

**PROPAGATION AND SEED VIABILITY OF THE ENDANGERED ORCHID  
*PRASOPHYLLUM CORRECTUM* D.L. JONES (GAPING LEEK-ORCHID)**

**Final report to the Australian Flora Foundation**

**Project 97/98-14**

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**December 1999**

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## SUMMARY

The Gaping leek orchid, *Prasophyllum correctum* D.L. Jones, is a Critically Endangered (IUCN 1994) taxon with a total population in Victoria of approximately 130 plants. *P. correctum* regenerates from seed, and occasionally by tuber division, but little recruitment has been observed in the wild.

Seed viability, isolation of fungal symbiont, and germinability of seed at 5 days, 90 days, 150 days, 270 days and 365 days after collection, using symbiotic and asymbiotic methods, were investigated. Future efforts to increase population size in Victoria may rely on *ex-situ* cultivation and translocation, so that methods to evaluate the propagation potential of the taxon are required.

Results from viability tests using fluorescein diacetate (FDA) were lower overall than germinability. Triphenol tetrazolium chloride (TTC) tests were difficult to prepare and interpret, and the method was abandoned. Mycorrhizal fungus (Basidiomycetes) was isolated from *P. correctum* roots and effectively promoted germination of fresh (5 days old) seed (38%). However, it was not effective using 90, 150 or 270 day old seed. Germination trials using 365 day old seed were destroyed by mites. Sporulation could not be induced in culture and the fungus remained unidentified.

Asymbiotic methods were more successful overall in germinating stored seed. Although not effective on fresh seed, there was significantly higher germination compared to media inoculated with fungal culture, at 90, 150 and 270 days. However, percentage germination was relatively low (<20%). Mites also destroyed 365 day old seed used in trials.

Overall, seed viability appears to be highest immediately after collection (38%) but is significantly lower at 90 days (19%). However results were variable, and are likely to have been influenced by storage conditions of both seed and fungal culture. The literature suggests that symbiotic methods are more likely to be appropriate if plants are to be translocated. Future efforts to propagate *P. correctum* should use either fresh seed and cultures, or biological material stored in liquid nitrogen, to maximise viability and effectiveness.

## INTRODUCTION

*Prasophyllum correctum* is one of the most critically endangered orchids in Victoria. Two populations are known from remnant grassland and grassy woodland on rail-line easements in Gippsland, Victoria, and one population occurs in Tasmania. In Victoria, 137 plants have been recorded, while the Tasmania population consists of at least 1,000 plants (Coates *et al.* 1999). The population ecology and management requirements of *P. correctum* are currently being investigated and direct seeding trials using buried seed to establish new recruits have been initiated (Coates *et al.* 1999). However, there is an urgent need to develop propagation methods for *P. correctum* in Victoria, as a preliminary step toward evaluating the efficacy of re-introducing plants to existing populations, and to sites where the orchid is already extinct. As a first step, it is necessary to determine whether plants can be propagated from seed.

This information is pivotal to the development of techniques for recovery of populations, where orchids are not known to readily regenerate vegetatively, as in *Prasophyllum*. Propagation of endangered terrestrial Australian orchids is emerging as a vital component of their conservation but has been relatively neglected in Victoria. The aims of this project were to determine *P. correctum* seed viability and germinability.

## METHODS

### Seed and endophyte collection

Capsules were collected immediately prior to dehiscence from 1 population near Munro, Victoria (37° 55' 10" S 147° 11' 04" E), in late November 1997 (6 plants), and late November 1998 (18 plants). Following collection, capsules were placed in paper bags and stored at room temperature for 5 days in a cardboard box, then transferred to a domestic refrigerator and stored at 5°C until used for germination trials.

Root sections were collected from three plants from the Munro population in late July 1998. Plants were gently excavated and short sections of root material excised. Plants were then replaced and watered. Continued monitoring of the population in 1998 and 1999 showed that flower development was uninterrupted and there was no plant mortality.

### *Viability testing*

Seeds collected in 1997 were soaked in filtered triphenol tetrazolium chloride (TTC) solution (1 g in 100 ml phosphate buffer, pH 6.5-7.0) for 48 hours in darkness at 20°C and rinsed five times in sterile distilled water. Seeds were agitated between coverslips to remove the testa and viewed using a light microscope. Embryos completely coloured pink to red were considered viable, whilst seeds with embryos partially coloured, white, yellow or brown were assumed not viable (Van Waes and Deberg 1986).

