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## Pollen characteristics of grevillea species determined by in vitro germination

Rebecca Parsons  
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**Pollen Characteristics of *Grevillea* Species  
Determined by *in vitro* Germination**



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## ABSTRACT

Germination of pollen *in vitro* is a common technique used to assess the ability of pollen to germinate under a variety of conditions. The ability to determine the viability of a pollen grain, and optimal conditions for maximum germination and storage are important for hybridisation. A means of storing pollen, while maintaining viability, enables inter-species hybridisation between species that are spatially and temporally (flowering time) separated. The ultimate aim of hybridisation is seed set and in order to increase this in a genus renowned for a very low fruit to flower ratio, maximum pollen germination must be obtained. Therefore, by determining the ideal conditions for *Grevillea* pollen germination on the style (*in vivo*) using laboratory (*in vitro*) techniques may allow the highest possible germination and subsequent seed set for an individual species.

All *Grevillea* species displayed a significant difference ( $P = <0.001$ ) in pollen germination when pollen was collected and germinated one day before anthesis, the day of anthesis and up to three days post anthesis. Desiccation up to 24 hours before *in vitro* germination did not produce a difference in germination for *G. saccata* ( $P = 0.911$ ), however, *G. fililoba*, *G. pinaster* and *G. thelemanniana* responded significantly within 12 hours of desiccation with increased germination ( $P = <0.001$ ). The effect of temperature on pollen germination was significant for all species ( $P = <0.001$ ) with optimum temperatures being species specific. For example, *G. thelemanniana* displayed its' highest germination at 30 °C, with all other species investigated displaying optimum germination at 25 °C. The storage potential of untreated *Grevillea* species was low and there was a significant difference ( $P = <0.001$ ) in germination between freshly collected pollen and that which was stored for approximately seven months. Results suggest that *Grevillea* pollen may require special treatment to maintain viability during storage as only one species, *G. baxeri*, showed no difference between treatments ( $P = 0.064$ ) and lost little viability.

Dark conditions during germination *in vitro* significantly increased pollen germination ( $P = <0.001$ ) for all species, compared to light conditions. Using Brewbaker and Kwack as the germination medium, without a coverslip during the incubation period, also promoted a general increase in germination with the exception of *G. preissii* ( $P = 0.035$  and  $0.127$  respectively). The effect of sucrose content in Brewbaker and Kwack medium was significant for all *Grevillea* species with an optimum concentration range between 10-20% depending on the species. Any concentration above 40% sucrose caused germination to drop below 10% for all species. Sucrose was also demonstrated to have the greatest effect on *G. fililoba* germination when compared to the osmotica mannitol and polyethylene glycol (PEG).

Inter-species hybridisation between three distantly related *Grevillea* species produced no fruit set. Geitonogamous crosses in *G. rhyolitica* produced fruit, however, all fruit aborted before maturation. The removal of the stigma and its incompatibility barriers did not encourage fruit set for any of four crosses, geitonogamous or inter-species hybrid.

In conclusion, it was found that *Grevillea* pollen displays the highest germination *in vitro* when incubated in the dark, without a coverslip, on Brewbaker and Kwack medium containing between 10-20% sucrose. Osmotica such as mannitol and PEG did not increase germination, suggesting that sucrose is primarily a food source for pollen during germination with osmotic balance capabilities that are important for germination. The highest pollen germination was obtained when collected on the day of anthesis for four of the five *Grevillea* species investigated. Ideal temperature ranged between 22.5-27 °C depending on the species, and *G. saccata* was the only species in the study to show no difference in germination after desiccation prior to germination.

## **DECLARATION**

I certify that this thesis does not, to the best of my knowledge and belief:

- (i) incorporate without acknowledgement any material previously submitted for a degree or diploma in any institution of higher education;
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-

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## CHAPTER 1: INTRODUCTION

### 1.1 Introduction

#### 1.1.1 Phylogeny and Habitat

The genus *Grevillea* belongs to the subfamily of Grevilleoideae in the family Proteaceae, which has its greatest diversity in Australia with particular species richness within the south west of Western Australia. As of the year 2000, in the *Flora of Australia* 452 taxa (357 species) of *Grevillea* were recognised (Makinson, 2000) with new species being described since (Olde and Marriott, 2002). Of the 359 species identified to date, approximately 190 occur in the South West Botanical Province, most of which are endemic to the region. *Grevillea* species are found in a range of habitats including: foredunes (*G. lanigera* A. Cunn. ex R. Br), desert regions, just below the snowline on mountains in NSW (*G. victoriae* F. Muell.) as well as the tropical rainforests of North Queensland (Elliot and Jones, 1990). In Western Australia, most species are found in laterite or sandy soils that are well drained and devoid of nutrients, particularly nitrogen and phosphorous (Wrigley and Fagg, 1989).

#### 1.1.2 *Grevillea* Morphology

Morphologically, grevilleas vary substantially throughout their range and have various habits from groundcovers, to shrubs and trees. Leaves are arranged alternately along the stem and can be entire, lobed, deeply lobed, pinnate or bipinnate. Leaf shapes are highly varied between species, ranging from terete through to linear, oblong, ovate, obovate, lanceolate, oblanceolate, falcate and broadly elliptic to almost circular (Wrigley and Fagg, 1989; Olde and Marriott, 1994). A particular characteristic of the group is the presence of numerous trichomes over the plant body. These vary in length, shape, colour and can occur on the leaves, perianth, style, and fruits. There are four types: biramous, glandular, simple and papilloid;

these can be used to distinguish individual species (Olde and Marriott, 1994; Makinson, 2000).

Flowers are borne in pairs along the rachis (uniflorescence) which can be very long such as in *G. nudiflora* Meisn., where flowers are borne on an extremely long peduncle with flower pairs scattered along its length. Most, however, have multiple uniflorescences packed tightly together in a raceme either in an umbel or a toothbrush like structure. These are known as conflorescences and can be axillary or terminal with flowers generally opening from the basal end, towards the apex (Wrigley and Fagg, 1989; Olde and Marriott, 1994).

Most grevilleas have zygomorphic shaped perianths, however, actinomorphic forms can be found in primitive species. Grevilleas have four tepals (perianth segments), with anthers attached to a concave recess at the tip (epitepalous). Pollen dehiscence directly onto the presenter, and after anthesis the four tepals gently bow outwards to release the pollen presenter. The tepals roll backwards at anthesis and often separate (Wrigley and Fagg, 1989; Olde and Marriott, 1994). For the purpose of this study, anthesis is classified as the day the presenter is released from the tepals. The entire perianth dehiscence shortly after anthesis, which is a major problem for the cut flower industry because flowers rarely look presentable after seven days.

The style in most *Grevillea* species is long and protruding with the stigma tucked into the tepals until anthesis. However, great variation in style length and colour occurs in the genus, which is related to pollinator behaviour. Pollen presenter shape and stigma position is also highly varied (see Olde and Marriott, 1994), which can also be attributed to pollinator behaviour. The pollen presenter can either be flat, triangular, conical or disc like, with the stigma centrally or distally flattened or protruding.



The fruit is in the form of a follicle that splits down one side to release the seed when mature. Generally one or two seeds are found per follicle, however it is common to have sterile fruit with no seed (Olde and Marriott, 1994).

### **1.1.3 Pollinators**

Insects and birds are the main pollination vectors for *Grevillea*. Of the various insect pollinators, bees (melittophily) are probably the most utilized vector (Richardson *et al.*, 2000). Unfortunately, the exotic honey bee (*Apis mellifera* L.) has been shown in several studies to reduce the efficiency of pollen transfer and successful pollination compared to the native bee and bird pollinators. Other insect pollinators such as beetles (cantharophily), butterflies (psychophily), moths (phalaenophily), ants (myrmecophily) and flies (myophily) may pollinate grevilleas that have evolved specialised mechanisms, such as flower colour, odour and morphology, to entice these pollinators over melittophilous and ornithophilous pollination. Mammals also pollinate *Grevillea* species, which have developed specialised adaptations to accommodate these alternative vectors. Ornithophilous species often display limited scent, nectar availability, strong peduncles or branches. The nectary is placed at the base of the perianth which is brightly coloured (usually shades of red). The placement of flowers above the foliage on long protruding branches, as seen in *G. petrophiloides* Meisn., has been suggested as a typical adaptation to bird pollination (Olde and Marriott, 1994). Wind does not play a vital role because *Grevillea* pollen is classified as heavy (Olde and Marriott, 1994), therefore unable to be carried long distances.

### **1.1.4 Pollination and Fertilisation**

Grevilleas are protandrous, where gynoecium receptivity is delayed until after the androecium is receptive. This means that the stigma will become receptive after pollen is shed onto the pollen presenter (Collins

and Rebelo, 1987). Some species secrete a sticky substance after self pollen is removed to maximise pollen capture and adhesion. The stigma is receptive when the stigmatic groove has opened to display a 'V' shaped opening. Pollen grains become lodged in this groove where they hydrate and develop a pollen tube down the style (Olde and Marriott, 1994; Kalinganire *et al.*, 2000). Several pollen tubes germinate at the top of the stigma where transmitting cells are abundant. These cells begin to thin out near the ovary in order to allow only one or two pollen tubes entry into the ovary chamber. The pollen tube is then diverted to the micropylar end of the ovule where it enters and is guided to the embryo sac via the secretion of a chemotactic substance. Double fertilisation occurs and the ovary swells as the seed/s develop.

### 1.1.5 Natural Hybrids

Natural hybridisation is not a common occurrence in wild populations, with increases in hybrid production being a consequence of an environmental disturbance (Olde and Marriott, 1994). However, localised hybrid swarms have been reported to occur in New South Wales, Victoria and Queensland (Olde and Marriott, 1994). Often hybrids are derived from closely related parents that are within a close proximity and flower within a similar time frame. Western Australia has very few occurrences of natural hybrids. This may be due to reproductive isolation as suggested by Olde and Marriott (1994) or via mis-identification, particularly if a hybrid closely resembles one parent.

Occasionally, natural hybrids produce a stunning specimen. For example, the prostrate growing *G. laurifolia* Sieber ex Spreng. and the shrub *G. acanthifolia* subsp. *acanthifolia* A. Cunn. produced *G. x gaudichaudii* R. Br. ex Gaudich; a natural hybrid that has both a prostrate and an open shrubby form (Elliott and Jones, 1990; Olde and Marriott, 1994). In Western Australia, on the Canning Stock Route, a hybrid

between *G. spinosa* McGill. and *G. eriostachya* Lindl. was discovered by Peter Olde and Neil Marriott with confluences up to 30cm long. *Grevillea eriostachya* has also been reported to hybridise with closely related *G. excelsior* Diels. when the two species are within close proximity (Elliott and Jones, 1990).

#### 1.1.6 Commercial hybrids

Despite the low levels of hybridisation evident in the wild, under horticultural conditions, grevilleas hybridise relatively easily. It is possible that under natural conditions, species that have the ability to hybridise under horticultural conditions are spatially or temporally separated in the wild, therefore unable to ever cross pollinate. Because grevilleas hybridise easily, enthusiasts have created numerous varieties of varying quality. As a consequence of this, many of the cultivars available have not undergone testing and evaluation prior to being released into the market. Elliot and Jones (1990) have suggested a need to develop a planned breeding program to produce high quality hybrids that have advantages or colour variation not found in one or both parents.

There are over 100 *Grevillea* cultivars registered with many more available on the market. One of the most common known cultivars is 'Robyn Gordon', an artificial hybrid between *G. bipinnatifida* R. Br and *G. banksii* R. Br., developed by David Gordon. He also developed the cultivar 'Sandra Gordon' (Wrigley and Fagg, 1989). Another well known group of cultivars have been developed by Leo Hodge and are commercially available under the label of 'Poorinda' (Anon, 1977; Wrigley and Fagg, 1989).

### **1.1.7 *Grevillea* pollen morphology**

*Grevillea* produces triangular, triporate and binucleate pollen. Most pollen grains have three circular apertures located at the apex of the triporate grain. Occasionally a few individual pollen grains (within a species) are tetraporate. Tetraporate grains have a circular aperture located at each of the grains apices. Pollen tubes only grow from the apertures. The exine of viable pollen grains is rough and pitted, with smooth apertures.

In general, binucleate pollen has a higher storage potential due to a slower respiration rate compared to trinucleate pollen. Brewbaker (1967) and Dajoz *et al.* (1992) suggest that pollen grains with three apertures are longer lived than those with four apertures. This is related to the reduced thickness of the pollen wall around apertures which makes the pollen grain vulnerable to water stress.

### **1.2 Factors that influence pollen viability**

In any species, there are several main factors that relate to the viability of pollen once it has been produced by the plant. These include: the time of anthesis, desiccation and subsequent rehydration after anthesis, temperature before and after anthesis and, for the purpose of hybridization, storage.

Despite the attention pollination experiments have received over the past few decades, few have taken into account the age of pollen used. Some studies mention that pollen was freshly collected, but fail to mention how long the pollen grains had been exposed to the external environment. An assessment by Stone *et al.* (1995) revealed that of the 283 articles surveyed, only 14% mentioned the use of fresh pollen, or pollen collected from newly opened flowers. Kalinganire *et al.* (2000) is one of the only studies on Proteaceae pollen to investigate the viability of pollen pre- and

post- anthesis, while remaining on the stigma. Other studies to observe pollen pre and post anthesis are mainly on crops, investigating the effect of high temperature stress on the pollen (Kakani *et al.*, 2002; Young *et al.*, 2004; Kakani *et al.*, 2005; Liu *et al.*, 2006).

The effect of desiccation in regards to *in vitro* pollen germination for Australian flora has not received much attention. Pollen desiccation studies often review the benefits of desiccation before long term storage (Tyagi *et al.*, 1992; Hanna and Towill, 1995; Yan *et al.*, 2001). However, desiccation as a pre-treatment for *in vitro* pollen germination is not frequently reported for species used in horticulture or floriculture. There are, however, many studies on pollen from crop species, particularly those sensitive to desiccation (e.g. rye pollen requires little desiccation to germinate successfully on a receptive stigma). Lin and Dickinson (1984) have linked the desiccation processes in seeds to what might be important in the germination of pollen. Desiccation in seeds can help increase germination when followed by rehydration. It has been suggested that the desiccation stage helps activate germination processes. In some legume and cereal species, seed will not germinate until a desiccation stage has occurred, Lin and Dickinson (1984) suggest it is possible that similar mechanisms apply to pollen.

The effects of temperature on *in vitro* pollen germination has been investigated in many species. Studies in avocado (Loupassaki *et al.*, 1997), tomato (Pressman *et al.*, 2002), mango (Sukhvibul *et al.*, 2000), nashi pear (Rohitha and Klinac, 1994), walnut (Luza *et al.*, 1987; Polito and Weinbaum, 1992), groundnut (Prasad *et al.*, 1999; Kakani *et al.*, 2002) and cotton (Kakani *et al.*, 2005; Liu *et al.*, 2006) have described optimum temperatures, shown variation between cultivars and shown a decrease in pollen tube growth and length with an increase in temperature above the optimum.

To date, there has been no investigation on the effects of temperature on *in vitro* pollen germination in *Grevillea*. Previous studies conducted on Australian plants have investigated temperature in relation to storage. The effects of temperature on pollen storage in Proteaceae has been reviewed by Shchori *et al.* (1992) and Yan *et al.* (2001) for *Banksia* and *Leucadendron* respectively and the effects on *Verticordia* (Myrtaceae) pollen stored at a range of temperatures was investigated by Tyagi *et al.* (1992).

The ability to store pollen while maintaining viability is critical for plant breeding and hybridisation. Species that are separated temporally or spatially are can be cross pollinated if pollen viability is successfully maintained during the allocated storage period (Smith-Huerta and Vasek, 1984; van der Walt and Littlejohn, 1996; Yan *et al.*, 2001).

