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Seed Germination and Propagation of *Arachnorchis formosa*©

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Symbiotic and asymbiotic seed germination methods were investigated to maximise the production of the endangered orchid *Archnorchis formosa* (G. W. Carr) D.L. Jones et M.A. Clem. (Orchidaceae) for the re-establishment phase of its recovery plan. Mycorrhizal fungi were isolated from adult plants in the wild at various stages in the orchid life cycle (budding, leafing, flowering, capsule production, and senescence). Seed was germinated on minimal (oatmeal agar) and complex (PA5 containing coconut water) media with and without mycorrhizal fungi under axenic conditions. Germination and subsequent growth was recorded at monthly intervals for a period of 12 months. Mycorrhizal status of seedlings was determined by microscopic examination using SEM. Seed grown on minimal media inoculated with mycorrhizal fungi from the leafing, budding, and flowering stages gave fastest (within 1 month) and highest (>50%) germination rates. Seed grown on the complex media did not germinate in the 1st month, however those seeds that did germinate later achieved higher rates (>95%) than those on minimal media.

Symbiotic seedlings grown on minimal medium were able to be deflasked and survived to produce tubers under nursery conditions. Although seedlings produced on complex media had higher germination percentages than their counterparts on minimal media, none survived the deflasking process.

This study has shown that minimal media and mycorrhizal fungi isolated from actively growing adult plants (i.e., budding, leafing, and flowering stages) can enhance the germination and subsequent growth of seedlings of the endangered spider orchid *A. formosa.* This information can now be used to produce plants for re-introduction in the wild.

INTRODUCTION

Orchid seed is minute with minimal nutrient reserves so that the embryo relies on external sources of nutrients for growth and development (Rasmussen, 1995). Previous researchers have suggested that all orchid seeds can be germinated as long as essential nutritional requirements are met, extending to undefined complex compounds that can be artificially supplied (Knudson, 1946; Vacin and Went, 1949; Arditti and Krikorian, 1996). However, no single solution applies to all orchids, with some requiring specific compounds too complicated for laboratory analysis or horticultural practicality.

Alternatively, nutrients can be supplied via mycorrhizal fungi (Rasmussen and Whigham, 1993; St. George, 1998). Several studies have traced nutrient exchanges between orchid and fungus indicating a beneficial relationship (Arditti, 1992; Smith et al., 1994; Smith and Read, 1997). Others have observed larger and faster growing protocorms are produced with mycorrhizal fungi than asymbiotic controls (Jorgensen, 1995; Jusaitis and Sorensen, 1993). Populations of Victorian native orchids are declining due to factors such as habitat disturbance and low seedling recruitment. Recovery plans exist for rare and endangered orchid species, and an essential part of any recovery plan is to re-establish populations in the wild (F. Coates, pers. comm., 2001). However re-establishment is hampered by difficulties in propagation and failure of re-establishment plantings (Clements, 1981; Bohm, 1991).

The aim of this study was to investigate the effect of mycorrhizal inoculum and type of media on the germination and subsequent growth of the endangered spider orchid, *A. formosa*.

MATERIALS AND METHODS

Seed Collection and Fungal Isolation. Ten whole seed capsules were collected in the field in Nov. 2000. Air-dried seed was then gently scraped from all capsules and combined before being stored in paper envelopes in sealable plastic bags at 4°C until required. Fungal isolates were obtained from single pelotons in adult plants of *A. formosa* at the leafing (LOA), budding (BOA, BOB), flowering (FOA), capsule formation (COA), and senescence (SOA) stages over one season in 2000.

Germination. Seed was soaked overnight in 1% Tween-80® and water (v/v) before surface sterilisation with 0.5% $Na(OCl)_2$ for 3 min. Sterilised seed was rinsed clean with sterile water three times and plated onto a sterile filter paper on top of either OMA (2.5 g oatmeal, 12 g sucrose, 0.1 g yeast extract, 6 g agar in 1-litre distilled water, pH 5.3) or PA5 (Collins and Dixon, 1992) media.

The plated seed was then inoculated with the fungal isolates. An uninoculated asymbiotic control was also included. Plates were then placed at 20°C under artificial light and examined for germination at monthly intervals. Germination was graded as 0=no germination, +=<1 cm leaf, ++=>1 cm leaf, and +++=tuberisation.

