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THESIS

DEVELOPMENT OF *IN VITRO* AND CRYOGENIC PROTOCOLS FOR *EX SITU* CONSERVATIONOF ORCHIDS

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This study has derived a novel *in vitro* method for asymbiotic clonal propagation of *Caladenia latifolia* R.Br. (an endemic terrestrial orchid species from Western Australia) for horticulture and breeding from seedling explants. The highest induction of protocorm rate and size were achieved in MS medium plus 10 µM 2,4-D for a 90 day incubation period. The most effective medium for protocorm proliferation was ½ MS plus 10 µM BAP. The best shoot induction and protocorm size were obatined on PGR-free Thomale GD medium. The development of a cryopreservation protocol for orchid protocorms of *C. latifolia* was showed that large cryopreserved protocorms had the highest viability and potential survival rate. There were no significant differences in potential survival among desiccation medias, cryoprotectant solutions, and incubation time at 0 °C. Potential protocorm survival was also not significantly different among recovery media treatments.

Development of *in vitro* propagated-protocorms of *Paphiopedalum insigne* (Wall. ex Lindl.) Pfitzer (endangered Thai slipper orchid) was aslo investigated. The result revealed that ½ MS medium containing 10 and 40 µM BAP induced the greatest multiple shoot numbers. Nevertheless, both BAP and TDZ had no significant effect on the shoot length. Although the ½ MS plus 5 µM TDZ or 20 µM BAP gave the maximum root length, both BAP and TDZ inhibited root formation at above 10 µM. *In vitro*-propagated protocorms cultured on ½ MS medium plus 0.8 M glycerol showed the highest potential survival of both non- and encapsulated protocorms following cryostorage. Protocorm exposure to PVS2 solution $\geq$ 20 minute is sufficient for a great survival rate of non-encapsulation, the 90 minute air-drying time gave the best maximum survival rate of encapsulated protocorms.

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Patcharawadee Wattanawikkit
June 2014

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LIST OF ABBREVIATIONS

ANOVA = Analysis of Variance

BAP = N6-benzyladenine phosphate

^{o}C. = Degree celcius

CRD = Completely Randomized Design

2,4-D = 2,4 –dichlorophenoxyacetic acid

DMRT = Duncan’s multiple range test

min. = minute

mm. = milimetre

mL. = mililitre

½ MS = 1/2 – strength macro and micro elements of Murashige and Skoog (1962)

NAA = α-naphthaleneacetic acid

PGR = plant growth regulator

PGRs = plant growth regulators

PLB = protocorm-like body

PLBs = protocorm-like bodies

PSV2 = Plant vitrification solution 2 (30% glycerol (w/v) + 15% ethylene glycol (w/v) + 15% (w/v) DMSO in MS medium with 0.4 M sucrose)

PSV4 = Plant vitrification solution 4 (35% (w/v) glycerol, 20% (w/v) ethylene glycol in MS medium with 0.6 M sucrose)

TDZ = 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea

Thomale GD = THOMALE GD (1954)

DEVELOPMENT OF *IN VITRO* AND CRYOGENIC PROTOCOLS FOR *EX SITU* CONSERVATION OF ORCHIDS

INTRODUCTION

Orchids are one of the most important ornamental plants in Thailand. They exhibit an incredible range of diversity in size, shape and color of their flowers (Dressler, 1981; Lurswijidjarus and Thammasiri, 2004; Wannakrairoj, 2007). At present, many of them are endangered due to many causes, such as environmental changes, deforestation, the wild orchid trade and other factors such as introduced pests and diseases (Thammasiri, 2000; Lekawatana, 2010). As with orchids worldwide, there is an urgent need for conservation of Thai orchid species (Thammasiri, 2000; Lurswijidjarus and Thammasiri, 2004; Thammasiri, 2008).

The uses of micropropagation techniques have revolutionized the commercial orchid industry. Orchids are one of the few flowering plant of commercial value to be propagated *in vitro* both through seed and tissue culture (Bajaj, 1992). Aseptic germination of seed is essential, as orchid seeds lack metabolic machinery and have no endosperm, no cotyledons, and no root initials (Singh, 1981, 1988). They do not have the capacity for directly utilizing the substrates available in nature (Savina, 1974; Sheeham, 1983; Singh, 1981, 1988).

In vitro culture techniques have been used to successfully propagate many orchids including a number of southern Australian terrestrial species (Cross, 1997; Dixon, 1991; Collins and Dixon, 1992; McKendrick *et al*., 2000; Batty *et al*., 2002; Nigel and Dixon, 2009). Most published protocols on *in vitro* propagation of Australian terrestrial orchid taxa describe symbiotic or asymbiotic seed germination (Batty *et al*., 2001; Dowling and Jusaitis, 2012). However, the number of species for which *in vitro* clonal or shoot proliferation procedures have been published remains relatively small (Arditti and Ernst, 1993). Indeed, clonal propagation of Australian terrestrial orchids is only occasionally reported e.g. *Diuris longifolia* R. Br., a terrestrial orchid species endemic to Western Australia was clonally multiplied through adventitious shoot production using immature flower explants cultured on modified Burgeff N3f medium containing BAP (Collins and Dixon, 1992). *In vitro* propagation of *Caladenia* spp. with BM1 (#B141; Van Waes and Debergh, 1986) medium has been documented (Dowling and Jusaitis, 2012).

Various tissues from *in vitro*-germinated seedlings have been successfully induced to produce callus, shoots, and plants (Huang, 1988; Lin *et al*., 2000; Huang *et al*., 2001; Chen *et al*., 2004; Ng *et al*., 2010; Ng and Saleh, 2011). Recently, callus and protocorm-like bodies capable of regenerating into plants were also induced directly from seeds of several *Paphiopedilum* species (Hong *et al*., 2008; Long *et al*., 2010) and a threatened Mexican orchid (Santos Díaz and Álvare, 2009).

In general, the growth and development of *in vitro*-grown plants depends on factors such as macro- and micro-element composition, carbon source, and plant growth regulator (Murashige and Skoog, 1962; Xiong and Wu, 2003). Although sucrose is the most utilized carbon source for plant tissue culture, other sugars, such as glucose, fructose, maltose, and mannitol, are also effective (Li *et al*., 2002). The frequencies of *in vitro* seed germination of *P. insigne* var. *sanderae* (Rchb. f.) Stein and *P. armeniacum* are also influenced by seed maturity and organic nutrient additives, such as banana, potato, and coconut juice (Nagashima, 1982; Pierik *et al*., 1988; Lee, 1998; Ding *et al*., 2004; Lee, 2007).

The progress made on *in vitro* propagation of *Paphiopedilum* orchids so far clearly indicates that genotype difference between these orchids dictates the response of explants on different media formulations (Ng *et al*., 2010; Ng and Saleh, 2011; Wattanawikkit *et al*., 2011). This emphasizes that further investigation of *in vitro* propagation with *Paphiopedilum insigne* is needed in order for tissue culture technology to play a greater role in *ex situ* conservation.

Ex situ conservation in the form of seed banking and container collections of plants in botanic gardens can help save endangered orchid species. *In vitro* collections and cryopreservation of seed, mycorrhizal fungi and shoot apices complete the available *ex situ* options currently available for conservation of a wide range of plants species including orchids (Dixon, 1994; Swarts and Dixon, 2009a, 2009b). Cryopreservation is an important tool for long-term storage of biological materials and offers a safe and cost-effective option for long-term conservation of genetic resources in many plant species (Grout, 1995; Engelmann, 1997, 2000; Gonzalez-Arnao *et al*., 2008). At the temperature of liquid nitrogen (LN, -196 ^{o}C), all the metabolic activities of cells are at a standstill, thus, they can be preserved in stasis for long periods (Engelmann, 2004; Gonzalez-Arnao *et al*., 2008). Many cryopreservation techniques such as programmed freezing, vitrification, encapsulation/dehydration and encapsulation/vitrification have been reported as successful for many cells, tissues and organ of plant species (Engelmann, 1997, 2000; Reed, 2000, 2008). However, for successful cryopreservation, many factors are involved, such as starting materials, pretreatment conditions, time of exposure to LN, cryoprocedures and post-thaw treatment (Reed, 2000, Reinhoud *et al*., 2000; Reed, 2008). Therefore, in order to accomplish successful cryopreservation for each species and cultivar, a separate study must be carried out (Lurswijidjarus and Thammasiri, 2004).

Cryogenic storage or cryopreservation in particular offers superior efficiency with long term *ex situ* germplasm storage for endangered or threatened species and minimizes accidental losses due to disease and contamination, reduces storage space and maintenance costs, and reduces or eliminates somaclonal variation associated with long-term tissue cultures maintained under standard conditions (Kaczmarczyk *et al*., 2012; Turner *et al*., 2001a, 2001b; Harding, 2004; Harding *et al*., 2009). While there are some published protocols for cryostorage of Australian terrestrial orchid seeds and orchid mycorrhizal fungi (Batty *et al*., 2001) and on cryopreservation of non-Australian orchids using seed protocorms and shoot tips, (Lurswijidjarus and Thammasiri, 2004), evidence for successful cryostorage of somatic orchid tissues of Australian terrestrial orchid spp. has not been reported to our knowledge.

Therefore, this research was aimed at establishing more efficient *in vitro* orchid propagation and cryopreservation techniques. Using a common species of Western Australian wild orchid, the aim was to investigate a range of plant growth regulators for maximizing protocorm and shoot multiplication from seedling explants incubated on defined basal nutrient media.

In this study *Paphiopedilum insigne* (Rong thao nari chiang dao), which is endemic to Thailand, and *Caladenia latifolia* (one of the many endemic terrestrial orchids of Western Australia) are used as model species *Caladenia* to represent temperate herbaceous terrestrial orchids and *Paphiopedilum* representing tropical terrestrial orchids. The aim was to develop protocorm cryopreservation using the encapsulation/dehydration technique.

OBJECTIVES

1. To develop an *in vitro* method for clonal propagation of *Caladenia latifolia* R.Br., and *Paphiopedilum insigne* (Wall. ex Lindl.) Pfitzer by using seedling explants to achieve induced proliferation of protocorm and shoots for *ex situ* conservation.

2. To develop a cryogenic protocol for *in vitro*-propagated protocorm material of *C. latifolia*, and *P. insigne* to assist with co-development of cryoprotocols for cryopreservation of rare and endangered orchid taxa.

LITERATURE REVIEW

The family Orchidaceae

The orchid family (Orchidaceae) is one of the largest families in the plant kingdom, comprising over 35,000 species worldwide (Dressler, 1993). In Thailand, orchids are found in different habitats ranging from high elevation evergreen forests at 2,565 metre in the north to sea level in the south, with approximate 170 genera and 1,230 species of which 150 species are considered endemic species (Nanakorn and Indharamusika, 1999). Over 80 percent are epiphytic species and most of the rest are terrestrials, with only few saprophytic species (Cribb, 1987; Nanakorn, 2002).

1. Pink Fairy Orchid

Caladenia latifolia (Pink Fairy Orchid), is one of the endemic terrestrial orchids of Western Australia. The specific characteristic is a single flat, hairy leaf, as shown in Figure 1, 80-180 mm. long and 15-25 mm. wide, its rhizome has numerous hairs, and the pseudo-stem may attain a height to 450 mm. It flowers in spring (September) and presents bright pink flowers (narrow pink labellum with yellow calli). The mature capsule turns grey when drying and splits, releaseing large numbers of minute seeds (David, 1994) as shown in Figure 1 and 2.

Figure 1 *Caladenia latifolia* R.Br. (Pink Fairy Orchid).

Table 1 Botanical lineage of 'Pink Fairy Orchid' and slipper orchid.

	'Pink Fairy Orchid'	**Slipper orchid**
Kingdom	Plantae	Plantae
Division	Magnoliophyta	Magnoliophyta
Class	Liliopsida	Liliopsida
Subclass	Base Monocots	Base Monocots
Order	Asparagales	Asparagales
Family	Orchidaceae	Orchidaceae
Subfamily	Orchidaceae	Cypripedioideae
Tribe	Diurideae	Cypripedieae
Subtribe	Caladeniinae	Paphiopedilinae
Genus	*Caladenia*	*Paphiopedilum*

Source: Cribb (1987); Hopper and Brown (2004)

Quay *et al*. (1995) investigated methods for *ex situ* germination of *Caladenia latifolia* R.Br. via a potting mix primarily of leaf litter of *Allocasuarina fraseriana* (Miqq.) L., and further reported that this method can be used for commercial production of *Caladenia* seedlings, although, a lower percentage of seed germination is obtained compared to *in vitro* techniques. They also found that *in vitro* seedlings of orchids may encounter major problems during their transfer to soil and frequently do not establish well or do not produce tubers. Cross (1997) also reported that the plantlets of *Caladenia* species propagated through *in vitro* symbiotic germination for reintroduction into natural habitats, have less than 10% survival *ex situ*, which may be due to altered anatomy, physiology and biochemistry caused by *in vitro* environment (Preece and Sutter, 1991). In addition, the percentage germination of *C. tentaculata* was highest when seeds were exposed to ambient levels of CO_2 and low light levels *in vitro*. It has been found that seedling germination and survival *in situ* is generally highest close to adult plants, possibly due to the presence of an appropriate fungus (Dixon, 1991; Batty *et al*., 2002; McKendrick *et al*., 2000).

Figure 2 The development of *Caladenia latifolia* R.Br., a) Stem, b) Leaf, c) Flower and d) Pod of *Caladenia latifolia* R.Br.

2. Slipper orchids

Genus *Paphiopedilum*, or slipper orchids are native to Tropical Asia. They are placed in the subfamily Cypripedioideae. *Paphiopedilum* species are clearly distinguishable from other members of the orchids, by the unique 'slipper-like' flower due to the unusual shape of the pouch-like labellum of the flower (Cribb, 1998; Sheehan and Sheehan, 1994). The leaf can be short and rounded or long and narrow, and typically have a mottled pattern and it is persistent for more than one season (hence the common epithet). There are about 75 species of *Paphiopedilum* ranging from Sri Lanka, Southern India, Northeast India, Nepal, Bhutan, and Myanmar across to southern China, Hong Kong, Southeast Asia, the Malay Archipelago, New Guinea, the Philippines and the Solomon Irlands. A total of 27 species of *Paphiopedilum* have been reported to be present in China (Cribb, 1998; Liu *et al*., 2009a, 2009b). Wild populations are under increasing pressure from overcollection and habitat destruction, and all species are listed in the Convention on International Trade in Endangered

Species of Wild Fauna and Flora (CITES), Appendix I, thus restricting trade of these plants. Most species are terrestrials but there are a few lithophytes. Leaves are closely set at ground level and are either tessellated and alternately dark and pale green, or in some cases predominantly yellow-green. The sympodial growth produces new pairs of leaves each season, which causes dense leaf formation. A single terminal flower stem arises from the centre of the leaves bearing one flower, rarely two or more (Mark, 1987; Cho and Valmayor, 1988; Pederson *et al*., 2011) as shown in Figure 3.

Figure 3 Some examples of *Paphiopedilum* (Slipper) orchids: (a) *Paphiopedilum collosum* (Rchb.f.) Stein, (b) *Paphiopedilum insigne* (Wall. ex Lindl.) Pfitzer.

Apart from the slipper-shaped lip, which is about half the total flower length another distinctive feature of this orchid is the fusion of the lateral sepals to form the synsepalum, which is narrow and nearly always hidden behind the lip. The dorsal sepal is usually large and the petals spread out horizontally on either side of the flower. Another distinctive feature is the column, the end of which points forwards and has an odd structure called a staminode clearly visible at the base of the lip. This staminode protects the two others and stigma and is often hairy. On the lower side of the column is the large fleshy disc of the stigma, which is really the end of the column, but is normally hidden by the staminode and the incurving side lobes of the lip as shown in Figure 3 (the example of *Paphiopedilum*). The flowers are not usually scented but they are very long-lasting if not visited by insects. Most *Paphiopedilum* species are mountain plants, preferring slightly cooler temperatures and often found growing near streams, rock crevices or amongst ferns and mosses in cool locations (Mark, 1987).

Field investigation and ecological studies indicated that *Paphiopedilum* often reproduced by clonal growth (Cribb *et al*., 1999; Tsi *et al*., 1999). Therefore, with both sexual and asexual reproductive strategies the species has sufficient ability to propagate successfully in the wild as long as habitats are no longer disturbed seriously and overcollection is forbidden (Li *et al*., 2002). Liu *et al*. (2006) also reported that the conservation of *Paphiopedilum* (*P. armenicacum*) can be done by *ex-situ* conservation and by replanting the *ex-situ* reproduced ramets to original habitat. Slipper Orchids appear to have a capacity for both asexual and sexual reproduction, and even an ‘emergency’ mechanism consisting of enhanced production of rhizomes to cope with physical damage in some taxa. When *ex-situ* methods are needed for *Paphiopedalum* spp, sampling should be able to cover all populations across its distribution even if populations are disjunct due to habitat loss or disturbance (Cribb, 1998).

Unfortunately, propagation of *Paphiopedilum*s is difficult in that there is no reliable method for vegetative propagation. As expected, seedlings are often not true-to-type and seed set and germination rates of many cultivars and hybrids are extremely low (Arditti, 2008; Lin *et al*., 2000). Liu *et al*. (2006) also reported that in *P. armeniacum*, the interval between seed germination and tiller production is about four years, although some orchid hybrids and tropical orchids initiate tiller production within six months of germination and tiller yearly (Chen and Tsi, 2003). Although propagation has been entirely via seeds (Lin *et al*., 2000), most *Paphiopedilums* have stringent requirements for seed germination, but little is known about their specific requirements (Arditti and Ernst, 1993). At present, information concerning embryo development in *Paphiopedilum* species is limited, except for work on *P. insigne* (Zinger and Poddubnaya-Arnoldi, 1966; Lee *et al*., 2006).

In vitro propagation

In vitro culture techniques have been used to successfully propagate many orchids including some terrestrial species. Most published protocols on *in vitro* propagation of Australian terrestrial orchid taxa describe symbiotic or asymbiotic seed germination (Batty *et al*., 2001; Dowling and Jusaitis, 2012), however the number of species for which *in vitro* clonal or shoot proliferation procedures have been published remains relatively small (Arditti and Ernst, 1993). Indeed, clonal propagation of Australian terrestrial orchids is only occasionally reported, e.g. *Diuris longifolia* R. Br., a terrestrial orchid species endemic to Western Australia, which was clonally multiplied through adventitious shoot production using immature flower explants cultured on modified Burgeff N3f medium containing BAP (Collins and Dixon, 1992). *In vitro* propagation of *Caladenia* spp. with W3 (Western Orchids Laboratory, Blackwood, South Australia) medium (Hay *et al*., 2010) and BM1 medium (Van Waes and Debergh, 1986; Dowling and Jusaitis, 2012) has been documented. The former group observed that W3 medium had successfully germinated species of *Caladenia* species like *C. arenicola, C. flava, C. huegelii* (Hay *et al*., 2010). While, the latter indicated that asymbiotic germination on BM1 medium is an effective technique for testing the performance of Australian terrestrial orchid seeds, *Caladenia tentaculata* Schltdl. Mycobiont specificity was shown to play an important role in symbiotic orchid propagation and is thought to play a critical role in the establishment of orchids into field sites (Batty *et al*., 2006a, 2006b). They found that some specie (*Caladenia arenicola* Hopper & A.P.Brown) failed to produce tubers (from modified roots or droppers) necessary for plant survival through the summer dormancy period. The initiation of tubers on droppers by this species was inversely correlated with leaf size, with smaller plants more likely to form tubers. However, little information exists on asymbiotic seed germination of Australian terrestrial species, *Caladenia* (Hay *et al*., 2010; Dowling and Jusaitis, 2012), so there remains a need for reliable protocols to be developed to support *ex situ* orchid conservation strategies.

The following two major problems contribute to the unsuccessful micropropagation of slipper orchids: (1) explants from mature plants of *Cypripedioideae* species are recalcitrant to shoot induction and plant regeneration (Arditti and Ernst, 1993); (2) it is difficult to obtain aseptic explants from mature plants through normal surface sterilization steps for *in vitro* culture (Chugh *et al*., 2009) because of the long maturation time and high humidity growth conditions that are conducive to microbial contamination. To circumvent these obstacles, immature seeds have been used for shoot induction (Tomita and Tomita, 1997). Unfortunately, these seeds easily lose their germinability within a short time once capsules are harvested (De Pauw and Remphrey, 1993). Mature seeds can maintain their germinability for a long time (Lauzer *et al*., 1994; Whigham *et al*., 2006), and the resultant germinated seedlings can be kept sterile in culture. For these reasons, seeds and seedlings have been the most favorable explant sources for experimentation in tissue culture regeneration. Various tissues from *in vitro*-germinated seedlings have been successfully induced to produce callus, shoots, and plants (Huang, 1988; Lin *et al*., 2000; Huang *et al*., 2001; Chen *et al*., 2004; Ng *et al*., 2010; Ng and Saleh, 2011). Recently, callus and protocorm-like bodies capable of regenerating into plants were also induced directly from seeds of several *Paphiopedilum* species (Hong *et al*., 2008; Long *et al*., 2010) and a threatened Mexican orchid (Santos Díaz and Álvare, 2009). However, tissue culture clones derived from seeds or seedling tissues often suffer a serious drawback because their genotypes are highly variable and produce phenotypes that are unpredictable and lack uniformity. Furthermore, it commonly takes five years of greenhouse care to produce mature plants before their flower phenotypes are revealed. Even then, the few selected mature plants that are deemed valuable have not been able to be successfully micropropagated (Liao *et al*., 2011).

A few reports dealing with *in vitro* culture of *Paphiopedilum* have been published (Arditti and Ernst, 1993). Shoot tips have been used as explants to induce the growth of auxillary shoots (Huang, 1988; Arditti and Ernst, 1993). Nevertheless, development of tissue culture procedures for *Paphiopedilum* has been broadly characterized by great difficulty and lack of success (Stewart and Button, 1975; Huang *et al*., 1988). Similar to previous research, Pierik *et al*. (1988) also reported that *Paphiopedilum* seeds are very difficult to germinate *in vitro*. Efforts to establish long- term callus cultures from stem apices have been without much success. For the conservation and commercial production of endangered *Paphiopedilum* species, information concerning reproductive biology and improved methods of *in vitro* propagation are thus of great importance. Furthermore, a few plantlets occasionally regenerate from shoot apex-derived callus in an undefined medium, but the calli are difficult to maintain and eventually failed to survive during subcultures (Stewart and Button, 1975). However, the development of protocols for rapid and large scale clonal multiplication of selected elicits is of considerable commercial value and callus induced from seed-derived protocorms of a *Paphiopedilum* hybrid resulted in a few plantlets forming via protocorm-like-bodies (PLBs) from the callus (Lin *et al*., 2000). Therefore, poor callus proliferation and low regeneration capacity of callus cultures were the major impediments limiting the utility of *in vitro* culture for large-scale *Paphiopedilum* propagation.

It has been reported that the growth and development of *in vitro*-grown ornamental plants depends on several factors such as carbon source (sugar concentration), macro- and micro-inorganic and organic elements like vitamins, plant growth substance and plant growth regulators including cytokinins and auxins (Murashige and Skoog, 1962; Xiong and Wu, 2003; George *et al*., 2008; Wattnawikkit *et al*., 2011). Persson *et al*. (2006) demonstrated that plant cell has higher capacity to uptake, metabolize and reallocate nitrogen from organic sources than inorganic sources. It has been shown that sucrose is the most utilized carbon source for plant tissue culture, however Li *et al*., (2002) showed that other sugars, such as glucose, fructose, maltose, and mannitol, are also effective in some cases. There are relatively few published reports on the factors affacting micropropagation of *Paphiopedilum* species using tissue culture technology, i.e. *in vitro* seed germination of *P. insigne* var. *sanderae* (Rchb. f.) Stein and *P. armeniacum* are influenced by maturity and organic nutrient supplements like banana, potato, and coconut juice (Nagashima, 1982; Pierik *et al*., 1988; Lee, 1998; Ding *et al*., 2004; Lee, 2007). Lee *et al*. (2006) concluded that asymbiotic seed germination of fully mature orchid seeds is often difficult, with the frequency ranging from 35% to 68% in

12 *Paphiopedilum* spp. (Tay *et al*., 1988; Lee, 1998; Chen *et al*., 2004; Zeng *et al*., 2006; Lee, 2007). The germination frequency is 97% and 100% for *P. ciliolare* (Rchb. f.) Stein and *P. callosum* (Rchb. f.) Stein, respectively (Stimart and Ascher, 1981; Pierik *et al*., 1988). This variation is thought to be due to key variables affecting *in vitro* germination from species to species (Nhut *et al*., 2006).

Long *et al*. (2010) reported the *in vitro* propagation of four threatened species (Orchidaceae), *P. villosum* var. *densissimum*; *P. insigne* (Lindl.) Stein; *P. bellatulum* (Rchb.f.) Stein and *P. armeniacum*. They investigated whether their micropropagation frequency was affected by seed maturity, medium composition, sugars and organic additives (e.g. Knudson C (KC) medium supplemented with glucose and coconut milk). Explants of *Paphiopedilum* showed a two-fold increase in the frequency of shoot development when the cultured medium was supplemented with a combination of PGRs, i.e. *P. villosum* treated with 5 mg/L 6-benzyladenine (BAP) plus 0.5 mg/L α-naphthaleneacetic acid (NAA), and *P. insigne* (Lindl.) Stein treated with 0.2 mg/L BAP plus 0.1 mg/L NAA. The result also found that *P. bellatulum* explants induced the highest shoot formation in medium containing 5.5 mg/L BAP plus 0.5 mg/L NAA, while the treatment consisting of 4 mg/L BAP with 0.1 mg/L NAA resulted in the most gain in shoot length in *P. armeniacum*.

The propagation of *Paphiopedilum* orchids through *in vitro* shoot multiplication and direct shoot-bud formation and the regeneration of *Paphiopedilum* plants through PLB formation from callus culture have been reported (Lin *et al*., 2000; Huang *et al*., 2001; Chen *et al*., 2002, 2004; Hong *et al*., 2008). Lin *et al*. (2000) established a repeatable procedure to obtain normal plants of *Paphiopedilum* hybrid (*P. callosum* 'Oakhi' x *P. lawrenceanum* 'Tradition') from sub-cultured protocorm-derived calli on a ½ strength Murashige-Skoog (MS) medium plus 1-10 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1-1 mg/L 1-phenyl-3-(1.2.3-thiadiazol-5-yl)urea (TDZ). They found that these calli grew well, but proliferated more on ½ MS medium plus 5 mg/L 2,4-D and 1 mg/L TDZ. Calli developed further along a route of production of protocorm-like bodies and eventually formed plantlets that could be transported to pots and also grew well. However, poor callus formation rate, slow growth of callus and low regeneration capacity are still the major impediments limiting the utility of *in vitro* culture for *Paphiopedilum* propagation. Because of the limited success in tissue culture protocols, *Paphiopedilum* propagation is still almost entirely by asymbiotic germination (Chen *et al*., 2004).

In 2001, Huang *et al*. reported the new combination of optimal supplements for shoot multiplication and rooting of *Paphiopedilum* hybrids (*P. bellatulum* 'Big spot' x *P*. Jo Ann's Wine; *P. micranthum* x *P. glaucophyllum*. It is the modified MS based medium supplemented with Murashige and Tucker (MT) vitamins, glycine and inositol (13 µM BAP/N6-benzyladenine phosphate), 1.6 µM NAA/α-napthaleneacetic acid, 0.15 µM adenine sulfate.$2H_2O$, 1.23 mM $NaH_2PO_4.H_2O$), 0.18 M sucrose, and 15% (v/v) coconut milk. They found that addition of 1 g/L casein hydrolysate or 10 g/L potato tuber sections also promoted shoot and root induction of *Paphiopedilum* hybrids. This protocol enabled doubling of the number of *Paphiopedilum* species every 12 weeks. In contrast, TDZ addition inhibited both shoot proliferation and rooting of *Paphiopedilum*, while rooting was depressed in the presence of maltose.

