Micropropagation and Cryopreservation for Conservation of Western Australian Terrestrial Orchids

Betty Mauliya Bustam M.Sc.

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School of Plant Biology
Faculty of Science
The University of Western Australia
SUMMARY

Western Australia has a unique flora that includes a rich highly endemic terrestrial orchid component. However, many of these orchid species are threatened or rare, or in some cases extinct in the wild. Although efforts to conserve endangered species have been forthcoming, much research is still needed to gain a better understanding of the complexities of orchid conservation. The thesis outlines key studies that have been conducted for establishing micropropagation and cryopreservation protocols for Western Australian terrestrial orchids, an ex situ conservation tool. *Caladenia latifolia* R.Br., a common Western Australian terrestrial orchid was used for establishing initial in vitro and cryopreservation techniques and protocols (due to readily available seed), followed by research to optimise protocols for *Caladenia huegelii* Rehb.f., a threatened Western Australian species.

The first study aimed to optimise a simple and reliable asymbiotic germination medium that could be used with a broad range of terrestrial orchid species. The study investigated 19 asymbiotic media variations comprising four commonly used orchid basal media - half-strength Murashige and Skoog (½ MS), Knudson C (KC), Pa5 and Vacin and Went (VW); with combinations of the plant growth regulators (PGR), 6-benzylaminopurine (BA) and α-naphthalene acetic acid (NAA) or coconut water (CW) and these were compared with germination performance on a standard symbiotic germination medium, Oat Meal Agar (OMA). Percentage germination of seeds was recorded every two weeks for a total of eight weeks (five replicates per treatment), along with time to germination and growth and development phases in seedlings. ½ MS with 5% (v/v) fresh coconut water delivered germination of 93%, with seedling vigour and development indistinguishable from OMA (95% germination). The same protocol was applied to a further nine genera (including *Caladenia huegelii*), demonstrating high asymbiotic germination performance (60%-93%) across a wide phylogenetic range of terrestrial orchid species.

The second study aimed to determine the most suitable medium as well as the most suitable primary protocorm stage of development to facilitate secondary protocorms for use in cases where seeds may be scarce, especially with rare and threatened taxa. Seeds of *C. latifolia* were germinated asymbiotically on the optimised medium from the first study (½ MS medium fortified with 5% (v/v) coconut water). Resulting protocorms at
three, five and seven weeks (post-sowing) growth were subcultured to protocorm proliferation media treatments consisting of ½ MS basal salts medium with 6-benzylaminopurine (BA) and α-naphthalene acetic acid (NAA) singly or in combination. Percent germination on ½ MS (with 5% v/v CW) from seeds to primary protocorms was high (87%-92%) as expected from prior experiments. The highest secondary protocorm proliferation percentage was 40%, using five week old protocorms (early stage four of protocorm development) as explants and cultured on ½ MS with a combination of 5 µM NAA + 2 µM BA. ½ MS containing only a single PGR (BA or NAA) was relatively ineffective for protocorm proliferation (only one treatment exceeded 5% proliferation).

The third study aimed to develop approaches for cryopreserving primary protocorms and implement this basic protocol with further research to optimise procedures to effectively cryostore secondary protocorms. To develop such a cryopreservation approach for primary protocorms, three studies were conducted. The first investigated development of a suitable plant vitrification solution (PVS) by evaluating three known types of PVS (PVS 2, 3, and 4) as well as determining the most suitable primary protocorm stage for cryopreservation. The second study tested the effectiveness of using a pre-culture medium step prior to cryopreservation, while the third study involved consolidating and optimising the entire cryopreservation protocol for primary orchid protocorms. The cryopreservation protocol developed for primary protocorms was then implemented and optimized for secondary protocorms. All experiments used an asymbiotic approach for in vitro germination and proliferation using the optimised medium from previous studies, and various modifications of the droplet vitrification technique for cryopreservation. The optimized cryopreservation protocol developed has increased the percentage of survival of primary protocorms from 68% to 85% and regeneration from 17% to 48%; with survival of secondary protocorms from 63% to 84% followed by regeneration increasing from 11% to 26% in 14 weeks.

The fourth and final experimental study aimed to apply successful protocols developed using the common orchid species (C. latifolia) to an indicative rare species (C. huegelii). Applying the protocols derived with C. latifolia to C. huegelii required specific adjustment due to differences in protocorm size and time of protocorm development between species. For primary protocorms, the protocols for protocorm proliferation and cryopreservation were applied according to the most suitable
protocorm development stage (early Stage 4). *Caladenia latifolia* reach Stage 4 at five weeks after sowing while *C. huegelii* reach Stage 4 eight weeks after sowing. Moreover, *C. huegelii* required incubation in a constant 20°C incubator after cryopreservation until it regenerated and developed into plantlets. In addition, plantlets of *C. huegelii* were transferred to soil and acclimatized under greenhouse conditions with very promising results, relevant to other rare orchid species conservation programs.

Overall, micropropagation and cryopreservation protocols developed in this study are visible to be implemented to other rare and threatened terrestrial orchid species, particularly Western Australian species.
THESIS DECLARATION

This thesis was completed during the course of my enrolment in a PhD degree at School of Plant Biology, the University of Western Australia. This thesis contains no experimental material that has been previously presented for my degree at this university or any other institutions. The thesis contains published work which has been co-authored where design of experiments, experimental work and the preparation of the manuscripts were done by myself, under the supervision of Prof. Kingsley Dixon and Dr. Eric Bunn.

Chapter 3 of this thesis is the original chapter submitted for examination, with revisions made as suggested by examiners. Published work on the chapter is in Appendix 2.
PUBLICATIONS ARISING FROM THIS THESIS

Published paper:


Papers to be submitted:

Bustam BM, Dixon KW, Bunn E. Cryogenic approaches for conserving Western Australian terrestrial orchids. This paper will be submitted to a selected journal.

Bustam BM, Dixon KW, Bunn E. *Ex situ* conservation of threatened Western Australian terrestrial orchid, *Caladenia huegelii*, through micropropagation and cryopreservation. This paper will be submitted to a selected journal.

Conference presentation:

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUMMARY</td>
<td>i</td>
</tr>
<tr>
<td>THESIS DECLARATION</td>
<td>iv</td>
</tr>
<tr>
<td>PUBLICATIONS ARISING FROM THIS THESIS</td>
<td>v</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENT</td>
<td>vi</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>viii</td>
</tr>
</tbody>
</table>

## CHAPTER 1
**Introduction and General Literature Review**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>3</td>
</tr>
<tr>
<td>WORLD ORCHID CONSERVATION</td>
<td>3</td>
</tr>
<tr>
<td>AUSTRALIAN ORCHID CONSERVATION</td>
<td>4</td>
</tr>
<tr>
<td>MICROPROPAGATION</td>
<td>6</td>
</tr>
<tr>
<td>PROTOCORM PROLIFERATION</td>
<td>12</td>
</tr>
<tr>
<td>ORCHID SPECIES INVESTIGATED – <em>Caladenia latifolia</em></td>
<td>12</td>
</tr>
<tr>
<td>CRYOPRESERVATION</td>
<td>13</td>
</tr>
<tr>
<td><em>Vitrification</em></td>
<td>14</td>
</tr>
<tr>
<td><em>Encapsulation-dehydration</em></td>
<td>15</td>
</tr>
<tr>
<td><em>Encapsulation-vitrification</em></td>
<td>16</td>
</tr>
<tr>
<td><em>Droplet vitrification</em></td>
<td>16</td>
</tr>
<tr>
<td>OBJECTIVE AND AIMS OF THIS STUDY</td>
<td>20</td>
</tr>
<tr>
<td>STRUCTURE OF THIS THESIS</td>
<td>21</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>23</td>
</tr>
</tbody>
</table>

## CHAPTER 2
**In vitro propagation of temperate Australian terrestrial orchids: revisiting asymbiotic compared with symbiotic germination**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>34</td>
</tr>
<tr>
<td>MATERIAL AND METHODS</td>
<td>34</td>
</tr>
<tr>
<td><em>Seed</em></td>
<td>34</td>
</tr>
<tr>
<td><em>Mychorrizal fungus media</em></td>
<td>34</td>
</tr>
<tr>
<td><em>Mychorrizal isolates</em></td>
<td>34</td>
</tr>
<tr>
<td><em>Symbiotic culture medium</em></td>
<td>35</td>
</tr>
<tr>
<td><em>Asymbiotic culture media</em></td>
<td>35</td>
</tr>
<tr>
<td><em>Seed germination</em></td>
<td>35</td>
</tr>
<tr>
<td><em>Symbiotic</em></td>
<td>35</td>
</tr>
<tr>
<td><em>Asymbiotic germination</em></td>
<td>35</td>
</tr>
<tr>
<td>MEDIUM EFFECTIVENESS</td>
<td>36</td>
</tr>
<tr>
<td>DEVELOPMENT OF DROPLET SEED STERILIZATION METHOD</td>
<td>36</td>
</tr>
<tr>
<td>EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS</td>
<td>36</td>
</tr>
<tr>
<td>RESULTS</td>
<td>36</td>
</tr>
</tbody>
</table>
CHAPTER 3
Proliferation and harvesting of secondary protocorms as a novel means for improving propagation of terrestrial orchids

ABSTRACT
INTRODUCTION
MATERIAL AND METHODS
Asymbiotic culture medium
Proliferation process
Proliferation media
Experimental design and statistical analysis
RESULTS
Asymbiotic seed germination
Proliferation
DISCUSSION
CONCLUSIONS
ACKNOWLEDGEMENTS
REFERENCES

CHAPTER 4
Cryogenic approaches for conserving Western Australian terrestrial orchids

ABSTRACT
INTRODUCTION
MATERIAL AND METHODS
Developing primary protocorms from seeds
Cryopreservation of primary protocorms
Selection of plant vitrification solution (PVS) and protocorm stage
Preculture medium
Temperature preconditioning and recovery medium
Cryopreservation of secondary protocorms
Statistical analysis
RESULTS
Plant vitrification solution (PVS) and suitable protocorm stage selection
Effectiveness of preculture medium
CHAPTER 1

Introduction and General Literature Review
Chapter 1

Introduction and General Literature Review

INTRODUCTION

Many plant species are currently threatened with extinction mainly due to human activities such as clearing natural vegetation habitat for agriculture or other land use e.g. mining (Reed et al., 2011). Conserving rare and threatened organisms, including plants is of international importance due to dramatic declines in many species (Walter and Gillet, 1998). Recently, climate change is also increasingly cited as a new threat for many species (Mc Carty, 2001; Thuiller et al., 2005; Liu et al., 2010). According to the International Union for Conservation (IUCN, 2010), more than 8,000 plant species are threatened worldwide, including many species of orchids. Compared to most other plant families the Orchidaceae is in the ‘front line’ for plant extinctions (Nicholls, 2004).

WORLD ORCHID CONSERVATION

Orchidaceae is the most diverse angiosperm family consisting of more than 25,000 species (Dressler, 1993; Mobberley, 1997, Cribb et al., 2003). Many orchid species around the world are endangered for a variety of reasons including natural habitat destruction to accommodate agricultural crops, illegal collection from vulnerable wild populations and more recently, fears that climate change will almost certainly impact negatively on many already rare and threatened orchid species (Swarts and Dixon, 2009).

Myers et al., (2000) has described the tropic regions as distinct orchids hotspots and variability is high between continents and within regions. Tropical regions therefore have the greatest potential of orchid loss from a species perspective owing to the greatest numbers of orchid species present coupled with high human populations and human impact (Cribb et al., 2003). While orchids in the northern Andes (South America), Madagascar, Sumatra and Borneo are mostly epiphytic, Australia has limited tropical areas remaining and due in no small part to its long isolation from other land masses, is one of the few major centres of terrestrial orchid biodiversity in the world (Cribb et al., 2003).
AUSTRALIAN ORCHID CONSERVATION

Australia has one of the richest and most diverse endemic temperate terrestrial orchid floras in the world with 1,300 named species in 190 genera (Backhouse, 2007). As in many other parts of the world, Australia is also facing the threat of extinction of many plant species, including orchids. The southwest of Western Australia is a recognized Biodiversity Hotspot (Mittermier et al., 2004) and the Orchidaceae in WA carry the highest number of threatened taxa in any plant family (Batty et al., 2002). As more and more orchid species are expected to become endangered in the near future, the requirement for more research programs on integrated conservation will need to intensify or these species are likely to become extinct quite rapidly without human intervention (Batty et al., 2006).

In an effort to prevent extinction of rare and threatened plants, the Department of Parks and Wildlife (DPaW) in Western Australia manages the preservation of natural habitats as an in situ conservation strategy (Macneal, 2010). However, conserving threatened plant species in situ by itself has its risks whether abiotic (drought, frequent fire and potentially climate change) or biotic (e.g. diseases, weeds and feral pests) in origin, and both can result in genetic erosion over time, thus reducing the effectiveness of in situ only protection (Panis and Lambardi, 2005). Therefore, ex situ conservation methods become a necessary approach for integrated conservation of endangered taxa, including endemic Western Australia orchids.

In vitro propagation is the best available means of achieving mass propagation of native orchids while also enabling ex situ storage of key genetic material (Pedroza-Manrique et al., 2005; Stewart and Kane, 2006; Deb and Temjensangba, 2006; Millner et al., 2008; Roy et al. 2011; Zeng et al., 2011). Once in vitro material is readily available, cryopreservation becomes feasible and will be fundamental to developing secure long term ex situ collections of propagation material (Bunn et al., 2007; Kaczmarczyk et al., 2011). While orchid seeds can fill this requirement with more common species, with rare and threatened species there may be few individuals. Therefore, seeds can be in very short supply and in vitro generated material is a valuable additional reserve. However, in vitro propagation is comparatively labor-intensive and can be expensive per propagule depending on how difficult any particular species is to micropropagate.
Moreover, long-term maintenance of culture lines under standard incubation conditions and continuous subculturing is costly and prone to losses due to accidental contamination, somaclonal variation and loss of culture vigour over long periods of time in continuous culture cycles (Fay, 1992; Panis and Lambardi, 2005). Therefore, there is ample argument for developing optimal micropropagation protocols and combining these with cryopreservation protocols to offer the most effective means for *ex situ* conservation of threatened orchid species.

One endemic Western Australian terrestrial orchid genus that consists of many threatened taxa is *Caladenia* (Swarts and Dixon, 2009). Many *Caladenia* species are in urgent need of propagation research for effective *ex situ* conservation as this genus has experienced dramatic reductions in both habitat and numbers of plants in recent decades (Department of Environment and Conservation, 2009). According to Backhouse (2007), *Caladenia* consists of 234 taxa of which 97 are threatened. However, Dixon and Hopper (2009) have suggested that, based on current evidence (Hopper and Brown, 2004; Jones *et al.*, 2001; Kores *et al.*, 2001), this genus should be credited with at least 300 species.

Some researchers have suggested integrated conservation as the best option for conserving targeted orchid species (Dixon and Batty, 2003; Swarts, 2007). Examples of integrated conservation with terrestrial orchid species are not common but have been reported for some species such as *Caladenia huegelii* (Swarts and Dixon 2009). This species is found in Perth to Jarrah Forrest (FloraBase, 2014 – Fig. 1). However, to be sure of conserving the genus, many more research programs still need to be conducted that address (a) the problem of attaining the most efficient use of rare seed stocks and (b) the need for efficient and reliable long-term storage methods for seed, mycorrhizal fungus and/or other propagating material *i.e.* tissue cultures. While suitable micropropagation and cryopreservation methods might be able to supply these materials, there has been no conclusive research so far that comprehensively resolves these issues for threatened *Caladenia* species.
Chapter 1

**Fig 1.** The distribution map of *Caladenia huegelii* (FloraBase, 2014)

MICROPROPAGATION

Micropropagation is a procedure for regenerating many new plants from small amounts of tissue (usually shoots) on a defined nutrient medium maintained under axenic conditions (Razdan, 2003). To conserve terrestrial orchids through micropropagation, particularly seed propagation, research has been conducted around the world using different media formulations with various combinations of plant growth regulators, and natural products such as coconut water, etc. (de Faria *et al.*, 2002; Ket N.V. *et al.*, 2004; Mahendran and Bai, 2009; Godo *et al.*, 2010). This type of research has also included some Australian terrestrial orchid species (Collins and Dixon, 1992; Huynh *et al.*, 2002).

Some researchers have suggested that *in vitro* seed germination is a suitable and most efficient propagation method for orchid conservation, including native terrestrial orchids (Zettler and McInnis, 1993; Kauth *et al.*, 2006; Steward and Kane, 2006). In general, however, terrestrial orchid seeds are more difficult to germinate and grow than epiphytic orchids. This might be because terrestrial orchid seeds have a hardened seed
coat and need more requirements for germination in vitro (Lee, 2011). In addition, many species of terrestrial orchids, including Australian species, are depending on specific mychorrizal fungi to grow in the wild (Rasmussen, 2008; Nurfadillah, 2010, Philips, 2010). However, attempted to germinate Australian terrestrial orchids species in vitro either symbiotically (using specific mychorrizal fungi) or asymbiotically (without mychorrizae) have been reported for some species (Warcup, 1973; Collins and Dixon, 1992; Huynh et al., 2002; Batty et al., 2006). Symbiotic germination has been reported for Thelymitra, Diuris and Pterostylis in agar media containing sodium nitrate, potassium dihydrogen phosphate, magnesium sulphate, potassium chloride and powdered cellulose (Warcup, 1973). Moreover, modified VW medium (Vacin and Went, 1949), Pa5 200 and Pa5 10BA (modified Burgeff’s N3f - P. Milon pers.com), and Pa5 were chosen for initiating explants of Diuris longifolia (Collins and Dixon, 1992); and symbiotic and asymbiotic culture of Arachnorchis (Caladenia) formosa using oat meal agar (OMA) and Pa5 media was conducted by Huynh et al. (2002). In addition, Batty et al. (2006) has also reported symbiotic germination in three native Western Australia terrestrial orchids, Caladenia arenicola Hopper and A.P Brown, Diuris magnifica D.L Jones, and Thelymitra crinita Lindley using OMA media before transferring them to soil. More details of worldwide research into in vitro propagation of orchids in the last twelve years can be seen in Table 1.
Table 1. Summary of worldwide orchid *in vitro* propagation research (2002-present)

<table>
<thead>
<tr>
<th>No.</th>
<th>Species</th>
<th>Type of <em>in vitro</em> Propagation</th>
<th>Media</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Paphiopedilum</em> sp.</td>
<td>Protocorm like bodies (PLB) proliferation from the primary PLB developed from stem-derived callus</td>
<td>½ MS + 4 µM Kinetin</td>
<td>Ng Yih and Saleh (2011)</td>
</tr>
<tr>
<td>2.</td>
<td><em>Eulophia alta</em></td>
<td>Asymbiotic and Symbiotic mature seeds <em>in vitro</em> propagation</td>
<td>KC, MM, P723, ½ MS, VW (Asymbiotic), and OMA (Symbiotic)</td>
<td>Johnson <em>et al.</em> (2007)</td>
</tr>
<tr>
<td>7.</td>
<td><em>Grammatophyllum speciosum</em> Blume</td>
<td>Shoot tips from <em>in vitro</em> seedlings</td>
<td>½ MS, MS, and VW solid and liquid</td>
<td>Sopalun <em>et al.</em> (2010)</td>
</tr>
<tr>
<td>8.</td>
<td><em>Dendrobium</em> sp.</td>
<td>Protocorm like bodies</td>
<td>VW, KC, ½ MS, NP</td>
<td>Akter <em>et al.</em> (2007)</td>
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<td>Media</td>
<td>Authors</td>
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<tr>
<td>18.</td>
<td><em>Syngonium podophyllum</em></td>
<td>Generating protocorm like bodies from leaf and petiole</td>
<td>MS</td>
<td>Cui <em>et al.</em> (2008)</td>
</tr>
<tr>
<td>20.</td>
<td><em>Dactylorhiza baltica</em> (Klinge) Orlova</td>
<td>Protocorm like bodies</td>
<td>MS</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Dactylorhiza ruthel</em> (M. Schultze ex Ruthe) Soo</td>
<td>Asymbiotic immature seeds <em>in vitro</em> propagation</td>
<td>MS, Heller, Lindemann, Norstog</td>
<td>Vaasa and Rosenberg (2004)</td>
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<td><em>Dactylorhiza praetermissa</em> (Druce) Soo</td>
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<td>No.</td>
<td>Species</td>
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*Cuitlauzina pendula* La.Llave & Lex

**Abbreviations:**

| MS        | Murashige & Skoog                  | NP       | New *Phalaenopsis*          |
| ½ MS      | Half strength Murashige & Skoog    | P723     | PhytoTechnology Orchid Seed Sowing Medium |
| KC        | Knudson C                          | BM-1     | Terrestrial Orchid Medium   |
| MM        | Malmgren Modified Terrestrial Orchid Medium | W        | Western                   |
| VW        | Vacin Went                         | KCM      | Modified Knudson C         |
| OMA       | Oat Meal Agar                      | ND       | New Doghasima              |
| PM        | Phytamax Orchid Medium             | ML       | Modified Luke’s            |
| M         | Mitra Orchid Medium                | P668     | PhytoTechnology orchid Maintenance media with charcoal |
PROTOTOCORM PROLIFERATION

For many orchid species that are rare or limited in seed production (including quantity and viability of seed) their conservation remains problematic (Coates and Dixon, 2007). While some researchers have reported successful results in germinating orchid seeds after cryopreservation (Nikishina et al., 2001; Popova and Nikishina, 2003; Hirano et al., 2005); other reports indicate that problems do occur during the germination phase such as seed infection during germination - i.e. for reasons unknown the mychorrhizal fungus becomes pathogenic (Nikishina et al., 2001). Therefore to protect valuable seed resources in situations like these, and still ensure sufficient propagation materials (e.g. protocorms) for experiments, it is essential to develop protocols for proliferation of secondary protocorms from primary (seed-derived) protocorms. In this way continuously-replenishable propagating material can be generated from a minimum quantity of seed for experiments that may (initially) have a high risk of failure. Some studies have attempted mass-proliferation of secondary protocorms from primary protocorms (David et al., 2008; Hossain et al., 2010; Latip et al., 2010; Roy et al., 2011), however there is only one recent report that mentions in vitro propagated adventitious (secondary) protocorms of an Australian terrestrial orchid species (Watanawikkit et al., 2012).