To determine mycorrhizal status the emergent seedling and protocorms were sectioned and prepared as follows for viewing by SEM, fixation in 2.5% gluteraldehyde, postfixed in 1% osmium tetroxide, dehydrated in an alcohol series, and critical point dried. Samples were mounted onto stubs, gold coated, and viewed with a Philips XL30 scanning electron microscope (Beyrle et al., 1995).

Deflasking. Seedlings at stage + were transferred to culture tubes with fresh media and inoculum. At stage ++, seedlings were potted in seedling punnets and acclimatised through fogging, misting, and shade houses. After 3 weeks, punnets were taken to an outside growing area with an overhead watering system. Twelve months after germination punnets were upturned and examined for tuberisation.

RESULTS

Data was analysed by ANOVA. All values were average percentages with standard deviations in parenthesis. Due to heavy seedling losses, tuberisation was based on observational estimates only (Table 1).

Successful germination of greater than 50% (stage +) occurred within 1 month only on minimal OMA medium with isolates LOA, BOB, and FOA (Table 1, Fig. 1A). Protocorm quality (growth and development) was superior and resulted in tuber formation after 12 months from germination (Table 1, Figs. 1B-C). Tuberisation was less than 30% (observational estimates).

Germination occurred on complex PA5 media only after 12 months (Table 1) but none progressed beyond stage + and few tuberised (<1%) in the laboratory (Fig. 1D). Losses of 100% were experienced during deflasking stages and seedlings did not survive without PA5 media in tissue culture conditions (Table 1).

Fungal isolates BOA, COA, and SOA inhibited germination on both minimal and complex media. Microscopic examination of seedlings germinated symbiotically with isolates LOA, BOB, and FOA on OMA and PA5 contained mycorrhizal colonisation (Fig. 1E). Twelve-month-old asymbiotic seedlings germinated on PA5 were devoid of pelotons (Figure 1F).

Table 1. Seed germination and propagation percentages ± standard deviations of *Archnorchis formosa* on minimal (OMA) and complex (PA5) media#.

Fungi	1 month		12 Month°	
	PA5	OMA*	PA5	OMA
None	0	0	95 +	0
LOA	0	53.4 ± 11.7*	95 +	10 $^{+++}$
BOA	0	0	0	0
BOB	0	51.6 ± 5.6*	95 +	10 $^{+++}$
FOA	0	65.8 ± 2.5*	95 +	30 $^{+++}$
COA	0	0	0	0
SOA	0	0	0	0

Scoring definitions: 0=no germination, +=<1cm leafing, +++=tuberisation.
* No significant difference P >0.15 between LOA, BOB, FOA.
° observational estimates.

DISCUSSION

The results of this study show that mycorrhizal inoculum can improve the germination of seed and subsequent growth and development of seedlings of *A. formosa.* Interpretation of germination has become confused as researchers have applied the term to describe stages as varied as imbibed (swollen) seed to protocorm formation