Pollen longevity and vigour is species dependent under natural conditions, however, most *Grevillea* species loose viability within one to two days of anthesis. Therefore it is necessary to develop techniques to prolong longevity while maintaining viability and two common methods are freezing and drying pollen (Yan *et al.*, 2001). Previous storage experiments on Proteaceae species have been conducted on *Banksia* (Shchori *et al.*, 1992; Maguire and Sedgley, 1997), *Protea* (van der Walt and Littlejohn, 1996) and *Leucadendron* (Yan *et al.*, 2001). *Verticordia* (Myrtaceae) and *Acacia* (Leguminosae: Mimosoideae) pollen have also received attention with results suggesting cold temperatures (-20°C and below) are better for storage than ambient temperatures (Tyagi *et al.*, 1992; Sedgley and Harbard, 1993).

### 1.3 Factors that influence *in vitro* pollen germination

#### 1.3.1 Osmotica used *in vitro*

Pioneering studies into media composition for *in vitro* pollen germination found that specific elements were essential for pollen tube growth such as calcium (Schmucher, 1933, 1934; Johri and Vasil, 1961; Brewbaker and Kwack, 1964; Taylor *et al.*, 1998). Studies that followed showed that these constituents are required in different concentrations for various families (Brewbaker and Kwack, 1963; Kalinganire *et al.*, 2000). Hrabetova and Tupy (1964) found that out of 49 species tested, 41 produced a higher germination rate when sucrose was used as the carbohydrate source (compared to glucose and fructose). They also found that carbon from a sucrose source was taken into lipids and polysaccharides faster than carbon from other sugar sources.

A carbon source within a growth media is essential for pollen tube growth. Whether or not the pollen grain uses external sugar as a source for energy during pollen tube development is still debated (see Johri and Vasil, 1961; Herrero and Hormaza, 1996; Sanchez *et al.*, 2004), but the fact cannot be denied that there is an increase in pollen tube development and growth when a medium has a usable carbohydrate source (Herrero and Hormaza, 1996; Sanchez *et al.*, 2004). Pollen is unable to synthesize its own sugars because, for the majority of species, pollen does not contain chlorophyll. Therefore pollen must either convert stored carbon or absorb sugars from an external source (Johri and Vasil, 1961; Brewbaker and Kwack, 1964).

Sucrose, mannitol and polyethylene glycol (PEG) are osmotica and according to Stone *et al.* (2004) the key factor in obtaining the optimum germination of a pollen grain is the osmolarity of the germination medium. Sucrose is easily absorbed by a pollen grain, compared to other sugars and

is converted to fructose and glucose just prior to germination (Johri and Vasil, 1961; Vasil, 1964). Studies with germination media containing mannitol as a solitary sugar source often report low or zero pollen germination with (Vasil, 1964). PEG is often used in combination with sucrose or as a substitute carbohydrate source in germination media. Previous research has investigated the effects of differing concentrations of PEG on crop species. Generally PEG was found to increase pollen germination in *Nicotiana tabacum* L. and *Brassica* species (Shivanna and Sawhney, 1995; Taylor and Hepler, 1997).

### 1.3.2 Lighting conditions and media constituents

Very few *in vitro* investigations have reported using artificial or natural light during the incubation stage (Vasil, 1964; Taylor *et al.*, 1998), with the majority of experiments germinating pollen in the dark. *Conospermum* and *Verticordia* are two Australian native plants that have had pollen germinated in the dark using *in vitro* techniques (Tyagi *et al.*, 1992; Stone *et al.*, 2004), with the majority of other studies conducted on crop species (Shivanna and Cresti, 1989; Read *et al.*, 1993; Loupassaki *et al.*, 1997; Karapanos *et al.*, 2006).

There is more literature available for the constituents and concentrations of minerals and ions within germination media used for *in vitro* pollen germination as this is predominantly seen as one of the key factors to obtaining optimum germination. Most studies conducted on pollen germination use the standard Brewbaker and Kwack (1964) medium that comprises of 10% sucrose, 100 mg/L boric acid, 300 mg/L calcium nitrate, 200 mg/L magnesium sulphate and 100 mg/L potassium nitrate (Brewbaker and Kwack, 1963). Studies on *Borago officinalis* L. modified this recipe during a series of experiments to obtain optimal pollen germination and pollen tube growth specific to the species (Montaner *et al.*, 2003). The other common medium used for pollen



germination is a modification of the Brewbaker and Kwack medium known as Taylor's medium, consisting of 10% sucrose, 100 mg/L boric acid and 300 mg/L calcium nitrate (Taylor *et al.*, 1998; Stone *et al.*, 2004) with adjustments made to boron and calcium concentrations to cater to a species requirements (Chiang, 1974; Luza and Polito, 1987).

The use of adding a coverslip onto the pollen-medium mixture is commonplace for viability staining techniques such as tetrazolium salts, as it prevents oxygen reacting with the dye (Cook and Stanley, 1960). Taylor *et al.* (1998) placed a coverslip onto the mixture before the incubation period, however, most studies add a coverslip to the mixture just before microscope examination of the germinated grains (Shivanna and Rangaswamy, 1992; Kalinganire *et al.*, 2000). No previous literature has been published regarding the effect on germination when a coverslip is added before incubation.

#### **1.4 Significance of pollen for the development of hybrids**

Jensen and Wetzel (1992) stated "Pollen has a highly significant mission: It must develop a tube and deposit two sperm within the embryo sac", however, this is only half of the mission the male gametophyte must accomplish. The male gametophyte is small in comparison to the megagametophyte and once shed from the anther it must battle environmental extremities and travel to the gynoecium all the while maintaining viability. On arrival at the *Grevillea* stigma, pollen must become lodged into the stigmatic groove and begin the germination process down the style, into the ovary. At any point, the viability of the microgametophyte can be compromised. Extreme temperatures, prolonged desiccation or premature hydration before pollen has reached a receptive stigma may result in the inability for the pollen grain to germinate. Knowing the nutrient and physiological characteristics of pollen is imperative to ensure the success of hybridisation, particularly when two

species are known to be difficult to cross pollinate because of failed pollen germination.

#### 1.4.1. Floriculture / Horticulture

The use of Australian flora in horticulture and floriculture (cut flower industry) has steadily increased worldwide. In Australia, the boom comes in response to water restrictions and the demand for low maintenance landscaping. The natural drought tolerance of Australian species, combined with their tolerance to low nutrients within the soil, makes them ideal candidates for most areas. Australian flora in the cut flower industry holds ornamental value due to their unique form.

Annual wildflower exports amounted to approximately \$30 million in 1999/2000 with the major export markets identified in Japan, USA and the Netherlands (48%, 27% and 11% respectively) (Brooks, 2001; Sutton, 2002). Western Australia was identified as the dominant state for wildflower production and export (between 55 and 18%), followed by Victoria and New South Wales. During 1999/2000, exotic Proteaceae accounted for 11% of the market (Brooks, 2001). Proteaceae do not show high export percentages largely due to post harvest problems such as low flower longevity and fragility, with *Grevillea* being no exception. Economically they are not viable in growth and production. Foliage, however, holds a strong market for *Grevillea*.

Many members of the Proteaceae are cultivated in horticulture, indeed one member, *Macadamia*, produces edible fruit. Some of the more commonly known Australian genera in horticulture are *Banksia*, *Grevillea*, *Conospermum* (Smokebush), *Adenanthos* (Albany woolly bush), *Dryandra*, *Isopogon* (Pixie mops) and *Telopea* (Waratah). Other commonly known species such as *Leucadendron*, *Protea* and *Leucospermum* originate from South Africa.

Plant breeding programs allow cultivators to isolate ideal features from species that will maximise their appeal to a market that is largely subject to the fad of a fashion conscious market (Fuss and Sedgley, 1991a). Breeding programs for Proteaceae species began approximately 35 years ago with the South African *Protea* leading the way. Since then, breeding programs in Australia, America and South Africa have developed some highly valued *Banksia*, *Leucadendron*, *Protea* and to a degree *Grevillea* cultivars (Yan *et al.*, 2001). There is a need for scientific based breeding programmes where plant breeders and geneticists are able to combine technologies in order to replace the element of chance that is the foundation of current breeding programmes.

The need to identify markets wants and requirements (product demand) is essential in the design of a new product or cultivar. *Grevillea* cultivars that have been identified as 'best bet export cut flower crops' for the floriculture industry include G. 'Sylvia' and G. 'Yu-lo' for their colour, terminal flowering form and good performance post harvest (Slater and Carson, 2003). *Grevillea baileyana* McGill. has been identified for its foliage potential.

#### **1.4.2 Brief history of *Grevillea* horticulture**

Possibly the most widely cultivated of the Proteaceae, *Grevillea* has great potential for the horticulture market. Many species have been cultivated from wild populations / stock without any need for further breeding. *Grevillea fililoba* (McGill.) Olde & Marriott, a species native to Geraldton, is marketed under the name 'Ellendale Pool', *G. rhyolitica* Makinson is marketed as the 'Deua Flame Grevillea' named after its distribution through Deua National Park in NSW.

The question of who introduced *Grevillea* into cultivation remains debatable. However, most evidence suggests William Paterson, a Captain

who arrived in Australia in 1791 and an avid botanist, is recognized for introducing *G. buxifolia* (Sm.) R. Br., the first *Grevillea* to flower in cultivation in Britain during 1795 (Olde and Marroitt, 1994). *Grevillea* seeds were collected in Australia by commissioned or passionate botanists and sent to nurserymen in England and Europe where they were germinated. Live transportation of plant specimens was also conducted, however, many died on route due to lack of water, fungal attack and increased levels of salt from the sea spray. In Europe and England all members of the Proteaceae declined in popularity after the mid 1830's since they were difficult to propagate and grow, and by 1900 only the most enthusiastic botanists continued their propagation and cultivation (Olde and Marriott, 1994). Those species that survived both the world wars can still be found scattered around the Botanical Gardens of England and Europe.

In Australia, *G. hilliana* F. Muell. and *G. robusta* A. Cunn. were among the first grevilleas to be cultivated and sold as ornamentals during the early 1800's. *Grevillea robusta* was the first species to flower in cultivation in Sydney during 1835. Australia followed European trends and Proteaceae popularity waned from the mid 1800's through to the mid 1900's. However, via enthusiasts like Leo Hodge and David Gordon, grevilleas have made a come back in the horticultural industry.

*Grevillea rosmarinifolia* A. Cunn. was classified as extinct in the wild until a specimen was found in the Royal Botanic Gardens in Edinburgh. Cuttings were transported back to Australia and successfully propagated. Ironically, it's now used for grafting as root stock and is a parent of many beautiful hybrids (eg: Canberra Gem)

### 4.1.3 Cultivation

Grevilleas respond well to cuttings and grafting. Using seed for propagation is not common due to problems with collection, germination and the requirement of pre-treatments before planting. Most species have evolved chemical and physical barriers that ensure survival of the seed until appropriate conditions occur (Elliot and Jones, 1990; Olde and Marriott, 1994).

However, plants germinated from seed develop a deep tap root which is a desirable trait for Australian conditions (dry climate) as these plants are more drought resistant compared to plants grown from cuttings (Olde and Marriot, 1994). Most importantly when plants are grown from seed, genetic diversity increases. This is important when rehabilitating areas and maintains genetic difference within the species.

During hybrid development, using cross pollination and seed germination ensures variation in each plant's characteristics due to genetic differences. For example, the cultivar 'Bonnie Prince Charles' has the same parents (*G. alpina* Lindl. and *G. rosmarinifolia*) as 'Austraflora McDonald Park'. Both cultivars look very similar in flower colour, but 'Bonnie Prince Charles' is more compact in form and has larger flowers and leaves than 'Austraflora McDonald Park' (Elliot and Jones, 1990).

### 1.4.4 Conservation

Amazingly, despite Australia's many environmental problems threatening the native flora, no *Grevillea* to date has been classified as extinct. One species, *G. batrachioides* F. Muell. ex McGill. was presumed extinct as it had last been collected in 1850. However, a small population was found in 1991 near Mt Lesueur (Makinson, 2000). Olde and Marriott (1994) list 78 *Grevillea* species as endangered, 23 of which are found in

WA. A further 48 species are classified as vulnerable, with 81 *Grevillea* species classified as rare, but not threatened.

The grevilleas are clearly of economic value and have also been identified as a group that may be threatened by future land development with many wild populations rapidly decreasing. *Grevillea rara* Olde & Marriott. was last recorded in jarrah forest just north of Collie, however, the current existence of this small population is not known with most individuals known only in cultivation (Olde and Marriott, 1995; Makinson, 2000). *Grevillea scapigera* A. S. George. has five individuals known in the wild. Introductions of ramets (tissue cultured clones) to the population began in 1996 to help increase outcrossing and seed production (Krauss *et al.*, 2002). For these reasons, understanding some of the basic aspects of the reproductive biology of *Grevillea* would be very useful for both its conservation would aid in the development of its ornamental value.

## 5.1 Aims

This project was established to examine different aspects of the requirements for *in vitro* germination of *Grevillea* pollen that could ultimately assist in the development of the group in horticulture, as well as aid conservation programs. In particular, this study aimed to determine the:

- ideal laboratory conditions to germinate *Grevillea* pollen using *in vitro* techniques,
- ideal greenhouse conditions to germinate *Grevillea* pollen during hybridisation (*in vivo*) using *in vitro* techniques,
- storage potential of *Grevillea* pollen and
- potential for inter-hybridisation between *Grevillea* 'groups'.

## CHAPTER 2: GENERAL MATERIALS AND METHODS

### 2.1 Plant material and maintenance

Plants were purchased from two local nurseries, Zanthorrea Nursery and Lullfitz Nursery. All *G. rhyolitica* plants were purchased during January 2006 and *G. fililoba*, *G. pinaster* Meisn., *G. preissii* Meisn., *G. saccata* Benth. and *G. thelemanniana* Hügel ex Endl. were purchased during July, August and September 2006. Plants were transplanted into 250 mm pots containing Balieys® premium potting mix and staked to provide support and easier access to the inflorescences.

All plants were maintained in a greenhouse at Edith Cowan University under natural light conditions. Temperatures ranged from 20 °C to 30 °C during the experiment.

Plants had dead flowers and inflorescences removed monthly. *Grevillea rhyolitica* plants were infested with mealy bug during February and were sprayed twice with the commercial insecticide Confidor®. Several plants had caterpillars and these were treated with an organic insecticide, Beat-a-Bug® or removed by hand when sighted. Any flowers with caterpillar damage were immediately removed.

During June 2006 *G. rhyolitica* plants were treated with a systemic fungicide (Pevicure®) due to the loss of several plants possibly caused by a fungal infection. Mancozeb plus® was applied at ten day intervals between June and August 2006 until the fungal infection symptoms cleared.

All *Grevillea* plants received weekly applications of Polyfeed® to increase flowering, however, due to the reduced nitrogen content of this

liquid fertilizer, monthly applications of Thive® was used to prevent chlorosis.

## **2.2 Pollen collection and storage**

For all laboratory experiments, pollen was collected on the day of anthesis and used on the same day to ensure freshness. All dead and open flowers were removed from plants using fine scissors one day before experimentation. The remaining closed flowers were left in the greenhouse overnight to open. This was done to determine how long pollen had been present on the pollen presenter. Inflorescences that had newly opened flowers were taken immediately to the laboratory.

All field collected pollen was obtained from the Roe Gardens within Kings Park Botanical Gardens, Perth, Western Australia. Using a scalpel blade, pollen was scraped from the pollen presenter of several flowers and placed into an Eppendorf tube. One tube was used per plant so as not to mix pollen or the different genotypes of individuals. Day, time of pollen collection, botanical and common plant name, location and the number of flowers collected were recorded as well as an identification number for individual plants. Pollen was placed into a cooler bag and transported to Edith Cowan University (Joondalup Campus) and stored at -20 °C without desiccation for 6-7 months depending on the collection date.