Seeds of the same plants were soaked in equal volumes of freshly filtered fluorescein diacetate (FDA) solution (0.5 g in 100 mL absolute acetone) and distilled water for 15 min and viewed using an Olympus BH-2 (UV light) fluorescence microscope. Seeds with embryos completely stained (fluorescent) were considered viable (Rasmussen 1995). The same test was repeated on seeds collected in 1998 at 5 days, 90 days and 150 days. There were insufficient seeds for testing at 270 days.

### *Fungal extraction and culture*

Root sections were washed in running tap water and soaked for 2 h in distilled water.

Method **A** removed the velamen to reveal inner and outer root sections. Cells were teased with sterile needles to release pelotons from the cortex, which were subsequently touched onto Melin-Norkrans agar (MMN) (Warcup and Talbot 1966).

Method **B** sterilised roots in 2% NaOCl for 5 minutes. Sections were rinsed twice and soaked for 30 minutes in autoclaved Milli-Q® water (Jusaitis and Sorensen 1993). Thin transverse sections of the root (2-5 mm) were cut, the velamen was removed, squashed, plated on MMN and stored at 25°C in darkness. After one week, subcultures were plated onto liquid and solid OMA. Pure cultures were obtained for inoculation and identification.

### *Germination trials and media*

Seeds aged 5, 90, 150, 270 and 365 days were used to evaluate the effect of storage time on viability and germinability. Each trial consisted of 6 - 12 replicates of about 100 seeds for each treatment.

Preliminary tests were conducted to investigate the effects of surface sterilant concentration (0, 0.1 % or 1 % Ca(OCl)<sub>2</sub>), photoperiod, temperature and medium (Huynh 1999). These suggested that the optimum environment for germination would be autoclaved (121°C, 15 psi) oatmeal medium (OMA) plates (2.5 g oatmeal, 12 g sucrose, 6 g agar in 1 L distilled water, pH 5.3), inoculated with fungal isolates for symbiotic trials. Asymbiotic trials were uninoculated.

Plates were incubated in 20°C darkness for 7 days and sterile (autoclaved at 121°C for 5 minutes) Miracloth® (Calbiochem, USA) circles placed on top. Seeds were placed onto Miracloth® using a chisel shaped probe and plates double sealed with Parafilm. These were stored in darkness at 20°C and checked for germination every week. Germination was scored as significant swelling of the embryo, rupture of the testa and rhizoid formation. Resulting protocorms were grown in a 12 h photoperiod, under Osram "Warm White" fluorescent tubes at 27  $\mu\text{E m}^{-2} \text{s}^{-1}$  at 25°C and developmental stages recorded.

All fresh and stored seeds were incubated at room temperature for 1 h before treatment. Pre-treatments were 24 h in sucrose (1% w/v) containing Tween-80® (1% w/v). After

several washes in sterile water, the seeds were surfaced sterilised in 1%  $\text{CaOCl}_2$  for 5 min twice, rinsed clean with water and soaked overnight.

### *Statistical analysis*

Percentages of viable seeds from asymbiotic and symbiotic germination trials were tested for normality and analysed statistically using Analysis of Variance (ANOVA) to compare mean percentage germination of seed age for each medium. Results were considered statistically significant at  $p < 0.05$ .

## **RESULTS**

### *Viability testing*

Interpretation of seed viability using TTC was very difficult. Viewing of the embryo was only possible after removing the testa, a tedious and delicate process since orchid seeds are minute. Staining colours that distinguished viability were very difficult to determine and the method was abandoned.

By contrast FDA was an excellent stain for observing viable seeds. Fluorescence was easily recognised and stained seeds were easily counted without removing the testa, which is transparent.

Seed viability was highest at 5 days (26%), but was significantly less at 90 days (11%), 150 days (5%) and 365 days (10%). There was insufficient seed for testing at 270 days (Table 1).

### *Fungal extraction and culture*

Fungi were present in *P. correctum* roots in defined regions of the outer cortex and velamen. Most pelotons were intact, although some were collapsed.