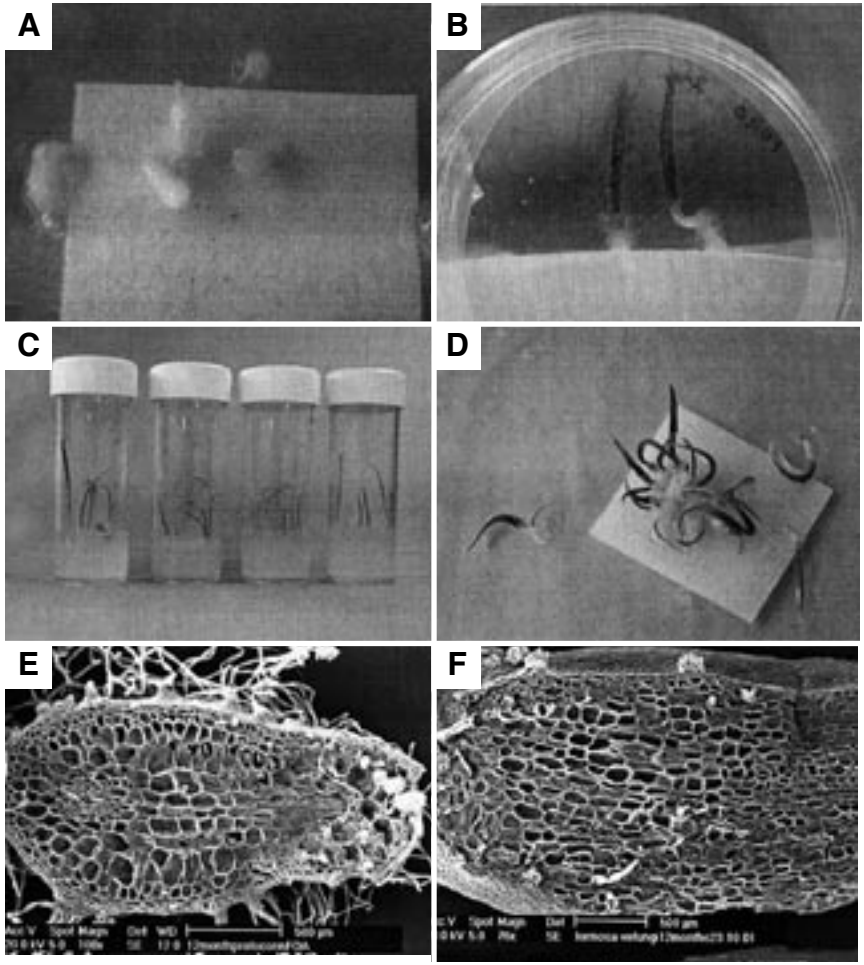

Figure 1. Stage + protocorms grown with isolate FOA on OMA media within 1 month (A). Stage ++ seedlings grown with isolate FOA on OMA media within 6 months (B) and 12 months (C). Stage + seedlings grown asymbiotically on PA5 in 12 months (D). Stage + seedlings grown with isolate FOA on PA5 in 12 months (E). Asymbiotically germinated seedling after 12 months on PA5 (F).

and leaf production stages (Knudson, 1946; Warcup, 1973; Bohm, 1991; Masuhara et al., 1993; Oddie et al., 1994; McKendrick, 1996) which makes comparison between studies difficult. Imbibition of many temperate and tropical orchid seed readily occurs without mycorrhiza however in this study germination was defined as formation of a protocorm and leaf production.

Asymbiotic germination did occur after 12 months on the complex medium (Fig. 1F), however, seedlings did not survive the deflasking process (Table 1). This could be due to asymbiotic protocorms being unable to fight off attack from pathogens as well as an inability to access nutrients without a mycorrhizal fungal partner in media with low nutrient levels. Beyrle et al. (1995) showed that asymbiotically grown protocorms had low levels of defensive chemicals (PAL and orchinol) in their tissues compared to symbiotic protocorms, and did not form mycorrhizal relationships when nutrients were readily available. PA5 is a complete medium containing high nutrient levels which may have lowered orchid defences and discouraged mycorrhizal symbiosis so that when the plants were deflasked into a low nutrient medium they were unable to access nutrients and did not survive. Similar observations have suggested using simple media (oatmeal) for symbiotic germination and complex media (PA5) for asymbiotic germination (Jusaitis and Sorensen, 1994; Oddie et al., 1994) to encourage fungal symbiosis.

In contrast three mycorrhizal isolates significantly shortened the length of time to reach deflasking stage and produced better quality plants (faster growth rate, bigger leaf and protocorm production) that were able to survive deflasking (Table 1, Figs. 1A-C). Mycorrhizal fungi have previously been shown to accelerate orchid germination (Clements, 1981; Jusaitis and Sorensen, 1993, 1994) and improve survival of symbiotic orchid seedlings (Arditti, 1967; Masuhara et al., 1993; Oddie et al., 1994; Perkins et al., 1995).

Fungi able to form beneficial mycorrhizal relationships can be manipulated to upsurge the symbiotic balance; becoming accustomed to supplied nutrients and competing with seeds when nutrient levels decline. Such opportunistic fungi have responded favourably to media alternations from being passively mycorrhizal to invasively pathogenic especially when supplied with high nitrogen (Beyrle et al., 1995; Dijk and Eck, 1995) or rich medium (Beardmore and Pegg, 1981). Chang and Chou (2001) suggest that complex media allowed for fungal invasion rather than colonisation, thereby dominating orchid defences and resulting in orchid death. However evidence of pelotons in confined regions indicate a mycorrhizal relationship, but failure to develop beyond leafing suggest inhibitory rather than detrimental factors being exhibited.

The seedling losses at deflasking seen in this study have been commonly encountered by others (Clements, 1981; Bohm, 1991) however the production of tubers by laboratory germinated seedlings is encouraging, as tubers are required for plant re-emergence (Clements, 1981; Rubluo et al., 1989). This procedure can now be used to increase the population of *A. formosa* and may be appropriate for other rare and endangered orchids.

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