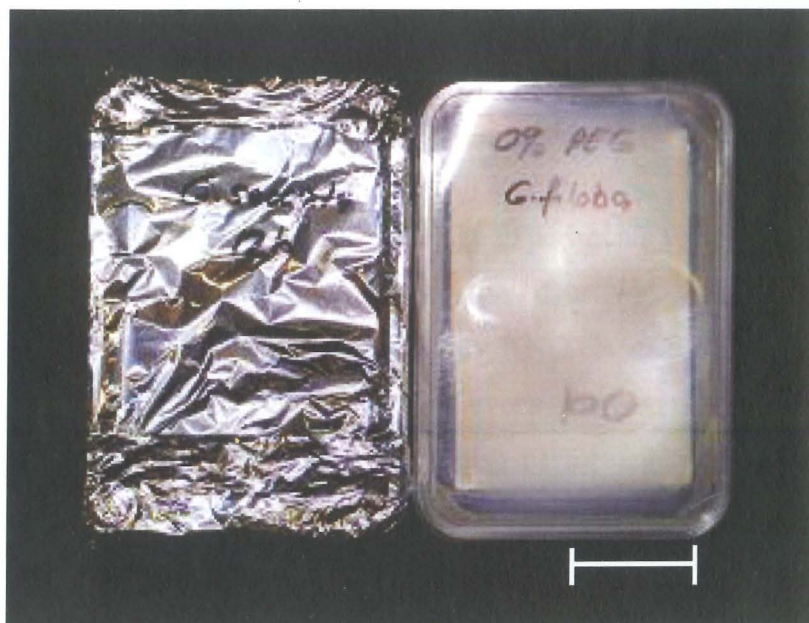
## **2.3 *In vitro* germination**

Three drops of either modified Brewbaker and Kwack or Taylor's medium was placed onto a microscope slide. One hundred milliliters of modified Brewbaker and Kwack (1963) medium was made up of 100 mg/L of boric acid, 300 mg/L calcium nitrate, 200 mg/L magnesium sulphate, 100 mg/L potassium nitrate and 20% sucrose. The modified Taylor's medium consisted of 100 mg/L boric acid and 300mg/L calcium



chloride di-hydrate and 20% sucrose (Brewbaker and Kwack, 1963; Taylor *et al.*, 1998; Stone *et al.*, 2004).

Pollen from one *Grevillea* species was placed into the drop and mixed using a dissecting needle to ensure a uniform distribution of the pollen. Each slide contained pollen from only one flower and a total of five slides (replicates) per treatment were placed into a plastic container (16.5cm x 10.5cm x 3.5cm). A sheet of moist paper towel was placed on the bottom of the container to maintain humidity. Treatments that required dark conditions were wrapped in aluminum foil (Fig 1). All treatments were germinated at  $24 \pm 1^\circ\text{C}$  (unless otherwise stated) for 48 hours, after which pollen was scored.



**Figure 1:** Set up for *in vitro* pollen germination both dark and light treatments. Paper towel was placed on the bottom of the plastic container (16.5cm x 10.5cm x 3.5cm) for both conditions and sealed, aluminum foil was used to create a dark environment for pollen germinated in the dark. Bar indicates 1 cm.

Slides were viewed at x100 magnification on a Leica DMLB compound microscope using dark field illumination. The field of view was randomly shifted 10 times from left to right to avoid scoring the same area twice (Shivanna and Rangaswamy, 1992; Kalinganire *et al.*, 2000). The total number of grains within each field of view was counted along with those displaying pollen tube development and any sterile grains. A pollen grain was considered to have germinated when its tube length was at least the same as the grain's diameter (in accordance to Shivanna and Rangaswamy (1992)). Pollen tubes that had broken away from the pollen grain were not included in the count. Sterile grains were either clear as opposed to semi-transparent or were small and considered immature, therefore incapable of germinating. No coverslip was applied (unless stated) before viewing slides as this had a tendency to break pollen tubes from the pollen grain.

## **2.4 Statistical Analysis**

All data from the *in vitro* and storage experiments were transformed using an Arcsine transformation because data was collected as a percentage then analysed using a one way ANOVA using SPSS version 11.0 with descriptive statistics and Levene homogeneity test. Non parametric tests (Kruskal-Wallis) were also performed (and reported in results) on all data as treatments were generally not homogeneous according to Levene's test.

## CHAPTER 3: POLLEN CHARACTERISTICS

### 3.1 Introduction

Before a pollen grain lands on a receptive stigma it must have undergone sufficient dehydration for its successful germination with its water content being dependent on how long the grain has been exposed to the external environment. After anthesis abiotic factors such as wind, humidity and temperature dehydrate pollen and Lin and Dickinson (1984) suggested that it may be a prerequisite for pollen to undergo desiccation before germination as it is often an important process in seed germination. Heslop-Harrison (1979) suggested that dehydration is important to lower the water potential of the pollen grain to increase water flow and subsequent rehydration when it lands on a receptive stigma or is placed onto germination medium. Hence to reach optimal germination, pollen must undergo species specific dehydration. However, excessive water removal can be detrimental to a pollen grain if cell membranes are damaged. During desiccation the cytosolic fluid converts to a gel-like substance and upon rehydration it is converted back to a liquid form via enzyme processes (Hoekstra *et al.*, 1992; Piffanelli *et al.*, 1998). It is thought that severe dehydration damages cellular membranes, possibly causing leakage which prevents water absorption. Turgidity is imperative for germination and if a pollen grain cannot absorb sufficient water to rehydrate, become turgid and convert the intracellular fluid to a liquid again, the grain becomes sterile (Hoekstra *et al.*, 1992).

No previous studies have been conducted on the effects of desiccation on *Grevillea* pollen prior to *in vitro* germination to determine the tolerance level of the genus. The longevity of pollen, or how long it can remain on the pollen presenter after anthesis, while retaining viability,

has been investigated for several Proteaceae species including *Grevillea* (Vaughton and Ramsey, 1991; Smith and Gross, 2002). Longevity also appears to be species specific and has been investigated by Kalinganire *et al.* (2000) for *G. robusta* pollen only. Temperature aids in the natural desiccation process (on the anther) with high temperatures increasing water loss from the pollen grain. This would be expected to alter the longevity of pollen and has not previously been investigated for *Grevillea*.

### **3.1.1 Aims**

The aims of the work reported in this chapter relate to factors that may affect pollen desiccation specifically to determine:

- when *Grevillea* pollen has the highest ability to germinate both pre- and post- anthesis,
- the optimum temperature *Grevillea* pollen requires for *in vitro* germination,
- if *Grevillea* pollen requires artificial desiccation prior to *in vitro* germination and
- the storage potential of untreated pollen collected from several *Grevillea* species.

## **3.2 Materials and Methods**

### **3.2.1 Anthesis**

To examine anthesis, *Grevillea* plants were prepared in the glasshouse by removing all dead and open flowers as described in chapter 2. Newly opened flowers from *G. fililoba*, *G. preissii*, *G. rhyolitica*, *G. saccata* and *G. thelemanniana* were culled to one or two per inflorescence with at least 10 flowers remaining on each plant. Pollen was collected one day before anthesis (-1), on the day of anthesis (0), 1, 2 and 3 days after anthesis (Kalinganire *et al.*, 2000). Flowers that opened overnight were

classified as day 0 or at anthesis and were collected immediately. Flowers taken from one row or whorl below the day 0 flowers during the late looping stage (Fig 2) were classified as 1 day pre anthesis and these were also collected immediately. All remaining pollen was removed on the corresponding day i.e. 1, 2 or 3 days after anthesis (Kalinganire *et al.*, 2000). Pollen was germinated *in vitro* in the dark without a coverslip for 48 hours on Brewbaker and Kwack as described in chapter 2.

### **3.2.2 Effect of desiccation on pollen germination**

To examine the effects of pollen desiccation, flowers were removed from *G. fililoba*, *G. preissii*, *G. rhyolitica*, *G. saccata* and *G. thelemanniana* on the day of anthesis as previously described, and placed into individual Eppendorf tubes. Lids were cut off from the tube using fine scissors to ensure all samples of pollen were able to desiccate evenly. Tubes containing flowers were desiccated over silica gel for 0, 12, 24, 36 and 48 hours inside a sealed plastic container (16.5cm x 10.5cm x 3.5cm) at room temperature (Tyagi *et al.*, 1992; Yan *et al.*, 2001). After desiccation flowers were removed from the Eppendorf tube and pollen was germinated on Brewbaker and Kwack medium for 48 hours and scored as described in chapter 2.

### **3.2.3 Effect of temperature on pollen germination**

To examine the effect of temperature, five flowers per treatment were collected from *G. fililoba*, *G. preissii*, *G. rhyolitica*, *G. saccata* and *G. thelemanniana* on the day of anthesis and pollen germinated at 15, 20, 22.5, 25, 27.5, 30 and 35°C. Clayson incubation cabinets were set three hours prior to the experiment to allow temperatures to stabilize. Treatments were germinated in the dark without a coverslip for 48 hours. *In vitro* germination of pollen was conducted on Brewbaker and Kwack media as described above in chapter 2, at the selected temperatures. Pollen was scored as described in chapter 2.

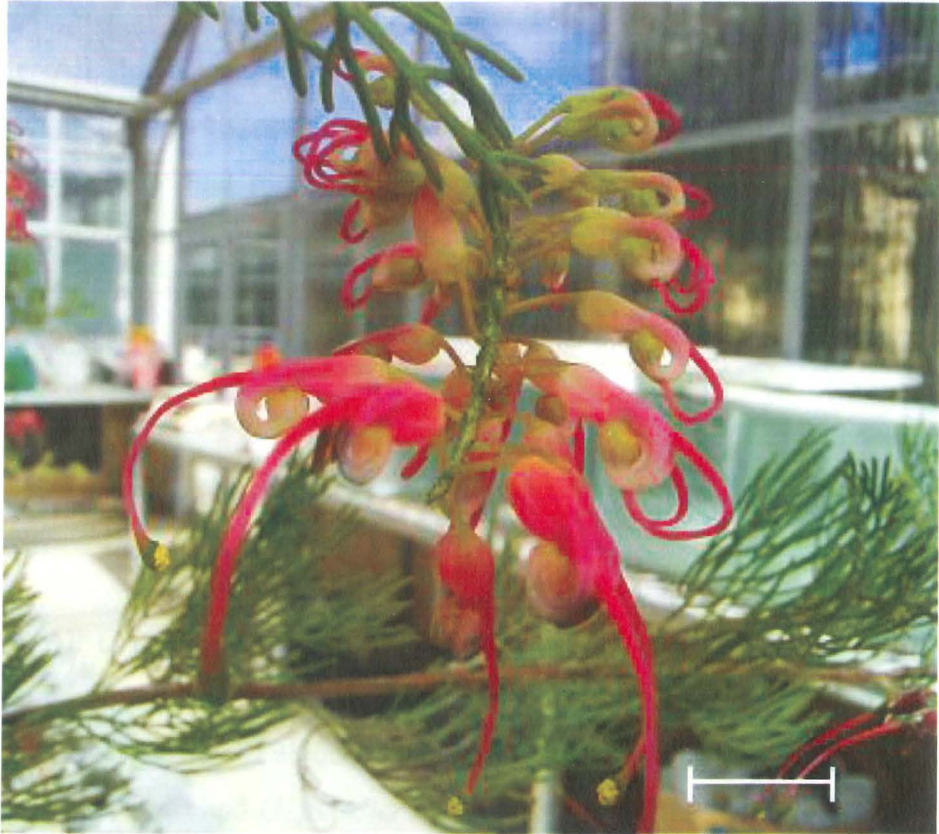


Figure 2: *Grevillea fililoba* conflorescence displaying the day of anthesis (0) flowers with pollen on the pollen presenter ready for collection. Flowers in the late looping stage are one whorl above newly opened flowers and these are classified as one day pre- anthesis (-1). Scale represents 1cm.

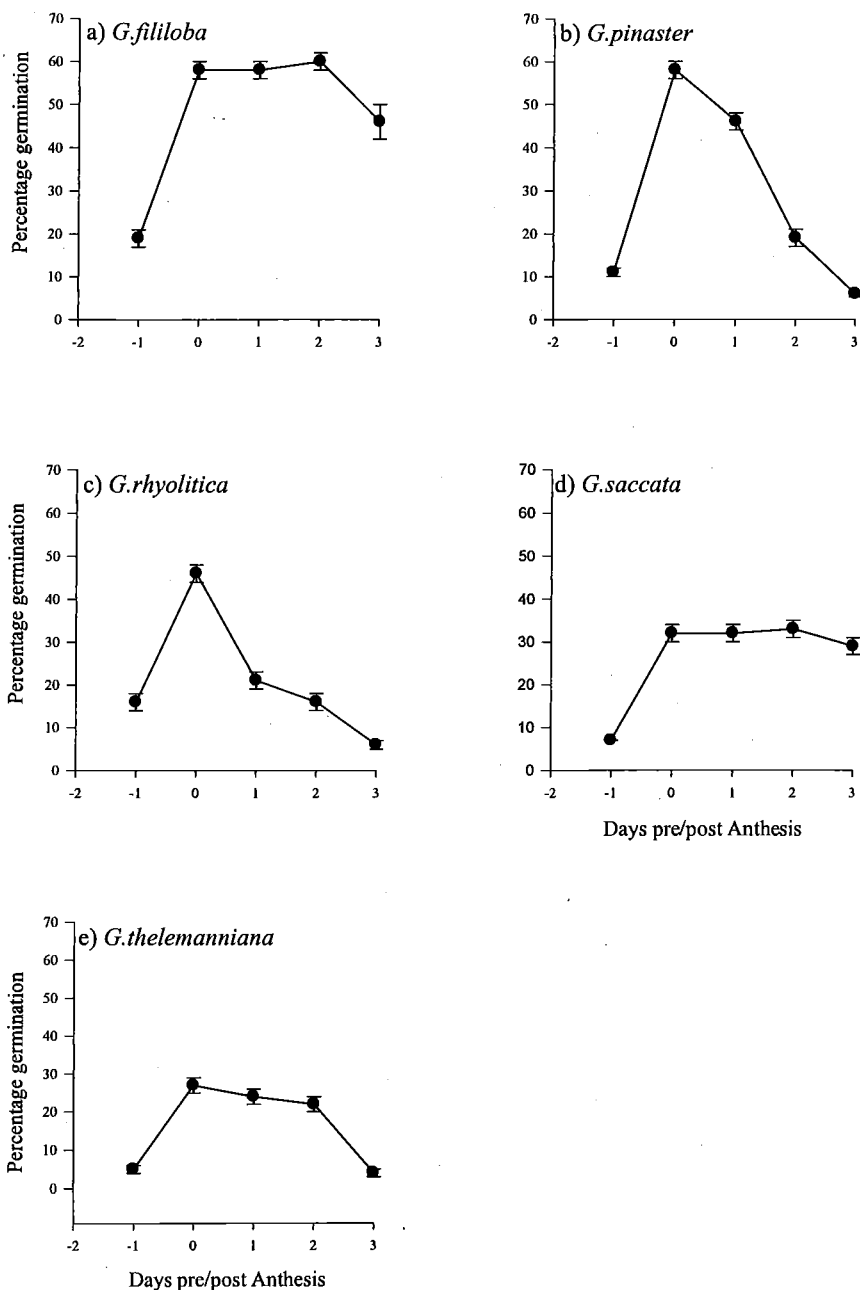
### 3.2.4 Storage potential of *Grevillea* pollen

To test for storage potential, pollen was collected as described in chapter 2, from Kings Park (Roe Gardens) during January/February 2006. Pollen was stored at -20 °C for 6-7 months depending on the collection date. All stored samples were germinated *in vitro* during August 2006. Fresh pollen was collected from the same location in August 2006. Where possible, individual plants that were collected during January/February were re-collected in August using the same technique. Fresh pollen samples were immediately germinated *in vitro* on arrival at ECU for 48 hours and scored as previously described in chapter 2.

