Asymbiotic *in vitro* germination and seed quality assessment of Australian terrestrial orchids

Nicole Dowling[A,B] *and Manfred Jusaitis*[A,B,C]

[A]South Australian Seed Conservation Centre, Botanic Gardens of Adelaide, Department of Environment and Natural Resources, North Terrace, Adelaide, SA 5000, Australia.
[B]School of Earth and Environmental Sciences, University of Adelaide, SA 5005, Australia.
[C] Corresponding author. Email: jusaitis.manfred@sa.gov.au

Australian Journal of Botany **60:** 592-601 (2012).

Abstract. Determining the seed quality and germination requirements for threatened orchid species in storage is vital for future conservation efforts. Seeds of many Australian terrestrial orchid species are held in conservation collections around the country, but few have been germinated *in vitro*, fuelling concerns over their long-term viability. This study tested three methods of assessing orchid seed quality; asymbiotic germination was compared with vital staining using triphenyltetrazolium chloride (TTC) or fluorescein diacetate (FDA). Six culture media were examined for efficacy in promoting asymbiotic seed germination of four Australian terrestrial orchid species (*Pterostylis nutans*, *Microtis arenaria*, *Thelymitra pauciflora* and *Prasophyllum pruinosum*). Germination occurred on all media but germination rates were consistently highest on BM1 and development was most advanced on BM1, P723 and Malmgren media. Subsequent trials tested the efficacy of BM1 for asymbiotic germination of additional genera (*Caladenia*, *Calochilus* and *Diuris*), several congeneric species, and two species collected from several different provenances within each of their ranges. The results indicate that asymbiotic germination on BM1 medium is an effective technique for testing the performance of Australian terrestrial orchid seeds. The efficacy of vital stains to determine seed viability, however, remains uncertain, as significant disagreement between degree of staining and germinability was observed for some species.

Introduction

Australia's temperate terrestrial orchids represent one of our most threatened plant groups with many taxa restricted to bushland remnants, road sides and unprotected bushland (Batty *et al.* 2001a; Swarts and Dixon 2009a). *In situ* protection and management of severely denuded or isolated populations is not always adequate to ensure species survival (Seaton *et al.* 2010) and must be integrated with *ex situ* conservation programs such as plant propagation and seed banking (Swarts and Dixon 2009a; Volis and Blecher 2010). The long-term storage of orchid seed is an important *ex situ* strategy currently employed by many Botanic Gardens around the world as they aim to conserve the natural genetic diversity of their wild species (Crane *et al*, 2009; Swarts and Dixon 2009a, b; Hosomi *et al.* 2012). To be of conservation value, seed banks need to systematically assess the quality of their seed, so routine germination and viability testing protocols must be established for each species in a collection (Offord *et al*. 2004; Cochrane *et al.* 2007; Seaton *et al.* 2010). This process has proven difficult for temperate terrestrial orchids because of their tiny, relatively undifferentiated seeds with impermeable coats, complicated dormancy patterns, and intricate biotic and abiotic dependencies (Lauzer *et al.* 2007; Thompson *et al.* 2007).

The most common methods for assessing orchid seed quality *in vitro* include germination testing (symbiotic or asymbiotic) and/or the use of vital stains such as triphenyltetrazolium chloride (TTC) or fluorescein diacetate (FDA) (Wood *et al.* 2003; Faast *et al.* 2011). Historically, Australian temperate terrestrial orchids have usually been tested by symbiotic germination with a compatible mycorrhizal fungus (Ramsay *et* al. 1986; Batty *et al.* 2001a; Brundrett 2007). The advantages of this method are that it produces rapid germination and growth (Rasmussen *et al.* 1991; Markovina and McGee 2000), seedlings are often superior to those grown asymbiotically (Clements 1981) and it allows co-introduction of the appropriate mycorrhizal fungus if the orchid is to be grown-on or reintroduced into the wild (Clements 1981; Wright *et al*. 2009). Disadvantages include the extreme mycorrhizal-specificity of some orchids (Clements 1981; Brundrett 2007), the requirement for time-consuming fungal isolation, culture and maintenance, and the need for specialist mycological skills and facilities (Wright *et al*. 2009).

Asymbiotic germination, on the other hand, does not require fungal isolation and culture, so it constitutes a faster test routinely applicable across a range of species. Seeds are cultured on nutrient rich media containing minerals, carbohydrates (typically sucrose) and organic compounds. It can yield relatively high germination rates and produce seedlings with a long *in vitro* flask life (Wright *et al*. 2009). Unfortunately, this method has proven difficult for many Australian terrestrial species (Clements 1982; Oddie *et al.* 1994). Studies have cited an uncompromising reliance on mycotrophy (Rasmussen 1995; Batty *et al.* 2001b), physiological or morphological seed dormancy mechanisms (Lauzer *et al.* 2007; Kauth *et al*. 2008, 2011), or more stringent requirements for nutrients (Oliva and Arditti 1984) as causes of poor or non-existent asymbiotic germination. Recently asymbiotic germination protocols have been developed for a range of terrestrial orchid species native to North America, Europe and Asia (Stewart and Kane 2006; Yamazaki and Miyoshi 2006; Kauth *et al.* 2008; Mahendran and Bai 2009; Godo *et al.* 2010; Pierce and Cerabolini 2011). However, little information exists on asymbiotic seed germination of Australian terrestrial species (Oddie *et al.* 1994; Hay *et al.* 2010), so there remains a need for reliable protocols to be developed to support *ex situ* orchid conservation strategies.

Results of vital staining are often correlated or used in parallel with germination tests to determine seed quality. The topographical tetrazolium test (Lakon 1949; Hosomi *et al.* 2011) and the FDA test (Pritchard 1985; Wood *et al.* 2003) have been used to determine orchid seed viability. TTC has been used successfully on tropical epiphytic orchid seed (Singh 1981), and adapted for several European (Van Waes and Debergh 1986a) and American terrestrial and epiphytic species (Lauzer *et al*, 1994; Shoushtari *et al.* 1994), although results have been inconsistent between species due largely to variation in the permeability of seed coats (Van Waes and Debergh 1986a, b). An alternative viability stain, FDA, freely enters living cells where it becomes hydrolysed by esterase enzymes to produce fluorescein. Fluorescing cells signify the presence of active enzymes and an intact plasma membrane and therefore a viable seed. However FDA staining has also had variable success with orchid seed (Pritchard 1985; Batty *et al.* 2001a; Wood *et al.* 2003; Vendrame *et al.* 2007). To be effective, these stains need to make direct contact with seed embryo cells, so removal or rupture of the water-repellent testa by mechanical or chemical means may assist (Pritchard 1985; Miyoshi and Mii 1988; Rasmussen 1995).

The aim of this study was to develop a reliable asymbiotic method of assessing germinability of Australian terrestrial orchid seed and to compare outcomes with viability assessment using two vital stains. Experiments were conducted to (i) evaluate germination of four genera of Australian terrestrial orchids (*Pterostylis*, *Microtis*, *Thelymitra* and *Prasophyllum*) on six asymbiotic culture media, (ii) compare vital staining using TTC and FDA with asymbiotic seed germination as an assessment of seed viability, and (iii) test the

optimal asymbiotic germination medium with several congeners, additional genera (*Caladenia, Calochilus* and *Diuris*), and conspecifics collected from different provenances to assess its universality.

Materials and methods

Study taxa

Four species from four genera were chosen to be the focus of this study. They were *Pterostylis nutans* R.Br., *Microtis arenaria* Lindl., *Thelymitra pauciflora* R.Br., and *Prasophyllum pruinosum* R.S.Rogers, collected from Scott Creek Conservation Park, Callington Hill, Aberfoyle Park and Wadmore Park respectively. All species are common except for *P. pruinosum* which is rated Vulnerable under the National Parks & Wildlife Act 1972, and Endangered under the Australian Commonwealth Environment Protection and Biodiversity Conservation Act 1999 (EPBC Act).

