ESTABLISHING *EX SITU* CONSERVATION METHODS FOR *DACTYLORHIZA EBUDENSIS* AND *D. TRAUNSTEINERIOIDES*, A COMBINATION OF *IN SITU* TURF REMOVAL AND *IN VITRO* GERMINATIONS

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ABSTRACT

Orchidaceae is one of the most diverse flowering plant families in the world, occupying a diverse range of habitats from epiphytes to terrestrial forms. It is also one of the most vulnerable to changes in land use because of its complex ecological requirements. In nature, orchid seed will only grow if infected with a compatible fungus which provides all the carbohydrates and nutrients needed for its development. This mycotrophic mode of nourishment can persist underground for years in some orchids, which makes them difficult to observe in the wild. Understanding their behaviour is essential for their successful propagation and conservation. In an investigation looking into conservation and propagation, turves were lifted from wild populations of two rare Scottish orchid species in order to ensure the best possible association between these species and their growing environment. A combined *in vitro* experiment was set up for the wild harvested seeds under different media to compare their effects. Two different successful *ex situ* conservation methods for *Dactylorhiza ebudensis* and *D. traunsteinerioides* are presented.

INTRODUCTION

Orchidaceae is the most species-rich plant family and undoubtedly one of the most horticulturally important – orchids fascinate and delight many people (Cribb *et al*., 2003). The key elements responsible for this enormous diversity are their ability to hybridise, their often tight pollination syndromes with specific invertebrate partners and mycorrhizal associations with specific fungal partners (Bonnardeaux *et al*., 2007; Waterman & Bidartondo, 2007). These same traits of specialisation in pollinators and in fungal partners also contribute to the rarity of many species (Swarts *et al*., 2010). Orchid seeds are produced in large quantities, they are minuscule (often referred to as 'dust-like') and they have very small food reserves to fuel germination. In nature, they will not grow unless supported by a suitable fungus, which provides all the carbohydrates and nutrients needed until the plants are large enough to become autotrophic. This nutritional system, referred to as mycotrophic, is obligate during germination and early seedling stage but

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becomes supplementary in most green orchids when they start producing their own food by means of phototrophy (Rasmussen, 1995). Furthermore, orchid–fungal relationships are dynamic through the life of the plant (Bidartondo & Read, 2008), with the fungus that promotes germination not necessarily persisting as the later mycorrhizal associate. These levels of interdependency make orchids very sensitive to changes in their natural environment (Bundrett, 2007), and can make propagation and cultivation particularly challenging. Habitat loss and changes in land use have caused decline and extinction in many wild populations (Swarts & Dixon, 2009). This also makes orchids valuable bioindicators, with their disappearance in a habitat being an indication of further-reaching, below-ground ecological change. Understanding these relationships is fundamental to our ability to propagate and cultivate these orchids for *ex situ* collections and *inter situ* conservation programmes (see Burney, 2009 for a description of *inter situ* conservation).

In the laboratory, orchids can be grown from seed by two different methods: asymbiotically, which makes use of a recipe that contains all the sugars, nutrients and vitamins in a form readily available to the orchid seed, and symbiotically, which uses a simpler recipe, low in carbohydrates and inoculated with a suitable fungus to aid germination and support seedling development. Whilst many tropical epiphytic orchids can be successfully micropropagated by asymbiotic techniques, temperate terrestrial orchids have remained rather difficult. The latter seems to be more successful using symbiotic techniques, especially for rare British orchids (Mitchell, 1989; Muir, 1989). One reason is because temperate terrestrials are more dependent on mycorrhizal associates than their epiphytic counterparts (Yoder *et al.*, 2000). They germinate in the dark and can remain white underground even for years supported mycotrophically (Rasmussen, 1995). Epiphytic orchids, in contrast, germinate in the light with no dormancy and with a green protocorm they start to photosynthesise at a very early stage. Symbiotically propagated plants develop more quickly and seem to be healthier and stronger compared to asymbiotically propagated plants, making them ideal for reintroduction programmes (Mitchell, 1993; Seaton *et al*., 2012). Rasmussen & Whigham (1993) and McKendrick (2000) have

Fig. 1 *Dactylorhiza ebudensis.* Photo: Sidney Clarke.

pioneered methods to understand the symbiotic behaviour in the wild and capture symbionts that can then be used in culture. These researchers used a seed-packet capture method in which orchid seeds are used as 'bait' for fungal symbionts. Specificity of fungal partner is thought to be one factor in the rarity of some terrestrial orchid species (Bonnardeaux *et al*., 2007; Waterman & Bidartondo, 2007); consequently, it is important to capture and characterise the fungal partners *in situ* to successfully conserve them.