ORCHID SPECIES INVESTIGATED – Caladenia latifolia

Caladenia latifolia (pink fairy orchid) is a common and widespread species and is found in southwest Western Australia (WA.) from Geraldton to Esperance, although mainly confined to coastal regions (FloraBase 2014 – Fig. 2). Caladenia latifolia was utilized to provide important test material for establishing protocols for rare orchid taxa from WA.
Seed for this species is readily available and it was considered an ideal test species with which to run the proposed experimental research in this study. Initial experiments were considered to require a lot of ‘trial and error’, therefore potentially requiring a plentiful (and easily replenishable) supply of seed. The strategy was to utilize *Caladenia latifolia* to develop basic in vitro and cryogenic protocols to certain level of repeatability before trials on threatened species, such as *Caladenia huegelii*.

**CRYOPRESERVATION**

Cryopreservation is the storage of germplasm in Liquid Nitrogen (LN) (-196°C) (Hamilton et al., 2009). This method is becoming a very important tool for long-term storage of plant germplasm because it requires minimum space and maintenance. Moreover, preservation of cultured cells and somatic embryos is becoming more important due to an increase in genetic engineering of plants (Sakai and Engelman, 2007). It is reported that cryopreservation offers real hope for long term preservation of threatened species (Sakai and Engelman, 2007).
Chapter 1

Classical and new cryopreservation techniques are available. Classical cryopreservation techniques involve slow cooling down to a defined prefreezing temperature, followed by rapid immersion in LN, whereas new cryopreservation techniques are based on desiccation and/or vitrification then direct cooling in LN. Seven different vitrification-based procedures (methods) can be identified: (1) Encapsulation-dehydration; (2) Vitrification; (3) Encapsulation-Vitrification; (4) Desiccation; (5) Pregrowth; (6) Pregrowth-Desiccation, and (7) Droplet Freezing (Droplet Vitrification) (Engelmann, 2000). Pregrowth consists of cultivating samples in the presence of cryoprotectants, then freezing them rapidly by direct immersion in LN while pregrowth-desiccation is preculturing of the explants on a medium with high concentration of sucrose or ABA or Proline and deciccation/drying followed by freezing in liquid LN (Dixit et al., 2004).

Added to this are various pre-treatments or acclimation where incubation temperature may be varied prior to cryopreservation to enhance freezing tolerance (Kaczmarczyk et al., 2011). Theoretically, any plant germplasm tissue can be cryopreserved i.e. seeds, zygotic embryos or axes, vegetative tissue (shoot tips), cultured tissue (callus, suspension cell cultures, embryogenic cultures or somatic embryos) and pollen (Hamilton et al., 2009).

Reports of cryopreservation protocols for orchid species (including some hybrids) are listed in Table 2. However, few of these reports detail protocols for Australian orchid species (see entry numbers 16-18 in Table 2). The initial cryopreservation experiment for Caladenia latifolia has been conducted using protocorms derived from asymbiotically germinated seed (Watanawikkit et al., 2012). In addition, Watanawikkit et al. (2012) determined protocorm viability using a fluorescein diacetate (FDA) test. However, the FDA test indicates potential viability but is not necessarily a good indicator of actual germinability (Vujanovic et al., 2000; Johnson et al., 2007).

Vitrification, encapsulation-dehydration, encapsulation-vitrification, and droplet vitrification are the most commonly used cryo-protocols for orchid protocorms (as listed in Table 2). There are some advantages as well as disadvantages of those protocols, listed in Table 3.

Vitrification

Vitrification refers to the physical process by which a highly concentrated cryoprotective solution supercools to very low temperatures and finally solidifies into a
metastable glass, without undergoing crystallization at a practical cooling rate. Different vitrification solutions have been developed by various research teams around the world. However, the most commonly used solutions are the glycerol-based vitrification solutions designated PVS2 and PVS3 (Sakai and Engelmann, 2007). Fig. 3. shows the general procedure of vitrification.

![Diagram of vitrification procedure](image)

**Fig. 3. Cryopreservation procedures, vitrification (Bunn et al., 2007)**

**Encapsulation-dehydration**

This method was developed by Fabre and Dereuddre (1990) and involves encapsulating desired plant materials (samples) for cryopreservation within alginate beads that are then dehydrated typically in a sterile air flow (or in osmotically adjusted solutions) for a specific interval to achieve a pre-determined tissue water content, then immersion in LN (Kaczmarczyk et al., 2012).
Encapsulation-vitrification

Encapsulation-vitrification is a combination of two methods: encapsulation-dehydration and vitrification. This combination gives advantages of encapsulation-dehydration (ease of manipulation of encapsulated explants) and of vitrification (rapidity of implementation) (Sakai and Engelmann, 2007).

Droplet vitrification

This is a very recent vitrification method derived from the droplet-freezing technique developed by Kartha et al. (1982). In the droplet-vitrification protocol, samples (i.e. protocorms) are placed within a droplet of 1-10 µl of cryoprotective solution on a piece of aluminium foil before immersion in liquid Nitrogen (Sakai and Engelmann, 2007; Kaczmarczyk et al., 2012). Fig. 4. shows the general procedure of droplet vitrification.

Fig. 4. Cryopreservation procedures, droplet vitrification (Kaczmarczyk et al., 2012)
<table>
<thead>
<tr>
<th>No</th>
<th>Species/Hybrid</th>
<th>Material used for cryopreservation</th>
<th>Cryopreservation methods</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.</td>
<td><em>Dendrobium candidum</em> Wall. ex Lindl.</td>
<td>Protocorm like bodies</td>
<td>Encapsulation vitrification</td>
<td>Yin and Hong (2009)</td>
</tr>
</tbody>
</table>

Table 2. Use of cryopreservation in orchid conservation
<table>
<thead>
<tr>
<th>No</th>
<th>Species/Hybrid</th>
<th>Material used for cryopreservation</th>
<th>Cryopreservation methods</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>Technique</td>
<td>Advantages</td>
<td>Disadvantages</td>
<td>References</td>
</tr>
<tr>
<td>-----</td>
<td>-----------------------------</td>
<td>-------------------------------------------------</td>
<td>---------------------------------------------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>1.</td>
<td>Vitrification</td>
<td>No special equipment needed</td>
<td>Vitrification solutions are toxic to many plants</td>
<td>Reed (2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fast procedure</td>
<td>Sample fracturing is possible</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fast recovery</td>
<td>Requires careful timing of solution changes</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Encapsulation-dehydration</td>
<td>Easy to treat large number of samples simultaneously because the large size of alginate beads</td>
<td>Longer time to implement, compared to vitrification Not suitable for all species</td>
<td>Sakai and Engelmann (2007)</td>
</tr>
<tr>
<td>3.</td>
<td>Encapsulation-vitrification</td>
<td>No special equipment needed Non-toxic cryoprotectants Simple thawing procedures Fast Recovery</td>
<td>Requires handling each bead several times Some plants do not tolerate the high sucrose concentration</td>
<td>Reed (2001)</td>
</tr>
<tr>
<td>4.</td>
<td>Droplet-vitrification</td>
<td>Use very small volumes of cryoprotectant Achieves higher cooling and rewarming rates High post-cryo recovery</td>
<td>Relatively new method Need to experiment with each new plant species</td>
<td>Kaczmarczyk et al. (2012)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

**Table 3.** Comparisons of approaches used in cryopreservation of plants
OBJECTIVE AND AIMS OF THIS STUDY

The main objective of this study is to develop micropropagation as well as cryopreservation protocols from primary and secondary protocorms as a means for establishing conservation collections of rare orchid species. The protocols are based on asymbiotic germination approaches. However, due to limited seeds for experiments a common native Western Australian terrestrial orchid species, *Caladenia latifolia* was chosen for establishing basic techniques and protocols (more detail about this species is explained in ‘Orchid Species Investigated – *Caladenia latifolia*’ section). Once basic micropropagation and cryopreservation protocols were established, these protocols were tested and optimised for a threatened species, *Caladenia huegelii*.

Hypotheses of the study are:

1. Primary protocorms generated from asymbiotic germination are equal to protocorms obtained via symbiotic germination - if they are generated in a suitable asymbiotic medium.
2. Primary protocorms derived from asymbiotically germinated orchid seeds are suitable for continuous secondary protocorms regeneration *in vitro*.
3. Primary and secondary (regenerated) protocorms derived from asymbiotically germinated orchid seeds are suitable for cryopreservation.
4. Viable orchid plantlets can be grown from primary and secondary (regenerated) protocorms derived from asymbiotically germinated orchid seeds following cryopreservation.
5. *In vitro* protocorm propagation coupled with cryopreservation are viable options for *ex situ* conservation of threatened Australian orchid spp.

*Aims*

1. Develop optimal asymbiotic seed germination for the study species (*C. latifolia* initially, followed by *C. huegelii*) to provide primary protocorm material for experiments on *in vitro* protocorm production for cryopreservation experiments.
2. Using primary protocorms of *C. latifolia* conduct experiments on continuous *in vitro* protocorm production (secondary protocorms) and plantlet development from protocorms from asymbiotic culture lines.
3. Utilize primary and secondary protocorms of *C. latifolia* to establish first principals for cryopreservation.
4. Conduct experiments to optimise cryopreservation of *C. latifolia* protocorms (primary and secondary) and establish repeatable protocols that enable high survival of protocorms following cryostorage.

5. Investigate the application of micropropagation protocols (asymbiotic germination and protocorm proliferation) to *Caladenia huegelii*.

6. Investigate the application of cryopreservation protocols with primary and secondary protocorms of *Caladenia huegelii*.

7. Investigate plantlet regeneration from post-cryopreserved protocorms and establish weaned plantlets in soil.

**STRUCTURE OF THIS THESIS**

This thesis is structured as a series of papers with a total of six chapters that consist of four experimental chapters (Chapter 2 – Chapter 5) and two additional chapters as Introduction and Literature Review (Chapter 1) and General Discussion (Chapter 6). The intent is for experimental chapters to be submitted to selected refereed scientific journals. Therefore, the chapters are arranged as close to a manuscript format as possible. Although repetition on every chapter has been minimized, it is inevitable that some repetition will occur in introductions, materials and methods, and references sections. Detail of thesis structure is in Table 4.
**Table 4.** Detail of this thesis structure

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Introduction and Literature Review</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><em>In vitro</em> propagation of temperate Australian terrestrial orchids: revisiting asymbiotic compared to symbiotic germination</td>
<td>This chapter has been published online in <em>Botanical Journal of the Linnean Society</em> (DOI: 10.1111/boj.12216: 12 November 2014).</td>
</tr>
<tr>
<td>3</td>
<td>Proliferation and harvesting of secondary protocorms as a novel means for improving propagation of terrestrial orchids</td>
<td>This chapter has been published online in <em>Australian Journal of Botany</em> (DOI: 10.1071/BT14291: 2014) (Published article is in Appendix 2)</td>
</tr>
<tr>
<td>4</td>
<td>Cryogenic approaches for conserving Western Australian terrestrial orchids</td>
<td>This chapter is in preparation to be submitted to a selected journal</td>
</tr>
<tr>
<td>5</td>
<td><em>Ex situ</em> conservation for a rare Western Australian terrestrial orchid, <em>Caladenia huegelii</em>, through micropropagation and cryopreservation</td>
<td>This chapter is in preparation to be submitted to a selected journal</td>
</tr>
<tr>
<td>6</td>
<td>General Discussion</td>
<td></td>
</tr>
</tbody>
</table>
REFERENCES


Chapter 1


CHAPTER 2

In vitro propagation of temperate Australian terrestrial orchids: revisiting asymbiotic compared with symbiotic germination
**In vitro** propagation of temperate Australian terrestrial orchids: revisiting asymbiotic compared with symbiotic germination

BETTY M. BUSTAM1,2,3, KINGSLEY W. DIXON1,2 and ERIC BUNN1,2,4

1School of Plant Biology, Faculty of Natural and Agricultural Sciences, The University of Western Australia, Crawley, WA 6009, Australia
2Kings Park and Botanic Garden, Fraser Avenue, West Perth, WA 6005, Australia
3Biography Department, Mathematics and Natural Sciences Faculty, Syiah Kuala University, Jl. SyechAbdurrauf no. 3, Darussalam 23111, Banda Aceh, Indonesia

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Using a common temperate herbaceous terrestrial orchid from Australia (**Caladenia latifolia**) this study investigated 19 asymbiotic media variations comprising four commonly used basal media [half-strength Murashige and Skoog (½MS), Knudson C (KC), Pa5, and Vacin and Went (VW), with combinations of the plant growth regulators 6-benzylaminopurine (BA) and α-naphthalene acetic acid (NAA) or coconut water (CW) and compared their performance with germination on a standard symbiotic germination medium, oatmeal agar (OMA). Percentage germination of seeds every 2 weeks for a total of 8 weeks (five replicates per treatment), time to germination, and growth and development phases in seedlings were recorded. ½MS with 5% (v/v) fresh CW delivered 93% germination, with seedling vigour and development indistinguishable from OMA (95% germination). The same protocol was applied to a further ten species (including the endangered **Caladenia huegelii**), demonstrating high asymbiotic germination performance (69–93%) across a wide phylogenetic range of terrestrial orchid species. © 2014 The Linnean Society of London, Botanical Journal of the Linnean Society, 2014, 176, 556–566.

**ADDITIONAL KEYWORDS:** **Caladenia** – optimized growth medium – orchid conservation – rare and threatened species.

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**INTRODUCTION**

Orchidaceae are among the most threatened of any plant families, due to human land use and more recently the threat of climate change (Batty et al., 2002; Backhouse, 2007; Seaton et al., 2010). Hence developing ex situ conservation approaches, such as in vitro culture, provides an important adjunct for conserving rare and threatened taxa, particularly when translocation programmes are involved.

Terrestrial orchids require mycorrhizal partners for in situ germination and seedling development (Batty et al., 2001), with a number of approaches now used for ex situ germination using the mycorrhizal agent (McKendrick et al., 2000; Batty et al., 2002; Brundrett et al., 2005). Germination of some terrestrial orchids can be problematic using in vitro methods (Arditti, Michaud & Oliva, 1981; Robinson, Balakrishnan & Britto, 2005) particularly if seed is limited or mycorrhizal endophytes are difficult to obtain or culture. However, in vitro seed germination and seedling culture remain the most effective means for propagating terrestrial orchids (Zettler & McInnis, 1993; Knuth, Vendrame & Kane, 2006; Stewart & Kane, 2006).

Symbiotic germination of orchids first requires isolation of efficacious symbiotic fungi and a means for vital storage of the symbionts. If the fungal extraction and purification process proves complex, seed must be stored and germination experiments delayed until the mycorrhizal fungus is available. In addition, storage and genetic stability of mycorrhizal fungi of some orchid species may be problematic (Brundrett, 2007; Wright et al., 2009).
Asymbiotic germination, i.e. germination without mycorrhizal fungi, is used extensively to propagate many millions of orchids for the horticultural trade (Johnson et al., 2007). Although asymbiotic germination protocols have been reported for terrestrial orchid species (Collins & Dixon, 1992), our preliminary experiments with some Australian species, e.g. Caladenia latifolia R.Br., resulted in a slow germination response accompanied by a low final germination percentage (unpubl. data). Thus, this study has the aim of improving in vitro seed germination for terrestrial orchids to provide reliable asymbiotic protocols comparable to (or better than) current symbiotic methods, with C. latifolia as the test species.

Although many media have been reported to promote orchid seed germination, a key aim of this study was the development of a simplified asymbiotic medium that could be used for a broad range of terrestrial taxa. Therefore, we screened a selection of well-known basal media (that are relatively easy to formulate) for in vitro germination (with addition of growth supplements) using the test species (C. latifolia) to compare symbiotic with asymbiotic germination and early growth of seedlings. The most efficient protocol was then applied to a wide phylogenetic range of orchid taxa, including endangered species for which problems exist with isolation and maintenance of fungal symbionts. The additional test taxa were Diuris magnifica D.L.Jones, Thelymitra benthamiana Rech.f., Spiculaea ciliata Lindl., Cyanicula gnomata (Lindl.) Hopper & A.P.Br., Elythranthera brunnonis (Endl.) A.S.George, Ericseionella saccharata (Rech.f.) Hopper & A.P.Br., Philadenia deformis (R.Br.) D.L.Jones & M.A.Clem, Eriochilus dilatatus Lindl., Microtis medica R.Br. and the rare and endangered Caladenia huegelli Rech.f. This study also includes nutrient comparison of the asymbiotic media to assist with determining why preliminary studies with C. latifolia resulted in low percentage germination when using a medium that has been reportedly used widely for terrestrial orchids (Paš; Collins & Dixon, 1992).

MATERIAL AND METHODS

SEED
Mature seeds were collected in 2011 from naturally pollinated, wild C. latifolia plants growing in remnant native bushland in Kings Park near Perth in Western Australia (Fig. 1). Sixty capsules (average of three capsules per plant) were harvested from 20 plants. Seeds were combined and dried over silica gel for 24 h at 15 °C then stored in air-tight containers at 4 °C for 6 weeks before being used for experiments.

MYCORRHIZAL FUNGUS MEDIA
Fungal isolation medium (FIM) was used for isolation of pelotons and initiation of fungal symbiont cultures. Potato dextrose agar (PDA) was used to maintain an active fungal collection for experimental use. FIM was prepared as described by Clements, Muir & Cribb (1986). Streptomycin sulphate solution was added to post-autoclaved media (cooled to -50 °C) using a 0.22-µm syringe-driven filter unit to a final concentration of 1% (w/v) (Bonnardeaux et al., 2007). PDA medium was prepared by combining 6.8 g L⁻¹ potato dextrose agar powder (Pythotechnology Laboratories) with 6 g L⁻¹ agar (Gelita Australia Pty Ltd).

MYCORRHIZAL ISOLATES
Stored cultures of mycorrhizal fungus and a freshly isolated fungus from C. latifolia from natural bushland were used for symbiotic germination of C. latifolia seeds. Mycorrhizal fungi were isolated from pelotons (single hyphal coils) extracted from the stem collar region of wild adult C. latifolia, based on the method of Rasmussen (1995). FIM was used for initial isolation before transfer to PDA.

![Figure 1](image_url) A. Caladenia latifolia plants growing in natural habitat in Kings Park and Botanic Garden bushland. Scale bar = 2 cm. B. Caladenia latifolia flower. Scale bar = 1 cm.

Symbiotic Culture Medium

Modified oat meal agar (OMA) was used for assessing symbiotic germination of *C. latifolia* seeds and was prepared by combining 2.5 g L⁻¹ crushed oats and 8 g L⁻¹ agar, with pH adjusted to 5.5 before autoclaving. This modified modified OMA (minus yeast extract) was utilized because it was found to be effective for *in vitro* germination and growth of orchid seedlings (Swarts, 2007).

**Asymbiotic Culture Media**

Asymbiotic culture was based on the following basal media: Pa5 (Collins & Dixon, 1992), Murashige and Skoog basal salts (Murashige & Skoog, 1962) at half-strength (½MS) with modifications (as described by Lau et al., 2014; Knudson C (Knudson, 1946), and Vacin and Went (VW) (Vacin & Went, 1949)). Pa5 medium was used without further modification. Details of all basal media formulations were taken from original papers (or from George, Puttock & George, 1987) and are presented in Table S1.

Plant growth regulators (PGRs) including 6-benzylaminopurine (BA) and c-naphthalene acetic acid (NAA) were added in various combinations and fresh coconut water (CW) was also added (at two concentrations) to three basal salt formulations (½MS, KC and VW). PGR treatments were 0.2 μM BA + 0.5 μM NAA (A); 0.4 μM BA + 1.0 μM NAA (B); 0.6 μM BA + 1.5 μM NAA (C) and no PGR (D). Fresh CW was added at 5% (v/v) (E) and 10% (v/v) (F). CW was extracted from eight fresh young coconuts obtained locally, mixed and filtered through nylon mesh to remove particulate matter, filter-sterilized (Bonnardeaux et al., 2007), frozen (−20 °C) in aliquots of 50 and 100 mL, thawed as required and aseptically added to prepare 1 litre of warm (−50 °C) post-autoclaved basal media (½MS, KC and VW) for final concentrations of 5% (50-mL aliquot, treatment series 'E') and 10% (100-mL aliquot, treatment series 'F') (v/v). All media treatments contained 8 g L⁻¹ agar and 20 g L⁻¹ sucrose. The pH of media was adjusted to 6.0 before autoclaving, except for Pa5 medium which was adjusted to 5.6. Testing the pH of media after autoclaving showed that the pH of modified OMA was 5.5 and the pH of asymbiotic media ranged from 5.5 to 5.8. These values were considered acceptable given the pH changes that autoclaving has been shown to induce in plant tissue culture media (George, 1993). All media (FIM, PDA, modified OMA and asymbiotic culture media) were prepared using deionized water, autoclaved for 20 min at 121 °C and 15–20 p.s.i., then dispensed into sterile 90-mm polystyrene Petri dishes (Techno-Plas Pty, Ltd) at 25 mL per plate in a laminar flow cabinet. Details of all prepared asymbiotic culture media and the abbreviations used are given in Table 1.

### Table 1. Asymbiotic culture media treatments

<table>
<thead>
<tr>
<th>Media</th>
<th>Composition</th>
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<tbody>
<tr>
<td>Pa5*</td>
<td>Pa5</td>
</tr>
<tr>
<td>½MS A</td>
<td>½MS + 0.2 μM BA + 0.5 μM NAA</td>
</tr>
<tr>
<td>½MS B</td>
<td>½MS + 0.4 μM BA + 1.0 μM NAA</td>
</tr>
<tr>
<td>½MS C</td>
<td>½MS + 0.6 μM BA + 1.5 μM NAA</td>
</tr>
<tr>
<td>½MS D†</td>
<td>½MS</td>
</tr>
<tr>
<td>½MS E</td>
<td>½ MS + 5% v/v CW</td>
</tr>
<tr>
<td>½ MS F</td>
<td>½ MS + 10% v/v CW</td>
</tr>
<tr>
<td>KC A</td>
<td>KC + 0.2 μM BA + 0.5 μM NAA</td>
</tr>
<tr>
<td>KC B</td>
<td>KC + 0.4 μM BA + 1.0 μM NAA</td>
</tr>
<tr>
<td>KC C</td>
<td>KC + 0.6 μM BA + 1.5 μM NAA</td>
</tr>
<tr>
<td>KC D†</td>
<td>KC</td>
</tr>
<tr>
<td>KC E</td>
<td>KC + 5% v/v CW</td>
</tr>
<tr>
<td>KC F</td>
<td>KC + 10% v/v CW</td>
</tr>
<tr>
<td>VW A</td>
<td>VW + 0.2 μM BA + 0.5 μM NAA</td>
</tr>
<tr>
<td>VW B</td>
<td>VW + 0.4 μM BA + 1.0 μM NAA</td>
</tr>
<tr>
<td>VW C</td>
<td>VW + 0.6 μM BA + 1.5 μM NAA</td>
</tr>
<tr>
<td>VW D†</td>
<td>VW</td>
</tr>
<tr>
<td>VW E</td>
<td>VW + 5% v/v CW</td>
</tr>
<tr>
<td>VW F</td>
<td>VW + 10% v/v CW</td>
</tr>
</tbody>
</table>

*Pa5 medium prepared without modification as per Collins & Dixon (1992).
†The three basal salts (minus CW or PGRs) are referred to as KC D, VW D and ½ MS D.

