outer bracts are usually keeled above with narrow, usually brown, membranous margins. The inner bracts are 2-keeled and usually also have brown membranous margins (MANNING & GOLDBLATT, 2001). The plants flower in July to September during which they may have 1 to 4 flowers (DE VOS, 1972; MANNING & GOLDBLATT, 2001). Plants are 120 to 400 mm in length with a subterranean stem (MANNING & GOLDBLATT, 1997; MANNING & GOLDBLATT, 2001). The plant has 3 to 5 filiform leaves, all of which are basal (MANNING & GOLDBLATT, 2001).

R. sabulosa is a local endemic that grows in renosterveld on clay and in the Bokkeveld Escarpment west of Nieuwoudtville on sandy soil (MANNING & GOLDBLATT, 1997; MANNING & GOLDBLATT, 2001). MANNING & GOLDBLATT (2001) places *R. sabulosa* in the subgenus *Spatalanthus*.

2.2.16 *Romulea tabularis*

Flowers are lavender blue to white or bluish-mauve. The flower has a yellow cup and the lower half of the tepals are yellow (DE VOS, 1983; MANNING & GOLDBLATT, 2001). They are sometimes fragrant with tepals that are elliptic (MANNING & GOLDBLATT, 2001). The fruiting peduncles are arching and later erect. Outer bracts have inconspicuous membranous margins. The inner bract is submembranous with wide brown-speckled membranous margins (MANNING & GOLDBLATT, 2001). These plants flower from July to August with 2 to 4 flowers (DE VOS, 1972; MANNING & GOLDBLATT, 2001). Plants are 100 to 350 mm in height with a stem that reaches 100 to 350 mm above ground. They have 3 to 5 leaves of which 1 or 2 are basal (DE VOS, 1983; MANNING & GOLDBLATT, 2001).

R. tabularis occurs from northern Namaqualand to Cape Agulhas. Here it grows in wet, often waterlogged, sandy soils or limestone flats from Clanwilliam to Bredasdorp (DE VOS, 1983; MANNING & GOLDBLATT, 1996; MANNING & GOLDBLATT, 2001). Very closely allied with *R. leipoldtii*, *R. tabularis* however has smaller flowers and bicoloured tepals (MANNING & GOLDBLATT, 2001). MANNING & GOLDBLATT (2001) places this species in the subgenus *Romulea*.

2.3 PHYLOGENY AND TAXONOMY

The Iridaceae is a large family of petaloid monocotyledons. It includes over 1 630 species in about 77 genera (GOLDBLATT, 1990). Within the Iridaceae there are the subfamilies of Isophysidoideae, Nivenoideae, Iridoideae and Ixioideae. *Romulea* belongs to the subfamily Ixioideae (GOLDBLATT, 1990). Ixioideae in turn contains the tribes Pilansiae, Watsonieae and Ixieae. *Romulea* is a member of Ixieae (GOLDBLATT, 1990). Ixioideae is centered in Southern Africa, but some species such as *Gladiolus*, *Crocus* and *Romulea* also occur in Eurasia. The subfamily comprises of over 860 species of which 760 belong to the tribe Ixieae (GOLDBLATT, 1990).

Within Ixieae there are the subtribes of Ixiinae, Tritoniinae, Gladiolinae, Radinosiphon, Hesperanthinae, Melasphaerula, Babianinae, Romuleinae, Freesiinae and Anapalinae. *Romulea* belongs to the subtribe of Romuleinae (GOLDBLATT, 1990). The other members of this subtribe are *Crocus* and *Syringodea* (GOLDBLATT, 1990).

Other genera in Ixieae includes *Anomatheca*, *Babiana*, *Chasmanthe*, *Crocosmia*, *Crocus*, *Devia*, *Dierama*, *Duthieatrum*, *Freesia*, *Geissorhiza*, *Gladiolus*, *Hesperantha*, *Ixia*, *Melasphaetula*, *Radinosiphon*, *Shizostylis*, *Syringodea*, *Tritonia*, *Tritonopsis* and *Zygotritonia* (GOLDBLATT, 1990).

REEVES *et al.* (2001) showed the close association of the two genera *Romulea* and *Crocus.* It also confirms the classification of GOLDBLATT (1990) with molecular techniques. This classification was obtained by REEVES *et al.* (2001) by combining sequencing work in the Iridaceae done by other studies with their own sequencing data and grouping these in a combined parsimonious tree with bootstrap percentages and Fitch weights.

Maratti did a small taxonomic study on *Romulea* species in 1772 (DE VOS, 1972). In this work he described *Romulea* after a species growing in the neighbourhood of Rome. He proposed that this species was distinct from *Crocus*, *Colchicum*, *Sisyrinchium*, *Bulbocodium* and *Ixia* (DE VOS, 1972).

2.4 CONSERVATION STATUS

As it is a moral duty of every scientist to be concerned about the conservation of species of which they are using reproductive material harvested from the wild, a section on the conservation status of this genus is therefore included.

RAIMONDO *et al*. (2009) list *Romulea* as having fewer endangered species than previous studies (HILTON-TAYLOR, 1996). Although this is explained, it does not mention the status of one attractive species listed by HILTON-TAYLOR (1996) as

extinct, *R. papyracea*. According to RAIMONDO *et al*. (2009) there are 3 critically endangered species, 7 endangered species, 23 vulnerable species, 4 non-threatened, 4 rare, 2 data deficient, and 59 species of least concern.

Most of the species used in this study are classed by RAIMONDO *et al*. (2009) as species of least concern or species with a very low risk of extinction. Exceptions are *R. pearsonii* and *R. sabulosa* which are listed as being vulnerable to extinction.

2.5 THE CLIMATE OF *ROMULEA* SPP. HABITATS

The Namaqualand, where this genus has its centre of diversity, has a unique climate (DESMET, 2007). This is partially due to the fact that it is under the influence by two pronounced geographical rainfall gradients (DESMET, 2007). These are a latitudinal gradient of aridity and a longitudinal gradient of precipitation. The former decreases in precipitation towards the north into the Namib dessert and the latter brings precipitation from the winter-rainfall coastal areas and some summer-rainfall inland areas (DESMET, 2007). As a result, Namaqualand has highly reliable rainfall when compared to other arid regions with similar mean annual precipitation. Although rainfall in Namaqualand is low, it arrives between May and September almost every year. More than 60% of this rain is recorded during winter (DESMET, 2007). The cold Atlantic Ocean stabilises the climate of this region by preventing the intrusion of unstable air and by cooling this area with the aid of a pervasive south-westerly sea breeze (DESMET, 2007).

The climatic conditions of regions where 8 of the species used in this study are commonly found are further described in more detail in the following paragraphs. Linking this data to data of flowering time and assuming that the flower will take a month to set seed gives a valuable insight into the temperature regime required for germination. Although only the above mentioned climatic conditions are discussed here, looking at the data a month before flowering also provides an estimation of what the most suitable temperature for further growth could be, whereas the climate at the time of flowering provides insight into possible physical stimuli for flowering.

R. camerooniana has been known to occur in the Drakensberg region (MANNING & GOLDBLATT, 2001). The data in Figures 2.5 to 2.7 describes the climate of Royal National Park, a park situated within the Drakensberg range. This has been known to flower from May to February, although it mostly flowers from December to April (MANNING & GOLDBLATT, 2001; BURROWS & WILLIS, 2005), therefore it would be expected to set seed during May and July.

In Figures 2.6 and 2.7 there is a large drop in average total monthly rainfall and a corresponding drop in average daily humidity for the month of April. These conditions, which are not suitable for germination, continue until September. During September, the average daily minimum and maximum temperatures (averaged for 2004 to 2009) at the weather station at Royal National Park has been reported to be 7.4±0.8℃ and 24.5±0.8℃ respectively.

The climate of Calvinia, where *R. diversiformis* is found, is described in Figures 2.8 to 2.10. *R. diversiformis* also occurs around the area of Sutherland, Fraserburg and Beaufort west (Figures 2.11 to 2.19). During the time in which this species is expected to set seed (October to November) there is an increase in average daily temperature and average total monthly rainfall and a decrease in average daily humidity. According to this data, seeds should germinate well with a regime of night temperatures between 0 and 15℃ and day temperatures between 20 and 30℃.

R. flava is widespread across a large part of the southern-African winter-rainfall zone, including areas near Nieuwoudtville (Figures 2.20 to 2.22). *R. monadelpha* and *R. sabulosa* and many other species in this genus also occur in this area (MANNING & GOLDBLATT, 2001). Here rainfall increases sharply in the month of October and average total monthly rain remains the same for November. The time in which these species are expected to set seed, October and November, appears to be a suitable time for seed germination in Nieuwoudtville, the rainfall also increases in December, providing moist conditions which are suitable for seedling establishment. Minimum and maximum temperatures are 8.2±0.3 and 25.6±0.4℃ for October and 10.1±0.3 and 27.5±0.4℃ for November in Nieuwoudtville.

R. leipoldtii occurs in areas near Malmesbury (Figures 2.23 to 2.25) (MANNING & GOLDBLATT, 2001). Here there is an increase in temperature from October to November with a corresponding increase in rainfall and decrease in humidity. In November the average daily temperature increases, but average total monthly rainfall and average daily relative humidity decreases. It is therefore more likely that these plants would set seed in October, when conditions are moist. This means that a minimum and maximum temperature of 9.3± 0.4 and 24.2±0.4°C should be suitable for germination.

R. minutiflora is also widespread across a large part of the southern-African winter-rainfall zone, occurring as far east as Grahamstown (Figures 2.26 to 2.28) (MANNING & GOLDBLATT, 2001). During October the average daily minimum and maximum temperatures are 10.4±0.3 and 23.0±0.7°C, while the average daily minimum and maximum temperatures are 11.7±0.3 and 23.2±0.4°C during November. *R. rosea* is found throughout the Cape region and therefore any of these environments should be suitable for its germination. This is apparent when considering its distribution as illustrated in Figure 2.1. This species also has a long flowering time, lasting 4 months (July to October) (MANNING & GOLDBLATT, 2001). Estimation of conditions favourable for germination, such as those made for other species, is therefore unsuitable for this species.

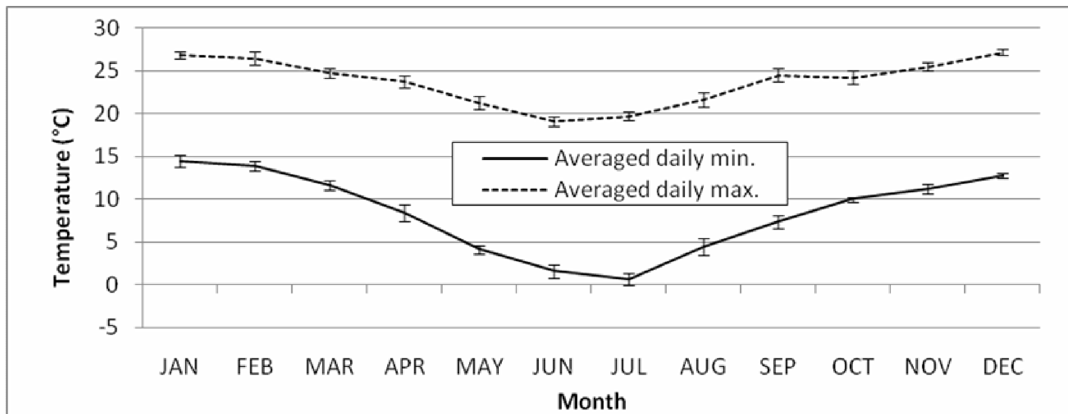

Figure 2.5: Royal National Park weather station (28° 57' E, 28° 41' S, 1392 m above sea level) average daily minimum and maximum monthly temperatures (Error bars indicate standard error of the mean of last 5 years).

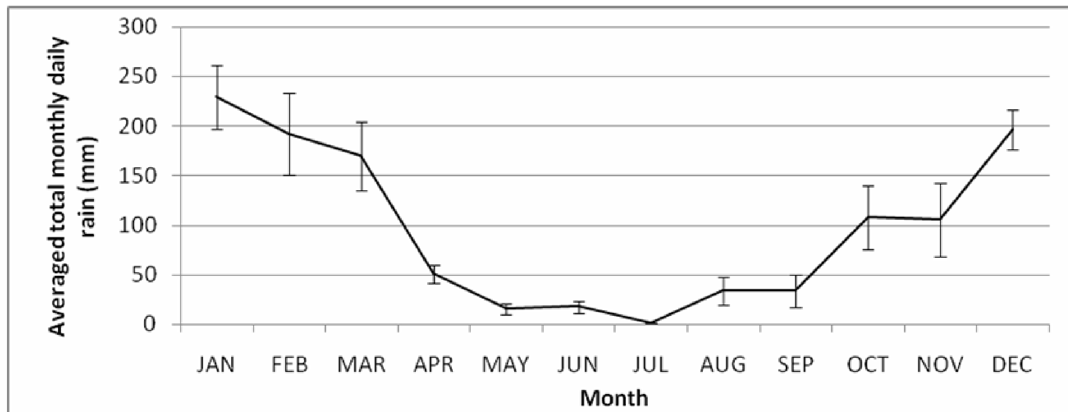

Figure 2.6: Royal National Park weather station (28° 57' E, 28° 41' S, 1392 m above sea level) average total monthly daily rain (Error bars indicate standard error of the mean of last 5 years).

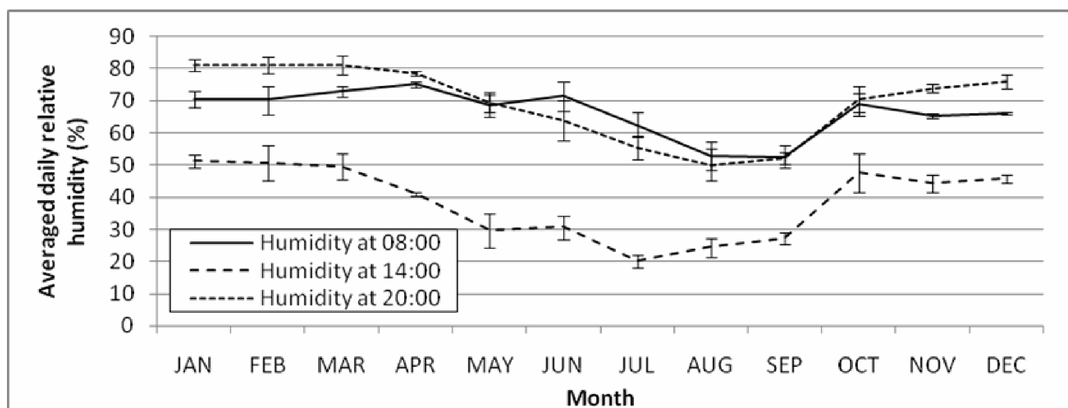

Figure 2.7: Royal National Park weather station (28° 57' E, 28° 41' S, 1392 m above sea level) average daily relative humidity (Error bars indicate standard error of the mean of last 3 years).

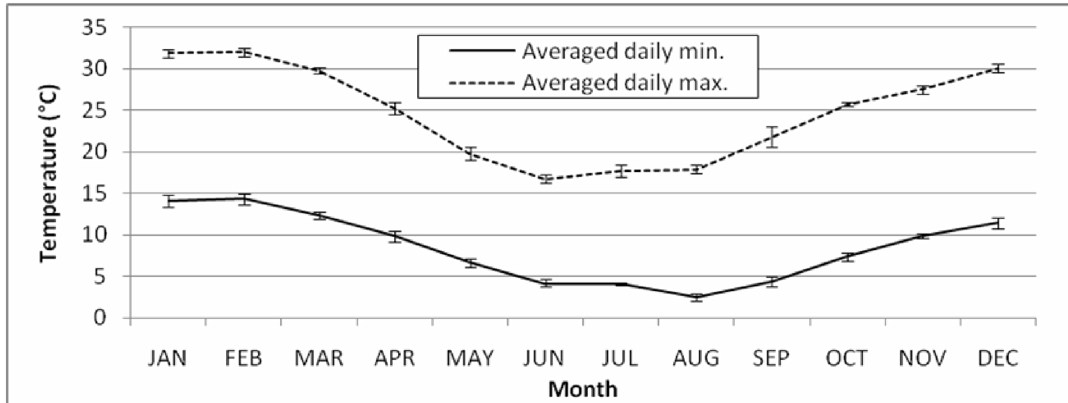

Figure 2.8: Calvinia (19° 56' E, 31° 29' S, 977 m above sea level) average daily minimum and maximum monthly temperatures (Error bars indicate standard error of the mean of last 5 years).

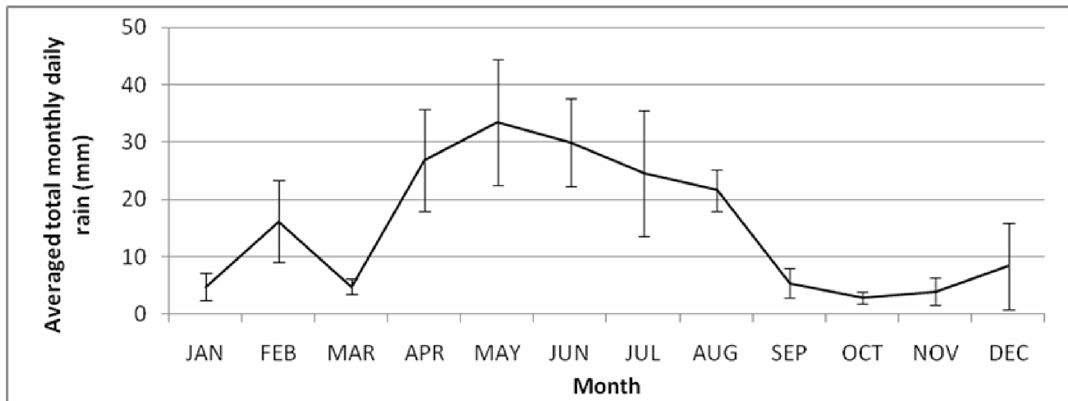

Figure 2.9: Calvinia weather station (19° 56' E, 31° 29' S, 977 m above sea level) average total monthly daily rain (Error bars indicate standard error of the mean of last 5 years).

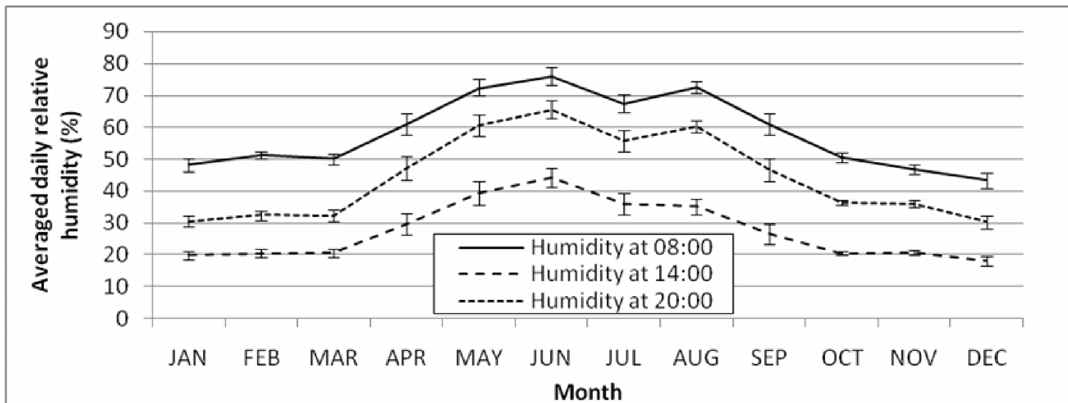

Figure 2.10: Calvinia weather station (19° 56' E, 31° 29' S, 977 m above sea level) average daily relative humidity (Error bars indicate standard error of the mean of last 5 years).

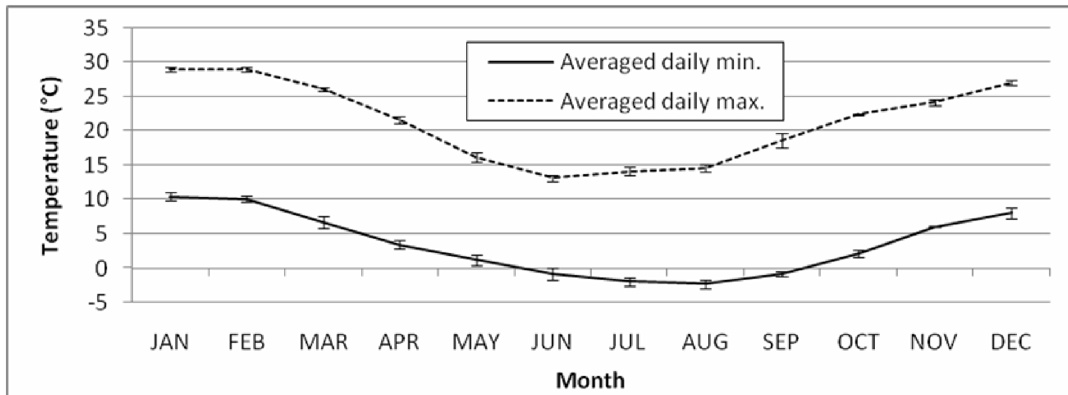

Figure 2.11: Sutherland weather station (20° 4' E, 32° 24' S, 1458 m above sea level) average daily minimum and maximum monthly temperatures (Error bars indicate standard error of the mean of last 5 years).

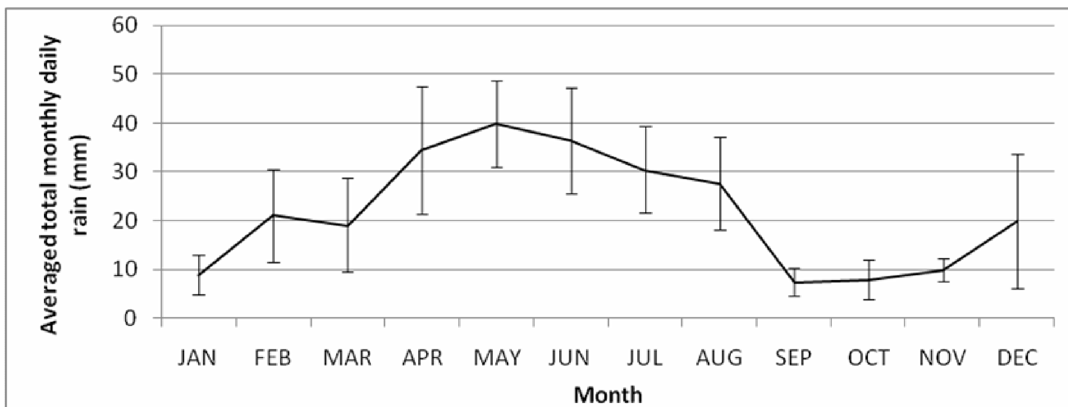

Figure 2.12: Sutherland weather station (20° 4' E, 32° 24' S, 1458 m above sea level) average total monthly daily rain (Error bars indicate standard error of the mean of last 5 years).

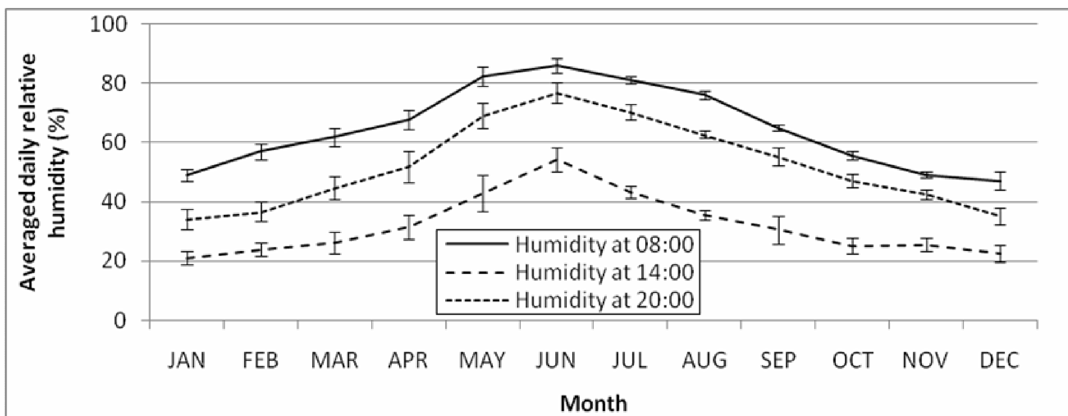

Figure 2.13: Sutherland weather station (20° 4' E, 32° 24' S, 1458 m above sea level) average daily relative humidity (Error bars indicate standard error of the mean of last 5 years).

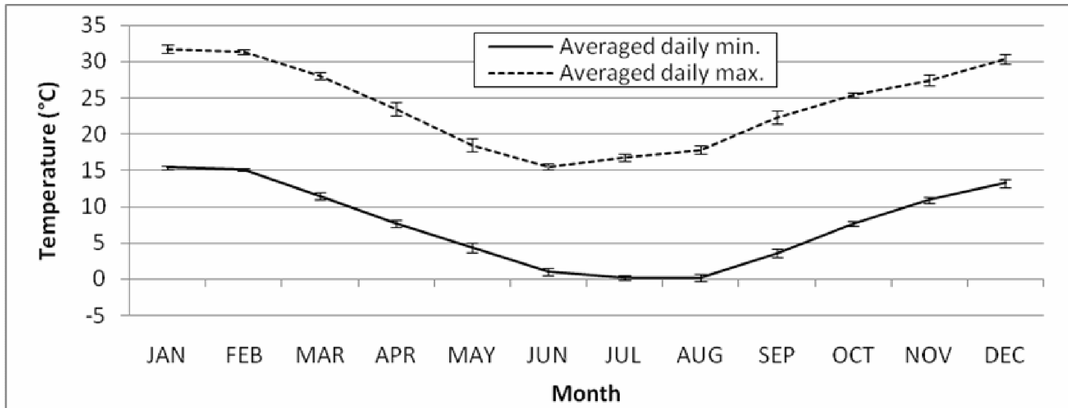

Figure 2.14: Fraserburg weather station (31° 55' S 21° 30' E, 1267 m above sea level) average daily minimum and maximum monthly temperatures (Error bars indicate standard error of the mean of last 5 years).

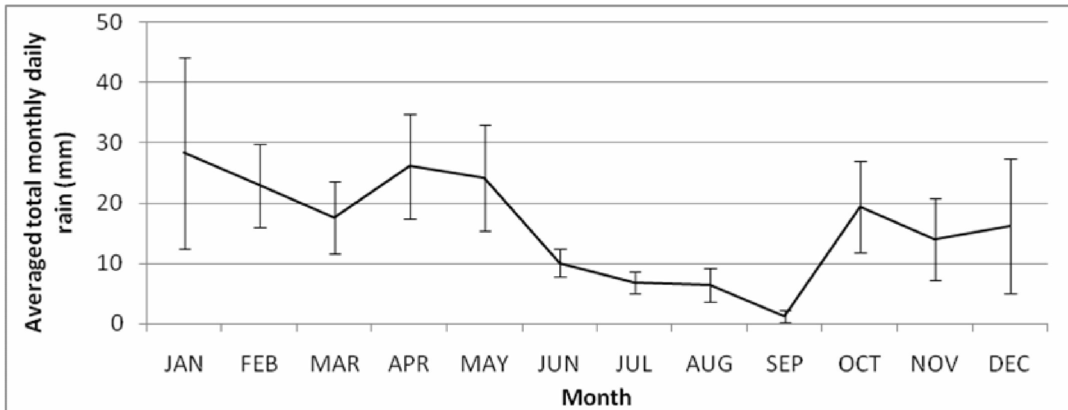

Figure 2.15: Fraserburg weather station (31° 55' S 21° 30' E, 1267 m above sea level) average total monthly daily rain (Error bars indicate standard error of the mean of last 5 years).

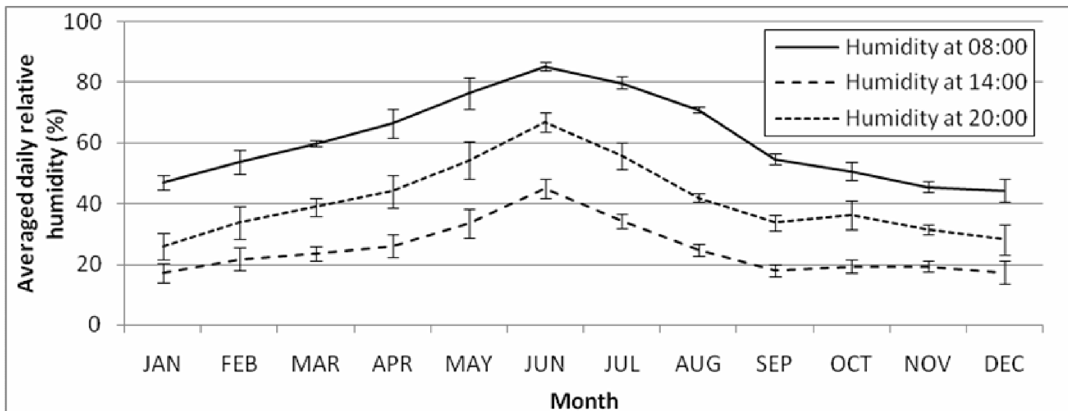

Figure 2.16: Fraserburg weather station (31° 55' S 21° 30' E, 1267 m above sea level) average daily relative humidity (Error bars indicate standard error of the mean of last 3 years).

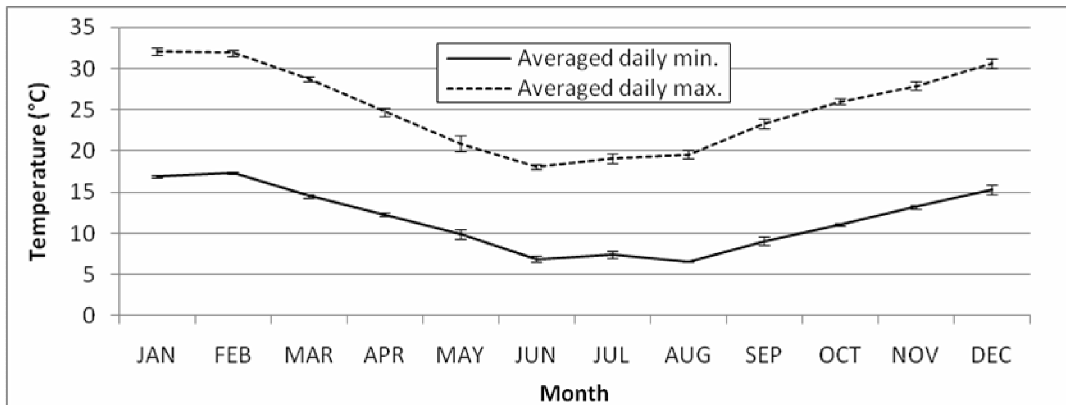

Figure 2.17: Beaufort West weather station (22° 35' E, 32° 21' S, 899 m above sea level) average daily minimum and maximum monthly temperatures (Error bars indicate standard error of the mean of last 5 years).

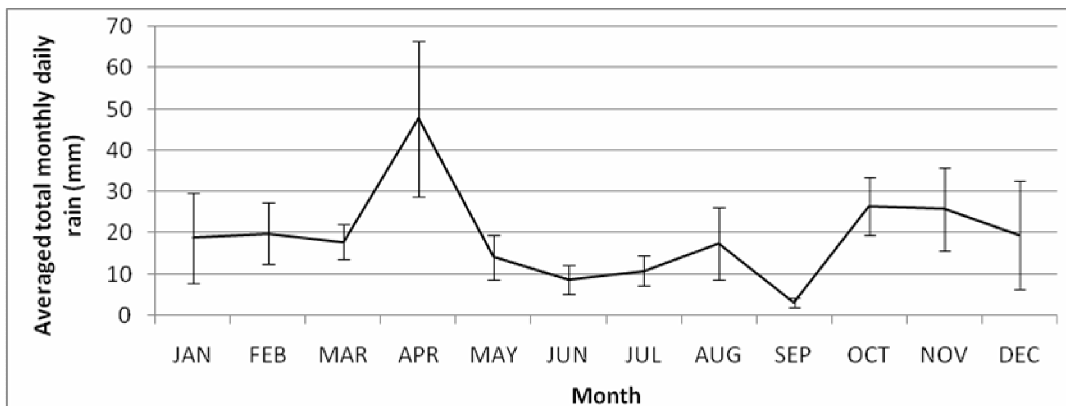

Figure 2.18: Beaufort West weather station (22° 35' E, 32° 21' S, 899 m above sea level) average total monthly daily rain (Error bars indicate standard error of the mean of last 5 years).

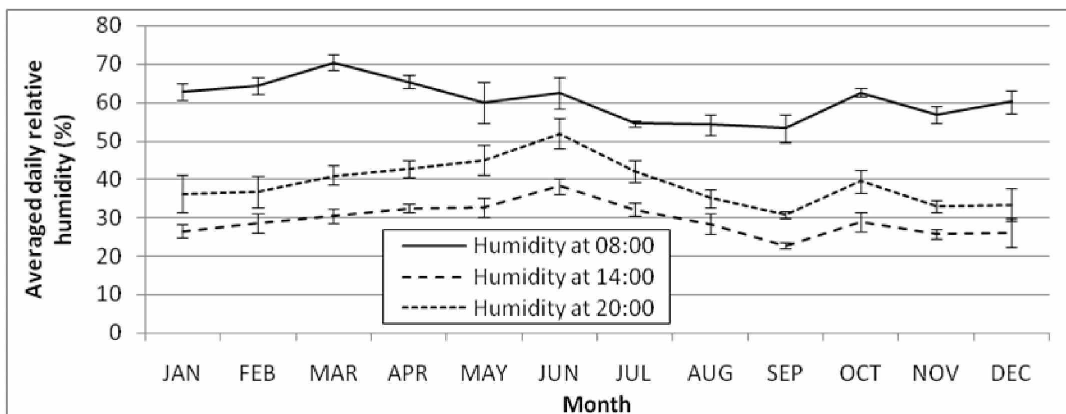

Figure 2.19: Beaufort West weather station (22° 35' E, 32° 21' S, 899 m above sea level) average daily relative humidity (Error bars indicate standard error of the mean of last 3 years).

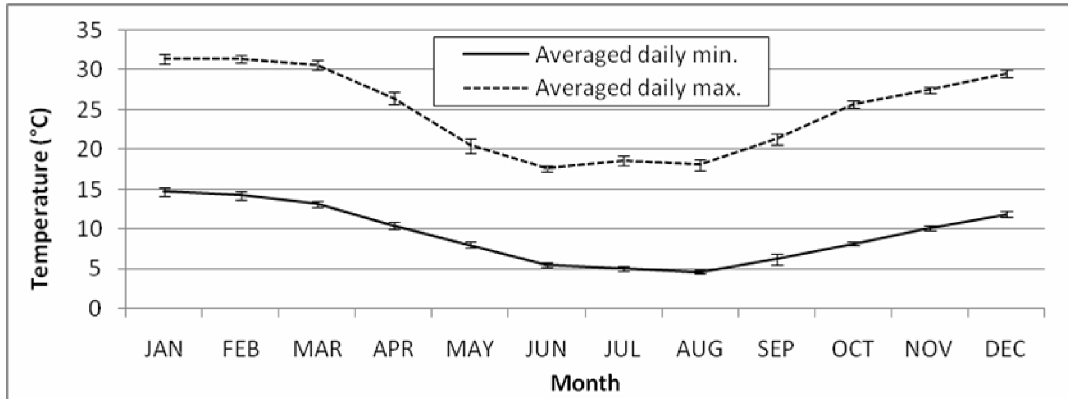

Figure 2.20: Nieuwoudtville weather station (19° 53' E, 31° 21' S, 731 m above sea level) average daily minimum and maximum monthly temperatures (Error bars indicate standard error of the mean of last 5 years).

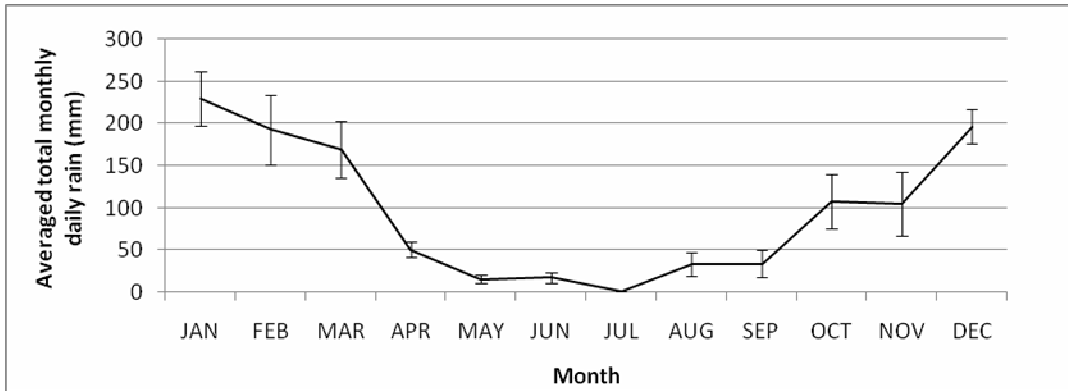

Figure 2.21: Nieuwoudtville weather station (19° 53' E, 31° 21' S, 731 m above sea level) average total monthly daily rain (Error bars indicate standard error of the mean of last 5 years).