Chen *et al*. (2002) investigated plant regeneration of *Paphiopedilum* through multiple shoot formation from stem node explants. Afterwards, they established plant regeneration of *Paphiopedilum* through direct shoot bud formation from leaf segments (Chen *et al*., 2004). They reported that induction of multiple shoots could be achieved from stem node explants of *P. philippinense* hybrids (hybrid PH59) cultured on a modified ½ MS (1962) medium supplemented with 4.52 µM 2,4-D plus 0.45 µM TDZ, a combination of 2,4-D (4.52 and 45.25 µM) and TDZ (0.45 and 4.54 µM) for six months. In a hybrid (PH60), although 4.52 µM 2,4-D and 0.45 µM TDZ promoted shoot formation, the highest shoot number was found with 4.52 µM 2,4-D alone. Plantlets, each having several roots, were obtained from regenerated shoots after transferring onto hormone-free basal medium for three months. The plantlets were further potted in sphagnum moss, and acclimatized well in greenhouse conditions.

Later, Chen *et al*. (2004) reported that leaf explants of *P. philippinense* hybrids (hybrid PH59 and PH60) directly formed adventitious shoot from wound regions within one month, when cultured on MS medium (½ strength macro-and full-strength micro-elements) free of plant growth regulator in darkness. In hybrid PH59, 4.54 µM TDZ increased mean numbers of shoots per explants with leaf segment explants. Whereas, 4.52 µM 2,4-D plus 0.45 µM TDZ promoted direct shoot bud formation from leaf culture explants in hybrid PH60. In addition, the supplementation of each 4.52 µM 2,4-D, 22.71 µM TDZ or 4.52 µM 2,4-D plus 4.54 µM TDZ gave a higher response than control (0 µM 2,4-D and TDZ) on mean numbers of shoots per explants. Healthy plantlets each with 1-3 roots were obtained from leaf-derived shoots after transfer onto hormone-free medium for 22 months. Theses plantlets were acclimatized in a greenhouse and grew well with 100% survival rate.

The recent investigation of Hong *et al*. (2008) showed that tiny seeds from 5-month-old green capsules of a maudiae type slipper orchid, *Paphiopedilum* Alma Gavaert, were also induced to form totipotent callus on ½ strength MS medium supplemented with 22.6 µM 2,4-D and 4.54 µM TDZ in darkness. The callus proliferated and could be maintained without any morphogenesis on the same medium with a 2-month interval of subculture for more than two years. When transferred to ½ MS medium supplemented with 26.85 µM NAA, an average of 4.7 protocorm-like bodies (PLBs) or shoot buds formed from each explants after 120 days of culture. After another 72 and 240 days of culture on the same medium, 25 shoot buds and eventually 75 plantlets were obtained through shoot multiplication from the original culture. Kinetin at 4.65 µM was suitable for shoot multiplication and could induce an average of three shoots from a single young shoot after 60 days of culture. In addition, the regenerated plantlets grew normally when transplanted to containers with sphagnum moss in a shaded greenhouse.

Ng *et al*. (2010) investigated the clonal propagation of *P. rothschildianum* through *in vitro* formation of multiple shots from stem nodal and single shoot explants cultured onto ½ MS medium supplemented with different types of organic nitrogen additives, i.e. casein hydrolaysate, peptone and tryptone-peptone (at 0.5, 1.0 and 2.0

g/L). The result showed that the addition of these organic additives slightly enhanced the number of multiple shoots formed on both types of explants when compared to additive-free MS medium. After 16 weeks, an average of 2.9 shoots per stem nodal explants and 2.8 shoots per single shoot explants were obtained on ½ MS medium supplemented with 1.0 g/L peptone and 2.0 g/L tryptone-peptone, respectively. Plantlets with 3-4 roots were acclimatized and transferred to a glass house after an additional 12 weeks of culturing on similar medium and resulted in 90% survival rate.

The formation of protocorms from germinated seed and the subsequent induction of protocorm-like bodies (PLBs) or callus from the protocorm, stem-node, shoot-tip, leaf, root-tip, or root-tuber explants has become a reliable method for propagating orchids (Park *et al*., 2003; Košir *et al*., 2004; Anjum *et al*., 2006; Kalimuthu *et al*., 2007; Roy *et al*., 2007; Hong *et al*., 2008; Medina *et al*., 2009; Udomdee, *et al*., 2012). Propagation through protocorm-like bodies (PLBs) formation is preferred by commercial growers of most orchid genera due to the large number of PLBs that can be obtained within a relatively short period of time. The large-scale propagation of PLBs can also be achieved using a bioreactor system (Park *et al*., 2000). PLBs are also the most common target tissue for genetic transformation studies in orchids because they can proliferate rapidly and have high capabilities to regenerate into complete plantlets (Liau *et al*., 2003; Subramaniam *et al*., 2008). In addition, PLBs can also serve as plant material for cryopreservation (Yin and Hong, 2009). PLBs are well-differentiated tissues that are sometimes regarded as orchid embryos that develop with two discrete bipolar structures, namely, the shoot and root meristem. Thus, these structures are able to convert to plantlets easily when grown on PGR-free medium. Moreover, the PLBs directly formed from meristem tissue will exhibit a higher genetic stability than those produced by callus (Lee and Phillips, 1988).

The propagation of *P. rothschildianum* was achieved through the *in vitro* formation of secondary protocorm-like bodies (PLBs) from the primary PLB that developed from stem-derived callus (Ng and Saleh, 2011). They demonstrated that the highest number of secondary PLBs formed was obtained on ½ MS medium supplemented with 4.0 µM kinetin, with an average of 4.1 PLBs per explant after 8 weeks of culture. The secondary PLBs continued to proliferate further and formed 9.5-12.1 new PLBs per secondary PLB after being subcultured onto half-strength plant growth regulator-free MS medium supplemented with 60 g/L banana homogenate. These tertiary PLBs were subcultured onto media containing different organic additives for plantlet regeneration. The result clesarly showed that the addition of 20% coconut water to ½ MS medium resulted in the best average plantlet regeneration percentage from the PLBs, 67.9%, after 8 weeks of culture.

Wattanawikkit *et al*. (2011), reported that *P. callosum* seedlings were grown on ½ MS medium with TDZ, 2,4-D or BAP compared to hormone- free medium. Overall, BAP appears to elicit the best shoot multiplication in response with *P. callosum* shoot explants compared to either 2,4-D or TDZ. Therefore, combined effects of TDZ and BAP may be worthwhile investigating in future shoot proliferation experiments. Root induction appears to be restorable if TDZ is removed and BAP reduced as the presence of BAP at the lower concentrations tested does not appear to completely inhibit root induction. The current study will hopefully assist with future development of *ex situ* conservation methods with endangered Thai orchids.

Recently, *in vitro* shoot induction and plant regeneration from flower buds (FBs) in *Paphiopedilum* species, *Paphiopedilum* Deperlen and *Paphiopedilum* Armeni White, were investigated (Liao *et al*., 2011). They found that sections of FBs between 1.5-3.0 cm. cut from *Paphiopedilum* Deperlen were able to produce shoots, but only sections of > 2.5 cm. size from *Paphiopedilum* Armeni White were regenerable. In addition, the microscropic observation revealed that the small bract at the FB base harbored a new miniature FB, which further harbored a primitive FB with dome-shaped meristem- like tissues that presumably led to the plant induction. The reiteration of this pattern resulted in a scorpioid cyme inflorescence architecture in the multifloral *Paphiopedilum* species and its failure to reiterate resulted in a single flower. The induction rates were 57-75%, and all plants survived in a greenhouse.

More recently, the effect of *in vitro* cutting stem methods and medium composition on efficient shoot multiplication of *Paphiopedilum* Hsinying Rubyweb was investigated by Udomdee *et al*. (2012). They found that its vertical cutting was able to produce more new shoots than horizontal and cross cutting when cultured on Hyponex based medium. Plantlets regenerated from vertical cutting were further able to produce new healthy and well-rooted shoots at a higher frequency than uncut stems on the same medium, after 12 weeks of culture. Moreover, the newly-formed shoots which were divided into single plantlets and subcultured onto ½ MS medium without PGRs could retain a higher shoot multiplication rate than in other media. Table 2 summarises observations on the *in vitro* propagation of *Paphiopedilum* species from various literature sources.

Table 2 Summary of tissue culture of *Paphiopedilum* spp.

Plant	Explant	Media	Result	References
Paphiopedilum spp.	shoot	Thomale GD + 1 mg/L 2,4-D	Callus	Morel (1974)
P. callosum	Shoot, leaf	Thomale GD + 1 mg/L IAA + 1 mg/L kinetin	Callus	Morel (1974)
P. villosum	shoot	Heller (1965) + 1 mg/L 2,4-D	Callus	Stewart and Button (1975)
Paphiopedilum spp.	Seed	Thomale GD + 2 mg/L peptone	Germinated seedling	Flamee (1978)
P. philipinense	Seed	MS + 15 % coconut milk	Germinated seedling	Cho and Valmayor (1987, 1988)
P. sukhakulii	Seed	Norstog and Dark room	Germinated seedling	Tay *et al*. (1988)
P. villosum	Seed	Vacin and Went + 100 g/L banana or 100 g/L tomato	Germinated seedling	Kornthong (1991)
P. concolor	Seed	Vacin and Went and Thomale G MS	Germinated seedling	Pakaew (1995)
P. bellatulum	Shoot	VW + 5 µm TDZ	Shoot	Tochareon (1996)
P. exul	Shoot	WS + 1 mg/L 2,4-D	Callus	Polthampitak (1997)
P. godefroyae	Shoot	WS + 1 mg/L 2,4-D + 1 mg/L kinetin	Callus	Polthampitak (1997)

Table 2 (Continued)

Plant	Explant	Media	Result	References
Paphiopedilum hybrids	Seedling	½ MS + 5 mg/L 2,4-D + 1 mg/L TDZ	Callus	Lin *et al*. (2000)
Paphiopedilum hybrids	Shoot	MS + Murashige and Tucker (1969) + 13 µM BA + 1.6 µM NAA	Shoot, Root	Huang *et al*. (2001)
P. philippinense hybrids 59 hybrids 60	Leaf, Stem node	½ MS + 4.54 µM TDZ ½ MS + 4.52 µM 2,4-D + 4.54 or 22.71 µM TDZ	Shoot Shoot	Chen *et al*. (2002, 2004)
Paphiopedilum Alma Gavaert	Seedling Callus	½ MS + 22.6 µM 2,4-D + 4.54 µM TDZ ½ MS + 26.85 µM NAA	Callus Protocorm like body	Hong *et al*. (2008)
P. rothschildianum	Stem node	½ MS + 1 g/L peptone or 2.0 g/L tryptone-peptone	Shoot	Ng *et al*. (2010)
P. rothschildianum	Callus	½ MS + 4 µM kinetin or 60 g/L banana homogenate	Protocorm like body	Ng and Saleh (2011)
P. villosum var. densissimum	Seed Protocorm	Knudson C (KC; 1946) + glucose + coconut water ¼ MS + 5 mg/L BA + 0.5 mg/L NAA	Germinated seedling, Protocorm Shoot	Long *et al*. (2010)
P. insigne (Lindl.) Stein	Protocorm	¼ MS + 0.2 mg/L BA + 0.1 mg/L NAA	Shoot	Long *et al*. (2010)

Table 2 (Continued)

Plant	Explant	Media	Result	References
P. bellatulum (Rchb. f.) Stein	Protocorm	¼ MS + 5.5 mg/L BA + 0.5 mg/L NAA	Shoot	Long *et al*. (2010)
P. armeniacum S. C. Chen et F. Y. Liu	Protocorm Rhizome	¼ MS + 4 mg/L BA + 0.1 mg/L NAA	Shoot	Long *et al*. (2010)
Paphiopedilum Deperlen and Armeni White	Flower bud	½ MS	Shoot	Liao *et al*. (2011)
P. callosum	Seedling Shoot	½ MS without PGRs ½ MS + 10-50 µM BAP	Shoot, root Shoot multiplication	Wattanawikkit *et al*. (2011)
Paphiopedilum Hsinying Rubyweb	Seedling Shoot	Hyponex (P2) ½ MS	Shoot, root Shoot multiplication	Udomdee *et al*. (2012)

Cryopreservation

Ex situ conservation, in the form of seed banking and container collections of plants in botanic gardens, can help to save endangered species, and *in vitro* culture of plant material and mycorrhizal fungi completes the *ex situ* options currently available for the conservation of a wide range of plants, including orchids (Batty *et al*., 2001; Touchell *et al*., 2002). Cryogenic storage in liquid nitrogen (LN, -196 °C) offers superior efficiency for long-term *ex situ* germplasm storage for endangered or threatened species, minimizes accidental losses as a result of disease and contamination of active growth collections, reduces storage and maintenance costs, and reduces or eliminates somaclonal variation associated with long-term tissue cultures maintained under standard conditions (Turner *et al*., 2001a; Kaczmarczyk *et al*., 2011). Cryopreservation offers the potential for reliable long-term storage capability, high stability of phenotypic and genotypic characters, plant genetic resources using minimum space with lower labor and maintenance costs and maintenance requirements, and deemed ideal for long-term storage of germplasm (Engelmann, 1997; Yin and Hong, 2009). In the last two decades, cryopreservation has been widely explored as an alternative for germplasm preservation.

Encapsulation-vitrification (use of alginate to enclose explants in a bead prior to dehydration, treatment with vitrification solutions and LN storage) have been developed and used with varying degrees of success to preserve diverse species of plants (Maruyama *et al*., 2000; Xue *et al*., 2008). The advantages of using somatic embryos in breeding programs would increase significantly if the embryo could be preserved during field evalution and selection of different genotypes (Von Arnold *et al*., 1996). The subculture routines of tissue and embryogenic cell lines involve much handing, time, and increase the risk of loss in embryogenic potential of cells over time. Cryopreservation is the best method for preservation of embryogenic cultures because it permits long-term storage and maintains juvenility of donor tissue, which is desirable for a stable plant regeneration system (Maruyama *et al*., 2000). As reported by Xue *et al*. (2008), who successfully observed the methods for cold storage and cryopreservation of hairy root cultures of the medicinal plant *Eruca sativa* Mill. *Astragalus membranaceus* and *Gentiana macrophylla* Pall.

Cryopreservation of from various plantlets of *Dendrobium candidum,* i.e. seeds (Wang *et al*., 1998), protoplasm and germplasm (Chen, 2000; Chen *et al*., 2001), protocorms (Wang *et al*., 1998), and protocorm-like-bodies (Bian *et al*., 2002) by either vitrification or air-drying has been successful. However, these two methods have resulted in both low and slow rates of regrowth of plantlets (Wang *et al*., 1998; Chen, 2000; Chen *et al*., 2001; Bian *et al*., 2002). Thus, developing an alternative protocol for long-term preservation of *D. candidum* is valuable for germplasm conservation, breeding programs, and the orchid floricultural industry. Cryostorage of terrestrial orchid seeds and orchid mycorrhizal fungi (Batty *et al*., 2001) has been well described, but the storage of somatic tissues of terrestrial orchids arising from protocorms has rarely been reported (Datta *et al*., 1999), although recent success has been described with some epiphytic species, hybrid orchid Bratonia (Popova *et al*., 2010) and *Grammatophyllum speciosum* (Sopalun *et al*., 2010).

Encapsulated, and so call “artificial seeds”, have been reported as having advantages over non-encapsulated explants (Das *et al*., 2011) hence have wider applications for germplasm storage in cryopreservation studies (Hirai and Sakai, 1999; Yin and Hong, 2009; Subramaniam *et al*., 2011). However, encapsulation-vitrification has been mainly used for cryopreservation of shoot-tips and few protocorm-like bodies (small vegetative parts of orchids that develop into whole plants) (Engelmann, 1997; Khoddamzadeh *et al*., 2011). A number of studies has been reported on cryopreservation of protocorms and PLBs of some orchids (Na and Kondo, 1996; Ishikawa *et al*., 1997; Thammasiri, 2000; Chen *et al*., 2001; Lurswijidjarus and Thammasiri, 2004; Nikishina *et al*., 2007; Pornchuti and Thammasiri, 2008; Yin and Hong, 2009; Anthony *et al*., 2010; Pouzi *et al*., 2011; Subramaniam *et al*., 2011). Though there is a report on the cryopreservation of protocorms of *Dendrobium nobile* (Vendrame and Faria, 2011), the emphasis was restricted to the use of phloroglucinol in the regrowth medium.

Yin and Hong (2009) revealed that the best result in the encapsulation-vitrification method of PLBs of *Dendrobium candidum* Wall. ex Lindl., orchid, were successfully obtained when PLBs were subjected to a 5-day preculture with liquid MS medium containing 0.2 mg/L NAA, 0.5 mg/L BAP and 0.75 M sucrose at 25±1 °C under continuous light (36 µmol m^2/s). Precultured PLBs were encapsulated in an alginate gel beads and then osmoprotected with MS medium plus 2 M glycerol and 1 M sucrose for 80 min. at 25 °C before dehydration with the PVS2 solution, pH 5.8, for 150 min. at 0°C. Encapsulated and dehydrated PLBs were plunged directly into liquid nitrogen for 1 hr. Cryopreserved PLBs were then rapidly rewarmed in a water bath at 40 °C for 3 min. and then washed with MS medium containing 1.2 M sucrose for three times at 10 min intervals. Within 60 days, plantlets obtained from cryopreserved PLBs developed normal shoots and roots.

MATERIALS AND METHODS

Methods

Experiment I. Development of *in vitro* methods for *ex situ* conservation of *Caladenia latifolia* R.Br.

Explant preparation of *Caladenia latifolia* R.Br.

Germination of seeds of *C. latifolia* achieved by method of Batty *et al*. (2001) were prepared. Mature undehisced seed capsules (‘green pods’) of *C. latifolia* (Fig. 2d) were obtained from hand-pollinated, outcrossed plants (sourced originally from local wild plants with an appropriate permit) growing at Kings Park and Botanic Garden, Perth, WA, Australia. Capsules were washed for 1 min. in 0.01% v/v Tween-80 detergent and water, dipped in 95% ethanol flamed, and then dissected under aseptic conditions. Seeds were removed and placed on on MS medium (Murashige and Skoog, 1962) containing 100 mg/L myo-inositol, 0.1 mg/L thiamine, 0.1 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 2 mg/L glycine HCl, and 30.8 mg/L sucrose with pH adjusted to 6.0. They were further incubated in the dark at 24 ± 2 °C for up to five months, or until sufficient seeds had germinated to provide seedlings for experiments.

1. *In vitro* propagation of *C. latifolia*

Germination of seeds of *C. latifolia* achieved by method of Batty *et al*. (2001) were used as explants of *in vitro* propagation experiments as follows.

1.1 Effect of 2,4-D on protocorm induction

Seedlings germinated *in vitro* (length, 5-10 mm.) were cultured on MS medium containing 100 mg/L myo-inositol, 0.1 mg/L thiamine, 0.1 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCL, 2 mg/L glycine HCl, and 20.5 g/L sucrose, and 2.5 g/L Phytagel®. Plant growth regulator 2,4-D (2,4-dichlorophenoxyacetic acid) was added at different concentrations of 0, 1, 5 and 10 µM with pH adjusted to 6.0. Eight replicate petridishes, each one consisting of ten explants, were used in this experiment. The cultures were incubated in darkness at 25 ± 2°C for 3 months. Protocorm induction of original explants was assessed as the sum of percentages of protocorm formation per dish and recorded. Average protocorm clump diameter (mm.) were then recorded.

1.2 Effect of plant growth regulators (PGRs) on protocorm multiplication

Protocorm clumps *in vitro* (diameter size, 5 mm.) obtained from experiment 1.1 were subcultured on ½ MS medium containing 100 mg/L myo-inositol, 0.1 mg/L thiamine, 0.1 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCL, 2 mg/L glycine HCl, and 20.5 g/L sucrose, 2.5 g/L Phytagel®, and 150 mL/L coconut juice. Plant growth regulators TDZ (1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea) and BAP (6-benzylaminopurine) were added at similar concentration of 5, 10 and 20 µM in comparison to the control treatment (without PGR), and the media was adjusted to pH 6. Explants were incubated under a 16/8-h photoperiod (40 µmol/m^2/s (white fluorescent lamps Philips TLD 36W/54) at 25 ± 2 ^{o}C, in order to multiply *in vitro* protocorm. Ten replicate petridishes consisting of ten protocorm explants for each, were used. Average derived protocorm size (mm.) was determined after 30 days of incubation.

1.3 Impact of TDZ on shoot induction

In vitro protocorm explants (diameter, 5 mm.) obtained from 1.2 were placed on Thomale GD medium (Thomale, 1954) containing 100 mg/L myo-inositol, 0.1 mg/L thiamine, 0.1 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 2 mg/L glycine

HCl, and 20.5 g/L sucrose, 2.5 g/L Phytagel®, and 2 g/L activated charcoal. The TDZ (1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea) at 0, 5, 10, 20 and 40 µM was added, with pH adjusted to 6.0. Explants were incubated under similar condition described in 1.2 to initiate shoot formation. Ten replicate Petri plates, each one consisting of ten explants, were used. Percentage of shoot formation, shoot number and derived protocorm size were assessed after 90 days.

Experimental design

Statistical analysis was conducted by analysis of variance (ANOVA) with treatment means separated using Duncan's multiple range test (DMRT) at the 95% confidence level (Duncan, 1955).

2. Cryopreservation of *in vitro*-propagated protocorms of *C. latifolia*

2.1 Preparation of protocorm induction

Seedlings germinated *in vitro* (length, 5-10 mm.) were cultured on MS medium containing 100 mM myo-inositol, 0.5 mM nicotinic acid, 2 mM glycine HCl, 0.5 mM pyridoxine HCl, 0.1 mM thiamine HCl, 20 g/L sucrose, 10 mmol BAP and 2.5 g/L Phytagel®, with pH adjusted to 6.0. Protocorms developed on this medium were subsequently used as explant source material for cryopreservation trials.

2.2 Cryopreservation of *in vitro*-propagated protocorms

The following cryopreservation procedure (modified from Touchell *et al*., 2002) was used. Protocorms were incubated on desiccation medium (½ MS with 0.8 M glycerol and 8 g/L agar, adjusted to pH 6.0) for two days of dark incubation at 25 °C. Protocorms were then placed into 1-mL cryotubes (Nunc®, Roskilde, Denmark) containing PVS2 cryoprotectant solution (Sakai *et al*., 1990) at 0 °C for 30 min. Cryotubes containing 10 protocorms per tube were attached to canes and immediately plunged into liquid nitrogen, where they remained for one day, and were then warmed (one tube at a time) in a water bath at 40 °C for 1 min. Under aseptic conditions, the contents of each cryotube were emptied into a sterile Petri dish and washed four times with unloading solution (½ MS + 1 M sucrose). Cryopreserved protocorms were placed on recovery medium (MS + BAP 0.5 mM) and incubated in the dark at 25 °C for up to 90 days for potential survival assessment [using fluorescein diacetate (FDA) staining; Batty *et al*., 2001]. This basic procedure (unless otherwise specified) was used to test cryo-capability responses in terms of protocorm size, desiccation treatments, cryopreservation solutions and recovery media, as followed.

A. Optimal protocorm size for cryopreservation

Protocorms were sorted into three sizes: small (S), $\leq$ 1 mm.; medium (M), >1 < 4 mm; large (L), > 4 mm. All protocorms were subcultured onto ½ MS medium without plant growth regulators for 3 weeks prior to cryostorage (as above). Potential protocorm survival and colour were assessed after 60 days. Medium protocorms were used for Experiments B–D as these were the most abundant protocorm size formed on MS medium with BAP supplement obtained from 1.2.

B. Effect of different desiccation media

Medium protocorms were transferred to five desiccation media treatments (0, 0.4, 0.6, 0.8 and 1.0 M glycerol for 2 days) and incubated in the dark.

C. Optimization of cryoprotectants for cryopreservation

After two days of desiccation treatment, medium protocorms were placed into 1-mL cryotubes containing two different cryoprotectant solutions (PVS2 or PVS4; Sakai *et al*., 1990) for 15, 20, 25 and 30 min. at 0 ^{o}C.

D. Recovery of cryopreserved protocorms and potential survival

Cryopreserved medium protocorms were placed onto three different recovery media [½ MS + 0.5 mM BAP, 6-furfurylaminopurine (kinetin) or 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (TDZ)], and incubated for up to 90 days in the dark at 25 °C. Potential survival of cryopreserved protocorms was assessed in each experiment using the FDA test (following Batty *et al*., 2001).

Experimental design

Five replicates consisting of 10 observations for each replicate were used in each experiment. Statistical analysis was conducted by ANOVA with treatments means separated using DMRT at 95 % confident level (Duncan, 1955).

Experiment II Development of *in vitro* methods for *ex situ* conservation of *Paphiopedilum insigne* (Wall. ex Lindl.) Pfitzer

Preparation of *P. insigne* explant

Mature seed capsules of *P. insigne* (Figure 4) were washed in 0.01% (v/v) Tween-80 detergent for one min., dipped in 95% ethanol flamed and dissected under aseptic conditions. Sterilized seeds were removed and placed on ½ MS medium (Murashige and Skoog, 1962) containing 555 mM myo-inositol, 4 μM nicotinic acid, 2.5 μM pyridoxine HCl, 1 μM thiamine HCl, 2 mg/L glycine HCl, 60 mM sucrose, and 0.7% (w/v) agar without phytohormones, at pH 6.0. All seeds were incubated under a 16/8-h photoperiod at 40 µmol/m^2/s (white fluorescent lamps Philips TLD 36W/54) at 25 ± 2 ^{o}C for two months. Germinated seedlings (*in vitro*-propagated shoots and protocorms) were used as explants for shoot multplication and root induction as well as a source of protocorms for cryopreservation in the following experiments.

Figure 4 Stem flower pod and seed of *Paphiopedilum insigne* (Wall. ex Lindl.) Pfitzer.

1. Influence of plant growth regulators (PGRs) on growth and development of *in vitro* propagated *P. insigne* seedlings.

Germinated seedlings were grown on ½ MS medium (Murashige and Skoog, 1962) containing 100 µM myo-inositol, 0.5 µM nicotinic acid, 2 µM glycine, 0.5 µM pyridoxine HCl, 0.1 µM thiamine HCl, 1,000 µM peptone, 170 µM NaH_2PO_4, 2.2 g/L sucrose, 2.2 g/L Phytagel®. Different plant growth regulators (PGRs); BAP (N6-benzyladenine phosphate) or 2,4-D (2,4–dichlorophenoxyacetic acid) were supplemented at 0, 5, 10, 20, 40 and 80 µM. The media was adjusted to pH 6.0-6.1 and incubated in darkness at 25 ± 2°C, with the aim to induce shoot multiplication, root and protocorm formation. Number and length of *in vitro* shoot and root induction as well as protocorm formation were assessed over three months of incubation period.

Experimental design as previously stated in experiment I section.

2. Cryoperservation of *in vitro*-propagated protocorms of *P. insigne*

Preparation of protocorm induction

Seedlings germinated *in vitro* (length, 5-10 mm.) were subcultured onto basal ½ MS medium (Murashige and Skoog, 1962) plus optimal concentration of PGRs (BAP or 2,4-D) obtained from experiment 1, (with pH adjusted to 6.0-6.1) and incubation in darkness at 25 ± 2°C. The *in vitro*-propagated protocorms developed on this medium were then used as explants for cryopreservation trials as followed.