**Seed Germination**

Symbiotic

Approximately 20 mg of *C. latifolia* seeds were placed in nylon mesh sachets before sterilizing for 10 min in 2% available chlorine solution [3 g 100 mL⁻¹ calcium hypochlorite granules (85% available chlorine) plus two drops of polyoxyethylene-sorbitan mono-oleate surfactant] after which seeds changed colour from dark green to pale yellow (prior experience determined this change in seed colour to be optimal for sterilization while retaining maximum viability). This 10-min sterilization period was initially used for larger quantities of seed placed in nylon bags, prior to the ‘droplet sterilization’ method being developed for small quantities of seed (see below). The sachets containing the seeds were then washed in sterile water three times. Sachets were opened and seeds spread under sterile conditions onto modified OMA medium in sterile 90-mm-diameter Petri dishes (five dishes per sachet). Each Petri dish was inoculated with two cubes (2 mm³) of mycorrhizal fungus placed about 3 cm apart equidistant from the centre of each plate. After sowing, germination plates were incubated in the dark at 23 ± 1 °C. Light was excluded by wrapping plates in aluminium foil. Five replicate plates were prepared for each treatment. Plates were examined weekly and germinated seeds were scored.
Chapter 2

Figure 2. A, stages of germination used in this study to assess germination rate: 0, unimbibed seed; 1, imbibed seed with fissured testa; 2, germination and development of trichomes; 3, enlargement of protocorm and initiation of leaf primordium; 4, further enlargement of protocorm with first green leaf; 5, seedling with green leaf and initiation of dropper/roots (redrawn from Batty et al., 2001); B, stage 3 seed germination in C. latifolia; scale bar = 1 mm.

every 2 weeks for up to 8 weeks with the aid of a dissecting microscope (Olympus SZX16). A 90-mm-diameter plastic grid divided into 2 × 2-cm squares was placed over plates to help with the counting accuracy. At least 100 seeds were assessed from each plate. Seeds without embryos were excluded. Germination was assessed according the germination stages of (Batty et al. (2001) (Fig. 2A). Seeds that reached stage 2 were scored as germinated. Plates were exposed to light (16-h photoperiod, photosynthetic photon flux density approx. 50 μmol m⁻² s⁻¹) when approximately 30% of seeds reached stage 3 germination (Fig. 2B).

Asymbiotic germination
The same seed preparation and sowing procedures were used as detailed above for symbiotic germination of C. latifolia.

Medium effectiveness
An additional experiment was conducted to test the broader effectiveness of the best performing asymbiotic medium obtained from the initial experiment (1/2MS E). Seeds of ten terrestrial orchid species from nine genera were obtained from an orchid seed bank maintained at Kings Park and Botanic Garden (stored for 3–5 years at 15 °C and 15% relative humidity).

Development of droplet seed sterilization method
Due to limited quantities of seeds for many of the study species, adjustments were made to optimize seed recovery during seed sterilization and sowing using a ‘droplet sterilization’ method developed during this study. The same sterilization solution as above was used. A small volume (200 μL) of sterilization solution was placed in the centre of a 55-mm sterile Petri dish, then the end of a toothpick was briefly immersed in the sterilizing solution before dipping it in a seed storage vial so that a small sample of seeds adhered to the tip of the toothpick. The toothpick was then immersed in the sterilization solution to release the seeds and sterilization timing began at this point. Sterilization continued for varying times (2–8 min) depending on species until the seed colour changed to a uniform pale yellow (6.5 min for Diuris magnifica, 6.2 min for Thelymitra benthamiana, 8.1 min for Spiculina ciliata, 5.1 min for Cyanicula gemmata, 4.2 min for Elythranthera brunonis, 3.4 min for Ericksonella saccharata, 4.1 min for Pheladenia deformis, 7.3 min for Eriochilus dilatatus, 5.3 min for Microtis media and 2.4 min for Caladenia huelligii). Seeds were then washed in three changes of sterile water (200 μL each). A 5-mm inoculation loop was used to spread seeds onto plates of asymbiotic medium (three replicate plates were prepared). Approximately 150–200 seeds were spread on each plate per species. Seed germination was assessed using the same method as described above and recorded after 8 weeks.

Experimental design and statistical analysis
A completely randomized design (CRD) was used in the experiment. Germination percentages were calculated by dividing the number of germinated seeds by the total number of seeds with an embryo ×100. All percentage data were arcsine transformed to normalize variation. One-way ANOVA was used to test for significance, followed by a Tukey post-hoc test at $P \leq 0.01$. Statistical analyses were performed using R freeware package (Institute for Statistics and Mathematics, 2014; http://www.rproject.org).

Results
Initial tests using stored and freshly isolated mycorrhizal fungus for C. latifolia symbiotic germination

determined that the stored fungus performed poorly compared with the newly isolated fungus (data not presented), and only newly isolated mycorrhizal fungus inoculum was used for all subsequent symbiotic germination in this study.

Caledonia latifolia seeds began germinating 2 weeks after sowing in symbiotic and asymbiotic treatments. Nine asymbiotic media produced some germination (2–20%) in the first 2 weeks with ½ MS E (containing 5%, v/v, CW) showing the highest germination of 20%, identical to symbiotic germination on modified OMA. At week 4, all treatments registered at least some germination of C. latifolia seeds although this varied considerably, with Pa5 remaining lowest (~1%) and ½ MS E highest at ~70%, again almost identical to symbiotic germination on modified OMA. At 6 weeks most asymbiotic media showed >20% germination except for Pa5 and VW A treatments, whereas ½ MS E had >90% germination which was again comparable to symbiotic germination in modified OMA. The rapid germination response to ½ MS E medium starting from week 2 to week 6 stands out from the other asymbiotic media tested over the same time frame (highlighted in Fig. 3). By 8 weeks, all asymbiotic media except Pa5 (<5%) gave >40% germination, with ½ MS E still the best performing asymbiotic medium treatment with >90% germination (Fig. 3). Final mean germination percentages are shown in Table 2. Asymbiotic germination with C. latifolia seeds essentially peaked from week 6 in ½ MS E media treatment with only a minor further increase by week 8. The final percentage germination in ½ MS E was not significantly different from the final symbiotic germination percentage with modified OMA (~95%) (Table 2).

Overall, the addition of fresh coconut water in ½ MS, KC and VW (E and F series) resulted in significantly improved germination compared with additional BA and NAA (A, B and C series) (Table 2). The greatest beneficial germination in response to CW was in combination with ½ MS, followed by KC and then VW. The addition of PGRs to ½ MS and VW media produced a negative effect on germination.
Table 2. Final germination (percentage) of C. latifolia seeds in all media treatments at the conclusion of the experiment (8 weeks)

<table>
<thead>
<tr>
<th>Media</th>
<th>Germination (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modified OMA</td>
<td>94.94 ± 0.03</td>
</tr>
<tr>
<td>Pa5</td>
<td>3.42 ± 0.12</td>
</tr>
<tr>
<td>½MS A</td>
<td>45.92 ± 0.06</td>
</tr>
<tr>
<td>½MS B</td>
<td>44.25 ± 0.13</td>
</tr>
<tr>
<td>½MS C</td>
<td>54.80 ± 0.08</td>
</tr>
<tr>
<td>½MS D</td>
<td>64.90 ± 0.04</td>
</tr>
<tr>
<td>½MS E</td>
<td>93.43 ± 0.02</td>
</tr>
<tr>
<td>½MS F</td>
<td>89.83 ± 0.01</td>
</tr>
<tr>
<td>KC A</td>
<td>65.81 ± 0.09</td>
</tr>
<tr>
<td>KC B</td>
<td>61.89 ± 0.04</td>
</tr>
<tr>
<td>KC C</td>
<td>63.60 ± 0.04</td>
</tr>
<tr>
<td>KC D</td>
<td>61.18 ± 0.02</td>
</tr>
<tr>
<td>KC E</td>
<td>71.91 ± 0.01</td>
</tr>
<tr>
<td>KC F</td>
<td>68.29 ± 0.01</td>
</tr>
<tr>
<td>VW A</td>
<td>48.62 ± 0.09</td>
</tr>
<tr>
<td>VW B</td>
<td>45.98 ± 0.05</td>
</tr>
<tr>
<td>VW C</td>
<td>58.89 ± 0.05</td>
</tr>
<tr>
<td>VW D</td>
<td>60.15 ± 0.05</td>
</tr>
<tr>
<td>VW E</td>
<td>68.39 ± 0.03</td>
</tr>
<tr>
<td>VW F</td>
<td>68.80 ± 0.02</td>
</tr>
</tbody>
</table>

Mean values (± SEM) followed by the same letter are not significant at *P* ≤ 0.01 (Tukey’s post-hoc test).

(significantly in the case of ½MS) compared with basal media treatments (½MS D and VW D).

These results contrasted with those for KC, for which some PGR amendments (B and C) were slightly but consistently positive throughout the experiment compared with the base KC D treatment, whereas KC A (0.2 μM BAP + 0.5 μM NAA) treatment had a significantly higher final germination result (65%) than KC D (no PGR) at 61%. It is also apparent that time plays a role, with many of the symbiotic media treatments improving substantially towards the end of the experiment (weeks 6 and 8) but still being much slower to reach their maximum germination percentages compared with modified OMA and ½MS E (and maximum germination still remained significantly lower than modified OMA or ½MS E at the conclusion of the trial). Importantly there were no major observable differences in terms of morphological appearance and rate of hypocotyl development between C. latifolia seeds germinated symbiotically in modified OMA and germinated asymbiotically in ½MS E. Some variation in size of hypocotyls was experienced in all treatments but did not appear to cluster in any particular treatment.

An experiment in which a further ten species were germinated on the best performing asymptomatic medium (½MS E, as determined with C. latifolia) provided further evidence for the efficacy of the protocol devised. All species had high germination performance, with Thelymitra benthamiana, Spiculoa ciliata, Cyanicul a gemmata, Elytranchera brunonis, Eriochonella saccharata, Pheladenia deformis, Eriochilus dilatatus, Microtis media and Caladenia huegelii all equalling or exceeding 90% germination of viable seed after 8 weeks. With other species tested (Diuris magnifica, Cyanicula gemmata, Elyranchera brunonis, Erichonella saccharata, Pheladenia deformis and Eriochilus dilatatus), germination ranged from 60 to 73% after 8 weeks. All results in Table 3 are approximately equal to or better than symbiotic germination observed for the above species (K. W. Dixon, pers. comm.). Germinated seeds of the above species continued to develop into vigorous seedlings (including the threatened taxon C. huegelii), indicating that the asymptomatic ½MS E medium is capable of high germination and sustainable seedling development for a range of native terrestrial orchids (Fig. 4).

**DISCUSSION**

Terrestrial orchid species rely on mycorrhizal fungi associations to facilitate seed germination and growth (Warcup, 1973; Clements et al., 1986; Rasmussen, 1995). However, since Knudson (1922) successfully germinated seeds of Cymbidium Sw. asymptotically, many studies have followed this technique and a number of asymptomatic media were developed for orchid seed germination (Knudson, 1946; Van i & Went, 1949; Arditi & Krikorian, 1996; Johnson et al., 2007). Asymptotic germination media such as BM1 (Van Waes & Deberg, 1986), Pa5 (Collins & Dixon, 1992) and MalMgren modified terrestrial orchid medium (MM) (MalMgren, 1996) were in popular use, but due to different germination requirements among...
orchid species, the effectiveness of basal media has been reported to vary widely between species (Hoque et al., 1994). Results in this study indicate that an asymptotic medium is capable of providing a germination rate (percentage and morphological development) indistinguishable from symbiotic germination (in modified OMA, with freshly isolated mycorrhizal fungi) using the study test species C. latifolia. It has been reported that larger and more quickly growing protocorms are produced in symbiotic germination compared with protocorms from asymptotic germination in terrestrial orchid species (Jasaitis & Sorenson, 1993; Jorgensen, 1995). In addition, some researchers have reported species-specific differences in media constituents for orchid seed germination (Bhadra et al., 2002; Kauth et al., 2008; Roy et al., 2011). Therefore, to test the broader effectiveness of the protocol for C. latifolia, the best performing basal medium (½MS E) developed in the first experimental trials was utilized for germination with seeds of a further ten species of Western Australian terrestrial orchids (Table 3) with germination ranging from 60 to 95%. It is not often that an asymptotic germination protocol developed for one orchid species (in this case C. latifolia) is able to provide effective asymptotic germination of seed of a wide phylogenetic range of other terrestrial taxa, comprising three of the five tribes in the terrestrial subfamily Orchideae (Pridgeon et al., 2001), including the diverse Diurideae and Cranichideae. However, further testing is required to test the efficacy of ½MS E in all tribes and across a broader geo-climatic cline of taxa. Some reports compare basalt salts with or without modification for either terrestrial or epiphytic orchids but not with simultaneous amendment of PGR and undefined growth additives such as CW (Stewart & Kane, 2006; Kauth et al., 2008; Dutra, Kane & Richardson, 2009; Dowling & Jasaitis, 2012; Mahendran et al., 2013). Other reports indicate modifications of terrestrial orchid asymptotic media that include additions of both PGRs and undefined organic supplements such as pineapple juice and CW (Kitsaki et al., 2004; Wang et al., 2009; Panwar, Ram & Harish Shekhawat, 2012; Zeng et al., 2012), but no comparison is made with comparable germination under symbiotic conditions. Johnson et al. (2007) compare symbiotic and asymptotic germination with Eulophia

Chapter 2

AUSTRALIAN ORCHID SEED GERMINATION

40

alta (L.) Fawc. & Rendle, in which only one of ten fungal symbionts isolated produced a high rate of germination, but resulted in more rapid protocorm development following germination than asymbiotic germination treatments. The study by Johnson et al. (2007) also illustrated the difficulties with asymbiotic orchid germination where fungal isolates can be problematic in a variety of ways, even if they do have certain benefits.

CW is one of the most frequently cited undefined organic additives for in vitro production of orchids from seed germination to proliferation phases (Piria, Rajmohan & Suresh, 2008), and is generally added to media as a source of sugar(s), natural plant growth supplements including vitamins and some PGRs (Sarma, 2002). Although C. latifolia seeds germinated in all asymbiotic media by the conclusion of the experiment, the three basal salt formulations that contained additional CW resulted in higher germination than the same basal media containing only combinations of PGRs (Table 2). CW is known to contain various phytohormones (auxin, cytokinins, gibberellins), amino acids and vitamins that assist plant growth, but the concentrations of these may be highly variable from one coconut to the next (Yong et al., 2009). Such naturally occurring phytohormones may not always be easily replaced by synthetic PGRs.

Pa5 medium, which also contains 5% (v/v) CW, exhibited the lowest percentage of germination. This indicates a degree of species variability in response to basal media nutrients and additives such as CW. As CW is a natural product it could be that it is difficult to ensure that CW is consistent from coconut to coconut (CW used in this study was extracted from fresh young coconuts), and therefore use of CW should allow for the possibility of product variability. Although the Pa5 medium formulation has been used for asymbiotic seed germination it was originally used for tissue micropropagation of Diuris longifolia R.Br. utilizing immature inflorescence sections (Collins & Dixon, 1992). As seen in Table S1, Pa5 has the lowest total mineral salts (28.82 mM) compared with other formulations (KC, Vw and ½MS). Moreover, it has also the lowest total inorganic N (12.42 mM). It is possible that this low overall mineral salts concentration and low total inorganic N could be responsible for the relatively slow and poor germination experienced with Pa5 in this study. In other studies it has been reported that germinating C. latifolia can take up to 10 months (continuous observation after the initial experiment finished), and 12 months is required to germinate Arachnochis formosa (G.W.Carr) D.L.Jones & M.A.Clem. (Caladenia formosa G.W.Carr) on Pa5 medium (Huynh, McLean & Lawrie, 2002). In addition, Dowling & Jusaitis (2012) reported that seeds of four Australian terrestrial orchids (Pterostylis nutans R.Br., Microtis arenaria Lindl., Thelymitra pauciflora R.Br and Prosperphillum prunorum R.S.Rogers) germinated on Pa5 medium took 12 weeks to reach stage 2 protocorm development (Jusaitis & Sorensen, 1993) which is equivalent to stage 1 according to Batty et al. (2001). Thus, the slow germination response found in this study with the Pa5 formulation has been experienced in past studies with other orchid taxa, including Caladenia species.

Comparison of mineral nutrients in basalt salts (Table S1) indicated that ½MS has the highest inorganic nitrogen (30.01 mM) and nitrate (NO₃, 19.70 mM), almost twice that of KC (10.49 mM) and four times higher than of Vw (6.19 mM). Even so it would appear that the C. latifolia seeds do not necessarily derive maximum benefit (as indicated by seed germination) from this relatively high nitrogen concentration in ½MS, at least until an organic supplement, in this case CW, is added. The addition of CW (at 5%, v/v) certainly stimulates C. latifolia seed germination in the presence of the higher nitrogen titre of the ½MS formulation, but this effect is not as pronounced with the other basal media trialled that have lower overall total nitrogen concentrations (and different ratios of NH₄/NO₃) by comparison (as noted in Table S1). It is possible that the ½MS E formulation (among those media tested) more closely mimics inorganic (particularly total N and/or NH₄/NO₃ ratio) and organic nutrient availability (provided by CW) that is accessible by C. latifolia seeds germinated in conjunction with a mycorrhizal symbiont.

The increase in germination with the addition of CW (5%, v/v) is consistent across all basal media trialled (with the exception of Pa5), but the higher concentration of CW (10%, v/v) reduces or has no apparent impact on seed germination with the three basal media tested, at least during the time scale of this study. This suggests a specific balance between 'sufficient' organic supplement (CW at 5%, v/v) and the 'right' basal medium nutrients (½MS in this study) under in vitro conditions, and it is possible that this reflects the intimate relationship between orchid seeds and their mycorrhizal symbionts as regards nutrient availability and acquisition, although this remains to be proven for the species tested.

The medium developed also provides a ready tool to test for the germination effectiveness (or loss as such) of mycorrhizal fungi and could potentially be assessed by using a similar protocol to the one described here, as efficacy of a fungal symbiont may change over repeated culture cycles, whereas an asymbiotic protocol remains relatively constant (but with possible variation in CW quality and variability in orchid seed batches).

The study has also shown that the usually superior speed and percentage of symbiotic germination can be matched by an asymbiotic germination protocol, while still producing morphologically ‘normal’ seedlings up to and beyond stage 5 protocorms. This study has illustrated the potential for improving in vitro propagation of Australian and potentially other terrestrial orchids via asymbiotic methods. Such an approach reduces the reliance on the more complex process of symbiotic germination [isolating to culturing the fungus (Wright et al., 2009), problems with fungal specificity (Brundrett, 2007) and maintenance of mycorrhizal fungal collections]. Studies at Kings Park (unpubl. data) have shown that asymbiotically generated species can be either planted out, i.e. transferred to soil that is deliberately inoculated with agar- or millet-based fungal inoculums (useful for stem-infected members of Caladeniaceae), or where soil from near an adult plant has been incorporated into the soil mix. For other root-infected orchids, e.g. *The-lymitra* J.R.Forst. & G.Forst., these taxa have been shown to locate suitable mycorrhizal fungi in pasteurized mixes containing organic materials such as composted leaf mulch.

CONCLUSIONS

The asymbiotic medium developed in this study, V2MS E (V2MS basal salts with additional 5%, v/v, CW) has produced an equivalent germination response with seeds of an Australian terrestrial orchid compared with a standard symbiotic germination method. The same asymbiotic germination protocol has shown encouraging results with other terrestrial orchids. Seeds of 11 species representing major clades have been germinated asymbiotically using the protocol described, with no observed abnormalities in seedling morphology compared with symbiotic germination. The array of taxa responding positively to the V2MS formulation in this study includes taxa that can be problematic germinators (e.g. *Caladenia*). This outcome provides confidence that the asymbiotic medium developed here is applicable to a broad range of terrestrial orchid taxa. This protocol also has potential for testing germinability of rare and threatened terrestrial orchids prior to long-term storage without having to wait for mycorrhizal fungi to become available.

ACKNOWLEDGEMENTS

We thank the Indonesian Higher Education Commission for providing the first author with a PhD scholarship. We also thank our colleagues at Kings Park and Botanic Garden including Belinda Newman, Wei-Han Lim and Alex Faber Castell for valuable discussions concerning experiments and Wolfgang Lewandrowski and Bryn Funnekotter for assisting with data analysis.

REFERENCES


Chapter 2

AUSTRALIAN ORCHID SEED GERMINATION


SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Table S1. Details of macro- and micronutrients, organic compounds, total mineral concentration, total nitrogen (N) and ammonium (NH₄) to nitrate (NO₃) molar ratio of the basal media used for asymbiotic seed germination of Caladenia latifolia.