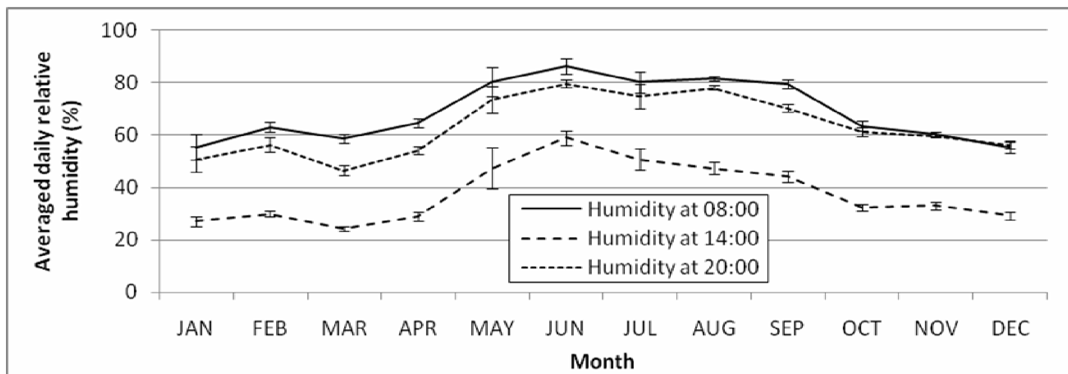

Figure 2.22: Nieuwoudtville weather station (19° 53' E, 31° 21' S, 731 m above sea level) average daily relative humidity (Error bars indicate standard error of the mean of last 3 years).

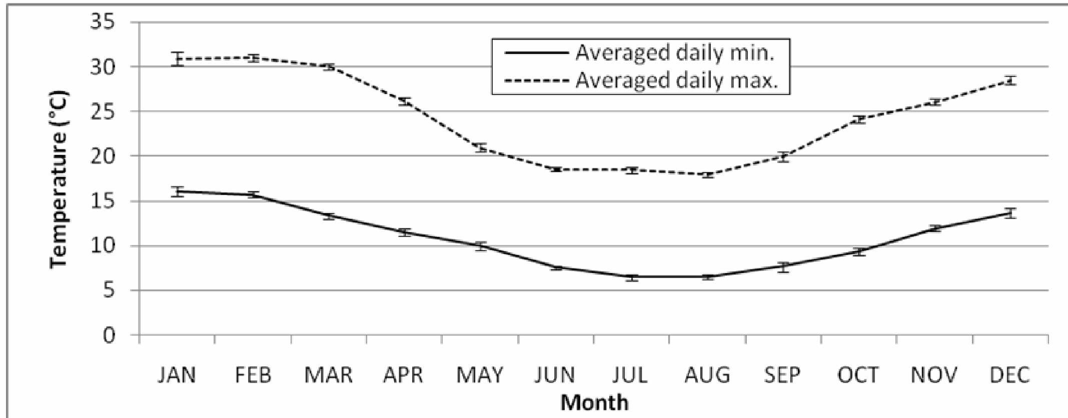

Figure 2.23: Malmesbury weather station (18° 43' E, 33° 28' S, 108 m above sea level) average daily minimum and maximum monthly temperatures (Error bars indicate standard error of the mean of last 5 years).

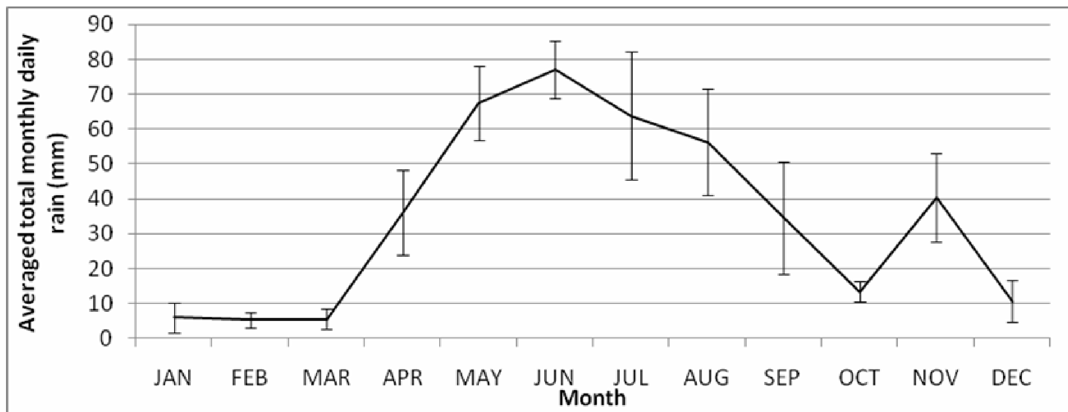

Figure 2.24: Malmesbury weather station (18° 43' E, 33° 28' S, 108 m above sea level) average total monthly daily rain (Error bars indicate standard error of the mean of last 5 years).

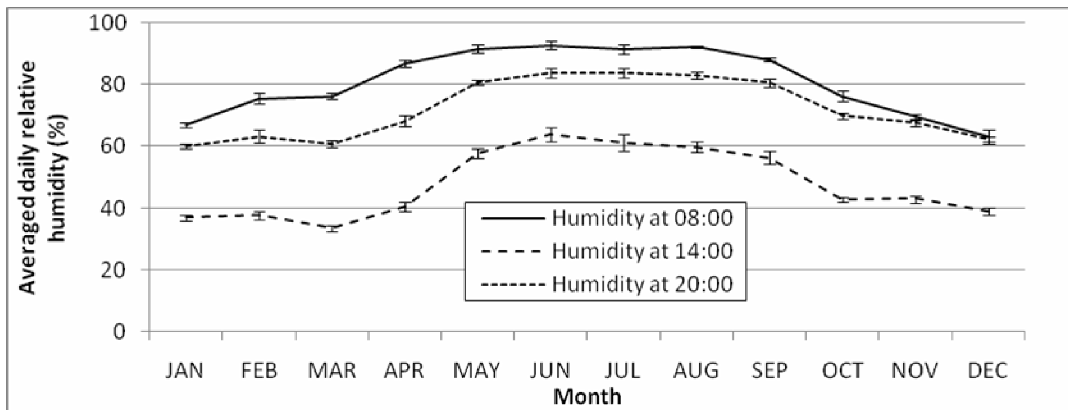

Figure 2.25: Malmesbury weather station (18° 43' E, 33° 28' S, 108 m above sea level) average daily relative humidity (Error bars indicate standard error of the mean of last 3 years).

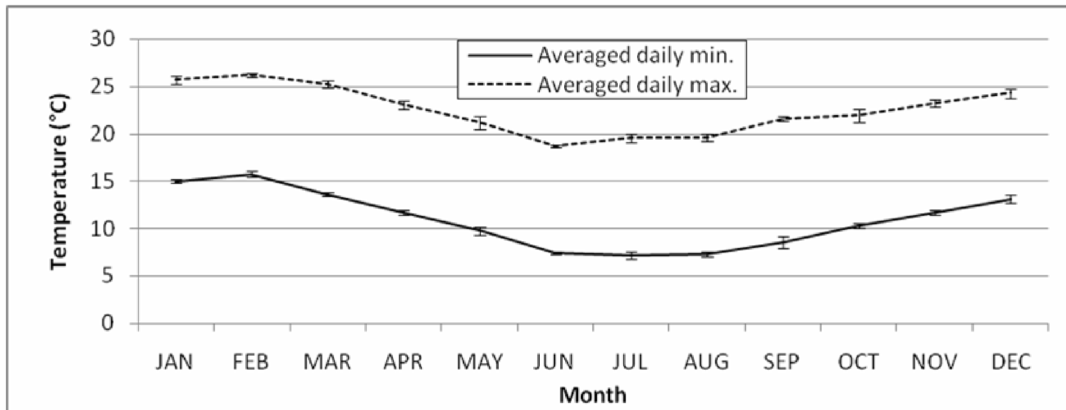

Figure 2.26: Grahamstown weather station (26° 30' E, 33° 17' S, 642 m above sea level) average daily minimum and maximum monthly temperatures (Error bars indicate standard error of the mean of last 5 years).

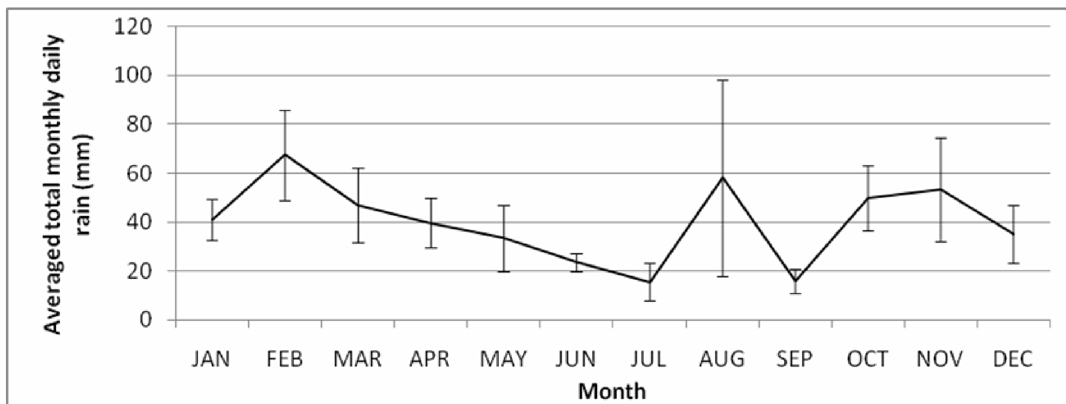

Figure 2.27: Grahamstown weather station (26° 30' E, 33° 17' S, 642 m above sea level) average total monthly daily rain (Error bars indicate standard error of the mean of last 5 years).

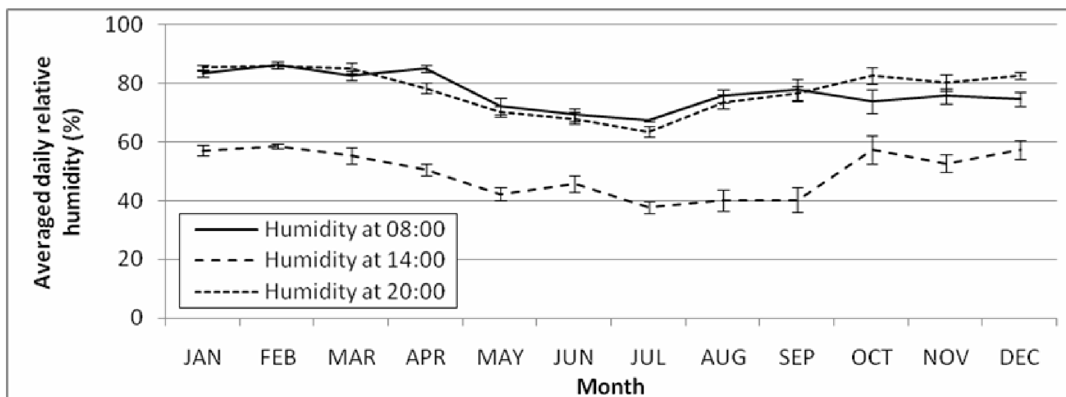

Figure 2.28: Grahamstown weather station (26° 30' E, 33° 17' S, 642 m above sea level) average daily relative humidity (Error bars indicate standard error of the mean of last 5 years).

2.6 SOIL SAMPLING AND ANALYSIS

Soils are complex systems high in spatial variation. It universally consists of three phases; solid, liquid and gas (GLASS, 1989). The solid phase contains the major inorganic reserves of the soil, the liquid phase contains a source of nutrients that are immediately available for uptake by the roots and the gaseous phase permits exchange of gases, the most important of these gases being oxygen, carbon dioxide and nitrogen (GLASS, 1989). In addition to these physical phases soil also contains a wide variety of biota including a diverse community of interdependent plants, animals and microorganisms. Other definitions are that soils generally consists of the rocks and their weathering products, substances formed by reactions within the soil profile and material from plants and animals (SLEEMAN & BREWER, 1988) and that soil is a multilayer granular composite originating from larger rocks (LECLERC, 2003). Just by looking at the diversity in these explanations and definitions, it is clear that soil is very complex, and its study is very interdisciplinary. Although soil is so complex and creating a complete list of all the constituents of soil would be very difficult and has not been attempted, Figure 2.29 shows some of the general constituents of soil (SLEEMAN & BREWER, 1988)

The mineral content of soil is the result of this weathering of larger rocks, climate, necrosis and decomposition of biota and the action of soil micro biota (LECLERC, 2003). The organic matter content of soil originates from the necrosis and decomposition of cells, tissues, organs and whole organisms (LECLERC, 2003). The nature and type of a soil is determined by the rock it originates from, the climate and the surrounding biota (LECLERC, 2003).

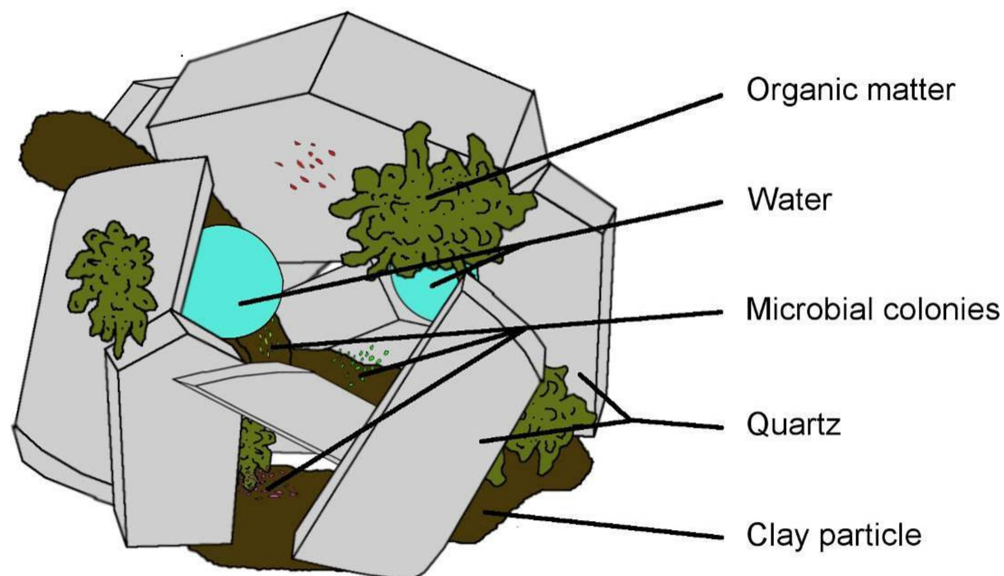

Figure 2.29: Diagrammatic representation of a small cluster of soil illustrating the complexity of organic soil. Also note the air spaces between the various components illustrated. Modified from descriptions of SLEEMAN & BREWER (1988).

2.6.1 Physical properties of soil

The physical properties of soils, which are greatly influenced by surface to volume relations of the soil particles, are important in determining the availability of various important ions to the plant roots (GLASS, 1989). Soil particles often have inorganic particles bound to their surface by a specific charge, making the ions unavailable to the plant for uptake (GLASS, 1989). When bulk flows are insufficient to supply the plant demand, diffusion-limited zones might develop around the roots. This results in fluctuations in the amounts of various mineral nutrients available to the plant (GLASS, 1989).

The physical properties of soil include texture, structure, density, porosity, water content, consistency, temperature and colour (DONAHUE *et al.*, 1983). These influence root penetration and the availability of water and oxygen to the root surface (DONAHUE *et al.*, 1983). Only soil texture and water content will be discussed here.

2.6.1.1 Soil texture

Soil is generally comprised of soil particles of various sizes (DONAHUE *et al.*, 1983). Soil texture can be described by granulometry, which is the quantification of the distribution of these soil particles in the different size classes (LECLERC, 2003). These size classes are called the soil separates and they can be placed in three general classes: Sands, silts and clays (Table 2.1) (DONAHUE *et al.*, 1983).

Table 2.1: Names of the soil separates and the particle diameters which define them (Modified from DONAHUE *et al.* (1983)).

Soil separate name	Diameter range (mm)
Stones	> 254
Cobbles	75 to 254
Gravels	2 to 75
Very coarse sand	1.0 to 2.0
Coarse sand	0.5 to 1.0
Medium sand	0.25 to 0.5
Fine sand	0.10 to 0.25
Very fine sand	0.5 to 0.15
Silt	0.002 to 0.5
Clay	< 0.002

The texture of a soil can be determined after the percentage of each separate within a sample is known and these are then grouped into percentage sand, silt and clay (DONAHUE *et al.*, 1983). These percentages can then be plotted on a triangular graph (See Figure 2.30). The description of the soil is determined by drawing three lines each perpendicular to a side of the triangle and arranged on the axes according to the relative percentages. The description at the point where these lines meet can then be read off. This method can also be used to determine the percentage content of a third separate group if the percentage content of 2 is known. Soil texture has a large influence on plant growth due to its effect on soil water retention capacity, oxygen capacity and thermal conductivity (LECLERC, 2003). Particles larger than 2 mm but less than 250 mm also play a large role in soil texture (DONAHUE *et al.*, 1983). When classifying a soil, the names of these separates precede the rest of the name (e.g. 'Stony', 'silty' and 'clay').

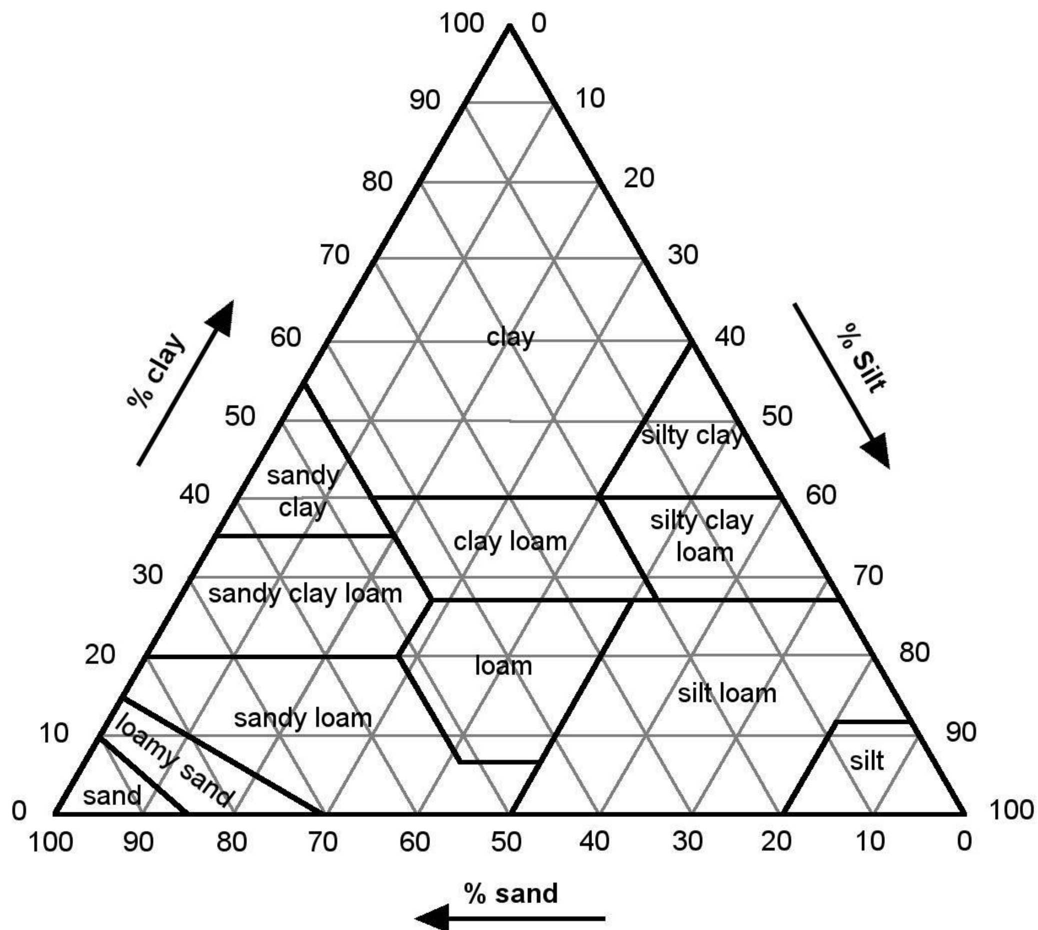

Figure 2.30: A textural triangle showing the range of variation in sand, silt, and clay for each soil textural class (Modified from DONAHUE *et al.* (1983) and LOVELAND & WHALLEY (1991)).

2.6.1.2 Soil water content

Water is a solvent for the soil solution and is essential for plant growth. This depends on the available surface area within the soil, which is determined by soil texture (LECLERC, 2003). In general, soil water content refers to the water that is evaporated by heating soil at 100 to 110°C until no further weight loss is observed (GARDNER *et al.*, 1991). This measurement does however not include structural water, which usually requires heating between 400 and 800°C to evaporate. An example of such structural water is the water molecules which are incorporated with hydroxyl groups in clay lattice structures (GARDNER *et al.*, 1991). In soil water

content studies involving organic soils, some inaccuracy is probable. In such cases some of the observed decrease in weight may be caused by changes in the composition of the organic matter (GARDNER *et al.*, 1991)

Field capacity is defined as the soil moisture content where gravitational drainage ceases in a soil that was saturated with water (DONAHUE *et al.*, 1983). Wilting point is defined as the specific soil moisture content at which the plant can no longer absorb water. The water available to the plant can thus be calculated by the difference between field capacity and wilting point (DONAHUE *et al.*, 1983).

2.6.2 Organic matter

This includes living, dead and decomposed biotic matter (DONAHUE *et al.*, 1983). Soil organic matter is a very important factor in soil fertility (DONAHUE *et al.*, 1983). It is a reservoir of essential plant nutrients, including nitrogen and phosphorous. Soil organic matter also loosens up the soil to provide aeration (DONAHUE *et al.*, 1983).

2.6.3 Soil nutrients

Sixteen chemical elements are known to be important to a plant's growth and survival. The sixteen chemical elements can be divided into two main groups, non-mineral and mineral, according to chemical structure. The non-mineral nutrients are hydrogen (H), oxygen (O), & carbon (C). The mineral nutrients are divided into two groups; macronutrients and micronutrients (See Table 2.2). The macronutrients are considered to be the nutrients essential to plant growth.

Table 2.2: Classification of mineral elements into macro- and micronutrients (Modified from (MARSCHNER, 1995)).

Classification	Element
Macronutrient	N, P, S, K, Mg, Ca
Micronutrient	Fe, Mn, Zn, Cu, B, Mo, Cl, Ni, Na, Si, Co

Macronutrients can be divided into two more groups; primary and secondary nutrients. The primary nutrients are nitrogen (N), phosphorus (P), and potassium (K). These major nutrients usually are lacking from the soil first because plants use large amounts of these elements. The secondary nutrients are calcium (Ca), magnesium (Mg), and sulphur (S). There are usually enough of these nutrients in the soil. Micronutrients are those elements essential for plant growth which are needed in only very small quantities. These are boron (B), copper (Cu), iron (Fe), chloride (Cl), manganese (Mn), molybdenum (Mo) and zinc (Zn) (MARSCHNER, 1995).

2.6.4 pH

The abbreviation for pH comes from the French term pouvoir hydrogéne or "hydrogen power" (DONAHUE *et al.*, 1983). This is because the amount of hydrogen ions is the variable measured by instruments used to determine the pH of a solution.

The pH of a soil depends on the original weathered rock content and the C/N ratio of the surrounding biota and decomposing organic matter (LECLERC, 2003). The occurrence of ions of Ca and Mg in a soil is often correlated with a increase in pH (pH of 7.5 to 8.5) (DONAHUE *et al.*, 1983; LECLERC, 2003). This is also correlated with a decrease in the concentrations of K and Na (DONAHUE *et al.*, 1983). A very acidic soil is usually a result of extensive leaching, a high proportion of sesquioxide and kaolinite, slow microbial activity and a low concentration of exchangeable basic cations (DONAHUE *et al.*, 1983).

The pH of the nutrient solution affects the solubility and availability of nutritional elements (DONAHUE *et al.*, 1983). Most minerals are more soluble in acidic soils (DONAHUE *et al.*, 1983). Soluble forms of aluminium and manganese are commonly found in soils with a high acidity (pH of 4 to 5) (DONAHUE *et al.*, 1983). A high acidity also negatively affects many nitrogen fixing bacteria (DONAHUE *et al.*, 1983).

A highly basic soil can also affect plant growth negatively (DONAHUE *et al.*, 1983). Examples of such soils include soils that have a high calcium content and soil which have not been leached (DONAHUE *et al.*, 1983). These are common in low rainfall areas such as Namaqualand (DONAHUE *et al.*, 1983)

Because processes that causes the production of H^+ ions contributes to acidity, anything that reduces the activity of H^+ ions in the soil solution would thus neutralize soil acidity. Sources of such alkalinity include low rainfall, resulting in less leaching of non-acid cations, increased sodium levels and plant uptake of nutrients. An effective neutralizer of acidity is calcium. Each mole of $CaCO_3$ neutralizes 2 moles soil acid. It is the carbonate in $CaCO_3$ that acts to neutralize soil acidity. Other neutralizers include magnesium carbonate, calcium oxide, calcium hydroxide and wood ash. An acid soil with a high cation exchange capacity needs a greater amount of limestone than a low cation exchange capacity soil of the same pH, because of the much greater number of reserve H^+ ions held in the soil with the high cation exchange capacity (HEWITT & SMITH, 1974).

2.6.5 Salinity

A saline soil is defined as a nonalkali soil containing soluble salts in quantities that interfere with the growth of common crop plants (GAUCH, 1972). Soil is generally seen as saline if it contains more than 0.1% soluble salts (GAUCH, 1972).

2.6.6 Cation and anion exchange capacity and surface charges

The cation exchange capacity of soil can be defined as the sum of positive charges of the absorbed cations that a soil can absorb at a specific pH (MARSCHNER, 1995). The more clay and organic matter a soil contains, the higher its cation exchange capacity and the stronger the cations are held.

2.6.7 Soils of Namaqualand

The soils of Namaqualand are very diverse. The occurrence of a impenetrable hardpan layer in most plain or valley landscapes is however a common feature of this area (DESMET, 2007). The soils of Namaqualand can be broadly classified into 3 groups according to DESMET (2007).

These are the weakly structured grey, yellow or red medium sands of the Sandveld and Bushmanland, the shallow, undifferentiated and free-draining red and yellow, variably grained, sandy to loamy soils of the Kamiesberg and Richtersveld ranges and the red, base-rich, granite-derived colluvial soils rich in clay of the inland margin of the coastal plain below the Hardeveld range (DESMET, 2007).

The soils of the Sandveld and Bushmanland are derived from aeolean reworking of marine or fluvial deposits, whereas the soils of the Kamiesberg and Richtersveld range is derived from *in situ* weathering of the underlying parent material (DESMET, 2007). The sands of the inland margin of the coastal plain below the Hardeveld range has been reworked through time to produce a variety of sand types, ranging from white, calcareous sands to deep red, acidic sands (DESMET, 2007).

2.6.8 Soils of Nieuwoudtville

R. sabulosa is restricted to the clay and sandy soils of the Bokkeveld escarpment west of Nieuwoudtville in renosterveld (MANNING & GOLDBLATT, 2001). Many other species in this genus including *R. monadelpha* and *R. flava* also occur in the vicinity of Nieuwoudtville, making this an area of interest.

Not only the plant life, but also the soils of the Nieuwoudtville region are diverse (BRAGG *et al.*, 2005). In the west, along the Bokkeveld escarpment exposed Table Mountain Sandstone (TMS) is prominent, while tillite and shales covers the underlying TMS in the east (BRAGG *et al.*, 2005). Tillite is defined as a sedimentary rock composed of indurated (rendered hard by heat, pressure or cementation) till, which is an unstratified and unsorted sediment carried or deposited directly by or under a glacier. Shale can be defined as finely stratified consolidated sediment mainly composed of clay-sized particles (SOIL CLASSIFICATION WORKING GROUP, 1991).

A dolerite sill intrudes into tillite in the vicinity of Nieuwoudtville (BRAGG *et al.*, 2005). Dolerite is an igneous rock that has risen from under the earth crust as magma and mostly solidified as intrusions such as dykes and sills before reaching the surface (SOIL CLASSIFICATION WORKING GROUP, 1991). In this case the sill is visible as a rocky ridge. On the moist east slopes of this ridge, the dolerite has weathered through time to form self-mulching clay soils (BRAGG *et al.*, 2005).

2.7 PROPAGATION OF *ROMULEA* SPECIES

There are very few publications on the propagation of *Romulea* species. In a book by DENO (1993), it was reported that *R. bulbocdium* and *R. luthicii* geminated at 10℃, with no germination at 20℃. It also stated that 96% germination was obtained during winter in Tauranga, New Zealand with fresh *R. hantamnensis* seeds, whereas seeds which had been in dry storage for an unknown time showed no germination (DENO, 1993). The only scientific paper that could be found on this topic is a study on the seed dispersal and germination of *R. rosea* by EDDY & SMITH (1975). Some preliminary tests done by EDDY & SMITH (1975) suggests that application of KNO_3 and prechilling for 5 days at 2℃ does not increase the germination of this species. These experiments indicated an optimum temperature of 10 to 11℃. This was confirmed by larger experiments, which showed that its germination has an optimum in the range of 9.5 to 15℃ and is inhibited by temperatures exceeding 16.5℃. They found that germination of this species is quite slow relative to other species occurring in the pastures which they studied (EDDY & SMITH, 1975). They also showed that seed collected in 1969 had a higher percentage germination and shorter time to germination than seed collected in 1968 (EDDY & SMITH, 1975). This and the experiments on *R. hantamnensis seeds* described by DENO (1993) suggest that fresh seeds of species of this genus should be used for germination.

2.8 GERMINATION PHYSIOLOGY

2.8.1 Seed structure

An angiosperm seed is typically comprised of the embryo, which is the result of fertilization of the egg cell in the embryo sac by a male pollen tube nucleus; the endosperm, which arises from the fusion of two nuclei in the embryo sac with the other pollen tube nucleus; the perisperm, which is developed from the nucellus; and the protective testa or seed coat, which is formed from one or both the integuments around the ovule (HARTMANN & KESTER, 1965; BEWLEY & BLACK, 1994).

The embryo is the diploid result of fertilization and is a minute autotrophic plant (EDMOND *et al.*, 1964). The embryo principally consists of an embryonic axis and at least one cotyledon (BEWLEY & BLACK, 1994). The axis further consists of the embryonic root (radicle), the hypocotyl with attached cotyledons and the shoot apex with the first true leaves (plumules) attached (BEWLEY & BLACK, 1994).

The nourishing tissue is generally either the endosperm or cotyledons (EDMOND *et al.*, 1964). In the case of endospermic seeds, the endosperm, which is present in the mature seed, serves as a food storage organ (HARTMANN & KESTER, 1965). Here the testa and endosperm are the two layers covering the embryo (BEWLEY & BLACK, 1994). In non-endospermic seeds the cotyledons serve as the sole food storage organ (BEWLEY & BLACK, 1994). During development, the cotyledons absorb the food reserves from the endosperm. Here the embryo is enclosed by the testa and the endosperm is all but completely degraded in the mature seed (BEWLEY & BLACK, 1994).

In some cases the testa exists in a rudimentary form only and the prominent and outermost structure is the pericarp or fruit coat derived from the ovary wall (HARTMANN & KESTER, 1965). In such cases the embryo is also encased in a fruit (BEWLEY & BLACK, 1994). The seed coverings provide mechanical protection to the embryo and make transportation and storage of seeds possible (HARTMANN & KESTER, 1965).

Hairs or wings, which aid in seed dispersal, sometimes develop as a modification of the enclosing fruit coat (BEWLEY & BLACK, 1994). These are attached via the hilum (BEWLEY & BLACK, 1994). The hilum is a funicular scar on the seed or fruit coat that marks the point at which the seed was attached via the funiculus to the ovary tissue (LAWRENCE, 2000). In many cases a small hole, called the micropyle, can be seen at the end opposite to the end with the hilum (BEWLEY & BLACK, 1994).

Seeds store various substances that are important for germination and early seedling growth. These primarily include carbohydrates, fats and oils, and proteins (MAYER, 1977; BEWLEY & BLACK, 1994). Other important substances that are only stored in small amounts include alkaloids, lectins, proteinase inhibitors, phytin, and raffinose

oligosaccharides (RAGHAVAN, 1976; BEWLEY & BLACK, 1994). Most seeds store their major food reserves within the embryo; usually the cotyledons (BEWLEY & BLACK, 1994). Some plants also have their seed storage reserves within extra-embryonic tissues. These extra-embryonic tissues used for storage include the endosperm (Gymnosperms) or the perisperm (*Coffea arabica*). Both embryonic and extra-embryonic tissues can also be used for storage; such as in maize (BEWLEY & BLACK, 1994).

2.8.2 Seed germination

Germination starts with the uptake of water by a seed and ends with the onset of elongation of the embryonic axis, usually the radicle (BEWLEY & BLACK, 1994). It also includes the steps of protein hydration, sub cellular changes, respiration, macromolecular syntheses and cell elongation (RAGHAVAN, 1976; BEWLEY & BLACK, 1994). The combined effect of these steps is to transform a dehydrated, dormant embryo into an embryo which grows actively and accumulates water (MAYER, 1977; BEWLEY & BLACK, 1994). A seed in which none of these processes have taken place is said to be quiescent. They characteristically have low moisture content (5-15%) and an extremely slow metabolic rate (BEWLEY & BLACK, 1994). Seeds are able to survive in this state for a number of years. Quiescent seeds require an environment of suitable temperature, hydration and the presence of oxygen in order to germinate (BEWLEY & BLACK, 1994). The major cellular processes involved in the initiating and facilitating of radicle emergence include respiration, RNA and protein synthesis and enzyme and organelle activity (RAGHAVAN, 1976; BEWLEY & BLACK, 1994).

During imbibition various structural and physical changes occur (BEWLEY & BLACK, 1994). The completion of imbibition requires a small amount of water (not more than three times the seed's dry weight). For successful subsequent root and shoot growth a larger and more constant supply of water is essential (BEWLEY & BLACK, 1994). The uptake of water by the seed can be seen as triphasic (Figure 2.31) (BEWLEY & BLACK, 1994). Phase 1 involves imbibition, where a strong osmotic gradient results in the uptake of water from the soil (BEWLEY & BLACK, 1994). Different areas of the testa and different organs within the seed often absorb variable amounts of water.

Phase 2 is seen as a lag phase. Here the matrix forces of the seed cells that caused the strong osmotic gradient in phase 1 are no longer active (BEWLEY & BLACK, 1994). During this phase major metabolic events take place in preparation for radicle emergence (BEWLEY & BLACK, 1994). Only germinating seeds, and not dormant seeds, enter phase 3. This stage is associated with changes in the cells of the radicle and radicle elongation (JANN & AMEN, 1977; BEWLEY & BLACK, 1994). These changes are facilitated by the uptake of water (JANN & AMEN, 1977). This uptake of water is a result of the production of low-molecular-weight osmotically active substances (BEWLEY & BLACK, 1994). These substances are produced as a result of hydrolysis of stored reserves (BEWLEY & BLACK, 1994). The kinetics of water uptake is however more complex than this, as many seeds distribute water to different seed parts at different rates.

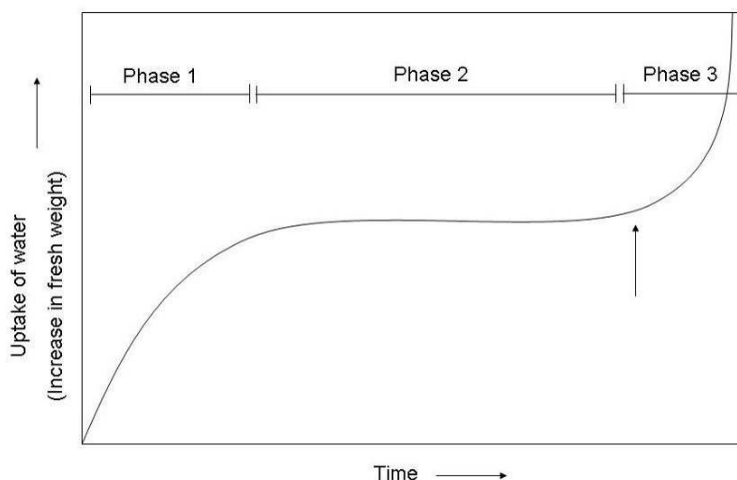

Figure 2.31: The triphasic pattern of water uptake by germinating seeds, with arrow showing the time of radicle protrusion (BEWLEY & BLACK, 1994).

For germination to be completed, the radicle must expand and penetrate the surrounding structures (BEWLEY & BLACK, 1994). This does not require cell division. Instead, the radicle cells elongate as the radicle penetrates through the surrounding tissues and cell division starts some time after the testa is eventually ruptured. There are a number of possible requirements for radicle elongation (BEWLEY & BLACK, 1994). One such requirement is the lowering of the osmotic potential as a result of the accumulation of solutes within the radicle; this increases water uptake and raises the turgor pressure, which facilitates cell elongation. The

initial growth of the seedling follows one of two distinct patterns (HARTMANN & KESTER, 1965). The seedling either follows the pattern of epigeous germination, where the hypocotyl elongates and raises the cotyledons above the ground, or hypogeous germination, where the lengthening of the hypocotyl does not cause the cotyledons to rise above the ground and only the epicotyl emerges (HARTMANN & KESTER, 1965).