## 3.3 Results

### 3.3.1 Anthesis

All *Grevillea* species showed a statistically significant ( $P = <0.001$ ) in response to anthesis with a peak in germination value when pollen was collected on the day of anthesis (0). Germination was lower on other days. *Grevillea fililoba* and *G. pinaster* had the highest germination at 58% and *G. pinaster* displayed the sharpest decline from 46% to 19% between 1 and 2 days after anthesis. *Grevillea rhyolitica* germination also declined from 46% to 21% between 0 and 1 day post anthesis. *Grevillea fililoba* and *G. thelemanniana* displayed a less dramatic decline in germination between 0-2 days post anthesis with germination decreasing rapidly thereafter (Fig 3). *Grevillea saccata* displayed a distinctly different trend with no difference in the germination between the day of anthesis and 3 days post anthesis (32, 32, 33 and 29% respectively). All germination was below 20% 1 day pre anthesis (-1), with all species increasing to between 27-46% the day of anthesis (0). The largest increase from -1 day to day 0 was displayed by *G. pinaster* from 11% to 58% germination.



**Figure 3:** *In vitro* germination rates for pollen collected 1 day before to 3 days post- anthesis (-1, 0, 1, 2 and 3 days anthesis). Pollen was germinated under dark conditions at  $24 \pm 1$  °C using Brewbaker and Kwack media without coverslips. Mean percentages are based on 50 replicates and error bars represent standard error.



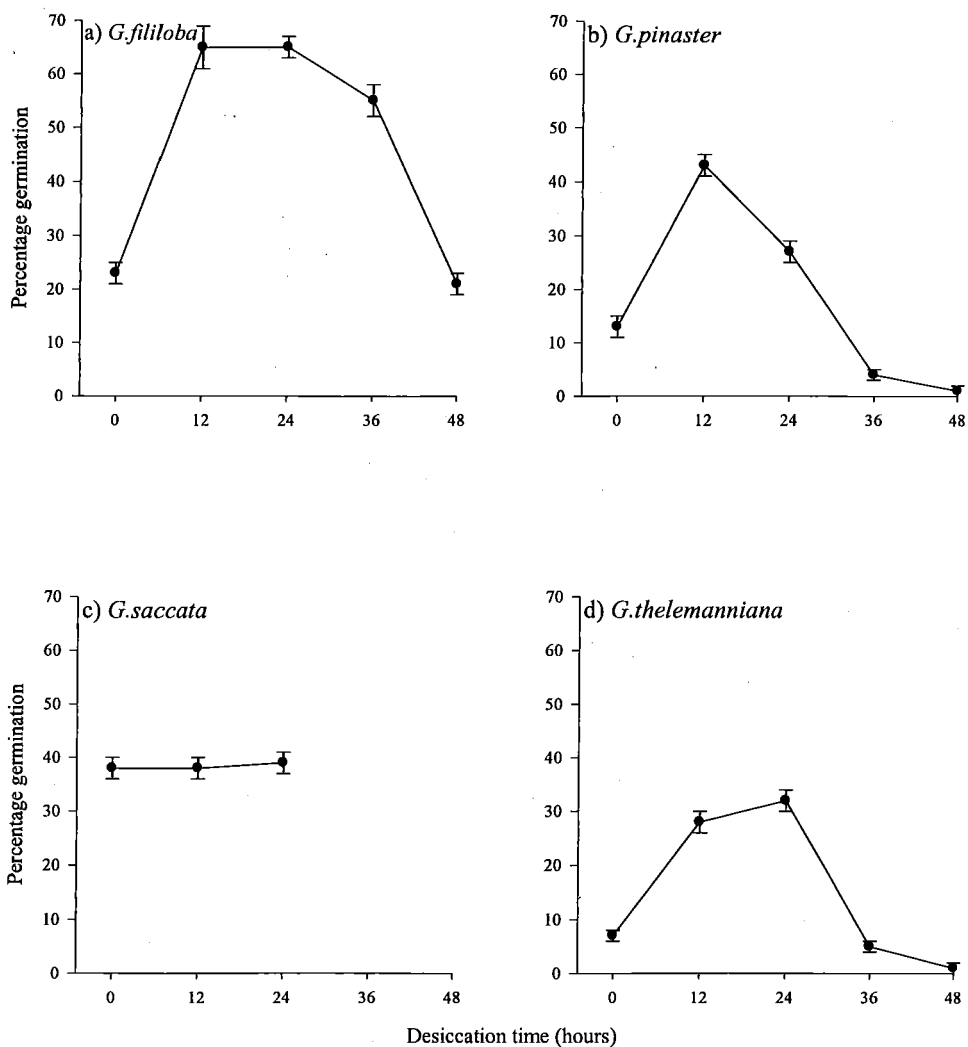
### 3.3.2 Effect of desiccation on pollen germination

*Grevillea fililoba* and *G. pinaster* displayed a significant difference ( $P < 0.001$ ) in response to desiccation prior to *in vitro* germination with all three species having highest germination at 12 or 24 hours of desiccation (Fig 4). *Grevillea saccata*, however, did not show a difference ( $P = 0.911$ ) over a 24 hour desiccation period. *Grevillea fililoba* showed the highest germination of 65% for both 12 and 24 hours desiccation while the lowest germination value was 1% for *G. thelemanniana* at 48 hours of desiccation. For species where desiccation produced a significant change, peak germination occurred when pollen was desiccated for either 12 or 24 hours. *Grevillea fililoba* germination peaked at 65% after 12 hours of desiccation and this remained unchanged after 24 hours; *G. pinaster* had its highest germination of 43% after 12 hours of desiccation, and *G. thelemanniana* required between 12-24 hours of desiccation to reach its highest germination of 32%.

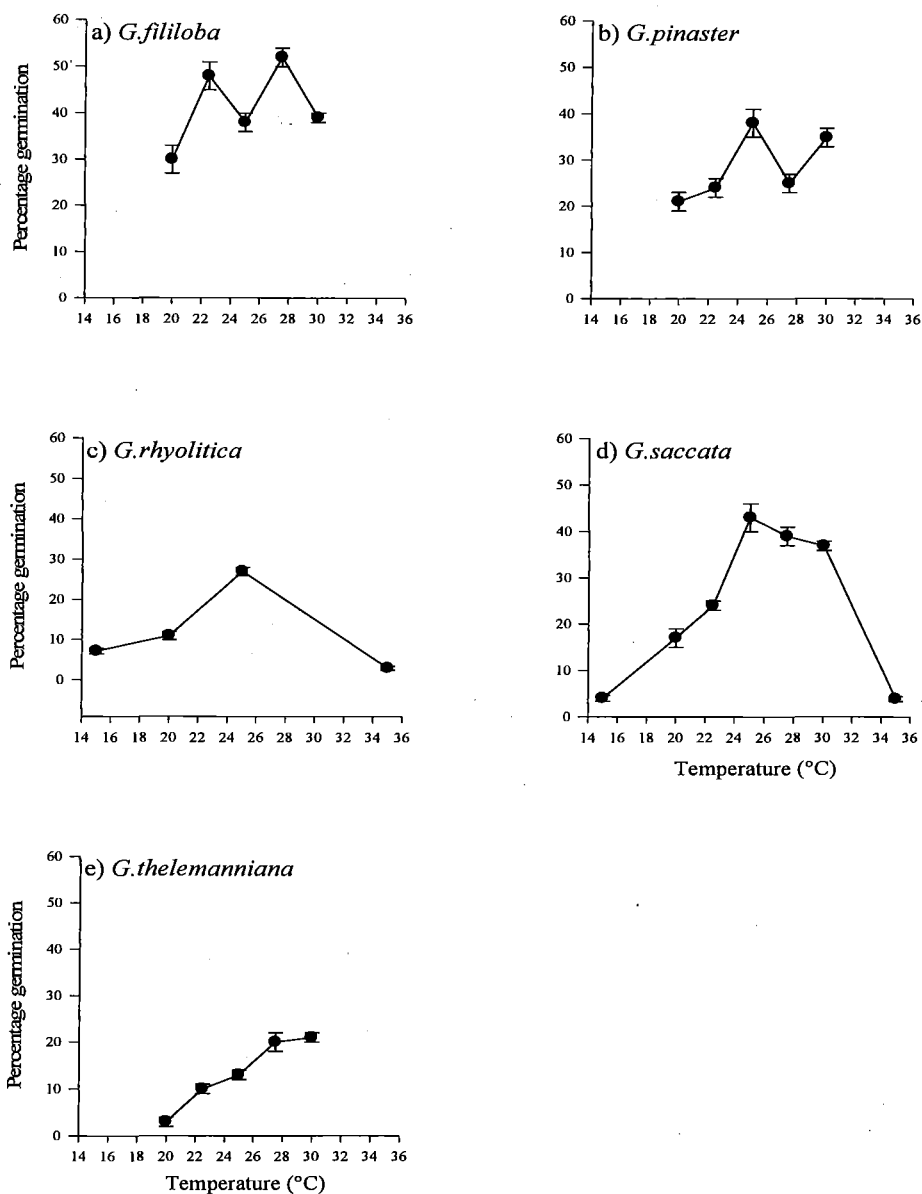
*Grevillea saccata* did not show a significant difference in pollen germination when subjected to desiccation prior to *in vitro* germination. It showed little variation over the 24 hour desiccation period and was only desiccated for only 24 hours due to a limited supply of pollen.

### 3.3.3 Effect of temperature on pollen germination

Pollen germination in response to temperature was statistically different ( $P < 0.001$ ) for each *Grevillea* species with peak germination of 38%, 27% and 43% recorded at 25°C for *G. pinaster*, *G. rhyolitica* and *G. saccata* respectively. *Grevillea fililoba*, however, experienced peak germination at 27.5°C with 52% and *G. thelemanniana* displayed its highest germination of 21% at 30°C (Fig 5). *Grevillea thelemanniana* did not display any decrease over the selected temperature range and had its highest germination at 30°C. *Grevillea rhyolitica* and *G. saccata* showed



**Figure 4:** Mean percentage pollen desiccated at 0, 12, 24, 36 and 48 hours before *in vitro* germination. Pollen was collected at the day of anthesis, and germinated under dark conditions at  $24 \pm 1$  °C using Brewbaker and Kwack media without coverslips. Mean percentages are based on 50 replicates and error bars represent standard error. (NB: did not include data for 36 and 48 hours of desiccation for *G. saccata*).



**Figure 5:** Mean percentage of *in vitro* germination for pollen germinated at various temperatures (15, 20, 22.5, 25, 27.5, 30 and 35 °C). *Grevillea* pollen was germinated for 48 hours in Brewbaker and Kwack medium, under dark conditions without coverslips. Mean percentages are based on 50 replicates, error bars represent standard error.

a decrease in germination after 25°C. Trends were difficult to distinguish for *G. fililoba* and *G. pinaster* due to apparent fluctuations in germination rate between 22.5 and 30°C.

#### 3.3.4 Storage potential of *Grevillea* pollen

All *Grevillea* species collected except *G. baxeri* R. Br., showed a significant difference ( $P < 0.001$ ) between freshly collected pollen germinated *in vitro* compared to pollen that was collected and stored for approximately seven months before germination. There was also a difference in germination between individual plants (Table 1).

Both *G. macrostylis* F. Muell. individuals displayed the highest germination for freshly harvested pollen with no significant difference ( $P = 0.426$ ) between *G. macrostylis* 1 at 68% and *G. macrostylis* 2 at 66% (Table 1). The species with the lowest germination for fresh pollen was *G. baxeri* 2 at 10%. *Grevillea macrostylis* also had the highest rate of germination (16%) while *G. patentiloba* subsp. *platypoda* (F. Muell.) Olde & Marriott had the lowest germination of <1%.

Pollen collected from two *G. baxeri* individuals (*G. baxeri* 1 and *G. baxeri* 2) showed no significant difference between the germination of stored pollen when compared fresh pollen ( $P = 0.065$  and  $0.063$  respectively). Germination for stored pollen was 13% for both *G. baxeri* individuals while *G. baxeri* 1 displayed a slight increase to 15% germination when fresh pollen was used and *G. baxeri* 2 showed a small decrease in germination of 10% which was found to be significantly different ( $P < 0.001$ ).

There was a significant difference ( $P < 0.001$ ) in germination between individual plants of *G. batrachioides* for both stored and fresh pollen. *Grevillea baxeri* only showed a difference for freshly collected pollen

Table 1

The mean germination (*in vitro*) of stored and fresh pollen for Grevillea species collected from Kings Park, Roe Gardens between January – February 2006 and August 2006.

<i>Grevillea</i> Species	Germination (%) of stored pollen (approx 7 months)	Germination (%) of fresh pollen
<i>G.armigera</i>	1.37	13.90
<i>G.asparagoides</i>	1.88	33.30
<i>G.batrachoides</i> 1	1.55	34.40
<i>G.batrachoides</i> 2	4.14	20.99
<i>G.baxeri</i> 1	12.89	15.39
<i>G. baxeri</i> 2	12.67	9.59
<i>G.bronwenae</i>	1.10	12.35
<i>G. dryandroides</i> subsp. <i>dryandroides</i>	2.95	51.88
<i>G.eriostachya</i>	2.77	20.57
<i>G.leucopteris</i>	0.49	33.06
<i>G.macrostylis</i> 1	15.92	68.47
<i>G.macrostylis</i> 2	1.61	65.57
<i>G.patentiloba</i> subsp. <i>platypoda</i>	0.18	22.85
<i>G.saccata</i>	6.65	42.88

( $P < 0.001$ ) with no significant difference between individual plants for stored pollen ( $P = 0.751$ ) and *G. macrostylis* displaying a significant difference ( $P < 0.001$ ) between stored pollen germination for individuals.

### 3.3 Discussion

#### 3.3.1 Anthesis

Like many Proteaceae, grevilleas are protandrous where the stigma becomes receptive after pollen has matured. Most species must wait for pollen to be removed to initiate stigma receptivity and have longevity of pollen to match the timing of such processes (Collins and Rebelo, 1987). Generally, a reduction in pollinator activity or efficiency in removal of pollen will result in an increase in the longevity of the pollen grain (Dafni and Firmage, 2000). For example, a study with two *Banksia* species indicated *B. menziesii* R. Br. pollinators to be efficient in the removal of pollen from the presenter, however, pollen displayed a severe decrease in viability within 24 hours of anthesis. When pollinator activity was low and often incomplete, as is the case of *B. spinulosa* Smith var. *neoanglica* A. S. George, pollen displayed a greater longevity and remained viable for over a week (Ramsey and Vaughton, 1991). While pollinator activity of the four *Grevillea* species was not investigated in this study, further investigation into their ability to remove pollen may explain why *G. fililoba*, *G. pinaster*, *G. thelemanniana* and *G. rhyolitica* did not maintain the longevity displayed by *G. saccata*. If pollen presenter morphology can be related to a species' pollinator (Collins and Rebelo, 1987), then the similarities between *G. fililoba*, *G. pinaster*, *G. thelemanniana* and *G. rhyolitica* with their long styles and a convex stigma enabling easier access of pollinators to pollen, may also explain the similarities in their pollen viability post anthesis. *Grevillea saccata* has a very short, concave pollen presenter that does not extend far from the perianth.

Pollen may be difficult to remove completely in one visit, requiring multiple visits from pollinators. Further studies into pollinator behaviour based on morphology and pollen longevity would be required to confirm this.

Dehydration may be required for some *Grevillea* species to reach optimal germination. For example *G. robusta* experienced peak germination at one day post anthesis (Kalinganire *et al.*, 2000). This suggests that between the time when pollen is first exposed to the external environment and one day after this event, enough water has been removed to initiate germination (Lin and Dickinson, 1984). If this is so, pollen from *G. fililoba*, *G. pinaster*, *G. thelemanniana* and *G. rhyolitica* would require a short period of desiccation, between the day of anthesis and one day post anthesis. Any longer than this and pollen may become severely dehydrated and unable to germinate. The time required after anthesis for pollen to display maximum germination may be attributed to the morphological differences of pollen between species. All species studied have a triangular, triporate pollen shape with three apertures, therefore, they have similar areas from which to lose water. However, differences in exine thickness may explain why some *Grevillea* species, such as *G. saccata*, are able to maintain the ability to germinate over a period of days while others lose viability within a day. *Grevillea saccata* pollen was the largest studied, this is generally related to a thicker exine and thus greater protection from natural desiccation.