A further three species from three different genera were selected for a subsequent germination trial on the medium that performed optimally in the initial screening trial. They were *Caladenia tentaculata* Schltdl., *Calochilus platychilus* D.L.Jones and *Diuris orientis* D.L.Jones, collected from Kaiserstuhl Conservation Park, Kuitpo and Belair respectively. In addition, four extra species from the original four genera were chosen for further germination trials and included *Microtis unifolia* (G.Forst.)Rchb.f., *Prasophyllum pallidum* Nicholls, *Pterostylis plumosa* Cady and *Thelymitra sp*., collected from Wadmore Park, Belair, Ironbank and Big Heath Conservation Park, respectively. The second *Thelymitra* could not be identified accurately at the time of collection, but is likely to be either *T. antennifera* (Lindl.)Hook.f. or *T. flexuosa* Endl. and will be referred to as *Thelymitra sp*. in this study. Seeds from two additional populations of *P. nutans* (Kuitpo and Myponga) and of *T. pauciflora* (Blackwood Forest and Coromandel Valley) were also germinated. All additional species are common, except for *P. pallidum* which is rated Rare under the South Australian National Parks & Wildlife Act 1972, and Vulnerable under the EPBC Act (1999).

All orchid seed collection sites were located in South Australia, specifically in the Mt Lofty Ranges or the south-east of South Australia. Seed capsules were harvested at maturity from orchid populations between October and December 2010. Capsules were collected from 1-20 plants of each species depending on availability, and were stored under dark conditions in porous paper bags at 15˚C and 15% relative humidity until dried. Seeds of each species/population were removed from capsules and pooled to reflect the variability within each population rather than that of individual plants. The proportion of seed with full embryos was estimated for *P. nutans*, *M. arenaria*, *T. pauciflora* and *P. pruinosum* by assessing 15 x 100 seeds of each species under a stereo microscope.

Asymbiotic culture media

Six asymbiotic culture media were screened for their efficacy to support seed germination and protocorm development of *P. nutans*, *M. arenaria*, *T. pauciflora* and *P. pruinosum*. The media were: BM1 (#B141; Van Waes and Debergh 1986b), Malmgren (#M551; Malmgren 1996), ½MS inorganic salts (#M524; Murashige and Skoog 1962), Pa5 (modified Burgeffs N3f, Collins and Dixon 1992), P723 (#P723; *Phyto*Technology Orchid Seed Sowing Medium,) and W3 (Proprietary Formulation, Western Orchids Laboratory). All media were purchased from *Phyto*Technology Laboratories (*via* Austratec Pty Ltd, Victoria) with the exception of W3 (Western Orchids Laboratory, Blackwood, South Australia) and Pa5 which was prepared with 10 µM 6-benzylaminopurine using the formulation of Collins and Dixon (1992) (see Supplementary Table I for chemical composition of these culture media).

Coconut water, added to both Pa5 and W3 media (50 ml/l), was prepared from fresh mature (brown) coconuts. The liquid endosperm was extracted and filtered (Whatman No.1 filter paper), the filtrate boiled for 10 minutes (to precipitate proteins), cooled and filtered again until clear (Dodds and Roberts 1985). Banana pulp added to W3 medium (50 g/l) was prepared by blending one ripe banana in a beaker with a little sterile deionised water. The agar concentration of all media was standardised to 8 g/l by the addition of agar (#A111, *Phyto*technology Laboratories) to the formulation where necessary. All media were adjusted to pH 5.5 before autoclaving for 35 minutes at 117.7 kPa and 121°C. Media were dispensed in a laminar-flow hood to sterile 90 mm polystyrene Petri dishes (Techno-Plas Pty Ltd) as 30 ml aliquots and allowed to solidify before use.

Seed germination

Approximately 0.02 g of seed was surface sterilized in a solution of 1% (v/v) NaOCl (fresh stock containing 8-12.5% available chlorine) + 0.01% Triton (X-114) by agitating in a 25 ml conical flask for 10 minutes. Suspended seed was dispersed onto sterile (autoclaved and soaked in 70% ethanol before use) filter paper (Whatman No. 1, 55 mm) using a Buchner funnel to remove excess solution under suction. The filter paper with adhering, evenly dispersed seeds was then transferred, without rinsing, to the surface of the test medium in a Petri dish. Preliminary experiments had shown that withholding rinsing at this point reduced microbial infection on plates without adversely affect seed germination. Plates were sealed with Parafilm (Pechiney Plastic Packaging Company) and incubated in darkness at 20 ± 3°C. The initial screening trial was replicated eight times, and subsequent trials testing additional species/populations on the selected optimal medium were replicated five times.

Seed germination and protocorm development were recorded after 10 weeks. Seedling development was recorded on a scale of 1-5, where 1 = testa intact; 2 = testa ruptured, embryo approximately doubled in size forming a spherical protocorm; 3 = rhizoids developing; 4 = start of shoot differentiation; 5 = emergence of first leaf (Jusaitis and Sorensen 1993). Germination was considered to have occurred when the embryo had emerged from the testa, and approximately doubled in size (i.e. Stage 2), enabling rapid assessment of viability for routing seed testintg (Pritchard 1985; Lauzer *et al.* 1994; Vujanovic *et al.* 2000; Pierce and Cerabolini 2011).

Unless otherwise stated, all seed observations were made using an Olympus SZH zoom stereo microscope fitted with an ILLD illumination base. To aid counting accuracy a 60 x 60 mm acetate grid (divided into 10 mm squares) was placed over plates and only seeds in the inner 16 cells of the grid were counted. At least 100 seeds were assessed from each plate. Seeds without an embryo were not included in the calculation of germination percentage.

Tetrazolium assay

Seeds from each of the four species *P. nutans, M. arenaria, T. pauciflora* and *P. pruinosum* were subjected to TTC staining to assess biochemical viability (Van Waes and Debergh 1986a). A 1% TTC solution was prepared by dissolving 1g of 2,3,5-triphenyltetrazolium chloride in 100 ml of sterile deionised water. The pH was adjusted to 6.5 with 1M NaOH.

Seeds were pre-treated in small flasks with 20 ml of a 5% NaOCl + 1% Triton (X-114) solution for 0, 1 or 2 h. Following pre-treatment, seeds were rinsed then allowed to soak in deionised water (10 ml) for 24 hr at 20°C in darkness. Seeds were then transferred to 1% TTC solution (10 ml) for 24 hours at 30°C in darkness, then dispersed onto filter paper using a Buchner funnel under suction and washed twice with sterile water to remove

excess stain. Seeds were examined under a stereomicroscope and scored as viable (pink or red embryo) or unviable (unstained embryo). For each species/treatment combination five replicates of at least 100 seeds each were used to determine staining percentages.

Because some embryo damage was observed after 1 or 2 h pre-treatment with 5% NaOCl, the viability test was repeated on the same four species using a pre-treatment of 1% NaOCl + 0.01% Triton (X-114) for 0, 0.5 or 1 h. Pre-treated seeds were rinsed and soaked in water as before, but this was followed by a 48 hour soak in 1% TTC solution at 30˚C in darkness. Seeds were transferred to a microscope slide using a micropipette and embryos were extracted from their testae by gently rotating a cover slip over the seed. Seeds were examined and scored as before. For each species/treatment combination three replicates were used to determine staining percentages.

Fluorescein diacetate assay

Seed viability of each of the four species *P. nutans M. arenaria*, *T. pauciflora* and *P. pruinosum* was assessed using a modification of the FDA staining method of Pritchard (1985). Seeds were surface sterilized with 1% NaOCl + 0.01% Triton (X-114) for 10 minutes, rinsed twice and then soaked for 16 h at room temperature in sterile deionised water. FDA was prepared at 0.5% (w/v) in absolute acetone and applied 1:1 with sterile deionised water to seeds on a microscope slide. The stain reaction was allowed to develop for 15-30 minutes before seeds were examined under an Olympus BX51 epi-fluorescent microscope with a UMWIB2 filter cube (excitation 460-495 nm). Embryos exhibiting a bright yellowish-green staining reaction over the whole embryo were considered viable. This experiment was repeated with the inclusion of a gentle cover slip rotation to remove testae immediately before addition of FDA. In both experiments three replicates of each species (of 50–100 seeds per replicate) were used to calculate staining percentages.

Statistical analyses

Germination and protocorm development percentages were determined after 10 weeks incubation. Germination percentages were calculated by dividing the number of germinated seeds by the total number of seeds with an embryo (x100). The percentage of protocorms at a developmental stage was calculated by dividing the number of seeds in that stage by the total number of seeds with an embryo (x100). Viability estimates were determined by dividing the number of stained embryos by the total number of embryos counted for each species (x100).