2.8.3 Measuring germination

It is incorrect to equate germination to seedling emergence from soil, as germination ends sometime before this (BEWLEY & BLACK, 1994). Emergence of the axis can however be used as a precise measurement of termination of germination (BEWLEY & BLACK, 1994).

The progress of germination is expressed as a percentage of the total number of seeds tested at time intervals throughout the germination period (BEWLEY & BLACK, 1994). When this relationship is expressed graphically it ordinarily yields a sigmoid curve. Some valuable conclusions can be drawn from variations in the shape of such a curve. If the curve flattens off when only a low percentage of the seeds have germinated it indicates that the seeds have a low germinating capacity (BEWLEY & BLACK, 1994). The shape of the curve also describes the uniformity of germination (BEWLEY & BLACK, 1994).

Mean germination time can be calculated by the following equation: $MGT=\Sigma(n \times d)/N$. Here n=number of seeds germinated on each day, d=number of days from the beginning of the test, and N=total number of seeds germinated at the termination of the experiment (ELLIS & ROBERTS, 1981). When this is combined with a measure of germination, data can be displayed in a more concise way (KULKARNI *et al.*, 2007).

2.8.4 Promotion and inhibition of germination

<u>*2.8.4.1 Gibberellin and abscisic acid*</u>

Numerous studies have shown that GA promotes germination in dormant and non-dormant seeds (JONES & STODDART, 1977). It has also been shown that levels of

endogenous gibberellins increase during low temperature treatments (JONES & STODDART, 1977).

Gibberellins (GA) promote the induction of cell wall hydrolases and thereby promote endosperm weakening and endosperm rupture (MARION-POLL, 1997). Abscisic acid (ABA) inhibits the induction of cell wall hydrolases and thereby inhibits this weakening and rupture (MARION-POLL, 1997).

GA promotes and ABA inhibits the embryo growth potential. ABA, however, also plays an important role in seed development and germination, acquisition of desiccation tolerance, accumulation of proteins and lipid reserves, and induction and maintenance of seed dormancy (MARION-POLL, 1997).

The expression of genes encoding enzymes that mobilise food reserves is induced by several known GA signalling factors. These food reserves include the starches, proteins and lipids that are stored in the endosperm (PENG & HARBERD, 2002). During protein rehydration GA biosynthesis is induced by a phytochrome-mediated light signal and the newly synthesised GA down-regulates the expression of protein repressors of germination. These are also activated by rehydration, by protein degradation, suppression of transcription or mRNA degradation (MARION-POLL, 1997). This newly synthesized GA also initiates signals to induce the expression of hydrolytic enzymes that modify the cell wall and weaken the endosperm cap, thus facilitating germination (PENG & HARBERD, 2002).

It has also been proposed that GA could promote the formation of low molecular weight mono- and disaccharides, which assist the intracellular generation of negative water potentials, thus aiding radicle emergence (TIAN *et al.*, 2003).

2.8.4.3 Cytokinins and auxins

Cytokinins are involved in cell division and thus both radicle and cotyledon expansion (EMERY & ATKINS, 2006). Cytokinins accumulates mainly in the endosperm of seeds (LEUBNER-METZGER, 2006). Auxins are involved in the coordination of correct cellular patterning after the globular stage in embryogenesis (LEUBNER-METZGER, 2006).

2.8.4.2 Potassium nitrate

This chemical is commonly used to encourage seed germination, with solutions of 0.1 and 1.0% frequently being used in germination tests (COPELAND, 1976). KNO_3 has been shown to act synergistically with factors such as temperature and media supplementation with kinetin and gibberellic acid. Seeds sensitive to KNO_3 are often also sensitive to light (COPELAND, 1976).

2.8.4.3 Ethylene

Increased ethylene evolution accompanies seed germination of many species (BASKIN & BASKIN, 1998). Ethylene promotes its own biosynthesis during pea seed germination by positive feedback regulation of 1-aminocyclopropane-1-carboxylic acid oxidase.

2.8.4.4 Smoke

Many plant species have shown increased percentage germination and vigour after exposure of seeds or seedlings to smoke water (MINORSKY, 2002). These include plants naturally adapted to areas of high fire frequency as well as some species that are not specifically adapted to these conditions. These include agriculturally useful species, such as maize (SPARG *et al.*, 2006) and lettuce (BROWN & VAN STADEN, 1997), as well as many aesthetically useful species, such as those naturally occurring in fynbos (BROWN & VAN STADEN, 1997). Species originating from natural fire-prone habitats include many species of South African fynbos such as the fire-climax grass, *Themeda triandra* (Poaceae) and members of the Mesembryanthemaceae (BROWN & VAN STADEN, 1997). Other examples of such smoke water stimulated plants include species of the California chaparral and many other fire-prone communities (BLANK & YOUNG, 1998). It has been suggested that the promotive effect of smoke is independent of seed size and shape, plant life form and fire sensitivity (BROWN & VAN STADEN, 1997). Smoke can also be utilised as an effective seed pre-sowing treatment, as the stimulatory effect of smoke is irreversible and cannot be leached (LIGHT *et al.*, 2002). Seeds treated with smoke are known to retain this stimulatory effect even after a year of storage (MINORSKY, 2002). The inhibitory effects of high smoke concentrations appear to be reversible and seeds grow with increased vigour after the smoke has been leached to a

tolerable level (LIGHT *et al.*, 2002). This effect would be a favourable adaptation to a post-fire environment as the inhibitory compounds will only leach with sufficient rainfall (LIGHT *et al.*, 2002). Smoke thus enables seeds to germinate at the right time, grow faster and have a more robust root system and so have a major competitive advantage in their natural environment (BLANK & YOUNG, 1998).

In early experiments by DE LANGE and BOUCHER (1990), smoke was generated by burning a mixture of fresh and dry plant material in a metal drum. This smoke was then fed into a polythene tent and allowed to settle on the soil where the seeds were stimulated to germinate. One disadvantage is that the germination cue of smoke is easily confused with the effect of temperature on germination. This is because temperatures slightly higher than ambient temperature significantly increase seedling emergence in many species (BASKIN & BASKIN, 1998). Due to the complications of separating smoke from high temperatures, direct exposure to smoke is not recommended.

Aqueous smoke extracts were pioneered by DE LANGE and BOUCHER in 1990 and since then many authors have demonstrated that the active component of airborne smoke is soluble in water (BROWN & VAN STADEN, 1997; TAYLOR & VAN STADEN, 1998; SPARG *et al.*, 2005). The method for preparing such a solution usually involves forcing smoke that has been generated in a drum to bubble through water (BROWN & VAN STADEN, 1997). Combustion usually proceeds slowly and the burning material is made to smoulder, thus releasing relatively large quantities of smoke (BROWN & VAN STADEN, 1997).

In an experiment conducted by JÄGER *et al.* (1996) it was found that aqueous smoke extracts prepared from a range of plants, as well as extracts prepared by heating agar and cellulose contained compounds which stimulated the germination of Grand Rapids lettuce seed. They also demonstrated that the same active compound is produced by burning *Themeda triandra* leaves, agar and cellulose by providing evidence obtained by thin-layer chromatography and high-performance liquid chromatography. This was also demonstrated with the same methods by BROWN and VAN STADEN (1997).

BLANK and YOUNG (1998) reported that smoke increases the permeability to solutes of a subdermal seed membrane for some species of California chaparral. They also stated that these specific mechanisms of fire cue stimulation may be species dependent. This response involves triggering via elevated nutrient content or via the presence of stimulating gases in the smoke or triggering chemicals that permeate the embryo and induce enzymatic changes that trigger germination. In an experiment done by BROWN and VAN STADEN (1997) the dormancy of celery seeds was broken by a combination of plant-derived smoke, benzyladenine and gibberellins in the dark at temperatures between 18 and 26°C. From these results it could be argued that smoke extracts act in a similar way to cytokinins in the celery seed as it enhances gibberellin activity.

In a study conducted at the ultrastructure level by EGERTON-WARBURTON (1998), the causal factor(s) associated with seed dormancy and the stimulation of germination were investigated for *Emmenanthe penduliflora* (Hydrophyllaceae) seeds. It was found that a short exposure to smoke resulted in two major morphological changes. These changes are closely associated with the stimulation and acceleration of germination. The first major and most visible smoke induced morphological change observed was an intense chemical scarification of the external cuticle (EGERTON-WARBURTON, 1998). This has a direct and destabilising effect on the external cuticle and is manifested as the formation of oil-like spheres or micelles. These micelles increase the surface area of the seed for the exchange of water and solutes, as well as altering the hydrophobicity of the seed surface (EGERTON-WARBURTON, 1998).

The second, more important smoke induced morphological change occurred at the internal cuticle. Here the exposure to smoke stimulus caused a significant increase in the number and diameter of permeate channels in the cuticle of *Emmenanthe penduliflora* seeds (EGERTON-WARBURTON, 1998). The creation of such channels within the cuticle increases the permeability of this layer. It was shown through the use of the fluorescent apoplastic tracer dye, lucifer yellow (carbohydrazide), that

these channels permit the rapid exchange of water and solutes between the external environment and the seed.

Even though these major morphological changes have a large influence on the function of the cell, they do not significantly alter the general shape or dimensions of the cells (EGERTON-WARBURTON, 1998). EGERTON-WARBURTON (1998) speculated that the mechanism by which such morphological changes benefit the plant may be a synergy between the observed increased cuticular permeability and a simultaneous leaching of endogenous inhibitors of germination during imbibition.

They also further described the mechanism of formation of permeate channels. Because the permeate channels occurs in the cuticle, the formation of channels has to be the result of a preceding reaction between certain constituents of smoke and the semi-crystalline structure of waxes (EGERTON-WARBURTON, 1998). This is a rather promising argument as the pyrolysis of cellulose alone produces a collection of compounds, such as aromatic hydrocarbons, ketones and a number of organic acids, which all have the potential to dissolve or modify waxes.

Smoke also contains a number of compounds that may act as surfactants (e.g. alcohols) (EGERTON-WARBURTON, 1998). These surfactants modify cuticular layers by plasticizing the molecular structure of waxes (EGERTON-WARBURTON, 1998). The sorption of such surfactants to the cuticle also creates hydrophilic channels. This increases the area of channels within the cuticle that leads to accelerated transcuticular penetration of solutes in several species.

Using liquid chromatography, BLANK and YOUNG (1998) discerned over 30 organic anions in aqueous extracts of soil heated between 250 to 450°C. BLANK and YOUNG (1998) speculated that these unknown compounds or combinations of compounds could be cueing agents.

FLEMATTI *et al.* (2004) identified the UV absorbance maximum and molecular weight of a germination-enhancing compound in smoke. They also assigned a molecular formula of $C_8H_6O_3$ to the compound. They also identified the compound as the butenolide, 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one and were able to synthesize it.

They compared the activity of this synthesized butenolide with that of smoke water dilutions and found a high similarity in the germination delivered by these two compounds. Since this study, many experiments have been conducted using butenolide. This compound has been shown to enhance germination in a similar way to smoke water dilutions in many genera including *Acacia*, *Eucomis* and *Dioscorea* (KULKARNI *et al.*, 2006b; KULKARNI *et al.*, 2006a; KULKARNI *et al.*, 2007). In these experiments a 10^{-7} M butenolide solution was used.

2.8.5 Phytochromes and light quality

Photo-sensitive substances that are responsible for photoperiodic control of germination, in some species, were first discovered in 1959 (COPELAND, 1976).

There are two photo-reversible forms of phytochrome in plants (COPELAND, 1976). The first is P_R phytochrome, which is sensitive to orange-red light (600-680 nm), and the second is $P_{F\text{-}R}$ phytochrome, which is sensitive to far-red light (700-760 nm). In most species the greatest promotion of germination occur after exposure to light in the red area (660 to 700 nm) with a peak of germination often being observed at 670 nm (COPELAND, 1976). Wavelengths at 440 nm, above 700 nm and below 290 nm are known to inhibit germination. No studies has shown an effect of wavelengths of 290 to 400 nm on germination (COPELAND, 1976)

2.8.6 Scarification

Mechanical scarification

To mechanically scarify seeds a small hole is made in the seed coat using a needle or a scalpel or the whole seed coat is scarified with sandpaper or specialized equipment (BASKIN & BASKIN, 1998).

Acid scarification

This is usually done by soaking seeds in concentrated sulphuric acid for a short period of time after which they are rinsed several times with distilled water (BASKIN & BASKIN, 1998). By using acid scarification, both the testa and the seed pores are scarified (BASKIN & BASKIN, 1998).

2.8.7 Seed dormancy and the influence of temperature and stratification

The imposition of dormancy is normally controlled endogenously and germination is initiated in response to a certain combination of environmental variables. These environmental variables include temperature, availability of minerals, light and weathering (KOLLER, 1972). With the termination of dormancy, the metabolic processes of synthesis and growth are resumed (KOLLER, 1972). Some seeds exhibit no dormancy, such as mangrove seeds which germinate on the tree itself, while other seeds such as lotus or lupin seeds may remain dormant for centuries or millennia (THIMANN, 1977).

There are, generally speaking, two types of organic seed dormancy; endogenous and exogenous (BASKIN & BASKIN, 1998). In endogenous dormancy there is some characteristic of the embryo that prevents germination, while in exogenous dormancy it is some characteristic of the structures that cover the embryo that prevents germination. These structures include the endosperm, perisperm, testa and fruit walls (BASKIN & BASKIN, 1998). Seeds may, for example, be unable to germinate because of seed or fruit coats that are impermeable to water (BASKIN & BASKIN, 1998). Before water uptake and subsequent germination can take place these blocks to germination must be removed. There are a number of endogenous and exogenous dormancy types (Table 2.3).

Endogenous physiological dormancy is generally caused by a physiologically inhibiting mechanism of the embryo that prevents germination. The structures that cover the embryo may however also play a substantial role (BASKIN & BASKIN, 1998). Physiological dormancy can be differentiated into non-deep, intermediate and deep physiological dormancy (BASKIN & BASKIN, 1998). Embryos of seeds in non-deep and intermediate dormancy tend to germinate when isolated from the surrounding tissues while those of seeds in deep physiological dormancy do not (BASKIN & BASKIN, 1998).

Table 2.3: Organic seed endogenous and exogenous dormancy types (Modified from BASKIN & BASKIN (1998)).

	Type	Cause	Broken by
Endogenous	Physiological	Physiological inhibiting mechanism (PIM) of germination	Warm/cold stratification
	Morphological	Underdeveloped embryo	Appropriate conditions for embryo germination/growth
	Morphophysiological	PIM of germination and underdeveloped embryo	Warm/cold stratification
Exogenous	Physical	Seed/fruit coats impermeable to water	Opening of specialized structures
	Chemical	Germination inhibitors	Leaching
	Mechanical	Woody structures restrict growth	Warm/cold stratification

The causes of non-deep physiological dormancy are factors relating to the covering structure (BASKIN & BASKIN, 1998). These factors include the physical barrier created by these structures, the resulting oxygen supply to the embryo, inhibitors within the covering structures and changes in the covering structures (BASKIN & BASKIN, 1998). *Iris lorteti* is an example of a species with seeds exhibiting non-deep physiological dormancy as a result of the physical restriction caused by their seed coats (BASKIN & BASKIN, 1998). It takes a force of 133.2 MPa, which can only be overcome by an embryo with sufficient growth potential, to break the seed coat of this species (BASKIN & BASKIN, 1998).

The dormancy of such seeds can often be broken by a cold or hot stratification treatment (COPELAND, 1976). Such a treatment is performed by placing moistened seeds at low temperatures (3 to 10℃) for a certain period of time (COPELAND, 1976). In some cases (European ash seed) dormancy can only be overcome by stratification (COPELAND, 1976). In such cases the growth-stimulating substance produced during stratification breaks dormancy caused by inhibitory chemicals within

the embryo (COPELAND, 1976). Stratification has also been known to decrease the time to germination and increase growth rate in other species (COPELAND, 1976). Stratification may also decrease the sensitivity to external conditions, so that a seed might, for example, germinate at a less suitable temperature (COPELAND, 1976). The germination rate may also be improved by exposing seeds to different day/night temperatures (COPELAND, 1976). In some cases embryonic dormancy may be broken by exposure to a certain light intensity, wavelength and/or photoperiod (COPELAND, 1976). In some cases dormancy caused by inhibitory chemicals within the embryo can be broken by gibberellic acid (COPELAND, 1976). Other chemicals which are known to break non-deep physiological dormancy include potassium nitrate, kinetin and ethylene. The effects of endogenous physical dormancy also diminishes as seed age increases (COPELAND, 1976).

In the case of morphological dormancy, germination is prevented at the time of maturity due to the morphological characteristics of the embryo. The embryo is underdeveloped or even undifferentiated at the time of dispersal and a period of growth, known as after-ripening, is required before the seed can successfully germinate (COPELAND, 1976; BASKIN & BASKIN, 1998). Morphological dormancy occurs in seeds with rudimentary and linear embryos. Most of the interior of these seeds is occupied by endosperm and the embryo may only be 1% of the seed volume or less (BASKIN & BASKIN, 1998).

Morphophysiological dormancy is essentially a combination of the two dormancy types. In this case the embryo must grow to a species-specific critical size and the physiological dormancy of the seed must occur before germination can take place (BASKIN & BASKIN, 1998).

The primary reason for dormancy of seeds with exogenous physical dormancy is the impermeability of their seed or fruit coats to water (BASKIN & BASKIN, 1998). Seeds with physical dormancy frequently have a palisade layer of lignified cells in the testa or pericarp (BASKIN & BASKIN, 1998). The breakdown of such hard seed coats in the natural environment occurs through the gradual processes of hydration and dehydration, exposure to hot and cold temperatures, scorching by fire and

degradation due to extreme soil acidity, microbial breakdown and digestion by animals (COPELAND, 1976). Another reason for exogenous physical dormancy is sometimes the impermeability of seed coats to gasses (KHAN, 1977). Physical dormancy is often found in combination with chemical dormancy.

Chemically dormant seeds do not germinate due to the presence of inhibitors in the pericarp. These inhibitors are usually removed by leaching (BASKIN & BASKIN, 1998). These inhibitors include chemicals such as ABA (Abscisic acid) and phenolic compounds (KHAN, 1977). Many studies have shown that there is a interaction between phytochromes activated by red light and inhibitors (KHAN, 1977).

Mechanical dormancy is usually the result of a hard, woody fruit wall or seed coat. This woody structure is usually the endocarp or the mesocarp (BASKIN & BASKIN, 1998). Upon germination the endocarp often splits into two halves (BASKIN & BASKIN, 1998). In such cases dormancy can sometimes be broken with a period of cold stratification (KHAN, 1977).

2.8.8 Seed longevity and viability

Some seeds are viable after several years, decades or even after a few hundred years (BEWLEY & BLACK, 1982). Longevity is largely dependent on storage conditions. Factors that influence the longevity of seeds in storage include temperature, moisture and oxygen pressure (BEWLEY & BLACK, 1982). A low temperature and moisture content usually equates to a longer period of viability (BEWLEY & BLACK, 1982). Higher oxygen pressure results in a shorter period of sustained viability (BEWLEY & BLACK, 1982). Unorthodox or recalcitrant seeds cannot withstand drying (BEWLEY & BLACK, 1982). These seeds must retain a relatively high moisture content to remain viable (BEWLEY & BLACK, 1982). Even in relatively moist storage conditions they are rarely viable for more than a few months (BEWLEY & BLACK, 1982). The majority of seed plants are however orthodox and can remain viable for a prolonged period under suitable storage conditions (BEWLEY & BLACK, 1982). Various mathematical equations have been derived to relate the viability of seeds with their storage environment (BEWLEY & BLACK, 1982). To test the longevity of seeds the relative number of normal seedlings produced by seed

germinated under controlled conditions each year serves as a comparative measure of seed viability (HARTMANN & KESTER, 1965).

The tetrazolium test for seed viability is used by soaking seeds in a solution of 2,3,5-triphenyltetrazolium chloride (TTC) (HARTMANN & KESTER, 1965). This chemical is absorbed by living tissue and changed into an insoluble red compound, formazan, by NADPH dehydrogenases (HARTMANN & KESTER, 1965; COPELAND, 1976; LEADEM, 1984; BAND & HENDRY, 1993). Non-living tissue remains uncoloured (HARTMANN & KESTER, 1965). The reaction takes place equally well in dormant and non-dormant seeds and results are obtained in less than 24 hours. The test is used as a rapid assessment of viability or as a viability test of dormant seeds that do not respond to other methods (HARTMANN & KESTER, 1965; INTERNATIONAL SEED TESTING ASSOCIATION, 1999a). A 1% solution is commonly used, although a 0.05% solution may sometimes be satisfactory (HARTMANN & KESTER, 1965; BAND & HENDRY, 1993). It should be used at a pH of 6.5 to 7.5 (INTERNATIONAL SEED TESTING ASSOCIATION, 1999a).

The intact seeds of some species may be soaked in a TTC solution whereas some seeds require to be soaked in water first so that tissues are hydrated (COPELAND, 1976). Some seeds should be soaked in a solution with a respiration stimulant such as hydrogen peroxide (COPELAND, 1976). Other seeds require procedures to be followed before staining that include the removal of any hard covering and sectioning of the seeds so that the embryo may be exposed to the TTC solution (HARTMANN & KESTER, 1965). The embryo is then incubated in 1% TTC for 2 hours in the dark after which the excess tetrazolium is washed off with water (COPELAND, 1976; BAND & HENDRY, 1993).

The amount of staining is then observed. The location and intensity of the formazan stain is important to accurately define the viability of the embryo (COPELAND, 1976; LEADEM, 1984). If the areas of cell division are unstained or abnormally stained the potential for germination to occur is lowered (COPELAND, 1976). A number of broad classes, defining the germinability of the embryo are given in Table 2.4.

Table 2.4: Topographic stain evaluation classes for the TTC test (LEADEM, 1984).

Class	Description	Viability
1	Embryo completely stained	Germinable
2	Very pale staining	Possibly germinable
3	Cotyledons unstained	Non-germinable or possibly germinable
4	Radicle unstained	Non-germinable or probably not germinable
5	No staining	Non-germinable

The meristems should be well stained in order to insure healthy germination and growth of the embryo (LEADEM, 1984). A typical viable embryo should be at least 75% stained and its tissue should be firm with a smooth surface (LEADEM, 1984).

The TTC stain test leaves a number of uncertainties. A sample may, for example, not be stained because the stain never penetrates the tissue and the non-enzymatic reduction of TTC is also possible in dead and living tissue (BAND & HENDRY, 1993). A second viability test should be used to confirm the tetrazolium result (BAND & HENDRY, 1993).

2.8.9 After-ripening

In the period of after-ripening individual or collective changes take place so that a seed that was once dormant can germinate (COPELAND, 1976). This period may be determined by physical and chemical stimuli in the environment such as a phytochrome response and or the balance of inhibiting and promoting substances in the environment (COPELAND, 1976). It may also be determined by the effect of the environment on the balance of inhibiting and promoting substances within the seed and embryo itself and morphological growth and development of the embryo (COPELAND, 1976).

After-ripening can sometimes be hastened by growth promoting substances, low-temperature stratification, alternating temperature and light exposure treatments

(COPELAND, 1976). Such growth promoting substances are synthesised by the seed during this after-ripening process (KHAN, 1977). These include gibberellins, cytokinins and auxins.

2.8.10 Embryo-excision as a tool for investigating mechanisms behind dormancy and testing viability

Embryo excision serves as a good test for determining whether seed dormancy is endogenous or exogenous. This test can also be used to test seeds in cases where embryos require long periods of after-ripening before germination will take place (HARTMANN & KESTER, 1965). It is also useful to determine the viability of slow germinating seed (INTERNATIONAL SEED TESTING ASSOCIATION, 1999b).

The embryo is essentially excised from the seed and germinated. Before excision is attempted, seeds must be soaked thoroughly, changing the water once or twice daily to avoid the accumulation of seed exudates, to retard the growth of contaminants and to circumvent the evolution of anoxic conditions (INTERNATIONAL SEED TESTING ASSOCIATION, 1999b).

During the excision the embryo should be kept moist and working conditions should be aseptic. Observations such as predated, empty, decayed and discoloured seeds and deformed embryos should be noted and included in a calculation of viability (INTERNATIONAL SEED TESTING ASSOCIATION, 1999b).

A viable embryo shows some indication of germination, whereas a non-viable embryo becomes discoloured and deteriorates (INTERNATIONAL SEED TESTING ASSOCIATION, 1999b). Signs of germination include the expansion of cotyledons, the development of chlorophyll and the growth of the radicle and plumules. The time required for this test ranges from 3 days to 3 weeks (HARTMANN & KESTER, 1965). Viability is calculated by dividing the number of viable embryos with the total number of seed tested and is reported as a percentage (INTERNATIONAL SEED TESTING ASSOCIATION, 1999b).

2.8.11 Germination, dormancy and germination ecology in Iridaceae

Geophytes are a very important component of Eurasian semi-deserts with cold winters. Geophytes belonging to Iridaceae in this biome exhibits morphophysiological dormancy (BASKIN & BASKIN, 1998). Such seeds are known to have underdeveloped embryos (BASKIN & BASKIN, 1998). Halophytes and emergent aquatics in the family of Iridaceae also exhibits morphophysiological dormancy (BASKIN & BASKIN, 1998). *Iris angustifolia, Iris pseudacorus*, *Iris versicolor* and *Iris virginica* are examples of such species (BASKIN & BASKIN, 1998). Two genera of the Iridaceae have been found in persistent seed banks (BASKIN & BASKIN, 1998). Accordingly, a species of Iridaceae could have morphological and/or morphophysiological dormancy. In a study by DIXON *et al.* (1995) it was found that a species in Iridaceae, *Patersonia occidental*, only germinated when treated with smoke.

2.8.12 Embryo and seedling morphology of Iridaceae

Embryo's of the Iridaceae are straight and poorly differentiated (TILLICH, 2003). Only at the seedling stage can three different groups, defined by their cotyledon morphology, be clearly distinguished (TILLICH, 2003). The three groups are the compact cotyledon group, the tubular cotyledon group and the assimilating cotyledon group (TILLICH, 2003).

At the event of germination the cotyledonary sheath, hypocotyl and radicle are pushed through the micropyle region of the testa (TILLICH, 2003). The cotyledon then immediately bends at 90° in most cases (TILLICH, 2003). In *Crocoideae* the typical cotyledon is characterised by a remarkable elongation of the cotyledonary sheath and / or the development of a long coleoptile (TILLICH, 2003).

The seedling morphology of *Crocus* and *Romulea* is quite rare (TILLICH, 2003). Here an elongated tubular structure is combined with a tubular cataphyll (TILLICH, 2003). Possible explanations for this is the pronounced hypogeous germination which is promoted by a strong contractile primary root (TILLICH, 2003). The cotyledons of these genera have also lost most of their ability to produce chlorophyll and appear white even in high light intensities (TILLICH, 2003).

2.9 BRIEF REVIEW OF *IN VITRO* CULTURE

The term ”tissue culture” is actually a misnomer inherited from the field of animal tissue culture. Plant micropropagation involves the culture of a whole individual from isolated tissues, while animal tissue culture involves the culture of isolated tissues (KYTE & KLEYN, 1996).

According to AHLOOWALIA *et al.* (2002), the process of micropropagation can be divided into five stages: the pre-propagation step (stage 0); the initiation of explants (stage I); the subculture of explants for multiplication or proliferation (stage II); shooting and rooting of the explants (stage III); and hardening off the cultured individuals (stage IV). The pre-propagation stage involves preparing the explant for aseptic culture.

The explant and its response *in vitr*o is significantly influenced by the phytosanitary and physiological conditions of the donor plant (KANE, 2004). Plant material used in clonal propagation should be taken from mother plants that have undergone the appropriate pre-treatment with fungicides and pesticides to minimize contamination in the *in vitro* cultures (AHLOOWALIA & PRAKASH, 2002). Such a piece of plant material is called an explant (SMITH, 2000b). A single explant can theoretically produce an infinite number of plants (KYTE & KLEYN, 1996). Explants can be obtained from meristems, shoot tips, macerated stem pieces, nodes, buds, flowers, peduncle pieces, anthers, petals, pieces of leaf or petiole, seeds, nucellus tissue, embryos, seedlings, hypocotyls, bulblets, bulb scales, cormels, radicles, stolons, rhizome tips, root pieces or protoplasts (KYTE & KLEYN, 1996). The explants should be surface decontaminated with antibiotic sprays before they are introduced into culture (AHLOOWALIA *et al.*, 2002; KANE, 2004).

In stage I an aseptic culture is initiated by inoculating the explant onto a sterile medium (AHLOOWALIA *et al.*, 2002). Once such an explant is established it can be multiplied a number of times (AHLOOWALIA *et al.*, 2002). The explants are then transferred to a contaminant free *in vitro* environment (AHLOOWALIA *et al.*, 2002).

In stage II or the propagation phase explants are cultured onto a medium that promotes the multiplication of shoots (AHLOOWALIA *et al.*, 2002). Propagation must be achieved without excessive mutation (AHLOOWALIA *et al.*, 2002). The culture of various organs in stage I lead to the multiplication of propagules in large numbers. These propagules can be cultured further and used for multiplication (AHLOOWALIA *et al.*, 2002). These cultured shoots are often placed onto different media for elongation (AHLOOWALIA *et al.*, 2002).

The result of stage III is the production of complete plants, as the shoots derived from stage II are rooted (AHLOOWALIA *et al.*, 2002). If shoot clumps are present, they should be separated after rooting. Many plants can be rooted on half strength Murashige and Skoog medium without any growth regulators (AHLOOWALIA *et al.*, 2002). Successful and sufficient rooting is essential for survival of the plant during hardening and transfer to the soil (AHLOOWALIA *et al.*, 2002).

The complete plants are weaned and hardened during stage IV (AHLOOWALIA *et al.*, 2002). The plants should at this stage be autotrophic. Hardening consists of gradually altering the humidity, light and nutrition available to the plant. The plant is moved gradually from a high to a low humidity, from a low light intensity to a high light intensity and the agar is removed by gently washing it away with water. After sufficient hardening, plants can be transplanted to a suitable substrate and hardened further (AHLOOWALIA *et al.*, 2002).

In vitro regeneration techniques are essential to the application of *in vitro* selection techniques (REMOTTI & LÖFFLER, 1995). This is not only because it enables the selected genotype to be regenerated, but also it aids commercialisation of new species and selected genotypes (DEBERGH, 1994).

Micropropagation enables the production of disease free plantlets at high rates and generally increases the efficiency of known breeding techniques (DEBERGH, 1994). It is also essential in the breeding of plants for which no breeding methods or procedures have been established (DEBERGH, 1994). *In vitro* selection techniques have been used to increase genetic variability and to broaden the gene pool

(DEBERGH, 1994). *In vitro* techniques such as embryo rescue, protoplast fusion and genetic transformation enable plant breeders to accomplish wider crosses (DEBERGH, 1994).

2.9.1 Explant selection

There are numerous variables that should be considered when selecting an explant for *in vitro* culture. These are mostly caused by the physiology of the plant and its environment.

Physiologically younger tissues are generally much more responsive to tissue culture (SMITH, 2000b). In many cases, older tissues will not form callus that is capable of regeneration. Younger tissue is usually the newest formed and therefore easier to surface disinfect (KYTE & KLEYN, 1996; SMITH, 2000b). Plant material at the base of a plant may, however be more suitable than explants higher up (KYTE & KLEYN, 1996). The smaller the explant, the harder it is to culture (SMITH, 2000b). Larger explants have more nutrient and plant growth regulator reserves to sustain the culture. A large explant is often more difficult to decontaminate (KYTE & KLEYN, 1996; SMITH, 2000b). Explants should be obtained from plants that are healthy as opposed to plants under nutritional or water stress or plants exhibiting disease symptoms (SMITH, 2000b). Plant material in a state of active growth is cleaner, and more suitable for aseptic culture, compared to dormant tissue. To control contamination, donor plants should be pre-screened for diseases (SMITH, 2000b; AHLOOWALIA *et al.*, 2002).

The season of the year can have an effect on contamination and the response of the explant in culture (SMITH, 2000b). Contamination tends to increase as summer progresses. Plant material obtained from the field is often more contaminated than material obtained from greenhouses or growth chambers (KYTE & KLEYN, 1996; SMITH, 2000a). Mother plants should ideally be maintained under dust, insect and disease free conditions (AHLOOWALIA *et al.*, 2002). These plants should also not be stressed and they should preferably be grown under controlled conditions that promotes active growth (KANE, 2004). Such conditions should preferably include conditions of low relative humidity. Drip irrigation should preferably be used as the

misting will facilitate contamination by wetting the foliage (KANE, 2004). Placing the plant material in a less humid and dry environment a few weeks prior to taking explant material can reduce contamination of cultures (SMITH, 2000b).

Plant materials growing in soil (roots, tubers, bulbs) or near the soil surface (stolons, rhizomes, orchid protocorms, etc.) are often harder to clean and disinfect than aerial plant material (SMITH, 2000b). Explants are much easier to clean if the plant has been growing in an artificial medium, such as washed sand or perlite (KYTE & KLEYN, 1996). After cutting the explant from the source plant, it should be placed in a plastic bag containing a moist paper towel and kept refrigerated until culture initiation (KYTE & KLEYN, 1996).

2.9.2 Explant preparation

Explants require surface-disinfection before they can be placed in culture on the nutrient agar for *in vitro* culture (SMITH, 2000b). Explants are washed in sterile water and rinsed in ethanol and the surface is sterilised using chemicals with a chlorine base (AHLOOWALIA *et al.*, 2002). There are a number of products used for surface disinfection, the most commonly used is commercial chlorine bleach (SMITH, 2000b).

For soft, herbaceous material a calcium or sodium hypochlorite based solution is often used at a concentration of 1-3% (AHLOOWALIA *et al.*, 2002). Before surface-disinfection any remaining soil or dead parts should be removed from the explant (PIERIK, 1997). An inexpensive and ready-made alternative is a 5-7% solution of Domestos® (a toilet disinfectant by Lever Bros. Ltd., UK), which contains 10.5% sodium hypochlorite, 0.3% sodium carbonate, 10.0% sodium chloride and 0.5% sodium hydroxide and a patented thickener (AHLOOWALIA *et al.*, 2002). Explants are washed in sterile water before and after sterilization (AHLOOWALIA *et al.*, 2002).

A general procedure for preparing the explant involves washing the explant in warm, soapy water after which it is rinsed in tap water (PIERIK, 1997; SMITH, 2000b). The explant is then rinsed in a freshly made chlorine bleach solution (PIERIK, 1997). One to 2 drops of wetting agent should be added to every 100 ml of bleach solution. The explant is then rinsed in sterile water three to five times.

PIERIK (1997) stated that a brief alcohol rinse or swab is needed with hairy or wax coated surfaces. Epidermal hairs may trap air bubbles, in such cases these have to be evacuated under vacuum.

Sterilized forceps and scalpels must be used for the transfer of explants to fresh solutions (AHLOOWALIA *et al*., 2002). Sterile containers must be used throughout the protocol of surface sterilization (AHLOOWALIA *et al*., 2002). If explants become brown or pale the strength of the sterilizing agent should be reduced (GAMBORG & PHILLIPS, 1995; PIERIK, 1997).

A cut explant such as a stem or leaf that is surface sterilised often shows tissue damage from surface sterilisation (PIERIK, 1997). The damaged tissue should be removed before culture (SMITH, 2000b).

GAMBORG & PHILLIPS (1995) suggest that a procedure for seed sterilization should include washing the seeds in detergent, after which they are rinsed with tap water and subsequently alcohol, a bleach solution and autoclaved demineralised water respectively.

Seeds can be germinated on filter paper in Petri dishes or on an agar medium (GAMBORG & PHILLIPS, 1995). A single seed should ideally be placed in each container so that a single contaminated seed does not contaminate other seeds.

Contamination resulting from improperly sterilised tissue will generally arise from the explant and be located in the medium adjacent to the explant (SMITH, 2000b). Contamination that is due to poor technique will generally appear over the entire agar surface (SMITH, 2000b). Examples of poor technique include contaminated transfer hood filters and culture cabinets and improperly sterilised media.

Contamination of cultures by fungi appear as fuzzy growth whereas bacterial contamination appears as smooth pink, white or yellow colonies and contamination

from insects appears as tracks across the medium which are visible due to surrounding fungal or bacterial growth (SMITH, 2000b).

Explant material may harbour internal micro-organisms (SMITH, 2000b). In such a case, it is very difficult to establish clean cultures. Explants that are least likely to harbour internal contaminants include explants taken from growing shoot tips, ovules of immature fruit, immature and mature flower parts and runner tips (SMITH, 2000b). Explants that are more likely to harbour internal contaminants include explants taken from bulbs, slow-growing shoots or dormant buds, roots, corms and underground rhizomes (PIERIK, 1997; SMITH, 2000b). In such cases seeds are often aseptically germinated to provide clean explants from the root, hypocotyl, cotyledon and shoot (PIERIK, 1997). In these situations the use of antibiotics or fungicides in the medium is generally not useful (SMITH, 2000b). Although these agents can repress the growth of some microorganisms, they can also suppress the growth of the plant tissue or even kill it (SMITH, 2000b).