In the UK, there are 56 species of native orchids and around 70 in total, all of which are terrestrial. A third of these are considered threatened and ten species are protected under Schedule 8 of the Wildlife and Countryside Act (1981). There are four orchids endemic to the UK: *Epipactis sancta*, *Dactylorhiza occidentalis*, *D. traunsteinerioides* and *D. ebudensis* (Harrap & Harrap, 2009).

Fig. 2 *Dactylorhiza traunsteinerioides.* Photo: Sidney Clarke.

D. ebudensis (Fig. 1) is found only in the Machairs of North Uist (see Fig. 4). These are a very specialised Atlantic coastal grassland characterised by cattle-grazed shelly, well-drained alkaline substrates with high levels of exposure and salt. Their populations are quite stable but because of its localised distribution, the species is considered vulnerable. *D. traunsteinerioides* (Fig. 2), in contrast, grows typically in wet fens raised by nutrient-poor and lime-rich springs. Their populations are scarce and very localised (see Fig. 5), being considered near-threatened, and they are protected under Schedule 8 of the Wildlife and Countryside Act (1981).

This paper reports on two methods for establishing *ex situ* conservation populations of these two species, including turf removal and *in vitro* germination of wild seed with universal fungal strains.

TURF REMOVAL

In order to ensure the best possible association between plants and their *ex situ* growing environment, turves (Fig. 3) were lifted from the wild in two sites for *Dactylorhiza ebudensis*, and three sites for *D. traunsteinerioides* (Table 1).

Fig. 3 *Dactylorhiza ebudensis* turf in Machair, North Uist. Photo: Berta Millàs Xancó.

All collecting permits and licences required were applied for in advance as follows:

- Consent from Scottish Natural Heritage (SNH) to remove plants of *Dactylorhiza ebudensis* from two Sites of Special Scientific Interest (SSSIs)
- Licence from SNH to uproot plants from *Dactylorhiza traunsteinerioides*, a species

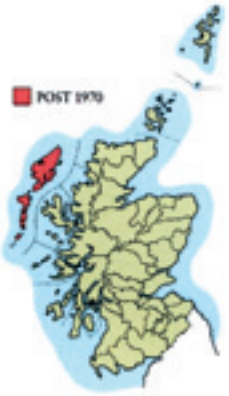

Fig. 4 Distribution map of *Dactylorhiza ebudensis.*

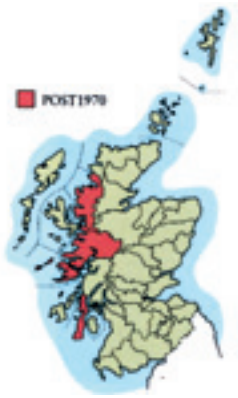

Fig. 5 Distribution map of *Dactylorhiza traunsteinerioides.*

protected under Schedule 8 of the Wildlife and Countryside Act (1981), from three different populations

- Permission from the farmers owning or renting the land

Population surveys were undertaken before any plant removal. Local experts were consulted to locate and identify the sites with the greatest number of individuals (aiming for 100 plants per population at least). No more than 10 per cent of the population was taken to ensure a minimum impact of uprooting in relation to the population size (Rae, 2006). All sites from which turves were harvested are considered to be stable and had large, representative populations.

Turves were dug out to a depth of 250mm with a minimum radius of 100mm from the basal rosette of each plant. Numbers of plant stems per turf varied depending on population density and ranged between one and four (Table 1). Following removal, turves were moistened and then packed in empty black compost bags for the return journey and wrapped up with mesh to prevent cross-pollinations between species.