Four isolates were obtained using Method A, and six isolates by Method B. Two isolates from Method B developed clamp connections, although dolipore septa were difficult to observe using light microscopy. Hyphal pigmentation (white growth) suggested fungi belonged to the Basidiomycetes. Both were used for germination trials.

### *Germination trials*

Symbiotically germinated seeds of different ages varied significantly in their germinability ( $F = 26.0$ ,  $p < 0.001$ ). The symbiotic method was most effective on seeds < 5 days old (38%) but germination was zero or near zero at 90 days, 150 and 270 days. Germination trials of seeds aged 365 days were lost as a result of mite infestation (Table 1).

There was no significant difference in asymbiotically germinated seed of different ages ( $p > 0.05$ ) other than between seed aged 90 days and 150 days (Table 1). At 5 days, no seeds germinated but at 90 days germination increased to 20%. Subsequently, germination dropped at 150 days (6%) but increased at 270 days to 16%. Mites destroyed the 365 day trial (Table 1). Overall percentages of seed germinated asymbiotically were significantly higher than seed germinated by symbiotic methods ( $p < 0,005$ ).

Germinated seeds developed into protocorms but no further development was observed.

**Table 1.** Mean percentage germination ( $\pm$ SE) for viability tests (FDA) and germination trials using media inoculated with fungal symbiont (symbiotic) and uninoculated media (asymbiotic).

Seed Age (days)	Trial		
	<i>FDA</i>	<i>Symbiotic</i>	<i>Asymbiotic</i>
5	26 $\pm$ 24	38 $\pm$ 7	0
90	11.4 $\pm$ 10	0	19 $\pm$ 3
150	5 $\pm$ 3	0.5 $\pm$ 0.2	6 $\pm$ 1
270	insufficient seed	0	16 $\pm$ 6
365	10 $\pm$ 9	lost due to mites	lost due to mites

## DISCUSSION

The data suggest that seed viability may decrease over time. Mean percentage of viable seeds tested using FDA was lower than germination using either symbiotic or asymbiotic methods, although tests using FDA did detect a gradual decline in viability. Difficulties with germination methods mean that results were not readily interpretable, although when combined, a trend of decreasing seed germinability may be apparent. Further work is clearly required to refine both symbiotic and asymbiotic techniques.

Differences between viability tests and germination support evidence in the literature. Viability tests using TTC (Harvais and Hadley 1967a, b; Hadley 1974) and FDA (A. Batty, Kings Park and Botanic Garden, pers. comm.) report a lack of correlation between germination and viability estimates and concluded that only germination gave a reliable estimate of viability. These results contradict Pritchard (1985), who achieved reliable viability estimates (using FDA) similar to germination.

One factor contributing to the discrepancy between germination and viability estimates may be testa permeability after surface sterilisation. Each seed batch may have different testa thicknesses that inhibit the uptake of viability stains and cause inaccurate estimates. Surface sterilisation was at one standard concentration, which may allow for stain uptake in some seeds but not others, since if the testa is thicker, surface sterilisation may not disrupt the testa sufficiently. Rasmussen (1995) suggested that surface sterilisation past its maximum limit may result in different viability tests overestimating germination and emphasised the importance of optimum surface sterilisation for uptake of viability stains.

Improvement to these tests may allow for more accurate estimates. Removal of the testa, as is done for TTC tests, may provide a more reliable estimate, and overcome problems associated with stain uptake due to surface sterilisation and testa permeability variables. However, this method physically damages the seed and is not desirable when dealing with threatened species where there is a limited seed supply.

Germination of *P. correctum* seeds also decreased with storage time, although storage conditions are likely to have affected the results. Due to the incompleteness of germination data caused by heavy mite infestation at 365 days, interpretations are limited. Nevertheless, seed did appear to lose germinability and by implication, viability, when stored in a domestic refrigerator without prior dehydration. This supports other research where lower germination for stored seeds compared to fresh seeds, have been reported (Ramsay and Stewart 1998). Consequently, use of fresh seed is more likely to yield better results for propagation unless facilities and staff are available to dry and store seed using standard protocols for germplasm storage (ANPCWG 1997).

The effectiveness of fungal cultures may also decrease over time, suggesting that using fresh cultures, or storing of cultures in liquid nitrogen is desirable. Although there was some germination, seeds did not develop past protocorm stage and were too small to observe peloton development. Maintaining fungal infection in developing protocorms may be important for ongoing cultivation, if cultivated populations are eventually reintroduced to sites.