CHAPTER 3

Proliferation and harvesting of secondary protocorms as a novel means for improving propagation of terrestrial orchids
Proliferation and harvesting of secondary protocorms as a novel means for improving propagation of terrestrial orchids

Betty Mauliya Bustam\textsuperscript{A,B,C}, Kingsley Dixon\textsuperscript{A,B}, Eric Bunn\textsuperscript{B}

\textsuperscript{A} School of Plant Biology, The University of Western Australia, 35 Stirling Hwy, Crawley, WA 6009, Australia
\textsuperscript{B} Kings Park and Botanic Garden, Fraser Ave., West Perth, WA 6005, Australia
\textsuperscript{C} Mathematics and Natural Sciences Faculty, Syiah Kuala University, Jl. Syech Abdurrauf No. 3, Darussalam, Banda Aceh 23111, Indonesia

ABSTRACT

This study investigated optimisation of media and primary protocorm development stages to enhance secondary protocorm production as a novel means for propagation of terrestrial orchids including taxa of conservation concern. Seeds of \textit{Caladenia latifolia} were germinated asymbiotically on $\frac{1}{2}$ MS medium fortified with 5\% (v/v) coconut water. Resulting protocorms at 3, 5 and 7 weeks growth were subcultured to protocorm proliferation media treatments consisting of $\frac{1}{2}$ MS basal salts medium with $\alpha$-naphthalene acetic acid (NAA) and 6-benzylaminopurine (BA) singly or in combination. Conversion of seeds to primary protocorms was high (87-92\%). The highest percentage of secondary protocorm proliferation was 40.1\%, using 5 week old protocorms (early Stage 4 of protocorm development) as explants and cultured on $\frac{1}{2}$ MS with a combination of 5 $\mu$M NAA + 2 $\mu$M BA. Half strength MS media containing single plant growth regulators (NAA or BA) were substantially less effective (<10\% protocorm proliferation). This study provides a novel approach to sequential protocorm production that will be of value particularly for threatened orchids with limited seed availability. Protocorm proliferation \textit{in vitro} enables a renewable supply of protocorms with which to conduct propagation, cryostorage and pilot restoration programs.

Keywords: protocorm proliferation, secondary protocorms, \textit{Caladenia latifolia}
INTRODUCTION

Human pressure on the world’s ecosystems has increased dramatically, particularly in the latter half of the past (twentieth) century, with accelerated deforestation and global warming adding to an already serious problem (Thuiller et al., 2005; Liu et al., 2010). Amongst terrestrial flora, orchids face an especially bleak future with many species at very high risk of extinction (Nicholls, 2004; Swarts and Dixon, 2009b). While orchid conservation research has addressed some immediate issues, many problems remain unresolved. One such problem concerns obtaining sufficient seed of endangered species for ex situ conservation purposes especially as terrestrial orchid seed is relatively short-lived compared to other species (Hay et al., 2010). A key difficulty is that with declining habitat quality coupled with a high degree of mycorrhizal and pollinator specificity, obtaining sufficient seeds of a rare orchid for restoration purposes is becoming increasingly challenging (Swarts and Dixon, 2009a). A solution to overcome this problem is enhancing the productivity of available seeds by producing secondary protocorms from primary protocorm explants.

These secondary protocorms can be utilized as renewable seed surrogates and can be generated from a minimum quantity of seed for experiments that may (initially) have a high risk of failure – thus protecting the bulk of the stored seed. Theoretically, a large number of plantlets can be obtained from a single protocorm (Mohanty et al., 2012). Some studies have attempted mass-proliferation of secondary protocorms from primary protocorms, of mainly epiphytic species (David et al., 2008; Hossain et al., 2010; Latip et al., 2010; Roy et al., 2011). However, there is only one recent report that mentions in vitro propagated protocorms of an Australian terrestrial orchid species (Watanawikkit et al., 2012).

In previous experimental work (Bustam et al., 2014) examined asymbiotic germination of Australian terrestrial orchid species in three basal salt formulations, i.e. Murashige and Skoog basal salts (MS) (Murashige and Skoog, 1962) at half-strength, Knudson C (KC) (Knudson, 1946) and Vacin and Went (VW) (Vacin and Went, 1949), with combinations of the plant growth regulators (PGR) NAA and BA. This study found that some protocorm cultures of ½ MS with additional PGR’s showed signs of secondary protocorm proliferation, but none were observed in KC and VW based media treatments. However, the proliferation process was of low frequency and very slow, and it was concluded that this could, at least in part, be because the PGR treatment were not
optimised to enhance the regeneration of protocorms. Type and concentration of PGR play an important role in the micropropagation of many orchids (Arditti and Ernst, 1993). Therefore, it was decided to continue using MS basal salts at half strength (½ MS) with the same PGR (NAA and BA, singly and in combination) as previously tested, while also evaluating PGR concentrations and combinations based on published studies (Park et al., 2002; David et al., 2008; Paudel and Bijaya, 2012; Watanawikkit et al., 2012).

Moreover, it has been reported that the specific type of explant also plays an important role in orchid micropropagation and proliferation (Chugh et al., 2009). Since (Australian) terrestrial orchids have five arbitrary stages of protocorm development recognised from seeds to seedlings (Batty et al., 2001), it was considered that different stages of the development may result in different proliferation outcomes. Therefore, the aim of this study was to research a simple and reliable protocorm proliferation protocol using the indicative genus *Caladenia*.

This genus has the largest number of threatened species of any orchid genus in Australia so it is appropriate to develop approaches for this group. Importantly of the major genera of Australia terrestrial taxa, *Caladenia* is also one of the most problematic in terms of reliable and scalable propagation solutions. The study species is *Caladenia latifolia*, a common Western Australian terrestrial orchid, with outcome of this work likely to be applicable to rare species such as *Caladenia huegelii*. Proliferation of *C. latifolia* protocorms has been reported previously however the focus of the study was on cryopreservation of protocorms (Watanawikkit et al., 2012). Moreover only one medium and one PGR (MS with addition of 10 µM BA) was reported. Experiments were therefore conducted to: (1) evaluate the most suitable primary protocorm stage(s) that would produce the highest yield of secondary protocorms, (2) assess the most effective concentration of PGR, and (3) evaluate the most effective PGR used, whether used singly or in combination.
Chapter 3

MATERIAL AND METHODS

All experiments were conducted without the use of mycorrhizal fungi (i.e. asymbiotically). Capsules containing naturally pollinated mature seeds were obtained in early August 2011 from *Caladenia latifolia* plants (20) in bushland located in Kings Park and Botanic Garden (KBPG). Seeds were combined and desiccated over silica gel (24 h at 22°C) before storage (at 4°C) in airtight containers. Nylon mesh sachets containing approximately 20 mg of seeds of *C. latifolia* were sterilised in 2% available chlorine solution (3g 100ml⁻¹ calcium hypochlorite granules [65% available chlorine] + two drops of polyoxyethylene-sorbitan mono-oleate surfactant) for 10 min, at which point seeds changed colour from dark green to pale yellow (prior experiments found this seed colour change to mark the optimal sterilization time, while still retaining high viability). The sachets containing the seeds were then washed in sterile water three times. Sachets were opened and seeds spread under sterile conditions onto germination medium with the contents of each sachet divided approximately equally between five replicates Petri plates. Sixteen sachets for a total of 80 germination plates were prepared to provide for required selective protocorm harvesting at each developmental stage. After sowing, germination plates were wrapped in aluminium foil to exclude light and incubated at 23±1°C.

Asymbiotic culture medium

Asymbiotic germination medium (optimised via previous experiments) consisted of ½ strength MS basal salts (Murashige and Skoog, 1962) with 100 µM NaFeEDTA, 60 mM sucrose, 500 µM myo-Inositol, 500 µM 4-morpholineethanesulfonic acid (MES), 1µM thiamine hydrochloride, 2.5 µM pyridoxine, 4 µM nicotinic acid, 20 g/L sucrose, 0.8% w/v agar (Gelita Australia Pty Ltd) and pH set to 6.0 prior to autoclaving at 121°C for 20 min. Nylon mesh was used to remove larger particulates from the milk from fresh young coconuts bought at a local supermarket. Aliquots of 50 mL were frozen (-20 °C) and thawed as required. The coconut water (CW) was filter-sterilized using 0.22 µm syringe-driven filter unit and added to warm (~50°C) post-autoclaved basal medium at 5% v/v concentration. Medium was dispensed into sterile 90-mm polystyrene Petri dishes (Techno-Plas Pty. Ltd, Australia) in a laminar flow unit to maintain sterility. Deionised water, unless otherwise specified, was used in all media preparation.
Proliferation process

Plates were examined at 3, 5, and 7 weeks after sowing on germination medium with the aid of a dissecting microscope (Olympus SZX16, Japan). Seeds were scored as germinated if they reached stage 2 according to Batty et al. (2001). A 90 mm diameter plastic grid divided into 2 cm x 2 cm squares was placed over plates to help with the counting accuracy. At least 250 seeds were assessed from each replicate plate to determine germination percentage. Seeds without an embryo (i.e. no proembryo inside the seedcoat) were excluded from both counting and subculturing. Protocorms corresponding to various developmental stages (Fig. 1) were selected at 3, 5 and 7 weeks after seed plating and subcultured to proliferation media treatments. Protocorms were subcultured from germination medium to proliferation media treatments using sterile forceps under a sterile airflow with the aid of a stereomicroscope (Nikon SMZ 800, Japan). Approximately 50-100 protocorms were transferred to each proliferation medium plate with each treatment consisting of five replicate plates (~250- 500 protocorms per treatment for each protocorm development stage) for a total of 240 plates for the entire experiments.

Plates were incubated in the dark at ± 23°C. After 4 weeks of incubation protocorms were subcultured to fresh proliferation media to continue the proliferation process. The percentage of protocorm proliferation was assessed at the conclusion of the second culture cycle on proliferation media treatments, as accurately counting individual protocorms amidst clumps of proliferating secondary protocorms was considered too difficult by the third culture cycle. The same procedure and counting aids as with seed germination were used and only those protocorms showing definite signs of secondary protocorm development were scored (Fig. 4a). Subculturing was continued every 4 weeks for six culture cycles to determine whether proliferation of secondary protocorms would continue and be adequate for future experiments.
Fig 1. Seeds and protocorms stages of *Caladenia latifolia* used in this study: (a) viable seeds (stained with tetrazolium) typical of those used for germination, scale bar = 500 µm; (b) 3 weeks after sowing (Stage 3), scale bar = 0.2 mm; (c) 5 weeks after sowing (Stage 4), scale bar = 0.5 mm; (d) 7 weeks after sowing (Stage 5), scale bar = 1 mm.

**Proliferation media**

Protocorm proliferation was undertaken using the same basal ½ MS minerals and organics as for germination medium (detailed above) with additional PGRs, BA and NAA, either singly or in combination (Table 1). CW was omitted but PGRs were added prior to autoclaving. Four concentrations of BA and two concentrations of NAA were used. Control treatments contain only ½ MS without addition of PGRs.
Table 1. Proliferation media

<table>
<thead>
<tr>
<th>Medium</th>
<th>Composition (+ ½ MS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>1 µM NAA</td>
</tr>
<tr>
<td>B1</td>
<td>5 µM NAA</td>
</tr>
<tr>
<td>C1</td>
<td>2 µM BA</td>
</tr>
<tr>
<td>D1</td>
<td>5 µM BA</td>
</tr>
<tr>
<td>E1</td>
<td>7 µM BA</td>
</tr>
<tr>
<td>F1</td>
<td>10 µM BA</td>
</tr>
<tr>
<td>A2</td>
<td>1 µM NAA + 2 µM BA</td>
</tr>
<tr>
<td>B2</td>
<td>5 µM NAA + 2 µM BA</td>
</tr>
<tr>
<td>C2</td>
<td>1 µM NAA + 5 µM BA</td>
</tr>
<tr>
<td>D2</td>
<td>5 µM NAA + 5 µM BA</td>
</tr>
<tr>
<td>E2</td>
<td>1 µM NAA + 7 µM BA</td>
</tr>
<tr>
<td>F2</td>
<td>5 µM NAA + 7 µM BA</td>
</tr>
<tr>
<td>G2</td>
<td>1 µM NAA + 10 µM BA</td>
</tr>
<tr>
<td>H2</td>
<td>5 µM NAA + 10 µM BA</td>
</tr>
</tbody>
</table>

Experimental design and statistical analysis

This experiment used a Completely Randomized Design (CRD) approach. Germination percentages were calculated by dividing the number of protocorms (germinated seeds) by the total seeds (x100) with data from all replicates pooled, while protocorm proliferation percentages were calculated by dividing the number of primary protocorms exhibiting secondary protocorm development by the total number of initial primary protocorms in each treatment, with data from all replicates pooled (x 100). Treatments used in this study were protocorm stage x medium treatments with a total of 48 treatments: 21 single PGR treatments and 27 combined PGR treatments. To normalise variation, all percentage data were arcsine transformed before being analysed with analysis of variance (ANOVA) followed by a Tukey post-hoc test at P ≤ 0.01. R freeware package (Institute for statistics and Mathematics of Wirtschaftsuniversitat Wein: www.r-project.org 2014) was used to perform statistical analysis.
RESULTS

Asymbiotic seed germination

Caladenia latifolia germinated reliably on ½ MS + 5% (v/v) coconut water with 80-93% germination starting as early as 2 weeks from sowing, with consistent protocorm development from week 3 up to 7 weeks. Three weeks after sowing, seeds were in Stage 2 and Stage 3 (majority, i.e. >90% of seeds developed to Stage 3). Five weeks after sowing, seeds were in late Stage 3 and Stage 4 (majority of seeds at Stage 4). Seven weeks after sowing, seeds were in late Stage 4 and stage 5 (majority of seeds at stage 5), as illustrated in Fig. 1. There were no significant differences in percent asymbiotic germination between all plated seeds. Germination (i.e. to Stage 2) ranged from 87-92% after 7 weeks.

Proliferation

The numbers of non-reactive protocorms was very low in the combined-PGR treatments but up to 50% in the single-PGR treatments in the first culture period. Protocorm proliferation for all single-PGR treatments was uniformly low, with the highest result of 5.3% with Stage 4 protocorms (5 weeks post-sowing) on C1 medium, ½ MS + 2 µM BA (Fig. 2). There were no significant differences among single-PGR treatments (Fig. 2). Combined-PGR treatments including treatments A2, C2 and, E2-H2 showed similarly low (<5%) proliferation with no significant differences between these treatments (Fig.3).

However two combined-PGR treatments resulted in significantly higher protocorm proliferation compared to other combined-PGR treatments: B2 (½ MS + 5 µM NAA + 2 µM BA) using protocorms at 5 weeks after sowing with 40.1%, followed by D2 medium (½ MS + 5 µM NAA + 5 µM BA) also using protocorms at 5 weeks after sowing with 23.3% (Fig. 3). On medium B2, the transition to the earliest signs of secondary protocorm development occurred after one subculture cycle (Fig. 4a) and developed to multiple secondary protocorms after three subculture cycles (Fig. 4b) and then finally to ‘soft’ secondary protocorm development after six subculture cycles (Fig. 4c).

Throughout this multi-cycle proliferation bulking process on B2 medium, protocorms remained vital and healthy with no sign of diminished proliferation or reduced growth and development. Moreover, although signs of proliferation could be seen as early as
the first subculture with single-PGR treatments, these tended to develop ‘hard’ secondary protocorms in dense, compact clumps (compared to combined-PGR treatments), with this tendency increasing with subsequent culture cycles (Fig. 5a,b). Most ‘soft’ secondary protocorms (approximately 75%) showed a greater abundance of trichomes when compared with ‘hard’ secondary protocorms (Fig. 5a,b). Primary protocorms for control treatments were subcultured once (from germination medium to control proliferation medium) because no sign of proliferation could be observed and most such primary protocorms (~95%) eventually proceeded to develop directly into plantlets after 12 weeks or longer (Fig. 6a).

‘Hard’ secondary protocorms formed in compact clumps and could only be separated (with difficulty) by scalpel and forceps, but rapidly became brown and most failed to proliferate further. In contrast, ‘soft’ protocorms formed on combined PGR treatments were easily separated and maintained proliferation capacity while ~20% on B2 medium (following six subculture cycles) turned green and developed into plantlets (Fig. 6b).

![Graph showing single-PGR treatments of secondary protocorm proliferation with Caladenia latifolia](image)

**Fig 2.** Single-PGR treatments of secondary protocorm proliferation with *Caladenia latifolia* expressed as percentage of primary protocorms (selected at 3, 5, and 7 weeks post-sowing). Media treatments indicated as A1-F1 relate to proliferation media with single PGR (see Table 1). There were no significant differences between treatments. Error bars represent standard error of mean.
Fig 3. Combined-PGR treatments of secondary protocorm proliferation with *Caladenia latifolia* expressed as percentage of primary protocorms (selected at 3, 5, and 7 weeks post-sowing). Media treatments indicated as A2-H2 relate to proliferation media with combined PGR (see Table 1). Mean values (± standard error) followed by the same letter are not significantly different at $P \leq 0.01$ (ANOVA and Tukey's test).
Fig. 4. Development of *Caladenia latifolia* primary protocorm proliferation on proliferation medium B2 (½ MS + 5 µM NAA + 2 µM BA): (a) first stage secondary protocorms (arrows) forming on a primary protocorm after one subculture cycle, scale bar = 2 mm; (b) multiple secondary protocorms (arrows) after three subculture cycles, scale bar = 1 mm; (c) soft secondary protocorms (arrows) after six subculture cycles, scale bar = 2 mm

Fig. 5. Two types of *Caladenia latifolia* secondary protocorms: (a) dense ‘hard’, difficult to separate secondary protocorms typical of single-PGR treatments; (b) soft, easily separated secondary protocorms typical of combined-PGR treatments (note increased presence of trichomes).
Fig. 6. *Caladenia latifolia* plantlets (a) control medium (½ MS, minus PGRs) formed from primary protocorms after 12 weeks incubation; (b) from secondary protocorms on B2 medium (5 µM NAA + 2 µM BA) after 7 weeks incubation on the sixth subculture cycle.

**DISCUSSION**

The current study aimed to improve upon the previous study conducted by Watanawikkit *et al.* (2012) by testing a wider range of media and investigating the most suitable stage of primary protocorm development to enhance secondary protocorm production. Micropropagation-related experiments with the main aim of mass production have been conducted around the world, using many different parts of orchids such as the flower stalk, axillary buds, leaf segments as well as protocorms (Chen *et al.*, 2002; Park *et al.*, 2002; David *et al.*, 2008; Huang and Chung, 2011; Sujarirutharakarn and Kanchanapoom, 2011; Khoddamzadeh *et al.*, 2011; Nambar *et al.*, 2012). However, these studies have mainly (with some exceptions, *e.g.* Collins and Dixon 1992) been conducted on epiphytic orchid species and not terrestrial taxa, it is also of paramount importance to develop protocols that suit Australian species. Germinated seeds (protocorms) were chosen as explants as seeds contain a diversity of genotypes and, therefore (theoretically at least) are more suitable for conservation programs. This assumes that proliferation capacity is not a genetically sensitive screen.

No significant differences in percentage seed germination (*i.e.* to Stage 2) were observed, with all seeds in this study being germinated asymbiotically on the same medium and incubated under the same conditions. The amount of secondary protocorm proliferation, however, showed clear differences among treatments (Fig. 2 and Fig. 3). It
is reported that orchids require auxins and/or cytokinins for neo-formation of protocorm like bodies (PLBs) and plantlet development (Roy et al., 2011). Although some studies showed PLBs can be effectively generated from media treatments using a single PGR (Sujjarithurakarn and Kanchanapoom, 2011; Khoddamzadeh et al., 2011), other studies showed otherwise, with the combinations of PGRs (including NAA and BA) producing more effective PLB proliferation (Park et al., 2002; Chen and Chang, 2006; David et al., 2008; Roy et al., 2011). Our results support those studies, because NAA and BA (5 µM NAA + 2 µM BA and 5 µM NAA + 5 µM BA) were the two optimal treatments for inducing proliferation of secondary protocorms (using primary protocorms as starting material) of *C. latifolia*.

Moreover, other studies that obtained successful seed and proliferation of PLBs using MS formulations (half or full strength) (Lin et al., 2000; Chen et al., 2002; Park et al., 2002; Huang and Chung, 2011; Mahendran and Bai, 2009; Khoddamzadeh et al., 2011; Moharty et al., 2012) provided support for our decision to continue using ½ MS for proliferation media (based on previous results in our laboratory). This may be due to the fact that MS based media generally contain higher amounts of inorganic nitrogen compared to other basal orchid media such as Vacin and Went (VW) and Knudson C (KC).

Another finding of this study was the difference between secondary protocorms formed from single-PGR compared to combined-PGR treatments. While many plates of single-PGR treatments produced hard protocorms with few or no trichomes, all combined-PGR treatments produced soft protocorms with abundant trichomes typical of healthy protocorms. ‘Hard’ protocorms when cut and separated before transferring to fresh media exhibited a browning reaction, possibly due to phenolic exudation (Chugh et al., 2009).

Some studies suggested using liquid media to enhance proliferation (Park et al., 1996; Van et al., 2011). Liquid medium was attempted (B2 minus agar) in some samples on the fourth subculture of B2 solid medium plates. However, browning of some secondary protocorms (and signs of possible contamination) resulted in early termination of the experiment. While further investigation of liquid protocorm proliferation medium would be worthwhile, our results indicated that solid media provide a reliable and easily manipulated approach for proliferation compared to liquid media used for the epiphytic
Doritaenopsis hybrids (Park et al., 2002) and Phalaenopsis bellina (Khoddamzadeh et al., 2011).

While time after sowing to reach Stage 4 protocorm development is likely to differ when this protocol is implemented with other orchid species, we expect that a similar stage of primary protocorm development combined with a suitable PGR/medium treatment will result in superior secondary protocorm proliferation. At the conclusion of this study it was observed that ~20% of secondary protocorms formed plantlets on B2 medium, which is encouraging but not as high as primary protocorm conversion to plantlets (>95%) with *C. latifolia*. However, the B2 medium, while ideal for secondary protocorm proliferation, is unlikely to be optimal for conversion to plantlets. Hence, further experimentation will be necessary to optimise the conversion rate of secondary protocorms to plants, which will be of maximum benefit for threatened species where seed is in short supply.