All data were arcsine transformed to normalize variation, but untransformed means are presented. An un-paired t test ($P = 0.05$) was used to compare the germination percentages and developmental stages of each species within the same genus. For all other experiments, data were subjected to one-way ANOVA to test for significance. Mean separation was performed using the Tukey-Kramer post-hoc test at $P = 0.05$. Statistical analyses were performed using Prism®5 (GraphPad Software Inc.) and Stata®12 (StataCorp LP) software.

Results

Asymbiotic media screen

Seeds of all four terrestrial orchid species tested in this experiment germinated on each of the six media screened, although the germination percentage and subsequent development of protocorms varied between species and media (Fig. 1). *P. nutans* reached developmental Stages 3 and 4 on all media except ½MS which failed to support growth beyond stage 2 within the time frame examined. Germination percentages differed significantly ($P < 0.001$) between media, with BM1 (90.7%), P723 (91.2%) and Malmgren (85%) proving optimal for this species (Fig. 2).

M. arenaria seed germinated on all media, most supporting growth up to Stage 3 (Fig. 1). Protocorm development was most advanced on BM1, with no Stage 2 protocorms remaining after 10 weeks and 45.5% of protocorms at Stage 4. While BM1 and Malmgren produced significant Stage 4 development, P723 and W3 retained many seed at Stage 1 after 10 weeks. Germination percentages were equally high for BM1 (99.2%), Malmgren (95.6%), ½MS (97.5%) and Pa5 (97.5%) (Fig. 2).

On all media, *T. pauciflora* retained a significant proportion of seed at Stage 1 (Fig. 1). P723 (3.5%) and Malmgren (3%) produced the highest percentages of protocorms at Stage 4, yet on both media over 80% of seed remained at Stage 1 after 10 weeks. Germination percentages for this species were optimal on BM1 (60.9%), ½MS (49%) and Pa5 (54.1%), although on all media, this species produced the lowest germination of all four species in this experiment (Fig. 2).

P. pruinosum showed a similar pattern of development on all media tested, most seeds reaching Stage 2, but only those on BM1 and Malmgren progressing to Stage 3 in significant numbers (>10%). W3 had the highest proportion of non-germinants (Fig. 1). Germination was highest on BM1 (73%) and did not differ significantly ($P = 0.05$) from that recorded on Malmgren (64.6%), ½ MS (57.6%), Pa5 (62.1%) or P723 (65.5%) media (Fig. 2). The germination and development of this species was slower, and Stage 3 protocorms had shorter and fewer rhizoids than the other three species tested in this experiment. Polyembryony (two embryos in one seed coat) was also observed in this species.

Of the four media tested, BM1 consistently supported optimal germination and protocorm development across all four species examined. It was also the medium that most consistently sustained ongoing development of all four species to Stage 3-4 protocorms over the 10 week incubation period (Fig. 3). For some individual species, P723, Malmgren and W3 performed equally well, yet failed to produce the consistency shown by BM1 across all species.

Seed viability testing

The vast majority of seeds examined microscopically for embryo development were found to be full. *M. arenaria* (94.8 ± 0.01%) and *P. nutans* (94.5 ± 0.01%) had the highest percentage of full seeds, followed by *T. pauciflora* (80.3 ± 0.02%) and *P. pruinosum* (43.2 ± 0.03%) which had the lowest seed set.

The percentage of embryos stained red with TTC solution varied between species and was influenced by the concentration and duration of NaOCl pre-treatment (Table 1). Stained embryos were readily distinguishable for all species tested, whether testae were present or absent. Pre-treatment with 5% NaOCl for 1-2 h appeared to bleach most seeds of *P. nutans* and *P. pruinosum* to the point of whitening and as a result, both failed to stain with TTC. *M. arenaria* and *T. pauciflora* were not similarly affected and the pre-treatment did not reduce staining. Pre-treatment with 1% NaOCl (for 0.5 or 1 h) resulted in a significant decrease in the staining percentage of *P. nutans* and *P. pruinosum,* but increased the staining percentages of both *M. arenaria* and *T. pauciflora* compared with untreated controls.

The highest staining percentages for *P. nutans* (50.9 ± 1.8) and *P. pruinosum* (58.4 ± 3.1) seed occurred in the absence of NaOCl pre-treatment and the results did not vary with TTC soaking times between 24 to 48 h (Table 1). Pre-treatment for 1 hour with 1% NaOCl produced the highest staining percentages for seeds of *M. arenaria* (25 ± 6.1) and *T. pauciflora* (62 ± 0.2), both being significantly higher than if pre-treated for only 30 minutes.

Removal of the testa did not significantly increase the proportion of FDA-stained embryos ($P > 0.05$), confirming that the stain was able to penetrate the seed coats of all four species tested. Therefore, results of the two experiments (with and without testae) were combined to give final staining percentages for each species. The viability estimates following FDA staining for *P. nutans* (85.5 ± 0.7%), *M. arenaria* (85.9 ± 2.3%) and *T. pauciflora* (81.1 ± 3.2%) seed did not differ significantly. However, the estimated viability for *P. pruinosum* was significantly ($P < 0.001$) lower at 68.7 ± 3%.

The relationship between seed viability as determined by TTC or FDA staining and the maximal germination percentage was not consistent between species (Fig. 4). For *P. nutans* the viability estimates of both vital stains differed significantly ($P < 0.001$) from the final germination percentage (91.2%), although FDA gave a closer approximation (85.5%) than did TTC (50.9%). Similarly for *M. arenaria,* FDA yielded a viability estimate much closer to maximal germination than did TTC, although differences were again highly significant ($P < 0.001$). For both *T. pauciflora* and *P. pruinosum* the differences between maximal germination and stain-determined viability were not significant ($P > 0.05$), suggesting that both staining procedures could potentially predict the seed viability of these species.

Further germination trials on BM1

The optimal germination medium from the screening trial (BM1), was further tested on species from three additional genera (*C. tentaculata, C. platychilus* and *D. orientis),* four additional species from the original genera (*M. unifolia*, *P. pallidum, P. plumosa, and Thelymitra. sp.*), and seed from two additional populations of *P. nutans* and *T. pauciflora.* Table 2 compares the seed development and germination results of all species after 10 wks on BM1. Nearly all seeds (99.5%) of *C. tentaculata* germinated on BM1, the majority reaching Stage 3-4 by 10 wks. Germination of *C. platychilus*, however, was very low (0.1%) with only a single seed progressing to Stage 3, even though 89.7 ± 1.1% of seeds had full embryos. Over half of *D. orientis* seeds germinated on BM1 with one third reaching Stage 3-4.

The two species of *Microtis* both germinated readily on BM1, with *M. unifolia* being the only species to reach Stage 5 of development within 10 weeks (Table 2). Nevertheless, *M. arenaria* had significantly higher germination that *M. unifolia* ($P = 0.002$). Germination of *Prasophyllum* ($P = 0.13$) and *Thelymitra* ($P = 0.79$) did not differ significantly between the two congeneric species tested. Both *Pterostylis* species yielded high germination, although *P. plumosa* was significantly higher than *P. nutans* ($P = 0.013$) even though protocorms of the latter were more advanced (almost 30% at Stage 4) than those of the former.

Seeds of *P. nutans* sourced from three populations demonstrated equivalent rates of germination after 10 wks on BM1 ($P = 0.48$), although Scott Creek seeds were most advanced with nearly 30% reaching Stage 4 (Table 2). *T. pauciflora* seeds from Aberfoyle Park and Coromandel Valley did not differ in germination response, but Blackwood Forest seed yielded significantly higher germination than both ($P = 0.006$). This was largely due to the higher proportion of non-viable or dormant seeds (about 30-40%) remaining at Stage 1 for these two cohorts.