2.9.3 Medium composition

A number of standard formulae for tissue culture media have been developed to provide optimum nutrients and growth regulators for specific plants (KYTE & KLEYN, 1996). The selection or development of a suitable culture medium is vital to the success of the culture (SMITH, 2000b). The approach to the development of a suitable medium will depend on the purpose of the culture (SMITH, 2000b).

The medium generally contains water, inorganic salts, plant growth regulators, vitamins, a carbohydrate and a gelling agent (SMITH, 2000b). High quality water should be used as an ingredient of the plant culture media (PIERIK, 1997; BEYL, 2005). Ordinary tap water contains cations, anions, particulates, micro-organisms and gases that may influence the reaction of the tissue culture media with the tissue (BEYL, 2005). The most commonly used method of water purification involves a deionization treatment followed by one or two glass distillations (PIERIK, 1997; BEYL, 2005).

The distinguishing feature of MURASHIGE & SKOOG (1962) inorganic salts is their high content of nitrate, potassium and ammonium in comparison to other salt formulations (SMITH, 2000b). Table 2.5 shows the composition of the Murashige and Skoog formula. MURASHIGE & SKOOG (MS) (1962) is the most suitable and the most commonly used basic tissue culture medium for plant regeneration from tissues and callus (BEYL, 2005).

Table 2.5: The standard MURASHIGE & SKOOG (1962) formula.

			mg/l
MAJOR SALTS	Ammonium nitrate	NH_4NO_3	1650
	Calcium chloride	$CaCl_2 \cdot 2H_2O$	440
	Magnesium sulphate	$MgSO_4 \cdot 7H_2O$	370
	Potassium nitrate	KNO_3	1900
	Potassium phosphate	KH_2PO_4	170
	Subtotal		4530
MINOR SALTS	Boric acid	H_3BO_3	6.2
	Cobalt chloride	$CoCl_2 \cdot 6H_2O$	0.025
	Cupric sulphate	$CuSO_4 \cdot 5H_2O$	0.025
	Manganese sulphate	$MnSO_4 \cdot H_2O$	16.9
	Potassium iodide	KI	0.83
	Sodium molybdate	$Na_2MoO_4 \cdot 2H_2O$	0.25
	Zinc sulphate	$ZnSO_4 \cdot 7H_2O$	8.6
	Subtotal		32.83
IRON	Ferrous sulphate	$FeSO_4 \cdot 7H_2O$	27.8
	Na_2EDTA		37.3
	Subtotal		65.1
	Total mg/l		**4627.93**

Plant growth and developmental processes are controlled by plant growth regulators (GABA, 2004). The study of plant growth regulator function is complex because several plant growth regulators usually work in concert with each other and their concentration within plant tissues changes with time, season and developmental stage (GABA, 2004). The effect of plant growth regulators on plant growth and development depend on the chemical structure of the plant growth regulators used, the plant tissue used and the genotype of the plant (GABA, 2004). The type and concentration of the plant growth regulators used will vary according to the culture purpose (PIERIK, 1997).

2.9.3.1 Auxins

Auxins (for example: IAA, NAA, 2,4-D, or IBA) is required by most plants for cell division and root initiation (PIERIK, 1997; SMITH, 2000b). IAA or indole-3-acetic acid, was the first plant growth regulator to be isolated (GABA, 2004). IAA is rapidly degraded in growth media and inside the plant (GABA, 2004). For this reason chemical analogues of IAA with similar biological activity are often substituted (GABA, 2004). These more stable synthetic auxins include 2,4-D, IBA and NAA (GABA, 2004). IAA is added at a concentration of 0.01 to 10 mg l^{-1}, while synthetic auxins, such as IBA, NAA and 2,4-D, are used at concentrations of 0.001 to 10 mg l^{-1} (PIERIK, 1997). IAA can be considered to be a weak auxin (PIERIK, 1997). Cultures in which a large quantity of IAA has been added are often less successful than cultures to which low concentration of a stronger auxin, such as NAA have been added (PIERIK, 1997).

At high concentrations auxin can suppress morphogenesis (SMITH, 2000b). Auxins have numerous effects on plant growth and differentiation, depending on their chemical structure, their concentration and the affected plant tissue (GABA, 2004). Auxins generally stimulate cell elongation, cell division in cambium tissue and, together with cytokinins, the differentiation of phloem and xylem and the formation of adventitious roots (PIERIK, 1997). High concentrations of auxins can induce somatic embryogenesis (GABA, 2004).

The essential function of auxins and cytokinins is to reprogram somatic cells that were in a state of differentiation (GABA, 2004). Such reprogramming causes dedifferentiation and then redifferentiation into a new developmental pathway (GABA, 2004).The mechanism of dedifferentiation is not understood (GABA, 2004).

The use of 2,4-D should be avoided, as it may induce mutations (PIERIK, 1997). This growth regulator is however important in callus initiation for many species.

2,4-D is widely used for callus induction, while IAA (0.6-60 µM), IBA (2.5-15 µM) and NAA (0.25-6 µM) are mainly used in root initiation (SMITH, 2000b; GABA, 2004). Higher than optimum levels of auxins causes callus production and a reduction in root growth and root quality (GABA, 2004). Combinations of auxins at low concentrations can sometimes produce better results than using individual auxins (GABA, 2004). High concentrations of auxins are sometimes necessary to induce rooting (GABA, 2004). This can however have undesirable side effects such as growth inhibition of induced roots (GABA, 2004). In such cases the elevated auxin levels should be administered as a pulse treatment (GABA, 2004). To do this the shoot is incubated with auxin for several days before it is transferred to a medium with no plant growth regulators to allow root growth and development (GABA, 2004).

Somatic embryogenesis is typically induced by auxins, sometimes in combination with cytokinins (GABA, 2004). 2,4-D is commonly used at this stage, although other auxins can be used (GABA, 2004). Auxins induce the cells to become embryogenic and promote subsequent repetitive cell division of embryogenic cells (GABA, 2004). High concentrations of auxins prevent subsequent cell differentiation and embryo growth (PIERIK, 1997).

2.9.3.2 Cytokinins

As the name suggests, cytokinins cause cell division (GABA, 2004). Such cell division can lead to shoot regeneration *in vitro*, by stimulating the formation of shoot apical meristems and shoot buds (PIERIK, 1997). This cell division caused by cytokinins can also cause the production of undifferentiated callus (GABA, 2004). A high concentration of cytokinins can cause the release of shoot apical dominance

and will block root development (PIERIK, 1997). Examples of cytokinins include kinetin, zeatin, 2-iP, BA and thidiazuron (PIERIK, 1997). Some new cytokinins, called topolins have been synthesised in the Laboratory of Growth Regulators, Palacký University and Institute of Experimental Botany AS CR, Czech Republic. These include Me*m*T [6-(3-methoxybenzylamino)purine, Me*m*TR [6-(3-methoxybenzylamino)-9-b-D-ribofuranosylpurine], *m*T [6-(3-hydroxybenzylamino)purine]and *m*TR [6-(3-hydroxybenzylamino)-9-b-D-ribofuranosylpurine] (BAIRU *et al.*, 2007).

A high concentration of cytokinins can cause many small shoots to initiate but fail to develop (GABA, 2004). Shoots are induced into forming roots by placing them in a regeneration medium, containing a high level of cytokinin, and then in a medium with no plant growth regulators (GABA, 2004). Cytokinins inhibit rooting and can be effectively removed from the plant material by placing shoots in a medium without plant growth regulators (GABA, 2004). Such a treatment can also be used to reduce endogenous cytokinin levels (GABA, 2004).

2.9.3.3 Gibberellins

Gibberellins are, in most cases, non-essential for plant development in *in vitro* culture (PIERIK, 1997). In tissue culture, gibberellic acid (GA_3) is used to stimulate either shoot elongation or the conversion of buds into shoots (PIERIK, 1997). Gibberellins reduce root formation and embryogenesis *in vitro* (PIERIK, 1997). Gibberellins are primarily used to stimulate cell elongation and to produce elongated shoots in plant tissue culture (GABA, 2004). Unwanted side effects caused by gibberellins include reduction in the number of buds produced, the elongation of leaf structures such as petioles and lamina, the excessive elongation of shoots and reduced root production (GABA, 2004).

YASMIN *et al.* (2003) found that GA_3 dissolved in 0.2% ethanol inhibited adventitious rooting of mungbean cuttings but when dissolved in water, GA_3 promoted adventitious rooting at 10^{-7} M and 10^{-8} M (YASMIN *et al.*, 2003). This indicates that ethanol suppresses the promoting effects of GA_3 (YASMIN *et al.*, 2003).

2.9.3.4 Abscisic acid

Abscisic acid is rarely used in tissue culture protocols and has a negative effect on growth in most cases (PIERIK, 1997). It is mainly used in plant tissue culture to facilitate somatic embryo maturation (GABA, 2004) but may also be used in some regeneration processes and rarely used to produce somatic embryos (GABA, 2004). ABA induces the formation of essential LEA (late embryogenesis abundant) proteins found at late stages of embryogenesis in somatic and sexual embryos (GABA, 2004). LEA proteins are associated with tolerance to water stress resulting from desiccation and cold shock (GOYAL *et al.*, 2005).

2.9.3.5 Ethylene

Ethylene or physiological reactions similar to that caused by ethylene, is produced by certain plastic containers, plant tissue and as a result of fire (PIERIK, 1997). This is the only gaseous natural plant growth regulator and it is naturally produced by all plant tissues in a controlled fashion (GABA, 2004). Endogenously produced ethylene can accumulate in a closed vessel to levels that negatively affect plant growth and development (PIERIK, 1997). The biological effect of ethylene depends on how air-tight the vessel is and the sensitivity of the plant material (GABA, 2004).

Ethylene is primarily known for its effects on fruit ripening (GABA, 2004). Exposure to ethylene also results in reduced stem length, restricted leaf growth, premature leaf senescence and may cause increased growth of axillary buds (GABA, 2004). An enhanced ethylene concentration can induce callus formation, while inhibiting bud and shoot regeneration (GABA, 2004). Low concentrations of ethylene stimulate somatic embryogenesis, while high concentrations of ethylene inhibit somatic embryogenesis (GABA, 2004). Explants need a low level of ethylene for correct biological functioning, but too high an ethylene concentration leads to symptoms of excess (GABA, 2004).

Such symptoms of excess include stunted growth, a reduction in leaf size and leaf drop (PIERIK, 1997; NOWAK & PRUSKI, 2002). These plants do not acclimatise well to the *in vivo* environment and often desiccate shortly after being transferred to soil (NOWAK & PRUSKI, 2002).

Endogenous ethylene has an important role in shoot and root growth and differentiation (PIERIK, 1997).

2.9.3.6 Combinations of plant regulators

A high ratio of auxin to cytokinin induces root formation in shoots of dicotyledonous plants and somatic embryogenesis (GABA, 2004). Intermediate ratios induce callus initiation and adventitious root formation from callus in dicotyledonous plants (GABA, 2004). Such intermediate ratios often involve high levels of both cytokinins and auxins (GABA, 2004). Low ratios of auxin to cytokinin induce adventitious shoot formation and axillary shoot production in shoot cultures (GABA, 2004).

The optimum cytokinin to auxin ratio can be established by using a matrix approach (Table 2.6) with two axes of increasing plant growth substance concentration (KYTE & KLEYN, 1996).

Table 2.6: Example of a matrix to establish optimal auxin to cytokinin ratios and their concentrations, where the rows represent auxin levels and the columns represent the cytokinin levels (Modified from Kyte and Kleyn (1996).

	0	0.5	1	3	5	10
0						
0.5						
1						
3						
5						
10						

2.9.3.7 Vitamins

The vitamin considered most important for plant cells is thiamine (B_1) (SMITH, 2000b). Other vitamins, such as nicotinic acid (B_3) and pyridoxine (B_6), are also added to culture media, as they may enhance cellular response (SMITH, 2000b).

2.9.3.8 Carbohydrates

Green cells in culture are generally not photosynthetically active and require a carbon source (SMITH, 2000b). Sucrose or glucose at 2-5% (w/v) is commonly used (SMITH, 2000b). Higher levels of sucrose leads to low levels of photosynthesis in the leaves (ROBERTS *et al.*, 1990). Higher levels may however be used for embryo culture (SMITH, 2000b). Sugars undergo caramelisation when autoclaved too long (SMITH, 2000b). When sugars are heated they degrade and form melanoidins, which are brown, high molecular weight compounds that can inhibit cell growth (SMITH, 2000b).

2.9.3.9 Gelling agent

The type of agar used to gel the medium can affect the response of experiments (SMITH, 2000b). To minimise problems that arise from agar impurities, washed or purified agar should be used (SMITH, 2000b).

2.9.4 Liquid culture

In liquid culture, the explant is covered by the medium; enlarging the surface area for absorbing nutrients and plant growth regulators (ASCOUGH & FENNELL, 2004). The medium can be changed automatically, this reduces labour costs of subculturing (ASCOUGH & FENNELL, 2004).

Liquid culture does however have a few side-effects (ASCOUGH & FENNELL, 2004). Because of the submersion of tissue, the explant may become oxygen deficient. This leads to the formation of elongated and hyperhydric leaves (ASCOUGH & FENNELL, 2004). There are a few methods to overcome hyperhydricity, they are however not universal and some of them retard growth and multiplication (ZIV, 1989; ASCOUGH & FENNELL, 2004).

Gladiolus bud explants have been propagated in an agitated liquid medium (ZIV, 1989). ZIV (1989) concluded that liquid cultures can be used to scale up the micropropagation of *Gladiolus* sp. and possibly other geophytes as it allows for a faster rate of cormlet production (ZIV, 1989).

2.9.5 Embryo-excision

Embryo rescue is known as one of the earliest and most widely used techniques for Iridaceae micropropagation (KRIKORIAN & KANN, 1986). Embryo culture is a well established branch of *in vitro* culture and is known as one of the oldest and most successful culture procedures (HU & ZANETTINI, 1995; REED, 2005). In embryo rescue, the artificial medium substitutes for the endosperm (REED, 2005). MURASHIGE & SKOOG (1962) is the most frequently used basal media for embryo culture.

Embryo development occurs in two phases, a heterotrophic and an autotrophic phase (REED, 2005). In the heterotrophic phase, the young embryo, or “proembryo”, requires a complex medium. *In vivo* grown embryos at this stage are dependent on the endosperm. Amino acids such as glutamine and asparagine are often added to the culture medium.

Young embryos require a medium with high osmotic potential (PIERIK, 1997). A high osmotic potential prevents precocious development and promotes normal embryogenic development (REED, 2005). Sucrose is usually added to serve both as an osmoticum and a carbon source (PIERIK, 1997). A medium with 8 to 12% sucrose is used for the culture of heterotrophic embryos (REED, 2005).

The autotrophic phase is usually initiated in the late heart-shaped embryo stage (REED, 2005). Embryos that are excised during this development stage are completely autotrophic (HU & ZANETTINI, 1995). Such embryos germinate and grow on a simple inorganic medium with a supplemental energy source (HU & ZANETTINI, 1995). An inorganic medium supplemented with 2 to 3% sucrose is used as a standard medium for the germination of autotrophic embryos (REED, 2005).

Growth regulators often have inconsistent effects on embryo culture (REED, 2005). They have however been extensively used in embryo rescue protocols, especially protocols involving heterotrophic embryos (REED, 2005). Low concentrations of auxins promote normal growth whereas gibberellins cause embryo enlargement and cytokinins inhibit growth (REED, 2005).

Hard-coated seeds are first soaked in water for a few hours up to a few days before dissection. Seeds are surface sterilised before and after soaking (HU & ZANETTINI, 1995). The most suitable point of incision into the ovule differs amongst species (REED, 2005). The embryos of some species can be extracted by cutting off the micropylar end of the ovule and applying gentle pressure at the opposite end of the ovule, so that the embryo is pushed through the opening (REED, 2005). Small seeds are dissected by making a longitudinal section using sterile microdissection needles (HU & ZANETTINI, 1995)

After excision, large embryos should immediately be transferred into culture vessels, using a pair of forceps (HU & ZANETTINI, 1995; REED, 2005). Small embryos can be handled using the moistened tip of a dissection needle (HU & ZANETTINI, 1995).

2.9.6 Callus culture

Explants, when cultured in the appropriate medium, usually with both auxin and cytokinin, give rise to a mitotically active, but unorganized mass of cells. It is thought that, under the right conditions, any plant tissue can be used as an explant (SLATER *et al.*, 2003). Callus culture concerns the initiation and continued proliferation of undifferentiated parenchyma cells from explant tissue on clearly defined semi-solid media (BROWN, 1990).

Callus initiation is the first step in many tissue culture experiments (BROWN, 1990; SMITH, 2000b). *In vivo*, callus is a wound tissue produced in response to injury or infestation (BROWN, 1990; MINEO, 1990; SMITH, 2000b). Not all the cells in an explant contribute to callus formation (SMITH, 2000b). Only certain callus types, which are competent to regenerate organised structures, display totipotency (SMITH, 2000b).

The level of plant growth regulators is a major factor that controls callus formation in the culture medium (BROWN, 1990; SMITH, 2000b). The correct concentration of plant growth regulators depends on the species, individual and explant source (SMITH, 2000b). Other culture conditions such as light, temperature and media composition are also important for callus formation and development (SMITH, 2000b).

Explants can be taken from various plant organs, structures and tissues (MINEO, 1990). Young tissues of one or a few cell types are most often used as explants (MINEO, 1990). The pith cells of a young stem are regarded as a good source of explant material for callus initiation (MINEO, 1990).

Callus growth is maintained, provided that the callus is subcultured onto a fresh medium periodically. During callus formation there is a degree of de-differentiation in both morphology (usually unspecialised parenchyma cells) and metabolism. As a consequence most plant cultures lose their ability to photosynthesise (SLATER *et al.*, 2003). This means that the metabolic profile does not match that of the donor plant

and the addition of compounds such as vitamins and a carbon source is necessary (SLATER *et al.*, 2003).

Callus culture is often performed in the dark as light can result in differentiation of the callus (SLATER *et al.*, 2003). The culture often loses its requirement for auxin and/or cytokinin during long-term culture (SLATER *et al.*, 2003). This process is known as habituation and is common in callus cultures from some species such as sugar beet (SLATER *et al.*, 2003).

By manipulating the auxin to cytokinin ratio whole plants can subsequently be produced from callus cultures (PHILLIPS *et al.*, 1995). Callus culture can also be used to initiate cell-suspension cultures (PHILLIPS *et al.*, 1995).

Endogenous levels of plant growth regulators and polar growth regulator transport can drastically influence callus induction (PIERIK, 1997; SMITH, 2000b). Explant orientation and different sectioning methods affect callus induction (SMITH, 2000b).

Callus cultures subcultured regularly on agar media exhibit a sigmoidal growth curve (PHILLIPS *et al.*, 1995). PHILLIPS *et al.* (1995) describe five phases of callus growth. Phase I is a lag phase, where cells prepare to divide. Phase II is an exponential phase, where the rate of cell division is the highest. Phase III is a linear phase, where cell division slows, but the rate of cell expansion increases. Phase IV is a deceleration phase, where the rates of both cell division and expansion decrease and phase V is a stationary phase, where the number and size of cells remain constant.

Callus growth can be monitored in a non-destructive manner using fresh weight measurements (STEPHAN-SARKISSIAN, 1990; PHILLIPS *et al.*, 1995). Dry weight measurements are more accurate, but involve the destruction of the sample (PHILLIPS *et al.*, 1995). Mitotic index measurements of cell division rates are not easy to perform as they require numerous measurements to be made at various time intervals with very small amounts of tissue (PHILLIPS *et al.*, 1995). Fresh weight measurements are performed by culturing a known weight of callus for a given time

(typically 4 weeks) and weighing the callus after this time using aseptic techniques (STEPHAN-SARKISSIAN, 1990).

2.9.7 Organogenesis

Organogenesis involves the *de novo* production of organs directly from an explant or through initial callus culture (SCHWARTZ *et al.*, 2004). In the Iridaceae organogenesis involves the regeneration of unipolar meristems (ZIV, 1997).

Organogenesis is regulated by altering the components of the culture medium (BROWN & CHARLWOOD, 1990). Most important of these components is the auxin to cytokinin ratio, which determines the developmental pathway the regenerating tissue will take (BROWN & CHARLWOOD, 1990). Shoots are usually induced to form first by increasing the cytokinin to auxin ratio of the culture medium (BROWN & CHARLWOOD, 1990). These shoots can then be easily rooted (SLATER *et al.*, 2003).

De novo organ formation via indirect organogenesis, which involves intermediate callus formation and a differentiation phase, may increase the possibility for somaclonal variation (SCHWARTZ *et al.*, 2004). Any stage in the process of organogenesis that involves callus growth should be minimized.

After dedifferentiation the explant acquires a state of competence, defined as its ability to respond to organogenic stimuli (SCHWARTZ *et al.*, 2004). The attainment of competence can not always be achieved with a single step. The induction phase occurs between the time of competence and determination (SCHWARTZ *et al.*, 2004). During induction, processes resulting from the expression of genes guides developmental processes and precede morphological differentiation. It has been suggested that such a genetically determined developmental process can be interrupted by certain physical and chemical stimuli (PIERIK, 1997). At the end of the induction phase, the cells are fully committed to the production of shoots or roots. At this point the tissue can be removed from the root or shoot producing medium and placed on a basal medium without plant growth regulators (PGR's), containing mineral salts, vitamins and a carbon source (SCHWARTZ *et al.*, 2004). The desired

organ is then produced on this medium. Successful determination is partially dependent on the chemical and physical environment to which they have been exposed. The result of failure of explant tissues to express totipotency is a failure of the explant tissues to achieve the state of competence for induction. This makes investigation of the effects of physical and chemical parameters difficult. The use of biochemical or genetic markers that can clearly indicate the developmental disposition of the primary explant tissue have not yet been discovered (SCHWARTZ *et al.*, 2004).

In the next phase the morphological differentiation and development of the nascent organ occurs (SCHWARTZ *et al.*, 2004). Organ initiation involves a rapid shift in polarity followed by a smoothing of this shift into a radially symmetrical organization and the concurrent growth along the new axis to form a characteristic bulge (SCHWARTZ *et al.*, 2004). There is not an absolute certainty as to which tissues are involved and the number of cells involved in meristem initiation (SCHWARTZ *et al.*, 2004).

The initiation of adventitious roots occurs in four stages (SCHWARTZ *et al.*, 2004). A meristematic locus is first formed by the dedifferentiation of a stem or other cells. These cells then multiply to form a spherical cluster. Further cell multiplication occurs with the initiation of planar divisions to form a recognizable bilateral root meristem. Lastly, the cells located in the basal part of the developing meristem elongate, resulting in the eventual emergence of the newly formed root (SCHWARTZ *et al.*, 2004). The production of a functional adventitious root system depends on the selection of a microcutting of the appropriate developmental stage and the ability of the *in vitro* environment to initiate the sequence of events described above (SCHWARTZ *et al.*, 2004).

2.9.8 Somatic embryogenesis

In plants, morphologically and functionally correct nonzygotic embryos can arise from an array of cell and tissue types at a number of different points within both the gametophytic and sporophytic phases of the plant life cycle (GRAY, 2005). Somatic (or asexual) embryogenesis involves the formation of embryo-like structures, which

have the potential to develop into whole plants in a way analogous to zygote embryos, from somatic tissues (SLATER *et al.*, 2003). Plant regeneration by somatic embryogenesis was first observed in carrot in 1958 (PHILLIPS *et al.*, 1995). These somatic embryos can be produced either directly or indirectly. In direct somatic embryogenesis, the embryo is formed directly from a cell or a small group of cells without the production of an intervening callus (FINER, 1995). Direct somatic embryogenesis is however rare and only common in reproductive tissues (SLATER *et al.*, 2003). In indirect somatic embryogenesis, a callus is first produced from the explant before embryos are produced.

Synthetic auxins, especially 2,4-D, are most often used in protocols involving somatic embryogenesis (GRAY, 2005). Somatic embryogenesis usually proceeds in two distinct stages. In the initial stage (embryo initiation), a high concentration of 2,4-D is used (FINER, 1995). In the second stage (embryo production) embryos are produced in a medium with no or little 2,4-D (SLATER *et al.*, 2003).

Auxins activate pathways that induce the formation of embryogenic cells (GRAY, 2005). Auxins promote division, while suppressing differentiation and growth of embryogenic cells (GRAY, 2005). When using embryogenic cells as an explant, auxins are often not required as there is no need for an induction step (GRAY, 2005). In many dicotyledonous species cytokinins are also required to induce embryogenesis (GRAY, 2005). BA is the cytokinin most often used in embryogenesis (GRAY, 2005). Other cytokinins that have been used include TDZ, kinetin and zeatin (GRAY, 2005). Somatic and microspore embryogenesis has been reported in *Tulipa* sp., *Gladiolus* sp. and *Nerine* sp. (ZIV, 1997).

2.9.9 Hardening

Micropropagation of a species is in some cases restricted due to unsuccessful *ex vitro* acclimatization, leading to a low survival rate of cultured plants (HUYLENBROECK *et al.*, 2000). During this period of *ex vitro* acclimatization, plants must acquire the morphological and physiological features required by the *in vivo* environment and develop new patterns of resource allocation (HUYLENBROECK *et al.*, 2000). *In vivo* cultured plants will otherwise not be able to cope with the

environmental stresses of the post-propagation environment (HUYLENBROECK *et al.*, 2000).

During acclimatization there is a switch to autotrophy and changes in stomatal functioning and cuticular composition (HUYLENBROECK *et al.*, 2000). Water is rapidly lost from the *in vitro* cultured plantlet because of the failure of the stomata to respond to stimuli that would normally induce their closure (ROBERTS *et al.*, 1990). The poorly developed cuticle results in a rapid loss of water (ROBERTS *et al.*, 1990).

Vitrified plants do not acclimatise well to *in vivo* conditions. Vitrified plants are common where liquid media and low agar concentrations are used (PIERIK, 1997). In vitrified plantlets there is a reduced deposition of cellulose and lignin, leading to an increase in water uptake by the cells and resulting in glassy swollen leaves and stems (ROBERTS *et al.*, 1990). Because of this, and the low rates of photosynthesis sustained by the *in vitro* cultured plants, they easily suffer from photoinhibition and water stress; leading to the production of reactive oxygen species (HUYLENBROECK *et al.*, 2000). It has been demonstrated that micropropagated plants develop antioxidant mechanisms during acclimatization (HUYLENBROECK *et al.*, 1998).

In vitro grown leaves are the only source of nutrition to cover metabolic demands and to sustain plant adaptation and regrowth during the first days after transplanting micropropagated plants to greenhouse conditions (HUYLENBROECK *et al.*, 1998). The good and sustainable health of leaves is therefore essential to the acclimatization and survival of the plant (HUYLENBROECK *et al.*, 1998).

Plant hardening is usually carried out under greenhouse conditions to increase the chance of survival (AHLOOWALIA & PRAKASH, 2002). A commonly used greenhouse is the Quonset type. This consists of movable or fixed benches with hardening tunnels on them (AHLOOWALIA & PRAKASH, 2002). It is also advantageous to acclimatise plants to lower humidities while they are still under *in vitro* conditions (AHLOOWALIA *et al.*, 2002). In this way, plants grown in strongly

aerated vessels often require little or no hardening (AHLOOWALIA & PRAKASH, 2002).

There are numerous differences between leaves formed *in vitro* and *ex vitro*: differences in wax composition, pigmentation content, stomatal response and photosynthetic performance (HUYLENBROECK *et al.*, 1998). In a study conducted by HUYLENBROECK *et al.* (2000) on *Calathea* plants (Marantaceae) it was shown that chlorophyll and carotenoid content in leaves formed *ex vitro* were almost three times higher than *in vitro*.

Stomatal aperture can be measured by applying nail varnish to the abaxial surface of the mature leaf (ROBERTS *et al.*, 1990). The image of the hardened film can then be analysed. This allows us to assess the degree to which the plant has hardened to *in vivo* conditions (ROBERTS *et al.*, 1990).

2.9.10 Applications of *in vitro* culture

In vitro culture and recombinant DNA technology enable plant improvement through sexual and para-sexual methods (AHLOOWALIA, 1997). Such methods include mutation induction, embryo rescue, anther and ovary culture, protoplast fusion and transgenic methods. Using these methods other flower colours, flower shapes and growth habits of species in the genus *Romulea* could possibly be obtained (AHLOOWALIA, 1997).

2.10 CORM PHYSIOLOGY

One main difference between corms and bulbs is that a corm is a stem base swollen with food reserves (HUSSEY, 1977a; KRIKORIAN & KANN, 1986). These stem bases have several nodes, each of which has its own superficial axillary bud (HUSSEY, 1977a). The leaves around a corm are distinctly different from those of bulbs. These leaves, which sheath the new corm, are thin and rarely useful as explants for culture initiation (HUSSEY, 1977a; KRIKORIAN & KANN, 1986).

2.11 *IN VITRO* FLOWERING

Florogenesis is divided into six phases for most geophytes. These include induction, initiation, differentiation, floral stalk elongation, maturation and growth of the floral organs, and anthesis. (FLAISHMAN & KAMENETSKY, 2006). Factors that affect florogenesis include the genetics of the plant and its environment (FLAISHMAN & KAMENETSKY, 2006). After induction of flowering the vegetative meristem ceases leaf production during flower initiation to allow for more resources for flower initiation (FLAISHMAN & KAMENETSKY, 2006).

Smoke and smoke solutions have been used to promote flowering in geophytes. These include *Narcissus tazetta*, a freesia hybrid and *Watsonia* spp. (IMANISHI, 1983; UYEMURA & IMANISHI, 1984; LIGHT *et al*., 2007). LIGHT *et al*., (2007) found that a 1:500 (v/v) smoke water solution promoted the flowering of corms of *Watsonia* spp., which is in the same subfamily as *Romulea*. Corms of *Freesia* sp., a species in the same tribe as *Romulea*, were stimulated to flower using a smoke treatment (UYEMURA & IMANISHI, 1984).

In a study of the plant-soil relationship in the habitat of *R. columnae* it was found that soil N, P and K was reduced during the generative growth stage whereas the soil organic matter, pH, and $CaCO_3$ levels were increased (KÖK *et al*., 2007).

It is better to use large plants with large, matured storage organs for florogenesis studies, as it is common for a plant with a small storage organ not to flower (FLAISHMAN & KAMENETSKY, 2006).

2.12 *IN VITRO* PROPAGATION OF GEOPHYTES

ZIV (1997) defines geophytes as plants pereniating by underground storage organs. Geophytes are generally capable of developing tubers, corms or bulbs *in vitro* (STEINITZ & LILIEN-KIPNIS, 1989). Many ornamental geophytes are used for gardening, pot plant production, flowering pot plant production, cut flower production and the production of phytochemicals (ZIV, 1997).

Plant biotechnology has provided geophyte production with clonal propagation, virus elimination, breeding and crop improvement through embryo rescue, *in vitro* fertilization, somaclonal variation, protoplast isolation and somatic hybridisation, and haploid production (ZIV, 1997). Genetic transformation is also aiding in geophyte development. Benefits include improving horticultural traits and induction of disease resistance (ZIV, 1997). Gene mapping and DNA fingerprinting are also additional developing areas (ZIV, 1997).

Micropropagation has been achieved by enhanced axillary bud development, organogenesis and adventitious bud formation or by somatic embryogenesis (ZIV, 1997). Explants used for propagation of bulbous, cormous and tuberous plants include the leaf lamina, petiole, mesophyll and epidermis (ZIV, 1997). Inflorescence peduncle, pedicel, tepals, petals, sepals, ovaries, anthers, ovules and embryos have also been used (ZIV, 1997). Other explants include the basal plate, scales, twin-scales and nodal and storage tissue of bulbs, corms and tubers (ZIV, 1997).

The use of the underground pereniating organ as an explant source is often associated with heavy pathogen contamination (ZIV, 1997). This is a destructive use of the pereniating organ and eliminates the possibility of further vegetative or horticultural evaluation (ZIV, 1997). Different parts of the young flower and inflorescence stem can be cultured (ZIV, 1997). This is a source of pathogen-free totipotent explants (ZIV, 1997). Totipotency depends on the developmental stage at time of excision and position from which the explant was isolated (ZIV, 1997). Explants isolated from tissue positioned immediately next to the basal plate have a

higher regenerative potential in some species of geophytes than explant obtained from tissue in the upper sections of the inflorescence stem (ZIV, 1997).

***2.13 IN VITRO* PROPAGATION OF BULBOUS PLANTS**

Micropropagation protocols are available for many bulbous plants. The only bulbous species for which a large scale micropropagation program is however functional is *Lilium* sp (DEBERGH, 1994). Producing cormlets has an advantage over producing plantlets, as it prevents the development of vitreous leaves (ZIV, 1989).

2.14 *IN VITRO* PROPAGATION OF IRIDACEOUS SPECIES

Within one family there are often similar requirements or difficulties in micropropagation (KYTE & KLEYN, 1996). Understanding the culture requirements of species in the same family (Iridaceae) could shed some light on the culture conditions needed for the successful propagation of species in genus *Romulea.* An excellent review of Iridaceae micropropagation protocols has recently been published by ASCOUGH *et al.* (2009), this section will therefore only be considering studies involving direct shoot and corm organogenesis. These were chosen because they are the micropropagation steps used most commonly in this study and for species of Iridaceae. Direct shoot organogenesis and corm induction is summarized separately in Table 2.7 and 2.8 and is discussed separately in relation to results for some *Romulea* species in chapter five and six.

Table 2.7: Explant sources and PGR's used by various authors for direct shoot or meristimoid organogenesis in genera of Iridaceae. Where the concentrations of PGR's are not mentioned, the study included multiple species within the genus, each reacted differently to various concentrations. A question mark indicates that the specific parameter is not included in the described publication. The genera are grouped phylogenetically, with vertical text on the right showing classification.

Subfamily	Tribe	Subtribe	Genus	Author(s)	Explant (s)	PGR(s)
Ixioideae	Ixieae	Romuleinae	*Crocus*	BHAGYALAKSHMI (2000)	Ovary	21.6 µM NAA and 22.2 µM BA
				HOMES, LEGROS and JAZIRI (1987)	Corm	0.5 µM kinetin and 4.5 µM 2,4-D; followed by subculture in 9.1 µM 2,4-D
				PLESSNER, ZIV and NEGBI (1990)	Corm	1.4 µM GA_3, 2.3 µM 2,4-D and 2.3 µM kinetin
		Babianinae	*Babiana*	MCALISTER, JÄGER and VAN STADEN (1998)	Hypocotyl	4.4 µM BA and 5.3 µM NAA
				JÄGER, MCALISTER and VAN STADEN (1995)	Hypocotyl	4.4 µM BA and 5.4 µM NAA
		Ixiinae	*Ixia*	MEYER and VAN STADEN (1988)	Corm, Leaf	5 or 10 µM BA
				SUTTER (1986)	Corm	4.4 µM BA
			Dierama	KOETLE, FINNIE and VAN STADEN (2010)	Seedling hypocotyl	1.0 µM zeatin
				MADUBANYA (2004)	Hypocotyl	2.2 µM BA
				PAGE and VAN STADEN (1985)	Corm	2.7 µM NAA / No PGR's
			Sparaxis	HUSSEY (1975)	Corm, Inflorescence	0.2 to 0.7 µM NAA
				HUSSEY (1976)	Shoot	0.5 µM BA

Subfamily	Tribe	Subtribe	Genus	Author(s)	Explant (s)	PGR(s)
Ixioideae (continued)	Ixieae (continued)	Gladiolinae	*Gladiolus*	DANTU and BHOJWANI (1987)	Corm	2.2 µM BA
				DANTU and BHOJWANI (1995)	Corm	2.2 µM BA
				DE BRUYN and FERREIRA (1992)	Corm	2.2 µM to 8.9 µM BA
				HUSSEY (1975)	Corm, Inflorescence	0.2 to 0.7 µM NAA
				HUSSEY (1976)	Shoot	0.5 µM BA
				HUSSEY (1977a)	Corm	0.5 to 1.3 µM BA
				JÄGER, MCALISTER and VAN STADEN (1998)	Hypocotyl	5.3 µM BA and 4.4 µM NAA
				KUMAR, SOOD, PALNI and GUPTA (1999)	Corm, Inflorescence	2.5 µM BA and 10.0 µM NAA
				LILIEN-KIPNIS and KOCHBA (1987)	Corm	kinetin/BA and NAA
				TAN NHUT, TEIXEIRA DA SILVA, HUYEN and PAEK (2004)	Corm	2.2 µM BA
				SEN and SEN (1995).	Corm	1.0 mg.l-1 BA
				STEINITZ, COHEN, GOLDBERG and KOCHBA (1991)	Corm	0.3 µM BA and 0.1 µM NAA
				STEINTZ and LILIEN-KIPNIS (1989)	Corm	0.3 µM BA and 0.1 µM NAA
				SUTTER (1986)	Corm	4.4 µM BA
				ZIV and LILIEN-KIPNIS (2000)	Inflorescence	10 µM kinetin and 5 µM NAA
				ZIV (1970)	Corm	2.7 µM kinetin and 2.3 µM NAA
				ZIV (1989)	Corm	10 µM BA and 0.25 µM NAA
				ZIV, RONEN and RAVIV (1998)	Corm	0.5 µM NAA, 0.5-5.0 µM BA and 1.7 µM PP3
		Freesiinae	*Freesia*	HUSSEY (1975)	Corm, Inflorescence	0.2 to 0.7 µM NAA
				HUSSEY (1976)	Shoot	2.2 to 35.5 µM BA
				HUSSEY (1977b)	Corm	2.2 to 8.9 µM BA

Subfamily	Tribe	Subtribe	Genus	Author(s)	Explant (s)	PGR(s)
Ixioideae (continued)	Ixieae (continued)	Hesperanthinae	*Shizostylis*	HUSSEY (1975)	Inflorescence	0.2 to 0.7 µM NAA
				HUSSEY (1976)	Shoot	0.5 µM BA
		Tritoniinae	*Tritonia*	ASCOUGH, SWART, FINNIE and VAN STADEN (2011)	Seedling	3.3 µM *m*T
			Crocosmia	GEORGE and SHERRINGTON (1984)	?	?
	Watsonieae	(Unknown)	*Watsonia*	ASCOUGH, ERWIN, VAN STADEN (2007)	Hypocotyl	BA and NAA
Iridoideae	Sisiyrinchieae	(Unknown)	*Sisyrinchium*	ASCOUGH, SWART, FINNIE and VAN STADEN (2011)	Seedling	4.1 to 20.7 µM *m*T
	Irideae	Iridinae	*Iris*	HUSSEY (1976)	Shoot	0.5 µM BA
				YABUYA, YOSHIHARA, INOUE and SHIMIZU (2006)	Shoot	4.4 µM BA and 5.4 µM NAA
				HUSSEY (1977b)	Corm	2.2 to 8.9 µM BA
	Tigrideae	Cipurinae	*Cipura*	SENGUPTA and SEN (1988)	Corm	4.5 µM 2,4-D, and 15% coconut milk

Table 2.8: Corm induction treatments for various genera in Iridaceae. Details on the media modifications, temperature and the hours of light (Photoperiod) during corm induction is included. The period it took for corms to form is also given in months. The genera are grouped phylogenetically, with vertical text on the right showing classification. A question mark indicates that the specific parameter is not included in the described publication.