2.1 Cryoperservation of *in vitro*-propagated protocorms of *P. insigne* by vitrification method without encapsulation

A. Impact of dessication media on non-encapsulated protocorm survival

Propagated-protocorms were transfered into five desiccation media treatments (0, 0.4, 0.6, 0.8 and 1.0 M glycerol). They were afterwards moved into 1-mL cryotubes (Nunc®, Roskilde, Denmark) containing PVS2 cryoprotectant solution (Sakai *et al*., 1990) at 0 °C for 30 min. before direct immersion in liquid nitrogen (LN) for one day. Protocorm cryotubes were warmed in a water bath for 1 min. at 40 °C, and placed on recovery medium (MS + BAP 0.5 mM) and then were incubated in the dark at 25 °C for 90 days. The potential survival of cryopreserved protocorms was examined. Three replicate treatments, each one consisting of ten propagated-protocorms, were used in the study.

B. Optimization of cryoprotectants for cryopreservation of *in vitro*-propagated protocorms

After two days of desiccation treatment obtained from experiment A., medium protocorms were placed into 1-mL cryotubes containing four levels of PVS2 cryoprotectant solution (Sakai *et al*., 1990) for 15, 20, 25 and 30 min.

(0 °C.) before cryopreservation in liquid nitrogen (LN) for one day. Protocorm cryotubes were warmed in a water bath at 40 °C. for 1 min., and placed on recovery medium (MS + BAP 0.5 mM) and then were incubated in the dark at 25 °C for 90 days. The potential survival of cryopreserved protocorms was checked. Four treatments containing three replicate, each one consisting of ten propagated-protocorms, were used in the study.

2.2 Cryoperservation of *in vitro*-propagated protocorms of *P. insigne* by vitrification method with encapsulation

Preparation of protocorm encapsulation

In vitro-propagated protocorms were suspended in sterile 1% sodium alginate (w/v) containing 90 mM sucrose. The mixture containing protocorms was dispensed as droplets using a sterile Pasteur pipette into sterile 50 mM calcium chloride solution at room temperature for 30 min., to form protocorm beads. The alginate beads of protocorms were placed onto sterile filter paper to dehydrate by air-drying in a laminar flow chamber at room temperature, and then stored in a sterile Petri dish. Alginate beads of encapsulated protocorms were subsequently used as explant source material for the following cryopreservation trials.

C. Impact of dessication media on encapsulated-protocorm survival

Encapsulated protocorms were transferred into five desiccation media treatments (MS basal nutrients with organic additives as described previously, with 0, 0.4, 0.6, 0.8 and 1.0 M glycerol) for 2 days and incubated in the dark. After two days of desiccation treatment, medium encapsulated protocorms were then placed into 1-mL cryotubes (Nunc®, Roskilde, Denmark) containing PVS2 cryoprotectant solution (Sakai *et al.*, 1990) at 0 °C for 30 min. Cryotubes containing encapsulated protocorms were directly immersed into liquid nitrogen (LN) for one day, then warmed in a water bath at 40 °C for 1 min. Under aseptic conditions, the contents of each cryotube were emptied into a sterile Petri dish and washed four times with unloading solution (½ MS + 1 M sucrose). Cryopreserved encapsulated-protocorms were placed on recovery medium (MS + BAP 0.5 mM) and incubated in the dark at 25 °C for 90 days. The potential for survival of cryopreserved encapsulated protocorms was tested. Each treatments were replicated five times consisting of five encapsulated-protocorms/replicate.

D. Effect of dehydration time on survival of encapsulated-protocorms

Alginate/encapsulated protocorms were placed onto sterile filter paper to gradually dehydrate by air-drying for 0, 30, 60, 90 and 120 min. in a laminar flow chamber at room temperature. They were afterwards transferred into 1-mL cryotubes (Nunc®, Roskilde, Denmark) containing PVS2 cryoprotectant solution (Sakai *et al.*, 1990) at 0 °C for 30 min., prior to cryopreservation in liquid nitrogen (LN) for one day. These cryotubes were warmed in a water bath at 40 °C for 1 min., and placed on recovery medium (MS + BAP 0.5 mM) and then were incubated in the dark at 25 °C for 90 days to find out the optimal dehydration time. The potential survival of cryopreserved encapsulated protocorm was recored. Three replicate treatments containing ten encapsulated-protocorms for each replicate.

Experimental design and data analysis

All experiment was based on a CRD. Statistical analysis was provided by ANOVA with treatment means separated using DMRT with significance determined at the 5% level (Duncan, 1955).

Places and Duration

Department of Botany, Faculty of Science, Kasetsart University, June 2004 - February 2014.

Botanic Gardens and Parks Authority, West Perth, WA, Australia, September 2005 – September 2007.

Funding Source

Strategic Scholarship Fellowships Frontier Research Networks, Office of the higher Education Commission, Thailand.

Ramkhamhaeng University, Bangkok, Thailand

RESULTS AND DISCUSSION

Results

Experiment I: Development of *in vitro* methods for *ex situ* conservation of *Caladenia latifolia* R.Br.

In vitro propagation of *Caladenia latifolia* R.Br.

1. Effect of 2,4-dichlorophenoxyacetic acid (2,4-D) on protocorm induction

Seedlings germinated *in vitro C. latifolia* were cultured on MS medium supplemented with 2,4-D at 0 (control), 5, 10 and 20 µM for three months to promote protocorm (PLB) induction (Figure 5). The results showed that all 2,4-D treatments promoted higher protocorm induction rate (63.75 ± 5.65, 63.75 ± 5.96 and 76.75 ± 7.54 %) and protocorm size (8.19 ± 0.94, 9.13 ± 0.94 and 7.54 ± 1.00 mm.) compared to the control treatment (without 2,4-D) (Table 3).

Table 3 Protocorm induction rate of *C. latifolia* obtained from explants cultured on MS medium supplemented with 2,4-D (0, 1, 5 and 10 µM) for 90 days.

2,4-D level (µM)	*Protocorm induction rate (%) ± SE	Mean protocorm clump diam. (mm.) ± SE
0	38.75 ± 3.50 ^{b}	4.68 ± 0.27 ^{b}
1	63.75 ± 5.65 ^{a}	8.19 ± 0.94 ^{a}
5	63.75 ± 5.96 ^{a}	9.13 ± 0.94 ^{a}
10	76.75 ± 7.54 ^{a}	7.54 ± 1.00 ^{a}

Values show the mean ± standard error of protocorm induction as a percentage of original shoot explants. Means followed by different superscript letters are significantly different ($P < 0.05$) between treatments according to DMRT.

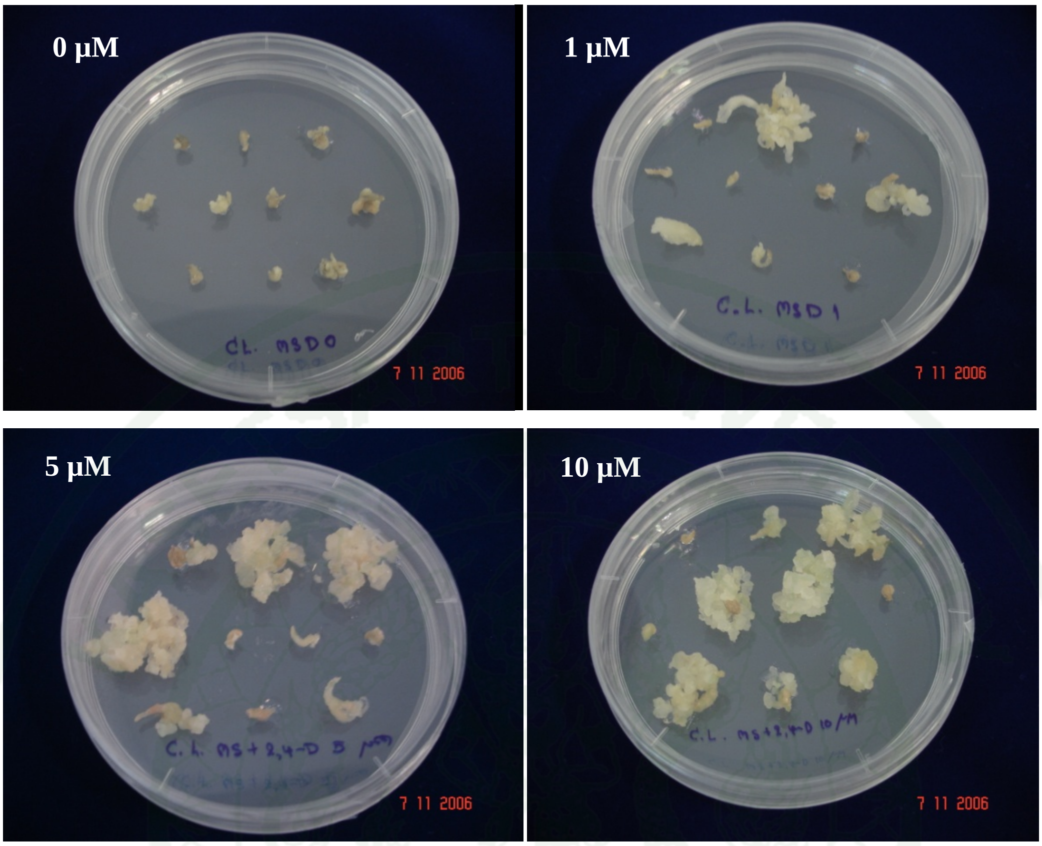

Figure 5 Protocorm induction of *C. latifolia* grown on MS medium supplemented with 2,4-D at concentrations of 0, 1, 5 and 10 µM for 90 days.

2. Effect of plant growth regulators (PGRs) on the protocorm multiplication

Figure 6 demonstrates protocorm proliferation on ½ MS medium with 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (TDZ) or N6-benzyladenine phosphate (BAP) at different levels (0, 5, 10 and 20 µM). The result showed that there was no significant difference of propagated protocorm size among all treatment ranging from 6.71 ± 0.30 mm. to 8.15 ± 0.95 mm. (Table 4). In addition, the protocorm size seemed to decrease slightly with increased concentration of TDZ. However, addition of 10 µM BAP tended to increase the protocorm diameter (8.15 ± 0.95 mm.).

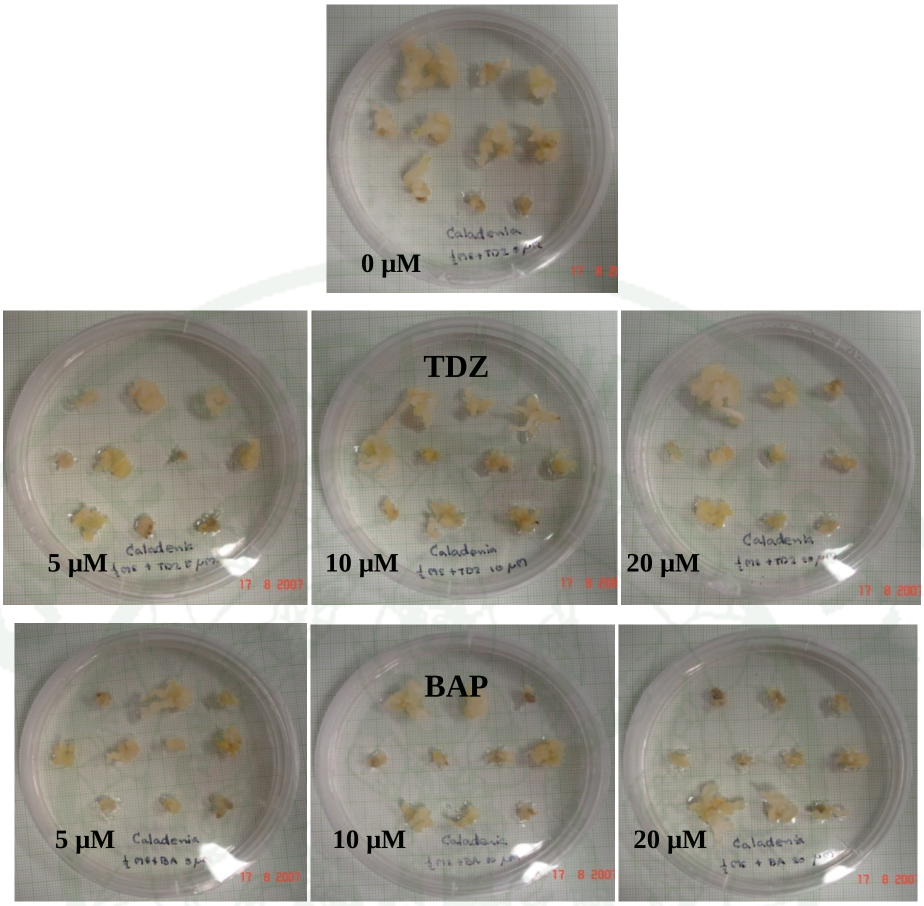

Figure 6 Protocorm mass multiplication of *C. latifolia* cultured on ½ strength MS medium supplemented with 0, 5, 10 and 20 µM TDZ compared to BA 5, 10 and 20 µM BA for 30 days.

Table 4 Mean protocorm clump size of *C. latifolia* cultured on ½ MS medium with TDZ compared to BAP addition at 0, 5, 10 and 20 µM after 30 days.

Plant Growth Regulators	Concentration (µM)	Protocorm clump diameter (mm.) ± SE
Control	0	8.09 ± 0.48^{a}
TDZ	5	7.10 ± 0.32^{a}
	10	7.10 ± 0.34^{a}
	20	6.92 ± 0.26^{a}
BAP	5	6.82 ± 0.18^{a}
	10	8.15 ± 0.95^{a}
	20	6.71 ± 0.30^{a}

Values show the mean ± standard error. Means followed by same superscript letters are not significantly different ($P > 0.05$) among treatments according to DMRT.

3. Impact of 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (TDZ) on shoot induction

In vitro propagated protocorms on Thomale GD medium supplemented with different TDZ (0, 5, 10, 20 and 40 µM) clearly induced adverse responses to shoot induction after 90 days (Figure 7 and Table 5**)**. It showed that the highest incidence of shoot induction (48.00 ± 13.93 %), shoot number (7.31 ± 0.89) and protocorm size (10.30 ± 0.86 mm.) occurred in the control treatment. The addition of TDZ (at all concentrations tested) significantly repressed the incidence of shoot induction and stall expansion of protocorm clumps.

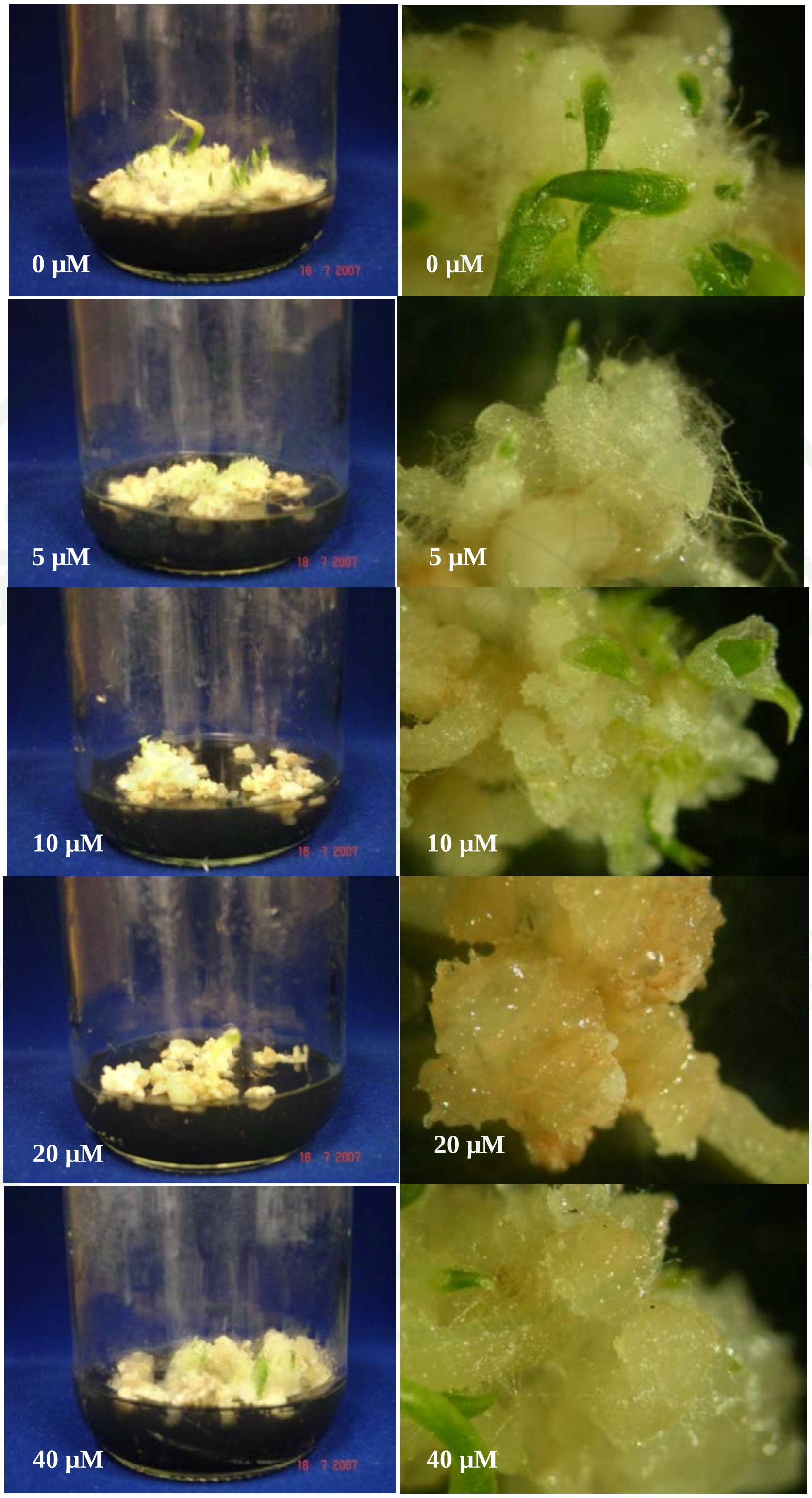

Figure 7 Shoots induction of *C. latifolia* grown on Thomale GD medium containing of TDZ at 0, 5, 10, 20 and 40 µM for 90 days.

Table 5 Shoot induction of *C. latifolia* on Thomale GD medium supplemented with TDZ (0, 5, 10, 20 and 40 µM) after 90 days.

TDZ (µM)	% Shoot induction ± SE	Shoot number ± SE	Protocorms clumb diameter (mm.) ± SE
0	48.00 ± 13.93 ^{a}	7.31 ± 0.89 ^{a}	10.30 ± 0.86 ^{a}
5	4.00 ± 4.00 ^{b}	0.70 ± 0.70 ^{b}	7.24 ± 0.72 ^{b}
10	4.00 ± 4.00 ^{b}	1.20 ± 1.19 ^{b}	8.48 ± 0.35 ^{b}
20	4.00 ± 2.47 ^{b}	0.40 ± 0.25 ^{b}	7.42 ± 0.54 ^{b}
40	8.00 ± 4.90 ^{b}	3.60 ± 2.21 ^{b}	7.78 ± 0.87 ^{b}

Values show the mean ± standard error. Means followed by different superscript letters are significantly different ($P < 0.05$) between treatments according to DMRT.

Cryopreservation of *Caladenia latifolia* R.Br.

4. Optimal protocorm size for cryopreservation of *C. latifolia*

Figure 8 demonstrates that protocorms of *C. latifolia* (derived from *in vitro*-germinated seedlings) proliferate well on MS medium containing 10 mM BAP and can be used to generate explants for other studies. A range of different sizes of these protocorms was used for the development of cryopreservation protocols.

A significant trend was evident between potential protocorm survival (%) of *C. latifolia* following cryostorage and increasing explant size (Table 6). The largest protocorm size (≥ 4 mm) gave the highest potential survival (96 ± 5.48%) following cryostorage. The largest cryopreserved protocorms were predominantly white or whitish in color, whereas those in the smallest size class (≤ 1 mm) were predominantly brown; medium-sized protocorms were yellowish (Figure 9).

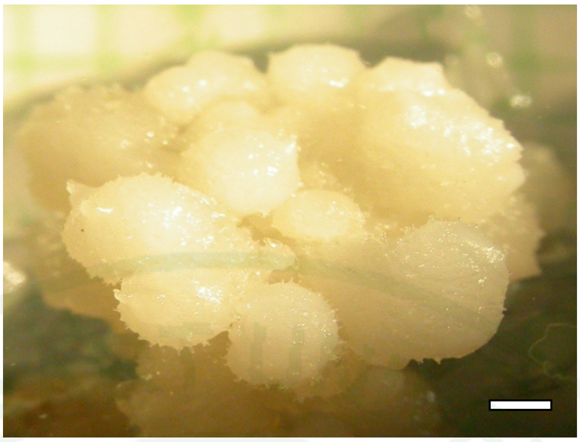

Figure 8 Protocorms of *C. latifolia* (derived from *in vitro*-germinated seedlings) proliferating on MS medium containing 10 mM BAP. A range of different sizes of these protocorms was utilized for the development of cryopreservation protocols. Bar, 1 mm.

Table 6 Effect of size [small (S), ≤ 1 mm.; medium (M), 2–3 mm.; large (L), ≥ 4 mm.] on potential survival of *C. latifolia* protocorms following cryostorage.

Protocorm Size	Percentage survival after 60 days*	Protocorm color**
S ≤ 1 mm.	8.00 ± 8.37 ^{c}	Brown
M = >1 < 4 mm.	76.00 ± 11.40 ^{b}	Yellow
L ≥ 4 mm.	96.00 ± 5.48 ^{a}	White
LSD.	12.07	
% C.V.	14.59	

Values show the mean ± standard error. Means followed by different superscript letters are significantly different ($P < 0.05$) between treatments according to DMRT.

* Determined by fluorescein diacetate (FDA) staining.

** Most prevalent colour of protocorms.

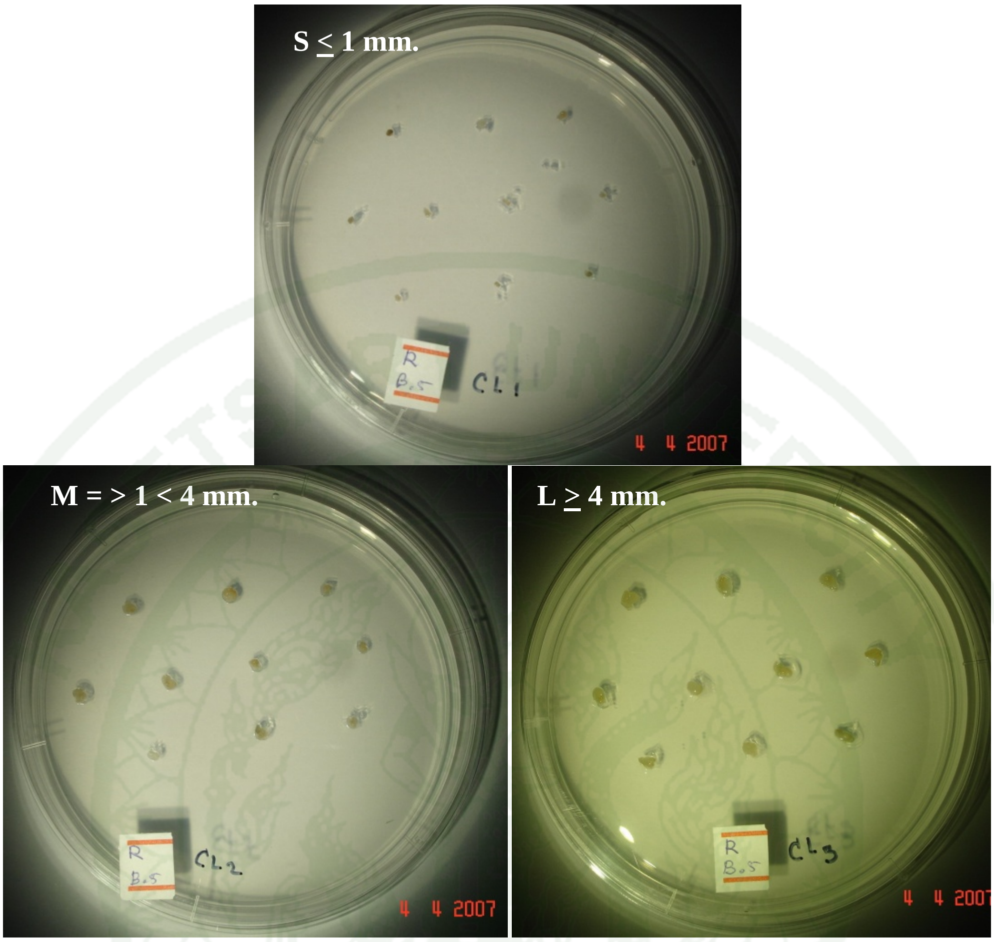

Figure 9 The most prevalent color of *C. latifolia* protocorm obtained from different size [small (S), ≤ 1 mm.; medium (M), 2–3 mm.; large (L), ≥ 4 mm.] following cryostorage.

5. Effect of differing desiccation media on cryopreserved protocorm survival

The effect of differing concentrations of desiccation media (glycerol, from 0 - 1 M) on cryopreserved protocorm survival was not significant, the value ranged from 74 ± 11.04 to 92 ± 13.04% (Table 7). The only treatment effect appeared to be a yellowing of protocorms in all glycerol concentrations relative to the control without glycerol (Figure 10).

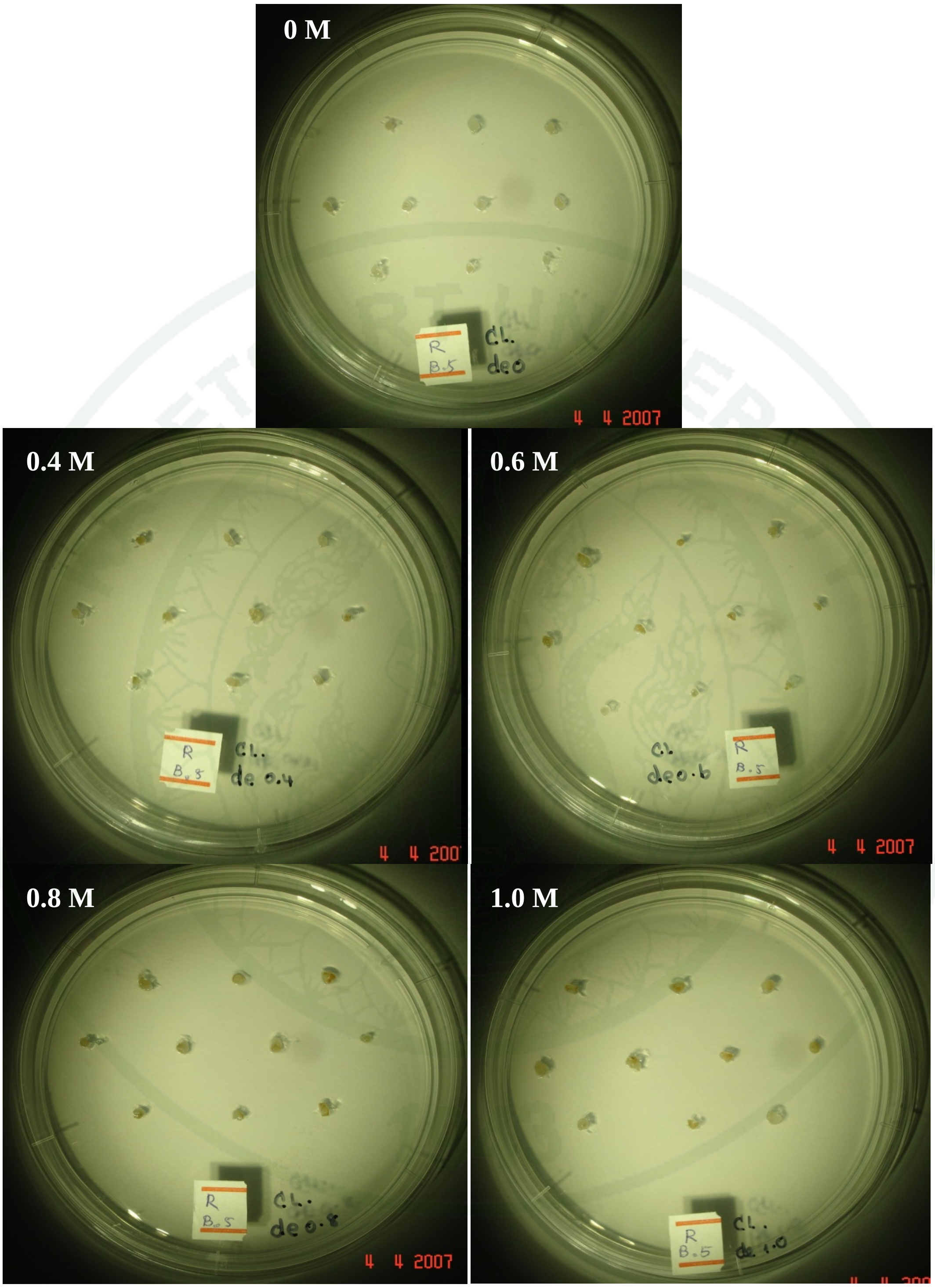

Figure 10 Protocorms of *C. latifolia* treated with different concentrations of desiccation media (glycerol) following cryostorage.