Discussion

An abundance of media have been tested to examine the nutritional and cultural requirements for *in vitro* germination of orchids (Rasmussen 1995; Hicks and Lynn 2010). However, to our knowledge, a comparative study to determine the optimal asymbiotic medium for routine seed testing of Australian temperate terrestrial orchids has never been

published. The six media assessed in this study were selected on the basis of a literature review of asymbiotic germination studies of terrestrial orchids from around the world. The medium P723 had proved successful with American species (Kauth *et al.* 2006; Johnson and Kane 2007; Dutra *et al.* 2009), ½ MS with American, Asian and South African species (Park *et al.* 2000; Johnson and Kane 2007; Thompson *et al.* 2007; Mahendran and Bai 2009; Lee 2011), and Malmgren (Malmgren 1996; Stewart and Kane 2006, 2010) and BM1 (Van Waes and Debergh 1986b; Kauth *et al.* 2008, 2011; Sgarbi *et al.* 2009) with American and European species. Pa5 had been used successfully with Australian terrestrial species, including *Pterostylis arenicola* (Jusaitis and Sorensen 1993), *Diuris longifolia* and *Elythranthera brunonis* (Collins and Dixon 1992; Oddie *et al.* 1994), and W3 had successfully germinated species of *Caladenia, Diuris, Microtis, Pterostylis* and *Thelymitra* (Hay *et al.* 2010).

Germination and subsequent protocorm development varied between these media and the four species tested. Final germination estimates showed that all media supported high germination rates for at least one of the four species tested. Stage 4 protocorms were recorded for three of these species after 10 weeks, and development was most advanced on BM1, Malmgren and P723 (Fig. 1). However, the medium supporting the most advanced development did not necessarily promote the highest germination rates. For example, the most advanced development of *T. pauciflora* after 10 weeks occurred on P723, yet the same medium recorded a very low germination percentage (< 12%) for the same species. The opposite was true for ½ MS, which supported high germination rates for all four species but no seedlings developed beyond Stage 3 (Fig. 3). Germination rates for all species were consistently highest on BM1. This medium was also found to yield a good germination response with 21 of 23 species of Western European orchids tested (Van Waes and Debergh 1986b), with the Mediterranean terrestrial orchid *Limodorum abortivum* (Sgarbi *et al.* 2009), and with seed from several populations of the North American terrestrial orchid, *Calopogon tuberosus* (Kauth *et al*, 2008, 2011).

Of the media screened, only BM1, Pa5 and P723 contained 20 g/l sucrose. Carbohydrates were also present in Malmgren (as pineapple powder) and W3 (as banana pulp and coconut water). Recent studies have suggested that rhizoids are only produced when seeds are cultured in the presence of carbohydrates (Stewart and Kane 2010; Johnson *et al.* 2011). However in this study, both *M. arenaria* and *T. pauciflora* developed protocorms with rhizoids (Stage 3) after 5 weeks in darkness on ½ MS without any source of exogenous carbohydrates. Even though they failed to develop any further, this result suggests that these two species either contained sufficient embryo reserves (lipids, proteins, sugars) to initiate protocorm (and rhizoid) development, or that they only require a supply of soluble sugars later in development. Both *P. nutans* and *P. pruinosum* also germinated on ½ MS, but neither developed beyond Stage 2. Seeds growing on a medium containing only minerals tend to use their limited reserves slowly, enabling survival for a year or more (Arditti 1967). In nature, such long survival periods allow increased opportunity for exposure to and infection by symbiotic fungi, further enhancing subsequent seedling development (Arditti and Ghani 2000).

Nitrogen was supplied to media as inorganic (½ MS, Pa5), organic (BM1, Malmgren), or a mixture of both forms (P723). Seed germinated on all media, suggesting that each of the four species was able to utilize a range of nitrogen forms and concentrations. However, protocorm development was reduced on media containing only inorganic nitrogen sources (Fig. 1). Several authors have reported that germination of terrestrial orchid seeds can be improved by replacing inorganic nitrogen with amino acids (Van Waes and Debergh 1986b; Vejsadova 2006; Johnson *et al.* 2007). Consistent with previous work (Malmgren 1996; Stewart and Kane 2006), the present study found that media containing organic

sources of nitrogen promoted the most advanced growth of Australian terrestrial orchids, indicating that this form is more readily available to developing seedlings than inorganic forms of nitrogen. BM1 is rich in L-glutamine and casein hydrolysate, both sources of organic nitrogen, which may have at least partially contributed to the efficacy of this medium (Van Waes and Debergh 1986b; Sgarbi *et al.* 2009).

Two additional species (*C. tentaculata, D. orientis*), four congeners (*M. unifolia, P. pallidum, P. plumosa, T. sp*) and seeds from two additional populations of *P. nutans* and *T. pauciflora* all germinated successfully on the selected optimal medium, BM1. *Calochilus platychilus* was the only species tested on this medium to record almost no germination (Table 2), suggesting that its seeds were unviable or had morphological or physiological dormancies which prevented germination (Lauzer *et al.* 2007). Poor germination may also reflect non-optimal storage or incubation conditions (Hay *et al.* 2010), presence of inhibitors in the seed or testa (Van Waes and Debergh 1986b; Thompson *et al.* 2006; Vejsadova 2006), deficiencies in culture media components (Johnson *et al.* 2011), limited endogenous seed reserves (Rasmussen 1995), or an obligate reliance on mycorrhizal fungi (Batty *et al.* 2001b; Thompson *et al.* 2006).

Congeners of *Microtis* and *Pterostylis* varied significantly in their germination and developmental responses on BM1, even though germination rates were consistently high (> 90%) for each species (Table 2). *Prasophyllum* and *Thelymitra* seeds had lower germination percentages but equal congeneric responses. While the three populations of *P. nutans* did not vary significantly in their germination response, *T. pauciflora* seed collected from Blackwood Forest produced significantly more germinants than seed from the other two populations. These results suggest that inherent differences in germination response may exist between species within genera, and within species collected from different provenances. The former are likely to be genetically based, while the latter potentially due to site-specific selection pressures influencing seed quality.

The correlation between observed germination and TTC staining is often variable and species specific for terrestrial orchids (Van Waes and Debergh 1986b; Shoushtari *et al.* 1994; Johnson and Kane 2007; Kauth *et al.* 2008) and may even vary within a species across its range (Kauth *et al.* 2011). In the present study TTC staining proved an effective method for assessing the seed viability of *T. pauciflora* and *P. pruinosum*, but was unsuccessful in determining the viability of either *P. nutans* or *M. arenaria* (Fig. 4). Orchid seeds are usually pre-treated with an oxidising agent such as NaOCl or $Ca(OCl)_2$ to weaken the testa and facilitate subsequent staining (Van Waes and Debergh 1986b; Johnson *et al.* 2011). The type of hypochlorite used will depend on the species to be treated, as some species may be more sensitive to NaOCl than to $Ca(OCl)_2$. Experimentation is usually necessary to optimise concentration and time of treatment while minimising sensitivity for each species. Longer soaking times or stronger hypochlorite solutions are needed for seeds with darker seed coats as they contain more suberin (Van Waes and Debergh 1986a). This may explain the variation in staining between the four species tested (Table 1), as 1% NaOCl pre-treatment produced the highest staining percentages for species with dark testae (*M. arenaria* and *T. pauciflora*), while species with lighter testae (*P. nutans* and *P. pruinosum*) required no pre-treatment to absorb stain. Stain influx may also be hindered if there is a large airspace between the testa and embryo (Pritchard 1985; Pritchard and Prendergast 1990). Visual inspection showed that the testa of *P. pruinosum* was closely adpressed to the embryo, allowing unrestricted stain uptake, in contrast with *P. nutans* which appeared to have a higher seed/embryo volume ratio. Some recent studies have shown that pre-conditioning seed with sucrose before TTC treatment could improve the correlation between TTC-assessed viability and seed germination for *Cattleya* species (Hosomi *et al.* 2011, 2012), but this was not tested here.

FDA staining has also produced variable results in assessing orchid seed viability. Positive correlations with germination have been recorded for some species (Wood *et al.* 2003; Wood and Pritchard 2004; Vendrame *et al.* 2007), as have overestimations (Batty *et al.* 2001a) and underestimations (Pritchard 1985; Wood *et al.* 2003) of viability. In this study, FDA proved effective in accurately estimating the viability of *T. pauciflora* and *P. pruinosum*. The viability of both *P. nutans* and *M. arenaria* was underestimated, although FDA estimates for all species were significantly closer to germination percentages than were TTC estimates, suggesting that FDA was a more accurate predictor of seed germination potential than was TTC. This may be due to the difficulty of accurately assessing topographical TTC staining of such small seeds, or to the development of stain being obscured by natural embryo coloration in some species of orchids (Wood *et al.* 2003; Wood and Pritchard 2004). While some workers have also attributed underestimates of seed viability to the presence of a non-permeable testa (Pritchard 1985; Wood *et al.* 2003; Lauzer *et al.* 2007), we found no evidence for such an effect on staining efficacy of the Australia terrestrial orchid seeds tested here.