**CONCLUSIONS**

This study was designed as a pilot project with the findings to be implemented with other terrestrial orchids, particularly rare species such as the nationally threatened species *Caladenia huegelii*. As such, this study has established a novel protocol for proliferating terrestrial orchids from primary protocorm explants, developed for an Australian terrestrial species, *Caladenia latifolia*. Primary protocorms of *C. latifolia* at 5 weeks following germination (Stage 4) appeared to be the most suitable stage at which to induce proliferation of secondary protocorms, with a solid basal medium (½ MS) and PGR treatment (5 µM NAA and 2 µM BA) achieving the highest proliferation percentage and the best type of secondary protocorms (easily separated ‘soft’ protocorms with abundant trichomes). Approximately 20% of these protocorms formed plantlets. While plantlet conversion remains to be optimised, the extended proliferation cycles achievable with secondary protocorms bode well for efficient production of rare and threatened terrestrial orchid species.
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REFERENCES


CHAPTER 4

Cryogenic approaches for conserving Western Australian terrestrial orchids
Cryogenic approaches for conserving Western Australian terrestrial orchids

Betty Mauliya Bustam\textsuperscript{A,B,C}, Kingsley Dixon\textsuperscript{A,B}, Eric Bunn\textsuperscript{B}

\textsuperscript{A} School of Plant Biology, The University of Western Australia, 35 Stirling Hwy, Crawley, WA 6009, Australia

\textsuperscript{B} Kings Park and Botanic Garden, Fraser Ave., West Perth, WA 6005, Australia

\textsuperscript{C} Mathematics and Natural Sciences Faculty, Syiah Kuala University, Jl. Syech Abdurrauf No. 3, Darussalam, Banda Aceh 23111, Indonesia

\textbf{ABSTRACT}

In a bid to better conserve Western Australian (WA) endemic and endangered terrestrial orchids, we detail experimental cryogenic research undertaken on somatic tissues using a common WA terrestrial orchid, \textit{Caladenia latifolia} for development of cryopreservation for primary protocorms, and also for secondary (adventitious) protocorms. Primary protocorm cryopreservation (using droplet vitrification) involved a number of experimental lines of inquiry: investigation of a suitable plant vitrification solution (PVS) by comparing three variants of a standard plant vitrification solution (PVS 2, 3, and 4), determining the most suitable primary protocorm developmental stage for successful cryopreservation, testing the effectiveness of a preculture medium treatment prior to cryopreservation, investigating temperature preconditioning and changing components of the recovery medium. Primary protocorms were generated using an asymbiotic \textit{in vitro} germination method developed by the authors specifically for the test species (half strength MS macro and micro elements with 5\% v/v fresh coconut water) and secondary protocorms using an \textit{in vitro} proliferation method (½ MS with 5 \textmu M \textalpha-naphthalene acetic acid + 2\textmu M 6-benzylaminopurine) developed previously. The cryopreservation protocol developed increased survival of primary protocorms from 68\% to 85\% and average regeneration of plants from primary protocorms post-cryostorage increased from 17\% to 48\%. Following similar development of the protocol devised for primary protocorms, survival of secondary protocorms was increased from 63 to 84\% with regeneration of plants from secondary protocorms after cryostorage increasing from 11\% to 26\% in 14 weeks.

\textbf{Keywords}: cryogenic, \textit{Caladenia latifolia}, primary protocorm, secondary protocorm, proliferation
INTRODUCTION

In Australia there are approximately 1,700 species of orchids of which approximately 95% are endemic with 424 species categorised as threatened, rare or in some cases extinct (Backhouse, 2007). As with many other specialised species, orchids worldwide are experiencing rapid decline due to loss of natural habitats to increasing take up of land for agriculture, over-harvesting from natural populations to satisfy horticultural demand for new products and in recent decades, climate change (Mc Carty, 2001; Nicholls, 2004; Thuiller et al., 2005; Swarts and Dixon, 2009; Liu et al., 2010). For those orchid species that are rare, limited seed production (both in quantity and viability) means that effective conservation remains problematic (Coates and Dixon, 2007). Efforts have been undertaken to conserve threatened terrestrial Australian orchid species including in situ and ex situ research initiatives (Batty et al., 2001; Brundrett, 2007; Swarts and Dixon, 2009). However, for many species, prospects for conservation are hampered by limited scope for conventional seed banking due to the poor storage characteristics of many Australian terrestrial orchid seeds (Merritt et al., 2014).

While some progress has been made with cryopreservation of orchid seeds (Popova et al., 2003; Hirano et al., 2005; Nikishina et al., 2001), and orchid symbiont fungi (Sommerville et al., 2008), there can be disadvantages, including germination problems that occur during the germination phase, such as seed infection for unknown reasons (Nikishina et al., 2001). In addition, limited seed availability hampers research on rare species, and there are still some problems encountered following different storage methods, including seed cryopreservation (Merritt et al., 2014). Hence, there is ample scope for developing protocols for maximising the use of limited seed stocks so that a small number of primary protocorms can provide secondary protocorms that can be used for developing in vitro and cryopreservation protocols.

We define secondary protocorms as any protocorms developing adventitiously from primary protocorms, or other secondary protocorms in successive culture cycles. By developing those protocols with secondary protocorms, more experiments can be conducted with a minimum amount of valuable seeds needing to be used. There have been some protocols for orchid cryopreservation that have involved seeds as well as protocorm like bodies (PLBs) but not for terrestrial orchid species, nor Australian orchids (Nikhisina et al., 2007; Maneerattanarungjoy et al., 2007; Yin and Hoy, 2009; Hooi et al., 2010; Khoddamzadeh et al., 2011). As far as is known, there are only three
reports of cryopreservation of Australian terrestrial orchids from seeds or protocorms (Batty et al., 2001; Sommerville et al., 2008; Watanawikkit et al., 2012) but none describe protocols specifically for secondary (adventitiously propagated) protocorms.

This paper describes a series of experiments conducted to develop cryopreservation protocols for primary and secondary protocorms including selection of a suitable plant vitrification solution (PVS), selection of the most appropriate stage(s) of primary protocorm development (according to Batty et al., 2001), evaluating the effectiveness of temperature preconditioning, changing various components of the recovery medium and finally, implementing an improved protocol with secondary (adventitious) protocorms. *Caladenia latifolia* R.Br., a common Western Australian terrestrial orchid, was used as a model species, with the protocol developed for *C. latifolia* to be used as a template for development of specific cryopreservation protocols for protocorms (primary and secondary) of rare orchid species. All experiments were conducted with the overall aim of achieving as simple and cost-effective protocols as possible that would be suitable to use in conservation programs in developing countries where funding is limited.

**MATERIAL AND METHODS**

**Developing primary protocorms from seeds**

Seeds were germinated *in vitro* asymbiotically to obtain desired protocorm stages for cryopreservation experiments. Seed sourcing and sterilizing were conducted as described by Bustam et al. (2014). Both sterilization methods *i.e.* nylon bags and droplet method, were used. Nylon bags sterilization was used for initial experiments where larger numbers of primary protocorms were required, while droplet sterilization was used in subsequent experiments where fewer primary protocorms were needed. ½ MS E, asymbiotic medium (Bustam et al., 2014), was used to germinate seeds. All other *in vitro* propagation (unless otherwise specified) was based on ½ MS E, as described in Bustam et al. (2014) but without coconut water (CW). Unless otherwise specified, all experiments were done twice with all results pooled for final analysis. Basal media and cryopreservation solutions were autoclaved except for heat labile components that were filter sterilized (syringed through a sterile 0.2µm microfilter) and added to other constituents post-autoclaving – the methods are described in Bustam et al. (2014) and Kaczmarczyk et al. (2013).
Cryopreservation experiments for primary protocorms

Cryopreservation experiments for primary protocorms were conducted using a droplet vitrification method as described by Kaczmarczyk et al. (2012) with some modifications to obtain better results. The experiments were conducted with primary protocorms to firstly establish a workable protocol that was then applied to secondary protocorms, with adjustments made as necessary.

Data collection was based on two parameters, i.e. survival, and regeneration. Absence of browning was indicative of protocorm survival as determined in prior studies (and as illustrated in Fig. 5A) while greening and development into plantlets (Fig. 6A) was indicative of protocorm regeneration. Survival and regeneration data sets are presented as percentages relative to the numbers of protocorms sampled, with at least 5 replicate samples (each consisting of at least 150 protocorms) taken from each plate. Survival percentages were calculated by dividing the number of protocorms that were not showing definite signs of browning by the total protocorms for pooled replicate samples (x100), while regeneration percentages were calculated by dividing the number of protocorms that were greening or developing into plantlets by the total numbers of protocorms (x100) for pooled replicated samples.

Selection of plant vitrification solution (PVS) and protocorm stage

Primary protocorms were selected at 3, 5, and 7 weeks after sowing asymbiotically, corresponding to early stages 3, 4, and 5 of protocorm development according to Batty et al. (2001). Three commonly used variants of plant vitrification solution or PVS were used based on methods as described in Suzuki et al. (2008) for PVS2 and Nishizawa et al. (1993) for PVS3 and PVS4 formulations respectively. Primary protocorms of desired stages were transferred onto solid preculture medium (½ MS + 20 g L⁻¹ sucrose + 0.8% w/v agar + 0.2 M raffinose) and incubated in the dark at 20°C for 48 hours.

Protocorms were immersed in 2 ml of loading solution (½ MS minus agar, with 0.4 M sucrose and 2 M glycerol) for 20 minutes at room temperature. A sterile mini-sieve was used as a tool to allow easier movement of protocorms from loading solution (LS) to PVS treatments. Protocorms were then treated with PVS for 20 minutes at 0°C while remaining in the sieve, after which they were transferred to aluminium foil strips (in a drop of PVS solution) in the last 10 minutes PVS incubation at 0°C. Five PVS droplets (each containing approximately ten protocorms) were placed on one 10 mm x 5 mm foil
strip (1 droplet contains ~1-2 µL of PVS), then strips were placed in cryovials
(Nunc®Cryotube vial, 1ml), with two foils strips/vial before being stored in Liquid
Nitrogen (LN) for 2 hours.

Control protocorms (not exposed to LN) were removed from PVS, treated with washing
solution (½ MS with 1 M sucrose, autoclaved) for 20 minutes and placed immediately
on recovery medium. Unless specified otherwise, control protocorm treatments
followed the same procedure in subsequent experiments. Recovery medium (RM)
consisted of ½ MS with 0.8% w/v agar, 2 mM chlorine chloride, 1 µM zeatin and 0.5
µM gibberellic acid. After 2 hours in LN, protocorms were warmed in a 40°C water
bath for 10 seconds, treated in washing solution (WS) and placed on RM (as described
for control protocorms). All protocorms were incubated in the dark at 20°C for two
weeks before being exposed to standard culture room light (16 hr photoperiod supplied
by 30W fluorescent lamps with PPFD 30 µmolm²s⁻¹). All experiments were conducted
twice with at least 5 replicates/treatment (1 plate = 1 replicate). Approximately 100
protocorms per plate were examined every week to check the survival, signs of
regeneration, and record the timing of regeneration phases (formation of new root and
shoot buds and development into plantlets).

Preculture medium

The efficacy of the preculture step in the cryopreservation process was tested separately.
Stage 4 primary protocorms (5 weeks after sowing) were used (based on results of
selection of PVS and protocorm stage experiment above), incubated without (second
control treatment) and with preculture medium (consisting of ½ MS + 20 g L⁻¹ sucrose
+ 0.8% w/v agar + 0.2 M raffinose for 48 hrs at 20°C) and then treated with LS (20 min,
at RT), then PVS2 and PVS4 cryoprotectant solutions only (results of selection of PVS
and protocorm stage experiment above suggested PVS3 solution did not result in post-
cryopreservation regeneration with primary protocorms, therefore was not tested in
subsequent experiments). All other procedures including number of protocorms/plate,
replication, standard control (minus LN) treatments, survival, and regeneration data
collection were as previously described.
Temperature preconditioning and recovery medium

The effects of temperature preconditioning and changing of the RM was investigated separately, but based on the first (selection of PVS and protocorm stage) and second (preculture medium) experimental data and experiences. Two different approaches were implemented simultaneously. Primary protocorms were dark incubated at 15°C in preculture medium (½ MS + 20 g L⁻¹ sucrose + 0.8% w/v agar + 0.2 M raffinose) for 48 hours (a 5°C reduction from 20°C used in prior experiments), then protocorms were treated in LS (20 min, RT), processed for cryopreservation, revived, washed, placed on RM and scored as already described.

The second experiment followed the same procedure as the first but the RM was changed after the first week of dark incubation (following cryopreservation) from the standard RM (½ MS with 0.8% w/v agar, 2 mM chlorine chloride, 1 µM zeatin and 0.5 µM gibberellic acid) to asymbiotic germination medium (½ MS + 5% (v/v) coconut water), then dark incubation at 20°C was continued for another week, after which protocorms were moved to standard culture conditions (as described earlier). All other procedures were as previously described for cryopreservation of primary protocorms except that a –LN treatment (standard control) was not applied, as the previous experiment (preculture medium) control treatment was considered sufficient considering that the seeds used were from an identical batch and the experiments were conducted concurrently.

Cryopreservation of secondary protocorms

Cryopreservation of secondary protocorms was conducted following the method for primary protocorms with some adjustments as necessary. Secondary protocorms were harvested from the proliferation of primary protocorms on ½ MS with 5 µM α-naphthalene acetic acid (NAA) + 2 µM 6-benzylaminopurine (BA). After six culture cycles (subculturing to fresh proliferation medium every four weeks) sufficient numbers of secondary protocorms were available for cryopreservation experiments.

Prior experiments (selection of PVS and protocorm stage, preculture medium and temperature preconditioning and changing of recovery medium) guided the base protocol to include the use of PVS2, a preculture stage and changing of the RM. Secondary protocorms were taken from proliferation medium (½ MS with 5µM NAA + 2 µM BA) and placed on preculture medium for 48 hours at 15 and 20°C, then
incubated in LS (20 min, RT) followed by PVS2 for 30 min, then LN immersion for 2 hr, after which protocorms were warmed at 40°C for 20 s, washed in WS for 20 min and plated to RM (½ MS with 0.8% w/v agar, 2 mM chlorine chloride, 1 µM zeatin and 0.5 µM gibberellic acid). After one week in darkness at 20°C one set (5 reps) of protocorms were transferred to proliferation medium, then dark incubation continued for another three weeks.

The other set of protocorms remained on RM in darkness for the whole 4 weeks. All secondary protocorms were then exposed to standard culture conditions i.e. 23±1°C with light (PPFD = ~ 30 µmolm⁻²s⁻¹ for 16/8 hr photoperiod) to induce regeneration into plantlets. Secondary protocorm survival and regeneration was recorded with absence of browning indicative of protocorm survival (Fig. 5B) while greening and development into plantlets (Fig. 6B) was indicative of protocorm regeneration. Following data collection (as per primary protocorms), survival and regeneration data sets are presented as percentages relative to the numbers of protocorms sampled, with at least 5 replicate plates (each consisting of 48 secondary protocorms) sampled per treatment. Survival percentages were calculated by dividing the number of protocorms that were not brown (or showing definite signs of browning) by the total secondary protocorms (x100) for pooled replicate samples, while regeneration percentages were calculated by dividing the number of secondary protocorms that were greening or developing into plantlets by the total numbers of protocorms (x100) for pooled replicated samples.

Secondary protocorms produced with the method described tended to be larger compared to primary protocorms, with an average primary protocorm measuring approximately 0.25 mm long while an average secondary protocorm was approximately 0.73 mm (see Fig. 4A and 4B). Moreover, the number of secondary protocorms available for experiments was considerably lower than experienced with primary protocorms, owing to slower multiplication and development. It was not feasible (in the time frame of the study) to synchronize the secondary protocorm proliferation process so that secondary protocorms at precisely the equivalent to Stage 4 development in primary protocorms, could be harvested in sufficient numbers.

Therefore necessary adjustments were made to five parts of the cryopreservation protocol: 1. the number of PVS2 droplets/foil was reduced from five to four; 2. the amount of PVS2/droplet increased from 1 to ~2-3 µL; 3. the number of secondary
protocorms/droplet was reduced to three (10 for primary protocorms); 4. the total number of secondary protocorms/plate sampled per treatment was reduced to 48 (down from ~100 primary protocorms/plate) and 5. warming time after cryopreservation increased to 20 s (10 s with primary protocorms). The LS step remained unchanged.

**Statistical analysis**

All data were analysed through ANOVA using R freeware package (Institute for statistics and Mathematics of Wirtschaftsuniversitat Wein: [www.r-project.org](http://www.r-project.org), 2014) to perform the statistical analysis. All percentage data were arcsine transformed before being analysed to normalise variation. Treatment means were separated with Tukey’s post-hoc test at (P ≤ 0.01).

**RESULTS**

*Plant vitrification solution (PVS) and suitable protocorm stage selection*

Survival and regeneration of protocorms in control treatments without Liquid Nitrogen (-LN) were not statistically different with percent survival and regeneration in the range of 68-81% (Fig. 1 and Fig. 2). However differences among treatments were evident with LN exposure. Generally, early stage three protocorms (3 weeks after sowing) performed less well than the other two stages (Stages 4 and 5) on both parameters (survival and regeneration) with three different types of cryoprotectant (see Fig. 1 and Fig 2). Although some Stage 3 protocoms did survive after cryopreservation the percentage of protocorms regenerating was very low (less than 3%). Stage 4 primary protocorms (5 weeks after sowing) gave the highest percent survival and regeneration, with 66% of these protocorms surviving after cryopreservation on PVS2 and 9% regenerated into seedlings. Stage 5 primary protocorms (7 weeks after sowing) showed a similar high percentage of survival but only 3% of these protocorms regenerated.

Although some protocorms in all three stages of development survived after being cryopreserved with PVS3 as cryoprotectant, none of these regenerated. Many protocorms showed early signs of browning after one week of dark incubation (Fig. 5B) with some of them turning completely brown by the second week of incubation, similar to the browning later observed with secondary protocorms (Fig. 5D).
**Fig. 1.** Percentage of *C. latifolia* protocorm survival following exposure to three plant vitrification solutions (PVS) and three different stages of protocorm development at 3, 5, and 7 week after sowing. C2 = control of PVS2 treatment, C3 = control of PVS3 treatment, C4 = control of PVS4 treatment. PVS2 = PVS2 treatment, PVS3 = PVS3 treatment, PVS4 = PVS4 treatment. Mean values followed by the same letter are not significant at $P \leq 0.01$ (Tukey post-hoc test).

**Fig. 2.** Percentage of *C. latifolia* protocorms regeneration following exposure to three plant vitrification solutions (PVS) and three different stages of protocorm development at 3, 5, and 7 week after sowing. C2 = control of PVS2 treatment, C3 = control of PVS3 treatment, C4 = control of PVS4 treatment. PVS2 = PVS2 treatment, PVS3 = PVS3 treatment, PVS4 = PVS4 treatment. Mean values followed by the same letter are not significant at $P \leq 0.01$ (Tukey post-hoc test).
Effectiveness of preculture medium

Results show that protocorms that were precultured appear to have higher survival and a better opportunity to regenerate but only with PVS2 as cryoprotectant. Percent survival after cryopreservation with PVS2 without and with preculture was in the range of 40-69% and for PVS4 from 43%-55% respectively. Percentage regeneration more than tripled (from 4.3% to >17%) when protocorms were incubated with preculture medium before being cryopreserved using PVS2 (see Fig. 3, PVS2 A and PVS2 B). The same preculture treatment with PVS4 as cryoprotectant did not result in a significant difference in regeneration (Fig. 3, PVS4 A, PVS4 B).

**Fig. 3.** Percentage of *C. latifolia* protocorm survival and regeneration during testing of the effectiveness of a preculture medium step. C2 A (–LN –PM, PVS2), C2 B (–LN +PM, PVS2), C4 A (–LN –PM, PVS4), C4B (–LN +PM, PVS4), PVS2 A (+LN –PM, PVS2), PVS2 B (+LN +PM, PVS2), PVS4 A (+LN –PM, PVS4), PVS4 B (+LN +PM, PVS4). LN = Liquid Nitrogen, PM = Preculture Medium. Mean values followed by the same letter are not significant at \( P \leq 0.01 \) (Tukey post-hoc test).

Temperature preconditioning, changing recovery medium and cryopreservation of secondary protocorms

Control treatments constantly showed no significant differences among treatment sets for both survival and regeneration of primary protocorms without temperature preconditioning or with temperature preconditioning (Table 1). Adding a preculture step resulted in increased survival with primary protocorms increasing from 68 to 85% with PVS2, and from 58% to 77% using PVS4. Similarly, a preculture step caused regeneration of primary protocorms to increase significantly from 17 to 48% using
PVS2, and from 6 to 11% using PVS4. Based on these results, only PVS2 was used as cryoprotectant for secondary protocorms.

Survival of secondary protocorms improved from 63 to 84% with temperature preconditioning and changing recovery medium and regeneration went from 11 to 26% (Table 1). Moreover, development of secondary protocorms into plantlets after cryopreservation was reduced from 20 to 14 weeks (Fig 6B), when the protocorms were preconditioned and the recovery medium changed. In comparison, it took approximately 6 weeks after cryopreservation for the majority of primary protocorms to develop into plantlets (Fig. 6A) both before and after temperature preconditioning and changing recovery medium. Based on the whole study, cryopreservation protocols for primary and secondary protocorms for *Caladenia latifolia* can be summarized as per Table 2.
Table 1. Comparison of survival and regeneration of primary and secondary protocorms of *C. latifolia* after cryopreservation with and without temperature preconditioning and with and without changes to recovery media

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<th>Treatment</th>
<th>-temp. preconditioning/-recovery</th>
<th>+temp. preconditioning/+recovery</th>
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<tr>
<td></td>
<td>Survival (%)</td>
<td>Regeneration (%)</td>
</tr>
<tr>
<td>Primary protocorm</td>
<td>Control</td>
<td>PVS2</td>
</tr>
<tr>
<td></td>
<td>87 ± 2.37a</td>
<td>82 ± 3.60a</td>
</tr>
<tr>
<td></td>
<td>80 ± 3.38a</td>
<td>79 ± 4.79a</td>
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<tr>
<td></td>
<td>68 ± 4.93ab</td>
<td>17 ± 2.44c</td>
</tr>
<tr>
<td></td>
<td>58 ± 6.34b</td>
<td>6 ± 1.08d</td>
</tr>
<tr>
<td>Secondary protocorms</td>
<td>Control</td>
<td>PVS2</td>
</tr>
<tr>
<td></td>
<td>79 ± 3.65ab</td>
<td>63 ± 5.67ab</td>
</tr>
<tr>
<td></td>
<td>63 ± 6.54ab</td>
<td>11 ± 1.27d</td>
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Mean values followed by the same letter are not significant at $P \leq 0.01$ by Tuckey contrast
Fig. 4. Primary and secondary protocorms of *C. latifolia* before cryopreservation. (a). Primary protocorms (5 weeks after sowing – early Stage 4), scale bar = 0.5 mm. (b). Secondary protocorms, 28 weeks after sowing, scale bar = 0.5 mm.

