Ex situ conservation of *Caladenia* - photoautotrophic micropropagation of *Caladenia*

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Acknowledgement

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Contents

Acknowledgement

Summary 1

1. Introduction 2

2. Materials and methods 3

2.1 Choice of species
2.2 Origin of plant and fungal material
2.3 Experimental design
2.3.1 Germination
2.3.2 In vitro *plantlet growth rate*
2.3.3 1 In vitro *plantlet anatomy*
2.3.4 Effect of photoautotrophic conditions on the growth of mycorrhizal fungi

3. Results 6

3.1 Germination
3.2 *In vitro* plantlet growth rate
3.3 *In vitro* plantlets anatomy
3.4 *Effect of photoautotrophic conditions on the growth of mycorrhizal fungi*

4. Discussion 10

References 12

Appendix

Summary

Many species of Australia's rich terrestrial orchid flora are under threat, and active intervention is required to ensure their continued existence. The genus *Caladenia,* which includes the Spider Orchids, has a particularly large number of species that are threatened. Plantlets of *Caladenia* species produced through *in vitro* symbiotic germination for reintroduction into natural habitats, have less than 10 percent survival ex vitro, which may be due to altered anatomy, physiology and biochemistry caused by the *in* vitro environment. Photoautotrophic micropropagation with its raised carbon dioxide and light levels, has been found to increase growth rates for the epiphytic orchid, *Cymbidium* (Kozai *et al.,* 1987) and for *Eucalyptus camaldulensis,* to increase both growth rates and plantlet survival *ex vitro*. In this study, the germination and growth of photoautotrophically micropropagated *Caladenia tentaculata* plantlets was compared with plantlets produced in the usual non-photoautotrophic way.

Photoautotrophic conditions did not result in improved symbiotic germination and growth of the common orchid species *C. tentaculata,* and therefore may not be suitable for threatened *Caladenia* species. It was found for *C. tentaculata* that optimum germination occurred with low light and ambient carbon dioxide levels rather than high light and raised carbon dioxide concentrations, and light and raised carbon dioxide levels inhibited the growth of the associated *Caladenia* mycorrhiza.

1. Introduction

The orchid flora in Australia is particularly rich with over 1000 species. The majority of species are terrestrial and these are found predominantly in the south-eastern and south-western parts of Australia. Many species are under threat, and active intervention is required to ensure their continued existence.

The genus *Caladenia,* which includes the Spider Orchids, has a particularly large number of species that are threatened. The Royal Botanic Gardens has established techniques to enable the *in vitro* symbiotic germination of *Caladenia* species as a part of an integrated conservation project with the Department of Conservation and Natural Resources. The production of plantlets, ultimately destined for reintroduction into their natural habitat, has been possible with these techniques. However, the survival rate of plantlets *ex vitro* has been less than ten percent. Many studies offer explanations for poor survival rates of micropropagated plants (Preece & Sutter, 1991), and these include the effect the *in vitro* micropropagation environment has on the anatomy, physiology and biochemistry of the plant.

Changing the *in vitro* environment that plantlets are produced in offers a solution to this problem. Kozai's (Kozai, 1991; Kozai *et al.,* 1994a; Kozai *et al.,* 1994b) development of photoautotrophic micropropagation for the production of plantlets has shown that this method's raised carbon dioxide and light levels, and the lack of sucrose in the medium, increases the growth rate of plantlets. Recent experimentation by Kirdmanee *et al.,* (1995) has shown that 100% of *Eucalyptus camaldulensis* plantlets micropropagated photoautotrophically with vermiculite as a supporting medium survived when moved from the *in vitro* to the *ex vitro* environment compared with 55% survival for plantlets produced in nonphotoautotrophic conditions using the usual agar as the support medium. It is thought that that the higher light and carbon dioxide levels, and the lack of sucrose in the medium of the photoautotrophic micropropagation method, stimulates photosynthesis in the plantlet and produces an anatomy, physiology and biochemistry more equivalent to plants growing in nursery or field conditions. The vermiculite provides a better support medium for the development of roots.

The aim of this study was to determine whether *Caladenia* plantlets produced in a photoautotrophic micropropagation environment have a higher percentage germination and improved growth, compared to plants grown under heterotropic conditions

2. Materials and Methods

2.1 Choice of species

The species *Caladenia tentaculata* was chosen as it is widespread, plant material was readily available, and it was considered to be an appropriate model for threatened species without adding additional threats to them.

2.2 Origin of plant and fungal material

Seed from a *Caladenia tentaculata* plant cultivated in the Royal Botanic Gardens Nursery since 1985 was used. The seed was collected in 1993 and stored at 4ºC until used.