In summary, ten species from six genera of Australian terrestrial orchids were successfully germinated on BM1 medium using asymbiotic techniques. The use of vital stains (TTC, FDA) to determine seed viability proved successful for two out of four species tested, with FDA generally providing a more accurate estimate of viability than TTC. Optimal seed pre-treatments should be determined for each species before testing viability by these methods, and viability estimates should always be corroborated with germination studies when working with temperate terrestrial orchids (Wood *et al.* 2003; Johnson *et al.* 2011). Although asymbiotic germination constitutes a rapid, routine and cost effective seed performance test for use by orchid seed banks, symbiotic germination should still be the method of choice for conservation or translocation outcomes.

Acknowledgements

We would like to thank A/Prof. David Paton and Dr Phil Ainsley for their assistance and constructive input during this project. Thanks also to Joe Quarmby, Kieran Brewer and Dan Duval for collecting the seed used in this project. Thank you to Carolyn Ricci (State Herbarium of South Australia) and Lyn Waterhouse (Adelaide Microscopy, University of Adelaide) for assistance with microscopy.

References

Arditti J (1967) Factors affecting the germination of orchid seeds. *The Botanical Review* **33**, 1-97.

Arditti J, Ghani AKA (2000) Tansley Review No. 110 – Numerical and physical properties of orchid seeds and their biological implications. *New Phytologist* **145**, 367-421.

Batty AL, Dixon KW, Brundrett M, Sivasithamparam K (2001a) Long-term storage of mycorrhizal fungi and seed as a tool for the conservation of endangered Western Australian terrestrial orchids. *Australian Journal of Botany* **49**, 619-628.

Batty AL, Dixon KW, Brundrett M, Sivasithamparam K (2001b) Constraints to symbiotic germination of terrestrial orchid seed in a Mediterranean bushland. *New Phytologist* **152**, 511-520.

Brundrett MC (2007) Scientific approaches to Australian temperate terrestrial orchid conservation. *Australian Journal of Botany* **55**, 293-307.

Clements MA (1981) The germination of Australian orchid seed. In 'Proceedings of the orchid symposium, 13th International Botanical Congress' pp. 5-8. (Harbour Press: Sydney)

Clements MA (1982) Australian native orchids (epiphytic and terrestrial). In. 'Orchid biology – reviews and perspectives , II' (Ed J Arditti) pp. 295-303. (Cornell University Press: Ithaca, NY)

Cochrane JA, Crawford AD, Monks LT (2007) The significance of *ex situ* seed conservation to reintroduction of threatened plants. *Australian Journal of Botany* **55**, 356-361.

Collins MT, Dixon KW (1992) Micropropagation of an Australian terrestrial orchid *Diuris longifolia* R. Br. *Australian Journal of Experimental Agriculture* **32**, 131-135.

Crane PR, Hopper SD, Raven PH, Stevenson DW (2009) Plant science research in botanic gardens. *Trends in Plant Science* **14**, 575-577.

Dodds JH, Roberts LW (1985) 'Experiments in plant tissue culture.' Second Edition (Cambridge University Press: Cambridge)

Dutra D, Kane ME, Richardson L (2009) Asymbiotic seed germination and in vitro seedling development of *Cyrtopodium punctatum*: a propagation protocol for an endangered Florida native orchid. *Plant Cell Tissue and Organ Culture* **96**, 235-243.

Faast R, Facelli JM, Austin AD (2011) Seed viability in declining populations of *Caladenia rigida* (Orchidaceae): are small populations doomed? *Plant Biology* **13** (Suppl. 1), 86-95.

Godo T, Komori M, Nakaoki E, Yukawa T, Miyoshi K (2010) Germination of mature seeds of *Calanthe tricarinata* Lindl., an endangered terrestrial orchid, by asymbiotic culture *in vitro*. *In Vitro Cellular and Developmental Biology – Plant* **46**, 323-328.

Hay FR, Merritt DJ, Soanes JA, Dixon KW (2010) Comparative longevity of Australian orchid (Orchidaceae) seeds under experimental and low temperature storage conditions. *Botanical Journal of the Linnean Society* **164**, 26-41.

Hicks AJ, Lynn K (2010) 'Orchid seed germination media: A compendium of formulations.' (The Orchid Seedbank Project: Chandler, AZ)

Hosomi ST, Santos RB, Custodio CC, Seaton PT, Marks TR, Machado-Neto NB (2011) Preconditioning *Cattleya* seeds to improve the efficacy of the tetrazolium test for viability. *Seed Science and Technology* **39**, 178-189.

Hosomi ST, Custodio CC, Seaton PT, Marks TR, Machado-Neto NB (2012) Improved assessment of viability and germination of *Cattleya* (Orchidaceae) seeds following storage. *In Vitro Cellular and Developmental Biology – Plant* **48**, 127-136.

Johnson TR, Kane ME (2007) Asymbiotic germination of ornamental *Vanda*: in vitro germination and development of three hybrids. *Plant Cell, Tissue and Organ Culture* **91**, 251-261.

Johnson TR, Kane ME, Perez HE (2011) Examining the interaction of light, nutrients and carbohydrates on seed germination and early seedling development of *Bletia purpurea* (Orchidaceae). *Plant Growth Regulation* **63**, 89-99.

Jusaitis M, Sorensen B (1993) Germination of *Pterostylis arenicola* – an endangered greenhood from South Australia. *The Orchadian* **11**, 18-22.

Kauth PJ, Vendrame WA, Kane ME (2006) *In vitro* seed culture and seedling development of *Calopogon tuberosus. Plant Cell, Tissue and Organ Culture* **85**, 91-102.

Kauth PJ, Kane ME, Vendrame WA, Reinhardt-Adams C (2008) Asymbiotic germination response to photoperiod and nutritional media in six populations of *Calopogon tuberosus* var. *tuberosus* (Orchidaceae): evidence for ecotypic differentiation. *Annals of Botany* **102**, 783-793.

Kauth PJ, Kane ME, Vendrame WA (2011) Comparative *in vitro* germination ecology of *Calopogon tuberosus* var. *tuberosus* (Orchidaceae) across its geographic range. *In Vitro Cellular and Developmental Biology – Plant* **47**, 148-156.

Lakon G (1949) The topographical tetrazolium method for determining the germinating capacity of seeds. *Plant Physiology* **24**, 389-394.

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

Lauzer D, Renaut S, St-Arnaud M, Barabe D (2007) *In vitro* asymbiotic germination, protocorm development, and plantlet acclimatization of *Aplectrum hyemale* (Muhl. ex Willd.) Torr. (Orchidaceae). *Journal of the Torrey Botanical Society* **134**, 344-348.

Lee YI (2011) In vitro culture and germination of terrestrial Asian orchid seeds. In 'Plant embryo culture; methods and protocols'. (Eds TA Thorpe, EC Yeung) *Methods in Molecular Biology* **710**, 53-62.

Mahendran G, Bai VN (2009) Mass propagation of *Satyrium nepalense* D.Don. – a medicinal orchid via seed culture. *Scientia Horticulturae* **119**, 203-207.

Malmgren S (1996) Orchid propagation: theory and practice. In 'North American native terrestrial orchids: propagation and production'. (Ed C Allen) pp. 63-71. (The North American Native Terrestrial Orchid Conference: Maryland, USA)

Markovina A-L, McGee PA (2000) Comparison of symbiotic and asymbiotic seed germination and plantlet development in *Sarcochilus* (Vandeae; Orchidaceae). *Lindleyana* **15**, 68-72.

Miyoshi K, Mii M (1988) Ultrasonic treatment for enhancing seed germination of terrestrial orchid, *Calanthe discolor*, in asymbiotic culture. *Scientia Horticulturae* **35**, 127-130.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-497.