Subfamily	Tribe	Subtribe	Genus	Author(s)	Media modifications	Temperature	Light (h)	Period (months)
Ixioideae	Ixieae	Romuleinae	*Crocus*	PLESSNER, ZIV and NEGBI (1990)	1.4 µM GA_3; 2.3 µM 2,4-D; 2.3 µM kinetin	15-25℃	?	3.6
				HOMES, LEGROS and JAZIRI (1987)	0 or 0.4 µM kinetin and 4.5 or 9.1 µM 2,4-D; 2% sucrose	30℃	0	2
		Ixiinae	*Ixia*	SUTTER (1986)	0.3 µM NAA	25℃	24	>2
			Dierama	MADUBANYA (2004)	17.0 to 34.0 µM PP3	25℃	16	3
			Sparaxis	HUSSEY (1976)	2% sucrose	20℃	16	6 -10
		Gladiolinae	*Gladiolus*	DANTU and BHOJWANI (1987)	10% sucrose	25℃	24	<1
				DANTU and BHOJWANI (1995)	6% sucrose	25℃	24	2
				DE BRUYN and FERREIRA (1992)	6% to 9% sucrose; 2 $g.l^{-1}$ agar	15 or 24℃	16	?
				GINZBURG and ZIV (1973)	2.2 µM kinetin and NAA	24℃	0	0.5
				HUSSEY, 1976 (1976)	2% sucrose	20℃	16	6 -10
				HUSSEY (1977a)	0.5 µM BA; 2% Sucrose	20℃	16	2.5
				JÄGER, MCALISTER and VAN STADEN (1998)	8.8 µM BA	25℃	16	?
				KUMAR, SOOD, PALNI and GUPTA (1999)	2% sucrose	25℃	12	?

Subfamily	Tribe	Subtribe	Genus	Author(s)	Media modifications	Temperature	Light (h)	Period (months)
Ixioideae (continued)	Ixieae (continued)	Gladiolinae (continued)	*Gladiolus* (continued)	SEN & SEN (1995)	4.4 µM BA or no PGR's	23 to 25℃	16	3
				STEINTZ and LILIEN-KIPNIS (1989)	34.0 or 170.2 µM PP3; 3%, 6% sucrose	25/20℃ (day/night)	16	1
				STEINITZ, COHEN, GOLDBERG and KOCHBA (1991)	6% sucrose	25/20℃ (day/night)	16	1
				SUTTER (1986)	4.4 µM BA	25℃	24	2
				TAN NHUT, TEIXEIRA DA SILVA, HUYEN and PAEK (2004)	1.0 µM BA	15 or 20℃	24	2
				ZIV (1989)	1/2 strength MS; 3.4 µM PP3	25℃	24	4 to 5
				ZIV, RONEN and RAVIV (1998)	8.7 µM PP3	24/22℃ (day/night)	16	2.5 - 3.5
		Freesinae	*Freesia*	HUSSEY (1976)	2% sucrose	20℃	16	12 -14
		Hesperanthinae	*Shizostylis*	HUSSEY (1976)	2% sucrose	20℃	16	6-10
		Tritoniinae	*Tritonia*	ASCOUGH, SWART, FINNIE and VAN STADEN (2011)	none	10 or 15℃	16	3
	Watsonieae	(Unknown)	*Watsonia*	ASCOUGH, ERWIN, VAN STADEN (2008)	6% sucrose; 1 mg l-1 GA_3	20℃	24	3
Iridoideae	Irideae	Iridinae	*Iris*	HUSSEY (1976)	2% Sucrose	20℃	16	6 -10
	Tigrideae	Cipurinae	*Cipura*	SENGUPTA and SEN (1988)	1/2 strength MS; 1% sucrose	22-25℃	16	1

3 Investigation into the habitat of *Romulea sabulosa* and *Romulea monadelpha*: Soil sampling and analysis

3.1 INTRODUCTION

R. sabulosa, one of the most attractive species of this genus, occurs west of Nieuwoudtville on sandy soil and in renosterveld on clay (MANNING & GOLDBLATT, 1997; 2001). In these areas of renosterveld populations of *R. monadelpha*, another attractive species, can also be found. Other species of *Romulea* used in this study also occur in this area (Figure 2.1).

In a study by KÖK *et al.* (2007) soil samples were taken during vegetative and flowering stages in the habitat of *R. columnae* in Turkey. This species is widely used as an ornamental plant (KÖK *et al*., 2007). The climate of this study area is somewhat similar to Namaqualand, with it being described as having a humid Mediterranean climate and a xeric period of 4 months. Analyses done on soil samples by KÖK *et al.* (2007) determined soil texture, pH, salinity and percentage nitrogen, phosphorous, potassium, organic matter and $CaCO_3$.

During a visit to the Nieuwoudtville Wildflower Reserve, Mr. Eugene Marinus showed me plants of *R. monadelpha* and *R. sabulosa* which he had grown from seed. Although not open, flower buds were visible on these plants and the plants appeared healthy and mature. He also mentioned that some of these plants had flowered in previous seasons. I enquired more about his methods and he subsequently showed me the soil he had used and allowed me to take samples. He had used a 1:1 mixture of soil from two locations about 20 m from each other.

Observations of plants in natural conditions will be useful to compare with the morphology and size of the plants propagated by *in vitro* techniques. Soil texture, pH, salinity and percentage nitrogen, phosphorous and potassium was expected to be similar to that obtained by KÖK *et al*. (2007) in Turkey.

The aims of this Chapter were to determine and measure the ecological variables in the soil of these plants so that these variables can be compared with observations made *in vitro* and *ex-vitro* for the same species in subsequent Chapters.

3.2 MATERIALS AND METHODS

Soil samples were taken from the Nieuwoudtville Wildflower Reserve (19° 8' E, 31° 24' S). Soil samples were divided into two groups based on locality (the two soils used by Mr. Marinus). Sample 1 was described by Mr. Marinus as dolerite soil and sample 2 as tillite soil.

Soils were analyzed by KwaZulu-Natal Department of Agricultural and Environmental Affairs Soil Fertility and Analytical Services, Pietermaritzburg. A texture test with 3 fractions produced the amount of sand (0.02 - 2 mm), fine silt (0.02 - 0.002 mm) and clay (<0.002 mm) particles present in the samples and grouped the samples into two texture classes. Fertility density tests produced measurements of density, pH, exchangeable cations, percentage nitrogen, phosphorous, potassium, magnesium, zinc, manganese and copper available to plants and carbon and clay content. Inorganic nitrogen tests measured the concentrations of NH_4^+ and NO_3^- concentrations. Total nitrogen and carbon tests by Dumus combustion using a CNS analyser measured the total nitrogen within the sample.

A salinity on saturation extract test measured the pH, the EC (mS/m), sodium adsorption ratio (SAR) and an assessment code based on two numbers. The SAR of a soil is calculated by the equation:

$$SAR = \frac{Na}{\sqrt{\frac{Ca + Mg}{2}}}$$

Where Na, Ca and Mg is the concentration of ions of these elements within the soil measured in mmol(+)dm^3. (SOIL CLASSIFICATION WORKING GROUP, 1991). In cases where the potassium content is very high, the ratio between the Na plus K content and the Ca plus Mg content, called the sodium and potassium adsorption ratio (SPAR) can be calculated (SARAH, 2004).

The first number of the assessment has a value from 1 to 3; 1 being non-saline, 2 potentially saline and 3 saline. The second number has a value from 4 to 6; 4 being non-sodic, 5 potentially sodic and 6 sodic. A sodic soil is defined as a soil with a low soluble salt content and a high exchangeable sodium percentage (ESP), with a usual ESP > 15 (SOIL CLASSIFICATION WORKING GROUP, 1991). A code 1,4 soil is described as suitable for irrigation, a code 2,5 soil as poorly drained and not suitable for irrigation and a code 3,6 soil as not suitable for irrigation.

A pH test and a water content test were done on both samples. The pH of three different replicate samples was measured. Soil was mixed 1:1 (v/v) with water and homogenized with a stirrer apparatus. After about an hour the soil samples were vacuum filtered through Whatman No.1 filter papers and their pH was measured. The water content was calculated by weighing the soil, placing the soil in a drying oven set at 110℃ and then subtracting the mass after no weight loss could be observed from the initial weight.

3.3 RESULTS

The colour of the two samples suggests that the drainage conditions of sample 1 is superior to that of sample 2 (DONAHUE *et al.*, 1983) (Figure 3.1). Sample 1 also had more leaf and root material than sample 2, suggesting that it has more organic matter, and therefore a higher nutrient content, than sample 2 (DONAHUE *et al.*, 1983). It is notable that sample 2 appears more aggregated and dense.

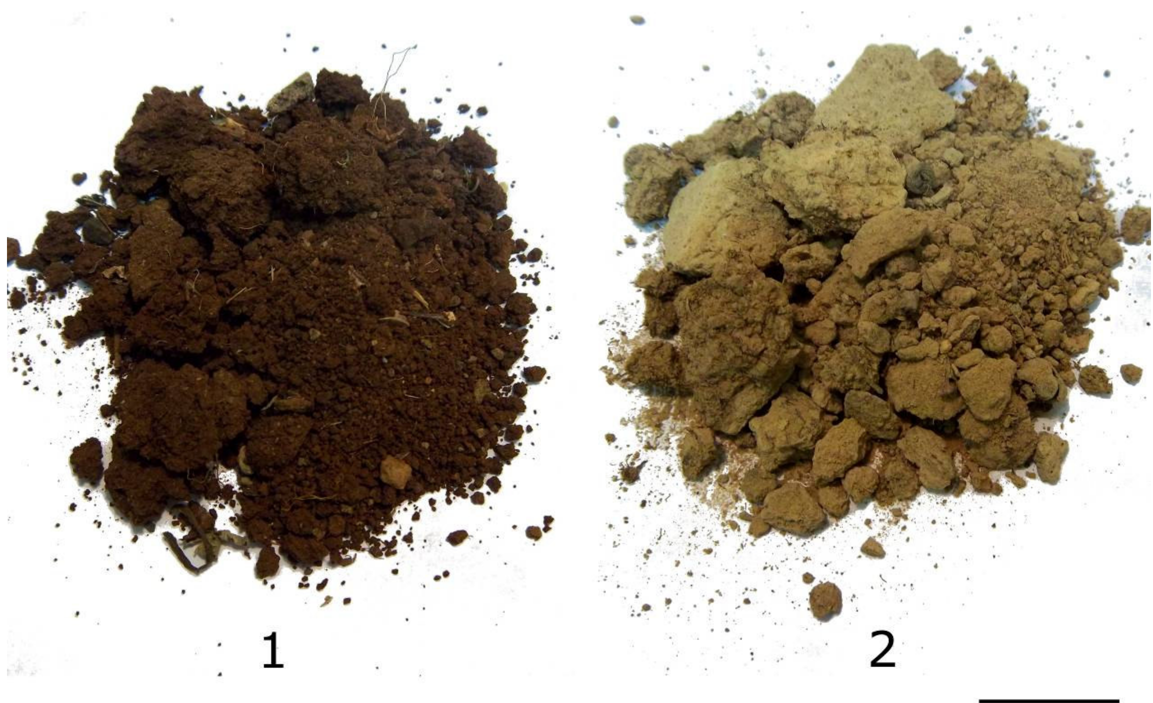

Figure 3.1: The colour and structure of samples 1 and 2. Horizontal bar = 20 mm.

The texture test revealed that sample 1 is a clay soil due to its high content of particles smaller than <0.002 mm and sample 2 is a sandy loam soil due to its high sand and silt content (Table 3.1). Sample 1 had a much higher percentage water content due to its larger clay and organic matter content. There was large variation in the pH readings although these and the exchangeable acidity all showed that sample 2 was more acidic than sample 1. Measurements of pH for sample 1 ranges from 6.66 to 7.81 and from 4.94 to 6.72 for sample 2.

As suggested by visual appearance, soil sample 2 had a higher density than sample 1. Nutritional elements were all in higher abundance within sample 1, with the exception of phosphorous and nitrogen. Sample 1 had a N:P:K ratio of 1.000:0.003:0.481, while sample 2 had a N:P:K ratio of 1.000:0.017:0.189. Despite the apparent higher nitrogen content of sample 2, sample 1 contained a greater concentration of the two ions mostly required for plant growth, NH_4^+ and NO_3^- and had a higher total nitrogen content.

More heavy metals were found in sample 2, whereas sample 1 was more saline. According to the assessment code, sample 2 is more poorly drained than sample 1, although sample 1 can also be described as poorly drained.

Table 3.1: Analysis results for two soil samples from the Nieuwoudtville Wildflower Reserve (19° 8' E, 31° 24' S).

Test	Test parameters	Sample 1	Sample 2
Texture	Sand %	31	63
	Fine Silt %	16	18
	Clay %	54	19
Water content	Wet weight (g)	439.3	392.0
	Dry weight (g)	393.5	384.7
	Water content (%)	10.4%	1.9%
pH	Measured with fertility density	6.66	4.94
	Measured with soil salinity	7.30	4.99
	Personal test	7.81±0.05	6.72±0.5
Fertility density	Sample density (g/ml)	1.13	1.23
	Phosphorous (%)	0.0003	0.0022
	Potassium (%)	0.0337	0.0246
	Calcium (%)	0.8796	0.0681
	Magnesium (%)	0.1587	0.0264
	Exchangeable acidy (cmol/l)	0.06	0.05
	Total cations	57.88	6.25
	Zinc (%)	0.0004	0.0006
	Manganese (%)	0.0002	0.0003
	Copper (%)	0.0003	0.0002
	MIR clay (%)	19	12
	MIR organic Carbon (%)	<0.5	0.7
	MIR Nitrogen (%)	0.07	0.13
Inorg. N	NH_4^+ (%)	0.0007	0.0006
	NO_3^- (%)	0.0059	0.0005
Total N & C	Total % Nitrogen	0.13±0.03	0.05±0.01
	Total % Carbon	2.15±0.36	0.71±0.21
Salinity	SAR	7.00	4.51
	Assessment code	3,5	3,4

3.4 DISCUSSION

R. culumnae grows in soils that are richer in nutrients and less saline compared to the soils in which *R. monadelpha* and *R. sabulosa* grows. The concentrations of nitrogen, phosphorous and potassium was higher in soil sampled from the natural habitat of *R. culumnae* in Turkey (KÖK *et al.*, 2007). The soil of the habitat of *R. culumnae* had a smaller N:P:K ratio (1.000:6.897:1.069) and a lower salinity. The pH of the soils from the Namaqualand and Turkey is however similar. *R. culumnae* also occurs in a loamy clay soil, which is essentially a combination between the clay soil (sample 1) and a sandy loam soil (sample 2) in which *R. monadelpha* and *R. sabulosa* occurs, except for the slightly higher sand content that such a combination would have.

Elements that are in abundance or that are lacking or low in the natural environment may have an effect on germination and growth in some species (BASKIN & BASKIN, 1998). In soil sample 1 and 2 calcium is abundant. Although the percentage Fe content was not tested, the red colour of the clay indicates that the soils are rich in Fe.

3.5 SUMMARY

- The pH and soil texture of soils in which *R. sabulosa* and *R. monadelpha*, and *R. culumnae* occur is similar.
- Soils in which *R. sabulosa* and *R. monadelpha* are found is more saline and has a much lower percentage nitrogen, phosphorous and potassium than soil in which *R. culumnae* is found.

4 Germination physiology

4.1 INTRODUCTION

Species in the Iridaceae may exhibit morphological and/or morphophysiological dormancy. These mechanisms of delaying germination is due to the underdeveloped embryo of species in this family (TILLICH, 2003). The only literature published so far in relation to the germination of *Romulea* species is that of DENO (1993) and EDDY & SMITH (1975). These studies indicates that species of this genus requires a low temperature of 10℃ for germination and temperatures between 16.5℃ and 20℃ may have an inhibitory effect on germination. EDDY & SMITH (1975) showed that application of KNO_3 and pre-chilling for 5 days at 2℃ did not increase germination of *R. rosea*. Seeds of this species are effectively dispersed by sheep and showed only 38% germination in its faecal pad compared to the normal germination of 96% (EDDY & SMITH, (1975). This finding suggests that scarification is not an ideal treatment to break the dormancy of these seeds. *R. rosea* is an invasive species on other continents and some islands. This is the only *Romulea* species which shows high germination under natural environmental conditions. On the other hand, there are several other important potential horticultural species that are not easily germinated and their seed biology remains unknown.

The aim of this Chapter was to understand the physiological mechanism behind dormancy and germination and to improve percentage germination of some important *Romulea* species with economic potential. Additionally, to test the germination mechanism of *R. rosea* in more detail, which can help in eradicating/controlling this species in countries where it is invasive.

4.2 MATERIALS AND METHODS

In South Africa, collection of many *Romulea* species is legally restricted due to the limited existence of natural populations. For this study, seeds were obtained from Silverhills Nursery, Kenilworth and African Bulbs, Napier. Both are South African

companies. The availability of seeds of *Romulea* species at these nurseries was very limited and therefore only a small quantity could be purchased. Seeds of all *Romulea* species examined in this study were about one-year-old. To understand the basic physiological functions of these seeds, water content, imbibition rate and the viability of seeds both *in vitro* and *ex vitro* were determined. Seed surface and micropylar regions of these species were observed using a scanning electron microscope.

4.2.1 Viability tests

2,3,5-Triphenyl tetrazolium chloride (TTC) and embryo excision tests were conducted to examine seed viability. For the TTC test, the seeds were soaked in 1% TTC solution for one week in a glass vial and kept under constant dark conditions at 25 ± 0.5°C. Subsequently, these seeds were cut into two and the percentage and degree of staining of the embryo and endosperm portions were recorded (INTERNATIONAL SEED TESTING ASSOCIATION, 1999). This test had four replicates of 10 seeds each.

To excise the embryos, the seeds were first surface-decontaminated with 1.75% sodium hypochlorite solution with a few drops of Tween 20 for 15 min. Thereafter they were rinsed three times with sterile distilled water (ASCOUGH *et al.*, 2007). Decontaminated seeds were placed in an autoclaved Petri dish with sterile distilled water. The seeds were left to imbibe in the laminar flow cabinet for 4 to 5 days. For embryo excision, slices of testa and endosperm were separated from the seed using a scalpel with a sterile blade, until the embryo was visible. Small pieces of endosperm around the embryo were carefully removed with a dissection needle. The embryo was then lifted from the endosperm with the help of a sterile dissecting needle and placed in an autoclaved Petri dish, filled with sterile distilled water to rinse off residual pieces of endosperm. Embryos were then placed into 33 ml culture tubes using an autoclaved Pasteur pipette filled with sterile distilled water. These tubes contained 10 ml Murashige and Skoog (MS) medium (MURASHIGE & SKOOG, 1962) supplemented with 100 $mg.l^{-1}$ *myo*-inositol and 3% sucrose, with pH adjusted to 5.7 and solidified with 0.8% agar. The experiment had four replicates of 5 seeds for each species. The culture tubes were sealed with Parafilm and placed under cool white fluorescent tubes (Osram L75 W/20X) with a 16-h photoperiod of 30.1 ± 3.4 $\mu mol\ m^{-2}\ s^{-1}$ irradiance at 25 ± 0.5°C.

4.2.2 Water content and imbibition rate

Water content was determined by weighing seeds before and after placing them in a drying oven set at 110℃. The weighing continued for six weeks until there was no further loss in seed weight. Per species, four replicates of 5 seeds each were used.

The imbibition rate was determined by weighing the seeds after imbibing them for 3, 6, 24, 48, 150, 168, 216, 264 and 336 h. Seeds were placed in 6.5 cm disposable plastic Petri dishes with filter paper (Whatman No.1) moistened with 3.5 ml of distilled water and subsequently kept moist by adding 1 to 3 ml of distilled water when needed. At each time interval, the seeds were removed from the Petri dishes, blotted dry, weighed and replaced in the respective Petri dishes. This experiment was conducted at room temperature (25 ± 0.5℃) using four replicates of 5 seeds for each species.

4.2.3 Scanning electron microscopy

Seeds were rinsed in 70% ethanol for 30 seconds and then allowed to dry on a paper towel. Seeds were mounted on 12 mm stubs and the micropylar regions and surfaces viewed using a scanning electron microscope *(*Zeiss EVO LS15 variable pressure).

4.2.4 *Ex vitro* germination experiments

Experiments were conducted to test the effect of temperature and light, cold and warm stratification, acid scarification, mechanical scarification, plant growth promoting substances and deficiency of nitrogen, phosphorous and potassium on germination of different *Romulea* species.

If not stated otherwise, a growth chamber set at 20℃ with a 16 h light: 8 h dark photoperiod and a photosynthetic photon flux density (PPFD) of 30.1 ± 4.0 µmol m^{-2} s^{-1} provided by cool-white fluorescent lamps was used. Seeds were decontaminated by placing them in 0.2% mercuric chloride for 5 min and rinsing them once with tap water and twice with distilled water. Unless otherwise stated, seeds were placed in 6.5 cm disposable plastic Petri dishes with two circles of filter paper (Whatman No.1) moistened with 3.5 ml of distilled water and test solutions. The filter paper was kept moist with the respective solutions when needed. In all germination experiments 10 seeds were placed in each Petri dish with 4 replicates per treatment. All germination experiments were terminated after 90 days. Germination was recorded every second day and was considered complete when the radicle had emerged up to 2 mm. Seeds

subjected to dark treatments were inspected every second day under a green “safe light” with a PPFD of 0.3 μmol m^{-2} s^{-1}. Mean germination time (MGT) was calculated using the equation: MGT = $\sum (n \times d)/N$, where ‘n’ is the number of seeds germinated on each day, ‘d’ is the number of days the experiment has been active and ‘N’ is the total number of seeds germinated after 90 days (ELLIS & ROBERTS, 1981).

To test the effect of temperature and light on germination of four *Romulea* species, decontaminated seeds were placed in growth chambers set at 10, 15, 20, 25, 30 and 30/15°C (day/night) with 16 h light: 8 h dark photoperiod. Petri dishes were covered with aluminium foil for the dark treatment and were only opened under a ‘safe light’ as indicated earlier.

For the stratification experiment, surface decontaminated seeds were placed between two layers of paper towelling moistened with distilled water. The towels were placed inside a plastic bag and were covered with aluminium foil to eliminate light. Seeds of all species were placed in a refrigerator at 5°C, except those of *R. rosea* which were placed at 30°C, as EDDY & SMITH (1975) reported pre-chilling did not improve the germination of this species. Seeds were kept under these conditions for 7, 14 and 21 days before placing them for germination in a growth chamber set at 20°C. Control seeds did not receive stratification treatment and were placed in the same growth chamber with the seeds of the 7 day stratified set.

For acid scarification, seeds were placed in 50% sulphuric acid for 5 min. Seeds were also mechanically scarified by rubbing them between two pieces of sand paper (P120 grade) for 1 min. Control seeds were not subjected to scarification treatment.

To test the effect of various plant growth promoting substances, seeds of *R. rosea* were placed in Petri dishes with filter papers moistened separately with 10^{-5}M of GA_3, Kinetin, KNO_3, IBA, NAA and IAA. Smoke water concentration of 1:500 (v/v) and butenolide (3-methyl-*2H*-furo[2,3-*c*]pyran-2-one) solution of 10^{-8}M were also used in this study. Butenolide is a compound isolated from plant-derived smoke water that enhances germination of many seeds, including *Eucomis* (KULKARNI *et al.*, 2006). Plant-derived smoke water was prepared according to BAXTER *et al.* (1994) and butenolide was isolated by the method of VAN STADEN *et al.* (2004). The effect of macro-nutrient deficiency was investigated by placing seeds on filter paper moistened with 3.5 ml of 50% Hoagland’s nutrient medium deficient of either nitrogen

(-N), phosphorous (-P) or potassium (-K) respectively. The seeds that were germinated with 50% Hoagland's medium consisting of all nutrients and with only distilled water served as controls. The filter paper was kept moist with these solutions throughout the duration of the experiment to maintain the levels of nutrients.

4.2.5 *In vitro* germination experiments

Seeds were decontaminated in accordance with the methods of ASCOUGH *et al.* (2007). Seeds were placed in 1.75% sodium hypochlorite solution with a few drops of Tween 20 for 15 min, after which they were rinsed three times with sterile distilled water. All seeds were placed in 33 ml culture tubes with 10 ml 1/10 strength MS media supplemented with 100 $mg.l^{-1}$ *myo*-inositol and no sucrose (ASCOUGH *et al.*, 2007). Tubes were placed in a growth chamber set at 15°C and a 16 h light: 8 h dark photoperiod with PPFD of 30.1 ± 4.0 $\mu mol\ m^{-2}\ s^{-1}$. Four replicates of 10 seeds (10 tubes) each of *R. autumnalis*, *R. citrina*, *R. cruciata*, *R. diversiformis*, *R. flava*, *R. leipoldtii*, *R. minutiflora*, *R. pearsonii*, *R. sabulosa* and *R. tabularis* were used. Considering *R. sabulosa*'s commercial potential, additional experiments were conducted by placing four replicates of 50 seeds each at 15 and 20°C.

4.2.6 Statistical analysis

Percentage germination data were arcsine transformed and analysis of variance (ANOVA) was calculated. Differences between means of percentage germination were tested with Least Significant Difference (LSD) at the 5% level. GENSTAT® Release 4.21 statistical package was used for analysis.

4.3 RESULTS

4.3.1 Viability tests

The results of the TTC and embryo excision tests are shown in Table 4.1. According to the TTC test, seed embryo and endosperm of *R. camerooniana* and *R. flava* showed < 61% staining indicating low to moderate viability respectively. All other tested species of *Romulea* exhibited over 90% staining of embryo and endosperm indicating high seed viability. The embryo excision test of *R. camerooniana* and *R. rosea* did not show any *in vitro* response. An *in vitro* response was however observed for all other species. Excised embryos of *R. diversiformis* and *R. leipoldtii* showed the highest percentage *in vitro* response. TTC and embryo excision tests were comparable for species *R. diversiformis*, *R. leipoldtii* and *R. monadelpha* (Table 4.1). The results of both tests differed for *R. flava*, *R. minutiflora* and *R. sabulosa*.

Table 4.1: Seed viability tests of different *Romulea* species.

Species	TTC (embryo + endosperm) (% staining)	Embryo excision (% response)
R. camerooniana	45 ± 19	0 ± 0
R. diversiformis	100 ± 0	100 ± 0
R. flava	60 ± 13	95 ± 5
R. leipoldtii	95 ± 6	100 ± 0
R. minutiflora	92 ± 12	70 ± 30
R. monadelpha	100 ± 0	90 ± 10
R. rosea	90 ± 6	0 ± 0
R. sabulosa	95 ± 5	54 ± 11

4.3.2. Water content and imbibition rate

R. leipoldtii seed had the highest initial water content (37 ± 1.4%) followed by *R. flava* (30 ± 2.1%). The lowest seed water content was determined for *R. camerooniana* and *R. diversiformis* (23%). Overall the range of seed water content was between 23 and 37% in the studied *Romulea* species (Figure 4.1). In most of the species the rate of imbibition sharply increased in the first three hours and was gradual up to 6 h, followed by a sharp increase in water uptake up to 48 h, after which it was slow. *R.*

rosea seeds however imbibed much more water between 3 h and 6 h and had a higher percentage water content at 6 h (89 ± 7.3 %) than any other of the species tested (Figure 4.1).

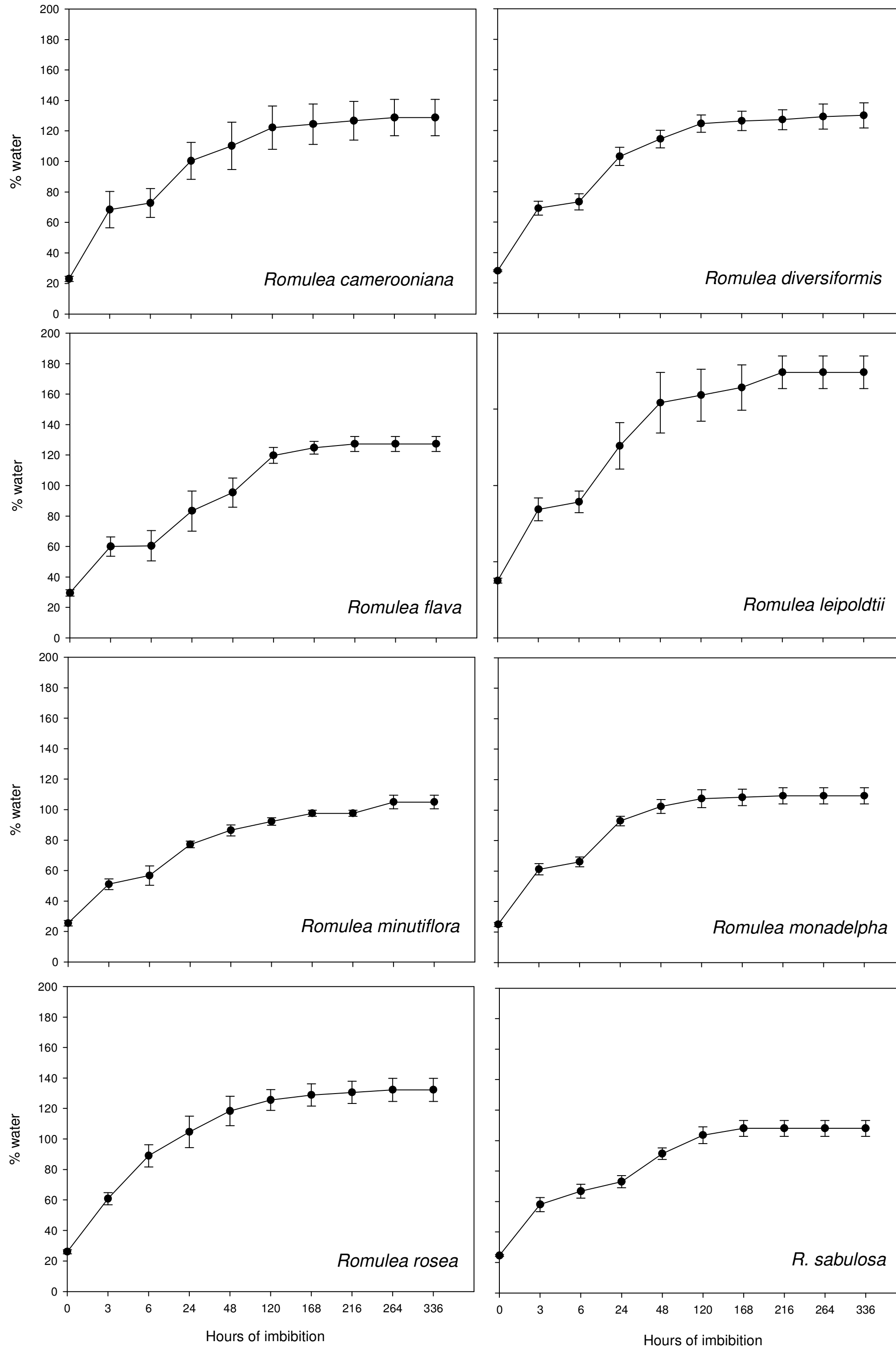

Figure 4.1: Water content (value at day zero) and imbibition rates of seeds of eight species of *Romulea*. Error bars indicate standard error of the mean.

4.3.3. Scanning electron microscopy

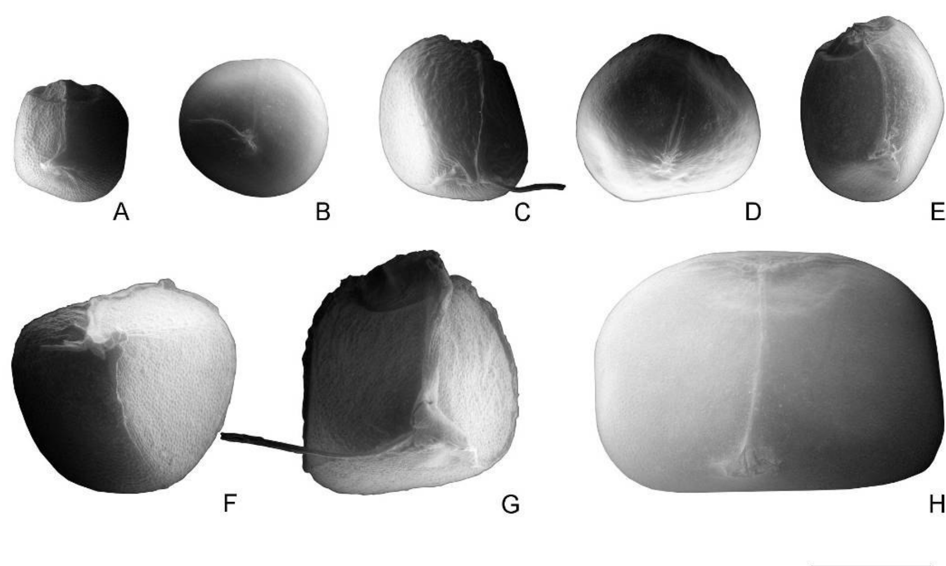

Figure 4.2: Scanning electron microscopic images of seeds arranged from the smallest to the largest for size comparison. *Romulea leipoldtii* (A); *R. flava* (B); *R. minutiflora* (C); *R. sabulosa* (D); *R. camerooniana* (E); *R. rosea* (F); *R. diversiformis* (G); *R. monadelpha* (H). Horizontal bar = 1 mm.