Mycorrhizal fungus was isolated from the collar of a *C. tentaculata* plant collected from Portland in 1995 and stored at Kings Park and Botanic Garden. The fungus was cultured on Fungal Isolating Medium (FIM).

2.3 Experimental design

2.3.1 Germination

Germination was recorded for four treatments, Treatment 1, the control, reproducing the standard *in vitro* micropropagation environment of uncontrolled $C0_2$ (sealed flask), and low light (30 µmol.$m^{-2}s^{-1}$). Photoautotrophic conditions were provided in Treatment 4, with elevated $C0_2$ and high light (a raised photosynthetic photon flux (PPF) of 230 µmol.$m^{-2}s^{-1}$). The remaining two treatments provided partial photoautotrophic conditions with carbon dioxide being at least maintained at ambient atmospheric levels or elevated. Table 1 summarises the treatments.

Table 1: Treatments to determine the effect of *in vitro* conditions on the germination of *Caladenia tentaculata* seed

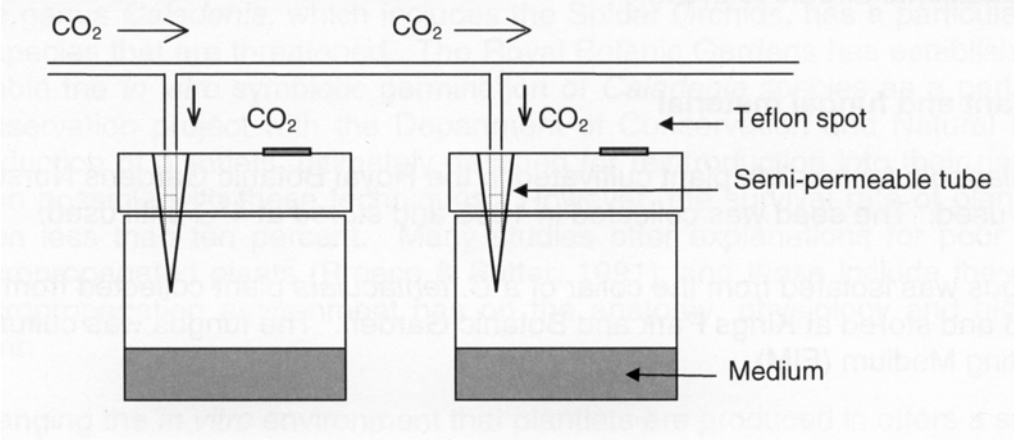
Treatment	$C0_2$ level in flask[1]			Light leve1[2]
	$C0_2$ not controlled (sealed flasks)	Ambient $C0_2$ (350ppm)	Elevated $C0_2$ (1OOO ppm)	
1 (Control)	+			Low
2		+		Low
3			+	Low
4			+	High

Note 1: Ambient CO_2 350 ppm; elevated $C0_2$ 1000 ppm
Note 2: Low light 30 µmol.$m^{-2}s^{-1}$; high 230 µmol.$m^{-2}s^{-1}$

Maintenance of ambient $C0_2$ in treatment 2, and elevation of $C0_2$ in treatments 3 and 4 was achieved by the flow of air or $C0_2$ respectively through semipermeable silicon tubing installed in each flask in such a way as to retain sterile conditions (Figure 1). The flask lid also had a 1O mm teflon spot to allow gaseous exchange. The air or $C0_2$ was supplied to the flasks through $C0_2$ impermeable tygon and nylon tubing. The concentration of the $C0_2$ in each flask was maintained at 1000 ppm by regulating the flow rate through the silicon tubing, and it was monitored by sampling the flask atmosphere and measuring the $C0_2$ concentration in a gas analyser against an air standard.

Low light levels were provided using OSRAM 36 watt cool white fluorescent tubes and high light levels were provided using Radium HRI-T 400 W/D metal halide lights. Light levels were measured using a Li-Cor Ll 250 Integrating Quantum Photometer.

Figure 1: Diagram representing the method for raising carbon dioxide levels in the flasks



Surface-sterilised *C. tentaculata* seeds on sterile Whatman No. 1 filter paper squares (25mrn x 25mm) were placed on oats agar (2.5gL^{-1} finely ground oats; 10 gL^{-1} agar; pH 5.5) in the sterile flasks. Four agar blocks (4mm x 4mm x 4mm) of the mycorrhizal fungi isolated from C. *tentaculata* were placed on each edge of the filter paper square.

Flasks were incubated at 18ºC in the dark for 28 days and then exposed to 12 hours of light per 24 hour period.