### **3.3.2 Pollen desiccation**

While 12-24 hours of desiccation showed a peak in pollen germination for *G. fililoba*, *G. pinaster* and *G. thelemanniana*, desiccation did not exhibit a higher germination than that recorded at the day of anthesis (0). This may be due to a difference in the water content within pollen desiccated naturally on the presenter or artificially in the laboratory using silica gel beads. Water

content has been shown to affect pollen germination of most crop species, specifically legumes and cereal crops with temperature, humidity and morphology affecting how quickly water is lost from the pollen grain (Lin and Dickinson, 1984). It must also be considered that natural desiccation for this study was an estimate of time in the form of days. Laboratory desiccation was an exact measurement of time in the form of hours. Therefore direct comparisons from one day to 24 hours must be made tentatively and this may further explain why there was no increase in germination between the treatments.

When pollen is released from the anther, it is partially dehydrated. The level of dehydration of pollen on release is species-dependent, ranging from 6-60% (Taylor and Hepler, 1997). The level of desiccation required before pollen is able to germinate is also species-dependent. It may have been beneficial to measure the moisture content of the pollen to help determine the optimal water content of germination and at what moisture percentage *Grevillea* pollen switches into a germination phase. For example, Easter lily pollen, that had a moisture content of 15% or less, displayed the highest pollen germination percentage whether pollen was desiccated naturally on the anther or artificially (Lin and Dickinson, 1984).

Results from this study are in accordance with those found by Lin and Dickinson (1984) in other species and, in addition some *Grevillea* species responded to desiccation more than others. Easter lily pollen collected on the day of anthesis and desiccated for 24 hours was found to increase pollen germination (Lin and Dickinson, 1984). *Grevillea fililoba*, *G. pinaster* and *G. thelemanniana* had a higher germination value after 12-24 hours of desiccation. Corn pollen, however, displayed little change in germination when desiccated for one hour compared to fresh pollen, 80-73% respectively (Lin and Dickinson, 1984) as does walnut pollen when desiccated over a



longer time period with desiccation having little effect on subsequent pollen germination (Luza and Polito, 1987). This was found to be the case with *G. saccata* when germinated over a 24 hour period and it may be suggested that *G. saccata* pollen does not require desiccation to increase *in vitro* germination.

There is evidence from previous studies that exposure of pollen to bulk water immediately after desiccation may cause 'imbibition damage' where a surge in water and ion concentration due to an increase in the osmotic potential alters membrane integrity (Taylor and Hepler, 1997). Whether this is caused by an increase in enzyme activity, leading to an incomplete or altered reconstruction of membrane lipid bilayers, or an incomplete transition from the gel phase into the liquid crystalline phase required for germination is not known (Hoekstra *et al.*, 1992; Taylor and Hepler, 1997). The results from this study suggest that *Grevillea* pollen was able to show germination after all time periods of desiccation without the need to be rehydrated slowly. This suggests that *Grevillea* pollen may not display a vulnerability to imbibition damage caused from dehydration. Further investigation would increase knowledge as to what mechanism *Grevillea* pollen exhibits that imbibition sensitive species do not.

### **3.3.3 Effect of temperature on pollen germination**

The distribution of the four West Australian *Grevillea* species ranges from the Geraldton Sandplains down through the Swan Coastal Plains and out towards the Avon Wheatbelt. All species flower from winter to spring. Olde and Marriott (1995a and 1995b) described the summer conditions of the areas as hot and dry, with winter conditions ranging from cool to mild and wet. From the results found in this study, it may be suggested that the temperatures of a species natural distribution may not necessarily relate to the optimum temperature found in *in vitro* laboratory germination. Polito and Weinbaum

(1992) found that walnut pollen, when germinated *in vitro* displayed a degree of plasticity in response to the temperatures experienced during development. Plants were maintained at a higher temperature during pollen development, and then germinated *in vitro* at various temperatures. Walnut pollen displayed a higher optimal temperature for *in vitro* germination when exposed to temperatures 2-3 °C higher than ambient conditions. This may describe why the optimal temperatures found in the laboratory are not the same as those found in the natural environment, as species with prolonged exposure under glasshouse conditions experienced an average of 27 °C during pollen development.

There was a noticeable decrease in *in vitro* germination when *G. rhyolitica* and *G. saccata* pollen was germinated at the upper and lower temperatures of 15 °C and 35 °C (as found in previous studies by Sukhvibul *et al.* (2000) for mango cultivars). The optimal temperature range of *G. fililoba*, *G. pinaster* and *G. rhyolitica* appear to be between 22.5 °C and 27.5 °C, while the optimum temperature for pollen germination for *G. saccata* appeared to be closer to 30 °C. In the main, these results concur with mango and avocado cultivars in previous studies, with optimum temperature for pollen to be between 20 and 25 °C (Sukhvibul *et al.*, 2000; Loupassaki *et al.*, 1997). *Grevillea thelemanniana*, however, appeared to have the highest optimum temperature range between 25 and 30 °C and this is possibly an underestimate because the species did not show any decline at the highest temperature. Of the five *Grevillea* species studied, three are closely related; indeed until 1993 they were classified as the species *G. thelemanniana* (Olde and Marriott, 1995b). Despite their close relationship *G. fililoba*, *G. pinaster* and *G. thelemanniana* exhibited differences in optimum temperatures and temperature tolerance ranges. Similar results have been described by Kakani *et al.* (2002) and Sukhvibul *et al.* (2000) for groundnut genotypes and mango varieties with variations in germination response to temperatures.

#### 3.3.4 Storage potential of *Grevillea* pollen

Sedgley and Harbard (1993) have suggested that where possible, fresh pollen should be used during pollination experiments. Results from this study reiterate their finding as fresh pollen had a higher germination in all *Grevillea* species collected with the exception to *G. baxeri* which was low for both fresh and stored pollen. Unlike *Protea* pollen, *Grevillea* pollen did not maintain a sufficient germination ability to be used for pollination purposes when stored at -20 °C (van der Walt and Littlejohn, 1996) and this may be due to a loss of enzyme activity during storage (Shivanna and Heslop-Harrison, 1981).

The response of *Grevillea* pollen to storage at -20 °C without desiccation compares with undesiccated *Leucadendron* pollen (Yan *et al.*, 2001). Of the six *Leucadendron* hybrids that Yan *et al.* (2001) investigated, five showed no germination after three months of storage at -20 °C without desiccation. When *Leucadendron* pollen was subjected to desiccation before storage at -20 °C, all species germinated with a range from 17-46%. After seven months in -20 °C storage, most *Grevillea* species showed very low (<6%) or no germination and it is possible desiccation of *Grevillea* pollen before storage would increase the potential of pollen to germinate after the allocated storage period.

Results from Shchori *et al.* (1992) showed that pollen viability drops within the first seven days of storage for non desiccated *Banksia* species and is maintained at this lower viability thereafter. This, however, doesn't occur with desiccated *Protea* pollen stored at -18 °C. Granted that relative humidity was taken into account, three of the four *Protea* species studied by van der Walt and Littlejohn (1996) displayed very little change to the germination percentage over a one year storage period when stored between -14 and -18 °C. This suggests that *Grevillea* pollen may require special treatment before

being placed into storage. *Grevillea* pollen is bicellular, a trait that confers a long storage potential due to a slower respiration rate compared to tricellular pollen (Brewbaker, 1967). This further suggests that it could be possible to store *Grevillea* pollen over a long period of time providing it is subjected to an effective pre-treatment. Sedgley and Harbard (1993) suggested that vacuum drying *Acacia* pollen was required before long term storage at -18 °C to reduce the occurrence of ice crystals within the cytoplasm, which in turn would damage the membrane preventing the pollen grain from germinating. Essentially desiccation would perform the same task; removing water from the pollen grain to prevent subsequent membrane damage.

*Leucadendron* (Yan *et al.*, 2001), *Protea* (van der Walt and Littlejohn 1996) and *Banksia* pollen (Shchori *et al.*, 1992) all displayed differences between species or hybrids in regards to storage potential and subsequent germination, as did *Grevillea* pollen in this study. *Grevillea baxeri* displayed no significant change between fresh or stored pollen germination values, and this may be caused by a morphological difference in *G. baxeri* pollen not displayed by the other species investigated.

The ability to optimise storage conditions for *Grevillea* species has major advantages for plant breeders and conservation biologists. When cross breeding species that do not flower at the same time or are separated over large distances the advantages of storage are obvious. Due to advances in storage capabilities, pollen from other species has been transported from one country to another and this is easier than using seeds as there are fewer quarantine restrictions for pollen (Johri and Vasil, 1961; Hanna and Towill, 1995). There is also an advantage for conservation efforts with many recovery plans published by the Department of Conversation and Land Management suggesting pollen storage is an option for recovering endangered *Grevillea* species.

## CHAPTER 4: OPTIMISATION OF LABORATORY POLLEN GERMINATION TESTS

### 4.1 Introduction

The most accurate assessment of pollen viability is fruit and seed set (Heslop-Harrison *et al.*, 1984; Smith-Huerta and Vasek, 1984). However, due to time limitations, many studies rely on other assessments that will give an estimation of a pollen grain's ability to germinate (viability). Furthermore, seed set does not solely reflect a pollen grains' ability to germinate. Rather it reflects that pollination has occurred and both sperm and ovule were able to fertilise and mature to produce a seed (Heslop-Harrison *et al.*, 1984). *In vitro* pollen germination is not a new technique, indeed it dates back to 1869 with work done by Van Tieghem. Since then, the technique has been reworked and refined for use under a range of conditions (Johri and Vasil, 1961) and species. The main problem with *in vitro* germination is that no technique to date has been able to replicate the results found during *in vivo* studies. For example, pollen tubes are longer *in vivo* than that found *in vitro* and, a low germination of pollen *in vitro* can be attributed to the media composition and germination conditions (Johri and Vasil, 1961; Taylor and Hepler, 1997). The percentage of sucrose or nutrients used in a germination medium, combined with lighting conditions, the presence or absence of a coverslip, the density of pollen grains on the slide during the germination period or even the time of incubation can affect pollen germination percentages (Shivanna and Rangaswamy, 1992; Firmage and Dafni, 2001). Therefore, manipulation of these factors is critical to attain results that mimic those found during *in vivo* germination.

Most investigations that have reviewed *in vitro* pollen germination in Proteaceae have failed to conduct and / or report on preliminary studies that

determine the optimal conditions required by the genus. For example, previous work investigating *in vitro* germination in *Grevillea* has been ambiguous as to the lighting conditions used during germination. Kalinganire *et al.* (2000) did not state if pollen was germinated under light or dark conditions, only that it was incubated at 22 °C. A study conducted on mango pollen is one of the few to investigate the effect of light and dark germination conditions before experimentation (Sukhvibul *et al.*, 2000). It is also rare for *Grevillea* studies to identify the ideal media constituents, sucrose concentration or the effect of alternative osmotica. Germination media used in previous studies for *Grevillea* pollen contained sucrose, boric acid, calcium nitrate, magnesium sulphate and potassium nitrate (Kalinganire *et al.*, 2000). It was not documented in Kalinganire *et al.* (2000) if a coverslip was placed onto the pollen medium mixture before the incubation period for *Grevillea* pollen, however, previous studies have included this technique in the methodology (Taylor *et al.*, 1998). Shivanna and Rangaswamy (1992) recommend the addition of a coverslip prior to microscope analysis.

The most common sugar source used during *in vitro* germination of *Grevillea* pollen is sucrose. Previous studies of *Grevillea* pollen have used a 20% sucrose concentration in the germination medium, however, no literature has been reported that identifies the optimum concentration of sucrose required for *Grevillea* pollen using stepwise concentration differences over a large range. Likewise, to date, there has been no study identifying the ideal concentrations of polyethylene glycol during *in vitro* germination of *Grevillea* pollen when used as a sucrose substitute, neither has there been any investigation to identify the effects of mannitol on *in vitro* pollen germination. *Grevillea* studies including mannitol within a germination media have been for propagation purposes.

#### **4.1.1 Aims**

This section of the study aimed to identify optimal conditions for the germination of *Grevillea* pollen *in vitro*. In particular, it investigated the effects of:

- lighting conditions for *Grevillea* germination *in vitro*,
- different germination media,
- adding a coverslip during germination,
- sucrose concentration on germination and
- different osmotica.

### **4.2 Materials and Methods**

#### **4.2.1 Media composition and lighting conditions**

To determine the effect of media composition, lighting conditions and the effect of a coverslip, pollen from *G. rhyolitica*, *G. thelemanniana* and *G. preissii* were subjected to the following treatments:

1. Light, Brewbaker and Kwack medium, without coverslip (L/BK/ -cp)
2. Light, Brewbaker and Kwack medium, with coverslip (L/BK/ +cp)
3. Dark, Brewbaker and Kwack medium, without coverslip (D/BK/ -cp)
4. Dark, Brewbaker and Kwack medium, with coverslip (D/BK/+cp)
5. Light, Taylor's medium, with coverslip (L/Taylor/-cp)
6. Light, Taylor's medium, without coverslip (L/Taylor/+cp)
7. Dark, Taylor's medium, with coverslip (D/Taylor/ -cp)
8. Dark, Taylor's medium, without coverslip (D/Taylor/ -cp)

The compositions of Taylor's medium and Brewbaker and Kwack were previously described in chapter 2. The methods of *in vitro* germination are also described within chapter 2 section. Treatments with coverslips had the coverslips placed on the medium pollen mixture before the 48 hour germination period began. This was to determine the effect of a coverslip on pollen tube growth during germination. Scoring technique follows chapter 2. *Grevillea saccata* was subjected only to treatments without a coverslip, (L/BK/-cp; D/BK/-cp; L/Taylor/-cp; D/Taylor/-cp). This was due to pollen limitation and it had been established at the time of experimentation that coverslips reduce the number of germinated pollen grains.

#### **4.2.2 Media and coverslip treatments in dark conditions**

To examine the effect of media composition and the addition of a coverslip before the incubation period, *G. preissii*, *G. rhyolitica* and *G. thelemanniana* pollen was germinated under dark conditions on either Taylor's medium and Brewbaker and Kwack, with and without coverslips. Methodology is as previously described in chapter 2. This experiment was repeated for *Petunia* pollen to ensure that both media had an effect on pollen.

#### **4.2.3 The effect of sucrose concentration**

Pollen from *G. fililoba*, *G. preissii*, *G. rhyolitica*, *G. saccata* and *G. thelemanniana* was germinated in Brewbaker and Kwack medium with varying sucrose concentrations from 100g to 1000g L<sup>-1</sup> (10-100% sucrose). Pollen was collected on the day of anthesis, germinated and scored as described in chapter 2.