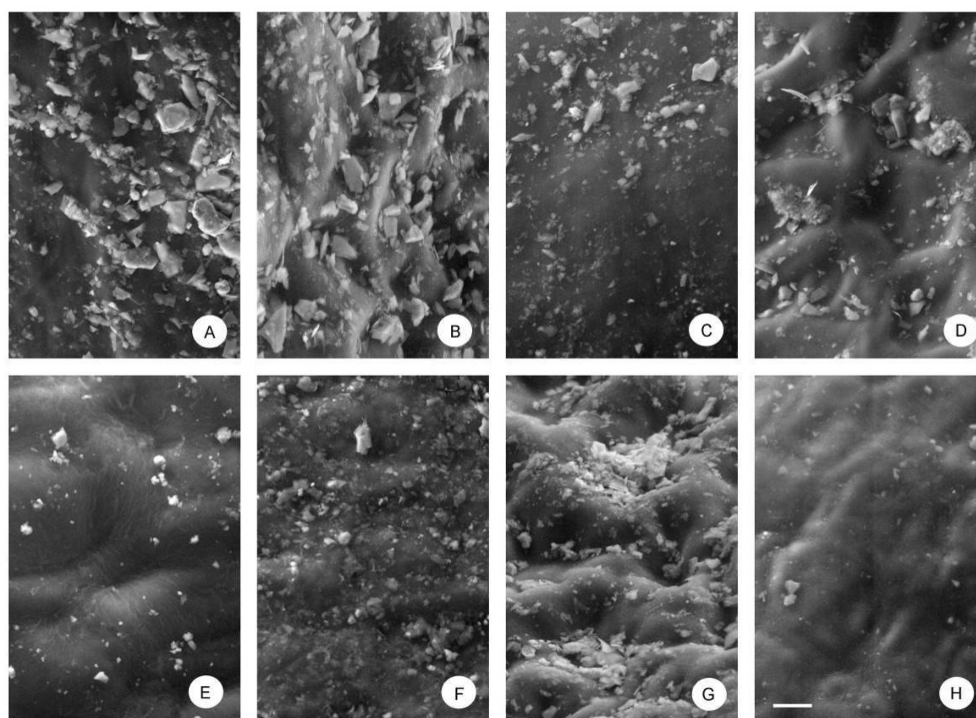

Figure 4.3: Scanning electron micrographs of the seed surfaces of *Romulea camerooniana* (A); *R. diversiformis* (B); *R. flava* (C); *R. leipoldtii* (D); *R. minutiflora* (E); *R. monadelpha* (F); *R. rosea* (G) and *R. sabulosa* (H). Horizontal bar = 10 µm (the same magnification was used for all species).

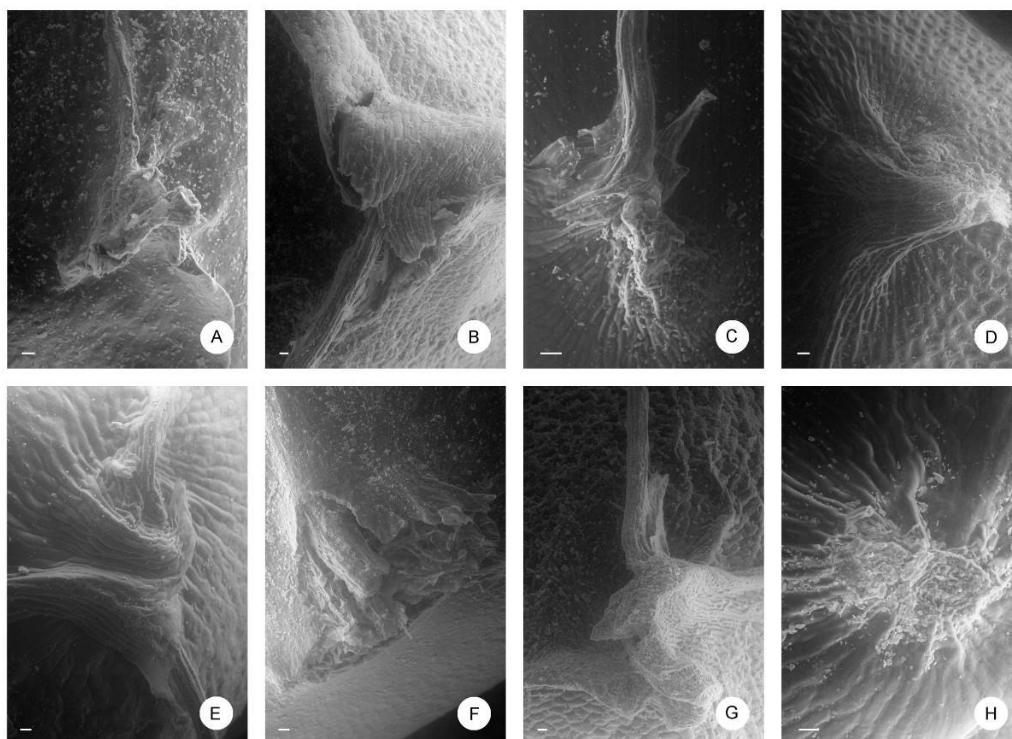

Figure 4.4: Scanning electron micrographs of the micropylar regions of seeds of *Romulea camerooniana* (A); *R. diversiformis* (B); *R. flava* (C); *R. leipoldtii* (D); *R. minutiflora* (E); *R. monadelpha* (F); *R. rosea* (G) and *R. sabulosa* (H). Horizontal bar = 20 µm.

The size and shape of seeds of different *Romulea* species showed large variations as seen in Figure 4.2. Both these parameters have a significant influence on water imbibition. *R. rosea* had the roughest seed surface, followed by *R. leipoldtii* and *R. diversiformis* (Figure 4.3). The seed surface of *R. flava* appears to be the smoothest, followed by *R. sabulosa* and *R. minutiflora*. In *Romulea* species, the sizes of the micropylar region are correlated to seed size with *R. monadelpha* and *R. diversiformis* having the largest micropylar regions (Figure 4.4). The micropylar regions of *R. minutiflora* and *R. rosea* appear more membranous than those of other species. The micropylar region of *R. sabulosa* seeds appears to be the densest, followed by *R. camerooniana.*

Table 4.2: Effect of different treatments on seed germination of four *Romulea* species. Asterisk (*) indicates seed germination under 16 h photoperiod at 20 ± 0.5°C. The number sign (#) indicates that the seeds initiated germination during stratification.

	Species							
	R. diversiformis		*R. flava*		*R. monadelpha*		*R. rosea*	
Treatment	Germination (%)	MGT	Germination (%)	MGT (days)	Germination (%)	MGT (days)	Germination (%)	MGT (days)
Temperature (°C)								
(16 h photoperiod)								
10	65 ± 0.9 a	41 ± 3 b	0 ± 0 d	0 ± 0 d	0 ± 0 b	0 ± 0 b	90 ± 1 a	32 ± 1 e
15	0.5 ± 0.3 d	30 ± 6 b	5 ± 1d	57 ± 0 a	0 ± 0 b	0 ± 0 b	68 ± 1 b	72 ± 2 b
20	0 ± 0 d	0 ± 0 c	0 ± 0 d	0 ± 0 d	3 ± 1 b	42 ± 0 a	15 ± 1 c	57 ± 1 c
25	0 ± 0 d	0 ± 0 c	0 ± 0 d	0 ± 0 d	0 ± 0 b	0 ± 0 b	0 ± 0 d	0 ± 0 f
30	0 ± 0 d	0 ± 0 c	0 ± 0 d	0 ± 0 d	0 ± 0 b	0 ± 0 b	0 ± 0 d	0 ± 0 f
30/15	0 ± 0 d	0 ± 0 c	0 ± 0 d	0 ± 0 d	0 ± 0 b	0 ± 0 b	0 ± 0 d	0 ± 0 f
(Constant dark)								
10	60 ± 1.5 ab	39 ± 1 b	8 ± 5 d	32 ± 1 b	3 ± 1 b	64 ± 0 a	95 ± 1 a	36 ± 1 e
15	48 ± 0.6 bc	41 ± 4 b	18 ± 5 c	53 ± 3 a	23 ± 1 a	43 ± 4 a	5 ± 1 d	57 ± 2 c
20	0 ± 0 d	0 ± 0 c	0 ± 0 d	0 ± 0 d	8 ± 1 b	56 ± 0 a	5 ± 1 d	76 ± 0 a
25	0 ± 0 d	0 ± 0 c	0 ± 0 d	0 ± 0 d	0 ± 0 b	0 ± 0 b	0 ± 0 d	0 ± 0 f
30	0 ± 0 d	0 ± 0 c	0 ± 0 d	0 ± 0 d	0 ± 0 b	0 ± 0 b	0 ± 0 d	0 ± 0 f
30/15	0 ± 0 d	0 ± 0 c	0 ± 0 d	0 ± 0 d	0 ± 0 b	0 ± 0 b	0 ± 0 d	0 ± 0 f
Cold stratification (5°C)*								
(Days)							Warm stratification (30°C)	
0	0 ± 0 d	0 ± 0 c	0 ± 0 d	0 ± 0 d	0 ± 0 b	0 ± 0 b	5 ± 1 d	77 ± 7 a
7	0 ± 0 d	0 ± 0 c	0 ± 0 d	0 ± 0 d	3 ± 1 b	52 ± 0 a	5 ± 1 d	62 ± 2 cd
14	18 ± 0.5 d	10 ± 5 c	43 ± 5 b	10 ± 6 c	0 ± 0 b	0 ± 0 b	70 ± 2 b	64 ± 2 c
21	38 ± 0.9 c #	1 ± 0 c	53 ± 9 a #	1 ± 0 d	0 ± 0 b	0 ± 0 b	73 ± 2 b	65 ± 1 c
Scarification*								
Control	0 ± 0 d	0 ± 0 c	0 ± 0 d	0 ± 0 d	0 ± 0 b	0 ± 0 b	0 ± 0 d	0 ± 0 f
Acid (50% H_2SO_4)	63 ± 0.8 ab	71 ± 3 a	0 ± 0 d	0 ± 0 d	0 ± 0 b	0 ± 0 b	0 ± 0 d	0 ± 0 f
Mechanical (sand paper)	0 ± 0 d	0 ± 0 c	0 ± 0 d	0 ± 0 d	0 ± 0 b	0 ± 0 b	0 ± 0 d	0 ± 0 f

Means (± SE) in the column with different letters are significantly different according to LSD at the 5% level ($P < 0.05$)

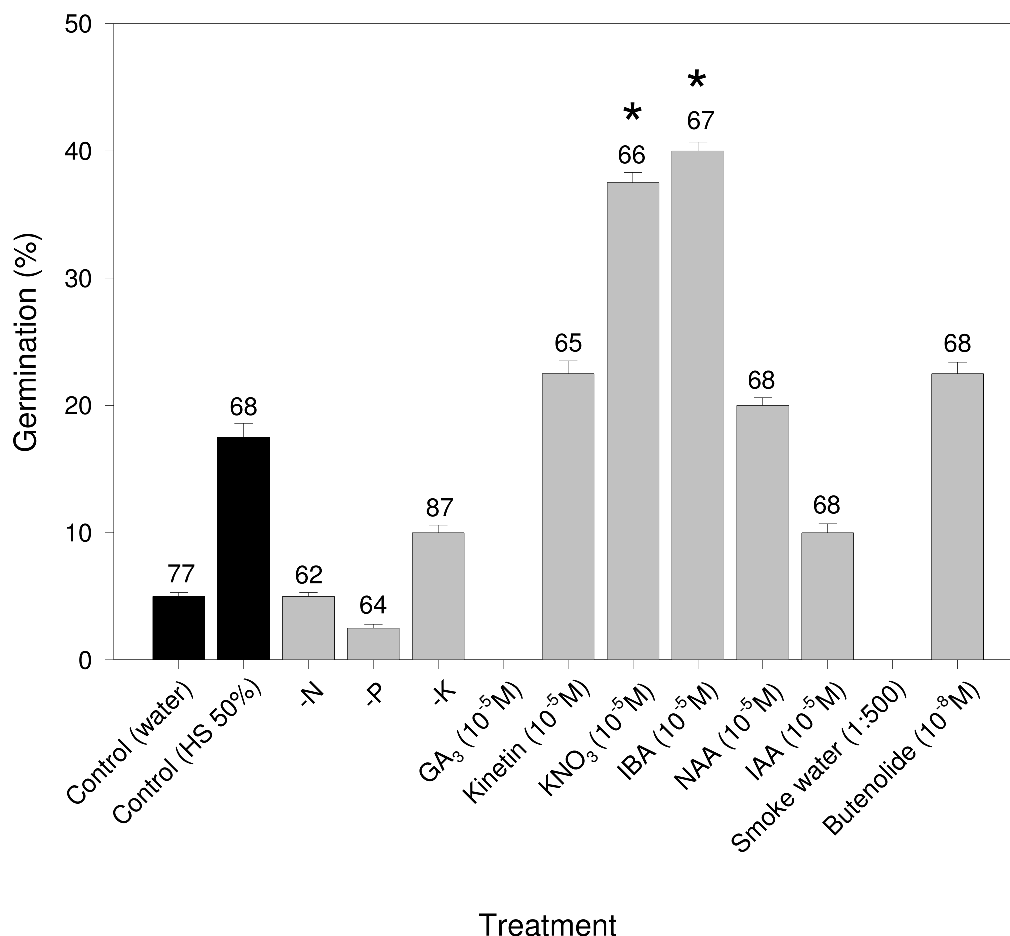

Figure 4.5: Effect of nutrients without N, P or K, plant growth promoting substances and smoke constituents on seed germination of *Romulea rosea* under 16 h photoperiod at 20 ± 0.5°C. A number above the standard error bar represents mean germination time and an asterisk denotes that the treatment was significantly different from the control (water) according to LSD test at the 5% level.

4.3.4. *Ex vitro* germination experiments

Germination was observed for the four tested *Romulea* species. *R. diversiformis* seeds showed the highest percentage germination when placed at 10°C under alternating light (16 h photoperiod), in the constant dark conditions and with seed scarification treatment (Table 4.2). These results were significantly different from other treatments for *R. diversiformis*, with the exception of constant dark at 15°C, where it did not differ significantly in some cases. Although acid scarification

exhibited higher percentage germination, the MGT was significantly greater than for the other treatments. Cold stratification of seeds for 14 and 21 days significantly increased percentage germination in comparison to the non-stratified seeds with significantly shorter MGT (some seeds germinated during stratification period) (Table 4.2).

R. flava seeds showed some germination both under 16 h photoperiod and constant dark conditions at 15°C. However, 14 and 21 days of stratification at 5°C of seeds achieved significantly greater percentage germination compared to all other treatments examined. Twenty-one day stratification had the shortest MGT of only one day due to the incidence of germination during the treatment period (Table 4.2).

In the case of *R. monadelpha*, placing seeds at 15°C in the dark was the only treatment that significantly increased germination compared to the other treatments (Table 4.2).

R. rosea seeds had significantly higher percentage germination when placed at 10°C both under 16 h photoperiod and constant dark in comparison to other treatments. Mean germination time was also significantly shorter for these treatments (Table 4.2). Warm stratification did not significantly increase percentage germination in comparison to the seeds incubated at 10°C. Seed germination of this species at 20-35°C was very low. These temperatures are generally favourable for many weed species, seeds of *R. rosea* were therefore tested for nutrients, plant growth promoting substances and smoke components at only 20 ± 0.5°C. In this experiment, seeds that were treated with KNO_3 and IBA solutions of 10^{-5}M yielded significantly higher percentage germination over the control (water) (Figure 4.5). Percentage germination at HS 50% (all nutrients), -K, kinetin, NAA, IAA and butenolide treatments showed an increase compared to control (water). However, these results were not significantly different to the control (water). No germination was recorded for GA_3 and smoke-water-treated seeds.

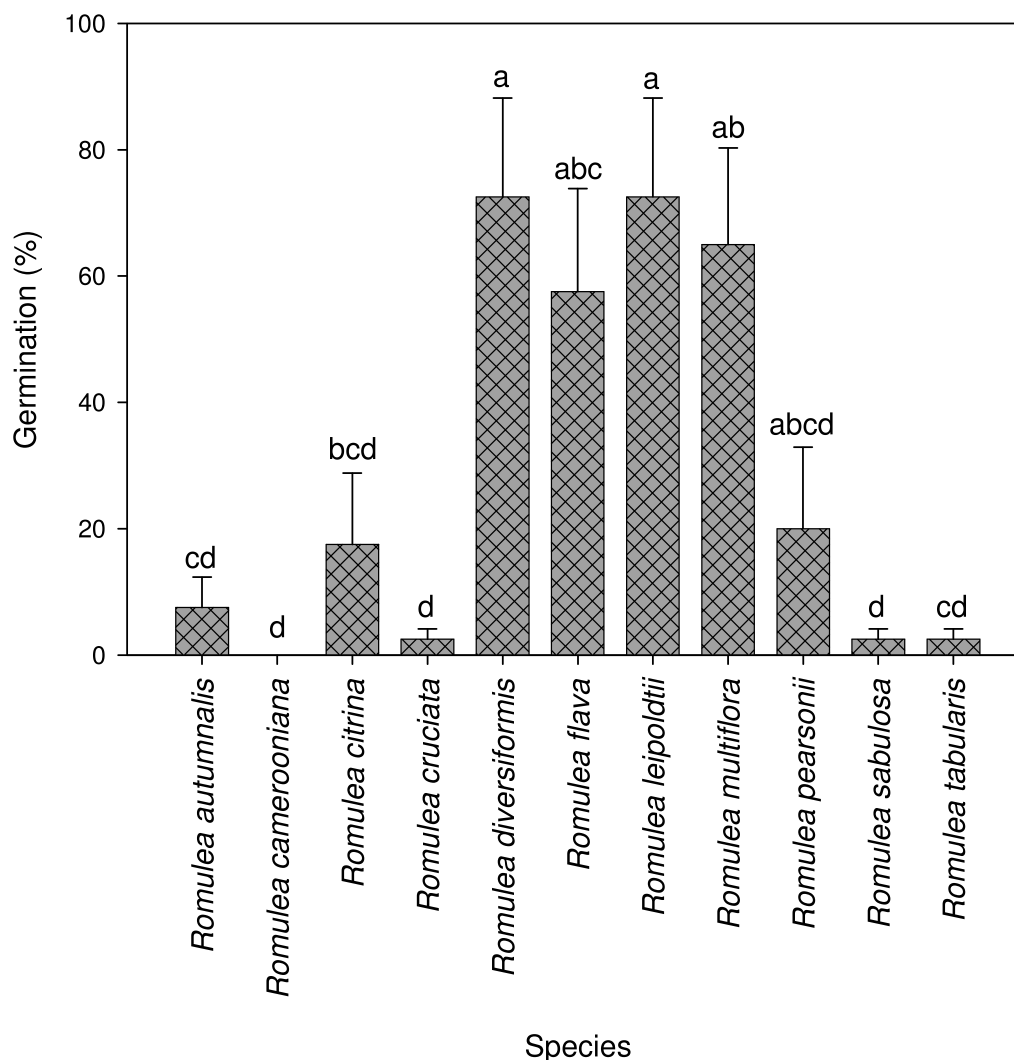

Figure 4.6: *In vitro* seed germination of different *Romulea* species at 15°C after 2 months. Standard error bars with different letters are significantly different according to LSD at the 5% level.

4.3.5. *In vitro* germination experiments

R. diversiformis, *R. flava, R. leipoldtii* and *R. minutiflora* showed the highest germination (> 55%) in comparison to other species, although in some cases these results were not significant (Figure 4.6). The lowest germination was recorded for *R. camerooniana*, *R. cruciata*, *R. sabulosa* and *R. tabularis*. Only few seeds of *R. sabulosa* germinated (9 out of 200 seeds) when incubated at 15°C, with no germination at 20°C. Seeds did not geminate at 15°C and 20°C in the case of *R. camerooniana*.

4.4 DISCUSSION

A short rainy season plays an important role for the wild flowers of Namaqualand. Plants must complete their life-cycle during this period, ensuring successful germination and subsequent establishment due to sufficient moisture. Even under moist environmental conditions there may be constrains on germination to the seeds that are on the soil surface compared to those seeds that are buried and which form part of the soil seed bank (MEEKLAH, 1958; EVANS *et al.*, 1967). This can be attributed to high fluctuations in moisture and humidity which results in unfavourable conditions for germination (MILLER & PERRY, 1968; DOWLING *et al.*, 1971). Seed surface characteristics in modifying the seed/soil interface has been emphasized by SEDGLEY (1963) and HARPER AND BENTON (1966). These workers showed that germination was promoted when a greater area was in contact with the substrate. Hence size, surface and micropylar region are important factors of the seed to be considered for the process of water-uptake.

Many *Romulea* species have a narrow and limited distribution and germination plays a significant role in their survival. It is therefore necessary to understand the seed structure of different *Romulea* species and their ecological relevance. In comparison to other species, high initial water content and fast imbibition of *R. leipoldtii* seeds can be attributed to its small seed size. Large surface area to volume ratio requires the seed to have more water reserves (as it is more prone to desiccation) and would allow the seed to absorb more water in a shorter time (when available). The small seed size of *R. flava* also requires it to have more water reserves to avoid dehydration. These species showed the roughest seed surface after *R. rosea*, *R. leipoldtii* and *R. diversiformis*, which further increase surface area and the risk of dehydration (but also allows it to imbibe more water in a shorter time). *R. leipoldtii* and *R. flava* are all found in areas with highly variable rainfall (Figure 2.21).

The seeds of *R. camerooniana* are smooth and their seeds are larger than those of *R. leipoldtii* and *R. flava*, resulting in a small surface area to volume ratio. These seeds are however, small enough to have a relatively high imbibition rate because of the resulting larger surface area to volume ratio. This species also occur in areas with higher and more consistent rainfall than other species of this genus (Figure 2.7). The seeds of *R. camerooniana* therefore do not require a high initial water content.

The relatively low initial water content and slow water imbibition of *R. monadelpha* seeds can be attributed to their large size and smooth surface compared to the seeds of other *Romulea* species, while the relatively low water content of *R. sabulosa* seeds can be attributed to their relatively smooth seed surface and compact micropylar region.

It is interesting to note that the eight species used in water content and imbibition experiments can be divided perfectly into their subgenera by looking at variability of their initial water content, as species in the subgenus *Romulea* (*R. camerooniana*, *R. flava*, *R. leipoldtii* and *R. minutiflora*) all had variable initial water content when compared to all studied species in the subgenus *Spatalanthus* (*R. diversiformis*, *R. monadelpha*, *R. rosea* and *R. sabulosa*). It was also noted that seed sizes of species in the subgenus *Romulea* had more variability compared to species in the subgenus *Spatalanthus*, which is a possible explanation for the variability in water content.

The rough seed surface and membranous micropylar regions of *R. rosea*, enables them to absorb a relatively large amount of water; they also initiated an increase in imbibition rate 3 h before seeds of other species. *R. minutiflora* has a smooth seed surface, compact micropylar region and small seed size to facilitate its low water absorption capacity. The larger the seed, the higher the relative water capacity and imbibition, these are possible explanations why *R. rosea* is a more successful invasive plant than *R. minutiflora* and other species.

The seeds of *R. diversiformis* and *R. rosea* showed high germination both under alternating and constant dark conditions at 10°C. These findings suggest that these seeds are not specifically light or dark requiring. Percentage germination of *R. monadelpha* seed was significantly higher under constant dark conditions at 15°C than any other treatment examined, which indicates a possible negatively photoblastic nature. However, this species had very low germination and therefore more investigation on seed physiology is required which may help improving germination of this species. On the other hand, *R. flava* seeds did not respond effectively when subjected to different temperatures under both light and dark conditions. Interestingly, this species exhibited increasing percentage germination as the cold stratification period was prolonged. It was observed that these seeds germinated during the stratification period. This suggests that *R. flava* seeds require a wet cold winter season for germination. *R. sabulosa* seeds did not respond to *ex vitro* germination but little germination was recorded in *in vitro* experiments. The

intact seed of these species may need a longer period of cold stratification, or culture conditions at 5℃. There is a period of about two months with average daily minimum temperatures of 5℃ in Nieuwoudtville where *R. sabulosa* is found (Figure 2.20). Although *R. diversiformis*, *R. flava*, *R. leipoldtii* and *R. minutiflora* all had *in vitro* germination percentages higher than 57% at 15℃, seeds of other species showed very low germination. *R. camerooniana* was the only species of which neither the seeds germinated nor the embryos responded. Low viability of *R. camerooniana* seeds was indicated by TTC. However, all species endemic to South Africa showed positive responses to either seed or embryo germination treatments.

The results of the temperature experiment for *R. rosea* confirms that of EDDY & SMITH (1975) who found that *R. rosea* seeds has a clear germination optimum in the temperature range of 9.5 to 13℃. These results suggest that invasive individuals of *R. rosea* could be eradicated (by mechanical or chemical control) when temperatures are low. EDDY & SMITH (1975) reported that pre-chilling of seeds or KNO_3 treatment does not increase germination of this species. However, in the present study KNO_3 significantly increased germination over the control. This result was confirmed where seed germination was low in Hoagland's nutrient solution without K in comparison to the control (HS 50%). These findings suggest that the use of fertilizers containing high potassium should be avoided to reduce *R. rosea* invasion which will be higher in agricultural areas.

It appears that seeds of *R. diversiformis*, *R. flava*, *R. leipoldtii*, *R. minutiflora*, *R. monadelpha, and R. sabulosa* all exhibit non-deep endogenous morphophysiological dormancy, as excised embryos showed a growth response and the causes could include a physiological germination inhibiting mechanism or an underdeveloped embryo (BASKIN & BASKIN, 1998). The percentage germination of *R. rosea* seeds on filter paper moistened with KNO_3 was significantly higher than the control, this response to potassium nitrate is a characteristic of non-deep morphophysiological dormancy (COPELAND, 1976). The causes of non-deep physiological dormancy include the physical barrier created by covering structures, the resulting low oxygen supply to the embryo, inhibitors within the covering structures and/or physical/chemical changes in the covering structures (BASKIN & BASKIN, 1998). COPELAND (1976) states that the dormancy of such seeds can often be broken by a cold stratification treatment. This happened in the case of *R. flava*. However, this was not tested for *R. leipoldtii* and *R. minutiflora* due to limitations in seed availability. The

seeds of *R. camerooniana* appears to have deep endogenous morphophysiological dormancy, as excised embryos showed no growth response and the apparent causes may include a physiological germination inhibiting mechanism or rudimentary embryos (BASKIN & BASKIN, 1998).

4.5 SUMMARY

- Germination experiments revealed that *R. diversiformis* and *R. rosea* seeds germinate best at 10°C. *R. flava* seeds germinate best when cold stratified for 21 days and *R. monadelpha* seeds showed some response at 15°C in the dark indicating photoblastically negative behaviour.
- An initial *in vitro* germination experiment showed germination above 57% for *R. diversiformis*, *R. leipoldtii*, *R. minutiflora* and *R. flava* seeds placed at 15°C; while seeds of other species placed at 15°C had less than 30% germination.
- Seeds of *R. diversiformis*, *R. flava*, *R. leipoldtii*, *R. minutiflora*, *R. monadelpha* and *R. sabulosa* may have non-deep endogenous morphophysiological dormancy, whereas seeds of *R. camerooniana* appear to have deep endogenous morphophysiological dormancy.

5 *In vitro* culture initiation and multiplication

5.1 INTRODUCTION

Due to the low germination observed for some species of horticultural importance such as *R. monadelpha* and *R. sabulosa*, an *in vitro* culture initiation protocol was developed for these two species and some other species of interest. Developing a micropropagation protocol will be essential in the commercialization of these species as it enables high propagation rates (LILIEN-KIPNIS & KOCHBA, 1987; PIERIK, 1997). Developing a micropropagation protocol for *R. sabulosa* may also aid in the conservation of this rare and beautiful flower, as it has been shown that micropropagation can play a significant role in plant conservation (WOCHOCK, 1981; SARASAN *et al.*, 2006; SHIBLI *et al.*, 2006; WITHERS, 2008).

Explant selection is an important step in the micropropagation process. Explant type can affect culture survival, vigour, regenerative capacity, bulking time, contamination rates and health. Explant size, for instance, influences survival and contamination rates (KYTE & KLEYN, 1996; SMITH, 2000). Explant maturity also affects survival and contamination rates as well as regenerative capacity and bulking time (KYTE & KLEYN, 1996; SMITH, 2000). Explant source affects survival, regenerative capacity, contamination rates and health (SMITH, 2000; AHLOOWALIA & PRAKASH, 2002). The preparation of the explant and the culture initiation medium is equally important (SMITH, 2000). See Table 2.6 for a review on explant types and media used for Iridaceae direct shoot culture initiation.

The main aim of this Chapter was to determine the most appropriate explant type and medium for *R. camerooniana*, *R. diversiformis*, *R. flava*, *R. leipoldtii*, *R. minutiflora, R. monadelpha, R. rosea* and *R. sabulosa* shoot culture initiation. Some embryos of *R. sabulosa* were also used in an experiment aimed to produce embryogenic tissue. Shoots of *R. sabulosa* were used in experiments aimed to identify physical and chemical stimuli to significantly increase shoot multiplication.

5.2 MATERIALS AND METHODS

Seeds were obtained from Silverhill Nurseries, Kenilworth, South Africa and African Bulbs, Napier, South Africa. Although using seedling organ explants is common for culture initiation in the Iridaceae, seeds of the attractive and vulnerable *R. sabulosa* did not germinate and some other attractive species had low germination. Because these experiments were done before the cold stratification experiments described in Chapter 4, germination was slow. In an attempt to obtain cultures of all species in a shorter time embryo rescue techniques were employed for eight species.

If not stated otherwise, a MS medium supplemented with 100 $mg.l^{-1}$ *myo*-inositol and 3% sucrose, with pH adjusted to 5.7 and solidified with 0.8% agar was used. All experiments were conducted in a laminar flow hood and cultures were placed in a growth room set at 25℃ under 4.3 µmol m^{-2} s^{-1} light using Osram® 75 W cool white fluorescent tubes with a 16/8 light/dark photoperiod. The duration of all experiments was two months. Shoots multiplied for further experiments were subcultured onto the medium that produced the best shooting response of the initial explant every two months.

5.2.1 Explants from seedlings

Seedlings from initial *in vitro* germination experiments described in Chapter 4 were used for a small experiment to assess the responsiveness of the species of which seeds germinated. Only 5 replicates were used per treatment, as the availability of seeds of *Romulea* species was very limited at the time and therefore only small quantities could be purchased.

Seedlings with stems longer than 30 mm were removed from the culture tubes using autoclaved forceps. The seedlings were then placed on Petri dishes and cut into three sections using a scalpel and blade. They were divided into shoot (>10 mm), hypocotyl (10 mm) and root (>10 mm) sections. For hypocotyl explants the remainder of the seed was removed. All seedling organs were placed in 33 ml culture tubes with 10 ml MS media supplemented with various plant growth regulators.

Seedling organs of *R. flava* and *R. leipoldtii* were placed in culture tubes with MS media with nine different plant growth regulator treatments; a control with no plant growth regulators, 4.4 µM BA, 22.2 µM BA, 4.5 µM 2,4-D, 22.6 µM 2,4-D, 4.4 µM BA and 4.5 µM 2,4-D, 4.4 µM BA and 22.6 µM 2,4-D, 22.2 µM BA and 4.5 µM 2,4-D, and 22.2 µM BA and 22.6 µM 2,4-D. Seedling organs of *R. diversiformis* and *R. minutiflora* were placed in culture tubes with MS media with 12 different plant growth regulator treatments; a control with no plant growth regulators, 2.3 µM kinetin, 23.2 µM kinetin, 5.4 µM NAA, 26.9 µM NAA, 53.7 µM NAA, 2.3 µM kinetin and 5.4 µM NAA, 23.2 µM kinetin and 5.4 µM NAA, 2.3 µM kinetin and 26.9 µM NAA, 23.2 µM kinetin and 26.9 µM NAA, 2.3 µM kinetin and 53.7 µM NAA, and 23.2 µM kinetin and 53.7 µM NAA.

5.2.2 Explants from embryos

The seeds were surface sterilised as with *in vitro* germination experiments described in Chapter 4 and imbibed for 5 days. Decontaminated seeds were placed in a Petri dish with sterile distilled water. The seeds were left to imbibe in the laminar flow cabinet and were dissected every 24 h to test for ease of dissection. Seeds could only be dissected after 4 days, as the blade could not penetrate the periderm (fruit coat) with a clean cut with less time for imbibition. The embryos that were dissected after 7 days of imbibition did not show any growth response when placed on media.

To excise the embryo some of the surrounding endosperm had to be cut away (See Figure 5.1).

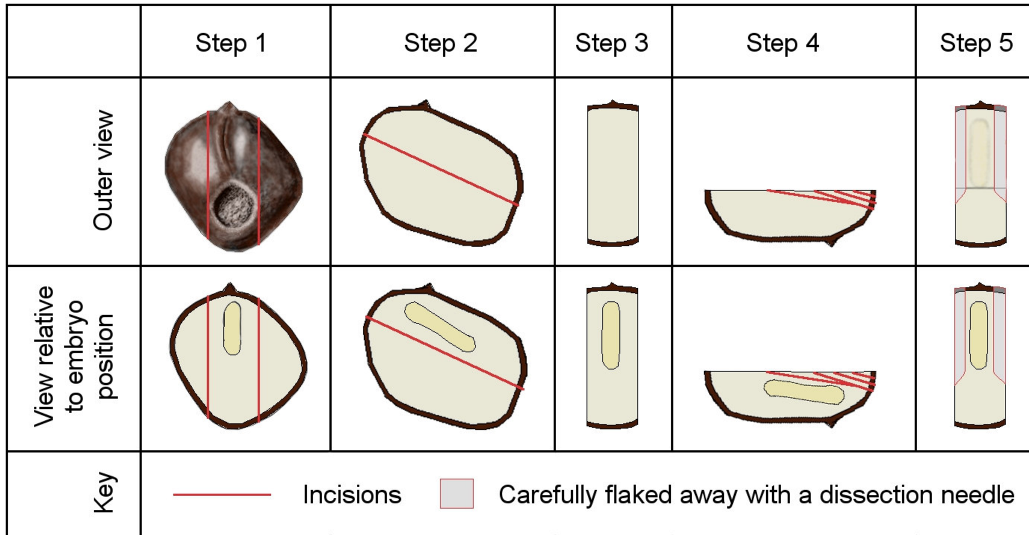

Figure 5.1: General embryo excision procedure for *Romulea* seeds. An outer view, as one would view it through a stereo microscope, as well as a view relative to the embryo is provided so that the importance of the placing of the incisions can be seen. Step 1 is viewed from the top, Step 2 is a side view, Steps 3 and 5 are bottom views 90° to the incision made in Step 2. Step 4 is a side view.

In Step 1 the seed was gripped with a pair of fine forceps and two incisions were made. The excess tissue was scrapped to the front of the Petri dish. In Step 2 the seed was turned onto one of the flat sides created by the incision done in Step 1. When turning on the bottom light of the dissection microscope the light radiated through the seed, making the embryo visible. Another incision was then made below the embryo. The seed was then turned onto the Petri dish as indicated in Step 3 to check for embryo visibility and to allow a better cutting angle. Small slices of tissue could then be removed as indicated in Step 4 until the embryo was clearly visible through the somewhat transparent endosperm tissue. An autoclaved dissection needle was then used to carefully flake away the sections indicated in Step 5 (Figure 5.1). The embryo was then lifted out of the endosperm tissue and placed in a Petri dish with sterile distilled water to rinse off residual pieces of endosperm.

Excised embryos were placed into 33 ml culture tubes with 10 ml nutrient media using an autoclaved Pasteur pipette with sterilised distilled water immediately after excision. Treatments with various concentrations of plant growth regulators in the growth media were prepared for each species.

R. diversiformis, *R. flava*, *R. minutiflora* and *R. monadelpha* embryos were placed on media with no plant growth regulators and media supplemented with 2.3, 4.7 and 23.2 µM kinetin. For *R. diversiformis* and *R. minutiflora* 10 embryos were used for each treatment, while 20 embryos were used per treatment for *R. flava* and *R. monadelpha*.

Embryos of *R. camerooniana* and *R. rosea* were placed on 13 media treatments, a medium with no plant growth regulators, media with 2.3, 4.7 or 23.2 µM kinetin or *m*TR and media supplemented with 2.3, 4.7 or 23.2 µM kinetin or *m*TR in combination with 0.5 µM NAA.

For *R. sabulosa* an experiment was designed to investigate the suitability of a set of PGR treatments for shoot culture initiation and embryogenesis. The experiment had 12 treatments and was repeated twice, first with 20 replicates and then with 10 replicates. The 12 treatments consisted of media supplemented with combinations of 0, 2.3, 4.7 and 23.2 µM kinetin, and 0, 2.2 and 4.5 µM 2,4-D. An experiment with 3 treatments, media supplemented with 4.4 µM BA, 22.2 µM BA and 2.3 µM kinetin with 5.4 µM NAA was also initiated. This experiment was only repeated once with 20 replicates. This is because of the vitrification and abnormal growth of shoots cultured on media supplemented with BA.

After two months the number of shoots formed per explant, the presence or absence of roots and the morphology was recorded. The cultures that appeared embryogenic where placed in culture bottles with 30 ml of MS media supplemented with 100 $mg.l^{-1}$ myo-inositol, 30 $g.l^{-1}$ sucrose and 8 $g.l^{-1}$ activated charcoal after 2 months.

Culture morphology was not only observed with the naked eye, but also under a light microscope. Samples of tissue suspected to have embryo initials were prepared by Epon resin embedding, sectioned using a LKB Ultrotome II microtome, stained with Ladd's multiple stain and viewed with an Olympus AX 70 stereo microscope.

5.2.3 Explant comparison

R. leipoldtii seeds were used, not only because of their high germination and *in vitro* shooting in preliminary seedling organ experiments, but also because this species has the smallest seeds. If embryo excision is possible for a species with such a small seed it shows that seed size is not a limitation to using embryo excision in a culture initiation protocol for *Romulea* species.