On return to the Royal Botanic Garden Edinburgh (RBGE), the pH of each turf was measured, and each entire turf was potted up in Airpots™ (Single and Single, 2010), which allowed us to tailor the size of the pot to the turf, minimising root disturbance. The turves were jacketed with sphagnum, which retains moisture and is pH-inert, and grit was used to fill the spaces between the sphagnum and walls of the pots. Pots were kept in a shade tunnel to allow exposure to rain and seasonal variations. Maximum care was taken to cultivate them as naturally as possible. Rainwater or water that had been standing in the open at least overnight was used to water the orchids. Vegetation surrounding the orchids was left undisturbed and trimmed regularly by hand to imitate grazing. Each plant was lodged on the *BG-BASE*™ database (Walter & O'Neal, 1985–2010) and given an RBGE accession number.

Accession number	Orchid species	Grid reference	Turf number	pH	Location
20100819	*D. traunsteinerioides*	NF579.419	T1	6.3	Druim an Aonaich, Isle of Raasay
20100820	*D. traunsteinerioides*	NF578.418	T2	6.5	
20100821	*D. traunsteinerioides*	NF577.415	T3	6.2	
20100822	*D. traunsteinerioides*	NF784.212	T4	6.4	Otter Haven Kylerhea, Isle of Skye
20100823	*D. traunsteinerioides*	NF784.212	T5	6.3	
20100878	*D. traunsteinerioides*	NF784.212	T6	6.3	
20100879	*D. traunsteinerioides*	NF374.659	T7	6.2	Totescore, Scuddaburgh farm, Uig, Isle of Skye
20100880	*D. traunsteinerioides*	NF374.659	T8	6.4	
20100881	*D. traunsteinerioides*	NF374.659	T9	6.4	
20100882	*D. traunsteinerioides*	NF374.659			
20100883	*D. traunsteinerioides*	NF374.659			
20100884	*D. ebudensis*	NF869.761	T10	6.6	Cemetery at Druim na Croise, North Uist
20100885	*D. ebudensis*	NF869.761			
20100886	*D. ebudensis*	NF869.761			
20100887	*D. ebudensis*	NF869.761			
20100888	*D. ebudensis*	NF870.761	T11	6.5	
20100889	*D. ebudensis*	NF870.761			
20100890	*D. ebudensis*	NF877.766	T12	6.6	Cemetery onto the Machair Robach, North Uist
20100891	*D. ebudensis*	NF877.766			
20100892	*D. ebudensis*	NF877.766	T13	6.5	
20100893	*D. ebudensis*	NF877.766			

Table 1 Collection data for the species studied.

In order to obtain seed from each species, they were hand-pollinated using tweezers, in each case outcrossing between the turves. The turves were then protected with mesh to prevent further cross-pollinations. Capsules were marked with red ribbon and then harvested when mature – brown, but not dehisced, to avoid loss of seeds and minimise environmental bacteria or fungi spore contamination. Ripening times were taken from published guidelines. In the case of *Dactylorhiza*, the framework was between six and ten weeks (Harrap & Harrap, 2009).

Results

Following two years of observation, the turves have established themselves well and there are currently fourteen stems (Table 2): eight of *Dactylorhiza ebudensis* and six of

D. traunsteinerioides. This means an increase of six rosettes since 2011. The reduction in numbers from harvest to the first year of *ex situ* cultivation could be due to the stress of the move and/or the plants remaining in dormancy. The period from germination to flowering time for most *Dactylorhiza* is four or five years (Harrap & Harrap, 2009) but this may vary subject to environmental conditions and soil nutritional levels (Vermeulen, 1947). The exact duration of the early underground stage remains unclear and can be varied between orchid groups (Rasmussen, 1995). *Dactylorhiza* spp. annually replace the underground tuber and roots by producing a new tuber that will sustain the next year's growth alongside the current one that will shrivel away gradually at the end of the season (Harrap & Harrap, 2009). It is interesting to see the increase in numbers of individuals in turf 13 beyond the numbers originally harvested, with one emerging with completely purple-flushed foliage. The newly observed shoots probably represent individuals that were in a mycotrophic underground phase. It seems clear that the fungal partners remain present and healthy. The vegetation surrounding the orchids which is still present and managed also serves as an indicator that the microorganisms held in most of the turves have remained sustainable.

Turves		Number of individuals – harvest 2010	Number of individuals – 2011	Number of individuals – 2012
T1	*Dactylorhiza traunsteinerioides*	1	1	1
T2		1	1	1
T3		1	–	–
T4		1	–	–
T5		1	1	1
T6		1	–	–
T7		1	–	1
T8		1	–	–
T9		3	2	2
T10	*Dactylorhiza ebudensis*	4	–	1
T11		2	–	2
T12		2	2	1
T13		2	1	4

Table 2 Numbers of observed individuals on each turf each year. Note the 2012 figure was taken on 4 May 2012, so more plants may emerge in summer 2012.