Table 7 Effect of desiccation medium concentration (0, 0.4, 0.6, 0.8 and 1.0 M glycerol) on potential survival of *C. latifolia* protocorms following cryostorage.

Glycerol treatment (M)	Percentage survival after 60 days*	Protocorm color*
0	92 ± 13.04 [a]	White
0.4	78 ± 8.37 [a]	Yellow
0.6	74 ± 11.04 [a]	Yellow
0.8	84 ± 15.17 [a]	Yellow
1.0	78 ± 16.43 [a]	Yellow
LSD.	17.40	
% C.V.	16.24	

Values show the mean ± standard error. Means followed by similar superscript letters are not significantly different ($P > 0.05$) among treatments according to DMRT.
* Most prevalent colour of protocorms.

6. Investigation of cryoprotectants for *in vitro* protocorm cryopreservation

The influence of cryoprotectant solutions (PVS2 or PVS4) applied for 15, 20, 25 and 30 min. is shown in Table 8 and Figure 11. There was a trend for higher potential protocorm survival with 30 min. of exposure to the PVS2, but it was not significantly different from the rest of PVS2 treatments. PVS4 treatments did not show any significant differences in potential protocorm survival when applied from 15 to 30 min. It would appear that at least 25-30 min. of exposure to PVS2 solution is sufficient for a high survival rate (> 90%) with protocorms of *C. latifolia*.

Table 8 Effect of the cryoprotectant solution (PVS2 and PVS4) and the exposure time (15, 20, 25 and 30 minutes) on the percentage of protocorm survival.

Treatment	Percentage survival after 60 days	Protocorm color*
PVS2		
15 min.	76 ± 13.42 [a]	Yellow
20 min.	76 ± 11.40 [a]	White
25 min.	92 ± 8.37 [a]	Yellow
30 min.	96 ± 8.94 [a]	White
PVS4		
15 min.	86 ± 8.94 [a]	White
20 min.	86 ± 11.40 [a]	White
25 min.	76 ± 19.49 [a]	Yellow
30 min.	80 ± 23.45 [a]	Yellow
LSD.	18.22	
% C.V.	16.94	

Values show the mean ± standard error. Means followed by similar superscript letters are not significantly different (P > 0.05) among treatments according to DMRT test.

* Most prevalent color of protocorms.

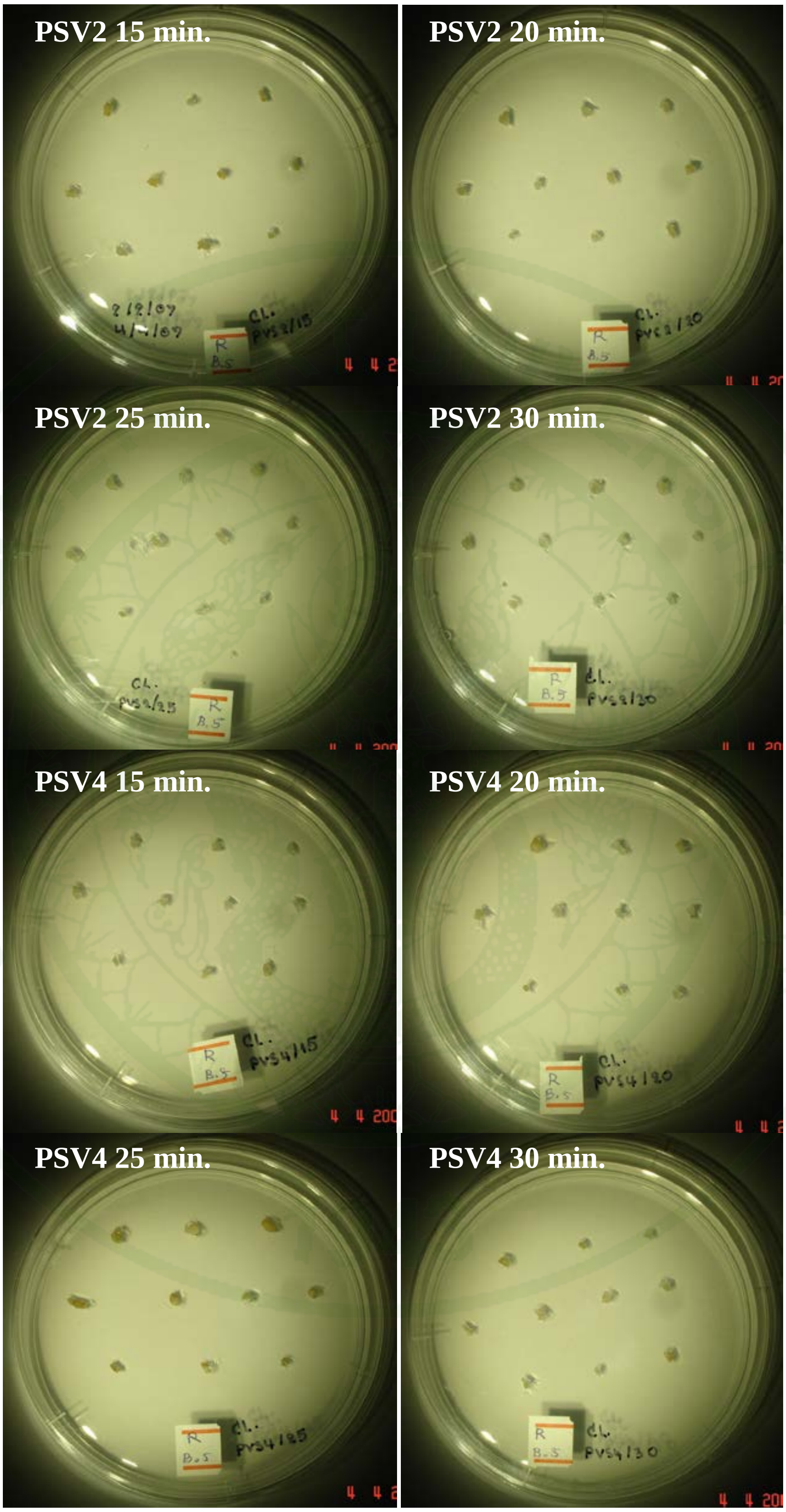

Figure 11 Protocorms of *C. latifolia* treated with different cryoprotectant solution (PVS2 and PVS4) and exposure time following cryostorage.

7. Recovery of cryopreserved protocorms and potential survival of *C. latifolia*

The application of different recovery media (½ MS + BAP, kinetin or 0.5 µM TDZ) did not alter significantly post-LN protocorm survival among treatments (Table 9 and Figure 13). The FDA staining technique indicated a level of protocorm survival in both control (no LN storage) and post-LN treatments, showing little evidence of any areas of pronounced cell loss. Nevertheless, small and brown protocorms showed an extensive absence of staining with FDA, indicating a high level of cellular death (Figure 12).

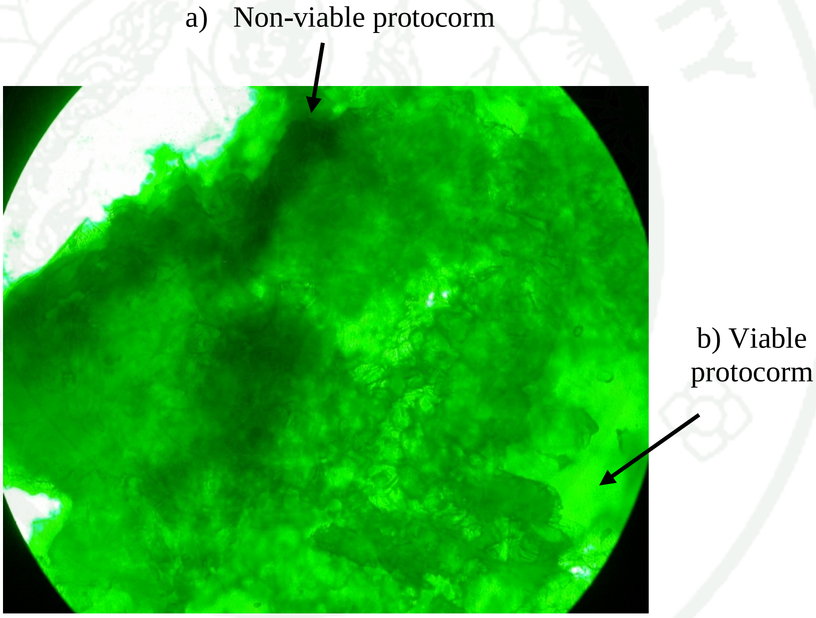

Figure 12 An example of FDA staining of viable and non-viable protocorms (green fluorescence and very dark green colour/no fluorescence, respectively) of *C. latifolia*.

Table 9 Effect of different recovery media (½ MS, ½ MS + BAP, kinetin and 0.5 µM TDZ) on potential survival of *C. latifolia* protocorms following cryostorage.

Treatment	Percentage survival after 90 days	Protocorm color*
½ MS	100 ± 0.0 ^{a}	White
½ MS + BAP	88 ± 13.04 ^{a}	White
½ MS + kinetin	100 ± 0.0 ^{a}	White
½ MS + TDZ	94 ± 13.42 ^{a}	White
LSD.	12.54	
% C.V.	9.79	

Values show the mean ± standard error. Means followed by similar superscript letters are not significantly different ($P > 0.05$) among treatments according to DMRT test.

* Most prevalent color of protocorms

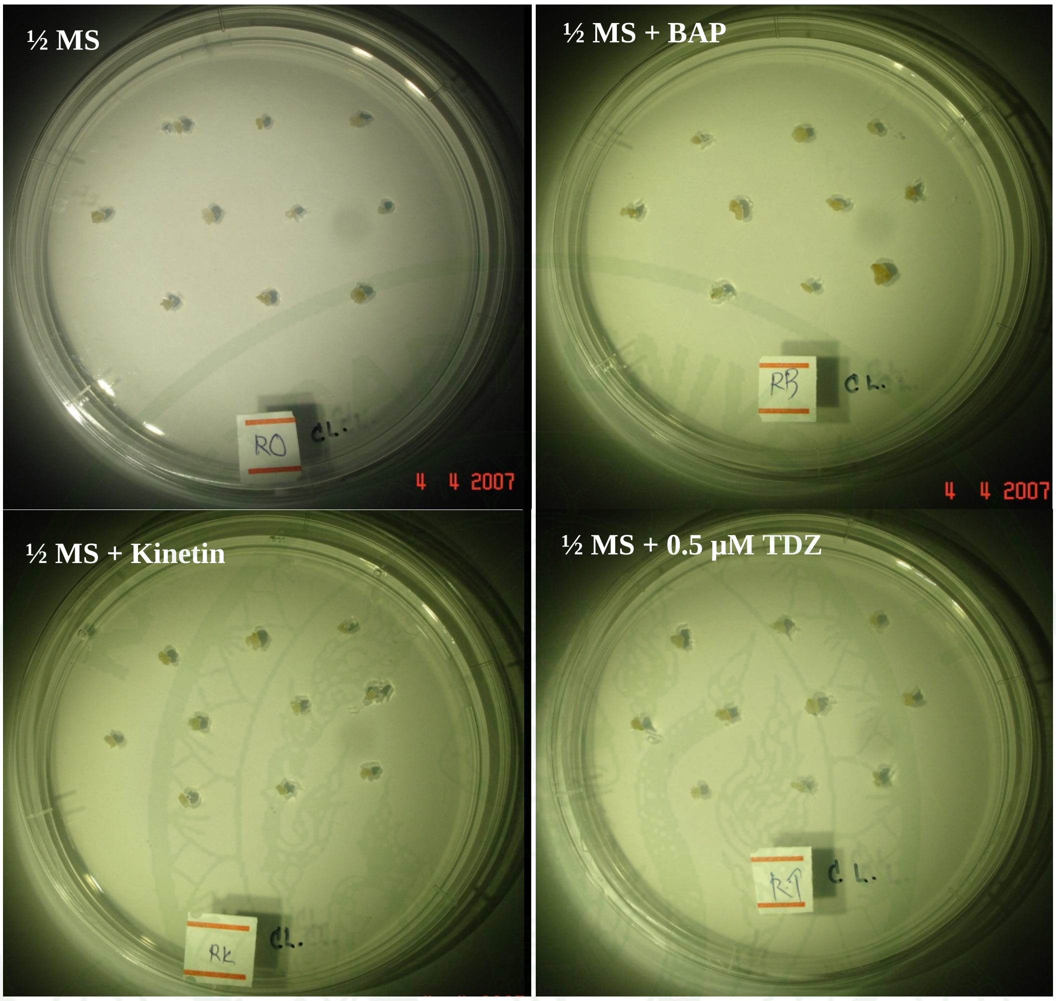

Figure 13 Protocorms of *C. latifolia* on different recovery media (½ MS + BAP, kinetin and 0.5 µM TDZ) following cryostorage.

Experiment II: Development of *in vitro* methods for *ex situ* conservation of *Paphiopedilum insigne* (Wall. ex Lindl.) Pfitzer.

1. Influence of plant growth regulators (PGRs) on growth and development of multiple shoots and roots of *P. insigne*

Seed of *P. insigne* was germinated *in vitro* (Figure 14 and 15) and derived shoots were then used as explants for shoot multiplication in the study. As shown in Table 10, BAP addition at 5, 10 and 40 µM resulted in the highest shoot multiplication of *P. insigne* (4.13 ± 0.89, 3.36 ± 0.33 and 2.88 ± 0.44, respectively). While various TDZ concentrations were tested none showed a distinctly different effect on shoot induction when compared together or with the control treatment (without PGR). The shoot length, although exhibiting some empirical differences among treatments, was highly variable, but all TDZ treatments were not significantly different from the control, despite the 40 µM TDZ treatment having the greatest shoot length of 20.76 ± 2.20 mm. Shoot multiplication of *P. insigne* on ½ MS medium plus various PGRs are shown in Figures 16 and 17.

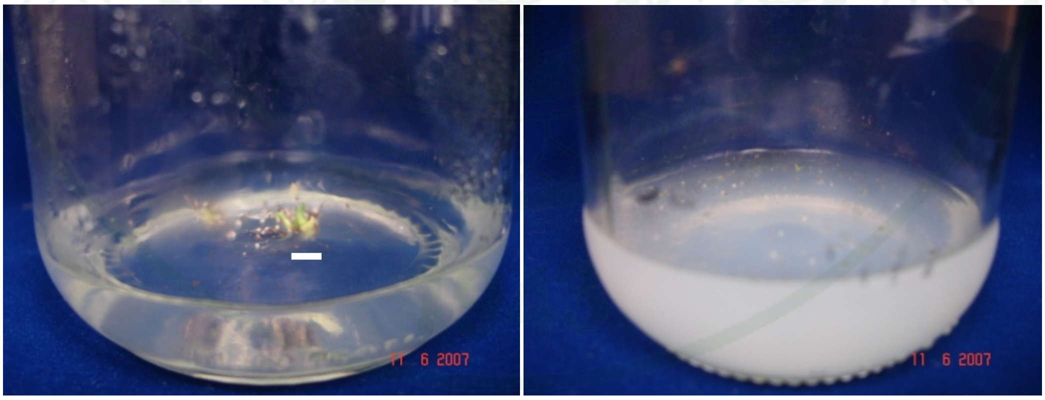

Figure 14 *In vitro* germination of *P. insigne* on ½ MS medium for two months. Bar, 1 mm.

Figure 15 *In vitro* germinated *P. insigne* seedlings on ½ MS medium for two months.

Table 10 Development of shoot multiplication of *P. insigne* shoots cultured on ½ MS medium plus differing concentrations of BAP and TDZ for three months.

Concentration (µM)	Shoot numbers/explant ± SE	Shoot length (mm.) ± SE
0	1.63 ± 0.37 ^{c}	19.22 ± 2.00 ^{a}
BAP 5	2.88 ± 0.44 ^{abc}	16.25 ± 1.80 ^{a}
BAP 10	4.13 ± 0.89 ^{a}	14.69 ± 1.80 ^{a}
BAP 20	2.25 ± 0.37 ^{bc}	17.48 ± 2.30 ^{a}
BAP 40	3.38 ± 0.33 ^{ab}	15.78 ± 1.00 ^{a}
BAP 80	2.38 ± 0.73 ^{bc}	16.89 ± 2.20 ^{a}
TDZ 5	2.00 ± 0.33 ^{bc}	17.60 ± 1.60 ^{a}
TDZ 10	2.38 ± 0.26 ^{bc}	12.13 ± 0.80 ^{a}
TDZ 20	1.50 ± 0.50 ^{c}	16.38 ± 1.60 ^{a}
TDZ 40	2.25 ± 0.45 ^{bc}	20.76 ± 2.20 ^{a}
TDZ 80	1.88 ± 0.40 ^{bc}	13.49 ± 1.70 ^{a}

Values show the mean ± standard error. Means followed by differing superscript letters are significantly different ($P < 0.05$), while means followed by similar superscript letters are not significantly different ($P > 0.05$) among treatments according to DMRT.

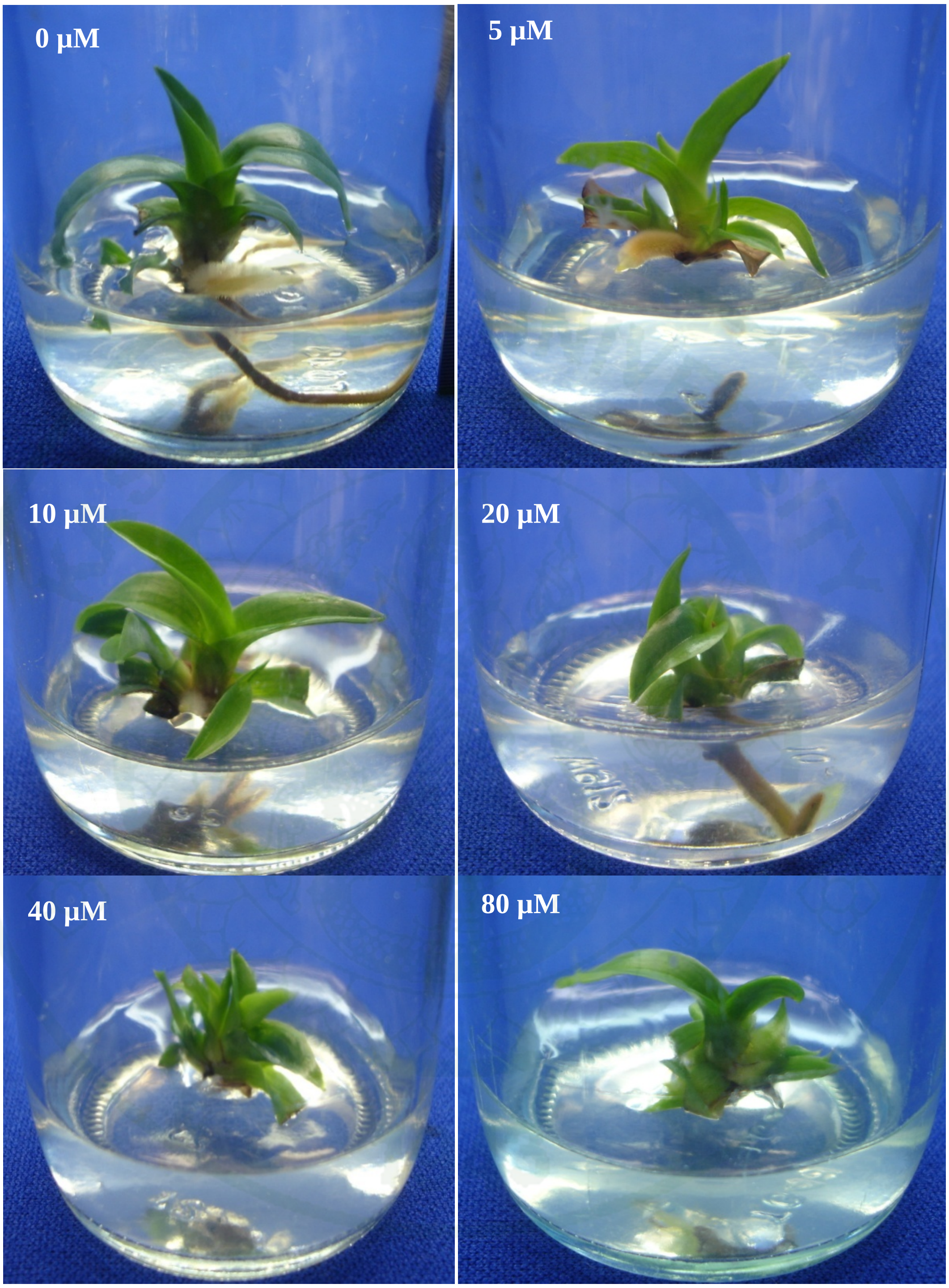

Figure 16 Shoot multiplication of *P. insigne* cultured on ½ MS medium containing 0, 5, 10, 20, 40 and 80 µM BAP for three months.

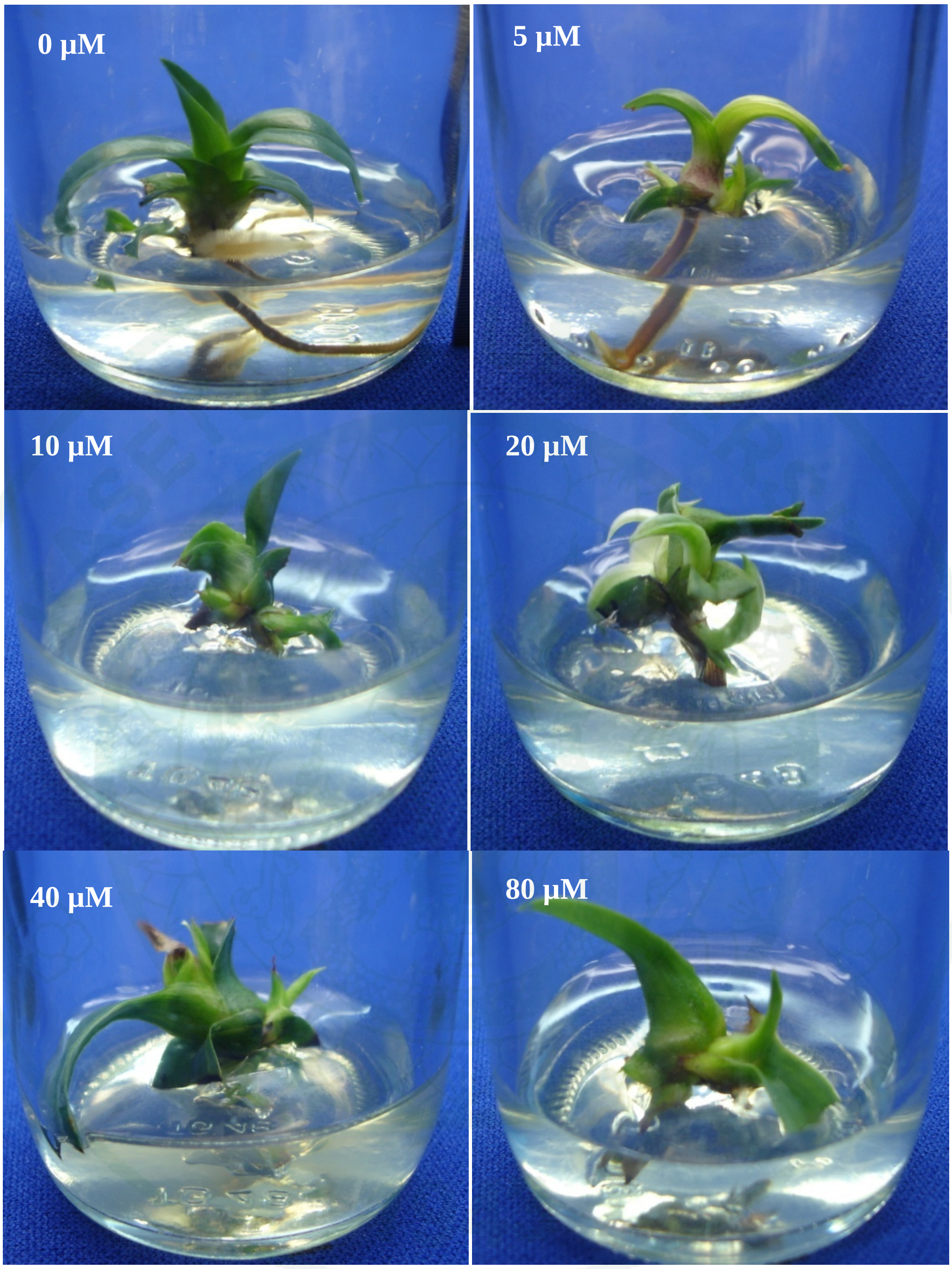

Figure 17 Shoot multiplication of *P. insigne* cultured on ½ MS medium containing 0, 5, 10, 20, 40 and 80 µM TDZ for three months.

The induction of root numbers and shoot lengths of *P. insigne* after subculturing on ½ MS medium plus different PGRs (BAP and TDZ) at 0, 5, 10, 20, 40 and 80 µM are demonstrated in Figure 18. The results clearly showed that supplementation of either BAP or TDZ decreased the induction of both root number and length (Table 11). Addition of 5 µM TDZ or 20 µM BAP gave greater root length than the control, while TDZ concentrations >10 µM completely inhibited root formation.

Table 11 Development of root induction of *P. insigne* shoots on ½ MS medium plus differing levels of BAP and TDZ for three months.