Fig. 5. Primary and secondary protocorms of *C. latifolia* after cryopreservation. (a). Survival of primary protocorms (no indication of browning). (b). Early indication of browning on primary protocorms. Scale bar (a and b) = 0.5 mm. (c). Survival of secondary protocorms. (d). Browning secondary protocorms. (c and d) = 0.5 mm.
Fig. 6. Regeneration and development of cryopreserved primary and secondary protocorms of *C. latifolia* into plantlets following temperature preconditioning + changing recovery medium protocol, with PVS2 cryoprotectant. (a). Plantlets from primary protocorms, 6 weeks after cryopreservation, scale bar = 0.5 mm. (b). Plantlets from secondary protocorms, 14 weeks after cryopreservation, scale bar = 1 mm.
Table 2. Summary of the protocols developed in this study.

<table>
<thead>
<tr>
<th>Cryopreservation protocol steps</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Preculture and preconditioning</strong></td>
<td>Primary protocorms (early Stage 4 for primary protocorms) or a clump (~3/clump) of secondary protocorms were precultured on solid preculture medium (½ MS + 20 g L⁻¹ sucrose + 0.8% w/v agar + 0.2 M raffinose) and incubated in the dark at 15°C for 48 hours. Protocorms were immersed in liquid loading solution (½ MS + 0.4 M sucrose and 2 M glycerol) at room temperature for 20 minutes.</td>
</tr>
<tr>
<td><strong>Cryopreservation</strong></td>
<td>Protocorms were immersed in PVS2 for 20 minutes then moved to PVS2 droplets on aluminium foil for another 10 minutes at 0°C. ~1-2 µL of PVS/droplet for primary protocorms and ~2-3 µL of PVS/droplet for secondary protocorms. Aluminium foil strips placed in 1ml cryovials (2/vial) and plunged in liquid nitrogen for 2 hours.</td>
</tr>
<tr>
<td><strong>Thawing</strong></td>
<td>Protocorms were warmed up by plunging cryovials in a 40°C water bath for 10 s for primary protocorms and 20 s for secondary protocorms.</td>
</tr>
<tr>
<td><strong>Survival and regeneration evaluation</strong></td>
<td>Protocorms were incubated in the dark at 20°C on solid recovery medium (½ MS + 0.8% w/v agar, 2 mM chlorine chloride, 1 μM zeatin and 0.5 μM gibberellic acid) for one week then moved to asymbiotic germination medium (½ MS + 5% (v/v) coconut water) for primary protocorms and proliferation medium (½ MS + 5µM NAA + 2 µM BA) for secondary protocorms, incubated for another one week for primary protocorms and three weeks for secondary protocorms. Survival defined by absence of browning in protocorms. Protocorms then exposed to light (PPFD = ~ 30 μmolm⁻²s⁻¹ for 16/8 hr photoperiod). Regeneration can be evaluated approximately 6 weeks after cryopreservation process for primary protocorms and 12 weeks for secondary protocorms. Regeneration was defined by greening and development into plantlets.</td>
</tr>
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</table>

81
Chapter 4

DISCUSSION

A prior study on *Caladenia latifolia* determined that cryopreservation of protocorms of this species was feasible but relied solely on an FDA staining method to assess protocorm survival following cryopreservation (Watanawikkit et al., 2012). The current work reported in this paper substantially extends the work by Watanawikkit et al. (2012), by reporting on post-cryopreservation survival, regeneration, and development into plantlets of both primary and secondary protocorms with *C. latifolia*.

Vitrification without encapsulation has been reportedly used for orchid cryopreservation in a number of cases (Ishikawa et al., 1997; Nikishina et al., 2007; Watanawikkit et al., 2012), while other studies report on encapsulation-vitrification (Yin and Hong, 2009; Mohanty et al., 2012) and encapsulation-dehydration techniques for orchid seed (Flachsland et al., 2006; Sommerville et al., 2008), protocorms (Maneerattanarungjoy et al., 2007; Jitsopakul et al., 2008; Gogoi et al., 2013) and protocorm like bodies (Antony et al., 2001; Yin et al., 2011; Khoddamzadeh et al., 2001).

Initial trials on vitrification and LN exposure with primary protocorms of *C. latifolia* in this study were not as successful as reported by Watanawikkit et al. (2012) with most protocorms turning brown and dying around two weeks following cryopreservation, despite following the methods (with minor adjustments) of Watanawikkit et al. (2012). It was therefore decided to try droplet vitrification as this method is relatively straightforward to perform, uses very small volumes of cryoprotectant, hence achieves very rapid freezing and consequently high post-cryo recovery with most species tested (Kaczmarczyk et al., 2012; Kim and Lee, 2012). Moreover, primary protocorms of *C. latifolia* are much smaller with an indistinguishable white colour compared to protocorms of most epiphytic species, hence it was thought that encapsulating such small protocorms was a less practical proposition than placing them in a small amount of cryoprotectant with aid of sterile forceps and a mini-sieve.

This assumption influenced the decision of this study to try another approach, i.e. preconditioning at a lower temperature (cold acclimatization) and changing the recovery medium, with *C. latifolia* using a droplet vitrification method without encapsulation. As can be seen from the results (Table 1), preconditioning at a lower temperature (cold acclimatization) and sub-culturing to the suitable regeneration medium significantly improved regeneration results. Several reports on other plant species support the
findings that cold acclimatization increases survival and regeneration following cryopreservation (Chang and Reed, 2000; Reed et al., 2003; Senula et al., 2007).

Up to the present approximately five main cryoprotectant mixtures are known and have been used in plant cryopreservation, i.e. PVS1 (Uragami et al., 1989; Towill, 1990), PVS2 (Suzuki et al., 2008), PVS3, PVS4, and PVS5 (Nishizawa et al., 1993). Among those cryoprotectant solutions, PVS2, PVS3, and PVS4 were widely used (Nishizawa et al., 1993; Ashmore et al., 2001; Ellis et al., 2006; Towill et al., 2006; Heringer et al., 2013). More studies reported successful cryopreservation results using PVS2 as cryoprotectant (Rong and Hua, 2012; Sinniah and Gantait, 2013; Yin et al., 2014). Those studies led to the decision to use PVS2, PVS3, and PVS4 in the first set of experiments of this study. Although PVS3 was reported as suitable for other plant species with some regeneration occurring (Kim et al., 2009; Barraco et al., 2011; Heringer et al., 2013), no regeneration occurred with C. latifolia primary protocorms and PVS3 was therefore deleted from further experiments.

It was considered that the size (volume) of the sample being cryopreserved would impact on successful survival and regeneration. It has been observed that larger (within the range used) orchid protocorms perform better after cryostorage than smaller examples (Antony et al., 2011; Watanawikkit et al., 2012). Moreover, for cryopreservation of terrestrial orchid primary protocorms, despite variations in sample size, we postulated that distinct developmental stages would perform differently and in turn would have different effects on survival and regeneration. Our assumptions were supported by the results in this study that indicated strongly that Stage 4 primary protocorms (corresponding to 5 weeks after sowing) gave the best survival and regeneration results following cryopreservation.

There are a number of studies determining developmental stages of primary protocorms, from seeds to plantlets (Clements et al., 1986; Ramsey et al., 1986; Jusaitis and Sorensen, 1993; Batty et al., 2001). However, we have not located any reports regarding the developmental stages of secondary (adventitious) protocorms. The latter part of this study details the first attempts at cryopreserving secondary protocorms (generated from asymbiotically germinated primary protocorms) and subsequent plantlet regeneration for Western Australian terrestrial orchids. To achieve this within the time frame of the study, we concentrated solely on developing sufficient secondary protocorms for
establishing first principals for cryopreservation, without seeking to select discrete secondary protocorm developmental stages.

From a practical perspective, the cryopreservation protocols outlined in this study have delivered promising results and could be implemented with other terrestrial orchid species (in particular Western Australian taxa). However, further studies are needed to increase regeneration success for both primary and secondary protocorms following cryopreservation. Moreover, additional studies for establishing whether secondary protocorms actually undergo the same developmental patterns as primary protocorms is also necessary, to determine if there is a preferred developmental stage for cryopreservation as was determined for primary protocorms. In addition, further refinement of the preconditioning and recovery phases of the protocol for both primary and secondary protocorms remain to be concluded to maximise recovery and plant regeneration.

CONCLUSIONS
Results from the series of experiments conducted in this study have established successful cryopreservation protocols for primary and secondary protocorms for *Caladenia latifolia*. PVS2 was found to be the most suitable cryoprotectant solution and early Stage 4 (5 weeks after sowing) primary protocorms development was determined to be the most suitable stage for cryopreserving *C. latifolia* primary protocorms. Preconditioning protocorms on solid preculture medium for 48 hours at 15°C and amending the standard recovery protocol to include subculture to asymbiotic germination medium (½ MS + 5%v/v CW) for primary protocorms and protocorm proliferation medium (½ MS + 5µM NAA + 2 µM BA) for secondary protocorms, significantly improved post cryopreservation regeneration. The protocol will be used as a template for other terrestrial orchid taxa, including rare species in future studies.

ACKNOWLEDGEMENTS
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CHAPTER 5

Ex situ conservation of a threatened Western Australian terrestrial orchid, *Caladenia huegelii*, through micropropagation and cryopreservation
Ex situ conservation of a threatened Western Australian terrestrial orchid, Caladenia huegelii, through micropropagation and cryopreservation

ABSTRACT

The main aim of this study was to develop micropropagation and cryopreservation protocols for threatened Western Australian terrestrial orchid species, Caladenia huegelii, to enhance ex situ conservation. Five sets of experiments were set up, including asymbiotic germination, protocorm proliferation, cryopreservation of primary protocorms, cryopreservation of secondary protocorms, and a preliminary experiment of transfer to soil, and acclimatization (weaning) under glasshouse conditions. Asymbiotic germination on optimised medium, ½ MS E (½ MS fortified by 5% (v/v) coconut water) resulted in an average germination of 94%. Protocorm proliferation was conducted using Stage 4 protocorms development (eight weeks after sowing asymbiotically) with 43% of primary protocorms proliferating on B2 medium (½ MS + 5µM NAA + 2 µM BA). Cryopreservation experiments were carried out using both primary Stage 4 protocorms and secondary protocorms developed during protocorm proliferation experiments. Constant incubation of protocorms at 20°C after cryopreservation resulted in an increase in regeneration of both primary and secondary protocorms. Seedlings regenerated after primary protocorms were cryopreserved were successfully acclimatized in soil under glasshouse conditions after twelve weeks. 88% seedlings survived and grew well with an average leaf length increase of approximately 4.1 cm in twelve weeks. Cryopreservation of secondary protocorms was also achieved, with viable seedlings generated 15 weeks after revival from LN storage. These results are encouraging and will be a useful additional means to implement successful ex situ orchid conservation that can also be extended to many other terrestrial orchids, especially endangered species.

Keywords: ex situ conservation, Caladenia huegelii, micropropagation, cryopreservation.
Chapter 5

INTRODUCTION

*In situ* plant conservation describes retaining species in their natural habitats via management strategies to preserve habitats and whole ecosystems with their constituent populations of species in reserved lands, while *ex situ* plant conservation describes conservation outside the natural habitat, commonly via seed banking, micropropagation and cryopreservation of shoots, seeds or seed embryos (Cochrane *et al.*, 2007) and is considered to be an important complimentary component of integrated conservation programs (Seaton, 2007). It has been suggested that integrated conservation (the melding of *in situ* and *ex situ* disciplines) is the ideal way to conserve rare and threatened orchid species (Dixon, 1994; Dixon and Batty, 2003; Swartz, 2007; Seaton, 2007).

*Caladenia* is an endemic Western Australian terrestrial orchid genus that consists of at least 300 species (Hopper and Brown, 2004; Koreset *et al.*, 2001; Dixon and Hopper, 2009) with approximately one third of these species are considered as rare and endangered (Backhouse, 2007). One of these threatened species is *Caladenia huegelii* Rchb.f. This tuberous, perennial terrestrial orchid species, with a height ranged from 0.25 – 0.6 m, can be found in the Jarrah Forest, Perth, Swan Coastal Plain, and Southern Jarrah Forrest (FloraBase, 2014 - Fig. 1). This species has been previously used as a model species in orchid conservation programs (Swartz, 2007).

![Caladenia huegelii](image)

*Fig. 1. Caladenia huegelii* (FloraBase, 2014)
This study was conducted with the main aim to develop micropropagation and cryopreservation protocols for *Caladenia huegeli*. Five experiments were undertaken: 1. asymbiotic germination, 2. protocorm proliferation, 3. cryopreservation of primary protocorms, 4. cryopreservation of secondary protocorms, 5. preliminary assessment of plantlet transfer to soil and acclimatization under glasshouse conditions.

Assessing the ability of *C. huegelii* plantlets to adjust to transfer from *in vitro* conditions to soil with accompanying acclimatization under glasshouse conditions also offered the opportunity to gain information as to the feasibility of introducing the mychorrhizal fungus as soil inoculum at the seedling stage of plants generated from cryopreserved protocorms (derived from seeds germinated asymbiotically).

**MATERIAL AND METHODS**

*Seed sources and asymbiotic germination*

Mature seeds of *Caladenia huegelii* were harvested from mature plants grown in a glasshouse at Kings Park and Botanic Garden, collected in spring 2013. There were approximately 20 capsules collected and seeds were mixed then put in a 1ml cryotube (Nunc®) and kept in a seed drying room (15°C and 15% RH). All experiments were conducted using the same seed batch. All germination experiments were conducted following the optimum techniques and media from previous experiments using *Caladenia latifolia* (Chapter 2-4). To use as little seeds as possible yet remain effective, the droplet sterilization technique developed from previous experiments (Bustam *et al.*, 2014) was used to sterilize seeds. Optimised asymbiotic germination medium, ½ MS fortified by 5% (v/v) coconut water, was used to germinate seeds asymbiotically and the percent germination was scored as described in Chapter 2. This experiment used 15 replicate plates with approximate seed numbers/plate as described in Chapter 2. The experiment then continued to the second set of experiments (protocorm proliferation) after germination data was collected at early Stage 4 (eight weeks after sowing) (Fig. 3a).

*Protocorm proliferation*

The two best performing protocorm proliferation media (developed in prior experiments outlined in Chapter 3), and containing combined plant growth regulators (PGRs), were B2 (½ MS + 5µM NAA + 2 µM BA) and D2 (½ MS + 5µM NAA + 5 µM BA). These
media were used to develop secondary protocorms from primary protorms through the proliferation process. Protocorms at Stage 4 (from the asymbiotic germination experiment) were subcultured to proliferation media. There were five replicates/treatment. Protocorms that showed signs of proliferation (Fig 4a, Chapter 3) were subcultured to fresh proliferation medium every three weeks for eight cycles to generate sufficient secondary protocorms for cryopreservation experiments. Protocorms were maintained in a constant 20°C incubator. The percentage of protocorm proliferation was as described in Chapter 3. Analysis of variance (ANOVA) was used to analyse the data. Percent data was arcsin transformed before performing analysis using R freeware package (Institute for statistics and Mathematics of WirtshaftuniversitatWein: www.r-project.org, 2014). Tukey post-hoc test at P ≤ 0.01 was used to test for significant treatment differences.

Cryopreservation of primary and secondary protocorms

This set of experiments was conducted based on previous primary protocorm cryopreservation experiments completed using Caladenia latifolia (Chapter 4). The optimum developmental stage for cryopreservation of primary protocorms was determined to be Stage 4 (Batty et al., 2001), (Fig. 1, Chapter 3). C. huegelii seeds were germinated asymbiotically in ½ MS E (½ MS fortified by 5% (v/v) coconut water) in a constant 15°C incubator, in the dark (plates were wrapped up with aluminium foil) for eight weeks, when the majority of protocorms reached Stage 4 (Fig. 3a).

A droplet cryopreservation method, with temperature pre-treatment as well as changed recovery medium (as detailed in Chapter 4), was used. This experiment was divided into two groups. Both of them were treated using the same cryopreservation techniques as described in Chapter 4. The difference was in the light incubation stage after cryopreservation at the regeneration stage. The first group was moved to culture room conditions when they were exposed to light (PPFD = ~ 30 µmolm⁻²s⁻¹ for 16/8 hr photoperiod) and kept at 23 ± 1°C while the second group was kept in a 20°C incubator (PPFD = ~ 30 µmolm⁻²s⁻¹ for 16/8 hr photoperiod). Five replicates/treatment, including a control, were used in this experiment.

Cryopreservation of secondary protocorms was conducted following the same techniques as used for primary protocorms. Secondary protocorms were developed from primary protocorms during the proliferation experiment as outlined earlier. This experiment was also arranged with five replicates/treatment, with 40 secondary
protocorms/plate (for a total of 200 secondary protocorms/treatment). Data collected was percent survival, percent regeneration, and time required to develop into viable plantlets after cryopreservation (as described in Chapter 4). Survival for both primary and secondary protocorms was recognized as per methods in Chapter 4. Absence of browning was categorized as survival, while developing into plantlets was determined as regeneration. Statistical analysis was performed as in the protocorm proliferation experiment.

Transfer to soil and acclimatization under glasshouse conditions

Transfer to soil and acclimatization under glasshouse conditions was conducted with seedlings developed from primary protocorms after cryopreservation. The soil mixture consisted of five components: white sand, Canadian peat moss, perlite, proprietary orchid mix, and soil containing mycorrhizal inoculum.

The composition of soil mix was 50% fine white sand, 25% sieved Canadian peat moss, 20% perlite, 3% professional orchid mix (Osmocote, Scotts Australia), 2% soil from pots planted with *C. huegelii* (to ensure the presence of some *C. huegelii* mycosymbiont). Three compositions (fine white sand, sieved Canadian moss, and perlite) were mixed and steam pasteurized in stem trailers at 60°C for 30 minutes, and then put in a forestry tray (13 cm x 8 cm x 3.5 cm) (Fig. 2a).

The proprietary orchid mix and soil from pots of *C. huegelii* plants were mixed and put in the forestry trays after the pasteurised white sand, sieved Canadian moss and perlite components were added and the soil mix was ready to use. To prevent excessive moisture loss due to increasing temperature from incubator to glasshouse, seedlings of *C. huegelii* that were previously grown in a Petri dish (Fig. 2b), were treated with an antidesiccant/protective coating (AgroBest ‘Envy’- AgroBest Australia) at 10 ml L⁻¹, for approximately 1 minute (Fig. 2c) before being planted into the combined soil mixture.

Two forestry trays contained 60 seedlings/tray were prepared. Trays were placed in a fogged glasshouse with temperature in the range of 23 – 26°C for two weeks, and covered with a clear plastic canopy (Yates Garden products humidity cover) with a 3 cm hole to provide ventilation. After 3 weeks the plastic canopy was removed and plants were placed on a glasshouse bench. Watering was provided with an interval of three to four days, depending on temperature inside the glasshouse. Sowing trays were put
inside a plastic container (16 cm x 11 cm x 5 cm), with one plastic container per sowing tray. Approximately 200 ml of water was poured into the plastic container to provide bottom-up moisture for the soil medium and seedlings. The duration of this experiment was 12 weeks.

Survival and growth data were taken, with survival of seedlings monitored weekly. Seedlings remaining green and continuing to grow (enlarge) were recorded as surviving. Growth of seedlings was recorded by measuring leaf lengths fortnightly.

**Fig. 2.** Preparation of planting the *C. huegelii* seedlings from Petri dish to soil medium. (a). soil mix in a forestry tray, (b). *C. huegelii* seedlings in a Petridish (post cryopreservation), (c). *C. huegelii* seedlings in liquid anti desiccant.
RESULTS

Asymbiotic germination and protocorm proliferation

Average percent germination of *Caladenia huegelii* of this experiment was 94 % ± 1.58 (average germination in 15 plates), while percent proliferation is detailed in Table 1.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent Proliferation*</th>
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<tbody>
<tr>
<td>Control</td>
<td>(½ MS)</td>
</tr>
<tr>
<td>B2</td>
<td>(½ MS + 5µM NAA + 2 µM BA)</td>
</tr>
<tr>
<td>D2</td>
<td>(½ MS + 5µM NAA + 5 µM BA)</td>
</tr>
</tbody>
</table>

*Percent of primary protocorms undergoing secondary protocorm proliferation

Mean values followed by the same letter are not significant at $P \leq 0.01$ (Tukey post-hoc test)

Secondary protocorms of *C. huegelii* developed in this study were almost the same morphologically as those of *C. latifolia*, *i.e.* easily separated ‘soft’ protocorms (Fig. 5b Chapter 3) but with fewer trichomes on average (Fig. 3b and Fig. 4b). While there were few morphological differences between secondary protocorms developed in both proliferation media, percent proliferation on D2 was much lower (9%) than that of B2 (43%) (Table 1). No secondary protocorms developed in the control treatment indicating an absolute requirement for PGR presence to promote secondary protocorm initiation and proliferation (Table 1).

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**Fig. 3.** (a). *Caladenia huegelii* primary protocorm Stage 4 (eight weeks after sowing), (b). *Caladenia huegelii* secondary protocorm. Scale bar a and b = 500 µM.
Cryopreservation of primary and secondary protocorms

Comparison of survival and regeneration between primary and secondary protocorms in two different incubation temperatures after cryopreservation can be seen in the Table 2.

Table 2. Cryopreservation of *C. huagelii* primary and secondary protocorms

<table>
<thead>
<tr>
<th>Treatment</th>
<th>23 ± 2°C incubation</th>
<th>20 ± 0.5°C incubation</th>
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<tr>
<td></td>
<td>-LN</td>
<td>+LN</td>
</tr>
<tr>
<td></td>
<td>Survival (%)</td>
<td>Regeneration (%)</td>
</tr>
<tr>
<td>Primary protocorms</td>
<td>85 ± 2.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>83 ± 3.36&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>89 ± 1.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>89 ± 2.33&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Secondary protocorms</td>
<td>82 ± 1.63&lt;sup&gt;a&lt;/sup&gt;</td>
<td>80 ± 4.24&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>86 ± 3.48&lt;sup&gt;a&lt;/sup&gt;</td>
<td>86 ± 1.96&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Mean values followed by the same letter are not significant at P ≤ 0.01 by Tuckey contrast.
Survival of both primary and secondary protocorms can be evaluated one week after cryopreservation process. Constant incubation at $20^\circ \pm 0.5^\circ C$ after the cryopreservation process and during the transition from dark to light incubation was found to increase percent regeneration in primary protocorms from 46% to 79%; and also increased percent regeneration of secondary protocorms from 19% to 45% (Table 2). Moreover, $20^\circ \pm 0.5^\circ C$ incubation treatment also reduced the time to regeneration from nine weeks to seven weeks for primary protocorms and from 18 to 15 weeks for secondary protocorms.