In general plants have been pest free. At present, no additional nutritional additives have been employed to modify the microhabitats. It is intended to foliar-feed them with a dilute organic extract of seaweed so as not to upset the balance between the orchid and

the fungi. It is known that high levels of nitrogen can cause fungi to become too vigorous and orchids can suffer and die off as victims of parasitism (Mitchell, 1993). Another good layer of sphagnum moss will be added to the turves for water retention. Plants will be cross-pollinated by hand and seed harvested from all plants flowering this year.

In 2011 leaf material from the three different populations of *D. traunsteinerioides* was donated to the DNA barcoding project at RBGE. Seed donations and collection data for *D. ebudensis*, *D. traunsteinerioides* and *Platanthera bifolia* were lodged with Royal Botanic Gardens, Kew and The Millennium Seed Bank.

IN VITRO PROPAGATION

In order to compare symbiotic versus asymbiotic propagation for raising *Dactylorhiza ebudensis* and *D. traunsteinerioides* and determine which of the two methods was more successful, we used three known universal fungal symbionts obtained from RBG Kew cultures. Two other orchid species were included in the trial as a control: the common *D. purpurella*, and *Platanthera bifolia*, a UK Biodiversity Action Plan species.

Seeds from *Dactylorhiza ebudensis* and *D. traunsteinerioides* were harvested from the *ex situ* turves at RBGE on 9 September 2010, seeds from *Platanthera bifolia* were harvested near Loch Leven, in Kinross, on 20 August 2010 and seeds from *D. purpurella* on 21 August 2010 at RBGE from plants in the alpine collection.

Capsules were labelled and stored following Thompson (1977) methods in a desiccator at room temperature (20ºC) with a saturated solution of calcium chloride. This solution at this temperature reduces the humidity level of the seed to around 5–6 per cent, which is the optimum for seed storage. After four days, when seeds were optimally dry, they were cleaned following methods described by Seaton & Ramsey (2005). Prior to sowing, seeds were sterilised by soaking them in 0.1 per cent sodium hypochlorite solution and a drop of detergent for 20 minutes. Sterile deionised water was used to rinse the seeds and help to wash any sodium chloride away (Seaton & Ramsey, 2005).

Each orchid species was cultured with each of the three fungi, plus one standard asymbiotic growth medium (Table 3), used as a control. The symbiotic media were prepared with a basic receipt recommended by Clements *et al.* (1986) (Table 3). The

Asymbiotic media		**Symbiotic media**	
20 g/l	Sucrose	3.5g/l	Powdered oats
1g/l	Charcoal	0.1g/l	Yeast extract
6g/l	Agar	6 g/l	Agar
25mls/l	Pineapple juice		
28 mls/l	Minerals (macro and micro)		
0.75mls/l	Seaweed extract		

Table 3 Asymbiotic and symbiotic media recipes.

medium previously autoclaved was poured under the laminar flow into sterile plastic petri dishes (9cm diameter) and allowed to set. The symbiotic medium was inoculated with 5mm squares of fungus for each of the three species. Between four and six replicates were produced per species (Table 4). Between 25 and 200 seeds were sown on the set medium with a glass rod, and it was attempted to place the seeds in rows to facilitate scoring.

Cultures were sealed with laboratory film, arranged in trays and grown in the dark in a growing cabinet at a constant temperature of 18ºC. Seed germinations were examined under a light microscope monthly for a period of three months and scored to a standardised germination stage scale of 0–5 (Muir, 1986; Clements *et al.*, 1986) (Table 6). Germination was considered to have taken place when a rupture of the seed coat was noticed.

Observations were continued beyond this to monitor asymbiotic germinations, as they tend to take longer. After three months, means (+/- standard error) of total germinations were worked out for each of the treatments to determine whether the differences between treatments were significant.

Code	Isolate species	Host orchid/life cycle-stage	Origin/donor
F550	*Cerotobasidium cornigerum*	Unknown	International Mycological Institute (IMI) culture collection
F414	*Rhizoctonia* sp.	*Dactylorhiza iberica* (mature stage)	RBG Kew alpine collection
F21	*Tulasnella* sp.	*Liparis sp.* (seed germinating stage)	RBG Kew fungi culture collection

Table 4 Fungal isolates used to inoculate the symbiotic media.