Concentration (µM)	Percentage root induction	Average root numbers/shoot	Average root length (mm.)
0	100	2.75	1.67
BAP 5	75	1.67	1.25
BAP 10	25	2	1.25
BAP 20	25	1	2
BAP 40	12.5	2	1.25
BAP 80	12.5	1	1.5
TDZ 5	25	1	2.25
TDZ 10	12.5	1	1
TDZ 20	-	-	-
TDZ 40	-	-	-
TDZ 80	-	-	-

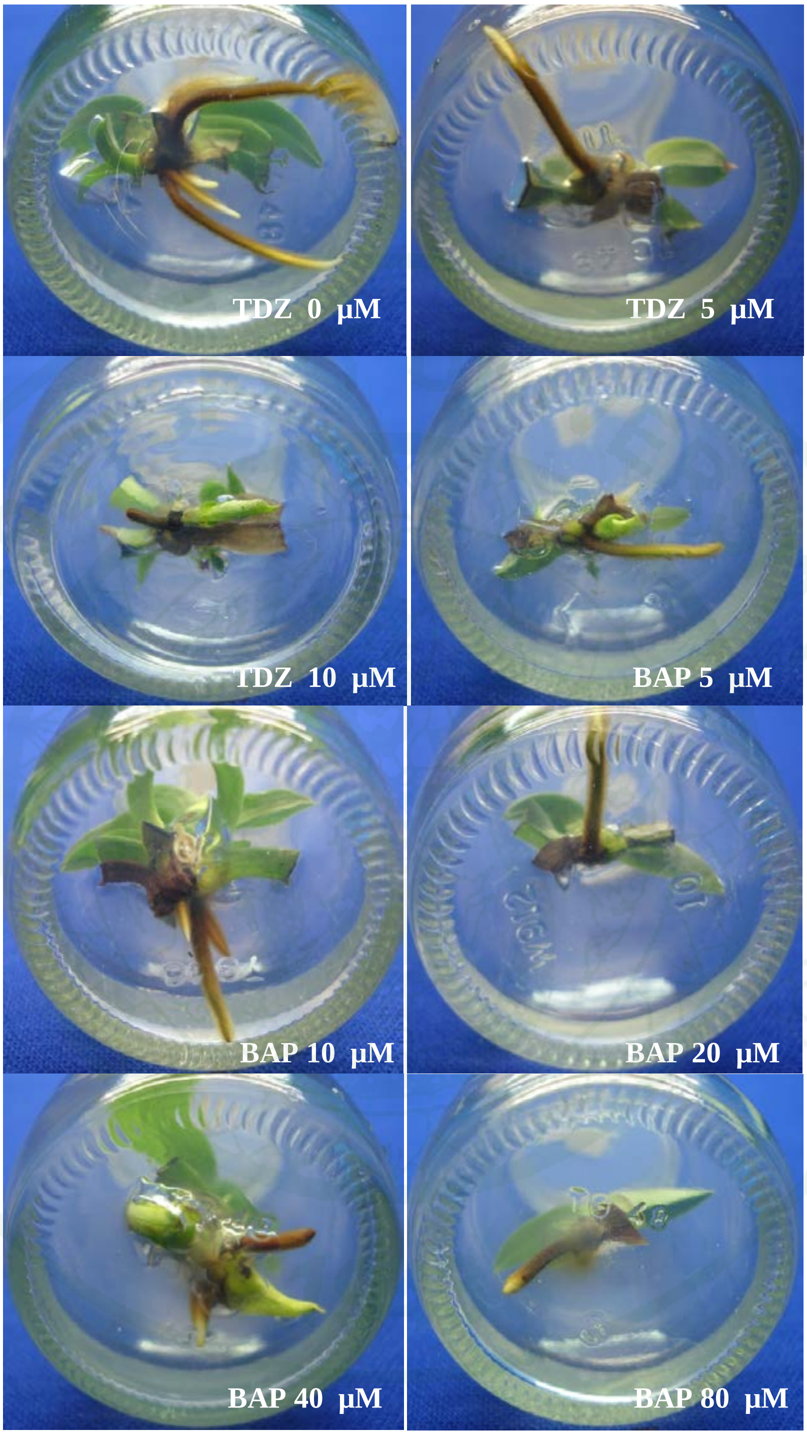

Figure 18 Root induction of *P. insigne* grown on ½ MS medium containing BAP and TDZ at differing concentrations for three months.

2. Cryoperservation of *in vitro*-propagated protocorms of *P. insigne*

The development of *in vitro*-propagated protocorm derived from *P. insigne* germinated seedlings on basal ½ MS medium (Murashige and Skoog, 1962) plus 10 µM BAP during 2 months is demonstrated in Figure 19. They were used as explants for cryopreservation trials.

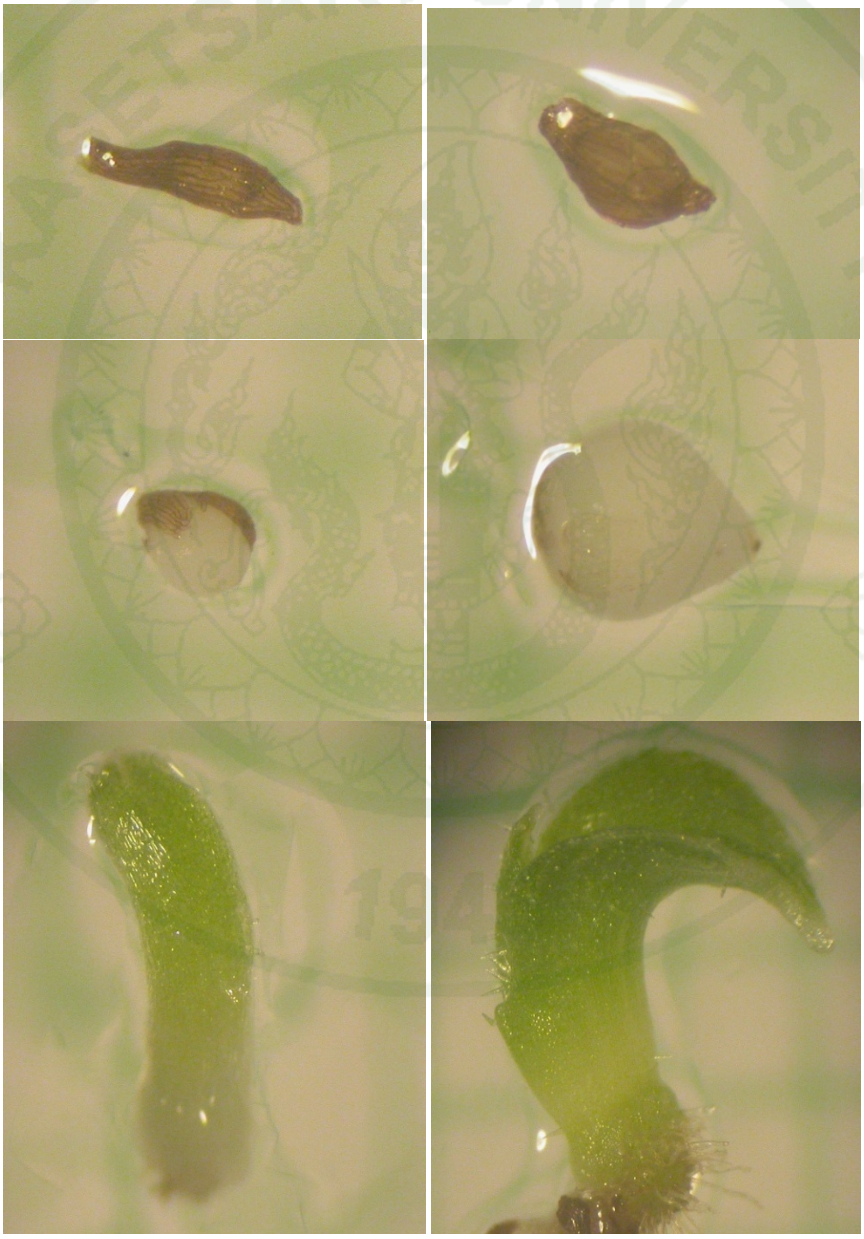

Figure 19 Development of *in vitro*-propagated protocorms of *P. insigne* seedlings cultured on ½ MS medium during two months.

2.1 Cryopreservation of *in vitro*-propagated protocorms of *P. insigne* without encapsulation

Impact of desiccation media (0, 0.4, 0.6, 0.8 and 1 M glycerol) added into ½ MS medium gave differing result on potential survival of of *P. insigne* following cryoprevation (Table 12). Protocorms incubated on ½ MS desiccation media showed varying potential survival, with the highest potential survival (100%) found in the 0.8 M glycerol and control treatments with lower survival in other treatments.

Results of the influence of PVS2 cryoprotectant solutions applied for 15, 20, 25 and 30 min. are shown in Table 13, indicating there was a trend for higher potential protocorm survival with 20 and 30 min. (96.67 and 100%, respectively) of exposure to PVS2. Therefore, it would reveal that at least 15 min. of protocorm exposure to PVS2 solution is most likely sufficient for a high survival rate (> 90%) with protocorms of *P. insigne*.

Table 12 Effect of desiccation medium concentration (0, 0.4, 0.6, 0.8 and 1.0 M glycerol) on potential survival of *P. insigne* propagated-protocorms following cryostorage without encapsulation.

Glycerol level (M)	Percentage survival after 90 days	Protocorm color*
0	100.00	White
0.4	83.33	Yellow
0.6	90.00	White
0.8	100.00	White
1.0	90.00	White

Table 13 Influence of the exposure time at 15, 20, 25 and 30 min. with PVS2 cryoprotectant solution on percentage survival of *in* vitro propagated *P. insigne* protocorms without encapsulation.

Exposure time in cryoprotectant solution	Percentage survival after 90 days	Protocorm color*
15 min.	93.33	White
20 min.	93.33	White
25 min.	96.67	White
30 min.	100.00	White

Surviving protocorms of *P. insigne* were mostly small and yellowish, while protocorms that were brown after immersion in liquid nitrogen did not revive (Figure 20). The FDA staining technique indicated a level of protocorm survival following cryoprevation, with little evidence of any areas of pronounced cell loss. Nevertheless, small and brown protocorms showed an extensive absence of staining with FDA, indicating a high level of cellular death.

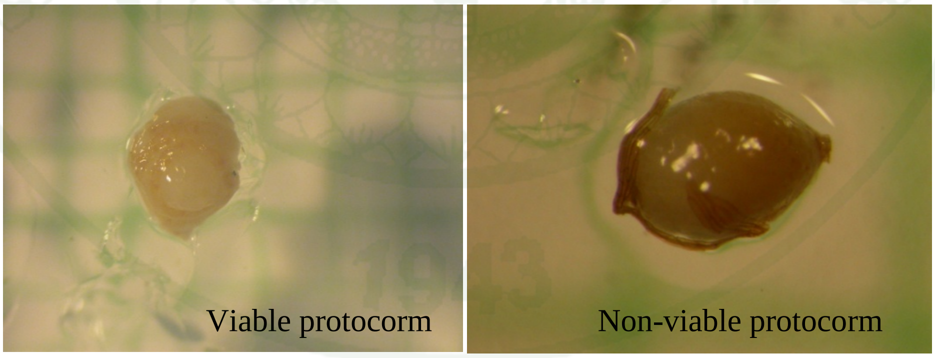

Figure 20 Viable and non-viable protocorms for checking of potential survival of *P. insigne* following cryostorage.

2.2 Cryopreservation of *in vitro*-propagated protocorms of *P. insigne* by encapsulation-desiccation and vitrification

The result in Table 14 demonstrated that potential survival of encapsulated protocorms via cryopreservation varied among desiccation media (glycerol) concentration used. The highest potential survival was found in the 0.8 M glycerol treatment, followed by the 0.4 M treatment at 91 and 81%, respectively. The only treatment effect appeared to be a yellowing of protocorms in all glycerol concentrations relative to the control without glycerol.

The dehydration time before immersion in liquid nitrogen had a varied impact on the potential survival of *P. insigne* encapsulated protocorms after cryopreservation. Protocorms air-dried for 90 min exhibited greater potential survival of protocorm (80%) after encapsulated-cryopreservation (Table 15). Whereas, the other dehydration times resulted in adverse effect on potential survival in comparion to the control treatment (without dehydration).

Table 14 Effect of desiccation medium concentration (0, 0.4, 0.6, 0.8 and 1.0 M glycerol) on potential survival of *P. insigne* encapsulated-protocorms following cryostorage.

Glycerol level (M)	Percentage survival after 90 days	Protocorm color*
0	76	White
0.4	81	White
0.6	57	Yellow
0.8	91	White
1.0	76	White

Table 15 Potential survival following cryostorage of *P. insigne* encapsulated-protocorms from differing time of air-drying dehydration (0, 30, 60, 90 and 120 min.).

Dehydration time (min.)	Protocorm survival after 90 days (%)	Protocorm color*
0	73.33	White
30	66.67	Yellow
60	70.00	Yellow
90	80.00	White
120	73.33	White

Discussion

Experiment I: Development of *in vitro* methods for *ex situ* conservation of *Caladenia latifolia* R.Br.

1. Optmial *in vitro* propagation of *Caladenia latifolia* R.Br.

Comparison of germinated seedlings grown on MS medium containing differing concentrations of auxin (2,4- D) indicated that higher 2,4-D concentrations enhanced the protocorm induction of *C. latifolia* (Table 3). The 10 µM 2,4-D treatment tended to give the greatest induction rate with no significant differences with other 2,4-D treatments (Figure 5). The largest protocorms were derived from the 5 µM 2,4-D treatment,. In agreement with other studies, the addition of exogenous plant growth regulators like 2,4-D to the medium appeared to increase germination and protocorm formation (Bektaş *et al*., 2013). There is some evidence that seedlings are capable of endogenous hormone production that may have synergistic effects with exogenous PGRs added to the medium and thus further induce protocorm formation (Mei *et al*., 2012). Similar basal MS medium with 2,4-D has been used previously for other orchid species (*Phalaenopsis*) and promoted protocorm-like bodies (PLBs) and callus induction (Chen *et al*., 2000). Dohling *et al*. (2012), also reported that explants cultured in media containing 2,4-D gave a more variable response by forming more PLBs and protocorms compared with media without PGR. In other reports MS medium containing 2,4-D resulted in low seed germination and protocorm formation of *Epidendrum ibaguense* Kunth (Hossain, 2008). Inhibitory effects of 2,4-D on seed germination and protocorm development have been reported in other orchid species (Fornnesbech, 1972; Kusumoto, 1978; Sharma and Tandon, 1986).

The cytokinins TDZ and BAP exhibited no significant differences within treatments on protocorm multiplication with *C.* *latifolia* and tended to inhibit protocorm mass proliferation at higher concentrations in this study (Table 4 and Figure 6). Nevertheless, it is evident that 10 µM BAP treatment increased avergage protocorm size with *C. latifolia*. Roy *et al*. (2007) reported that BAP (among other cytokinins investigated), showed significantly better development of protocorm/PLBs of *Dendrobium chrysotoxum*, compared to TDZ and PGR-free control treatments. Niknejad *et al*. (2011) demonstrated that TDZ in combination with NAA was better than TDZ alone for protocorm and PLB induction, in terms of producing higher quantity and quality. It has been also found that PLBs of *Dendrobium densiflorum* proliferated well on basal MS medium and completely converted into shoots on MS medium containing 2.0 mg/L BAP (Luo *et al*., 2008). Although, efficacy of BAP in PLB and protocorm proliferation on ½ MS basal medium has been demonstrated with different orchids, (Martin and Madassery, 2006); *in vitro* protocorms of *C. latifolia* showed slow growth with all cytokinin treatments, indicating a lack of specific response to exogenous cytokinins at the concentrations tested. This may possibly have been due to the protocorm explants possessing endogenous hormone that resulted in an unfavourable combination with exogenous PGRs, (e.g. TDZ and BAP), resulting in less protocorm multiplication. It has been suggested that such differential responses of orchid tissue cultures to exogenous cytokinins is related to the type-specific metabolism of cytokinins that determines their biological activity (Horgan, 1987; De Pauw *et al*., 1995). In our study, further proliferation and regeneration of protocorms and PLBs were devoid of continuous exposure to the exogenous PGR, as suggested by Roy *et al*. (2007). It has also reported that the addition of BAP was found to inhibit the induction of secondary PLB formation. In addition, the secondary PLBs of *Paphiopedilum* continued to proliferate further after being subcultured onto half-strength PGR-free MS medium (Ng and Saleh, 2011).

To improve shoot regeneration of *C. latifolia*, the Thomale GD medium was optimized by testing the effect of differring concentrations of TDZ (0, 5, 10, 20 and 40 µM) on shoot induction and growth (Table 5 and Figure 7). The Thomale GD formula is unique and has been reported as optimal for shoot regeneration and enlarging protocorms of *Paphiopedilum spp*. (Arditti, 1992; Arditti and Ernst, 1993). Other *Paphiopedilum spp*. have been reported to grow well on Thomale GD medium such as *P. concolor* (Paneerat, 1996), and *P. rothchildiana* (Haas-von Schmude *et al*, 1986). TDZ has been shown to exhibit a stronger effect than other PGR's on shoot proliferation and adventitious shoot organogenesis on plant species (Huetteman and

Preece, 1993). There appears to be some species specificity as regards positive response to TDZ on shoot formation and multiplication; for example 5 µM TDZ was sufficient for shoot multiplication of *P. bellatulum* (Paneerat, 1996), 4.52 µM 2,4-D plus 0.45 µM TDZ for shoot regeneration of *P. philippinense* (Chen *et al.*, 2002), 13.62 µM TDZ for shoot regeneration from seed-derived protocorms of *Phalaenopsis amabilis* (Chen and Chang, 2004), and 6.8 µM TDZ for shoot regeneration of *P. delenatii* (Nhut *et al.*, 2007). In contrast to this result, All TDZ treatments in this study showed an adverse response on shoot induction, number of shoots and stalled expansion of protocorms for *C. latifolia*. Huang *et al*. (2001) reported that TDZ inhibits shoot proliferation and rooting in *Paphiopedilum*. Results reported by Wattanawikkit *et al*. (2011) indicated that shoot development in *P. callosum* was less responsive to medium containing TDZ. The *in vitro* morphogenesis stimulated by the same hormone on different orchid species can be quite different, therefore the culture and PGR requirements for specific plants needs to be identified. In addition, the variable effects of PGR and organic supplements on different orchid species further emphasizes that the plant growth response depends largely on plant genotype and the formulation of the basal medium (Islam *et al*., 2003).

This study has derived a novel method for asymbiotic clonal *in vitro* propagation of *Caladenia latifolia* (an endemic terrestrial orchid species from Western Australia) for horticulture and breeding from seedling explants using MS medium. A basal MS medium containing 10 µM 2,4-D, was useful in obtaining high protocorm formation, and initial proliferation used germinated seedlings as donor explants with repeated subcultures required. Half-strength MS medium with 10 µM BA was most effective for inducing proliferation of larger protocorms, while the PGR-free Thomale GD medium produced the best shoot induction from *in vitro* protocorms.

2. Cryopreservation of *Caladenia latifolia* R.Br.

Protocorms used in this study were obtained from *in vitro* germinated seedlings of *Caladenia latifolia* R.Br. cultured on MS medium containing 10 mM BAP and further incubated under a 16/8-h photoperiod at 25 ± 2 ^{o}C. A range of different sizes of these protocorms was used for the development of cryopreservation protocols (Figure 8).

In this study, a significant trend was evident between potential protocorm survival of *C. latifolia* following cryostorage and increasing explant size (Table 6 and Figure 9). The largest protocorm size ($\geq$ 4 mm.) showed the greatest potential survival (> 95%) following cryostorage. Larger cryopreserved protocorms were predominantly white or whitish in color, whereas those in the smallest size class ($\leq$ 1 mm.) were predominantly brown following retrieval from cryostorage; while medium-sized protocorms were mainly yellowish (Figure 9). In accordance with Safrihah *et al*. (2009), who observed that larger shoots of the *Mokara* golden nugget orchid (derived from PLBs) from 1.0-1.5 cm long, survived better following cryopreservation compared to smaller sized shoots. Khoddamzadeh *et al*. (2011) reported success with cryopreserved protocorms of *Phalaenopsis Blume* that were 3-5 mm. in diameter (and encapsulated in alginate), but did not specify whether other sizes of protocorms were tested. In other reports, increasing size of protocorms of *Dactylorhiza* reduced survival after cryopreservation by vitrification (Nikishina *et al*., 2007). In cryopreservation studies with PLBs of *Dendrobium* Bobby Messina by vitrification method, PLBs of larger size (3-4 mm.) showed better viability for both cryopreserved and non-cryopreserved PLBs (Antony *et al*., 2010), while Zainuddin *et al*. (2011) found that the highest viability rate of PLBs of *Dendrobium* Bobby Messina after cryoprevation via encapsulation-dehydration technique was found in the smaller sized protocorm class (1-2 mm.). Smaller size PLBs (1-2 mm.) of other *Dendrobium* sonia-28 also resulted in the higher potential survival than a bigger size (3-4 mm.) following cryopreservation (Poobathy *et al*., 2013). As reported by Engelmann (2000), vitrification cryopreservation based procedures allow the use of samples of relatively large size (shoot-tips of 0.5 to 2–3 mm.) which can regrow directly without any difficulty. It is evident that the size of protocorms and survival after cryopreservation is very variable and possibly species-specific, but may also depend on the type of cryopreservation protocol utilized.

Results with other Australian monocotyledons have indicated that the optimal sizes of explants (e.g. shoot apices) for the cryopreservation of species of *Anigozanthos* Labill., for example, were small (1.0-1.5 mm.) and that larger apices were less likely to survive cryostorage (Turner *et al*., 2001b). Reports of shoot apices used for the cryopreservation of other (non-Australian) species have shown that small apical explants comprising relatively small, homogeneous and actively dividing cells containing few vacuoles with a high nucleocytoplasmic ratio enabled greater desiccation tolerance and subsequent higher levels of survival following cryopreservation (Quain *et al*., 2009; Varghese *et al*., 2009). The simplicity of the approach developed here indicates that a similar cryogenic regime might apply more broadly to the protocorms from other terrestrial orchids. Direct comparison of protocorms with shoot apices subjected to cryostorage has some limitations. First, protocorms require minimal manipulation, and hence there is likely to be less damage to tissues during preparation for cryopreservation when compared with excised shoot tips. Second, a protocorm is a discrete propagule perhaps more comparable with a somatic embryo than an isolated shoot apex.

Preconditioning or preculture stage refers to the culturing of explant material on medium added with osmotic agents at various concentrations and durations depending on plant species and type of plant material used (Shibli *et al*., 2006). In the preculture step, the osmotic agent(s) (i.e. sucrose, glycerol, manitol and/or sorbitol) concentrations in the plant material is raised considerably (Reed *et al*., 2008) and it can increase the efficiency of cryopreservation in many species (Shibli *et al*., 2006). Vaious concentration of sugar, particulary sucrose or combination of sucrose and glycerol, is the most cryoprotective solution used to increase desiccation and freezing tolerance for survival of cryopreserved tissues including orchids (Engelmann, 2000; Takagi, 2000; Flachsland *et al*., 2006). By contrast, Wang *et al*. (1998) found that another species of orchids, such as *Dendrobium*, was sensitive to high concentration of sucrose in an unexpected manner. Similar to this work, the effect of different concentrations of desiccation media (0-1 M glycerol) on potential survival of cryopreserved protocorm in this study was not significant (Table 7). The only treatment effect appeared to be yellowing of protocorms in all glycerol concentrations relative to the control (without glycerol) (Figure 10). However report of Koo and Aziz (2005), defines glycerol at 0.5 M as the best loading/cryoprotectant solution for protocoms of *Phalaenopsis gigantean* and *Paphiopedilum rothschildianum*, with the most viability of protocorms after cryopreservation. In other plant tissues, the viability of melon somatic embryos of medium and large sizes showed a higher survival rate than those with a small size for both desiccated and cryopreserved embryos (Shimonishi *et al*., 2000).

In the tests of the influence of cryoprotectant solutions (PVS2 or PVS4) applied for 15, 20, 25 and 30 min. (Table 8), there was a trend for higher potential protocorm survival with 30 min. of exposure to PVS2, although this was not significantly different from less exposure to PVS2 and PVS4 treatments. The potential survival of cryopreserved protocorms increased gradually with increasing time of exposure to PVS2 and reached a maximum (96%) for 30 min. exposure, with protocorm remaining white. The protocorms treated with PVS4 for up to 25 min. retained high rates of potential survival, however, longer exposures reduced the survival percentage with changes of color from white to yellowish compared to the the low exposure time (Figure 11). Protocorms that have been subject to cryostorage rarely display the characteristic white color that indicates viability. Whitish appearance indicates a quicker regrowth and higher viability, whereas appearance of yellowish colors, could be attributed to osmotic shock or unfavorable regrowth conditions as described by Moges *et al*. (2004). The exposure to PVS2 (30% glycerol, 15% ethylene glycol and DMSO in 0.4 M sucrose) prior to cryopreservation must be carefully controlled to minimize possible damage by chemical toxicity, osmotic stress and ice crystallization (Vendrame *et al*., 2007). Overexposure of plant tissues to the vitrification solution may lead to chemical toxicity and excessive osmotic stress on cell viability (Sakai, 2000; Hong *et al*., 2009). It is an essential step for successful vitrification to identify the minimum exposure time to the solutions to avoid the toxic effects but to obtain sufficient dehydration and cryoprotection of tissues (Yamada *et al*., 1991; Takagi, 2000). In this study, toxic effects caused by PVS2 were not readily observed at least with larger sized protocorms of the study species. It would appear that at least 15-20 min. of exposure to PVS2 solution is ample time for a high survival rate with protocorms of *C. latifolia*. Sakai *et al*. (1990) reported that some components of PVS2 do not readily permeate into the cytosol during dehydration. This finding is similar to results for cryopreservation of embryos of a Japanese terrestrial orchid (*Bletilla striata*), where more than one hour with PVS2 was necessary for adequate cryoprotection (Ishikawa *et al.*, 1997).

There are a number of reports on best cryopreservation in orchid species being found with PVS4 instead of PVS2, and comparatively long exposure times to vitrification solutions such as PVS2 and PVS4 being required with some species The viabilities of white protocoms of *P. rothschildianum* exposed to PVS2 before storage in LN, were higher than protocorms exposed to PSV4 (Koo and Aziz, 2005). Safrinah *et al*. (2009) found that *in vitr*o shoots of *Mokara* orchid had the highest viability when they were treated with PSV2 vitrification solution for 15-30 min. of exposure time at 0°C. PLBs of *Dendrobium candidum* treated with PVS2 at 0 °C for 150 min resulted in the highest post-LN survival rate at 89.4% (Yin and Hong, 2009). Mohanty *et al*. (2012) also revealed that a significance increase in survivability (78.1%) of *Dendrobium nobile* Lindl was noticed when PLBs were exposed in PVS2 solution at 0 °C for 115 min. prior to cryopreservation. In other reports, protocorms of *Dendrobium cariniferum* treated with PVS2 for 60 min. at 25 ± 2°C and cryopreserved by encapsulation–vitrification exhibited low (15%) survival frequency (Thammasiri, 2008). Poobathy *et al*. (2013) reported that a significantly high cellular viability of *Dendrobium sonia*-28 was recorded when PLBs were exposed to PVS2 for 20 min., suggesting that the PLBs can be effectively dehydrated at the mentioned duration without causing harmful effect to the cells. However, they also recorded a drastic decline of cellular viability in PLBs, when exposure to PVS2 was increased to 30 and 40 min., which was thought to be due to toxic effects of the PVS2 solution. Sakai (1997) reported that long exposure of explants to highly concentrated vitrification solutions is potentially injurious because of the phytotoxic effects of individual components or combined osmotic effect on cell viability.

Similar results have been observed in other plant species, where formation of vitrified shoot-tips increased gradually with increasing time of exposure to PVS2 (45 min.) to a maximum 75% post-LN survival of *in vitro*-grown shoot-tips from cassava (Charoensub *et al*., 2000). Meristems of some orchid, *Cymbidium,* tolerated dehydration for 30-60 min. exposure to PVS2, with 50-90% of surviving meristems producing protocorms, enabling a thorough dehydration of meristems by PVS2 with less post-cryostorage damage (Thinh and Takagi *et al*., 2000). The meristems of some species (*Cymbidium*, *Cymbopogon* and taro) could be treated with PVS2 at 0 °C for up to 20-40 min. with little effect on plant recovery survival rates (70-90%) depending on species (Thinh *et al*., 2000). Touchell (2000) also reported that excised shoot apices of *Grevillea scapigera* exposed to a PVS2 plant vitrification solution for 30 min. at 0 °C showed the best shoot apice surviving before directly immersing samples in liquid nitrogen. The result of PSV2 found in this study can therefore confirm observations by other researchers that the optimal exposure time for PVS2 varies considerably with plant species and also depends on the temperature during exposure (Hong *et al*., 2009). In addition, differences in the optimal cryoprotectant period for various plant species could also be attributed to cell volume size, developmental stage and physiological condition of explants (Tsukazaki *et al*., 2000).