Although mycorrhizal fungus has been isolated and seeds are viable, techniques for *ex-situ* cultivation, and requirements for successful reintroduction are entirely unknown, as is the case for much of the orchid flora of southeastern Australia. Work is urgently required in these areas, as translocation is likely to play a pivotal role in recovery of a number of Critically Endangered (*sensu* IUCN 1994) Orchidaceae in the future. The Tasmanian population of *P. correctum* was not known at the time this work began, and similar trials using material from these plants, which comprise a large seed resource, may be useful.

Attempts to cultivate *Prasophyllum* species have been mainly undertaken by amateur growers using asymbiotic methods, generally without success or at best have resulted in production of only a handful of individuals. However, fungal isolates have been shown to improve germination and/or progression through plantlet developmental stages, more rapidly than asymbiotic methods in a range of orchid species (Jusaitis & Sorensen 1994; Rasmussen 1995; A. Batty, Kings Park and Botanic Garden, unpub. data).

Mycorrhizal relationships and ecology, particularly in relation to fire, and conditions for seed germination and seedling establishment *in-situ* also require further investigation.

Isolation and consequentially the likelihood of a restricted gene pool at each of the populations suggests that the genetic potential of populations should be assessed. Variation in levels of seed viability between plants (Huynh 1999) indicates that outcrossing between Munro and Lindenow South populations may be advantageous to their long term survival.

## ACKNOWLEDGMENTS

The project benefited considerably as a result of advice and guidance from Associate Professor Ann Lawrie (RMIT, Melbourne), and from contributions by Mr Mark Clements and Ms Maggie Nightingale (Centre for Plant Biodiversity Research, Canberra). Funding was provided by the Australian Flora Foundation (project 97/98-14).

## REFERENCES

- Australian Network for Plant Conservation Working Group (1997) *Germplasm Conservation Guidelines for Australia*. Australian Network for Plant Conservation, Canberra.
- Coates, E, Lunt, I. and Wapstra, H. (1999) *Draft Recovery Plan 2000-2003 Prasophyllum correctum (Gaping leek-orchid)*. Unpublished report to Environment Australia. La Trobe University, Bundoora.
- Hadley, G. (1975) Organisation and fine structure of orchid mycorrhiza. In: *Endomycorrhizas* (Eds F. E. Sanders, B. Mosse and P. B. Tinker). pp. 335-367. Academic press, UK.
- Harvais, G. and Hadley, G. (1967a) The development of *Orchis purpurella* in asymbiotic and inoculated cultures. *New Phytologist* **66**, 217-230.
- Harvais, G. and Hadley, G. (1967b) The relation between host and endophyte in orchid mycorrhiza. *New Phytologist* **66**, 205-215.
- Huynh, T. (1999) *In vitro propagation of the endangered Gaping Leek Orchid, Prasophyllum correctum* Honours thesis, Bachelor of Applied Science, RMIT University, Melbourne.
- IUCN (1994) *IUCN red list categories*. IUCN Species Survival Commission, Kew.
- Jusaitis, M. and Sorensen, B. (1993) Germination of *Pterostylis arenicola* - an endangered greenhood from South Australia. *The Orchadian* **11**, 18-22.
- Jusaitis, M. and Sorensen, B. (1994) *Conservation Studies on Endangered Plant Species from South Australia's Agricultural Regions*. Black Hill Flora Centre, Botanic Gardens of Adelaide, South Australia.
- Pritchard, H. W. (1985) Determination of orchid seed viability using fluorescein diacetate. *Plant Cell and Environment* **8**, 727-730.
- Ramsay, M. M. and Stewart, J. (1998) Re-establishment of the lady's slipper orchid (*Cypripedium calceolus* L.) in Britain. *Botanical Journal of the Linnean Society* **126**, 173-181.



- Rasmussen, H. N. (1995) *Terrestrial orchids from seed to mycotrophic plant*. Cambridge University Press, Cambridge, Great Britain.
- Van Waes, J. M. and Deberg, P. C. (1986) 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.
- Warcup, J. H. and Talbot, P. H. B. (1966) Perfect states of some Rhizoctonias. *Transactions of the British Mycological Society* **49**, 427-435.