Results

The results from the trials after three months showed a clear higher percentage of germinations in symbiotic versus asymbiotic treatments (Table 6). *Ceratobasidium cornigerum* fungus (F550) promoted the highest percentage of germinations in *Platanthera bifolia*, *Dactylorhiza ebudensis* and *D. purpurella*, and *Tulasnella* sp. fungus (F21) in *D. traun-*

Stage number	Visible evidence
0	No germination
1	Germination of seed with rupture of testa
2	Production of rhizoids
3	Appearance of leaf primordium
4	Production of first chlorophyllous leaf
5	Production of root initial

Table 5 Seed development stages and visible evidence of the stages reached (Muir, 1986; Clements *et al*., 1986).

steinerioides. However, F21 appeared to stimulate plants to reach the most advanced protocorm stages (Table 6) in *D. purpurella* (stage 4), *D. traunsteinerioides* (stage 3) and *Platanthera bifolia* (stage 2). *D. ebudensis* reached its furthest development with F550 (stage 2). Asymbiotic germinations after three months were very low in all orchids with a maximum stage 2 being reached in *Platanthera bifolia*, *D. traunsteinerioides* and *D. purpurella* and with no germinations (stage 0) in *D. ebudensis*. Table 5 describes the development stage numbering and corresponding visible evidence of these.

	Platanthera bifolia		***Dactylorhiza ebudensis***		***Dactylorhiza traunsteinerioides***		***Dactylorhiza purpurella***	
		Stage		**Stage**		**Stage**		**Stage**
F550	18.8%±2.75	1	24.01%±9.49	2	36.16%±10.44	1	80.45%±3.79	2
F21	8.52%±1.63	2	0%±0	0	42.76%±12.05	3	28.40%±3.69	4
F414	3.90%±0.84	1	2.98%±1.78	1	9.56%±4.17	1	16.11%±2.69	2
Asymbiotic	1.00%±0.39	2	0%±0	0	2.85%±1.45	3	1.10%±1.10	2

Table 6 Germination trial results after three months, showing percentage germinations (mean ± standard error) and development stage reached in symbiosis (see Table 5).

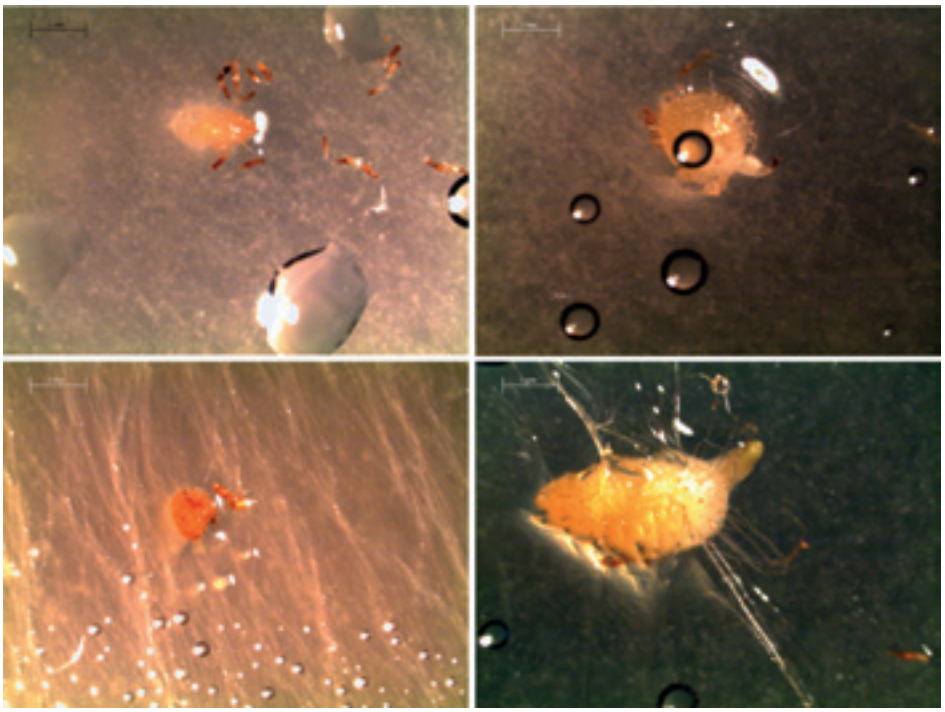

Fig. 6 Effect of f21 on each species: (top left) *Platanthera bifolia* reached stage 2; (top right) *Dactylorhiza traunsteinerioides* reached stage 3; (bottom left) *Dactylorhiza ebudensis* reached stage 2; and (bottom right) *Dactylorhiza purpurella* reached stage 4. All photos have a 1mm scale bar and were taken with the same magnification. Photo: Berta Millàs Xancó.