Recovery medium is often important whether cryopreserved meristems or cryopreserved cell cultures are involved (Reed, 1993). The composition of the recovery medium can influence the recovery after cryopreservation (Reinhoud *et al*., 2000). In this study, the application of different recovery media (½ MS, ½ MS + BAP, kinetin or TDZ at 0.5 mM) did not significantly alter post-LN protocorm survival of *C. latifolia* among treatments (Table 9 and Figure 13). However, protocorms cultured on basal ½ MS medium without PGRs and ½ MS medium plus kinetin tended to improve potential survival (as determined by FDA viability testing) compared to the rest of treatments. Reed (2000) reported higher survival of *Rubus* meristems grown on MS medium without PGRs following cryopreservation.

Flachsland *et al*. (2006) demonstrated optimal survival of cryopreserved protocorms of *Oncidium bifolium* Sims grown on ½ MS medium with 2-3% w/v sucrose and without PGRs. Antony *et al*. (2013) reported that the optimal recovery medium for cryopreserved PLBs of *Brassidium* (shooting star orchid) was ½ MS medium plus 2-3% w/v sucrose, 2.75 g/L GelriteTM without PGRs. In contrast, Escobar *et al*. (2000) reported that the use of cytokinins in the recovery medium influenced the recovery response of viable cassava shoot-tips after freezing, with kinetin being more effective than BAP, TDZ and adenine. When the BAP concentration was increased, a drastic effect on shoot recovery was noted (Escobar *et al.*, 1995). Turner *et al*. (2001a) also reported that recovery medium containing plant growth regulators such as kinetin, zeatin, IAA, GA_3 significantly increased survival of shoot apices of *Anigozanthos viridis* following cryopreservation.

The FDA staining technique indicated a level of survival in both control (no LN storage) and post-LN treatments, showing little evidence of any areas of pronounced cell loss. However small, brown protocorms showed extensive absence of staining with FDA, indicating a high level of cellular death (Figure 12). Although FDA staining has been reported to result in unreliable estimates of cell viability in some cases (Suzuki *et al*., 2008), FDA and triphenyl tetrazolium chloride (TTZ) were useful for indicating the viability of terrestrial orchid seed, albeit usually overestimating viability (Batty *et al*., 2001). In addition, there was a definite correlation between TTZ reduction viability testing and re-growth after cryostorage of *Phalaenopsis* protocorms (Khoddamzadeh *et al*., 2011). FDA was therefore used here to estimate the relative survival potential of protocorms, and further confirmation of survival is required from additional re-growth experiments using the full range of protocorm sizes.

The study indicates that the cryopreservation of terrestrial orchid protocorms is technically straightforward and provides a new and potentially highly beneficial tool in endangered terrestrial orchid conservation where seed may be limited (because of species rarity), or as a means of storing and later utilizing the large surpluses of protocorms generated in propagation programmes (e.g. Gale *et al*., 2010).

Further research is now needed to verify estimations of post-LN viability and to apply cryopreservation protocols to a broader range of terrestrial orchids and rare taxa to determine the applicability of cryopreservation as a general tool in orchid conservation.

Experiment II: Development of *in vitro* methods for *ex situ* conservation of *Paphiopedilum insigne* (Wall. ex Lindl.) Pfitzer.

1. Influence of plant growth regulators (PGRs) on growth and development of multiple shoots and roots of *P. insigne*

The use of PGRs for efficient micropropagation of orchids by stimulating induction and proliferation of shoots from various explants is well established. The type, concentration, and combination of PGRs exert differential influence on various explants (Arditti and Ernst, 1993). For *Paphiopedilum* orchids seeds are still commonly used for micropropagation of as multiplication rates from shoot explants are very low; in fact slow growth and poor multiplication rates are the most important limiting factors for *in vitro* culture of slipper orchids (Thongpukdee *et al*., 2013). In the present study, it was clearly demostrated that the addition of BAP at 5, 10 and 40 µM increased shoot numbers of *P. insigne* on ½ MS medium when compared to TDZ and control treatments (Table 10, Figure 16 and 17). However, at the higher concentrations of BAP tested (eg. 80 µM) a reduction in the number of shoots was observed, indicating that high levels of BAP may be supraoptimal. TDZ has been shown to be more effective for inducing high rates of regeneration and axillary shoot proliferation than conventional cytokinins over a wide range of plants including several orchid species (Huetteman and Preece, 1993; Chen *et al*., 2002; Chen *et al*., 2004; Ferreira *et al*., 2006). In this study, the presence of higher TDZ in the medium resulted in lower shoot formation with respect to shoot number and length. The shoot length, although exhibiting some empirical differences among treatments, was highly variable, but all TDZ treatments were not significantly different from the control (minus PGR), despite the 40 µM TDZ treatment having the greatest shoot length.

Huang *et al*. (2001) also found that TDZ inhibited shoot proliferation and rooting of *Paphiopedilum*.

In further observations on impact of PGRs on *in vitro* propagation of orchids Latha (1999) showed that a relatively low 4.44 µM BAP is effective in inducing a higher frequency of shoot induction in *Habenaria* crinifera, and similarly low levels of BAP are capable of inducing ample shoot multiplication in some species of *Cymbidium* and *Cattleya* (Nagaraju *et al*., 2003). Long *et al*. (2010) reported a relatively high BAP concentration was needed to increase shoot organogenesis in *P. villosum* var. densissimum and *P. armeniacum*. Wattanawikkit *et al*. (2011) reported that BAP appeared to elicit the best shoot multiplication in response with *P. callosum* shoot explants on ½ MS medium compared to TDZ, and that while TDZ resulted in similar shoot length compared to the control treatment (minus PGR), TDZ induced more shoot proliferation. Thwe *et al*. (2012) reported that 8.88 µM BAP was highly effective for shoot initiation, in terms of regeneration efficiency, shoot number, and shoot length of *Polygonum tinctorium*. However high concentrations of BAP resulted in a decrease in shoot induction of *Cymbidium devonianum* Paxt (Das *et al*., 2007). Hajong *et al*., (2013) recorded significant responses of *Dendrobium* explants cultured in MS medium with 5 or 10 µM BAP, or 5 µM TDZ, with the highest number of shoots/explant observed in MS plus 5 µM TDZ, while BAP was found to be more effective in boosting shoot elongation.

Chen *et al*. (2002) found that 0.45 µM TDZ in modified ½ MS medium enhanced the percentage of stem nodal explants of *Paphiopedilum philippenense* hybrids regenerating shoots. Thongpukdee *et al*. (2013) found that the most effective growth regulator for inducing multiple shoots of *Paphiopedilum* ‘Delrosi’ was TDZ at the low level (0.45 µM) in Hyponex medium (Kano, 1965; compositions: 3 g/L Hyponex, 8 g/L peptone, 2 g/L charcoal, 8 g/L agar and 30 g/L sucrose). However, Thongpukdee *et al*. (2013) also observed that the growth of *Paphiopedilum* shoots was very slow and numbers of new regenerated shoots were limited. It would appear that suitable media, types and concentrations of plant growth regulators used in tissue culture of *Paphiopedalum* is very varied, and with variable results depending on types of explants, species or whether plants are of hybrid origin.

The effect of cytokinins on root formation of *P. insigne* clearly showed that the presence of either BAP or TDZ decreased the induction of both root number and length (Table 11 and Figure 18). Although the 5 µM TDZ and 20 µM BAP treatments gave greater root length than the control, inhibition of root formation found in the treatment plus 10 µM BAP and treatments added with TDZ at level above over 10 µM. It has been also shown that rooting induction of micro shoots was most effective on PGR-free solidified medium (Skirvin and Chu, 1979). Chu *et al*. (1993) found that micro shoots of *Rosa chinensis* cultured in liquid medium with BAP were difficult to root and only shoots that were maintained on a medium without BAP developed roots. Wattanawikkit *et al*. (2011) reported that the root induction in *P. callosum* should be restorable if TDZ is removed and BAP reduced (as the presence of BAP does not appear to completely inhibit root induction, unlike TDZ). Retarding effects of TDZ on shoot growth and root formation of some orchid species i.e. *Acampe praemorsa* (Roxb.), *Cymbidium aloifolium* (L.), *Dendrobium aphyllum* (Roxb.) and *Dendrobium moschatum* (Buch.-Ham.), have been reported (Nayak *et al*., 1997a, 1997b; Erişen *et al*., 2011).

Based on results of this study, it can be suggested that explants of *P. insigne* were more responsive to PGR stimulation, with 10 and 40 µM BAP producing a better than two-fold shoot induction after a 90-day incubation period. Increasing the concentration of BAP beyond 20-40 µM would appear to have no particular advantage on shoot and root formation. Results with various TDZ treatments indicated that shoot and root induction were less responsive with this PGR in the medium, although TDZ is normally considered a highly phyto-active PGR. There appears to be some species specificity as regards optimal type and concentration of PGRs as regards *in vitro* propagation of *Paphiopedilum*, for example shoot regeneration of *P. bellatulum* and *P. philippinense* hybrids PH 60 was induced by 0.45-5 µM TDZ, while 6.8 µM TDZ was required for *P. delenatii* (Paneerat, 1996; Chen *et al*, 2002; Nhut *et al*, 2007). Eckardt (2003) suggested that cytokinin deficiency causes a major decline in shoot development, consequently leading to dwarfism, late flowering plants, enhanced root growth, and changes in reproductive development and that *in vitro* morphogenesis stimulated by phytohormones can be quite different between different genotypes of *Paphiopedilum*. Therefore, the culture requirements for specific selected plants need to be studied and identified.

This study confirmed that development of uniformly viable tissue culture protocols for *Paphiopedilum* is fraught with great difficulty and success is often limited (Stewart and Button, 1975; Huang, 1988; Arditti and Ernst, 1993). Pierik *et al*. (1988) also reported that *Paphiopedilum* seeds are very difficult to germinate *in vitro*. It has been noted that tissue culture clones derived from seeds or seedling tissues often suffer a serious drawback because their genotypes are highly variable and produce phenotypes that are unpredictable and lack uniformity (Liao *et al*., 2011). Because of the limited success in somatic tissue culture protocols, *Paphiopedilum* micropropagation remains almost entirely achieved by asymbiotic germination (Chen *et al.*, 2004).

This study has made significant progress in development of an *in vitro* protocol for regenerating *Paphiopedilum insigne* plants using explants from germinated seedlings that will facilitate *ex situ* conservation methods of endangered Thai slipper orchids with limited seed availability.

2. Cryoperservation of *in vitro*-propagated protocorms of *P. insigne*

In vitro propagated protocorms (derived from germinated seedlings) of *P. insigne* were grown on ½ MS plus 10 µM BAP at 25 ± 2 °C under continuous darkness and consequently regenerated to form shoots after two months of culture. *Paphiopedalum insigne* seed germination proceeded according to the developmental stages of embryos (Yamazaki and Kazumitsu, 2006) as shown in Figure 19. It shows that embryos firstly swell to fill the seed coat (pregermination stage), then emerge from the seed coat (germination stage) and are completely discharged from the seed coat to form a whitish protocorm (protocorm stage). Rhizoids are subsequently formed on the protocorm surface (rhizoid stage) and finally, shoots are differentiated from protocorms (shoot stage). *In vitro* propagated protocorms were then used for development of cryopreservation protocols. The use of germinated seeds and seedling-derived protocorms has been reported in *Paphiopedilum* orchids (Lin *et al*., 2000; Hong *et al*., 2008).

The effect of desiccation media level (0 - 1 M glycerol) on the potential survival of non-capsulated protocorms of *P. insigne* following cryopreservation was examined in the present study. It showed that micropropagated protocorms treated with glycerol lowered the potential survival following cryopreservation, although at 0.8 M glycerol was the optimal cryoprotectant concentration to give maximum potential survival was no significance to the control (Table 12). Matsumoto *et al*. (1995) considered that glycerol contributes to minimizing the injurious membrane changes resulting from severe dehydration in plant meristems. Similar to the previous study of *C. latifolia* protocorms, the maximum potential survival rate was found with the 0.8 M glycerol treatment. Koo and Aziz (2005) also reported that 0.5 M glycerol was the best cryoprotectant solution for protocoms of *Paphiopedilum rothschildianum* and *Phalaenopsis gigantean* with the greatest survival after cryopreservation. On the contrary, increased glycerol level had an adverse effect on potential survival of non-encapsulated protocorms of *C. latifolia* after cryostorage as previously described.

Successful cryopreservation relies on avoidance of lethal intracellular freezing which occurs during rapid freezing in liquid nitrogen or excess osmotic stresses during treatment with PVS2 (Sakai *et al*., 2008). The influence of PVS2 loading solutions on the viable protocorm following cryopreservation of non-encapsulated protocorms of *P. insigne* showed in Table 13. The result demonstrated that the potential survival of cryopreserved protocorm increased gradually with increasing time of exposure to PVS2 and reached a maximum (100%) for 30 min. exposure, with whitish color of protocorm. This is in agreement with the result of *Caladinia latifolia* protocorms, at least 25-30 min. exposure to PVS2 solution is sufficient for a high survival rate with non-encapsulated protocorms. It has been suggested that the exposure to PVS2 (30% glycerol, 15% ethylene glycol and DMSO in 0.4 M sucrose) prior to cryopreservation contributed to minimized any possible damage by chemical toxicity, osmotic stress and ice crystallization (Vendrame *et al*., 2007) and it does not permeate into the cytosol during dehydration (Sakai *et al*., 1990). Poobathy *et al*. (2013) also had similar result that a significant maximum cellular viability of *Dendrobium* PLBs was recorded in the 20 min. PVS2 exposure without causing harmful effect to the cells. Although, a gradual decline of cellular viability in PLBs occurred, with increasing exposure to PVS2 at 30 and 40 min., this could be due to a toxic effect of solution components or the whole solution. Recently, Antony *et al.* (2013) observed that cryopreserved PLBs of *Brassidium* shooting star orchid showed higher cellular viability with 20 min. in PVS2 solution prior to storage in liquid nitrogen. On the contrary, Tsukazaki *et al*. (2000) discovered that the use of PVS2 was detrimental to the survival of *Doritaenopsis* suspension culture. Sakai (1997) suggested that longer exposure of explants to highly concentrated vitrification solution is potentially injurious because of the phytotoxic effects of individual component or combined osmotic effect on cell viability. Loaded meristems and protocorm of some orchids tolerated quite well to the dehydration by 30-60 min. of PVS2 exposure time with little effect on plant recovery survival rates depending on species (Thinh and Takagi, 2000; Thinh *et al*., 2000). The result of PSV2 explosure revealed in present study can confirm some explanation that the optimal exposure time for PVS2 varies with plant species (Hong *et al*., 2009) and depends on cryopreservation technique.

Non-encapsulated protocorms of *P. insigne* that have been subjected to cryostorage rarely display the characteristic of white color that indicates viability with agreement to previous study in *Caladenia latifolia* R.Br. Whitish protocorm indicates a quicker regrowth and higher viability whereas appearance of other colors (yellow or brown, Figure 20) could be attributing to osmotic shock or unfavorable regrowth conditions as suggested by Moges *et al*. (2004). All the cryostored protocorms recovered in this study were initially light white when recovered from the liquid nitrogen and incubated in the dark at 25 °C. for 90 days, but underwent either bleaching or browning without dark incubation. Similar observation has been reported in orchids as well as other plant species (Yin and Hong, 2009; Sharaf *et al*., 2012;

Antony *et al.*, 2013), stating that this step is essential to reduce shock due to photo-oxidation of the cryopreserved plant tissues. In particular, dark incubation for a short time following post-thawing enhanced survival and this was presumably attributed to damage repair of tissues that might take place during darkness. The FDA staining technique was also used here to estimate the relative survival potential of *P. insigne* protocorms as described in *Caladinia latifolia*. The FDA staining technique indicated a high level of survival in post-LN treatments of most larger protocorms, showing little evidence of any areas of pronounced cell loss. However small, brown protocorms showed extensive absence of staining with FDA, indicating a high level of cellular death.

Glycerol, a critical component in cryoprotective solutions, has been reported to increase desiccation and freezing tolerance (Engelmann, 2000; Takagi, 2000; Flachsland *et al*., 2006) and was used to assist with cryopreservation of *Caladenia latifolia* R.Br. (this study). A similar approach was planned for enhancing post-cryogenic survival of *in vitro* propagated-protocorms of *Paphiopedilum insigne* via the encapsulation-vitrification technique. Preculture with cryoprotectants provides the opportunity to increase the desiccation and freeze-tolerance of the materials to be cryopreserved by contributing to improvement and stabilization of post-thaw tissue recovery and to development of protocols for materials, which are extremely sensitive to desiccation and cryogenic procedures (Bachiri *et al*., 2001). In present study, the potential survival of encapsulated protocorms via cryopreservation varied among desiccation media (0-1 M glycerol) concentration used (Table 14). The 0.8 M glycerol gave the highest potential survival percentage, followed by the 0.4 M treatment. The only treatment effect appeared to be a yellowing of protocorms in all glycerol concentrations relative to the 0.6 M glycerol. This result suggested that the induction of freezing tolerance by 0.8 M glycerol may be optimal and sufficient for *P. insigne* protocorms to survive following cryopreservation by encapsulation-vitrification. It has been considered that glycerol contributes to minimizing the injurious membrane changes resulting from severe dehydration in encapsulated lily meristems (Matsumoto *et al*., 1995). In contrast to previous results with *Caladenia latifolia* R.Br., increasing glycerol treatment tended to reduce potential survival of protocorms following cryostorage. In accordance with Koo and Aziz (2005), glycerol at 0.5 M was the best loading/cryoprotectant solution for protocoms of *Paphiopedilum rothschildianum* and *Phalaenopsis gigantean* with the highest viable protocorms after cryopreservation.

It has been reported that encapsulation-dehydration and encapsulation-vitrification are comparatively more appropriate methods for orchid cryopreservation with higher success rates (Yin and Hong, 2009; Subramaniam *et al*., 2011). However, the number of studies reported on cryopreservation of protocorms and PLBs of *Paphiopedilum* using encapsulation-dehydration and encapsulation-vitrification method is limited. The control of water content of plant samples before liquid nitrogen freezing is the key factor in developing successful cryoprotection protocols (Zhang *et al*., 2001). Bian *et al*. (2002) suggested that when cell beads are insufficiently dehydrated, intracellular ice is more likely to form resulting in cryoinjury during cold storage in liquid nitrogen and subsequent thawing, while over-dehydrating causes damaging osmotic stress. In this study, protocorms encapsulated in beads and dehydrated for 90 min. under a laminar airflow, achieved maximum potential survival following cryopreservation, while a longer (120 min.) dehydration treatment caused protocorms to change from white to yellow color indicating stress (Table 15). In other reported studies decreasing the water content of the beads containing *Dendrobium* PLBs, increased survival rates after cryopreservation but survival decreased markedly if beads were over-dehydrated (Xue *et al*., 2008; Maruyama *et al*., 1998). In cryopreservation of *Dendrobium* species by encapsulation/dehydration, the survival ratio and regrowth of encapsulated shoot tips decreased with decreasing bead water content and increasing dehydration time (Lurswijidjarus and Thammasiri, 2004). Maneerattanarungroj *et al*. (2007) observed that most of encapsulated protocorms that contained high water content were dead after freezing in liquid nitrogen, in contrast, beads with the lowest water contents after dehydration showed the highest levels of viability in *Cleisostoma areitinum* protocorm after cryopreservation. Jitsopakul *et al*. (2008) also reported that the regrowth rate of cryopreserved protocorms of *Vanda coerulea* depended on the water content of the precultured beads during dehydration. During dehydration, the water content changed, the longer the duration of the dehydration, the lower the water content. Thus, the duration of dehydration was an important factor for protocorms forming plantlets after cryopreservation. In addition, a dehydration step was necessary to avoid the formation of intracellular ice crystals even though increasing dehydration time led to damage of encapsulated protocorms. Therefore, in order to achieve the highest survival of a particular cultivar of a given orchid species, the optimum water content using dehydration technique of encapsulated explants should be carefully determined before any application of cryostorage.

There are few reports on the cryopreservation of *Paphiopedilum* protocorms using encapsulation-dehydration and encapsulation-vitrification method. This study reports on development of a protocol for cryopreservation of *P. insigne* using *in vitro* propagated protocorms as explants. The success of encapsulation-dehydration, a process where explants are encapsulated in alginate beads, treated with a concentrated desiccation media (e.g. 0.8 M glycerol), dehydrated by air-drying in an aseptic laminar airflow for various times and then plunged into liquid nitrogen, has been applied to a number of plant species including orchids (Maneerattanarungroj *et al*., 2007; Jitsopakul *et al*., 2008; Khoddamzadeh *et al*., 2011; Pouzi *et al*., 2011). Compared to other vitrification methods, the manipulation of encapsulated explants by this method is easy, and non-toxic cryoprotectants are applied to protect explants during the dehydration treatment. Encapsulation-dehydration appears to be a comparatively more successful method for orchid cryopreservation (including *Paphiopedilum* species) generally with high success rates (Yin and Hong, 2009; Subramaniam *et al*., 2011). The study on *P. insigne* potential survival of protocorms was demonstrated in both non-encapsulation-vitrification and encapsulation-dehydration methods following cryostorage. This report also opens up the possibility of recovering plants from seeds and protocorms from other *Paphiopedilum* species after cryostorage using the efficient, simple protocol for encapsulation/dehydration technique, and may be applicable to other species in the Orchidaceae. This procedure can be also used to replace the previous procedures that require cold-hardening or slow-freezing of the stock plants and, thus, avoid the use of programmable freezers or expensive growth chambers, enabling accurate low-temperature treatments.

CONCLUSION AND RECOMMENDATIONS

Conclusion

This study has derived a novel *in vitro* method for asymbiotic clonal propagation of *Caladenia latifolia* R.Br. (an endemic terrestrial orchid species from Western Australia) for horticulture and breeding from seedling explants using a basal MS medium. The highest induction of protocorm rate and size were achieved in MS medium supplemented with 10 µM 2,4-D for a 90 day incubation period. The most effective medium for protocorm proliferation of *C. latifolia* was ½ MS plus 10 µM BAP. The best shoot induction and the maximum protocorm size were obatined on PGR-free Thomale GD medium.

Cryopreservation is an important tool for the *ex situ* preservation of endangered plants. In this study the development of a cryopreservation protocol for orchid protocorms of *C. latifolia* is described. Protocorms generated asymbiotically each month on ½ MS medium containing 10 mM BAP provided explant sources for cryopreservation. The result showed that protocorm survival after cryopreservation was correlated significantly with explant size, with larger sized protocorms (L, $\geq$ 4 mm.) having .the highest potential viability. Changing glycerol concentrations (0-1 M), cryoprotectant solution (PVS2 or PVS4) and time of exposure to cryoprotectants (15, 20, 25 and 30 min. of incubation at 0 °C), did not significantly effect the potential survival percentage of protocorms (as measured by FDA staining). Potential survival of cryopreserved protocorm incubated on various recovery media (½ MS, ½ MS + BAP, kinetin and TDZ) under dark conditions at 25 °C for 90 days was also not significantly different (88-100%). The study indicates that the cryopreservation of terrestrial orchid protocorms is technically feasible and provides a new and potentially highly beneficial tool in terrestrial orchid conservation where seed may be limited (because of species rarity), or as a means of storing and later utilizing the large surpluses of protocorms generated in propagation programmes.

This study also revealed the effective development of *in vitro* propagation techniques obtained from *Caladenia latifolia* R.Br. result to assist with the conservation of endangered Thai slipper orchids, *Paphiopedalum insigne* (Wall. ex Lindl.) Pfitzer. Seedling explants induced the greatest multiple shoots when cultured on ½ MS medium plus 10 µM BAP for 3-month period in darkness at 25 ± 2°C. Nevertheless, PGRs had no significant effect on the shoot length in comparison to the control treatment without PGR. The addition of either BAP or TDZ decreased significantly the induction of both root number and length. Although the ½ MS plus 5 µM TDZ and 20 µM BAP gave the maximum root length. The result clealy revealed that both BAP and TDZ inhibited root formation at above 10 µM. It is hoped that these results will assist with further in vitro research for the future development of *ex situ* conservation methods with endangered Thai slipper orchids.

This report of the successful and efficient protocol for cryopreservation of propagated protocorms of *P. insigne* by encapsulation-dehydration in combination with a loading solution. The highest potential survival was achieved when either non-encapsulated or encapsulated protocorms were cultured on ½ MS medium plus 0.8 M glycerol in the darkness prior to cryopreservation. The air-drying time for 90 min. was the best dehydration for maximum survival rate of encapsulated protocorm following cryopreservation. The result also demonstrated that the potential survival of cryopreserved protocorm without encapsulation increased gradually with increasing time of exposure to PVS2 and reached a maximum (100%) for 30 minute exposure. The result of PSV2 explosure revealed in present study can confirm some explanation that the optimal exposure time for PVS2 varies with plant species and depends on cryopreservation technique. This report also revealed the possibility of recovering plants from seeds and protocorms from other *Paphiopedilum* species following cryopreservation using the efficient, simple protocol for encapsulation/dehydration technique, and may be widely applicable to other species in the Orchidaceae.

Recommendations

This study details *in vitro* propagation and cryopreservation of orchid somatic tissues (protocorms) for long-term storage of the terrestrial orchid species *Caladenia latifolia* R.Br. (Pink Fairy Orchid, an endemic species from the south-west of Western Australia) and *Paphiopedilum insigne* (Wall. ex Lindl.) Pfitzer. (a Thai slipper orchid). Cryopreservation is particularly useful when seed of rare species for example, may be in short supply and may not be amenable to storage, but renewable surplus protocorms generated via *in vitro* propagation are readily available. This study demonstrates the usefulness of such *in vitro* propagated protocorms as a renewable and continuous explant source for developing *in vitro* and cryogenic research programs for orchid species without having to rely on large numbers of valuable seed stocks. While future research will seek to verify the estimations of post-LN viability, this study reveals the possibility of cryopreserving a broader range of orchid species (including rare taxa) to determine the applicability of cryopreservation as a general tool in orchid conservation.

LITERATURE CITED

Anjum, S., M. Zia and M. F. Chaudhary. 2006. Investigations of different strategies for high frequency regeneration of *Dendrobium malones* ‘Victory’. **Afr. J. Biotechnol**. 5(19): 1738-1743.

Anthony, J. J. J., C. L. Keng, X. Rathinam, U. R Sinniah and S. Subramaniam. 2010. Preliminary study on cryopreservation of *Dendrobium* Bobby Messina protocorm-like bodies by vitrification. **Afr. J. Biotechnol**. 9 (42): 7063-7070.

Antony, J. J. J., H. Burkhan, U. R. Sinniah, R. Poobathy and S. Subramaniam. 2013. Effect of PVS2 vitrification on Brassidium shooting star orchid using protocorm-like bodies (PLBs). **Aust. J. Crop Sci**. 7 (8): 1078-1084.

Arditti, J. 1992. **Fundamentals of Orchid Biology.** Wiley-Interscience, New York.

Arditti, J. 2008. **Micropropagation of Orchids.** 2nd Edition. Blackwell Publishing Ltd., Oxford.

Arditti J. and R. Ernst. 1993. **Micropropagation of Orchids**. John Wiley & Sons. New York.

Bachiri, Y., G. Q. Song, P. Plessis, A. Shoar-Ghaffari, T. Rekab and C. Morisset. 2001. Routine cryopreservation of kiwifruit (*Actinidia spp*.) germplasm by encapsulationdehydration: importance of plant growth regulators. **Cryoletters** 22: 61-74.

Bajaj, Y. P. S. 1992. **Biotechnology in Agriculture and Forestry: High-tech and Micropropagation IV**. Springer-Verlag Berlin Heidelberg, Germany.