Oddie RLA, Dixon KW, McComb JA (1994) Influence of substrate on asymbiotic and symbiotic *in vitro* germination and seedling growth of two Australian terrestrial orchids. *Lindleyana* **9**, 183-189.

Offord CA, McKensy ML, Cuneo PV (2004) Critical review of threatened species collections in the New South Wales seedbank: implications for *ex situ* conservation of biodiversity. *Pacific Conservation Biology* **10**, 221-236.

Oliva AP, Arditti J (1984) Seed germination of North American orchids. II. Native California and related species of *Aplectrum*, *Cypripedium*, and *Spiranthes*. *Botanical Gazette* **145**, 495-501.

Park SY, Murthy HN, Paek KY (2000) In-vitro seed germination of *Calanthe sieboldii*, an endangered orchid species. *Journal of Plant Biology* **43**, 158-161.

Pierce S, Cerabolini BEL (2011) Asymbiotic germination of the White Mountain Orchid (*Pseudorchis albida*) from immature seed on media enriched with complex organics or phytohormones. *Seed Science and Technology* **39**, 199-203.

Pritchard HW (1985) Determination of orchid seed viability using fluorescein diacetate. *Plant, Cell and Environment* **8**, 727-730.

Pritchard HW, Prendergast FG (1990) Viability testing in terrestrial orchid seed. In 'Proceedings of the symposium on biology and ecology of European orchids'. pp. 11–16. (Acta Universitatis Wratislaviensis: Wroclaw, Poland)

Ramsay RR, Dixon KW, Sivasithamparam K (1986) Patterns of infection and endophytes associated with Western Australian orchids. *Lindleyana* **1**, 203-214.

Rasmussen HN (1995) 'Terrestrial orchids: from seed to mycotrophic plant'. (The University Press: Cambridge)

Rasmussen HN, Johansen B, Andersen TF (1991) Symbiotic in vitro culture of immature embryos and seeds from *Listera ovata*. *Lindleyana* **6**, 134-139.

Seaton PT, Hu H, Perner H, Pritchard HW (2010) Ex situ conservation of orchids in a warming world. *The Botanical Review* **76**, 193-203.

Sgarbi E, Grimaudo M, Del Prete C (2009) *In vitro* asymbiotic germination and seedling development of *Limodorum abortivum* (Orchidaceae). *Plant Biosystems* **143**, 114-119.

Shoushtari BD, Heydari R, Johnson GL, Arditti J (1994) Germination and viability staining of orchid seeds following prolonged storage. *Lindleyana* **9**, 77-84.

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

Stewart SL, Kane ME (2006) Asymbiotic seed germination and in vitro seedling development of *Habenaria macroceratitis* (Orchidaceae), a rare Florida terrestrial orchid. *Plant Cell Tissue and Organ Culture* **86**, 147-158.

Stewart SL, Kane ME (2010) Effects of carbohydrate source on the *in vitro* asymbiotic seed germination of the terrestrial orchid *Habenaria macroceratitis*. *Journal of Plant Nutrition* **33**, 1155-1165.

Swarts ND, Dixon KW (2009a) Terrestrial orchid conservation in the age of extinction. *Annals of Botany* **104**, 543-556.

Swarts ND, Dixon KW (2009b) Perspectives on orchid conservation in botanic gardens. *Trends in Plant Science* **14**, 590-598.

Thompson DI, Edwards TJ, van Staden J (2006) Evaluating asymbiotic seed culture methods and establishing *Disa* (Orchidaceae) germinability in vitro: relationships, requirements and first-time reports. *Plant Growth Regulation* **49**, 269-284.

Thompson DI, Edwards TJ, van Staden J (2007) A novel dual-phase culture medium promotes germination and seedling establishment from immature embryos in South African *Disa* (Orchidaceae) species. *Plant Growth Regulation* **53**, 163-171.

Van Waes JM, Debergh PC (1986a) Adaptation of the tetrazolium method for testing the seed viability, and scanning electron microscopy study of some Western European orchids. *Physiologia Plantarum* **66**, 435-442.

Van Waes JM, Debergh PC (1986b) In vitro germination of some Western European orchids. *Physiologia Plantarum* **67**, 253-261.

Vejsadova H (2006) Factors affecting seed germination and seedling growth of terrestrial orchids cultured in vitro. *Acta Biologica Cracoviensia Series Botanica* **48**, 109-113.

Vendrame WA, Carvalho VS, Dias JMM (2007) In vitro germination and seedling development of cryopreserved *Dendrobium* hybrid mature seeds. *Scientia Horticulturae* **114**, 188-193.

Volis S, Blecher M (2010) Quasi in situ: a bridge between ex situ and in situ conservation of plants. *Biodiversity Conservation* **19**, 2441-2454.

Vujanovic V, St-Arnaud M, Barabe D, Thibeault G (2000) Viability testing of orchid seed and the promotion of colouration and germination. *Annals of Botany* **86**, 79-86.

Wood CB, Pritchard HW, Mugambi GK (2003) Fluorescein diacetate and orchid seed viability in UK and Kenyan species. In 'Seed Conservation: turning science into practice'. (Eds RD Smith, JB Dickie, SH Linington, HW Pritchard, RJ Probert) pp. 494-506. (Royal Botanic Gardens: Kew, UK)

Wood CB, Pritchard HW (2004) Determination of intra-specific variation in orchid seed viability using fluorescein diacetate. *Seed Science and Technology* **32**, 629-635.

Wright M, Cross R, Dixon K, Huynh T, Lawrie A, Nesbitt L, Pritchard A, Swarts N, Thomson R (2009) Propagation and reintroduction of *Caladenia*. *Australian Journal of Botany* **57**, 373-387.

Yamazaki J, Miyoshi K (2006) *In vitro* asymbiotic germination of immature seed and formation of protocorm by *Cephalanthera falcata* (Orchidaceae). *Annals of Botany* **98**, 1197-1206.

Tables

Table 1. Effect of pre-treatment duration in 5 or 1% NaOCl on TTC staining of mature terrestrial orchid seeds.

Following pre-treatment, seeds were soaked in sterile deionised water for 24 h, followed by 1% TTC treatment for 24^{x} or 48^{y} h. Percentages are means of five^{x} or three^{y} replicates of at least 100 seeds. Within each row, percentages with identical superscripts are not significantly different (P = 0.05) based on the Tukey-Kramer post-hoc test.

Species	TTC staining (%)		
	(5% NaOCl pre-treatment)^{x}		
	0	1 h	2 h
Pterostylis nutans	50.9^{a}	0^{b}	0^{b}
Microtis arenaria	1.2^{a}	0.2^{a}	2.8^{a}
Thelymitra pauciflora	5.4^{a}	10.9^{a}	14.0^{a}
Prasophyllum pruinosum	51.6^{a}	0^{b}	0.4^{b}
	(1% NaOCl pre-treatment)^{y}		
	0	0.5 h	1 h
Pterostylis nutans	50.9^{a}	15.5^{b}	17.5^{b}
Microtis arenaria	2.0^{a}	7.4^{a}	25.0^{b}
Thelymitra pauciflora	5.8^{a}	36.3^{b}	62.0^{c}
Prasophyllum pruinosum	58.4^{a}	4.6^{b}	11.7^{b}

Table 2. Protocorm development and germination of terrestrial orchid seeds after 10 weeks incubation on BM1 medium.

Seeds that reached developmental Stage 2 were considered to have germinated. Numbers in parentheses represent standard errors. Species identified by an asterisk were $n = 8$, all other species were $n = 5$.