#### **4.2.4 Osmotica in germination medium**

Brewbaker and Kwack medium was modified with the total substitution of sucrose with either 0.61M mannitol or PEG (approximate molecular weight



3350) at concentrations of 0%, 0.5% 1%, 2% and 4% w/v. The level of mannitol was adjusted to apply similar osmotic pressure to 20% sucrose using the following equation.

$$\psi_s = -C \cdot i \cdot R \cdot T$$

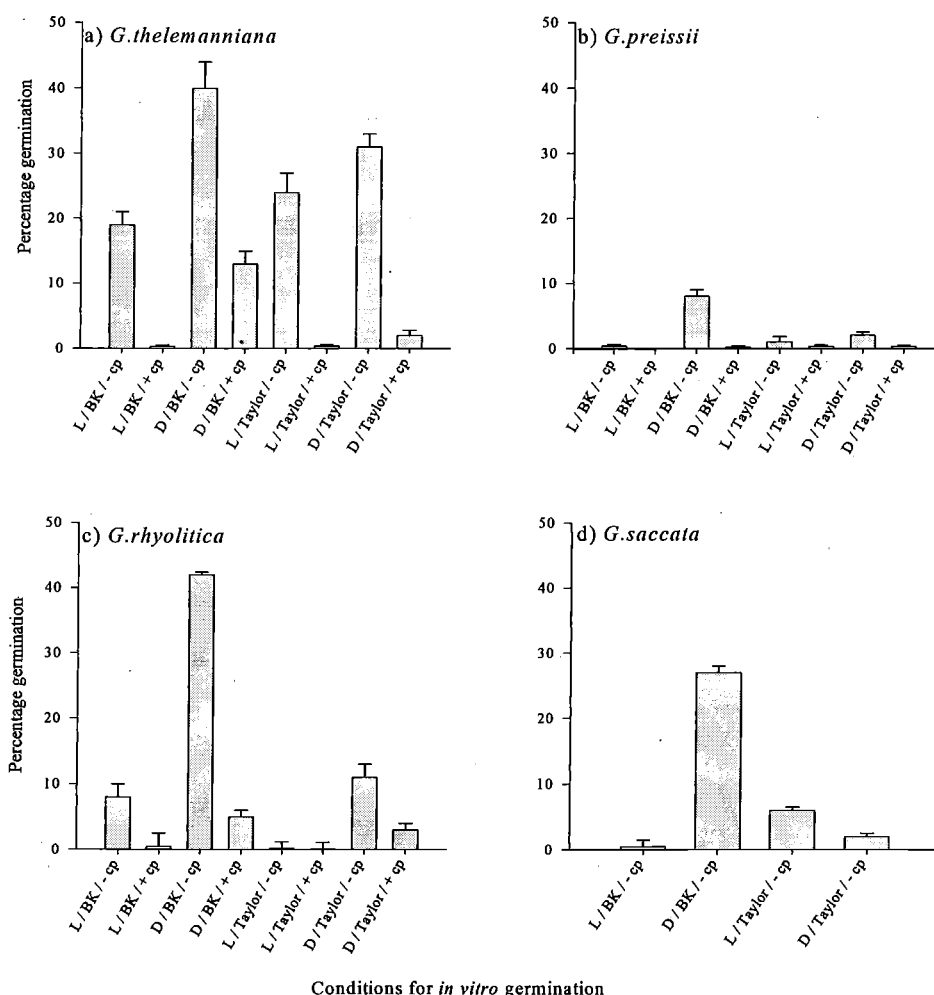
Where  $\psi_s$  is the osmotic potential (MPa), C is the solute concentration ( $\text{mol l}^{-1}$ ),  $i$  is the solute's ionisation constant (1.095 for sucrose and 1 for mannitol and polyethylene glycol), R is the universal gas constant ( $0.00831 \text{ kg MPa mol}^{-1} \text{ K}^{-1}$ ) and T is temperature (K) (Khuri and Moorby, 1995; Lipauska and Vreugdenhil, 1996). Pollen from *G. fililoba* was germinated under dark conditions with *in vitro* germination and scoring procedure described in chapter 2.

### 4.3 Results

#### 4.3.1 Media composition and lighting conditions

Light, medium composition and coverslips all had a significant effect on pollen germination. When all three variables are combined (Fig 6) the treatment that displayed the highest percentage of germination for the three *Grevillea* species was pollen germinated under dark conditions, without a coverslip using Brewbaker and Kwack media (*G. thelemanniana*: 40%; *G. preissii*: 8%; *G. rhyolitica*: 42%).

There was an increase in pollen germination under dark conditions, with increases up to 40 times for *G. thelemanniana*, 42 times *G. rhyolitica* and 8 times for *G. preissii* (Fig 6). Germination was higher on Brewbaker and Kwack than on Taylor's medium ( $P = <0.001$ ) with the germination rate of *G. thelemanniana* at 40% for Brewbaker and Kwack and 31% for Taylor's medium, *G. preissii* (8 and 0% respectively) and *G. rhyolitica* (42 and 11% respectively). The addition of a coverslip before the 48 hour germination period reduced the ability of pollen to germinate, with the germination



Conditions for *in vitro* germination

**Figure 6:** Mean percentage of *in vitro* pollen germination under light (L) and dark (D) conditions using two germination media; Brewbaker and Kwack (BK) and Taylor's (Taylor) at  $24 \pm 1$  °C. Both media had pollen germinated with (+ cp) and without (- cp) coverslips. Means were calculated from 50 replicates per treatment and error bars represent standard error. (NB: did not include with coverslips (+ cp) for *G. saccata* for both Taylor's and Brewbaker and Kwack media).

percentage for *G. thelemanniana*, *G. preissii* and *G. rhyolitica* all statistically significant ( $P = <0.001$ ). When a coverslip was added before the incubation period, germination percentages ranged from 0-13% (*G. preissii* and *G. thelemanniana* respectively). When a coverslip was not added to the germination mixture, germination values ranged from <1-42% (*G. preissii* and *G. rhyolitica* respectively). These values are subject to lighting conditions and media used.

The addition of a coverslip was not applied to *G. saccata*, however, the same trends occurred with regard to lighting conditions and medium composition. Treatments germinated under dark conditions using Brewbaker and Kwack medium had the highest germination of 27%. The lowest value for *G. saccata* was pollen germinated under light conditions on Brewbaker and Kwack medium (0.5%). When germinated under light conditions on Taylor's medium, pollen germination was 6% compared to 5% when germinated in the dark.

The addition of a coverslip after the 48 hour germination period for viewing under the microscope had a tendency to break the delicate pollen tube from the pollen grain. Therefore it became hard to identify which pollen tube germinated from which pollen grain in the sea of pollen grains.

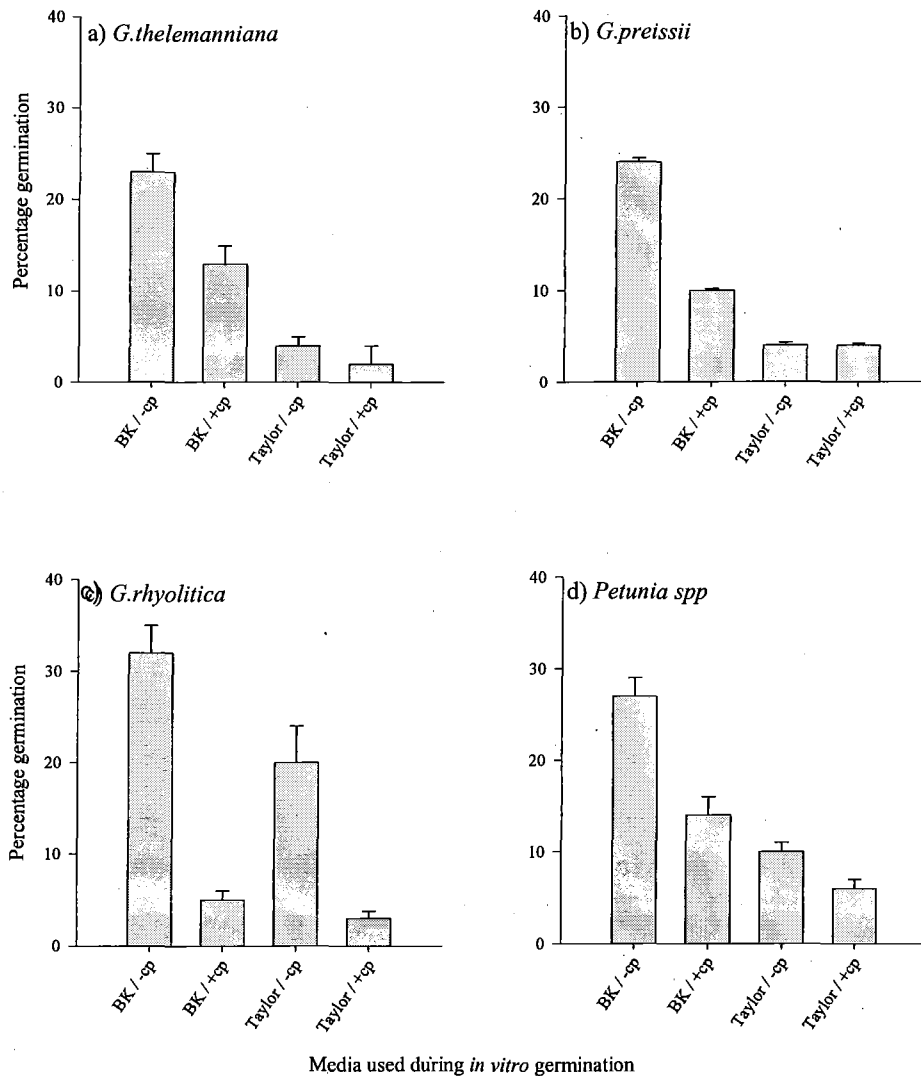
#### **4.3.2 Media and coverslip treatments in dark conditions**

Pollen from all three species displayed significantly higher germination on Brewbaker and Kwack medium than that found on Taylor's medium with germination with increases up to 2 times for *G. thelemanniana* and *Petunia* spp. ( $P = <0.001$ ) and 6 times for *G. rhyolitica* ( $P = 0.001$ ) (Fig 7). Medium composition and coverslips had a significant effect on *G. rhyolitica* and *Petunia* pollen ( $P = <0.001$ ) while *G. thelemanniana* only showed a

significant difference to medium composition with the addition of a coverslip producing no significant effect ( $P = 0.006$ ) on pollen germination. The addition of a coverslip to the medium pollen mixture before incubation significantly decreased germination in *G. rhyolitica* and *Petunia*, with pollen germination decreasing from 32% to 3% in *G. rhyolitica* when a coverslip was added and from 27% to 6% for *Petunia* pollen. Conversely *G. preissii* showed no significant difference to either the medium composition ( $P = 0.035$ ) or to a coverslip being added ( $P = 0.127$ ) prior to the 48 hour incubation period.

Considering that both of the treatments occur simultaneously (Fig 7), *G. thelemanniana*, *G. rhyolitica* and *Petunia* spp. showed a significant difference ( $P < 0.001$ ) in response to medium composition when combined with the use of coverslips. The highest germination for *G. thelemanniana*, *G. rhyolitica* and *Petunia* was 23%, 32% and 27% respectively for pollen germinated on Brewbaker and Kwack, without a coverslip. The lowest germination across these three species was 2%, 3% and 6% respectively for pollen germinated on Taylor's medium with a coverslip. However, *G. preissii* showed no significant difference between the four treatments ( $P = 0.137$ ) with Brewbaker and Kwack medium displayed both the highest and the lowest germination of 24% when germinated without a coverslip and 4% when germinated with a coverslip. Taylor's medium also had a pollen germination rate of 4% when a coverslip was applied and the germination value for pollen germinated on this medium without a coverslip was 10%.

*Petunia* spp. was used in this experiment as previous trials with *G. rhyolitica* produced very low germination (possibly due to poor plant condition) and to confirm that both media had the ability to germinate pollen when placed into dark conditions, *Petunia* spp was used as a control.



**Figure 7:** Mean percentage of *in vitro* pollen germination of three *Grevillea* species and one *Petunia* species using two different germination media. Media used was Brewbaker and Kwack medium (BK) or Taylor's medium (Taylor). Treatments with coverslips are represented by + cp (BK / + cp, Taylor / + cp), those without coverslips are represented by - cp (BK / - cp, Taylor / - cp). All treatments were incubated at  $24 \pm 1$  °C. Mean percentages are based on 50 replicates with error bars representing standard error.

### 4.3.3 The effect of sucrose concentration

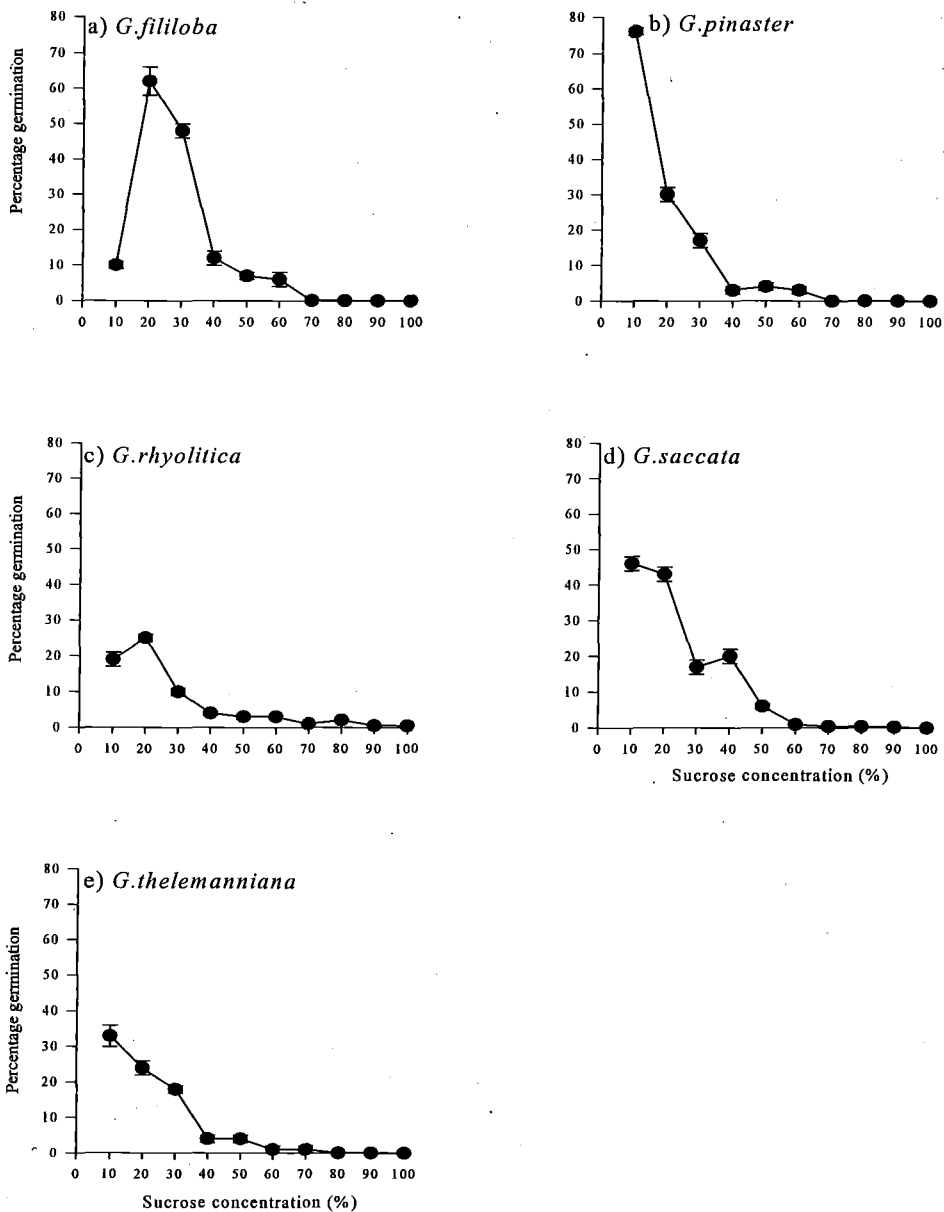
All *Grevillea* species showed a significant difference ( $P \leq 0.001$ ) in response of pollen to sucrose within Brewbaker and Kwack medium. *Grevillea fililoba* and *G. rhyolitica* had the highest germination value of 62% and 25% respectively when the sucrose concentration of Brewbaker and Kwack medium was 20%. Whereas *G. pinaster*, *G. saccata* and *G. thelemanniana* experienced the highest germination of 76%, 46% and 33% respectively, when the sucrose concentration in Brewbaker and Kwack was 10% (Fig 8).

Pollen germination decreased to less than 10% at sucrose concentrations between 40 and 60% for all species (Fig 8). After this no germination was recorded for *G. thelemanniana* and *G. saccata* while *G. fililoba*, *G. pinaster* and *G. rhyolitica* experienced zero germination at 70% sucrose concentration.