Batty, A. L., K. W. Dixon, M. C. Brundrett and K. Sivasithamparam. 2001. Long term storage of mycorrhizal fungi and seed as a tool for the conservation of endangered Western Australian terrestrial orchids. **Aust. J. Bot.** 49: 619-628.

Batty, A. L., K. W. Dixon, M. C. Brundrett and K. Sivasithamparam. 2002. Orchid conservation and mycorrhizal associations, pp. 205-235. *In*: K. Srivasithamparam, K.W. Dixon, and R.L. Barret, eds. **Microorganisms in Plant Conservation and Biodiversity.** Kluwer Academic Publishers, Netherlands.

Batty, A. L., M. C. Brundrett, K. W. Dixon and K. Sivasithamparam. 2006a. New methods to improve symbiotic propagation of temperate terrestrial orchid seedlings from axenic culture to soil. **Aust. J. Bot.** 54: 367-374.

Batty, A. L., M. C. Brundrett, K. W. Dixon and K. Sivasithamparam. 2006b. *In situ* symbiotic seed germination and propagation of terrestrial orchid seedlings for establishment at field sites. **Aust. J. Bot.** 54: 375-381.

Bektaş, E., M. Cüce and A. Sökmen. 2013. *In vitro* germination, protocorm formation, and plantlet development of *Orchis coriophora* (Orchidaceae), a naturally growing orchid species in Turkey. **Turk. J. Bot.** 37: 336-342.

Bian, H. W., J. H. Wang, W. Q. Lin, N. Han and M. Y. Zhu. 2002. Accumulation of soluble sugars, heat-stable proteins and dehydrins in cryopreservation of protocorm-like bodies of *Dendrobium candidum* by the air-drying method. **J. Plant. Physiol**. 159: 1139-1145.

Charoensub, R., S. Phansiri, A. Sakai and W. Yongmanitchai. 2000. Cryopreservation of *in vitro*-grown shoot-tips of cassava cooled to –196°C by vitrification, pp. 410-412. *In* F. Engelmann and H. Takagi, eds. **Cryopreservation of Tropical Plant Germplasm.** International Plant Genetics Research Institute , Rome, Italy.

Chen, Y. 2000. Protoplasm of *Dendrobium candidum* cryopreserved by vitrification. **J. Wenzhou Teachers College** 21: 40-41.

Chen, J. T. and W. C. Chang. 2004. Induction of repetitive embryogenesis from seed-derived protocorms of *Phalaenopsis amabilis* var. Formosa Shimadzu. ***In Vitro* Cell. Dev. Biol. Plant** 40 (3): 290-293.

Chen, S. C. and Z. H. Tsi. 2003. **The Orchids of China.** 2nd Ed. Chinese Forest Press, Beijing.

Chen, T. Y., J. T. Chen and W. C. Chang. 2002 . Multiple shoot formation and plant regeneration from stem nodal explants of *Paphiopedilum* orchids. ***In Vitro* Cell. Dev. Biol. Plant** 38: 595-597.

Chen, T. Y., J. T. Chen and W. C. Chang. 2004. Plant regeneration through direct shoot bud formation from leaf cultures of *Paphiopedilum* orchids. **Plant Cell Tissue Organ Cult.** 76: 11-15.

Chen, Y. C., C. Chang and W. C. Chang. 2000. A reliable protocol for plant regeneration from callus culture of *Phalaenopsis*. ***In Vitro* Cell. Dev. Biol. Plant** 36: 420-423.

Chen, Y., J. H. Wan and C.N. Huan. 2001. Germplasm cryopreservation of *Dendrobium candidum* by vitrification. **J. Zhejiang University (Agriculture & Life Science)** 27: 436-438.

Cho, M. S. and H. L. Valmayor. 1987. Embryo culture of *Paphiopedlum philippinense* (tropical orchid). **Korean J. Plant Cell Tiss.** 15: 15-26.

Cho, M. S. and H. L. Valmayor. 1988. **Embryo culture development Anatomy, Cytology and Isozyme Analysis of Some *Paphiopedlum* Species**. Ph.D. Thesis, University of the Philippines at Los Banos, Laguna.

Chu, C. Y., S. L. Knight and M. A. L. Smith. 1993. Effect of liquid culture on the growth and development of miniature rose (*Rosa chinensis*). **Plant Cell Tissue Organ Cult.** 32: 329-334.

Chugh, S., S Guha and I. U. Rao. 2009. Micropropagation of orchids: A review on the potential of different explants. **Sci. Hort**. 122: 507–520.

Collins, T. and K. W. Dixon. 1992. Micropropagation of an Australian Terrestrial orchid *Diuris longifolia* R.Br. **Aust. J. Exp. Agri.** 32: 731-735.

Cribb, P. 1987. **The Genus *Paphiopedilum***. Royal Botanic Gardens Kew, London.

Cribb, P. 1998. **The Genus *Paphiopedilum***. Natural History Publications, Borneo.

Cribb, J., Y. B. Luo, N. McGough and L. P. Siu. 1999. The distribution, ecology and conservation status of *Paphiopedilum micranthum* (Orchidaceae) in Southwest China. **Orchid Review** 1227: 149-152.

Cross, R. 1997. *Ex situ* conservation of *Caladenia*-photoautotrophic micropropagation of *Caladenia*. **Final Report to the Australian Flora Foundation.** Royal Botanic Gardens Melbourne. Australia.

Das, M. C., S. Kumaria and P. Tandon. 2007. Protocorm regeneration, multiple shoot induction and *ex vitro* establishment of *Cymbidium devonianum* Paxt. **Asian J. Plant Sci.** 6: 349-353.

Datta, B. K., B. Kanjilal and D. D. Sarker. 1999. Artificial seed technology: development of a protocol in *Geodorum densiflorum* (Lam) Schltr: an endangered orchid. **Current Science.** 74: 1142–1145.

Das, M. C., S. Kumaria and P. Tandon. 2011. Storage and high conversion frequency of encapsulated protocorm-like bodies of *Cymbidium devonianum* (orchid). **J. Hort. Sci. and Biotech**. 86: 611-615.

David, L. J. 1994. **Orchids-Australia.** Martin Ford Publisher, Sydney.

De Pauw, M. A., W. R. Remphrey and C. E. Palmer. 1995. The cytokinin preference for *in vitro* germination and protocorm growth of *Cypripedium candidum*. **Ann. Bot.** 75: 267-275.

De Pauw, M. and W. R. Remphrey. 1993. *In vitro* germination of three *Cypripedium* species in relation to time of seed collection, media, and cold treatment. **Canadian J. Bot.** 71: 879–885.

Ding, C. C., H. Wu and F. Y. Liu. 2004. Factors affecting the germination of *Paphiopedilum armeniacum*. **Acta Botanica Yunnanica** 26 (6): 673-677.

Dixon, K. W. 1991. Seeder/clonal concepts in Western Australian orchids, pp. 111-123. *In* T.C.E. Wells and J.H. Willems, eds. **Population Ecology of Terrestrial Orchids**. SPB Academic Publishing, The Hague, The Netherlands.

Dixon, K. W. 1994. Towards integrated conservation of Australian endangered plants-the Western Australian model. **Biodiversity & Conservation** 3 (2): 148-159.

Dohling, S., S. Kumaria and P. Tandon. 2012. Multiple shoot induction from axillary bud cultures of the medicinal orchid, *Dendrobium longicornu*. **AoB Plants** 1-22.

Dowling, N. and M. Jusaitis. 2012. Asymbiotic *in vitro* germination and seed quality assessment of Australian terrestrial orchids. **Aust. J. Bot.** 60: 592-601.

Dressler, R. L. 1981. **The Orchids: Natural History and Classification**. Harvard University Press, London.

Dressler, R. L. 1993. **Phylogeny and Classification of the Orchid Family**. Dioscorides Press, Portland, Oregons.

Duncan, D. B. 1955. Multiple range and multiple F test. **Biometrics** 11: 1-42.

Eckardt, N. A. 2003. A new classic of cytokinin research: cytokinin-deficient Arabidopsis plants provide new insight into cytokinin biology. **Plant Cell** 15: 2489-2492.

Engelmann, F. 1997. *In vitro* conservation methods, pp.119-161. *In* C. A. Callow, B. V. Ford-Lloyd and H. J. Newbury, eds. **Biotechnology and Plant Genetic**. Oxford.

Engelmann, F. 2000. Importance of cryopreservation for the conservation of plant genetic resources, pp. 8-20. *In* F. Engelmann and H. Takagi, eds. **Cryopreservation of Tropical Plant Germplasm.** Rome, Italy.

Engelmann, F. 2004. Plant cryopreservation: Progress and prospects. ***In Vitro* Cell. Dev. Biol. Plant** 40: 427-437.

Erişen, S., E. Atalay and M. Yorgancılar. 2011. The effect of thidiazuron on the *in vitro* shoot development of endemic *Astragalus cariensis* in Turkey. **Turk. J. Bot**. 35: 521–526.

Escobar, R. H., W. M. Roca and C. Guevara. 1995. Cryopreservation of cassava shoot tips in liquid nitrogen. **Biotech. Research Unit Ann. Rep.** 82-84.

Escobar, R. H., D. Debouck and W. M. Roca. 2000. Development of cassava cryopreservation, pp. 222-226. *In* F. Engelmann and H. Takagi, eds. **Cryopreservation of Tropical Plant Germplasm.** Rome, Italy.

Ferreira, W. D. M., G. B. Kerbauy and A. P. P. Costa. 2006. Micropropagation and Genetic Stability of *Dendrobium* Hybrid (Orchidaceae). ***In Vitro* Cell. Dev. Biol. Plant** 42: 568-571.

Flachsland, E., G. Terada, A. Scocchi, H. Rey, L. Mroginski and F. Engelmann. 2006. Cryopreservation of seeds and *in vitro*-cultured protocorms of *Oncidium bifolium* sims. (Orchidaceae) by encapsulation-dehydration. **Cryo Letters** 27 (4): 235-242.

Flamee, M. 1978. Influence of selected media and supplement on the germination and growth of *Paphipedilum* seedling. **Amer. Orchid Soc. Bull.** 47: 419-423.

Fornnesbech, M. 1972. Growth hormones and propagation of *Cymbidium in vitro*. **Physiol. Plant** 27: 310-315.

Gale, S. W., J. Yamazaki, M. J. Hutchings, T. Yukawa and K. Miyoshi. 2010. Constraints on establishment in an endangered terrestrial orchid: a comparative study of *in vitro* and *in situ* seed germinability and seedling development in *Nervilia nipponica*. **Bot. J. Linean. Soc.** 163: 166-180.

George, E. F., M. A. Hall and G. Jan De Klerk. 2008. **Plant Propagation by Tissue Culture**. 3rd Edition. Springer, Netherlands.

Gonzalez-Arnao, M. T., A. Panta, W. M. Roca, R. H. Escobar and F. Engelmann, 2008. Development and large scale application of cryopreservation techniques for shoot and somatic embryo cultures of tropical crops. **Plant Cell Tissue Organ Cult.** 92 (1): 1-13.

Grout, B. 1995. **Genetic Preservation of Plant Cells *In Vitro*.** Springer-Verlag Berlin Heidelberg, Germany.

Haas-von Schmude, N. F., E. Lucke, R. Ernst and J. Arditti. 1986. *Paphiopedilum rothschidianum*. **Amer. Orchid Soc. Bull.** 55: 579-584.

Hajong, S., S. Kumaria and P. Tandon. 2013. Effect of plant growth regulators on regeneration potential of axenic nodal segments of *Dendrobium chrysanthum* Wall. ex Lindl. **J. Agr. Sci. Tech.** 15: 1425-1435.

Harding, K. 2004. Genetic integrity of cryopreserved plant cells: a review. **Cryo Letters** 25: 3-22.

Harding, K. N., J. W. Johnston and E. E. Benson. 2009. Exploring the physiological basis of cryopreservation success and failure in clonally propagated in vitro crop plant germplasm. **Agri. Food Sci.** 18: 103-116.

Hay, F. R., D. J. Merritt, J. A. Soanes and K. W. Dixon. 2010. Comparative longevity of Australian orchid (Orchidaceae) seeds under experimental and low temperature storage conditions. **Bot. J. Linean. Soc.** 164: 26-41.

Hirai, D. and A. Sakai. 1999. Cryopreservation of *in vitro*-grown axillary shoot-tip meristems of mint (*Mentha spicata* L.) by encapsulation vitrification. **Plant Cell Reports** 19: 150-155.

Hong, P. I., J. T. Chen and W. C. Chang. 2008. Plant regeneration via protocorm-like body formation and shoot multiplication from seed-derived callus of a maudiae type slipper orchid. **Acta Physiologiae Plantarum** 30: 755-759.

Hong, S. R., M. H. Yin, X. H. Shao, A. P. Wang and W. H. Xu. 2009. Cryopreservation of embryogenic callus of *Dioscorea bulbifera* by vitrification. **Cryo Letters** 30: 64-75.

Hopper, S. D. and A. P. Brown. 2004. Robert Brown's *Caladenia* revisited, including a revision of its sister genera *Cyanicula*, *Ericksonella* and *Pheladenia* (Caladeniinae: Orchidaceae). **Aust. Syst. Bot.** 17 (2): 171 - 240.

Horgan, R. 1987. Cytokinin genes, pp. 119-130. *In* G.V. Hoad, J.R. Lenton, M.B. Jackson and R.K. Atkin, eds. **Hormone Action in Plant Development–A Critical Appraisal.** Butterworths, London, Boston.

Hossain, M. M. 2008. Asymbiotic seed germination and *in vitro* seedling development of *Epidendrum ibaguense* Kunth. (Orchidaceae). **Afr. J. Biotechnol.** 7 (20): 3614-3619.

Huang, L. C. 1988. A procedure for asexual multiplication of *Paphiopedilum in vitro.* **Amer. Orchid Soc. Bull.** 57: 274-278.

Huang, L. C., C. J. Lin, C. I. Kuo, B. L. Huang and T. Murashige. 2001. *Paphiopedilum* cloning *in vitro*. **Sci. Hort**. 91: 111-121.

Huetteman, C. A. and J. E. Preece. 1993. Thidiazuron - a potent cytokinin for woody plant tissue culture. **Plant Cell Tissue Organ Cult.** 33: 105-119.

Ishikawa, K., K. Harata, M. Mii, A. Sakai, K. Yoshimatsu and K. Shimomura. 1997. Cryopreservation of zygotic embryos of a Japanese terrestrial orchid (*Bletilla striata*) by vitrification. **Plant Cell Reports** 16: 754-757.

Islam, M. O., A. R. M. M. Rahman, S. Matsui and A. K. M. A. Prodhan. 2003. Effects of complex organic extracts on callus growth and PLB regeneration through embryogenesis in the *Doritaenopsis* orchid**. Jpn Agric Res Q**. 37: 229-235.

Jitsopakul, N., K. Thammasiri and K. Ishikawa. 2008. Cryopreservation of *Vanda coerulea* protocorms by encapsulation-dehydration. **Cryo Letteres** 29 (3): 253-260.

Kaczmarczyk, A., S. R. Turner, E. Bunn, R. Mancera and K.W. Dixon. 2011. Cryopreservation of threatened native Australian species – what have we learned and where to from here?. ***In Vitro*** **Cell. Dev. Biol. - Plant** 47: 17-25.

Kaczmarczyk, A., B. Funnekotter, A. Menon, P. Ye Phang, A. Al-Hanbali, E. Bunn and R. L. Mancera. 2012. **Current Issues in Plant Cryopreservation, Current Frontiers in Cryobiology**.Available Source: http://www.intechopen.com/books/currentfrontiers-in-cryobiology/current-issues-in-plant-cryopreservation, March 09, 2012

Kalimuthu, K., R. Senthilkumar and S. Vijayakumar. 2007. *In vitro* micropropagation of orchid, *Oncidium* sp (Dancing Dolls). **Afr. J. Biotechnol**. 6: 1171-1174.

Kano, K. 1965. Studies on the media for orchid seed germination. **Mem. Fac. Agri. Kagawa Univ**. 20: 1-68.

Khoddamzadeh, A. A., U. R. Sinniah, P. Lynch, M. A. Kadir, S. B. Kadzimin and M. Mahmood. 2011. Cryopreservation of protocorm-like bodies (PLBs) of *Phalaenopsis bellina* (Rchb.f.) Christenson by encapsulation-dehydration. **Plant Cell Tissue Organ Cult.** 107: 471-481.

Koo, G. C. and Z. A. Aziz. 2005. Development of cryopreservation systems for *Paphiopedilum rothschildianum* and *Phalaenopsis gigantean*, pp. 38-42. *In* D.A. Anton, V. Kumar and M. Wong, eds. **Biotechnology Technical Report.** University Malaysia Sabah, Malaysia.

Kornthong, S. 1991. **Effects of Banana, Tomato and Jew's Ear in Transflasking Media of *Paphiopedilum villosum* Pfitz**. Bachelor’s Degree Special Problem, Kasetsart University.

Košir, P., S. Škof and Z. Luthar. 2004. Direct shoot regenerated from nodes of *Phalaenopsis* orchids. **Acta Agri. Slovenica** 83: 233-242.

Kusumoto, M. 1978. Effect of combinations of growth regulating substances and organic matter on the proliferation and organogenesis of *Cymbidium* protocorms culture *in vitro*. **J. Jpn. Soc. Hort. Sci.** 47: 391-400.

Latha, P. G. 1999. Micropropagation and *in vitro* flower induction in *Habenaria crinifera* Lindl. **J. Orchid Soc. India** 13: 47-53.

Lauzer, D., M. St-Arnaud and D. Barabé. 1994. Tetrazolium staining and *in vitro* germination of mature seeds of *Cypripedium acaule* (Orchidaceae). **Lindleyana** 9: 197-204.

Lee, M. and R. L. Phillips. 1988. The chromosomal basis of somaclonal variation. **Annu. Rev. Plant Physiol. Plant Mol. Biol.** 39: 413-437.

Lee, Y. I. 1998. **The Study of Embryo Development and Seed Germination *In Vitro* in Slipper Orchids**. Master Thesis. National Taiwan University, Taiwan.

Lee, Y. I. 2007. The symbiotic seed germination of six *Paphiopedilum* species in relation to the time of seed collection and seed pretreatment. **Acta Horticulturae** 755: 381-385.

Lee, Y. I., E. C. Yeung, N. Lee and M. C. Chung. 2006. Embryo development in the lady's slipper orchid, *Paphiopedilum delenatii*, with emphasis on the ultrastructure of the suspensor. **Ann. Bot.** 98: 1311-1319.

Lekawatana, S. 2010. **Thai Orchid: Current situation**. *In* Taiwan International Orchid Symposium. 5 March 2010, International Conference Hall, Taiwan Orchid Plantation, Tainan, Taiwan. Available Source: http://www.orchid.mju.ac.th/orchid/uploadfiles/docs/23894.pdf, March 5, 2010

Li, A., Y. B. Luo and S. Ge. 2002. A preliminary study on conservation genetics of an endangerd orchid (*Paphiopedilum micranthum*) from Southwest China. **Biochemical Genetics** 40: 195-201.

Liao, Y. J., Y. C. Tsai, Y. W. Sun, R. S., Lin and F. S. Wu..2011. *In vitro* shoot induction and plant regeneration from flower buds in *Paphiopedilum* orchids. ***In Vitro* Cell. Dev. Biol. Plant** 47: 702-709.

Liau, C. H., S. J. You, V. Prasad, H. H. Hsiao, J. C. Lu, N. S. Yang and M. T. Chan. 2003. *Agrobacterium tumefacien*-mediated transformation of an *Oncidium orchid*. **Plant Cell Reports** 21: 993-998.

Lin, Y. H., C. Chang and W. C. Chang. 2000. Plant regeneration from callus culture of a *Paphiopedilum* hybrid. **Plant Cell Tissue. Org. Cult.** 62: 21-25.

Liu, Z., K. Liu, L. Chen, S. Lei, L. Li, X. Shi and L. Huang. 2006. Conservation ecology of endangered species *Paphiopedilum armeniacum* (Orchidaceae). **Acta Ecologica Sinica**. 26(9): 2791-2800.

Liu, Z. J., S. C. Chen, L. J. Chen and S. P. Lei. 2009a. **The Genus' *Paphiopedilum*' in China.** Science Press, Beijing, China.

Liu, Z. J., X. Q. Chen and P. J. Cribb. 2009b. *In* Z.Y. Wu, P.H. Raven, and D.Y. Hong (eds), ***Paphiopedilum*, Flora of China**. **Vol. 25 (Orchidaceae)**, pp. 33-34. Beijing, China: Science Press, and St. Louis: Missouri Botanical Garden Press.

Long, B., A. X. Niemiera and Z. Cheng. 2010. *In vitro* propagation of four threatened *Paphiopedilum* species (Orchidaceae). **Plant Cell Tissue Organ Cult.** 101: 151-162.

Luo, J. P., Y. Wang, X. Q. Zha and L. Huang. 2008. Micropropagation of *Dendrobium densiflorum* Lindl. ex Wall. through protocorm-like bodies: effects of plant growth regulators and lanthanoids. **Plant Cell Tissue Organ Cult.** 93: 333-340.

Lurswijidjarus, W. and K. Thammasiri, 2004. Cryopreservation of shoot tips of *Dendrobium walter* Oumae by encapsulation/dehydration. **ScienceAsia** 30: 293-299.

Maneerattanarungroj, P., S. Bunnag and M. Monthatong. 2007. *In vitro* conservation of *Cleisostoma areitinum* (Rchb.f.) Garay, rare Thai orchid species by an encapsulation-dehydration method. **Asian J. Plant Sci.** 6 (8): 1235-1240.

Mark, F. 1987. A Preliminary introduction to and cultivation of the Chinese slipper orchid, genus *Paphiopedilum*. **Orchid Digest** 51 (2): 63-69.

Martin, K.P. and J. Madassery. 2006. Rapid *in-vitro* propagation of *Dendrobium* hybrids through direct shoot formation from foliar explants, and protocorm-like bodies. **Sci. Hort.** 108: 95-99.

Maruyama, E., K. Ishii and I. Kinoshita. 1998. Alginate encapsulation technique and cryogenic procedure for long term storage of the tropical forest tree *Guazuma crinite* Mart. *In Vitro* cultures. **Jpn Agric Res Q**. 32: 301-309.

Maruyama, E., T. Tanaka, Y. Hosoi, K. Ishii and N. Morohoshi. 2000. Embryogenic cell culture, protoplast regeneration, cryopreservation, biolistic gene transfer and plant regeneration in Japanese cedar (*Cryptomeria japonica* D. Don). **Plant Biotechnol.** 17: 281-296.

Matsumoto, T., A. Sakai and K. Yamada. 1995. Cryopreservation in vitro-grown apical meristems of lily by vitrification. **Plant Cell Tissue Organ Cult.** 41: 237-241.

McKendrick, S. L., J. R. Leake, D. L.Taylor and D. J. Read. 2000. Symbiotic germination and development of myco-heterotrophic plants in nature: transfer of carbon from ectomycorrhizal *Salix repens* and *Betula pendula* to the orchid *Corallorhiza trifida* through shared hyphal connections. **New Phytologist** 145: 539-548.

Medina, R. D., E. A. Flachsland, A. M., Gonzalez, G. Terada, M. M., Faloci and L. A. Mroginski. 2009. *In vitro* tuberization and plant regeneration from multimodal segment culture of *Habenaria bractescens* Lindl., an Argentinean wetland orchid. **Plant Cell Tissue Organ Cult.** 97: 91-101.

Mei, T. A., M. Danial, M. Mahmood and S. Subramaniam. 2012. Exquisite protocol of callus induction and protocorm-like bodies (PLBs) regeneration of *Dendrobium sonia*-28. **Aust. J. Crop Sci**. 6: 793-800.

Moges, A. D., Shibli, R. A. and N. S. Karam. 2004. Cryopreservation of African Violet (*Saintpaulia ionantha* Wendl.) shoot-tips. ***In Vitro*** **Cell. Dev. Biol. Plant** 40: 389-395.

Mohanty, P., M. C. Das, S. Kumaria and P. Tandon. 2012. High-efficiency cryopreservation of the medicinal orchid *Dendrobium nobile* Lindl. **Plant Cell Tissue Organ Cult.** 109: 297-305.

Morel, G. M. 1974. Clonal multiplication of orchids, pp. 169-222. *In* C. L.Withner, ed. **The Orchids; Scientific Studies**. Wiley-Interscience Publication, New York.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. **Phisiologia Plantarum** 15: 495-497.

Na, H. Y. and K. Kondo. 1996. Cryopreservation of tissue cultured shoot primordia from shoot apices of cultured protocorms in *Vanda pumila* following ABA preculture and desiccation. **Plant Sci.** 118: 195-201.

Nagaraju, P., S. P. Das, P. C. Bhutia and R. C. Upadhyaya, 2003. Effect of media and BAP on protocorms of *Cymbidium* and *Cattleya*. **J. Orchid Soc. India** 17: 67-71.

Nagashima, T. 1982. Studies in the seed germination and embryogenesis in goeringii Rchb. f. and *Paphiopedilum insigne* var. *sanderae* Rchb. **J. Japan. Soc. Hort. Sci.** 51: 94-105.

Nanakorn, W. 2002. Status of Thai native orchids, p. 28. *In* **Program and Extended Abstract of The First International Orchid Conservation Congress**, September 2002, Perth, Australia.

Nanakorn, W. and S. Indharamusika. 1999. *Ex-situ* conservation of native Thai orchids at Queen Sirikit Botanic Garden, pp. 23-27. *In* **Proceeding of The International Conference on Biodiversity and Bioresources: Conservation and Utilization**, 23-27 November 1997, Phuket, Thailand.

Nayak, N. R., S. Patnaik and S. P. Rath. 1997a. Direct shoot regeneration from foliar explants of an epiphytic orchid, *Acampe praemorsa* (Roxb.) Blatter & McCain. **Plant Cell Reports** 16: 583-587.

Nayak, N. R., S. Patnaik and S. P. Rath. 1997b. *In vitro* propagation of three epiphytic orchids, *Cymbidium aloifolium* (L.) Sw., *Dendrobium aphyllum* (Roxb.) Fisch. and *Dendrobium moschatum* (Buch.-Ham.) Sw. through thidiazuron-induced high frequency shoot proliferation. **Sci. Hort.** 71: 243-250.

Ng, C.Y. and N. M. Saleh. 2011. *In vitro* propagation of *Paphiopedilum* orchid through formation of protocorm-like bodies. **Plant Cell Tissue Organ Cult.** 105: 193-202.

Ng, C. Y., N. M. Saleh and F. Q. Zaman. 2010. *In vitro* multiplication of the rare and endangered slipper orchid, *Paphiopedilum rothschildianum* (Orchidaceae). **Afr. J. Biotechnol.** 9 (14): 2062-2068.

Nhut, D. T., D. T. T. Thuy, N. T. Don, V. Q. Luan, N. T. Hai, K. T. T. Van and C. C. Chimmappa. 2007. *In vitro* stem elongation of *Paphiopedilum delenatii* Guillaumin and shoot elongation via stem node culture. **Prop. Ornamen. Plants** 7 (1): 29-36.

Nhut, D. T., N. T. Thuy, N. H. Don, Vu, N. Q. Thien, D. T. T. Thuy, N. Duy and J. A. Teixeira da Silva. 2006. Advanced technology in micropropagation of some important plants, pp. 325-335. *In* J.A. Teixeira da Silva, ed. **Floriculture, Ornamental and Plant Biotechnology Volume II.** Global Science Books, UK.

Nigel, S. D. and K. W. Dixon. 2009. Terrestrial orchid conservation in the age of extinction. **Ann Botany** 104 (3): 543-556.