**Fig. 4.** Plantlets from primary and secondary protocorms after cryopreservation. (a). Plantlets from primary protocorms, twelve weeks after cryopreservation. Scale bar = 0.5 cm. (b). Plantlets from secondary protocorms, seventeen weeks after cryopreservation. Scale bar = 1 mm.

*Transfer to soil acclimatization under glasshouse conditions*

Average leaf length of seedlings when first moved to the soil was $1.2 \pm 0.07$ cm and this increased to an average $5.3 \pm 0.39$ cm at the end of the experiment, indicating an average growth rate of 3 - 4 mm per week (Fig. 5a-5d).
Fig. 5. Transfer of seedlings, generated from cryostored primary protocorms of *Caladenia huegelii*, to soil and acclimatization to glasshouse conditions. (a). planting to soil. (b). one week after transfer to soil. (c). four weeks after transfer to soil. (d). Twelve weeks after transfer to soil. Scale bar (for all pictures) = 1 cm.

DISCUSSION

This study is the first conducted on a threatened Western Australian terrestrial orchid species in this case *Caladenia huegelii*, in terms of using an asymbiotic germination protocol to enhance *ex situ* conservation. A previous study with this species was based on symbiotic germination to generate plants for conservation purposes (Swartz, 2007). In another study with terrestrial orchids conducted using two endangered species in New South Wales, *Pterostylis saxicola* D.L. Jones & M.A Clem. and *Diuris arenaria* D.L. Jones, it was suggested that *ex situ* conservation of threatened terrestrial orchids
should, if possible, be done simultaneously with their mycosymbionts (Sommerville et al., 2008).

Sommerville et al. (2008) conducted the research using seeds with the mycorrhizal fungus encapsulated together and reported that while encapsulation per se did not impede seed germination, loss of co-encapsulated fungal viability significantly affected the ability of the seeds to germinate. Moreover, maintaining the right temperature and moisture content of simultaneously encapsulated mycorrhizae and seeds is also a challenge that has proved difficult to overcome (Wood et al., 2000). In addition, although the simultaneous encapsulated protocol can be implemented with other orchid species, the protocol needs to be optimized for each fungus and orchid combination due to different growth requirements of mycorrhizal fungi (Sommerville et al., 2008). The mycorrhizal fungus growth protocol, homogenisation process, duration of immersion in sucrose solution and duration of air drying (of the capsule) are among factors that need to be optimised to retain vigorous fungal symbionts (Block, 2003).

This study used asymbiotic approaches as an alternative way towards orchid ex situ conservation. It was thought that developing suitable asymbiotic approaches in vitro and introducing the mycorrhizal symbionts at a later stage bypasses difficulties with loss of fungal viability inherent in symbiotic methods as described above. This study has successfully germinated > 1000 C. huegelii seeds asymbiotically in ½ MS E (½ MS fortified by 5% (v/v) coconut water), with >90% germination success on average. Cryopreserved primary protocorms from asymbiotic germination were able to regenerate into viable plantlets/seedlings and these seedlings have been able to be planted in soil containing mycorrhizal fungal inoculum and then grown symbiotically under glasshouse conditions.

The trial on transfer to soil and acclimatization is promising because 88% of the seedlings survived and grew under the glasshouse conditions despite the fact that this experiment was conducted in late winter (mid August 2014), which is not the usual Autumn germination and seedling growth period for Australian terrestrial orchids. More experiments, however, need to be conducted to determine the ability of seedlings to produce tubers to survive during the Summer dormant period. Studies conducted by Swartz (2007) and Sommerville et al. (2008) suggested that late autumn (April-May) is
the right month to transfer seedlings to soil, so that seedlings are able to produce tubers by late October or early November of the same year.

Results of this study suggest that synchronizing the whole process, starting with seed harvesting in mid spring (September-October), asymbiotic germination of fresh seed and growth to Stage 4 protocorms (8 weeks), cryopreservation and post-cryopreservation regeneration of seedlings (12 weeks) followed by transfer of seedlings to soil (~ 2 wks) could be arranged for April-May as recommended by Swartz (2007) and Sommerville et al. (2008). In this way the whole cryopreservation process from seed collection, asymbiotic germination and primary protocorm production, cryostorage, revival and transfer of plantlets to soil (to verify the protocol for plant production for conservation purposes) is achievable within one year. Excess cryopreserved protocorms could remain stored in LN for varying lengths of time until needed, as long as revival from LN was begun in summer (December-January) to allow time for seedlings to develop sufficiently for transfer to soil in April-May.

Experiments with protocorm proliferation supported reports in the literature that type and concentration of PGRs play an important role in orchid micropropagation (Arditi and Ernst, 1993). The two best performing protocorm proliferation media, B2 (½ MS + 5µM NAA + 2 µM BA) and D2 (½ MS + 5µM NAA + 5 µM BA), developed with C. latifolia also resulted in protocorm proliferation with C. huegelii. 43% of Stage 4 asymbiotically germinated primary protocorms produced secondary protocorms in response to B2 medium and 9% with D2 medium. This result suggests that combining PGRs (specifically 5µM NAA + 2 µM BA in ½ MS basal medium) provided superior protocorm initiation and proliferation for both C. latifolia and C. huegelii. This medium could prove useful for protocorm multiplication with other Australian terrestrial orchid species, but remains to be tested.

Adjustments made during development of the cryopreservation protocol for C. huegelii enabled an increase in regeneration success for both primary and secondary protocorms as well as decreasing the time to achieve regeneration. Incubation temperature following cryopreservation appears to play an important role in improving protocorm regeneration. Previous studies conducted with C. latifolia (Chapter 4) suggested that temperature preconditioning significantly improved regeneration, as reported by Chang and Reed (2000); Reed et al. (2003) and Senula et al. (2007). This study suggests that
maintaining the right temperature after cryopreservation will also improve regeneration significantly, with a 23% increase in regeneration of primary protocorms post-cryopreservation (from 46 to 79%) and a similar result with secondary protocorms (from 19 to 45%) as well as a reduced time to achieve seedling regeneration (Table 2).

CONCLUSIONS

This study has successfully developed both micropropagation and cryopreservation protocols for a threatened Western Australian species, Caladenia huegelii. In addition, this species has also survived and grown well following experiments on transfer of seedlings (developed from primary protocorms after cryopreservation) to soil with acclimatization under glasshouse conditions.

The study has opened up the possibility for future application of the protocols developed here to other threatened orchid taxa (particularly Western Australian terrestrial taxa). During the development of these protocols, adjustments were made to procedures to further enhance success including changing the incubation temperature following recovery from cryopreservation (to a constant 20°C) to increase post-cryopreservation regeneration success. Further research will however be necessary to build on the progress made in this study, particularly for secondary protocorms, including their transfer to soil, re-introduction to mycorrhizal fungus and acclimatization.
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CHAPTER 6

General Discussion
General Discussion

INTRODUCTION

This study details new and more efficient protocols for *ex situ* conservation of Western Australian terrestrial orchids. Development of the protocols involved researching, improving and redeveloping some basic techniques that have been in use for quite some time to evolve more suitable techniques needed in orchid conservation. This study investigated *in vitro* symbiotic and asymbiotic orchid seed germination approaches to provide orchid protocorms with which to conduct *in vitro* and cryopreservation research streams.

It has been mentioned in previous chapters that orchids are a unique flora that have been in rapid decline worldwide due to various causes, from over harvesting wild plants as cut flowers to maximise profit (de Faria *et al.*, 2002), overuse as medicinal plants (Bhadra and Hossain, 2003; Mahendran and Bai, 2009), to various other factors such as changing habitat conditions with associated loss of specific pollinators and myosymbionts (Dixon and Phillips, 2007). Although orchid conservation programs have been instigated worldwide to overcome the problems (Debeljak *et al.*, 2002; Vaasa and Rosenberg, 2004; Robinson *et. al.*, 2009), many problems remain to be resolved, either via *in situ* or *ex situ* conservation as part of an integrated approach to conservation.

Some researchers (Dixon and Batty, 2003; Swartz, 2007, Seaton *et al.*, 2010) have suggested that adopting such an integrated conservation approach is the best, indeed the only way to effectively conserve the large numbers of threatened orchid species. However, it is not easy to establish and maintain *in situ* conservation programs for threatened species (Rosas and Rojas, 2009) and this is where *ex situ* conservation, is able to complement *in situ* conservation efforts in many cases (Ket *et al.*, 2004). Engaging in research for improving *ex situ* technology is a necessary task to enhance *ex situ* conservation effectiveness and increase its value as a complementary undertaking along side *in situ* conservation.
IMPROVEMENT AND DEVELOPMENT OF MICROPROPAGATION PROTOCOLS

This study involved revisiting some basic ex situ conservation techniques such as asymbiotic and symbiotic orchid seed germination including sterilization of orchid seeds and development of asymbiotic media; techniques that have been in use for many decades. At the beginning of the study, the commonly used formula Pa5 was used as the asymbiotic germination medium of choice, based on prior studies (Collins and Dixon, 1992; Huynh et al., 2002). However, when this medium was first tested in this study the results were not as expected. There were no signs of germination at approximately six weeks after sowing and when germination did occur (many weeks later) it was low and unreliable. This prompted the decision to set up experiments to try and develop a simple and reliable asymbiotic medium that could be used for timely germination of a broad range of Australian terrestrial orchid species. It was described in Chapter 2 that there are numerous asymbiotic germination media that have been developed (Knudsen, 1946; Vacin and Went, 1949; Van Waes and Deberg, 1986; Collins and Dixon, 1992; Arditti and Krikorian, 1996; Malmgren, 1996) but many of them cannot be used for a broad range of species (Hogue et al., 1994; Dowling and Jusaitis, 2012).

It was hypothesized that primary protocorms generated from asymbiotic germination are as good as that from symbiotic germination if they are generated in a suitable asymbiotic medium that provides sufficient nutrition throughout the germination and protocorm development processes (Chapter 1). This hypothesis was supported with experiments explained in Chapter 2.

Developing a simple and reliable medium is expected to overcome germination-related problems, including assessment of seed viability. Many studies used staining methods such as tetrazolium chloride, fluorescence diacetate and Evans blue to determine orchid seed viability with varying results (Batty et al., 2001b; Nikishina et al., 2001; Wood and Pritchard, 2004). However, other researchers suggested that estimation of terrestrial orchid seed viability should always be corroborated with germination studies (Johnson et al., 2007; Wood et al., 2003).
Moreover, seed sterilization using nylon bags (Fig. 1a) is a commonly used method at a number of plant research laboratories including Kings Park Botanic Garden (KPBG). However, after numerous experiments it was discovered that there are some disadvantages with this method including:

a. using large quantities of materials. It needs approximately 600 ml of sterile water for washing nylon bags to wash seeds free from liquid bleach while the droplet sterilization method only needs ~100 µL sterile water.

b. takes longer to sow seeds (using nylon bag sterilization results in approximately 30 minutes to sow 20 Petri dishes, from dissolving the bleach powder until the sowing process is complete, while it takes only 15 minutes for the droplet method).

c. high risk of wasting seeds when the nylon bags need to be torn apart after sterilization and washing.

In view of these disadvantages with the nylon bag method, a droplet sterilization method was developed (Fig 1b and Fig 2) (details in Chapter 2) because this method seems to be more suitable for orchid conservation programs when money, time and especially seeds are limited. Thus revisiting even a basic method such as seed sterilization was both necessary and ultimately effective as the new droplet method reduces resource wastage especially seeds of threatened species, with flow-on benefits to orchid conservation programs.

Fig 1. Nylon bag and droplet sterilization methods. (a). *Caladenia latifolia* seeds in a nylon bag (before sterilization). (b). *Thelymitra benthamiana* seeds were sterilized using droplet sterilization method.
In addition, the droplet sterilization method is easier to perform compared to the nylon bag method, as there are only three steps in the sowing process (Fig. 2).

**Fig. 2.** Droplet sterilization method.
A. orchid’s seed in a cryo vial
B. sterilization in liquid bleach (~100 µL) with the aid of a tooth pick until seeds colour was changed, then washing with sterile water (~100 µL)
C. Seeds spreading into asymbiotic germination medium in Petri dishes with the aid of sterile 5 mm nichrome wire loop.

The optimised asymbiotic germination medium from this study was ½ MS fortified with 5% (v/v) coconut water. There are some other studies using coconut water to enhance seed germination and proliferation (Zeng *et al.*, 2011; Abraham *et al.*, 2012; Zeng *et al.*, 2012; Zeng *et al.*, 2013; Shekarriz *et al.*, 2014). However, those studies did not mention the type (age) of coconut used. This study used young fresh coconuts bought at a local supermarket. Young coconuts were chosen because preliminary experiments using old coconuts did not give expected results.

Coconut water was frozen and thawed when necessary throughout the whole experiments in this study. To have all the nutrients in the coconut water, the thawing process avoided the use of a hot water bath because it was assumed that exposure to even moderately high temperature might compromise the efficacy of some of the nutrients present. Therefore, CW was thawed by moving it overnight from the freezer (-18°C) to a refrigerator (~ 4°C) before being used the next day for experiments. It is mentioned in Chapter 2 that this asymbiotic medium resulted in high percentage germination and unnoticeable differences in primary protocorm development compared to symbiotic germination (Fig. 3).
This medium has been investigated for asymbiotic germination with ten Western Australian terrestrial orchid species as described in Chapter 2 with addition of two more species along the way, *Caladenia arenicola* (a common species) and another rare species *Drakaea elastica* (Fig. 4). Average percent germination of seed of *C. arenicola* was 86% and reached stage three protocorm development three weeks after sowing, very similar to results achieved with *C. latifolia*. *Drakaea elastica*, on the other hand, took 13 weeks after sowing to reach stage three (Fig. 4).

*Drakaea* is, however, well known as one of the most difficult genera to germinate asymbiotically due to highly specific nutritional and mycorrhizal fungus requirements (Nurfadilah, 2010; Phillips, 2010). The percentage germination of *Drakaea* seeds only reached a maximum of 5% and took nine weeks to start to germinate, however this seed had been in storage at 15°C and 15% RH for 18 months posing the hypothesis that the percentage and time to germinate could be shortened if fresh seeds were available. A further hypothesis would be that seed of *Drakea* undergoes some form of dormancy transition during desiccation and storage. In addition, original viability and germinability data at the time of collection for the particular *Drakea* seed batch used was not available, therefore the possibility that the particular seed batch was of inferior quality cannot be ruled out. These hypotheses remain to be tested in future studies.
A part of this study was constructed to further the work started by Watanawikkit et al. (2012) as explained in Chapter 4. There are at least two advantages in developing secondary protocorms from primary protocorms, *i.e.* producing many plantlets directly from secondary protocorms with only a small amount of seed required for initial primary protocorms, and having the ability to conduct repeated research/pilot project conservation studies without having to deplete valuable seed stocks.

**Fig. 3.** Comparison in protocorm development of seeds germinating symbiotically and asymbiotically: a, c, e are seeds germinating symbiotically with modified OMA; b, d, f are seeds germinating asymbiotically on the optimal medium ½ MS E (½ MS + 5% v/v CW). Images taken at two weeks: a and b; six weeks: c and d; eight weeks: e and f. Scale bar is the same for a-h (1 mm).
Development of this protocorm proliferation protocol is feasible for implementation with rare orchid species, particularly terrestrial species that have similar protocorm development patterns (Batty et al., 2001a). However, results will almost certainly vary according to the species tested with some species still presenting difficulties e.g. Drakaea spp. until further studies on optimisation of both asymbiotic seed germination and secondary protocorm proliferation can be carried out.

**Fig. 4.** Drakaea elastica germinated asymbiotically on ½ MS E. (a). *D. elastica* just after sowing. (b). *D. elastica* stage three protocorm (13 weeks after sowing). (c). *D. elastica* early stage four protocorm (17 weeks after sowing). (d). *D. elastica* stage five protocorm (24 weeks after sowing). Scale bar (a-c) = 500 µM, d = 1 mm.

**DEVELOPMENT OF CRYOPRESERVATION PROTOCOLS**

Maintaining large collections of micropropagated (tissue culture) material is labour intensive and prone to accidental contamination and culture decline, and also to somaclonal variation over longer time periods of continuous culture cycles (Engelmann, 2000; Kaczmarczyk et al., 2011). Therefore, to minimise these risks with orchid culture material, this study also aimed to develop cryopreservation protocols to improve long
term storage efficiency of valuable orchid culture lines. These protocols were also developed to test the hypotheses generated in Chapter 1 that primary and secondary (regenerated) protocorms derived from asymbiotically germinated seeds are suitable for cryopreservation and viable orchid plantlets can be grown after protocorms have been cryopreserved.

Development of cryopreservation protocols were detailed in Chapter 4. Several experimental approaches were set up including verification of the most suitable plant vitrification solution (PVS) and determining the optimal stage of protocorm development for use in cryopreservation.

Cryopreservation protocols developed in this study with the test species *Caladenia latifolia* were verified with a threatened Western Australian terrestrial orchid, *Caladenia huegelii*, with some adjustment due to variations in protocorm size and development stages (Fig. 5). The implementation of the basic cryopreservation protocol developed with *C. latifolia* however, has been successful as per results explained in Chapter 5. In addition, preliminary acclimatization experiments and transfer to soil under glasshouse conditions shows that it is possible to ‘short cut’ the process from taking asymbiotically-generated seedling lines (even after cryopreservation) from the culture environment to the deflasking stage, by introducing mychorrhizal fungi directly to the soil mixture (via soil from pots containing *C. huegelii* plants).

**Fig. 5.** One week after sowing primary protocorms. (a). *C. latifolia* protocorms. (b). *C. huegelii* protocorms, scale bar (a-b) = 500 µM.
CONCLUSIONS AND FUTURE WORKS

The central theme of this study was the notion that it is feasible to minimize declining biodiversity of orchids through maximizing efforts for \textit{ex situ} conservation using micropropagation and cryopreservation as part of an integrated orchid conservation program. Moreover, this study suggested the possibility that commonly used symbiotic protocols for orchid propagation could be replaced with asymbiotic protocols, from seed germination to plantlet development after cryopreservation. Mychorrhizal fungi, which might still be needed as a symbiont, will be introduced after plantlets/seedlings have been transferred to soil.

All hypotheses and aims mentioned in Chapter 1 have been addressed, and In addition, transfer to soil and acclimatization under glasshouse conditions has been successfully carried out for the threatened species \textit{C. huegelii}. However gaps in knowledge still remain and further studies needing to be conducted for the future benefit of Australian terrestrial orchid ssp. are:

- Optimising the protocorm proliferation medium to match the percent conversion from seeds to primary protocorms (approximately 95\% primary protocorms develop into plantlets from seed germinated asymbiotically on asymbiotic medium (½ MS + 5\% (v/v) CW). However the rate of secondary protocorms developing into plantlets from proliferation medium (½ MS + 5 \mu M NAA + 2 \mu M BA) with \textit{C. latifolia} is approximately 20\%. It is expected that increased regeneration will follow after further improvements to the proliferation medium.

- Optimizing the conditions of transfer to soil and acclimatization to better align with conditions closer to the \textit{C. huegelii} natural habitat, particularly ambient temperature. As the transfer to soil and acclimatization experiment had to be conducted in mid August (late winter in Australia) due to study time constraints this timing was almost certainly asynchronous compared to orchid seed germination and seedling development in nature. Unfortunately glasshouse conditions including temperature/RH etc could not be fully controlled. Although the plants produced in this study are still growing well, it is assumed that they will grow even better in more suitable conditions. Plant survival still needs to be recorded over the summer period when the plants would normally be ‘resting’ as dormant tubers. It therefore remains to be seen whether plants produced
asynchronously or out of the normal seasonal growth pattern can survive and resume ‘normal’ growth patterns in subsequent seasons.

- Experiments on secondary protocorms after cryopreservation also resulted in viable seedlings for both *C. latifolia* and *C. huegelii*. Another study needs to be conducted to monitor their survival in soil under glasshouse conditions for extended periods up to first flowering.

- Conducting research on other Western Australian terrestrial orchid species to determine the applicability of the protocols developed in this study to a broad range of taxa.

It is hoped that this study has made some useful contributions to *ex situ* conservation research that will translate to significant enhancement of the efficiency and cost-effectiveness of biotechnology approaches to orchid propagation and cryopreservation for restoration purposes.
REFERENCES


APPENDICES
## Appendix 1

**Table S1**: supplement material from Chapter 2 paper.

**Table S1.** Nutrient composition of basal salts in the germination media used for asymbiotic seed germination of *Caladenia latifolia*.

<table>
<thead>
<tr>
<th>Media components</th>
<th>¹^KC</th>
<th>¹^VW</th>
<th>½^MS</th>
<th>³^Pa5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macronutrients (mM)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrogen-Ammonium</td>
<td>13.82</td>
<td>7.57</td>
<td>10.31</td>
<td>3.78</td>
</tr>
<tr>
<td>Calcium</td>
<td>2.12</td>
<td>1.93</td>
<td>1.50</td>
<td>4.32</td>
</tr>
<tr>
<td>Chlorine</td>
<td>3.35</td>
<td>1.50</td>
<td>3.1</td>
<td>3.35</td>
</tr>
<tr>
<td>Magnesium</td>
<td>1.01</td>
<td>0.62</td>
<td>0.75</td>
<td>1.01</td>
</tr>
<tr>
<td>Nitrogen-nitrate</td>
<td>10.49</td>
<td>5.19</td>
<td>19.70</td>
<td>8.64</td>
</tr>
<tr>
<td>Potassium</td>
<td>5.19</td>
<td>7.03</td>
<td>10.89</td>
<td>3.35</td>
</tr>
<tr>
<td>Phosphate</td>
<td>1.84</td>
<td>3.77</td>
<td>0.63</td>
<td>1.83</td>
</tr>
<tr>
<td>Sulphate</td>
<td>8.69</td>
<td>8.71</td>
<td>0.86</td>
<td>1.92</td>
</tr>
<tr>
<td>Sodium</td>
<td>0.20</td>
<td>0.10</td>
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<td></td>
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<tr>
<td><strong>Micronutrients (µM)</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Boron</td>
<td></td>
<td>50.0</td>
<td>40.0</td>
<td></td>
</tr>
<tr>
<td>Cobalt</td>
<td></td>
<td>0.053</td>
<td>0.04</td>
<td></td>
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<tr>
<td>Copper</td>
<td></td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iron</td>
<td>90.0</td>
<td>100.0</td>
<td>50</td>
<td>100.0</td>
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<tr>
<td>Iodine</td>
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<td></td>
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<tr>
<td>Manganese</td>
<td>30.0</td>
<td>30.0</td>
<td>50.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Molybdenum</td>
<td></td>
<td>0.50</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Zinc</td>
<td>14.95</td>
<td></td>
<td>12.0</td>
<td></td>
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<tr>
<td><strong>Organics (µM)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citric acid</td>
<td></td>
<td>450.0</td>
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<td></td>
</tr>
<tr>
<td>myo-Inositol</td>
<td></td>
<td>500.0</td>
<td></td>
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<tr>
<td>Thiamine hydrochloride</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Pyridoxine hydrochloride</td>
<td></td>
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<td>Nicotinic acid</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>MES buffer</td>
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<td></td>
</tr>
<tr>
<td>Coconut water (mL/L)</td>
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<td>50.0</td>
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<tr>
<td><strong>Total mineral salt concentration (mM)</strong></td>
<td>46.72</td>
<td>35.54</td>
<td>48.01</td>
<td>28.82</td>
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<tr>
<td><strong>Total inorganic N (mM)</strong></td>
<td>24.31</td>
<td>12.76</td>
<td>30.01</td>
<td>12.42</td>
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<tr>
<td>NH₄ NO₃ molar ratio</td>
<td>1.32</td>
<td>1.46</td>
<td>0.52</td>
<td>0.44</td>
</tr>
</tbody>
</table>

¹^Knudson C (*KC*), Vacin and Went (*VW*), *KC* and *VW* nutrients based on Table 1 (Dutra et al., 2009).