The highest germination rate overall was 76% for *G. pinaster* at 10% sucrose concentration with the lowest germination (within the optimal sucrose concentration range, before 40% sucrose concentration) being 10% for both *G. fililoba* and *G. rhyolitica* at 10 and 30% sucrose respectively.

### 4.3.4 Osmotica in germination medium

To determine if sucrose had an effect on *in vitro* pollen germination as an osmoticum or as a food source, solutions were made up using either mannitol or PEG, using a 20% sucrose solution as a standard. There was a significant difference between treatments ( $P \leq 0.001$ ) with the 20% sucrose Brewbaker and Kwack medium producing the highest germination of 66% for *G. fililoba*. Mannitol showed the lowest germination of 19%. Varying PEG concentrations had little effect (Fig 9).



**Figure 8:** Mean percentage of *in vitro* germination for five *Grevillea* species at sucrose concentration from 10-100% (10, 20, 30, 40, 50, 60, 70, 80, 90, 100%) using Brewbaker and Kwack medium under dark conditions without coverslips. All treatments were incubated for 48 hours  $24 \pm 1$  °C. Mean percentages are based on 50 replicates per treatment with error bars representing standard error.

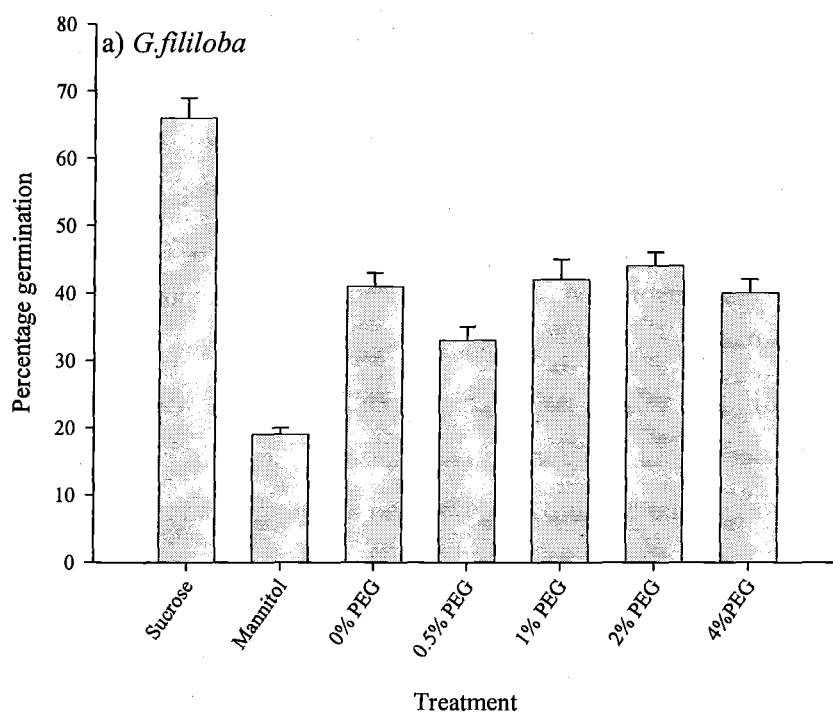


Figure 9: Mean percentage of *in vitro* germination for *G. fililoba* at varying concentrations of polyethylene glycol (0%, 0.5%, 1%, 2% and 4% PEG) and the effect of two different sugar compounds (0.6M sucrose and 0.61M mannitol) incubated at  $24 \pm 1$  °C. Mean percentages are based on 50 replicates and error bars represent standard error.



## 4.4 Discussion

### 4.4.1 Media, lighting conditions and coverslip treatment

The only difference between the two basal media used in this study is the addition of magnesium sulphate and potassium nitrate in Brewbaker and Kwack medium. Generally, all four *Grevillea* species had a higher germination when the medium contained the two inorganic ions of  $K^+$  and  $Mg^{2+}$ . This may suggest that *Grevillea* pollen requires potassium and magnesium during pollen germination and subsequent pollen tube growth. Boron, calcium, potassium and sucrose have also been identified as important elements for *in vitro* pollen germination in other, non related species (Brewbaker and Kwack, 1964; Feijo *et al.*, 1992; Taylor and Hepler, 1997; Fan *et al.*, 2001). Calcium, potassium and magnesium are closely associated with the plasma membrane. It has been suggested that the influx of potassium via  $K^+$  channels, may play a role in rehydration as well as protein synthesis once germination and pollen tube growth is underway (Fan *et al.*, 2001). While the role of magnesium during pollen germination is not fully understood, it appears to play a role within the plasma membrane and is also found in the highest concentration at the pollen tube base (Feijo *et al.*, 1992; Fan *et al.*, 2001). All of the ions found within the Brewbaker and Kwack medium play a role during initial pollen germination and/or subsequent pollen tube growth and they can have a detrimental affect on pollen germination if their concentration in a germination medium is too high or low. For example, *Arabidopsis* and *Brassica* pollen has been shown to be particularly sensitive to elevated calcium concentrations as  $K^+$  channels are regulated by external  $Ca^{2+}$  (Fan *et al.*, 2001).

Pollen from all four *Grevillea* species studied germinated better under dark conditions during the germination period, however, Sukhvibul *et al.*

(2000) reported no difference between pollen incubated in light or dark conditions for mango pollen. The response of pollen germination to light or darkness is not known. Despite this, most *in vitro* germination is carried out under dark conditions (Shivanna and Cresti, 1989; Tyagi *et al.*, 1992; Read *et al.*, 1993; Stone *et al.*, 2004; Karapanos *et al.*, 2006). There is a need to understand the light response of pollen and why *Grevillea* pollen germinates at a higher rate in the absence of light. This may be an evolutionary response to pollinator behaviour, in that pollinator activity in *Grevillea* is lower at night, therefore pollen grains would have a higher chance of being able to adhere, rehydrate and germinate on the stigma without being knocked off by a foraging bird or bee.

The data in this study regarding coverslip treatment of *Grevillea* pollen before incubation suggests that some species respond negatively to the addition of a coverslip whereas, others do not. *Grevillea thelemanniana* and *G. preissii* did not show a difference between treatments, whereas *G. rhyolitica* and *Petunia* spp. did. The pollen tube morphology of each of these pairs was similar, with *G. rhyolitica* and *Petunia* pollen having thicker pollen tubes compared to the thinner pollen tubes formed by *G. thelemanniana* and *G. preissii* pollen. A coverslip may inhibit cell elongation from the aperture in *G. rhyolitica* and *Petunia* spp. by applying pressure to the pollen tube or the pollen grain based on their wider tube diameter. A coverslip may also disrupt the osmotic movement of water into the grain in larger grains if pockets of air are formed near the pollen grain. Taylor *et al.* (1998) described the addition of a coverslip before incubating *Arabidopsis* pollen, but did not suggest why. Considering that the addition of a coverslip after germination had a tendency to break pollen tubes from the pollen grain, it would be recommended not to place a coverslip on a *Grevillea* pollen medium mixture at any point during the incubation or observation stage.

#### 4.4.2 The effect of sucrose concentration

Sucrose has the ability to increase as well as decrease pollen tube germination and growth. Because sucrose is an osmoticum, when its concentration is too high in a growth medium, pollen grains will cease to germinate. Alternatively, when it is too low pollen tubes burst. While burst pollen tubes were not recorded in this study, it can be seen from the data that high sucrose concentrations inhibit pollen germination. Most of the *Grevillea* species observed in the study had very little or no germination in medium containing 60% sucrose and above. Pollen from crop species in other studies were unable to germinate when the medium contained a high sugar content as it is often found to be detrimental to the pollen grain (Montaner *et al.*, 2003). *Conospermum* spp, are one of the few species reported to germinate in media containing 100% sucrose (Stone *et al.*, 2004), albeit very slowly.

The optimum concentration of sucrose has been shown in previous studies to be important in estimating the germination value or viability of pollen. The need to acquire the optimum sucrose concentration is important as the role of sugars in germination media has been suggested (and refuted) by many to provide nutrients to the developing tube, as well as provide an osmotic gradient (Johri and Vasil, 1961; Potts and Marsden-Smedley, 1989). Results from this study are in agreement with previous work on *in vitro* pollen germination by Kalinganire *et al.* (2000) and Herscovitch and Martin (1989) who investigated *in vitro* germination of *G. robusta* and *G. banksii* pollen respectively. However, despite that, they did not incorporate preliminary studies to determine the optimal sucrose concentrations of the medium, both studies used a sucrose concentration of 20%. *Grevillea* pollen used in this study had the highest germination when the sucrose concentration was 20%. This concurs with results found for *Borago* pollen (Montaner, 2003), maize (*Zea*) (Schreiber and Dresselhaus, 2003) and four species of *Eucalyptus* (Potts

and Marsden-Smedley, 1989). Crop species such as mango (Sukhvibul *et al.*, 2000), groundnut (Kakani *et al.*, 2002) and walnut (Luza and Polito, 1987) has also had pollen germinated on a medium with a 20% sucrose concentration. *Grevillea* pollen displayed a tolerance range, where pollen was able to maintain the ability to germinate (above 10% germination value) at sucrose concentrations between 10 and 30% and this is conclusive with studies on eucalypt pollen (Myrtaceae) which had a germination range from 10-40%, dependent on the species (Potts and Marsden-Smedley, 1989). However, results from another Proteaceae member, *Conospermum*, do not concur with *Grevillea* data. *Conospermum* pollen has a larger range of 10-80% sucrose where pollen was able to germinate above 10% germination (Stone *et al.*, 2004). From this, it can be assumed that the optimum sucrose concentration within a germination medium is genus (possibly species) specific and preliminary studies should be conducted to identify it.

#### 4.4.3 Osmotica in germination medium

The results for sucrose and mannitol concur with previous studies in unrelated species, with mannitol producing little or no germination and sucrose being the preferred carbohydrate source between the two (Johri and Vasil, 1961; Vasil, 1964; Rihova *et al.*, 1996; Karapanos *et al.*, 2006). This is because mannitol is metabolically inert and most pollen grains are able to absorb and use sucrose more readily than mannitol (Johri and Vasil, 1961). Even when sucrose is partially substituted with mannitol in a germination medium, as was done with tomato pollen, germination is still greatly reduced (Karapanos *et al.*, 2006). Kessler *et al.* (1960) suggested that mannitol acts as a metabolic inhibitor, possibly deactivating starch synthesis while allowing the metabolism of externally supplied sucrose. However, data from the current study using *Grevillea* pollen does not completely agree with this statement, as mannitol completely replaced sucrose in the germination

medium with some pollen still able to germinate. Yet, there is evidence to suggest that mannitol does inhibit pollen germination on an internal level with the 0% PEG medium containing only ions and minerals (boric acid, calcium nitrate, magnesium sulphate and potassium nitrate) being able to germinate a higher percentage of pollen compared to the mannitol medium. Further investigation into the inhibitory affects of mannitol in pollen is required to determine the processes mannitol disrupts.

From the results from this study, it can be suggested that *Grevillea* pollen requires sucrose for pollen tube growth because without its presence in the medium, germination was low. In previous studies, PEG is generally used in conjunction with sucrose (Read *et al.*, 1993; Karapanos *et al.*, 2006). PEG lowers the water potential of the germination medium allowing water to be drawn out of the pollen grain. When PEG completely replaces sucrose in a germination medium, pollen tubes are often reported to be longer and straighter. The sucrose in the medium provides a carbohydrate source for the pollen grain to produce thicker tubes (Reid *et al.*, 1993; Rihova *et al.*, 1996; Karapanos *et al.*, 2006). Externally supplied sugars are thought to act in the same manner that nutrients supplied by the style during pollen tube growth do. Each pollen grain has a small supply of energy to begin pollen tube growth, however, it is not enough to fully develop a pollen tube down a lengthy style. Nutrients from the style that include polysaccharides, free sugars and proteins are suggested to enter into the pollen grain providing energy which essentially is what sucrose does in a germination medium, providing energy to the pollen grain for pollen tube growth (Herrero and Hormaza, 1996; Lord, 2003; Sanchez *et al.*, 2004). The concentration of PEG did not alter germination for *Grevillea* pollen nor did it increase germination above that found for pollen germinated on a sucrose medium, suggesting that sucrose is required for the germination process in *Grevillea* species.

## CHAPTER 5: BREEDING AND HYBRIDISATION

### 5.1: Introduction

George (1984) described the *Grevillea* genus as one of the “most adaptable to cultivation than any other in the family, at least in Australia” and most *Grevillea* species have long, colourful periods of flowering. The genus responds well to cuttings and grafting and are easily hybridised under horticultural conditions. However, while the genus has flourished in the horticultural industry, its development in the cut flower industry has lagged somewhat. One of the major problems the floricultural industry has encountered in *Grevillea* development is the short vase life, which is approximately three to ten days depending on the variety. This makes *Grevillea* cultivars currently unsuitable for overseas markets (Olde and Marriott, 1994; Beal *et al.*, 1996; Joyce and Beal, 1999). There appears to be more of an economical market for grevilleas in the foliage industry rather than cut flower with stems lasting approximately 30 days (Elliott and Jones, 1990; Criley and Parvin, 1993).

Most of the published work on grevilleas with regard to hybridisation is produced either by enthusiasts or plant breeders who are most concerned with the end result rather than the processes involved in hybridisation (Fuss and Sedgley, 1991b; Abell and Olde, 2002). The exceptions being work produced by Herscovitch and Martin (1989) who examined inter-hybrid crosses *G. banksii* and Fuss and Sedgley (1991a) who investigated hand pollination as a method of hybridisation technique for *Banksia menziesii* R. Br. The horticultural industry needs to start using the scientific advances in genetics to improve cultivar development of *Grevillea*, such as using molecular markers to distinguish desired traits in seedlings (Olde and Marriott, 1994; Abell and Olde, 2002).

With the exception of Herscovitch and Martin (1989), there has been no investigation into inter-hybridisation of *Grevillea* species and there has been no study on the effect of removing the chemical and physical barriers in the stigma for *Grevillea*. This technique has shown positive results for some *Solanum* and *Borago* species when the stigma and its chemical barriers have been removed before pollination (Swaminathan, 1955; Montaner *et al.*, 2003).

### **5.1.1 Aims**

The work reported in the section examined inter-species hybridisation of *G. rhyolitica* with three other species of *Grevillea*: *G. hirtella* (Benth.) Olde & Marriott; *G. dielsiana* C. A. Gardner; and *G. baxeri*, in combination with the effects of stigma removal.

## **5.2 Materials and Methods**

Ten *G. rhyolitica* plants with at least 20 inflorescences were chosen as mother plants from the glasshouse for each experiment. All dead flowers or those that had opened were removed from inflorescences using fine scissors. This was done so that the stigmas became receptive at approximately the same time. Flowers in the late looping stage were opened manually and emasculated by removing the anthers in the end of the perianth using fine scissors. If self pollen was present on the pollen presenter, it was removed using a soft cotton cloth to initialise stigma receptivity and to prevent autogamy (Kalinganire *et al.*, 2000). Only four flowers were emasculated on each of the 20 inflorescences (one for each treatment). All remaining flowers were removed using fine scissors as described in Kalinganire *et al.* (2000). Inflorescences and flowers were chosen at random. One plant was emasculated as a control and did not undergo any treatment (not pollinated). The control was used to determine if pollination occurs via external pollination under greenhouse conditions.

One of the four flowers was chosen randomly from the inflorescence and assigned a coloured thread (red, blue, gold or purple) which represented the species from which the pollen was donated. Four pollen donors were used:

*G. baxeri* (blue)

*G. hirtella* (red)

*G. dielsiana* (purple)

*G. rhyolitica* (geitonogamous) (gold)

The thread was loosely tied into the peduncle of the flower so that nutrients supply would not be cut off (Fig 10 and 11).

Inflorescences from *G. baxeri*, *G. hirtella* and *G. dielsiana* were collected one day before they were required from King's Park (Roe Gardens) to be used as pollen donors. All open flowers were removed and closed flowers were placed in the glasshouse to open. Cut flowers were pushed through shade cloth that had been stretched over a bucket of water and sucrose. This allowed flowers to remain hydrated without the pollen becoming wet and allowed flowers to mature and open.