Nikishina, T. V., E. V. Popova, M. G. Vakhrameeva, T. I. Varlygina, G. L. Kolomeitseva, A. V. Burov, E. A. Popovich, A. I. Shirokov, V. Y. Shumilov and A. S. Popov. 2007. Cryopreservation of seeds and protocorms of rare temperate orchids. **Russ. J. Plant Physiol.** 54: 121-127.

Niknejad, A., M. A. Kadir and S. B. Kadzimin. 2011. *In vitro* plant regeneration from protocorms-like bodies (PLBs) and callus of *Phalaenopsis gigantean* (Epidendroideae: Orchidaceae). **Afr. J. Biotechnol.** 10 (56): 11808-11816.

Pakaew, S. 1995. **Study of Pod Ages and Media for Green Pod Culture of *Paphiopedilum concolor* Pfitz**. Master Thesis, Kasetsart University, Thailand.

Paneerat, T. 1996. **Asexual Micropropagation of *Paphiopedilum***. Master Thesis, Kasetsart University, Thailand.

Park, S. Y., H. N. Murthy and K. Y. Paek. 2000. Mass propagation of protocorm-like bodies using bioreactor system and subsequence plant regeneration in *Phalaenopsis*. **Plant Cell Tissue Organ Cult.** 63: 67-72.

Park, S. Y., H. N. Murthy and K. Y. Paek. 2003. Protocorm-like body induction and subsequent plant regeneration from root tip cultures of *Doritaenopsis*. **Plant Sci.** 164: 919-923.

Pedersen, H. A., H. Kurzweil, S. Suddee and P. J. Cribb. 2011. **Flora of Thailand, Orchidaceae 1 (Cypripedioideae, Orchidoideae, Vanilloideae)** 12: 277-279.

Persson, J., P. Gardestrom and T. Nasholm. 2006. Uptake, metabolism and distribution of organic and inorganic nitrogen sources by *Pinus sylvestris*. **J. Exp. Bot.** 57: 2651-2659.

Pierik, R. L. M., P. A. Sprenkels, B. Vanderharst and Q. G. Vandermeys. 1988. Seed germination and further development of plantlets of *Paphiopedilum ciliolare* Pfitz in vitro. **Scientia Horticulturae** 34: 139-153.

Polthampitak, T. 1997. **Axenic Clonal Propagation of *Paphiopedilum***. Master Thes, Kasetsart University, Thailand.

Poobathy, R., N. Izwa, A. L. Julkifle and S. Subramaniam. 2013. Cryopreservation of *Dendrobium* sonia-28 using an alternative method of PVS2 droplet freezing. **Emir. J. Food Agric.** 25 (7): 531-538.

Popova, E., N. Bukhov, A. Popov and H. H. Kim. 2010. Cryopreservation of protocorm-like bodies of the hybrid orchid Bratonia (*Miltonia flavescens* x *Brassia longissima*). **Cryo Letters** 31: 426-437.

Pornchuti, W. and K. Thammasiri. 2008. Cryopreservation of protocorms of *Dendrobium virgineum* Rchb.f. **Acta Horticulturae** 788: 63-68.

Pouzi, N. Z., X. Rathinam, A. J. J. James, R. Poobathy and S. Subramaniam. 2011. Early investigation on cryopreservation of *Dendrobium sonia*-28 using encapsulation-dehydration with modified Evan blue assay. **Afr. J. Biotechnol.** 10: 3534-3539.

Preece, J. E. and E. G. Sutter. 1991. Acclimatisation of micropropagated plants to the greenhouse and field, pp. 71-93. *In* P.C. Deburgh and R.H. Zimmerman, eds. **Micropropagation-Technology and Application**. Kluwer Academic Publishers, London.

Quain, M. D., P. Berjak, E. Acheampong and J. I. Kioko. 2009. Sucrose treatment and explant water content: critical factors to consider in development of successful cryopreservation protocols for shoot tip explants of the tropical species *Dioscorea rotundata* (yam). **Cryo Letters** 30: 212-223.

Quay, L., J. A. McComb and K. W. Dixon. 1995. Methods for *ex situ* germination of Australian terrestrial orchids. **Hort. Sci.** 30 (7): 1445-1446.

Reed, B. M. 1993. Responses to ABA and cold acclimation are genotype dependent for cryopreserved blackberry and raspberry meristems. **Cryobiology** 30: 179-184.

Reed, B. M. 2000. Genotype considerations in temperate fruit crop cryopreservation, pp. 200-204. *In* F. Engelmann and H. Takagi, eds. **Cryopreservation of Tropical Plant Germplasm.** Rome, Italy.

Reed, B. M. 2008. **Plant Cryopreservation: A Practical Guide**. Springer New York, New York.

Reinhoud, P. J., F. V. Iren and J. W. Kijne. 2000. Cryopreservation of undifferentiated plant cells, pp 91-102. *In* F. Engelmann and H. Takagi, eds. **Cryopreservation of Tropical Plant Germplasm.** Rome, Italy.

Roy, J., S. Naha, M. Majumdar and N. Banerjee. 2007. Direct and callus-mediated protocorm-like body induction from shoot-tips of *Dendrobium chrysotoxum* Lindl. **Plant Cell Tissue Organ Cult.** 90: 31-39.

Safrinah, R., R. Xavier, S. Uma and S. Sreeramanan. 2009. Optimisation of cryopreservation technique in *Mokara* golden nugget orchid using PVS2 vitrification. **Inter. J. Agri. Res**. 1-10.

Sakai, A. 1997. Potentially valuable cryogenic procedures for cryopreservation of cultured plant meristems, pp. 53-66. *In* M. K. Razdan and E. C. Cocking, eds. **Conservation of Plant Genetic Resources *In Vitro* General Aspects**. Science Publishers Inc., Enfield, USA.

Sakai, A. 2000. Development of cryopreservation techniques, pp. 1-7 *In* F. Engelmann and H. Takagi (eds), **Cryopreservation of Tropical Plant Germplasm**. Rome, Italy.

Sakai, A., S. Kobayashi and I. Oiyama. 1990. Cryopreservation of nucellar cells of naval orange (*Citrus sinesis* Osb. var. brasiliensis Tanaka) by vitrification. **Plant Cell Rep.** 9: 30-33.

Sakai, A., D. Hirai and T. Niino. 2008. Development of PVS-based vitrification and encapsulation-vitrification porotocols, pp 33-57. *In* B.M. Reed, ed. **Plant Cryopreservation: A Practical Guide.** Springer New York, New York.

Santos Diaz, M. S. and C. C. Álvare. 2009. Plant regeneration through direct shoot formation from leaf cultures and from protocorm-like bodies derived from callus of *Encyclia mariae* (Orchidaceae), a threatened Mexican orchid. ***In Vitro*** **Cell. Dev. Biol. Plant** 45: 162-170.

Savina, G. I. 1974. Fertilization in orchids, pp. 197-204. *In* H. F. Linskens, ed. **Fertilization in Higher Plants**. American Elsevier Co., New York.

Sharaf, S. A., R. A. Shibli, M. A. Kasrawi and S. H. Baghdadi. 2012. Cryopreservation of wild Shih (*Artemisia herba-alba* Asso.) shoot tips by encapsulation-dehydration and encapsulation-vitrification. **Plant Cell Tissue Organ Cult.** 108 (3): 437-444.

Sharma, S. K. and P. Tandon. 1986. Influence of growth regulators on asymbiotic germination and early development of *Coelogyne punctulata* Lindl, pp. 441-451. *In* **Biology, Conservation and Culture of Orchids.** New Delhi, India.

Sheehan, T. J. 1983. Recent advances in botany, propagation, and physiology of orchids, pp. 279-315. *In* J. Janick, ed. **Horticultural Reviews, Volume 5**. AVI Publishing Company Westport Connecticut, USA.

Sheehan, T. J. and M. Sheehan. 1994. **An Illustrated Survey of Orchid Genera**. Cambridge University Press, UK.

Shibli, R. A., M. A. Shatnawi, W. Subaih and M. M. Ajlouni. 2006. *In vitro* conservation and cryopreservation of plant genetic resources: a review. **World J. Agri. Sci**. 2: 372-382.

Shimonishi, K., M. Ishikawa, S. Suzuki and K. Oosawa. 2000. Cryopreservation of melon somatic embryos by desiccation method, pp. 167-171. *In* F. Engelmann and H. Takagi, eds. **Cryopreservation of Tropical Plant Germplasm.** Rome, Italy.

Singh, F. 1981. Differential staining of orchid seeds for viability testing. **American Orchid Society Bulletin** 50: 416-418.

Singh, F. 1988. Storage of orchid seed in organic solvents. **Gartenbauwissenschaft** 53: 122-124.

Skirvin, R. M. and M. C. Chu. 1979. *In vitro* propagation of “Forever Yours” rose. **Hort. Sci.** 14 (5): 608-610.

Sopalun, K., K. Thammasiri and K. Ishikawa. 2010. Vitrification-based cryopreservation of *Grammatophyllum speciosum* protocorms. **Cryo Letters** 31: 347-357.

Stewart, J. and J. Button. 1975. Tissue culture studies in *Paphiopedilum*. **Amer. Orchid Soc. Bull.** 44: 591-599.

Stimart, D. P. and P. D. Ascher. 1981. *In vitro* germination of *Paphiopedilum* seed on a completely defined medium. **Scientia Horticulturae** 14: 165-170.

Subramaniam, S., X. Rathinam, R. Poobathy and U. Sinniah. 2008. *In vitro* production of multiple bud clumps (Mbcs) from *Cavendish* banana cultivar, Brasilian (AAA). **Am.-Eurasian J. Sustain. Agric.** 2 (3): 300-307.

Subramaniam, S., U. R. Sinniah, A. A. Khoddamzadeh, S. Periasamy and J. J. James. 2011. Fundamental concept of cryopreservation using *Dendrobium sonia*-17 protocorm-like bodies by encapsulation-dehydration technique. **Afr. J. Biotechnol.** 10: 3902-3907.

Suzuki, M., P. Tandon, M. Ishikawa and T. Toyomasu. 2008. Development of a new vitrification solution, VSL, and its application to the cryopreservation of gentian axillary buds. **Plant Biotechnol. Rep.** 2: 123-131.

Swarts, N. D. and K. W. Dixon. 2009a. Terrestrial orchid conservation in the age of extinction. **Ann Botany** 104 (3): 543-556.

Swarts, N. D. and K. W. Dixon. 2009b. Perspectives on orchid conservation in botanic gardens. **Trends Plant Sci.** 14: 590-598.

Takagi, H. 2000. Recent developments in cryopreservation of shoot apices of tropical species, pp. 178-193. *In* F. Engelmann and H. Takagi, eds. **Cryopreservation of Tropical Plant Germplasm.** Rome, Italy.

Tay, L. J., K. Takeno and Y. Hori. 1988. Culture conditions suitable for *in vitro* seed germination and development of seedling in *Paphiopedilum*. **J. Jpn. Soc. Hortic. Sci.** 57: 243-249.

Thammasiri, K. 2000. Cryopreservation of seeds of a Thai orchid (*Doritis pulcherrima* Lindl.) by vitrification. **Cryo Letters** 21: 237-244.

Thammasiri, K. 2008. Cryopreservation of some Thai orchid species. **acta horticulturae** 788: 53-62

Thinh, N. T. and H. Takagi. 2000. Cryopreservation of in vitro-grown apical meristems of terrestrial orchids (*Cymbidium spp*.) by vitrification, pp. 453-455. *In* F. Engelmann and H. Takagi (eds), **Cryopreservation of Tropical Plant Germplasm.** Rome, Italy.

Thinh, N. T., H. Takagi and A. Sakai. 2000. Cryopreservation of *in vitro*-grown apical meristems of some vegetatively propagated tropical monocots by vitrification, pp. 227-232. *In* F. Engelmann and H. Takagi, eds. **Cryopreservation of Tropical Plant Germplasm.** Rome, Italy.

Thomale, H., 1954. **Die Orchideen: Einfuehrung in die Kultur und Vermehrung tropischer und einheimischer**. Eugen Ulmer, Stutgart, Germany.

Thongpukdee, A., E. Nisayan and C. Thepsithar. 2013. Multiple shoot formation of *Paphiopedilum* 'Delrosi'. **World Academy of Science, Engineering and Technology** 78: 6-21.

Thwe, A. A., Y. B. Kim, S. U. Kim and S. U. Park. 2012. *In vitro* shoot regeneration from stem internodes of *Polygonum tinctorium*. **Life Sci.** 9 (4): 2059-2062.

Tochareon, P. 1996. **Asexual Micropropagation of *Paphiopedilum***. Master Thesis, Kasetsart University, Thailand.

Tomita, M. and M. Tomita. 1997. Plant regeneration from immature seed-derived callus of *Cypripedium macranthos* Swartz var. taiwanianum (Masamune) F. Maekawa. **Breed. Sci.** 47: 279-281.

Touchell, D. H. 2000. Conservation of threatened flora by cryopreservation of shoot apices, pp. 269-272. *In* F. Engelmann and H. Takagi, eds. **Cryopreservation of Tropical Plant Germplasm.** Rome, Italy.

Touchell, D. H., S. R. Turner, E. Bunn and K. W. Dixon. 2002. Cryostorage of somatic tissues of endangered Australian species. pp. 357-372. *In* L.E. Towill and Y.P.S. Bajaj, eds. **Biotechnology in agriculture and forestry 50 Cryopreservation of Plant Germplasm II.** Springer-Verlag, Berlin.

Tsi, Z., Y. Luo, P.J. Cribb, N. McGough, G. Siu and L. Chau. 1999. A preliminary report on the population size, ecology, and conservation status of some *Paphiopedilum* species (Orchidaceae) in Southwest China. **Lindleyana** 14 (1): 12-23.

Tsukazaki, H., M. Mii, K. M. Tokuhara and K. Ishikawa. 2000. Cryopreservation of *Doritaenopsis* suspension culture by vitrification. **Plant Cell Rep.** 19 (12): 1160-1164.

Turner, S. R., T. Senaratna, E. Bunn, B. Tan, K. W. Dixon and D. H. Touchell. 2001a. Cryopreservation of shoot tips from six endangered Australian species using a modified vitrification protocol. **Ann. Botany** 87: 371-378.

Turner, S. R., D. H. Touchell, T. Senaratna, E. Bunn, B.Tan and K. W. Dixon. 2001b. Effects of plant growth regulators on survival and recovery growth following cryopreservation. **Cryo Letters** 22: 163-174.

Udomdee, W., P. J. Wen, S. W.Chin and F. C. Chen. 2012. Shoot multiplication of *Paphiopedilum* orchid through *in vitro* cutting methods. **Afr. J. Biotechnol.** 11 (76): 14077-14082.

Van Waes, J. M. and P. C. Debergh. 1986. *In vitro* germination of some Western European orchids. **Physiologia Plantarum** 67: 253-261.

Varghese, D., P. Berjak and N. W. Pammenter. 2009. Cryopreservation of shoot tips of *Trichilia emetica*, a tropical recalcitrant-seeded species. **Cryo Letters** 30: 280-290.

Vendrame, W. A. and R. T. Faria. 2011. Phloroglucinol enhances recovery and survival of cryopreserved *Dendrobium nobile* protocorms. **Scientia Horticulturae** 128: 131-135.

Vendrame, W. A., V. S. Carvalho and J. M. M. Dias. 2007. *In vitro* germination and seedling development of cryopreserved *Dendobium* hybrid mature seeds. **Scientia Horticulturae** 114: 188-193.

Von Arnold, S., D. Clapham, U. Egertsdotter and L. H. Mo. 1996. Somatic embryogenesis in conifers - A case study of induction and development of somatic embryos in *Picea abies*. **Plant Growth Regulation** 20: 3-9.

Wang, J. H., J. G. Ge, F. Liu, H. W. Bian and C. N. Huang. 1998. Cryopreservation of seeds and protocorms of *Dendrobium candidum*. **CryoLetters** 19: 123-128.

Wannakrairoj, S. 2007. Status of ornamental plants in Thailand. **Acta Horticulturae** 788: 29-36.

Wattanawikkit, P., E. Bunn, K. Chayanarit and S. Tantiwiwat. 2011. Effect of cytokinins (BAP and TDZ) and auxin (2,4-D) on growth and development of *Paphiopedilum callosum*. **Kasetsart Journal (Natural Science)** 45: 12-19.

Whigham, D. F., J. P. O. Neill, H. N. Rasmussen, B. A. Caldwell and M. K. McCormick. 2006. Seed longevity in terrestrial orchids-potential for persistent *in situ* seed banks. **Biological Conservation** 129: 24-30.

Xiong, L. and L. F. Wu. 2003. Ornamental tissue culture and large scale production. **Chemical Industry Press.** Beijing, China.

Xue, S. H., X. J. Luo, Z. H. Wu, H. L. Zhang and X. Y. Wang, 2008. Cold storage and cryopreservation of hairy root cultures of medicinal plant *Eruca sativa* Mill. *Astragalus membranaceus* and *Gentiana macrophylla* Pall. **Plant Cell Tiss. Org. Cult.** 92: 251-260.

Yamada, T., A. Sakai, T. Matsumura and S. Higucho. 1991. Cryopreservation of apical meristems of white clover (*Trifolium repens* L.) by vitrification. **Plant Sci.** 78: 81–87.

Yamazaki, J. and M. Kazumitsu. 2006. *In vitro* asymbiotic germination of immature seed and formation of protocorm by *Cephalanthera falcata* (Orchidaceae). **Ann. Botany** 98: 1197-1206.

Yin, M. and S. Hong. 2009. Cryopreservation of *Dendrobium candidum* Wall. ex Lindl. protocorm-like bodies by encapsulation-vitrification. **Plant Cell Tissue Organ Cult.** 98: 179-185.

Zainuddin, M., A. L. Julkifle, R. Pobathy, U. R. Sinniah, A. Khoddamzadeh, J. J. J. A. Pavallekoodi and S. Subramaniam. 2011. Preliminary analysis of cryopreservation of *Dendrobium* Bobby Messina orchid using an encapsulation-dehydration technique with Evans blue assay. **Afr. J. Biotechnol.** 10 (56): 11870-11878.

Zeng, S. J., Chen, Z. L. and J. Duan. 2006. Asepsis sowing and *in vitro* propagation of *Paphiopedilum hirsutissimum* Pfitz. **Plant Physiology Communications** 42 (2): 247.

Zhang, Y. X., J. H. Wang, H. Bian and M.Y. Zhu. 2001. Pregrowth-desiccation: a simple and efficient procedure for the cryopreservation of rice (*Oryza sativa* L.) embryogenic suspension cells. **Cryo Letters** 22: 221-228.

Zinger, N. V. and V. A. Poddubnaya-Arnoldi. 1966. Application of histochemical techniques to the study of embryonic processes in certain orchids. **Phytomorphology** 16: 111-124.

APPENDICES

Appendix A

Statistical analysis

Appendix Table A1 Statistical analysis of protocorm induction rate of *C. latifolia* obtained from explants cultured on MS medium supplemented with 2,4-D for 90 days.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Prob.
Treatment	3	5937.500	1979.167	7.244	0.0010 *
Error	28	7650.000	273.214		
Total	31	13587.500			

Coefficient of Variation = 27.26 %

* = significantly different (P < 0.05)

Appendix Table A2 Statistical analysis of mean protocorm clump size of *C. latifolia* cultured on ½ MS medium with TDZ compared to BAP addition after 30 days.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Prob.
Treatment	3	0.884	0.295	5.066	0.0063 *
Error	28	1.628	0.058		
Total	31	2.512			

Coefficient of Variation = 32.53 %

* = significantly different (P < 0.05)

Appendix Table A3 Statistical analysis of shoot induction of *C. latifolia* on Thomale GD medium supplemented with TDZ (0, 5, 10, 20 and 40 µM) after 90 days.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Prob.
Treatment	4	7453.552	1863.388	7.281	0.0009 *
Error	20	5118.640	255.932		
Total	24	12572.193			

Coefficient of Variation = 117.58 %

* = significantly different ($P < 0.05$)

Appendix Table A4 Statistical analysis of shoot number of *C. latifolia* on Thomale GD medium supplemented with TDZ (0, 5, 10, 20 and 40 µM) after 90 days.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Prob.
Treatment	4	167.672	41.918	5.480	0.0038 *
Error	20	152.993	7.650		
Total	24	320.665			

Coefficient of Variation = 104.45 %

* = significantly different ($P < 0.05$)

Appendix Table A5 Statistical analysis of protocorms clumb diameter of *C. latifolia* on Thomale GD medium supplemented with TDZ (0, 5, 10, 20 and 40 µM) after 90 days.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Prob.
Treatment	4	0.309	0.077	3.168	0.0361 *
Error	20	0.488	0.024		
Total	24	0.797			

Coefficient of Variation = 18.95 %

* = significantly different ($P < 0.05$)

Appendix Table A6 Statistical analysis of effect of size on potential survival of *C. latifoli*a protocorms following cryostorage.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Prob.
Treatment	2	21280.000	10640.00	138.783	0.0000 *
Error	12	290.000	76.667		
Total	12	22200.000			

Coefficient of Variation = 14.59 %

* = significantly different ($P < 0.05$)

Appendix Table A7 Statistical analysis of differring desiccation medium level on potential survival of *C. latifolia* protocorms following cryostorage.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Prob.
Treatment	4	984.000	246.000	1.414	0.2656 ns
Error	20	3480.000	174.000		
Total	24	4464.00			

Coefficient of Variation = 16.24 %

ns = not significantly different (P > 0.05)

Appendix Table A8 Statistical analysis of different cryoprotectant solution and the exposure time on the percentage of protocorm survival of *C. latifolia*.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Prob.
Treatment	7	2110.000	301.429	1.507	0.2003 ns
Error	32	6400.000	200.000		
Total	39	8510.000			

Coefficient of Variation = 16.94 %

ns = not significantly different (P > 0.05)

Appendix Table A9 Statistical analysis of different recovery media on potential survival of *C. latifolia* protocorms following cryostorage.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Prob.
Treatment	3	495.000	165.000	1.886	0.1727 ns
Error	16	1400.000	87.500		
Total	19	1895.000			

Coefficient of Variation = 9.79 %

ns = not significantly different (P > 0.05)

Appendix Table A10 Statistical analysis of shoot number of *P. insigne* shoots cultured on ½ MS medium plus differing concentrations of BAP and TDZ for three months.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Prob.
Treatment	10	48.318	4.832	2.462	0.0130 *
Error	77	151.125	1.963		
Total	87	199.443			

Coefficient of Variation = 57.88 %

* = significantly different (P < 0.05)

Appendix Table A11 Statistical analysis of shoot length of *P. insigne* shoots cultured on ½ MS medium plus differing concentrations of BAP and TDZ for three months.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Prob.
Treatment	10	4.782	0.478	1.905	0.0571 ns
Error	77	19.335	0.251		
Total	87	24.117			

Coefficient of Variation = 30.51 %

ns = not significantly different (P > 0.05)

Appendix B

Materials used in the present study

Materials

Laboratory equipments

1. Equipment for media preparation

Autoclave, bench, gas outlet, hot plate and magnetic stirrer, pH meter, refrigerator, freezer, water purification and storage system, storage facilities for glassware and chemicals, balance, beakers, cylinder, pipette, erlenmyer flask, and other equipments for tissue culture technique

2. Aseptic transfer area

Laminar air-flow carbinet, forcep, scalpel and diposable blades, petri dish

3. Equipment of culture incubation

Incubator, light source (cool white fluorescent, 36 w), timer, shelves, shaker

4. Equipment for cryopreservation

Pasteur pipettes, liquid nitrogen vessel, cryotubes (Nunc®, Roskilde, Denmark),

5. Stereo microscope
6. Plant materials

6.1 Mature undehisced seed capsules (‘green pods’) of *Caladenia latifolia* R.Br. obtained from hand-pollinated, outcrossed plants (sourced originally from local wild plants with an appropriate permit) growing at Kings Park and Botanic Garden, Perth, WA, Australia.

6.2 Mature seed capsules of *Paphiopedilum insigne* (Wall. ex Lindl.) Pfitzer obtained from a commercial orchid company (Paphanatics, unlimited, USA).

Chemical

1. Chemicals for MS media (Murashige and Skoog, 1962) preparation, mineral salts, myo-inositol, nicotinic acid, glycine, pyridoxine HCL, thiamine HCL, peptone, NaH_2PO_4, phytagel, sucrose, vitamins, gelling compounds (agar and gelrite), and plant growth regulators, i.e. N6-benzyladenine phosphate (BAP), 2,4 –

dichlorophenoxyacetic acid (2,4-D), 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (TDZ), 6-furfurylaminopurine (kinetin)

2. Chemicals for cryopreservation and encapsulation, i.e. sodium alginate, calcium chloride, liquid nitrogen, dimethylsulfoxide, polyethylene glycol (PEG), glycerol

3. Cryoprotect solution (Sakai *et al*., 1990) preparation, i.e. PVS2 (30% glycerol + 15% etylene glycol + 15% DMSO + 0,4M sucrose), PVS4 (35% glycerol + 20% etylcne glycol + sucrose O.6M)

Chemical and media preparation

1. Desiccation media (1.0 M Glycerol solution)

- Glycerol 1 M	92.09 g/L
- ½ MS Media	1 L
- Agar	8 g/L
- pH 6.0	

2. PSV2 solution (For 50 mL.)

- Glycerol (15% w/v)	14.3 mL
- Ethylene Glycol	7.4 mL
- DMSO	7.4 mL.
- 0.4M sucrose ½ MS stock solution	20.9 mL

3. PSV4 solution (For 50 ml)

- Glycerol (15% w/v)	14.3 mL
- Ethylene Glycol	7.4 mL
- DMSO	3.7 mL
- 1,2 Propandiol (Propylene Glycol)	3.7 mL
- 0.4M sucrose ½ MS stock solution	20.9 mL

4. Cryo-washing solution

- 1.0 M Sucrose	342.30 g/L
- ½ MS (no sugar addition)	1 L
- Adjust pH 6.00	

5. Murashige and Skoog media (1962)

Constituents	Concentration (mg/L)	Constituents	Concentration (mg/L)
NH_4NO_3	1,650	$CuSO_4.5H_2O$	0.025
KNO_3	1,900	Na_2-EDTA	37.3
H_3BO_3	6.2	$FeSO_4.7H_2O$	27.8
KH_2PO_4	170	Glycine	2.0
KI	0.83	Nicotinic acid	0.5
$Na_2MoO_4.2H_2O$	0.25	Pyridoxine-HCl	0.5
$CoCl_2.6H_2O$	0.025	Thiamine-HCl	0.1
$CaCl_2.2H_2O$	332.2	Myo-inositol	100
$MgSO_4.7H_2O$	370	Sucrose	20
$MnSO_4.4H_2O$	22.3	Phytagel	2.5
$ZnSO_4.7H_2O$	8.6		

6. Thomale GD media (Thomale, 1954)

Constituents	Concentration (mg/L)
KNO_3	400
KH_2PO_4	300
$(NH_4)_2SO_4$	60
NH_4NO_3	370
$Mg(NO_3)_2.6H_2O$	110
$FeSO_4.7H_2O$	20
Sucrose	20
Activated charcoal	2
Coconut water	150
Phytagel	2.5

Appendix C

Published papers obtained from the present study

Published papers obtained from the present study

Wattanawikkit, P., E. Bunn, K. Chayanarit and S. Tantiwiwat. 2011. Effect of cytokinins (BAP and TDZ) and auxin (2,4-D) on growth and development of *Paphiopedilum callosum*. **Kasetsart Journal (Natural Science)** 45: 12-19. (SJR = 0.031; SNIP = 0.100)

Wattanawikkit, P., Tantiwiwat, S., Bunn, E., Kingsley, W.D. and K. Chayanarit. 2012. Cryopreservation of in vitro-propagated protocorms of *Caladenia* for terrestrial orchid conservation in Western Australia. **Botanical Journal of the Linnean Society** 170: 277-282. (Impact factor: 2.589)

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