Species/provenance	**%** in developmental Stage					Germination
	1	2	3	4	5	(%)
Caladenia tentaculata	0.5 (0.5)	0.3 (0.3)	64.4 (5.2)	34.8 (5.3)	0	99.5 (0.5)
Calochilus platychilus	99.9 (0.1)	0	0.1 (0.1)	0	0	0.1 (0.1)
Diuris orientis	47.4 (7.7)	19.2 (3.7)	30.2 (5.6)	3.2 (0.8)	0	52.6 (7.7)
*Microtis arenaria**	0.8 (0.3)	0	53.8 (10.2)	45.5 (10.3)	0	99.2 (0.3)
Microtis unifolia	4.5 (0.6)	2.7 (1.6)	30.4 (13.3)	28.0 (5.3)	34.5 (10)	95.5 (0.6)
*Prasophyllum pruinosum**	27.0 (3.1)	62.9 (4.0)	10.0 (3.7)	0	0	73.0 (3.1)
Prasophyllum pallidum	37.5 (5.9)	51.9 (2.4)	9.7 (4.0)	0.9 (0.9)	0	62.5 (5.9)
Pterostylis nutans						
Scott Creek*	9.3 (1.6)	14.6 (2.2)	46.6 (2.4)	29.4 (3.6)	0	90.7 (1.6)
Kuitpo	7.8 (2.4)	9.7 (2.8)	72.2 (5.2)	10.3 (1.2)	0	92.2 (2.4)
Myponga	12.2 (3.6)	3.6 (0.8)	72.5 (3.8)	11.7 (1.9)	0	87.8 (3.6)
Pterostylis plumosa	3.3 (1.2)	11.3 (3.8)	84.6 (3.9)	0.9 (0.5)	0	96.7 (1.2)
Thelymitra pauciflora						
Aberfoyle Park*	39.1 (7.8)	1.0 (0.3)	57.1 (7.7)	2.7 (0.9)	0	60.9 (7.8)
Blackwood Forest	9.5 (2.6)	2.8 (2.2)	87.7 (2.9)	0	0	90.5 (2.6)
Coromandel Valley	32.1 (5.8)	1.1 (0.5)	66.8 (5.4)	0	0	67.9 (5.8)
Thelymitra sp.	43.7 (9.5)	6.9 (6.1)	46.5 (2.2)	2.8 (1.4)	0	56.3 (9.5)

Figures

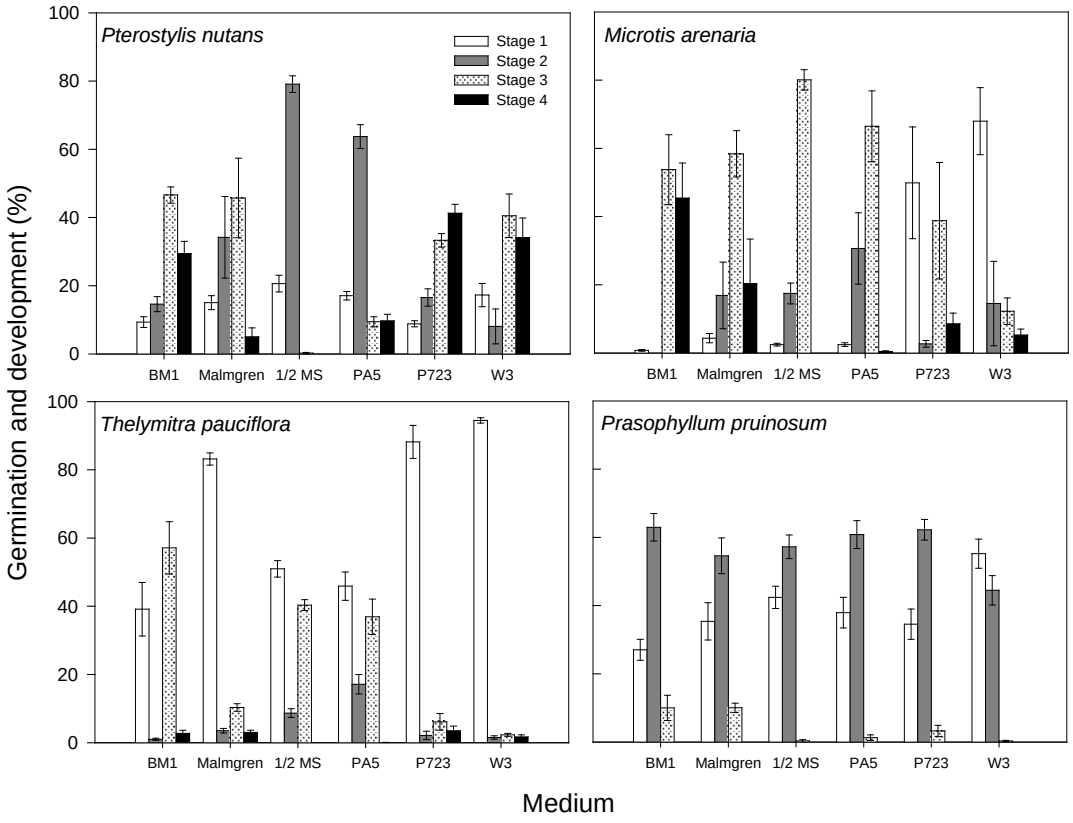

Fig. 1. Effects of culture medium on seed germination and early development of *P. nutans*, *M. arenaria*, *T. pauciflora* and *P. pruinosum* after 10 weeks asymbiotic *in vitro* culture. Vertical bars represent ±SE mean (n = 8).

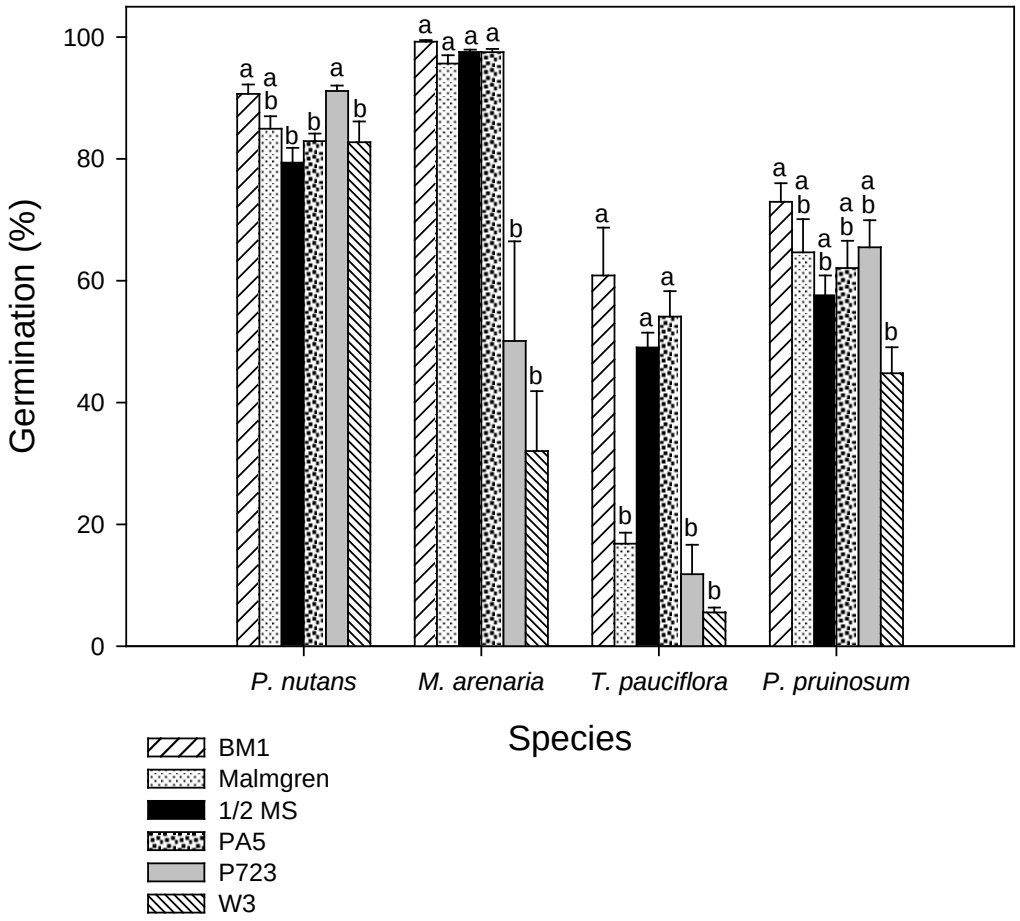

Fig. 2. Final germination percentages for *P. nutans*, *M. arenaria*, *T. pauciflora* and *P. pruinosum* seed after 10 weeks asymbiotic *in vitro* culture on six media. Within each species, histobars with the same letter are not significantly different ($P = 0.05$). Vertical bars represent ±SE mean ($n = 8$).