Seeds (200) were surface sterilised and germinated at 15°C as with *in vitro* germination experiments described in Chapter 4. After 2 months when sufficient seeds germinated, 130 seedling hypocotyls and 130 embryos were excised simultaneously. Seedlings with stems longer than 30 mm were then removed from tubes and placed on Petri dishes and cut into 3 sections using a sterilised scalpel and blade. For this experiment only the hypocotyl was used because of its higher percentage *in vitro* response. The seedling hypocotyls and embryos were then placed in 33 ml culture tubes with 10 ml nutrient media with 13 medium treatments, a medium with no plant growth regulators, media with 2.3, 4.7 or 23.2 µM kinetin or *m*TR and media supplemented with 2.3, 4.7 or 23.2 µM kinetin or *m*TR in combination with 0.5 µM NAA. Ten replicate explants were used per treatment and the experiment was repeated twice. After two months the number of shoots formed per explant and the presence or absence of roots was recorded.

5.2.4 Shoot multiplication

R. sabulosa shoots initiated and multiplied with 23.2 µM kinetin were placed in tubes containing MS media supplemented with three different kinetin concentrations (2.3, 4.7 and 23.2 µM) used for shoot initiation. These tubes were then placed at 25°C in a growth room with 16 hour light and a growth room with 24 hour light to test the effect of photoperiod on shoot multiplication rate. The effect of temperature was examined by placing tubes with shoots on MS media supplemented with 23.2 µM kinetin in growth chambers with a 16/8 light/dark photoperiod set at a range of temperatures from 10°C to 30°C under 3.4 µmol m^{-2} s^{-1} light Osram® 75 W cool white fluorescent tubes with a 16/8 light/dark photoperiod. These experiments were repeated twice and 10 explants were used per treatment. After two months the number of shoots formed was recorded.

Another experiment was conducted with *R. sabulosa* shoots to investigate the effect of other cytokinins and different concentrations of kinetin on shoot multiplication. *R. sabulosa* shoots initiated and multiplied with 23.2 µM kinetin were placed in 250 ml jars containing 30 ml MS media supplemented with no plant growth regulators (control) or 2.5, 5.0, 7.5 µM kinetin, BA, *m*T, *m*TR or *Mem*TR. For each treatment, 5 replicate bottles with 4 explants each were used. After 2 months the number of shoots formed was recorded.

5.2.5 Statistical analysis

Data were analyzed for significant differences by one-way analysis of variance (ANOVA), and means separated using either Tukey's HSD test or Duncan's multiple range test (DMRT) at 5% level of significance ($P \leq 0.05$). Percentage data were converted to proportion, arcsine transformed and then analyzed. GenStat® (VSN International, Hemel Hempstead, U.K.) version 11.1 statistical package was used to analyze the data.

5.3 RESULTS

5.3.1 Explants from seedlings

In the preliminary experiment with *R. diversiformis*, *R. flava*, *R. leipoldtii* and *R. minutiflora*, no shoots developed from root and shoot explants and shoots only developed from seedling hypocotyls (Results not shown due to low replication). Hypocotyls of *R. leipoldtii* placed on a medium supplemented with 22.2 µM BA developed the largest number of shoots. *R. flava* seedling hypocotyls produced less shoots per explant than those of *R. leipoldtii* on the same medium. *R. minutiflora* hypocotyls only formed shoots when placed on a medium supplemented with 23.2 µM kinetin and 5.4 µM NAA. *R. diversiformis* hypocotyls formed no shoots and only developed abnormal root-like structures. The shoots of *R. leipoldtii* appeared vitrified and when 50 shoots, multiplied on a medium supplemented with 23.2 µM kinetin, were placed in 33 ml culture tubes with 10 ml of MS media with no plant growth regulators, no rooting was observed after 2 months.

R. flava and *R. leipoldtii* produced callus with shoot initials on the media treatments with the highest concentrations of both cytokinins and auxins. These shoot initials and the shoots formed after one month from them, when transferred MS media with no plant growth regulators, also appeared vitrified and no roots or root initials were observed after 2 months.

5.3.2 Explants from embryos

R. diversiformis embryos only showed swelling, except for one embryo which only developed one rooted shoot (Figure 5.2).

R. flava embryos produced significantly more shoots when placed on a medium supplemented with either 2.3 µM kinetin or 4.7 µM kinetin compared to the control (Figure 5.3). The number of *R. flava* shoots observed on a medium with 4.7 µM kinetin was significantly higher than the number of shoots produced by all other treatments except on a medium supplemented with 2.3 µM kinetin. Single non-rooted shoots and swellings were observed on a medium with no plant growth regulators. Non-rooted shoots, rooted shoot clusters and swelling were observed on a medium supplemented with 2.3 µM kinetin. Only one of the shoot clusters on this medium did not produce a root, this shoot cluster had callus at its base. Non-rooted shoots, rooted and non-rooted shoot clusters and callus were produced on a medium supplemented with 4.7 µM kinetin. Shoot clusters and callus was observed on media supplemented with 23.2 µM kinetin. All but one of the shoot clusters formed on media supplemented with 23.2 µM kinetin did not root (Data not shown). Shoots generated from callus appeared vitrified and they also did not root on MS media with no plant growth regulators.

The number of shoots of *R. minutiflora* and *R. monadelpha* produced on media supplemented with kinetin was much lower than the number of shoots produced by *R. flav*a embryos and is not significantly different from the number of shoots produced on a medium with no plant growth regulators (Figures 5.4 and 5.5). Table 4.5 in Chapter 4 shows that most of the embryos of these species show an *in vitro* growth response. In all cases where shoots were not formed, only swelling of the embryo was observed for these two species. One swollen embryo of *R. minutiflora* rooted on a medium with no plant growth regulators. Single shoots and one small

shoot cluster (2 shoots) were formed on a medium supplemented with 2.3 µM kinetin. All shoots produced on this medium, except for one single shoot, were rooted. Only non-rooted single shoots developed from *R. minutiflora* embryos on a medium supplemented with 4.7 or 23.2 µM kinetin. For *R. monadelpha* embryos, only single rooted shoots were formed for all tested medium treatments.

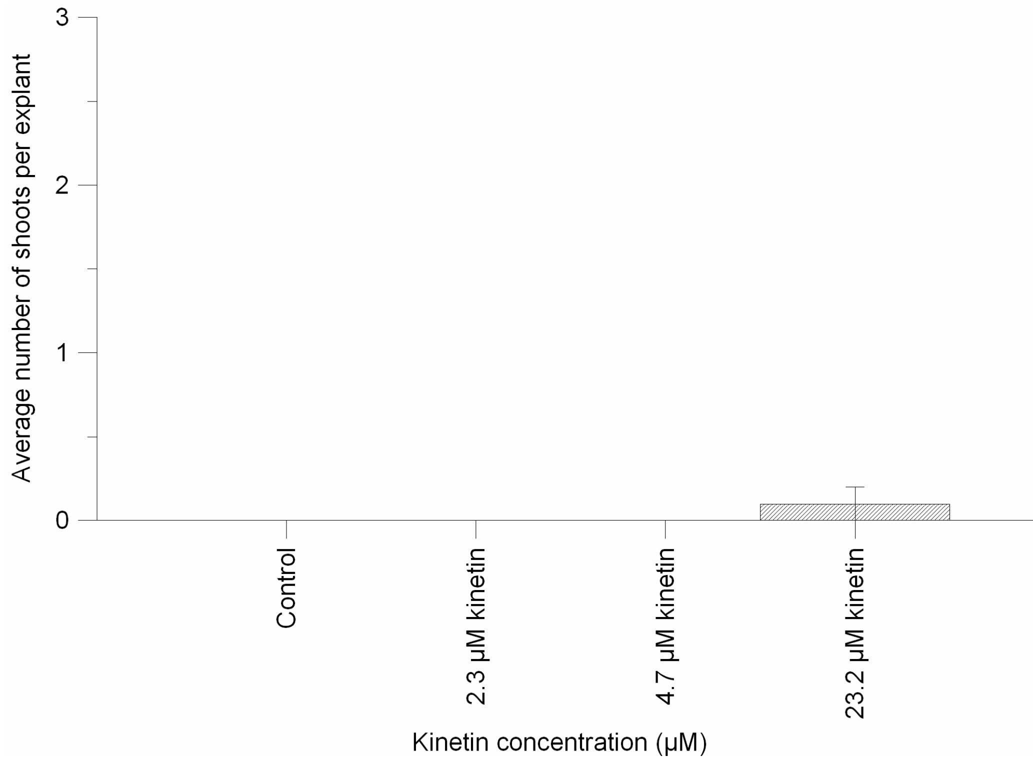

Figure 5.2: Effect of kinetin concentration on shoot production of *Romulea diversiformis* embryos after 2 months. Error bars indicate standard error of the mean.

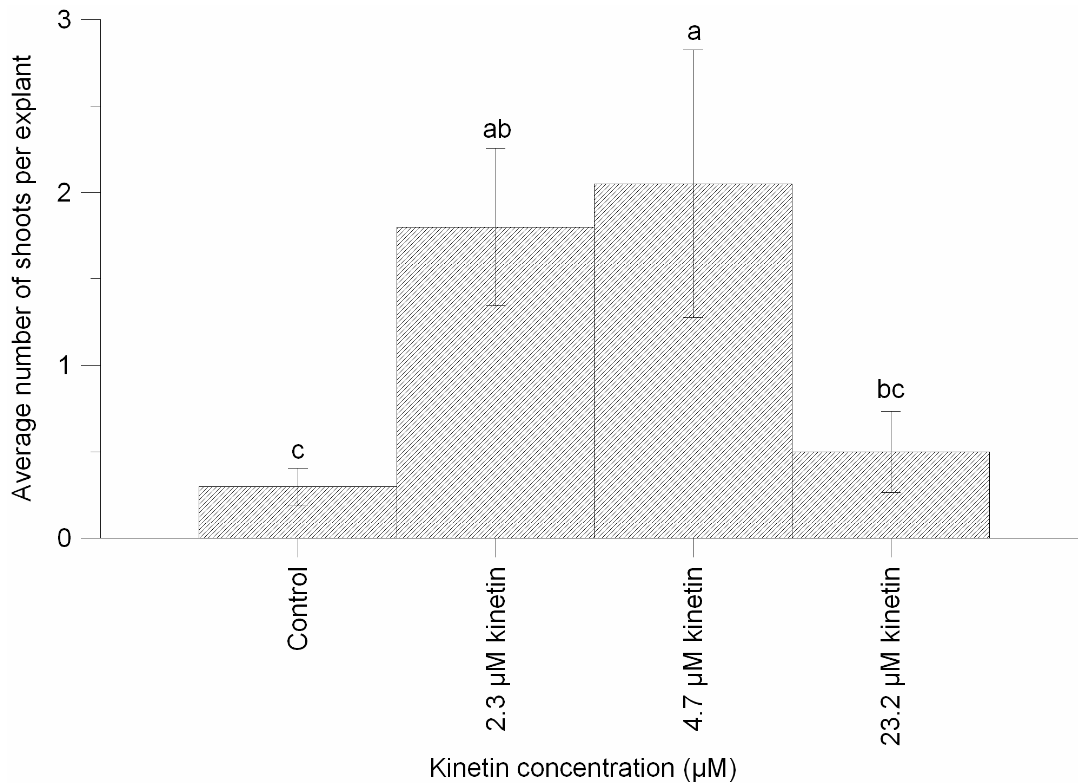

Figure 5.3: Effect of kinetin concentration on shoot production of *Romulea flava* embryos after 2 months. Error bars indicate standard error of the mean. Different letters indicates significance differences between treatments according to Duncan's multiple range test.

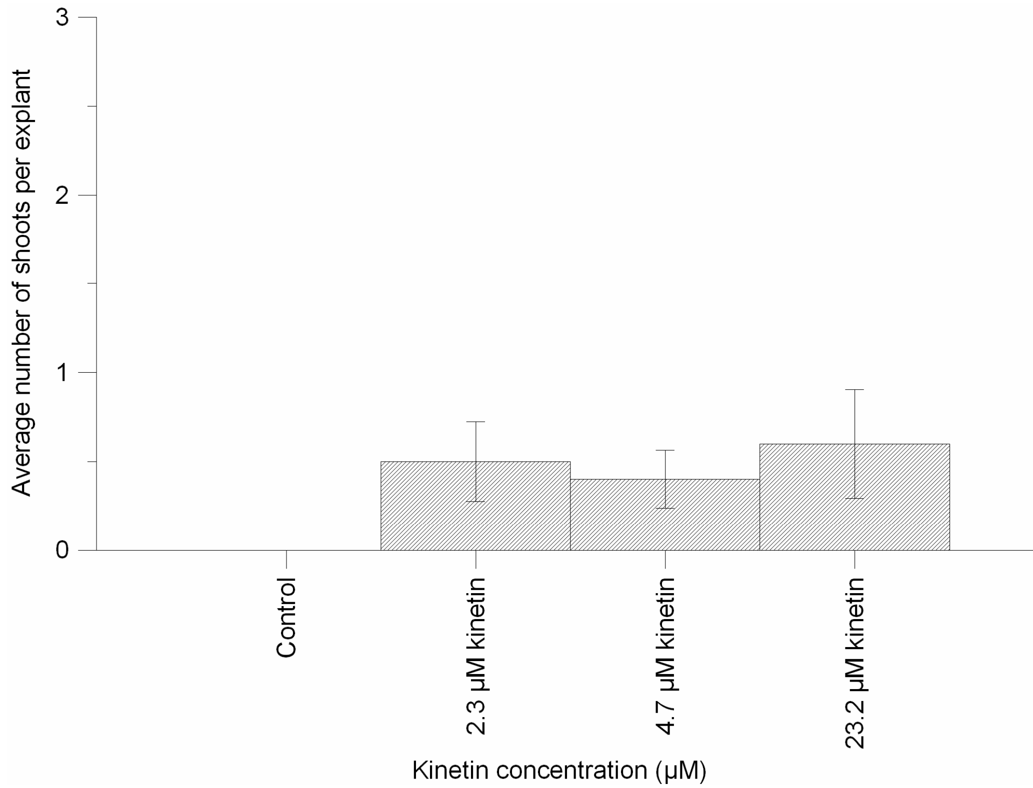

Figure 5.4: Effect of kinetin concentration on shoot production of *Romulea minutiflora embryos* after 2 months. Error bars indicate standard error of the mean.

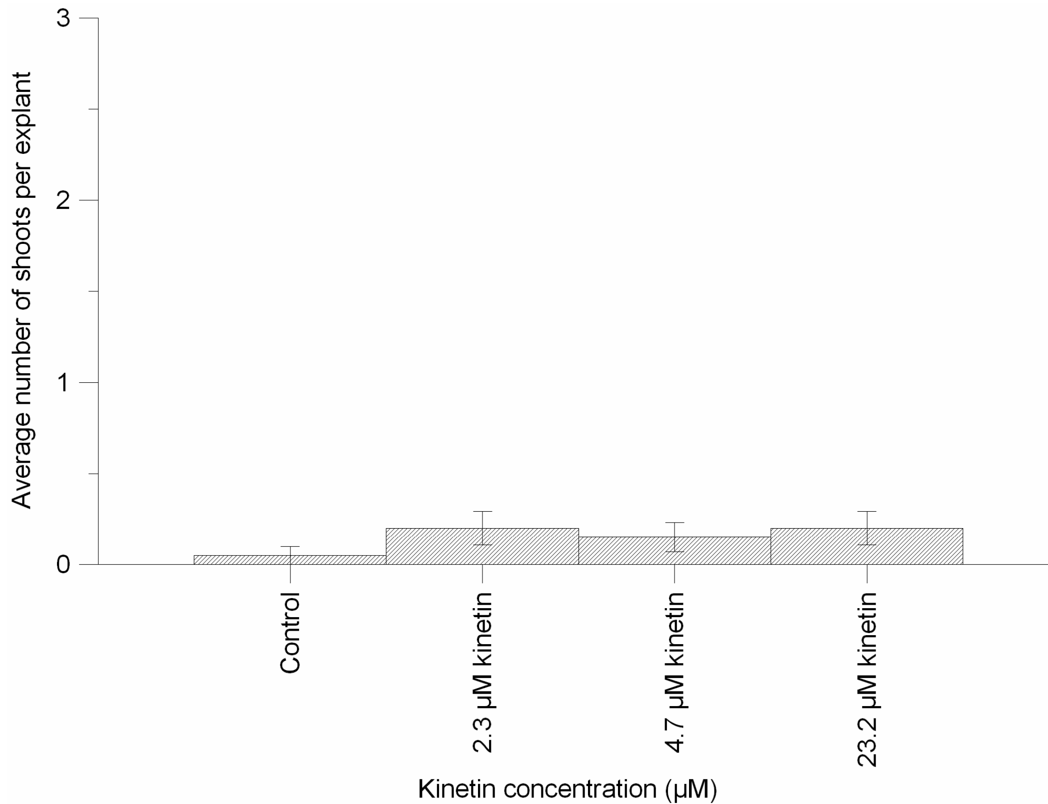

Figure 5.5: Effect of kinetin concentration on shoot production of *Romulea monadelpha* embryos after 2 months. Error bars indicate standard error of the mean.

No embryos of *R. camerooniana* or *R. rosea* showed any *in vitro* response. Embryos were completely dehydrated after 2 months.

Because of the larger amount of replicates used for the vulnerable and attractive *R. sabulosa* it was possible to examine the specific *in vitro* response of embryos to different plant growth regulator combinations with some statistical confidence (Table 5.1). The specific *in vitro* response for each treatment is therefore described in more detail for *R. sabulosa* than for other species.

As with embryos of other species of *R. sabulosa,* some cultures showed only swelling (Table 5.1). A significantly higher percentage of cultures showed swelling on the control medium than for other treatments and roots were also produced on this medium. Shoot initiation was only observed in cultures with kinetin only. Although swelling with shoot initials was produced on all media with kinetin only, this response was significantly higher for the 2.3 µM kinetin treatment compared to the control and all other treatments. Shoot and root formation was observed at the two lower kinetin concentrations. Shoot clusters were observed for the two higher kinetin concentrations. Significantly more shoot clusters were produced on a medium supplemented with 23.2 µM kinetin compared to all other treatments, as 36% of the embryos that showed a growth response on this media produced shoot clusters. When the data was pooled it also showed that a medium supplemented with 23.2 µM kinetin was best for shoot initiation. Among the embryos placed on media supplemented with 23.2 µM kinetin that showed a growth response, 89% showed a shoot initiation response. Callus with shoot cluster initials was observed for embryos placed on media supplemented with 23.2 µM kinetin. As with other species in *Romulea*, the shoots developed from these initials were however highly vitrified and did not develop roots when placed on a medium with no plant growth regulators.

Cultures placed on five of the media treatments containing both kinetin and 2,4-D produced small (<10 mm) corm-like structures (Table 5.1). These structures produced were in fact not corms, as they were hollow and had a texture analogous to callus. A number of these cultures placed on media containing both kinetin and 2,4-D also produced callus that appeared translucent and that was considered as unsuitable for organogenesis because of the vitrified shoot growth which was

observed when it was placed on media with activated charcoal. These cultures did not multiply well, or at all, and many became necrotic.

What appeared to be indirectly formed embryogenic tissue was observed on all media supplemented with both kinetin and 2,4-D and its induction percentage was highest on a medium supplemented with 2.3 µM kinetin and 2.3 µM 2,4-D (Table 5.1). Cultures that appeared to have direct embryogenesis was observed on media supplemented with 4.7 µM kinetin and 2.3 µM 2,4-D, and 23.2 µM kinetin, 4.5 µM 2,4-D. Most of the cultures that appeared to have initiated embryogenesis were observed on the latter medium.

The results of experiments that tested the effect of media supplemented with 5.4 µM NAA and 2.3 µM kinetin and media with two different concentrations of BA is not included. This is because of the vitrification observed in cultures placed on this media and the lack of subsequent explant response by these cultures when placed on media with no plant growth regulators.

Table 5.1: Effect of kinetin and 2,4-D on excised embryos of *Romulea sabulosa*. Mean values in a column followed by different letters that indicates significance differences between treatments according to Duncan's multiple range test (P ≤ 0.05). S = swelling of embryo; SR = swelling of embryo with rooting; SSI = swelling of embryo with shoot initials; SRF = shoot and root formation; SC = shoot cluster; SCR = shoot cluster with roots; CSCI = callus with shoot cluster initials; CIS = corm-like structure (< 10 mm); CAI = callus appearing incompetent; CPE = callus with potential embryogenesis; PDE = potential direct embryogenesis; CSGR = cultures showed growth response. Potential embryogenesis refers to cultures that appeared to develop embryo-like structures (Figure 5.7).

Treatment (µM)	Observed response (%)^{y}											
	S	SR	SSI	SRF	SC	SCR	CSCI	CIS	CAI	CPE	PDE	CSGR
control (no PGR's)	43 ± 6 a	20 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 e	0 ± 0 b	53 ± 8 abc
kinetin (2.3)	24 ± 3 bc	0 ± 0 b	24 ± 3 a	20 ± 0 a	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 e	0 ± 0 b	50 ± 10 abc
kinetin (4.7)	30 ± 4 b	40 ± 0 a	20 ± 0 b	20 ± 0 a	20 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 e	0 ± 0 b	50 ± 7 abc
kinetin (23.2)	20 ± 0 cd	0 ± 0 b	20 ± 0 b	0 ± 0 b	36 ± 8 a	20 ± 0 a	20 ± 0 a	0 ± 0 b	0 ± 0 b	0 ± 0 e	0 ± 0 b	63 ± 8 ab
2,4-D (2.3)	20 ± 0 cd	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	20 ± 0 a	20 ± 0 de	0 ± 0 b	27 ± 7 c
kinetin (2.3) + 2,4-D (2.3)	20 ± 0 cd	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	40 ± 7 ab	0 ± 0 b	47 ± 7 abc
kinetin (4.7) + 2,4-D (2.3)	20 ± 0 cd	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	30 ± 6 a	0 ± 0 b	43 ± 6 a	27 ± 5 a	70 ± 7 a
kinetin (23.2) + 2,4-D (2.3)	20 ± 0 bcd	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	20 ± 0 ab	0 ± 0 b	30 ± 5 abc	0 ± 0 b	50 ± 5 abc
2,4-D (4.5)	30 ± 4 bc	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	20 ± 0 ab	20 ± 0 b	26 ± 5 cde	0 ± 0 b	37 ± 13 bc
kinetin (2.3) + 2,4-D (4.5)	20 ± 0 cd	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	20 ± 0 ab	20 ± 0 a	35 ± 8 bcd	0 ± 0 b	47 ± 13 abc
kinetin (4.7) + 2,4-D (4.5)	20 ± 0 cd	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	20 ± 0 ab	0 ± 0 b	33 ± 7 ab	0 ± 0 b	47 ± 7 abc
kinetin (23.2) + 2,4-D (4.5)	20 ± 0 d	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b	20 ± 0 ab	20 ± 0 a	27 ± 5 cde	32 ± 5 a	57 ± 13 ab

Significantly more shoots were produced on a medium supplemented with 23.2 µM kinetin than a medium supplemented with 2.3 or 4.7 µM kinetin (Figure 5.6). Tukey's HSD test revealed that the number of shoots formed on a medium supplemented with 23.2 µM kinetin were significantly higher than on a medium supplemented with 2.3 µM kinetin (N = 14, 18; Mean difference = -3.42857; SE = 0.97832, p = 0.003). It also revealed that the number of shoots generated on a medium supplemented with 23.2 µM kinetin was significantly higher than on 4.7 µM kinetin (N = 16, 18; Mean difference = -3.12500; SE = 0.94330, p = 0.005).

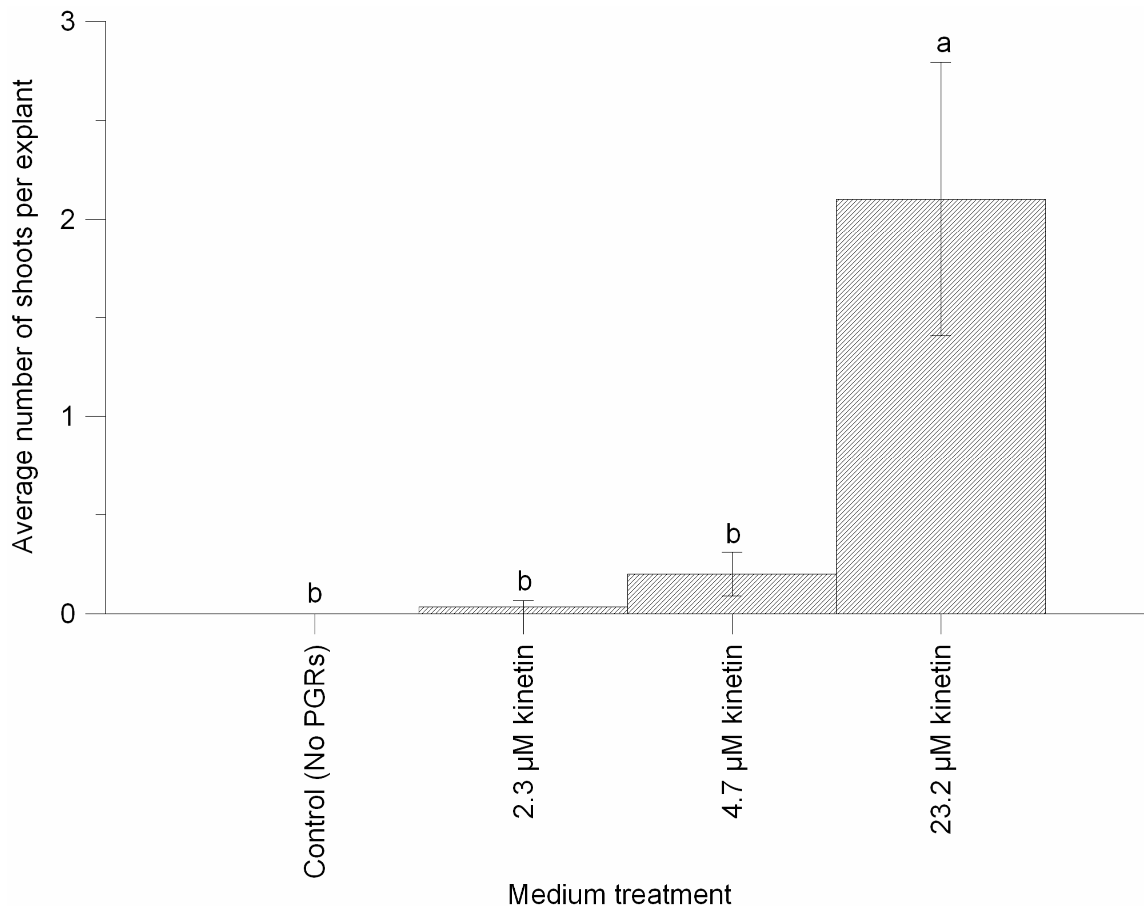

Figure 5.6: Effect of kinetin concentration on shoot production of *Romulea sabulosa* after 2 months. Error bars indicate standard error of the mean. Letters shows significance differences between treatments according to Tukey's HSD test.

Although it appeared that some cultures were embryogenic (Figure 5.7), sections of tissue presumed to exhibit direct and indirect embryogenesis did unfortunately not reveal any embryo initials and it was concluded that these cultures were not embryogenic, but formed abnormal shoot-like structures lacking chlorophyll.

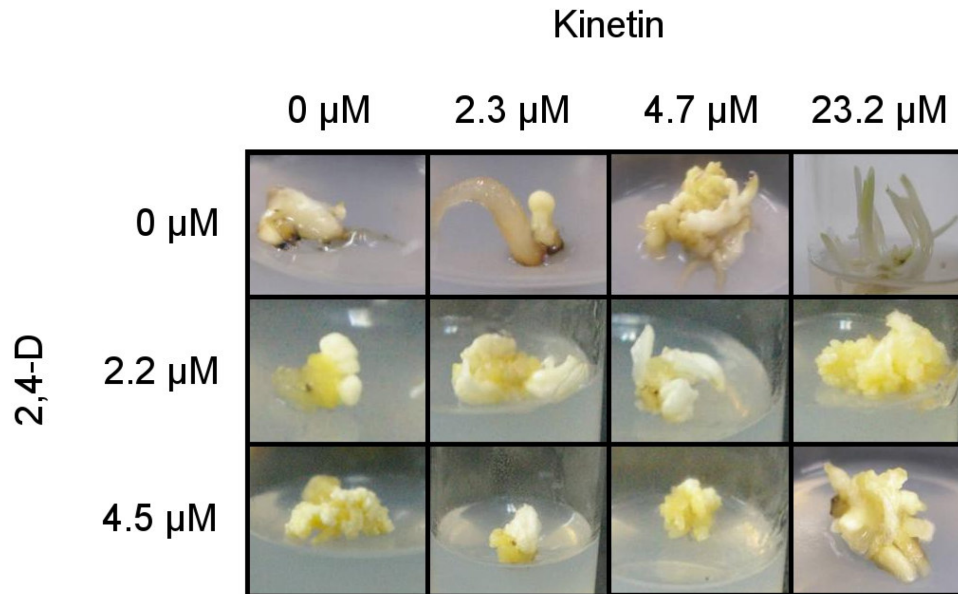

Figure 5.7: Visual observations of *Romulea sabulosa* cultures. Cultures including both kinetin and 2,4-D appears to exhibit embryo-like structures.

5.3.3 Explant comparison

Despite the apparent larger number of shoots produced between cultures in which embryos were used as explants compared to seedling organs, there is no significant difference between the numbers of shoots produced according to a paired t-test at 95% confidence limits. There was also no significant difference between the number of shoots formed on media supplemented with kinetin and the number of shoots formed on media supplemented with other plant growth regulator treatments (Figure 5.8). These results show that a medium supplemented with 23.2 µM *m*TR and 0.5 µM NAA significantly increases the amount of shoots formed per embryo compared to the control. This is not the case with seedling hypocotyls. Here cytokinins did not increase the number of shoots formed per explant (Figure 5.8). The number of shoots produced on a medium with 23.2 µM *m*TR and 0.5 µM NAA was not significantly different from the number of shoots observed on a medium supplemented with 2.3 µM kinetin or 2.3 µM *m*TR and 0.5 µM NAA.

Explant response data is not shown, as there were no significant differences in explant response between the two explant types. Although these differences are not

significant, they are important in comparing the two explant types and are described in the next few paragraphs.

The percentage cultures that showed some growth response was higher for hypocotyls (92.3%) than embryos (67.3%). Embryos showed a higher percentage growth response on medium supplemented with both cytokinins and auxins (78.3%) than on a medium with only cytokinins (60.0%). This effect was not as pronounced for seedling hypocotyls; although the highest percentage response was observed for media with both cytokinins and auxins, there was only a 5% difference between the percentage response on these media and the percentage response on media with only cytokinins.

The seedling hypocotyls showed higher rooting (53.8%) than the excised embryos (22.2%) on the control medium. A higher percentage of the shoots produced by embryos (33.3%) on a medium supplemented with 0.5 µM kinetin were rooted compared to seedling hypocotyl cultures (15.8%) on the same medium. On a medium supplemented with 2.3 µM kinetin, seedling hypocotyls produced a higher amount of rooted shoots (26.3% compared to 11.8% for embryos). None of the shoots produced by excised embryos on a medium supplemented with 23.2 µM kinetin rooted, whereas 36.8% of the shoots produced by seedling hypocotyls on this medium rooted. None of the shoots produced by excised embryos on a medium supplemented with 2.3, 4.7 or 23.2 µM kinetin or 0.5 µM NAA rooted, whereas at least 20% of shoots produced by seedling hypocotyls on these media were rooted. The seedling hypocotyls showed higher rooting percentage (>30%) than the excised embryos (<20%) on a medium supplemented with 2.3, 4.7 or 23.2 µM *m*TR. A higher percentage of the shoots produced by seedling hypocotyls (38.5%) produced roots on a medium supplemented with 2.3 µM *m*TR and 0.5 µM NAA compared to seedling hypocotyl cultures (21.4%) on the same medium. Embryo cultures (15.4%) produced more rooted shoots than seedling hypocotyl cultures (8.3%) on a medium supplemented with 4.7 µM *m*TR and 0.5 µM NAA. None of the shoots produced by excised embryos on a medium supplemented with 23.2 µM *m*TR and 0.5 µM NAA rooted, whereas 13.3% of the shoots produced by seedling hypocotyls on the same medium rooted.

For seedling hypocotyl and excised embryo cultures a large volume of callus was observed on more than 50% of cultures on media supplemented with cytokinins and auxins. For excised embryos, a small number (<15%) of cultures on media with the two lower cytokinin concentrations produced callus. This value rose to 50% on a media supplemented with 23.2 µM kinetin and *m*TR.

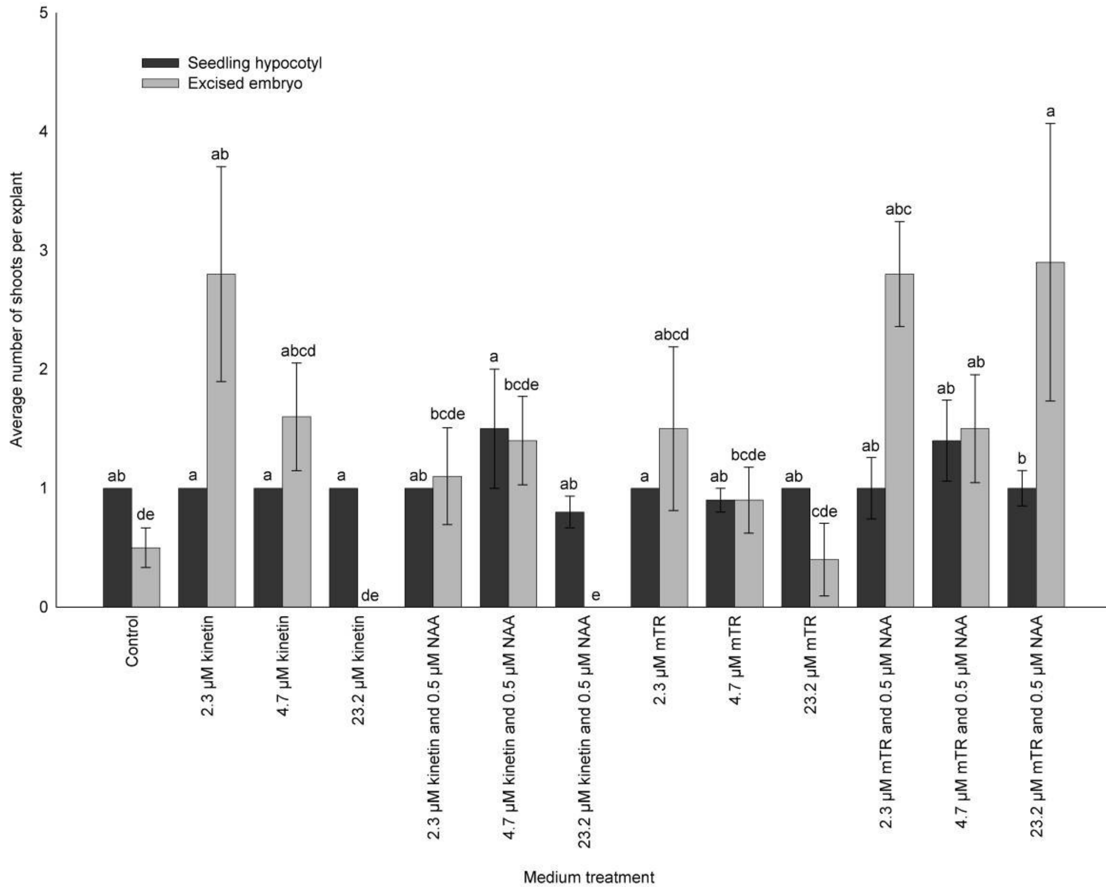

Figure 5.8: The effect of three different concentrations of kinetin and *m*TR either with or without 0.5 NAA on shoot production of *Romulea leipoldtii* seedling hypocotyls and embryos. Error bars indicate standard errors of the means. Letters show significant differences between treatments according to Duncan's multiple range test.

5.3.4 Shoot multiplication

There was no significant difference between the effect of the different kinetin concentrations, photoperiods or temperatures on the number of shoots formed per explant. When all the control replicates are grouped (cultures supplemented with 23.2 µM kinetin and placed at 25°C) it shows that an average of 2.4 ± 0.6 SE shoots was obtained after 2 months of culture.

There was a significant difference between the number of shoots produced on media supplemented with 2.5 µM *m*TR and the number of shoots produced on media supplemented with all the kinetin concentrations tested including 2.5 µM kinetin (Figure 5.9). Although the highest number of shoots were also produced on media supplemented with 2.5 µM *m*TR, there was no significant difference between the number of shoots produced on this medium and the number of shoots produced on a medium supplemented with 2.5, 5.0 or 7.5 µM BA, 5.0 or 7.5 µM *m*TR, or 2.5 or 5.0 *Mem*TR.

The shoots produced on a medium supplemented with 2.5 µM *m*TR appeared healthy and 40% of them were rooted. All other media treatments also produced healthy shoots of which at least 20% were rooted, except for media supplemented with BA, on which vitrified shoots were observed as with previous experiments.