Discussion

Seed germinations occurred in almost every treatment, except for *Dactylorhiza ebudensis*, which did not germinate in asymbiotic media or with symbiont F21. Otherwise each universal fungus supported germinations in *D. traunsteinerioides*, *D. purpurella* and *Platanthera bifolia*; with F21 promoting their further seedling development stage (see Fig. 6).

Germinations are indeed more prevalent in symbiotic treatments than asymbiotic treatments. Symbiotic germinations did not show much difference between month 2 and month 3 observations. Eight weeks is sufficient time to assess whether the symbionts have been compatible or not. However, observations were carried out for a further three months and it was apparent that symbiotic germinations seldom advanced beyond stage 3. This suggests that the universal fungi can promote germination, but a partner-switch may be required to support plants in later developmental stages. The prolonged observation showed that asymbiotic germinations are slower, ultimately producing 10.3 per cent germination in *D. purpurella* and 18.05 per cent in *D. traunsteinerioides*. In both of these cases, plants have reached stage 4, but will require subculture and prolonged periods of acclimatisation if they are to be cultivated in standard pots and grown outside. This is also the case for protocorms reaching stage 4 with f21 for these same species.

Platanthera has proved difficult to cultivate in the past due to strong dormancy mechanisms (Rasmussen, 1995). In this experiment, germination occurred and reached stage 2 (Muir, 1986). However, stage 2 is sometimes only induced by fungal contact and the fungus may become parasitic beyond this stage. This may have been the case in *P. bifolia*, as no germinations reached stage 3 within the observation period. *Ceratobasidium* (F550) appeared to do this in *D. traunsteinerioides* as well, with high germinations (36.16 per cent) and apparent initial symbioses shifting to pathogenesis. The fungus grew vigorously out of control for the orchid.

The rare Scottish endemic *D. ebudensis*, which is highly specialised and confined to Machair soils, did poorly in all treatments – this may be due to a tighter obligate symbiosis with a more specialised fungal partner adapted to the dry Machair. Consequently, it is particularly important to find the wild symbionts of this plant.

Broadly speaking, for orchid conservation basic research into the natural life cycle of species of interest and wild partners should be carried out. If no information is available, it is recommended to try both methods, asymbiotic and symbiotic, simultaneously (Seaton *et al*., 2012). In order to capture orchid-specific fungi, packets containing seeds of the two target species (*D. ebudensis* and *D. traunsteinerioides*) were buried in the respective field sites in October 2010 and harvested in November 2011, with a repeat set reburied at the same time. The intention for these is to capture specific fungal associates at the germination stage and/or seedling stage.

CONCLUSIONS

Although it might seem radical to remove entire plants and surrounding turf for rare orchids, this has proved to be a successful method of establishing an *ex situ* population. Removal of the plants is only possible in those populations where there are enough individuals to cope with the loss, in this case >100 individuals.

Although labour-intensive, it is evident that a combination of turf removal and micropropagation (both symbiotic and asymbiotic) is ideal, allowing orchids to be conserved and studied *ex situ*, as well as uncovering further information about their life cycles, which is a key component in their successful micropropagation. Developing these micropropagation techniques is essential for populations and species that are too few in number to support turf removal programmes. Isolation of wild fungal partners using seed trapping methods is also essential to these efforts in order to develop species-specific *ex situ* micropropagation protocols. The *ex situ* turf populations provide a ready supply of seed for such trapping experiments.

This method has been successful for *D. ebudensis*. Putative symbiotic fungal cultures have been isolated from wild seed-capture experiments and will be used for symbiotic germination and the fungi characterised using DNA sequences. This can then be compared with fungi isolated directly from adult plants in the turf cultures. Findings will be reported in a future paper.

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