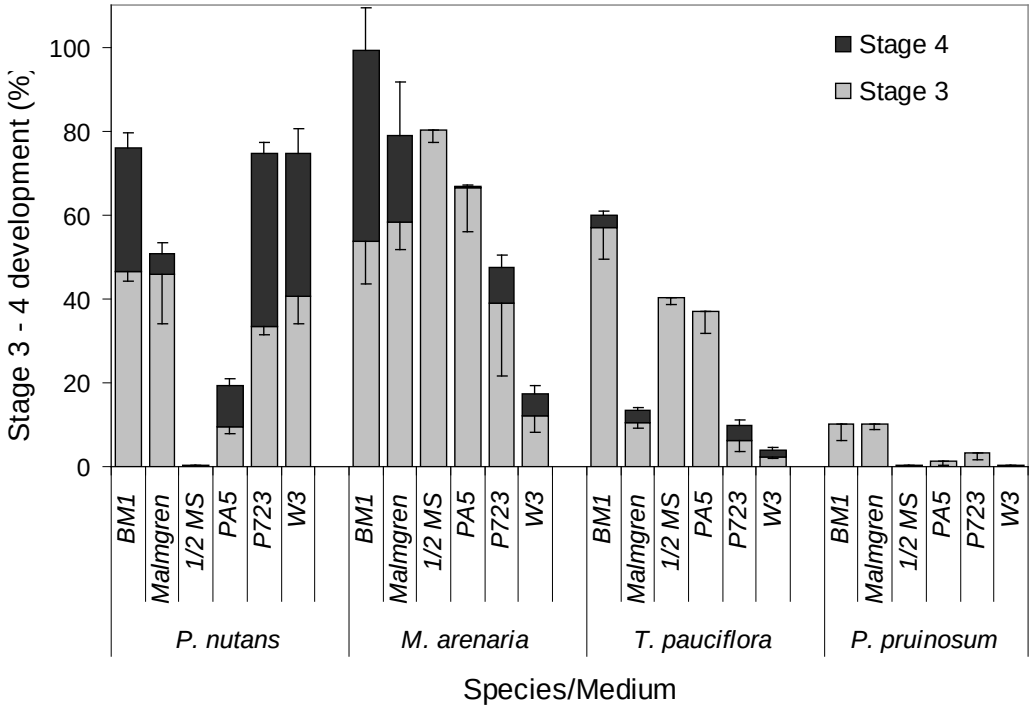

Fig. 3. Effects of culture medium on advanced protocorm development (Stages 3 and 4) for *P. nutans, M. arenaria*, *T. pauciflora* and *P. pruinosum* after 10 weeks asymbiotic *in vitro* culture. Vertical bars represent +SE mean for Stage 4 and –SE mean for Stage 3 (*n* = 8).

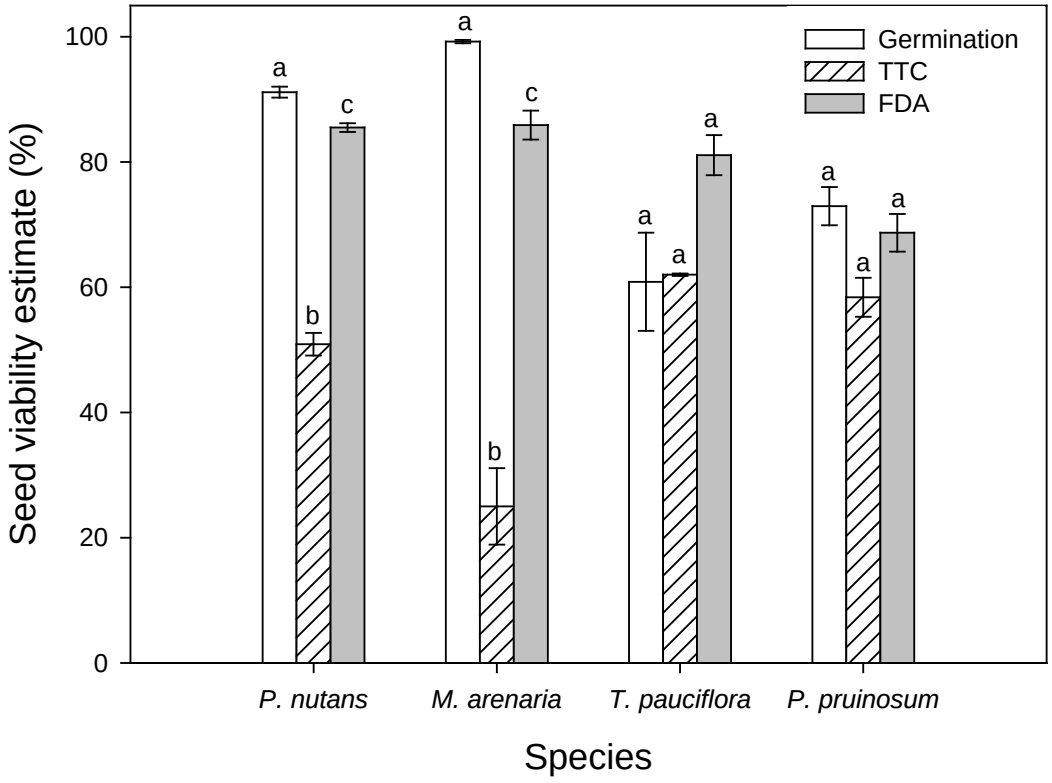

Fig. 4. Comparison of best estimates of seed viability derived from germination tests, TTC and FDA staining for *P. nutans*, *M. arenaria*, *T. pauciflora* and *P. pruinosum* seed. Within each species, histobars with the same letter are not significantly different ($P = 0.05$). Vertical bars represent ±SE mean ($n = 8$, 3 and 6 for germination, TTC and FDA respectively).

Supplementary Table I: Composition of asymbiotic culture media used for germination trial (mg/l unless otherwise specified).

	P723	W3	Malmgren	BM1	½ MS	Pa5 10BA
Ammonium Nitrate	412.5	Proprietary Formulation			825	
Ammonium Sulfate						250
Boric Acid	1.65			10	3.1	2.48
Calcium Chloride, Anhydrous	83				166.1	
Calcium Nitrate•$4H_2O$						1000
Calcium Phosphate, Tribasic			75			
Cobalt Chloride•$6H_2O$	0.0063			0.025	0.0125	0.0096
Cupric Sulfate•$5H_2O$	0.0063			0.025	0.0125	
Na2 EDTA•$2H_2O$	18.65		37.26	37.25	18.63	
FeNa EDTA						38.5
Ferrous Sulfate•$7H_2O$	13.93		27.8	27.85	13.9	
Magnesium Sulfate, Anhydrous	75.18		97.68	100	90.35	
Magnesium Sulfate•$7H_2O$						250
Manganese Sulfate•H_2O	4.23		1.54	25	8.45	
Manganese Sulfate•$4H_2O$						4.46
Molybdic Acid (Sodium Salt)•$2H_2O$	0.0625			0.25	0.125	0.1
Potassium Chloride						250
Potassium Iodide	0.2075				0.415	0.332
Potassium Nitrate	475				950	
di-Potassium Phosphate						250
Potassium Phosphate, Monobasic	42.5		75	300	85	
Zinc Sulfate•$7H_2O$	2.65			10	4.3	3.45
Ascorbic acid						1
D-Biotin			0.05	0.05		0.1
Casein, Enzymatic Hydrolysate			400	500		
Citric Acid (Free Acid) Anhydrous						90
Folic Acid			0.5	0.5		
L-Glutamine				100		
Glycine (Free Base)			2	2		
MES (Free Acid)	500					
myo-Inositol	100		100	100		100
Nicotinic Acid (Free Acid)	1		5	5		1
Pantothenic Acid						1
Pineapple Powder (g/l)			20			
Peptone from Meat	2000					
Pyridoxine•HCI	1		5	0.5		1
Thiamine•HCI	10		10	0.5		1
6-Benzylaminopurine (BA)						22.52
Sucrose (g/l)	20			20		20
Activated Charcoal (g/l)	1		1			
Coconut water (ml/l)		50				50
Banana Pulp (g/l)		50				
Agar (g/l)	8	8	8	8	8	8