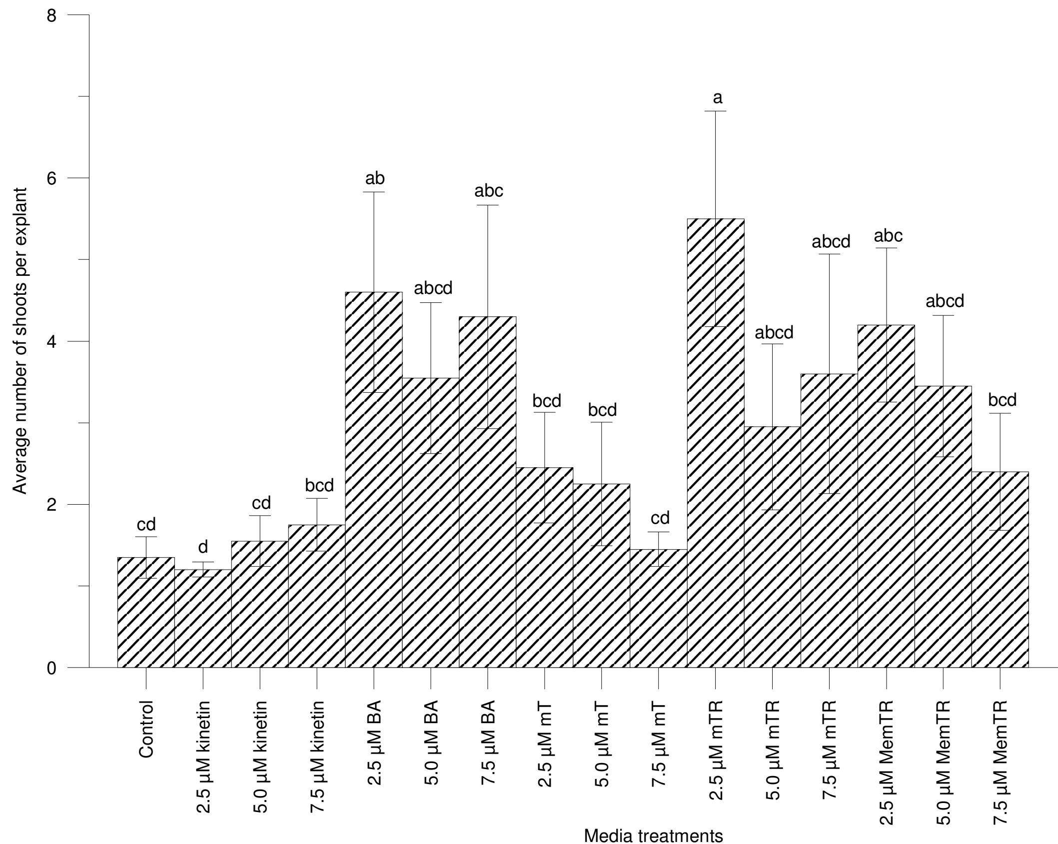

Figure 5.9: Effect of three different concentrations of five cytokinins on multiplication of *Romulea sabulosa* shoots after 2 months. Error bars indicate standard error of the mean. Letters shows significant differences between treatments according to Duncan's multiple range test.

5.4 DISCUSSION

Preliminary experiments with *R. diversiformis*, *R. flava*, *R. leipoldtii* and *R. minutiflora* seedlings showed that hypocotyls were the only seedling organs that gave an *in vitro* response for the *Romulea* species tested. They also show that *R. leipoldtii* and *R. flava* form the highest number of shoots per seedling hypocotyl compared to the other species tested. Because of possible vitrification and culture incompetence, the use of BA and shoots generated from callus should be avoided for this genus.

The *in vitro* response of *R. diversiformis* embryos and hypocotyls, although morphologically distinct, is equally unproductive, as no shoots were formed after 2 months. A higher concentration of cytokinins, or different cytokinins, is perhaps needed to generate a more pronounced shooting response for explants of this species.

The rooting of shoots formed *in vitro* of all species, except *R. diversiformis* and *R. monadelpha* was inhibited to a certain degree by a medium supplemented with 23.2 µM kinetin. Experiments performed with the aim of finding a concentration of kinetin high enough to induce shooting, but low enough to allow for normal root growth, is necessary for the efficient production of healthy shoots of these species. Such an experiment should investigate the effect of various kinetin concentrations between 7.5 and 23.2 µM.

Although *R. leipoldtii* embryos produced significantly more shoots on a medium with 23.2 µM *m*TR and 0.5 µM NAA, this medium and a medium supplemented with 2.3 µM *m*TR and 0.5 µM NAA resulted in a higher percentage of cultures with callus. This indicates that although these are the best media for culture initiation, they are not suitable for multiplication of shoots of *R. leipoldtii*. Shoots produced from this callus were vitrified.

The fact that there is no significant difference between the number of shoots produced by embryos and hypocotyls of *R. leipoldtii* suggests that excising the embryos of even the smallest seeds of all species presented here, except for *R.*

camerooniana and *R. rosea* (no *in vitro* response was observed for these species), can be used as a faster method for obtaining shoot cultures. The germination of the embryos of these species does not require the low temperature and stratification treatments that the seeds need as germination cues and the embryo geminates within about 2 weeks, whereas seeds require at least 2 months.

Although a higher percentage of *R. leipoldtii* hypocotyls than embryos showed some growth response because of their larger size, embryos were more responsive to the plant growth regulators in the medium because of the younger physiological age of these tissues (SMITH, 2000).

Although the rooting of embryo-derived shoots of *R. leipoldtii* was inhibited by high concentrations of cytokinins and developed more callus compared to hypocotyl-derived shoots it should be considered that the hypocotyls are essentially fully developed plants with trimmed shoots and roots, so that the development of an entire new shoot is not necessary. Following this logic, it also means the single shoots observed in some cultures for which hypocotyls is the initial explant was in fact the original hypocotyl, which did not form more shoots but only elongated and, in most cases, produced roots. When the data is viewed from this perspective, embryos produced more shoots than hypocotyls.

These factors make the choice of explant type very difficult. The explant type used should therefore rather depend on the species used.

These results show that the species response in *Romulea* is influenced to great extent by genetic factors, as media treatments or explant type could not increase the shooting of *R. diversiformis*, *R. minutiflora* and *R. monadelpha* to numbers similar to that of *R. flava* and *R. sabulosa* and the rooting of some species is not suppressed by a medium with a high kinetin concentration while the rooting of other species is inhibited on such a medium.

Although the kinetin concentrations tested in the *R. sabulosa* shoot multiplication experiment was not the same as those used in previous experiments, the number of shoots produced after two months on a medium supplemented with 2.5 µM *m*TR (5.5

± 1.3 SE) is much higher than the number of shoots produced by a medium supplemented with 23.2 µM kinetin (2.4 ± 0.6 SE) in previous experiments and the shoots appeared healthier. This medium is therefore better suited for *R. sabulosa* shoot multiplication. The effect of similar concentrations of *m*TR on shoot culture initiation from embryos of *R. sabulosa* should be tested.

It was expected that the direct shoot organogenesis requirements for *Romulea* species would be similar to that of *Crocus* species due to the small phylogenetic distance between these two genera compared to other genera in Iridaceae that has been micropropagated (REEVES *et al.*, 2001). The abnormal growth observed on media supplemented with BA and 2,4-D for *Romulea* cultures however shows that this is not the case. Most studies on direct shoot organogenesis in Iridaceae species, summarized in Table 2.6 in Chapter 2, also show BA and 2,4-D to be suitable plant growth regulators for direct shoot organogenesis. Kinetin has however been a component in a media for direct shoot organogenesis, in the absence of BA and 2,4-D, for a number of *Gladiolus* species (ZIV *et al.*, 1970; LILIEN-KIPNIS & KOCHBA, 1987; ZIV & LILIEN-KIPNIS, 2000). In a study on *Sisyrinchium laxum* and *Tritonia gladiolaris,* shoots generated on media supplemented with *m*T appeared healthier than those generated from media supplemented with BA, which had abnormal and stunted growth, and no roots (ASCOUGH *et al.*, 2011). This is analogous to positive effects of a topolin and the negative effect of BA observed in this study.

5.5 SUMMARY

- Before these experiments were conducted there were no studies published on the micropropagation of *Romulea* species.
- Both embryos and seedling hypocotyls can be used for *R. flava*, *R. leipoldtii* and *R. minutiflora in vitro* shoot culture initiation
- *R. sabulosa* shoot cultures can only be initiated by using embryos as explants, because the lack of germination for this species.
- Shoot cultures of *R. diversiformis*, *R. camerooniana* and *R. rosea* could not be initiated due to the lack of an *in vitro* explant shooting response.

- Shoot cultures was initiated on media supplemented with 2.3 to 23.2 µM kinetin for *R. flava*, *R. leipoldtii*, *R. minutiflora*, *R. monadelpha* and *R. sabulosa*, with the most suitable concentration depending on the species used.
- A medium supplemented with 2.5 µM *m*TR is suitable for *R. sabulosa* shoot multiplication.
- BA caused vitrification of shoots in all the experiments in which it was used and is not a suitable cytokinin for the micropropagation of these species.

6 *In vitro* corm formation and flowering and *ex vitro* acclimatization

6.1 INTRODUCTION

In vitro formation of storage organs increases the *ex vitro* survival rate during acclimatization and these organs serve as a more attractive product than seeds, as flowering of the plant can be enjoyed at a much earlier stage (ASCOUGH *et al*., 2009). Commercialising *in vitro* produced corms of *R. sabulosa* will also reduce the pressures on the vulnerable populations from which seeds are harvested (RAIMONDO *et al*., 2009).

In vitro flowering is an important tool in ornamental plant breeding, as it enables the breeder to see the floral traits in a much shorter time than in *ex vitro* conditions. Some factors that influence *in vitro* flowering and *ex vitro* survival is discussed in section 2.10 and 2.8.9 of Chapter 2 respectively.

The aims of this Chapter were to establish an *in vitro* corm induction protocol for *R. minutiflora, R. leipoldtii* and *R. sabulosa*, to investigate the effect of some physical and chemical stimuli on *in vitro* flowering of *R. minutiflora* and *R. sabulosa* corms and to establish an *ex vitro* acclimatization protocol for *R. minutiflora* and *R. sabulosa* corms and plantlets.

6.2 MATERIALS AND METHODS

6.2.1 Corm formation

In all experiments, the *in vitro* generated shoots were separated from each other, trimmed to 25 mm and all roots were removed for uniformity. If not stated otherwise a MS medium supplemented with 100 $mg.l^{-1}$ *myo*-inositol and 3% sucrose, with pH adjusted to 5.7 and solidified with 0.8% agar was used. All experiments were conducted in a laminar flow hood and cultures were placed in a growth chamber under 3.4 µmol m^{-2} s^{-1} light using Osram® 75 W cool white fluorescent tubes with a 16/8 light/dark photoperiod. The duration of all experiments was 6 months, as no

corm formation was observed after 2 and 4 months and no tunic development was seen after 5 months.

The shoots of *R. minutiflora* produced from seedling hypocotyls on a medium supplemented with 23.2 µM kinetin and 5.4 µM NAA were multiplied on the same medium for 6 months. These shoots were placed singly in 33 ml culture tubes on 10 ml of MS media supplemented with 3%, 6% or 9% sucrose or 5.0 $g.l^{-1}$ activated charcoal. The tubes were then placed in growth chambers maintained at 10℃, 15℃, 20℃, 25℃ or 30℃.

The remainder of these shoots were multiplied for a further 4 months and subsequently used in an experiment to test the effect of growth retardants on corm formation in *R. minutiflora*. The shoots were placed in 33 ml culture tubes on 10 ml of MS medium supplemented with 3.4, 17.0 or 34.0 µM paclobutrazol (PP3), or 4.9 µM abscisic acid (ABA). These tubes were placed at 25℃ under 4.3 µmol m^{-2} s^{-1} light supplied by Osram® 75 W cool white fluorescent tubes. Twenty replicates were used per treatment.

The shoots produced by seedling hypocotyls of *R. leipoldtii* on a medium supplemented with 22.2 µM BA were multiplied on this medium for 4 months. The shoots were placed in 33 ml culture tubes on 10 ml of MS medium supplemented with 3%, 6% or 9% sucrose or 5.0 $g.l^{-1}$ activated charcoal. These tubes were placed at 25℃ under 4.3 µmol m^{-2} s^{-1} light supplied by Osram® 75 W cool white fluorescent tubes and 50 replicates were used per treatment.

The shoots of *R. sabulosa* produced from excised embryos on a medium supplemented with 23.2 µM kinetin were multiplied on the same medium for 6 months. These shoots were placed singly in 33 ml culture tubes on 10 ml of MS media supplemented with 3%, 6% or 9% sucrose, 5.0 $g.l^{-1}$ activated charcoal or 23.2 µM kinetin. The tubes were then placed in growth chambers were maintained at 10℃, 15℃, 20℃, 25℃ or 30℃. Ten shoots were used per treatment and the experiment was repeated three times.

Corm induction percentage and corm diameter for different temperatures and media compositions were analyzed for significant differences with Duncan's multiple range

test using Genstat. To examine the combined effect of treatments on corm induction rate and corm weight, product analysis was done. This was done by multiplying the proportion of corm induction with the mass of the corms produced (mg) and dividing this number with hundred according to the methods of ASCOUGH *et al.* (2008). A high value indicates that the treatment resulted in a high proportion of induction and corm mass, while a low value indicates that either the proportion of induction, the corm mass or both are low.

6.2.2 *In vitro* flowering

After an *in vitro* flower was observed in the *R. minutiflora* corm formation treatments (9% sucrose at 20°C) further experiments to repeat this result were initiated. Information from UYEMURA & IMANISHI (1984), FLAISHMAN & KAMENETSKY (2006), LIGHT *et al*., (2007) and a study by KÖK (2007) on the difference in soil composition during vegetative growth and flowering in *R. columnae* was used to design an *in vitro* flowering experiment.

As it is better to use larger storage organs for florogenesis studies, only the largest corms were selected (FLAISHMAN & KAMENETSKY, 2006). These corms were placed in test tubes in a growth chamber with high light (190.1 µmol m^{-2} s^{-1}) set at 20°C. Medium treatments included a half strength MS medium, a half strength MS medium with 9% sucrose, a full strength MS medium with 9% sucrose, a full strength MS medium with 1:500 (v/v) smoke water and a full strength MS medium with 3% sucrose as a control. For each treatment 20 corms of *R. minutiflora* and *R. sabulosa* between 5 and 10 mm in diameter were used.

6.2.3 *Ex vitro* acclimatization and corm viability

Before all corms and plantlets were used for *ex vitro* growth studies they were rinsed in autoclaved distilled water to remove agar from roots. Corms were partially dried *in vitro* by placing them inside a Petri dish on a laminar flow bench for two weeks before planting.

The mist-house and greenhouse used in these experiments is situated in the University of KwaZulu Natal Botanical Garden in Pietermaritzburg (30° 24' E, 29° 37' S, 655 m above sea level). The mist-house had a misting interval of 15 min and a misting duration of 10 s, whereas the greenhouse did not have an automated

watering regime at the time during which these experiments where conducted and plants were watered every second day with 500 ml of water.

Corms of *R. minutiflora* and *R. sabulosa* were planted in plastic planting trays with a 1:1 ratio of potting soil and sand. For each species, 6 corms were placed in 6 trays. The trays were placed in the mist-house for 24 h, after which they were moved to the greenhouse. The trays were only placed in the mist-house for this short period of time because all corms were necrotic after only two weeks in an initial experiment were 2 trays with 6 corms each where placed in the mist-house, suggesting that these conditions are too moist.

In another experiment corms of *R. sabulosa* were planted in clay pots with a 1:1 ratio of potting soil and sand. Because of the limited number of pots, 4 corms were placed in each pot and 30 pots were used. These pots were placed in a growth chamber with high light (190.1 µmol m^{-2} s^{-1}) maintained at 20℃.

Healthy rooted plantlets of *R. minutiflora* and *R. sabulosa* that did not produce corms during corm formation experiments were planted in plastic planting trays with a 1:1 ratio of potting soil and sand. For each species, 6 plantlets were placed in 5 trays. The trays were placed in the mist-house for 24 h, after which they were moved to the greenhouse.

A small experiment was conducted with the remaining corms formed *in vitro* at 10 ℃. Four corms were placed in a clean plastic container with autoclaved vermiculite moistened with 50% Hoagland's nutrient solution. The containers were placed in a growth chamber set at 20℃ with a 16 h photoperiod of 12.5 µmol m^{-2} s^{-1} irradiance for two months. The rest of the corms were used for a viability study. The viability of ten corms larger than 5 mm in diameter were tested according to the method of WAGNER (1984).

6.3 RESULTS

6.3.1 Corm formation

In the first corm formation experiments with shoots of *R. minutiflora*, no corms were observed at temperatures of 25℃ and higher. The results of 15℃ are not reported as the growth chamber malfunctioned and the cultures had to be discarded. No significant differences in corm induction from shoot explants were observed either when changing the temperature or altering the sucrose concentration (Table 6.1). Corm mass, however, increased with increasing sucrose concentration. This was true at both 10 and 20 ℃. The addition of activated charcoal to media with 3% sucrose significantly increased corm mass under both temperature regimes (Table 6.1). The product of corm induction and corm mass indicates that placing the shoots at 10℃ on a medium with 6% sucrose leads to the best combination of both proportion of induction and corm mass, followed by the shoots placed at 20℃ on media with 9% and 3% sucrose respectively (Table 6.1).

Table 6.1: The effect of different temperatures and media composition on the *in vitro* formation and growth of *Romulea minutiflora* corms. Data shows the means ± the standard error. Letters indicates significant differences between treatments according to Duncan's multiple range test.

Culture temperature and medium treatment		Corm induction %	Corm mass (mg)	Product
10℃	3% sucrose	65.2 ± 9.2 a	108.7±21.9 b	70.9
	6% sucrose	80.4±6.9 a	279.0±114.0 ab	224.3
	9% sucrose	81.9±9.1 a	134.5±19.6 b	110.2
	5.0 $g.l^{-1}$ activated charcoal	56.7±5.6 a	285.2±33.7 a	161.7
20℃	3% sucrose	69.4±4.6 a	152.0±31.3 b	197.9
	6% sucrose	73.9±3.9 a	227.0±61.1 b	105.5
	9% sucrose	72.5±10.3 a	273.7±50.3 a	198.4
	5.0 $g.l^{-1}$ activated charcoal	63.8±5.9 a	239.3±45.0 a	157.7

PP3 and ABA both stimulated corm formation at 25℃ in the second experiment with *R. minutiflora*. The addition of 17.0 and 34.0 µM PP3 resulted in the largest percentage corm induction and corm size (Table 6.2).

Table 6.2: Percentage corm induction for *Romulea minutiflora* shoots cultured on medium supplemented with growth retardants.

Treatment	Basal swelling (%)	Corm induction (%)	Corms > 5 mm (%)
Control	70%	0%	0%
3.4 µM PP3	55%	15%	10%
17.0 µM PP3	50%	35%	20%
34.0 µM PP3	35%	35%	20%
4.9 µM ABA	45%	20%	5%

No corm formation was observed for any of the temperature and medium treatments tested for *R. leipoldtii*. When 100 bottles of shoots multiplied on media supplemented with BA placed at 25℃ were not subcultured for 6 months no corm formation or basal thickening was observed. This was not the case with the shoot cultures placed at 25℃ multiplied with media supplemented with kinetin and topolins. Here corms or basal thickening was observed for all shoots not multiplied after 6 months.

Such basal thickening was observed to a certain extent for non-subcultured shoot cultures of *R. minutiflora* and *R. sabulosa*, but no corm formation was observed in any cultures placed at 25℃ for these species.

The percentage corm induction for *R. sabulosa* after 6 months was significantly higher at 10 and 20 ℃ with all tested sucrose concentrations compared to 15 ℃ (Table 6.3). Corm induction was inhibited at 25 ℃ in this experiment (data not shown). The largest corm mass was recorded for shoots placed at 15 ℃ on a MS medium supplemented with 6% sucrose (Table 6.3). The mass of these corms were however not significantly different from the mass of the corms obtained for cultures placed at the same temperature on a medium with 9% sucrose or the mass of corms produced on a medium with activated charcoal and the mass of corms at 20 ℃ placed on a medium with 6% sucrose. Product analysis shows that corms at 15 ℃ with 6% sucrose achieved the highest value (Table 6.3).

Table 6.3: The effect of different temperatures and media composition on the *in vitro* formation and growth of *Romulea sabulosa* corms. Data shows the means ± the standard error. Letters indicate significant differences between treatments according to Duncan's multiple range test.

Culture temperature and medium treatment		Corm induction (%)	Corm mass (mg)	Product
10℃	3% sucrose	100.0 ± 0.0 a	325.5 ± 39.0 b	325.5
	6% sucrose	100.0 ± 0.0 a	346.5 ± 56.3 b	346.5
	9% sucrose	100.0 ± 0.0 a	294.1 ± 49.6 b	294.1
	5.0 $g.l^{-1}$ activated charcoal	87.5 ± 7.2 abc	336.3 ± 50.8 b	294.2
	23.2 µM kinetin	100.0 ± 0.0 a	371.1 ± 49.8 b	371.1
15℃	3% sucrose	71.3 ± 10.9 bc	325.2 ± 21.9 b	231.7
	6% sucrose	81.7 ± 6.9 bc	522.7 ± 61.7 a	426.9
	9% sucrose	83.8 ± 5.5 bc	439.3 ± 71.4 ab	348.9
	5.0 $g.l^{-1}$ activated charcoal	68.8 ± 12.6 c	409 ± 59.4 ab	281.2
	23.2 µM kinetin	20.0 ± 14.1 d	295.3 ± 39.2 b	58.5
20℃	3% sucrose	100.0 ± 0.0 a	310.9 ± 54.7 b	310.9
	6% sucrose	100.0 ± 0.0 a	383.5 ± 36.6 ab	383.5
	9% sucrose	100.0 ± 0.0 a	372.7 ± 57.8 b	372.7
	5.0 $g.l^{-1}$ activated charcoal	91.7 ± 8.3 ab	362.6 ± 63.8 b	332.4
	23.2 µM kinetin	18.8 ± 18.8 d	281.8 ± 52.9 b	52.2

Multiple corm formation from the same shoot was observed in some cultures of *R. sabulosa* (Table 6.4). The highest percentage of multiple corm formation was observed at 15℃ on a medium supplemented with 23.2 µM kinetin, while the highest number of corms per shoot was observed at 10℃ on a medium with 3% sucrose. Multiple corm formation also occurs in *Romulea* species under natural environmental conditions (DE VOS, 1972).

Table 6.4: Cultures with multiple corm formation for *Romulea sabulosa*. This shows the percentage of corm formation in cultures in which corm formation observed (Total cultures with corms) and the average number of corms produced in instances of multiple corm formation.

Culture temperature and medium treatment		Multiple corm formation (%)	Total cultures with corms	Average number of multiple corms
10℃	3% sucrose	6.3%	16	7.0
	9% sucrose	16.7%	18	2.3
	5.0 $g.l^{-1}$ activated charcoal	6.3%	16	3.0
15℃	6% sucrose	7.1%	14	3.0
	9% sucrose	12.5%	16	2.0
	5.0 $g.l^{-1}$ activated charcoal	7.7%	13	2.0
	23.2 µM kinetin	50.0%	4	2.0
20℃	3% sucrose	9.1%	11	2.0

6.3.2 *In vitro* flowering

Although an *in vitro* formed flower was observed for *R. minutiflora* during corm formation experiments at 20℃ on a medium with 9% sucrose (Figure 6.1), none of the further experiments yielded any flowers or flower initials.

Figure 6. 1: An in vitro formed flower of *Romulea minutiflora* observed in a test tube placed at 20℃ on a medium with 9% sucrose.

6.3.3 *Ex vitro* acclimatization and corm viability

None of the corms or plantlets survived more than one month. Four corms planted inside the plastic container however survived (Fig. 6.2) for two months, and 7 out of 10 corms tested were viable.

Figure 6.2: Corms of *Romulea sabulosa* growing in a modified plastic container with vermiculite after 2 months. Bar = 20 mm.

6.4 DISCUSSION

Temperature is the major factor that affects storage organ morphogenesis (ASCOUGH *et al.*, 2009). Low temperature significantly increased corm formation in *R. minutiflora* and *R. sabulosa*. The highest number of instances of multiple corm formation for *R. sabulosa* was also observed at 10 and 15℃.

In a study by ASCOUGH et al. (2008) on *Watsonia vanderspuyiae* (in the same subfamily, Ixioideae) the highest percentage corm induction was observed for shoots placed at low temperatures. This study showed no significant difference between corm induction percentages of shoots placed on MS media supplemented with sucrose at 10 and 20 ℃. Corm formation at low temperatures has been observed *in vitro* for many genera in the Iridaceae (ASCOUGH *et al.*, 2009). An exception to this is *Crinum macowanii*, where storage organ formation occurs above 25 ℃ and is inhibited at lower temperatures (SLABBERT *et al.*, 1993). The fact that corm induction was inhibited for *R. minutiflora* and *R. sabulosa* at 25 ℃ in this study correlates with the results of *Gladiolus* spp., where corm induction was inhibited at this temperature (TAN NHUT *et al.*, 2004).

In this study the requirements for corm formation of *R. leipoldtii* is similar to that determined in a study involving *Crocus sativus* corm formation (PLESSNER *et al.*, 1990). It was however shown by HOMES *et al.* (1987) that corm formation of *Crocus sativus* also occurred at 30℃, a temperature that totally inhibits the corm formation of *R. minutiflora* and *R. sabulosa* and that would probably inhibit the corm formation of *R. leipoldtii* as it occurs in the same regions as *R. minutiflora* (Figure 2.1).

When PP3 and ABA were added to the medium on which *R. minutiflora* shoots were placed, these shoots developed corms although they were placed at 25℃, a temperature that totally inhibits corm formation when these retardants that reduce leaf elongation and promote storage organ formation are not present. This effect of PP3 has also been shown for *Dierama* and *Gladiolus* species (MADUBANYA, 2004; STEINITZ & LILIEN-KIPNIS, 1989; ZIV, 1989; ZIV *et al.*, 1998).

The fact that corms were not observed in any *R. leipoldtii* shoot cultures supplemented with BA after 6 months show that this chemical inhibits corm formation for this genus. The same effect is expected for the other species in this genus, as the shoots of all species generated and multiplied on BA appeared abnormal and their root growth was stunted.

In *Watsonia* cultures, a correlation between corm mass and carbohydrate concentration was observed, with corm induction in some species decreasing as the carbohydrate concentration increases (ASCOUGH *et al.*, 2008). In the present study this was also observed to some extent, as the treatment that delivered the highest corm mass had a 6% sucrose concentration. It was however surprising that elevated levels of carbohydrates did not have a statistically significant effect on corm mass.

A two step corm formation protocol would work best for *R. sabulosa*, as the corms differentiate and accumulate carbohydrates under different temperatures. This two step system would involve placing corms at either 10 or 20℃ for a few months and then transferring these cultures to 15℃.

A similar two step program was proposed by ASCOUGH *et al.* (2011) for *Tritonia gladiolaris*. This two step system forms corms at lower temperatures (10 and 15℃) and promotes the accumulation of carbohydrates at higher temperatures (20℃). They suggest that the physiological mechanisms involved here are probably an adaptation for survival, so that the low temperature is perceived as a cue for corm induction and the onset of dormancy before the approach of unfavourable conditions.

Corm production is however promoted at 10 and 20℃ and corm mass increases at 15℃, a temperature flanked by the two former temperatures, for *R. sabulosa.* This phenomenon may be explained by comparing the life cycle of *R. sabulosa* with the temperature, rainfall and humidity observations for the last five years in Nieuwoudtville.

R. sabulosa flowers from August to September. Before flowering there are a few weeks of vegetative growth, facilitated by the increase in rainfall in July. During July and August the averaged daily minimum is 5.1 ± 0.3℃ and 4.7 ± 0.2℃ and the averaged daily maximum temperature is 18.6 ± 0.6℃ and 18.1 ± 0.7℃ respectively.

During this period new axillary buds are formed. The axillary buds gradually form their own corm at the base as conditions for growth becomes less suitable at the end of flowering in September. During September and October the averaged daily minimum temperatures are 6.3 ± 0.7℃ and 8.2 ± 0.3℃ and the averaged daily maximum temperatures are 21.3 ± 0.7℃ and 25.6 ± 0.4℃. The maximum temperature of periods of vegetative growth and corm induction is close to 15℃ and the minimum and maximum temperatures are close to 10 and 20℃ during periods of senescence, when accumulated carbohydrates may be reallocated to the corm in the natural habitat of *R. sabulosa*. Although such an explanation is plausible, the interactions of factors that influences the climate of Namaqualand is very complex and it is therefore a difficult topic (DESMET, 2007).

The corm production rate of various *Gladiolus* cultivars was also increased by a two step bud-culture technique, involving short-term exposure to a medium with plant growth regulators and subsequent withdrawal from plant growth regulators in a liquid medium (SEN & SEN, 1995).

The specific temperature needed for carbohydrate accumulation for *R. sabulosa*, compared to that of *Tritonia gladiolaris* may be explained by its very restricted distribution compared to that of the widespread *T. gladiolaris*. An adaptation to accumulate carbohydrates above a certain temperature and to induce corms below this temperature would allow a species to spread to a variety of habitats where this temperature is observed, whereas a specific carbohydrate accumulation temperature range will limit the populations to an area with periods of this temperatures long enough to accumulate sufficient carbohydrates to be able to survive the dry seasons underground in a dormant state and to produce enough vegetative growth in the next season for the accumulation of additional carbohydrates.

The fact that corms did not flower *in vitro* is probably because these plants need much colder minimum temperatures (below 5℃) such as in their natural environment for growth and development. The one corm of *R. minutiflora* which flowered *in vitro* at 20℃ was perhaps a mutation.

Plantlets of *Romulea* species develop viable corms after 6 months, which can be commercialized as propagation units. Corm formation speeds up the

micropropagation and seedling establishment process and it is also cost-effective, as there is no need for hardening or acclimatization of plants.

6.5 SUMMARY

- Low temperature significantly increased corm formation in *R. minutiflora* and *R. sabulosa*
- A two step corm formation protocol involving placing corms at either 10 or 20℃ for a few months and then transferring these cultures to 15℃ should be used for *R. sabulosa*
- When PP3 and ABA is added to the medium on which *R. minutiflora* shoots were placed, these shoots develop corms at 25℃, a temperature that totally inhibits corm formation when these growth retardants are not present
- BA inhibited corm formation in *R. leipoldtii*
- Corms of *Romulea sabulosa* formed at 10℃ are viable
- Corms can be commercialized as propagation units to grown in winter-rainfall areas with minimum temperatures below 5℃ during winter

7 Commercialization potential of *Romulea* species

Attributes that make species of *Romulea* attractive are their beautiful flowers and growth forms (Figure 7.1). The flowers are not only attractive because of their wide range of colours, including yellow to white, pink, orange, apricot, red, magenta, lilac and purple, but also because of the interesting shape of some flowers such as *R. diversiformis* (Figure 7.1 C) and *R. hantamnensis* (DE VOS, 1972; MANNING & GOLDBLATT, 2001). The flowers of some species are also scented. These include the honey scented flowers of *R. austinii* and the honey and coconut scented flowers of *R. schlechteri.* The growth form of many *Romulea* species is very reduced, with subterranean stems and a few filiform leaves. This accentuates the beautiful flowers of these species and creates a spectacular floral display when flowers are grown together.

Figure 7.1: Showing eight species used in propagation experiments arranged from the largest to the smallest growth form. From the left they are *Romulea minutiflora* (A), *R. camerooniana* (B), *R. diversiformis* (C), *R. rosea* (D), *R. flava* (E), *R. leipoldtii* (F), *R. monadelpha* (G) and *R. sabulosa* (H). Modified from DE VOS (1972) and photographs taken by Dr. John C. Manning. Horizontal bar = 50 mm.

Many horticulturalists I have spoken to during this study are not just interested in *Romulea* because of their beautiful flowers, but also because growing seeds of *Romulea* to the flowering stage is considered somewhat of a horticultural feat and they aspire to the challenge. There is a demand for propagative material of these plants and many companies supplying seeds via the internet. These companies often supply inadequate information on propagation. The seed germination protocols reported in Chapter 4 can now be used by horticulturalists as more effective methods for germinating seeds of *R. diversiformis*, *R. flava*, *R. leipoldtii*, *R. minutiflora*, *R. monadelpha* and *R. rosea*. Germination of the seeds of some of the most attractive species in this genus is however low.

In this study, micropropagation protocols that produce corms as an end-product have been established for *R. leipoldtii*, *R. minutiflora* and *R. sabulosa*. Corms of these species can now be commercialised. For the attractive *R. sabulosa*, one embryo produces 2.1 ± 0.7 SE shoots after 2 months; placing these shoots on a medium supplemented with 2.5 µM *m*TR for a further 2 months multiplies their number by 5.5 ± 1.3 SE. Each of these shoots can then be induced to produce a corm after 6 months. This means that 1 embryo can produce about 12 corms after 10 months or about 65 corms after 12 months (if shoots are subcultured to medium supplemented with 2.5 µM *m*TR for another 2 months). Although corms of *R. flava* and *R. monadelpha* were not produced, stems with basal thickening, like those observed for *R. leipoldtii*, *R. minutiflora* and *R. sabulosa,* were observed in shoot cultures not subcultured for 4 months. It is therefore expected that these species would also produce corms when placed at low temperatures.

Some other attractive species that were not micropropagated in this study include *R. citrina* and *R. tabularis*, which were used in initial germination experiments, and *R. eximia* for which seeds could not be obtained.

R. eximia flowers are old-rose pink to dark old-rose or deep red (DE VOS, 1972; MANNING & GOLDBLATT, 1996; MANNING & GOLDBLATT, 2001). The cup is greenish to pale yellow and is streaked purple. There are dark red blotches on each

segment around the cup. *R. eximia* flowers from August to September (MANNING & GOLDBLATT, 2001). According to DE VOS (1972) this plant has 2 to 3 flowers or more.

R. eximia is in the same subgenus and section as *R. sabulosa*, whereas *R. citrina* and *R. tabularis* are in the same subgenus, section and series as *R. leipoldtii* and *R. minutiflora* (MANNING & GOLDBLATT, 2001). *R. austinii* and *R. schlechteri,* species with scented flowers, are also in the same subgenus, section and series as *R. leipoldtii* and *R. minutiflora.*

It is therefore very likely that the culture requirements for *R. eximia* could be very similar to that of *R. sabulosa* and that the culture requirements for *R. citrina*, *R. tabularis, R. austinii* and *R. schlechteri* could be very similar to that of *R. leipoldtii* and *R. minutiflora*. This study can therefore be useful for the commercialization of these species.

Apart from the increased multiplication rate *in vitro* compared to conventional propagation, *in vitro* techniques such as embryo rescue established for these species will also be useful in the commercialization of these species. Embryo rescue enables the development of the embryo *in vitro* if the endosperm is underdeveloped (HARTMANN & KESTER, 1965). Crosses have been attempted *in vivo* on numerous species of *Romulea* by DE VOS (1972). She found that crosses between species that were not in the same section and with different chromosome numbers were largely unsuccessful. When crossing species in different subsections and sections she found that seeds were not viable. She speculated that this was because of a failure of endosperm development, suggesting that the embryo may be viable and that the techniques of embryo rescue and *in vitro* culture could be used to cultivate these hybrids (DE VOS, 1972). Weak plants were obtained in a few cases. These plants died within the first growth season, before flowering (DE VOS, 1972).

Using embryo rescue techniques, the beautiful and large flowered species in the subsection Spatalanthus (e.g. *R. sabulosa* and *R. monadelpha*) can therefore theoretically be crossed with species with scented flowers such as *R. austinii* and *R. schlechteri* in the subsection Romulea. Such a cross may result in a phenotype with

large, beautiful and scented flowers which can be cloned *in vitro* using protocols established in this study. Embryo rescue and *in vitro* culture techniques can also be used to cross such a phenotype with a widespread species such as *R. rosea*, so that cultivation requirements will be less specific. The commercialization potential of such a phenotype would be very high, as it could be cultivated in numerous geographical locations by somebody that does not have extensive horticultural experience.

Natural hybridization amongst the South African *Romulea* species are rare (DE VOS, 1972). Intermediates between *R. rosea* var. *australis* and var. *communis* have been found. A similar case of natural hybridisation has been reported for *R. rosea* var. *reflexa* and var. *australis*. DE VOS (1972) reported similar cases of natural hybridization of variants within species for *R. cruciata* and *R. obscura*. She also reported a case of natural hybridisation of *R. leipoldtii* and *R. tabularis* and speculated that *R. sabulosa* and *R. monadelpha* may also hybridise naturally. These hybrids resembled artificial hybrids obtained from crossing these varieties (DE VOS, 1972).

Polyploidy have been induced in a number of different monocotyledons (COHEN & YAO, 1996; GANGA & CHEZHIYAN, 2002). As polyploidy techniques are more affective with propagative material obtained from micropropagation, this study will also aid in the establishment of a protocol to generate polyploids of *Romulea* species.

The results produced in this study will be useful to consider before the design of experiments for the future horticultural development of species in this genus. It not only shows that species of this genus can be propagated, but also that there is potential for hybridisation and mass propagation within the genus of *Romulea*.

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