Inflorescences were chosen at random to determine if they would be pollinated with or without a stigma. The stigma was removed from all the flowers in the selected inflorescence using a scalpel. Stigmas were cut off at the top of the style where the two appendages are joined. Pollen-laden presenters were pressed onto the style immediately after the stigma was removed to ensure pollen tubes would not be blocked by callose plugs that occur in response to an open wound. Pollen laden presenters were also pressed onto stigmas to transfer pollen. Pollen was then reapplied on all flowers one day after initial pollination to ensure that pollen limitation would not affect results (Copeland and Whelan, 1989; Hermanutz *et al.*, 1998; Heliyanto *et al.*, 2005).



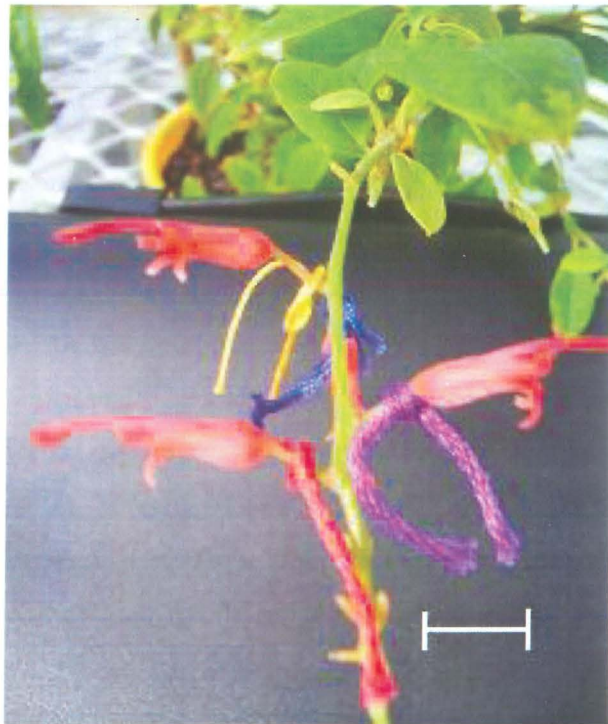


Figure 10: *Grevillea rhyolitica* flowers after being tagged with coloured thread representing different pollen donors. Bar = 1cm

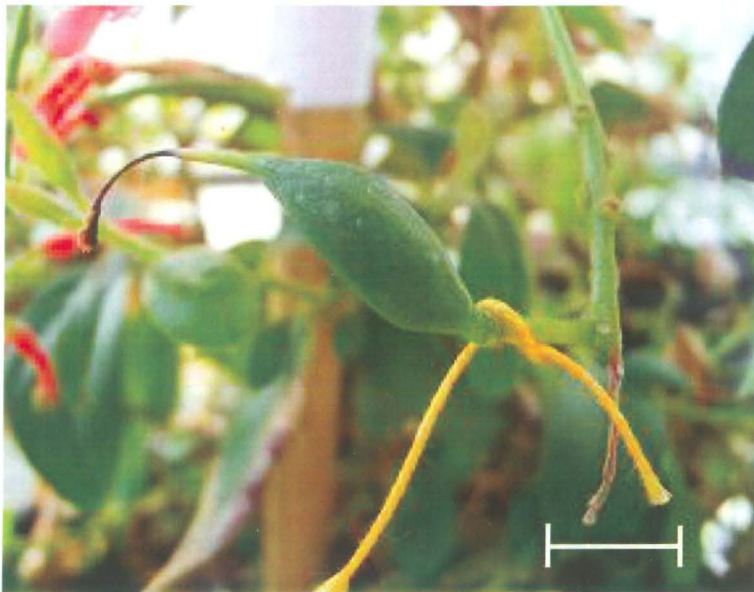


Figure 11: *Grevillea rhyolitica* fruit developed from a geitonogamous cross at 14 days after the final pollination. Bar = 1cm

### 5.3 Results for hybridisation trails

All fruit set recorded was a result of geitonogamous crosses with *G. rhyolitica*. Fourteen days after the final pollination (15 days after the first pollination), 70% of the plants bore between one or two fruit. Fruits from plant numbers 4 and 9 had begun to abort at this point and after 30 days from the final pollination only plant numbers 3 and 10 bore fruit, one fruit per plant. Plant number 3 originally had two fruit, of these two fruits, only one remained at 44 days after final pollination (Table 2). Fruit set was not measured after this point due to the severity of a fungal attack. Plant foliage was treated with Mancozeb® on the onset of the infection two weeks after pollination. No fruit set was recorded for inter-hybrid cross pollinations with *G. baxeri*, *G. hirtella* or *G. dielsiana*.

Fruit set was recorded for *G. rhyolitica* that had the stigma removed before pollination. Within 14 days after the final pollination all styles had turned black and shrivelled.

### 5.4 Discussion for hybridisation trails

The Proteaceae are known to have very low fruit to flower ratios, with successful pollination not always leading to successful fertilisation and fruit set (Hermanutz *et al.*, 1998). Natural fruit set is known to vary amongst *Grevillea* species, but is generally considered low (Collins and Rebelo, 1987). Xenogamous pollination has been reported to increase initial fruit set and subsequent fruit maturity in previous studies on other *Grevillea* species (Hermanutz *et al.*, 1998; Kalinganire *et al.*, 2000; Smith and Gross, 2002). Xenogamous pollination was not obtained in this study as it was assumed that all *G. rhyolitica* plants were clones as this is a common horticultural

Table 2

Number of fruit set for ten individual *G. rhyolitica* plants over a 44 day period. Time was measured after the final day of pollination at fortnightly intervals. (B) = beginning to abort (A) = aborted

Number of days after pollination	Plant number	Number of fruit
14 days post	1	1
	2	1
	3	2
	4	1 (B)
	5	0
	6	0
	7	1
	8	0
	9	1 (B)
	10	1
30 days post	1	0 (A)
	2	1 (B)
	3	1 (B)
	4	0 (A)
	5	0
	6	0
	7	0 (A)
	8	0
	9	0 (A)
	10	1
44 days post	1	0
	2	0 (A)
	3	0 (A)
	4	0
	5	0
	6	0
	7	0
	8	0
	9	0
	10	1

propagation method, particularly when the species is considered rare. As a result, all pollinations were with self pollen in a geitonogamous cross.

Smith and Gross (2002) suggest that self compatibility is a common trait within *Grevillea*, with most pollinators exhibiting a within conflorescence foraging pattern. Pollen rarely moves between populations, thus the ability to be self compatible would increase initial fruit set (Hermanutz *et al.*, 1998; Smith and Gross, 2002). The benefit of being self compatible, together with protandrous behaviour could be an evolutionary advantage for grevilleas. This would allow the megagametophyte to mature at a slower rate than the microgametophyte, providing the opportunity for self pollen to be removed from the pollen presenter. Any remaining pollen would be able to germinate and be utilised in an autogamous cross.

During previous experiments on *Grevillea* where autogamous and xenogamous crosses were used, the genus seems to prefer outcrossing with a higher rate of fruit set recorded when pollen is donated from an individual removed from the current population, such as *G. beadleana*, *G. macleayana* and *G. linearifolia* (Hermanutz *et al.*, 1998; Richardon *et al.*, 2000; Smith and Gross, 2002;). It has been suggested that the style inhibits self pollen tube growth to encourage xenogamous pollen to reach the ovary first (Hermanutz *et al.*, 1998).

The results found in this study do not conclusively suggest that *G. rhyolitica* is self incompatible. The species has the ability to initiate fruit set; however late acting incompatibility processes may have prevented fruit from maturing. Heliyanto *et al.* (2005) found similar results for *Banksia ilicifolia* R. Br. with the species displaying self pollen compatibility in the style, however, late-acting zygotic self incompatibility in the ovary lead to selective fruit abortion. Hermanutz *et al.* (1998) termed this 'partial self compatibility'.

Chemical barriers at the stigma generally prevent most self incompatible species from having pollen entering the style, however, in partially self compatible species the stigma tissues allow a small number of pollen tubes to develop through the transmitting tissue into the style (Hermanutz *et al.*, 1998). Partially compatible styles, as suggested previously, may slow self pollen tube growth without inhibiting it, carrying it down to the ovary and into the ovule. Harriss and Whelan (1993) suggest that selective fruit abortion enables a plant to terminate fruit/seed of a lesser genetic quality using late acting incapability mechanisms. Thus a plant can abort fruit from auto and geitonogamy crosses maintaining more vigorous outbred fruits.

Fruit may also have been aborted because of the reduced health of the *Grevillea* plants due to a fungal infection (or other factors leading to the deterioration of the plant's health) or the application of the fungicide. Other studies on the effect of phosphite based fungicides have been conducted on Proteaceae species found in jarrah forests with reports that phosphite reduces fruit and seed production (Fairbanks *et al.*, 2002).

Grevilleas will hybridise easily during inter-species hybridisation crosses with a closely related species. For example, the controlled pollination between *G. victoriae* and *G. rhyolitica* created the hybrid 'Lady O' with 'Ember Glow' was an inter-species hybridisation cross between *G. rhyolitica* and *G. juniperina* R. Br. All are species within the Linearifolia group. Both parents of 'Peaches and Cream' belong to the Pteridifolia group (*G. bipinnatifida* x *G. banksii*) and the parents of 'Ellabella' belong to Thelemannina group (*G. preissii* x *G. fililoba*).

The results found in this study further support this, as all species used during inter-hybrid cross pollinations were from different groups. No fruit developed when *G. rhyolitica* was emasculated and pollinated with pollen

from any of the less closely related species (*G. hirtella*, *G. dielsiana* or *G. baxeri*), suggesting that either the stigma, style or ovary possesses mechanisms that identify pollen from other 'groups' and prevent the pollen tube from fertilising the ovule. Herscovitch and Martin (1989) performed interspecific hybrid crosses between five *Grevillea* species from different groups (*G. banksii*, *G. triloba* Meisn., *G. buxifolia*, *G. thelemanniana* and *G. sericea* (Sm.) R. Br.) with all crosses having pollen tubes entering into the transmitting tissue and style after 24 hours. Subsequent fruit set results were not published. Therefore it may have been possible for pollen from *G. baxeri*, *G. dielsiana* and *G. hirtella* to have entered into the transmitting tissue and style of *G. rhyolitica* with late acting ovary mechanisms preventing fertilisation as with Herscovitch and Martin (1989). Further *in vivo* studies would be required to determine where pollination and fertilisation barriers occur within the *Grevillea* gynoecium.

Stigma removal did not encourage fruit set in *G. rhyolitica* as it has in other, unrelated, species such as *Brassica oleracea* L. and some *Solanum* species (Swaminathan, 1955; Hodgkin, 1987). *In vivo* analysis of the style may have determined if pollen was able to germinate on it without the presence of the stigma. Information from previous studies has suggested that the stigma may help adhesion and rehydration of pollen prior to germination, removal of which may also remove these essential stages of pollen germination (Taylor and Hepler, 1997). Generally, the stigma is removed in self incompatible species to remove the chemical barriers that prevent pollen from germinating into the style (Hodgkin, 1987), however, studies by Herscovitch and Martin (1989) on unrelated *Grevillea* species demonstrated that the incompatibility mechanisms in *Grevillea* may not be in the stigma or style, but within the ovary or ovule. Further research is required to determine this and discern how to overcome this barrier.

## CHAPTER 6: GENERAL DISCUSSION

Improvement of *in vitro* pollen germination has several benefits for horticulture, floriculture and conservation alike. As previously mentioned, no current *in vitro* germination technique has been able to replicate exactly what occurs during *in vivo* germination (nature) in terms of speed and percentage of pollen grains germinated. At present, seed germination is deemed as being the most accurate and time consuming way to determine pollen viability therefore it would be desirable to obtain another method such as *in vitro* germination that can represent the germination found in nature. By determining the ideal sucrose concentration, the effect that the presences or absence of inorganic ions such as  $K^+$  and  $Mg^{2+}$  has on pollen and the effects of alternative osmotica such as PEG and mannitol, germination values should become closer to the results obtained during *in vivo* germination. In turn, by increasing the validity and accuracy of *in vitro* germination, what is determined in the laboratory could be transferred to field with a greater probability of obtaining similar results. If conditions in the laboratory are able to closely mimic those found in nature, the success rate of *in vivo* germination and subsequent fertilisation would increase. Therefore by acquiring an *in vitro* germination medium that is able to replicate *in vivo* pollen tube germination rate and length in *Grevillea* would greatly increase the success rate of cross pollination and artificial hybridisation.

Once an optimum germination medium has been identified for *Grevillea*, determining a set of environmental conditions specific to the species would further increase pollen germination and subsequent fertilisation, providing that the conditions could be applied to *in vivo* and hybrid experiments outside of the laboratory. During artificial hybridisation, the ideal outcome is to obtain a viable seed and to increase the potential of this occurring. Results from this study suggest that *Grevillea* pollen should be

germinated in the dark with temperatures maintained at approximately 25 °C and donor pollen should be collected on the day of anthesis. Grevilleas, like most Proteaceae have a very low fruit to flower ratio (Ayre and Whelan, 1989; Walker and Whelan, 1991; Hermanutz *et al.*, 1998) and in order to increase the potential for pollen to germinate on the stigma and successfully travel down the style, the plant should be maintained at the species optimum conditions stated above during all stages of artificial hybridisation. By providing the optimal conditions for pollen to germinate, the maximum number of pollen tubes should develop and compete for an ovule ensuring that the fittest pollen tube fertilises the ovule first.

In horticultural and floricultural terms, *in vitro* pollen germination could be used to identify wild populations or individuals better suited to a warmer or cooler climate by subjecting pollen to specific temperatures during *in vitro* germination and selecting desired genotypes for further breeding. This technique has already been used to identify heat tolerant cultivars of cotton and groundnut (Kakani *et al.*, 2002; 2005; Liu *et al.*, 2006). However, this technique could be used to identify cold tolerant genotypes or species able to be grown in areas that are currently unsuitable because of temperature restrictions. Previous studies within the floriculture industry have suggested the need to identify cold tolerate genotypes of species used for cut flower and foliage, in order to meet demand during the low season when production has finished in warmer areas (Zamir and Gaddish, 1987; Petolino *et al.*, 1990; Slater and Carson, 2003). Therefore, testing pollen from individuals using *in vitro* germination prior to hybrid experimentation would increase the chances of desired traits such as cold and heat tolerance, being displayed rather than leaving it to chance.

For conservation efforts, storage and optimum pollen germination temperature are probably two of the most important aspects of this research.



Many conservation plans are beginning to suggest incorporation of *Grevillea* pollen storage into recovery efforts with results from this study providing important information that *Grevillea* pollen requires specific pre-storage treatment or storage under more specific conditions (than those used in this study) to maintain its ability to germinate after storage.

Temperature has been described as one of the most important factors for life with most organisms using temperature to regulate biological and biochemical functions with extreme temperatures being able to affect pollen during development, germination and fertilization (Kakani *et al.*, 2005). If extreme temperatures occur during any of these stages pollen quality, viability, longevity and vigour can be greatly reduced leading to a further reduction in seed set.

This study has provided data that suggest temperature can affect a pollen grain's ability to germinate *in vitro* and while *in vitro* technology cannot be directly correlated to what is found *in vivo*, the data should be used as a precaution. The ideal conditions found *in vitro* can easily be applied to *in vivo* germination of *Grevillea*, which should increase pollen germination, subsequent fertilisation and providing barriers within the ovule are overcome, increase subsequent seed set. This offers advantages for conservation efforts in a bid to increase the number of seeds stored in seed banks of rare and / or endangered species while aiming to maintain genetic diversity by using xenogamy crosses where possible. For horticulture and floriculture, an increase in seed set offers the potential for a range of desired characteristics to be displayed in seedlings from inter-hybrid crosses.

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