²½ MS based on a standard medium formulation used at KPBG.

Proliferation and harvesting of secondary protocorms as a novel means for improving propagation of terrestrial orchids

Betty Mauliya Bustam A,B,C,D, Kingsley Dixon A,B and Eric Bunn A,B

A.Kings Park and Botanic Garden, Fraser Avenue, West Perth, WA 6005, Australia.
B.School of Plant Biology, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia.
C.Mathematics and Natural Sciences Faculty, Syiah Kuala University, Jl. Syech Abdurrah No. 3, Darussalam, Banda Aceh 23111, Indonesia.
D.Corresponding author. Email: lyla1304@yahoo.com

Abstract. This study investigated optimisation of media and primary-protocorm development stages to enhance secondary-protocorm production as a novel means for propagation of terrestrial orchids, including taxa of conservation concern. Seeds of Caladenia latifolia were germinated asymbiotically on ½-strength Murashige and Skoog (MS) medium fortified with 5% (v/v) coconut water. Resulting protocorms at 3, 5 and 7 weeks of growth were subcultured to protocorm-proliferation media treatments consisting of ½-strength MS basal-salts medium with 6-benzylaminopurine (BA) and 6-naphthaleneacetic acid (NAA) singly or in combination. Conversion of seeds to primary protocorms was high (87-92%). The highest percentage of secondary-protocorm proliferation was 40.1%, using 5-week-old protocorms (early Stage 4 of protocorm development) as explants and cultured on ½-strength MS with a combination of 5 μM NAA + 2 μM BA. Half-strength MS media containing a single plant-growth regulator (BA or NAA) were substantially less effective (<10% protocorm proliferation). The present study has provided a novel approach to sequential protocorm production that will be of value particularly for threatened orchids with limited seed availability. Protocorm proliferation in vitro enables a renewable supply of protocorms with which to conduct propagation, cryostorage and pilot restoration programs.

Additional keywords: Caladenia latifolia, conservation, protocorm proliferation, restoration.

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Introduction

Human pressure on the world’s ecosystems has increased dramatically, particularly in the latter half of the past (20th) century, with accelerated deforestation and global warming adding to an already serious problem (Thiellert et al. 2005; Liu et al. 2010). Among terrestrial flora, orchids face an especially bleak future, with many species with a very high risk of extinction (Nicholls 2004; Swarts and Dixon 2009). Although orchid-conservation research has addressed some immediate issues, many problems remain unresolved. One such problem concerns obtaining sufficient seed of endangered species for ex situ conservation purposes, especially because terrestrial orchid seeds are relatively short-lived compared with those of other species (Hay et al. 2010). A key difficulty is that with declining habitat quality, coupled with a high degree of mycorrhizal and pollinator specificity, obtaining sufficient seeds of a rare orchid for restoration purposes is becoming increasingly challenging (Swarts and Dixon 2009a). A solution to overcome this problem is enhancing the productivity of available seeds by producing secondary protocorms from primary-protocorm explants. These secondary protocorms can be utilised as renewable seed surrogates and can be generated from a minimum quantity of seed for experiments that may (initially) have a high risk of failure, thus protecting the bulk of the stored seed. Theoretically, a large number of plantlets can be obtained from a single protocorm (Mohanty et al. 2012). Some studies have attempted mass-proliferation of secondary protocorms from primary protocorms, of mainly epiphytic species (David et al. 2008; Hossain et al. 2010; Latip et al. 2010; Roy et al. 2011). However, there is only one recent report that mentions in vitro-propagated protocorms of an Australian terrestrial orchid species (Watanavirkitt et al. 2012).

In previous experimental work, Bustam et al. (2014) examined asymbiotic germination of Australian terrestrial orchid species in three basal-salt formulations, namely, Murashige and Skoog basal salts (MS; Murashige and Skoog 1962) at half-strength, Knudson C (KC; Knudson 1946) and Vacin and Went (VW; Vacin and Went 1949), with combinations of the plant-growth regulators (PGR) NAA and BA. In a prior study the authors found that some protocorms cultures of ½-strength MS with additional PGRs showed signs of secondary-protocorm proliferation, but none was observed in KC- and VW-based media treatments. However, the proliferation process was of low frequency and very slow, and it was concluded that this could, at least in part,
be because the PGR treatments were not optimised to enhance
the regeneration of protocorms. Type and concentration of PGR
play an important role in the micropropagation of many orchids
(Arditti and Ernst 1993). Therefore, it was decided to continue
using MS basal salts at half-strength (1/2 MS) with the same PGR
(NAA and BA, singly and in combination) as previously tested,
while also evaluating PGR concentrations and combinations
based on published studies (Park et al. 2002; David et al. 2008;
Paudel and Bijaya 2012; Watanawikkit et al. 2012).
Moreover, it has been reported that the specific type of
explant also plays an important role in orchid micropropagation
and proliferation (Chugh et al. 2009). Because (Australian)
terrestrial orchids have five arbitrary stages of protocorm
development recognised from seeds to seedlings (Batty et al.
2001), it was considered that different stages of the
development could result in different proliferation outcomes.
Therefore, the aim of the present study was to research a
simple and reliable protocorm-proliferation protocol using the
indicative genus Caladenia. This genus has the largest number of
threatened species of any orchid genus in Australia, so it is
appropriate to develop approaches for this group. Importantly
of the major genera of Australian terrestrial taxa, Caladenia is
also one of the most problematic in terms of reliable and scalable
propagation solutions. The study species was Caladenia latifolia,
a common Western Australian terrestrial orchid, with outcome
of this work likely to be applicable to rare species such as
C. huegelii. Proliferation of C. latifolia protocorms has been
reported previously; however, the focus of the earlier study was
on cryopreservation of protocorms (Watanawikkit et al. 2012).
Moreover, only one medium and one PGR (MS with addition
of 10μM BA) was reported. Experiments were therefore
conducted to (1) evaluate the most suitable primary-protocorm
stage(s) that would produce the highest yield of secondary
protocorms, (2) assess the most effective concentration of
PGRs and (3) evaluate the most effective PGR used, whether
used singly or in combination.

Materials and methods
All experiments were conducted without the use of mycorrhizal
fungi (i.e. asymbiotically). Capsules containing naturally
pollinated mature seeds were obtained in early August 2011
from C. latifolia plants (20) in bushland in Kings Park and
Botanic Garden (KBP). Seeds were combined and desiccated
over silica gel (24 h at 22°C) before storage (at 4°C) in airtight
containers. Nylon-mesh sachets containing ~20 mg of seeds of
C. latifolia were sterilised in 2% available chlorine solution
(3 g 100 mL-1 calcium hypochlorite granules (65% available
chlorine) + two drops of polyoxyethylene-sorbitan mono-oleate
surfactant) for 10 min, at which point seeds changed colour
from dark green to pale yellow (prior experiments found this
seed-colour change to mark the optimal sterilisation time,
while still retaining high viability). The sachets containing the
seeds were then washed in sterile water three times. Sachets
were opened and seeds spread under sterile conditions onto
germination medium, with the contents of each sachet divided
approximately equally among five replicate Petri plates.
Sixteen sachets for a total of 80 germination plates were
prepared to provide for required selective protocorm
harvesting at each developmental stage. After sowing, germination plates were wrapped in aluminium foil to exclude
light and incubated at 23 ± 1°C.

Asymbiotic culture medium
Asymbiotic germination medium (optimised via previous
experiments) consisted of 1/2-strength MS basal salts (Murashige
and Skoog 1962) with 100 μM N-aFeEDTA, 60 mM sucrose,
500 μM myo-inositol, 500 μM 4-morpholineethanesulfonic acid
(MES), 1 μM thiamine hydrochloride, 2.5 μM pyridoxine, 4 μM
nicotinic acid, 20 g L-1 sucrose, 0.8% w/v agar (Gelita Australia
Pty Ltd) and pH was set to 6.0 before autoclaving at 121°C for
20 min. Nylon mesh was used to remove larger particulates
from the milk from fresh young coconuts bought at a local supermarket.
Aliquots of 50 mL were frozen (~20°C) and thawed as required.
The coconut water (CW) was filter-sterilised using 0.22-μm
syringe-driven filter unit and added to warm (~50°C) post-
autoclaved basal medium at 5% v/v concentration. Medium
was dispensed into sterile 90-mm polystyrene Petri dishes (Techno-
Plas Pty Ltd, Australia) in a laminar-flow unit to maintain sterility.
Deionised water, unless otherwise specified, was used in all media
preparation.

Proliferation process
Plates were examined at 3, 5, and 7 weeks after sowing on
germination medium with the aid of a dissecting microscope
(Olympus SZX16, Japan). Seeds were scored as germinated if
they reached Stage 2 according to Batty et al. (2001). A 90-mm-
diameter plastic grid divided into 2 cm × 2 cm squares was placed
over plates to help with the counting accuracy. At least 250 seeds
were assessed from each replicate plate to determine germination
percentage. Seeds without an embryo (i.e. no proembryo inside
the seedcoat) were excluded from both counting and subculturing.
Protocorms corresponding to various developmental stages
(Fig. 1) were selected at 3, 5 and 7 weeks after seed plating
and subcultured to proliferation-media treatments. Protocorms
were subcultured from germination medium to proliferation-media
treatments by using sterile forceps under a sterile airflow
with the aid of a stereomicroscope (Nikon SMZ 800, Japan).
Approximately 50–100 protocorms were transferred to each
proliferation-medium plate, with each treatment consisting of
five replicate plates (~250–500 protocorms per treatment for
each protocorm-development stage) for a total of 240 plates for
the entire experiment.
Plates were incubated in the dark at 23 ± 1°C. After 4 weeks
of incubation, protocorms were subcultured to fresh proliferation
media to continue the proliferation process. The percentage
of protocorm proliferation was assessed at the conclusion of the
second culture cycle on proliferation-media treatments,
because accurately counting individual protocorms amid
clumps of proliferating secondary protocorms was considered
too difficult by the third culture cycle. The same procedure and
counting aids as with seed germination were used and only
those protocorms showing definite signs of secondary-
protocorm development were scored (Fig. 4c). Subculturing
was continued every 4 weeks for six culture cycles, so as to
determine whether proliferation of secondary protocorms would
continue and be adequate for future experiments.
Proliferation media

Protocorm proliferation was undertaken using the same basal ½-MS minerals and organics as for germination medium (detailed above) with additional PGRs, namely BA and NAA, either singly or in combination (Table 1). CW was omitted but PGRs were added before autoclaving. Four concentrations of BA and two concentrations of NAA were used. Control treatments contained only 1/2 MS without addition of PGRs.

Experimental design and statistical analysis

This experiment used a completely randomised design (CRD) approach. Germination percentages were calculated by dividing the number of protocorms (germinated seeds) by the total seeds (×100), with data from all replicates pooled (x 100). Treatments used in the present study were protocorm stage × medium treatments, with a total of 48 treatments, including 21 single-PGR treatments and 28 combined-PGR treatments. So as to normalise variation, all percentage data were arcsine transformed before being analysed with a one-way ANOVA, followed by a Tukey post hoc test at $P = 0.01$. R freeware package (Institute for statistics and Mathematics of Wirtschaftsuniversitat Wein: www.r-project.org 2014) was used for statistical analysis.

Results

Asynbiotic seed germination

Caladenia latifolia germinated reliably on ½-strength MS + 5% (v/v) coconut water, with 80–93% germination,
starting as early as 2 weeks from sowing, with consistent protocorm development from Week 3 up to 7 weeks. Three weeks after sowing, seeds were in Stage 2 and Stage 3 (most, i.e. >90% of seeds having developed to Stage 3). Five weeks after sowing, seeds were in late-Stage 3 and Stage 4 (majority of seeds being at Stage 4). Seven weeks after sowing, seeds were in late-Stage 4 and Stage 5 (majority of seeds being at Stage 5), as illustrated in Fig. 1. There were no significant differences in percentage of viable germination among all plated seeds. Germination (i.e. to Stage 2) ranged from 87% to 92% after 7 weeks.

Proliferation

The numbers of non-reactive protocorms was very low in the combined-PGR treatments, but up to 50% in the single-PGR treatments in the first culture period. Protocorm proliferation for all single-PGR treatments was uniformly low, with the highest result of 5.3% with Stage 4 protocorms (5 weeks post-sowing) on C1 medium, 1/2 MS + 2µM BA (Fig. 2). There were no significant differences among single-PGR treatments (Fig. 2). Combined-PGR treatments, including Treatments A2, C2 and E2-H2, showed similarly low (<5%) protocorm proliferation, with no significant differences among protocorm stages within these particular treatments (Fig. 3). However, two combined-PGR treatments resulted in a significantly higher protocorm proliferation than did the other combined-PGR treatments, namely, B2 (1/2 MS + 5µM NAA + 2µM BA), using protocorms at 5 weeks after sowing, with 40.1%, followed by D2 medium (1/2 MS + 5µM NAA + 5µM BA), also using protocorms at 5 weeks after sowing, with 23.3% (Fig. 3). On Medium B2, the transition to the earliest signs of secondary-protocorm development occurred after one subculture cycle (Fig. 4d) and developed to multiple secondary protocorms after three subculture cycles (Fig. 4e) and then, finally, to ‘soft’ secondary-protocorm development after six subculture cycles (Fig. 4j). Throughout this multi-cycle proliferation bulking process on B2 medium, protocorms remained vital and healthy, with no sign of diminished proliferation or reduced growth and development. Moreover, although signs of proliferation could be seen as early as the first subculture with some single-PGR treatments, these invariably tended to develop ‘hard’ secondary protocorms in dense, compact clumps (compared with combined-PGR treatments), with this tendency increasing with subsequent culture cycles (Fig. 5a, b). Most ‘soft’ secondary protocorms (~75%) showed a greater abundance of trichomes than did ‘hard’ secondary protocorms (Fig. 5a, b).

Primary protocorms for control treatments were subcultured once (from germination medium to control proliferation medium), because no sign of proliferation could be observed and most such primary protocorms (~95%) eventually proceeded to develop directly into plantlets after 12 weeks or longer (Fig. 6a).

‘Hard’ secondary protocorms formed in compact clumps and could be separated (with difficulty) only by scalpel and forceps, and rapidly became brown and most failed to

Fig. 2. Single-plant growth regulator (PGR) treatments of secondary-protocorm proliferation with Caladenia latifolia expressed as percentage of primary protocorms (selected at 3, 5, and 7 weeks post-sowing). Media treatments indicated as A1–F1 relate to proliferation media with combined PGRs (see Table 1). Error bars represent standard error of the mean.

Fig. 3. Combined-plant growth regulator (PGR) treatments of secondary-protocorm proliferation with Caladenia latifolia expressed as percentage of primary protocorms (selected at 3, 5, and 7 weeks post-sowing). Media treatments indicated as A2–H2 relate to proliferation media with combined PGRs (see Table 1). Mean values (±standard error) within media treatments followed by the same letter are not significantly different at P = 0.01 (Tukey post hoc test).

131
proliferate further. In contrast, ‘soft’ protocorms formed on combined-PGR treatments were easily separated and maintained proliferation capacity, whereas ~20% on B2 medium (following six subculture cycles) turned green and developed into plantlets (Fig. 6b).

Discussion
The current study aimed to improve on the previous study conducted by Watanawikkit et al. (2012), by testing a wider range of media and investigating the most suitable stage of primary-protocorm development to enhance secondary-protocorm production. Micropropagation-related experiments with the main aim of mass production have been conducted around the world, using many different parts of orchids such as the flower stalk, axillary buds, leaf segments as well as protocorms (Chen et al. 2002; Park et al. 2002; David et al. 2008; Huang and Chung 2011; Khoddamzadeh et al. 2011; Sujariintharakarn and Kanchanapoom 2011; Nambiar et al. 2012). However, these studies have mainly (with some exceptions, e.g. Collins and Dixon 1992) been conducted on epiphytic orchid species and not on terrestrial taxa; it is also of paramount importance to develop protocols that suit Australian species. Germinated seeds (protocorms) were chosen as explants because seeds contain a diversity of genotypes and, therefore (theoretically at least), are more suitable for conservation programs. This assumes that proliferation capacity is not a genetically sensitive screen.

No significant differences in percentage seed germination (i.e. to Stage 2) were observed, with all seeds in the present study being germinated asymmetrically on the same medium and incubated under the same conditions. However, the amount of secondary-protocorm proliferation showed clear differences among treatments (Figs 2, 3). It is reported that orchids require auxins and/or cytokinins for neo-formation of protocorm-like bodies (PLBs) and plantlet development (Roy et al. 2011). Although some studies have showed that PLBs can be effectively generated from media treatments using a
Fig. 5. Two types of Caladenia latifolia secondary protocorms: (a) dense 'hard', difficult-to-separate secondary protocorms typical of single-plant growth regulator (PGR) treatments; and (b) soft, easily separated secondary protocorms typical of combined-PGR treatments (note increased presence of trichomes).

Fig. 6. Caladenia latifolia plantlets (a) on control medium (1/2-strength MS, minus plant growth regulators, PGRs) formed from primary protocorms after 12 weeks of incubation; and (b) those from secondary protocorms on B2 medium (5 μM NAA +2 μM BA) after 7 weeks of incubation on the sixth subculture cycle.

single PGR (Khoddamzadeh et al. 2011; Sujanthurakam and Kanchanapoom 2011), other studies have showed otherwise, with the combinations of PGRs (including NAA and BA) producing more effective PLB proliferation (Park et al. 2002; Chen and Chang 2006; David et al. 2008; Roy et al. 2011). Our results support those studies, because NAA and BA (5 μM NAA +2 μM BA and 5 μM NAA +5 μM BA) were the two optimal treatments for inducing proliferation of secondary protocorms (using primary protocorms as starting material) of C. latifolia.

Moreover, other studies that obtained successful seed and proliferation of PLBs by using MS formulations (half- or full-strength) (Lin et al. 2000; Chen et al. 2002; Park et al. 2002; Mahendran and Bai 2009; Huang and Chang 2011; Khoddamzadeh et al. 2011; Mohanty et al. 2012) provided support for our decision to continue using ½-MS for proliferation media (on the basis of previous results in our laboratory). This may be due to the fact that MS-based media generally contain higher amounts of inorganic nitrogen than do other basal orchid media such as Vacin and Went (VW) and Knudson C (KC).

Another finding of the present study was the difference between secondary protocorms formed from single-PGR and combined-PGR treatments. Whereas many plates of single-PGR treatments produced hard protocorms with few or no trichomes, all combined-PGR treatments produced soft protocorms with abundant trichomes typical of healthy protocorms. 'Hard' protocorms, when cut and separated before transferring to fresh media, exhibited a browning reaction, possibly owing to phenolic exudation (Chugh et al. 2009).
Some studies have suggested using liquid media to enhance proliferation (Park et al. 2002; Van et al. 2011). Liquid medium was attempted (B2 minus agar) in some samples on the fourth subculture of B2 solid-medium plates. However, browning of some secondary protocorms (and signs of possible contamination) resulted in early termination of the experiment. Although further investigation of liquid protocorm proliferation medium would be worthwhile, our results indicated that solid media provide a reliable and easily manipulated approach for proliferation, compared with liquid media used for the epiphytic *Dendrobium* hybrids (Park et al. 2002) and *Phalaenopsis bellina* (Khodatumzadeh et al. 2011).

Although time after sowing to reach Stage 4 protocorm development is likely to differ when this protocol is implemented with other orchid species, we expect that a similar stage of primary-protocorm development, combined with a suitable PGR and medium treatment will result in superior secondary-protocorm proliferation. At the conclusion of the present study, it was observed that ~20% of secondary protocorms formed plantlets on B2 medium, which is encouraging, although this value was not as high as that for primary-protocorm conversion to plantlets (~60%) with *C. latifolia*. However, the B2 medium, although ideal for secondary-protocorm proliferation, is unlikely to be optimal for conversion to plantlets. Hence, further experimentation will be necessary to optimise the conversion rate of secondary protocorms to plants, which will be of maximum benefit for threatened species where seed is in short supply.

**Conclusions**

The present study was designed as a pilot project, with the findings to be implemented with other terrestrial orchids, particularly rare species such as the nationally threatened species *Caladenia bispersiculata*. As such, the study established a novel protocol for proliferating terrestrial orchids from primary-protocorm explants, developed for an Australian terrestrial species, *C. latifolia*. Primary protocorms of *C. latifolia* at 5 weeks following germination (Stage 4) appeared to be the most suitable stage at which to induce proliferation of secondary protocorms, with a solid basal medium (1/2 MS) and PGR treatment (5 mM NAA and 2 mM BA) achieving the highest proliferation percentage and the best type of secondary protocorms (easily separated 'soft' protocorms with abundant trichomes). Approximately 20% of these protocorms formed plantlets. Although protocorm conversion remains to be optimised, the extended proliferation cycles achievable with secondary protocorms bode well for efficient production of rare and threatened terrestrial orchid species.

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