The number of germinated seeds was recorded and percentage germination calculated for the four treatments.

2.3.2 In vitro *plantlet growth rate*

Equal numbers of *Caladenia tentaculata* plantlets that had been germinated symbiotically with fungus in non-photoautotrophic conditions were placed in either non-photoautotrophic or photoautotrophic conditions 77 days after germination was initiated. Additional leaf growth for the plantlets under the two *in vitro* conditions was measured after a further 26 days of incubation.

2.3.3 In vitro *plantlet anatomy*

Plantlet anatomy was observed using light microscopy or scanning electron microscopy using a Hitachi S570 Scanning Electron Microscope (SEM).

2.3.4 *Effect of photoautotrophic conditions on the growth of mycorrhizal fungi*

As germination of Australian terrestrial orchid seed relies on mycorrhizal fungi, two trials were undertaken to determine the effect of photoautotrophic conditions on the growth of the fungus.

In the first trial, the orchid mycorrhizal fungi was grown for 42 days on oats agar medium *in vitro* with photoautotrophic light levels (230 µmol.$m^{-2}s^{-1}$) and without light, and in sealed and unsealed flasks (that is, with and without passive aeration). Growth was measured by colony diameter.

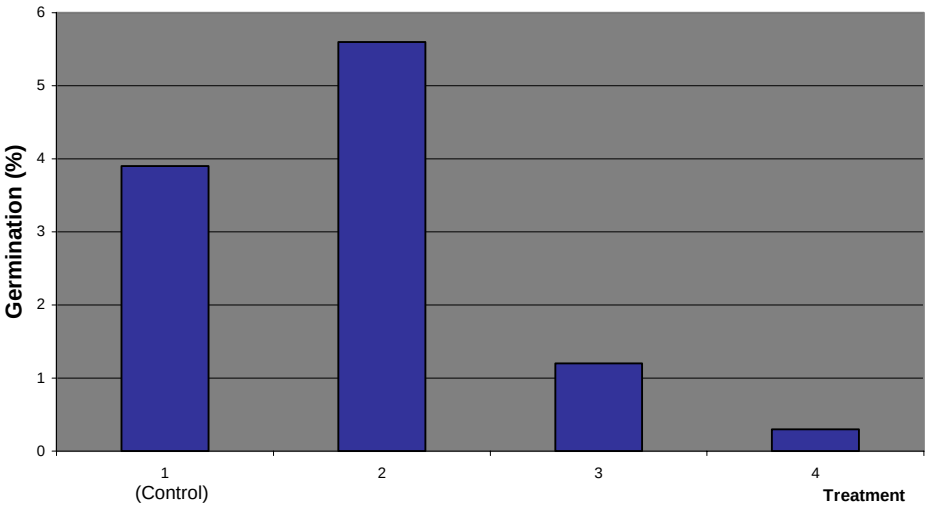
In the second trial, the orchid mycorrhizal fungus was grown for 28 days on oats agar medium *in vitro* with exposure to elevated $C0_2$ (1000 ppm) and no light, low light (30 µmol.$m^{-2}s^{-1}$) or high light levels (230 µmol.$m^{-2}s^{-1}$). Growth was measured by colony diameter.

3. Results

3.1 Germination

The percentage germination of *Caladenia tentaculata* was highest when seeds were exposed to ambient levels of carbon dioxide and low light levels *in vitro* (Treatment 2). Treatments exposed to elevated carbon dioxide levels, both with low and high light levels, had the lowest percentage germination (Treatments 3 & 4). Figure 2 summarises the germination results for the four treatments.

Figure 2: Effect of *in vitro* conditions on the germination of *Caladenia tentaculata* seed



3.2 *In vitro* plantlet growth rate

Established non-photoautotrophic *Caladenia tentaculata* plantlets (77 days post the initiation of germination) incubated over an additional 26 day period *in vitro* under nonphotoautotrophic conditions grew more rapidly than plantlets grown over the same 26 day period under photoautotrophic conditions (raised light and $C0_2$ levels). Leaves of nonphotoautotrophically grown plantlets had grown an average of 5.0 mm in length compared with photoautotrophically grown plantlets that had grown an average of 3.1 mm.

3.3 *In* vitro plantlets anatomy

Although the growth rate of plantlet leaves was on average greater in plantlets that remained in non-photoautotrophic *in vitro* conditions, the growth of the protocorm was slightly greater under photoautotrophic conditions. In addition, cells of the parenchymal tissue of the protocorm of photoautrophic plantlets developed red-violet pigmentation within six days of transfer to the photoautotrophic conditions (Figure 3). Protocorm

Figure 3: Cells of the protocorm of *Caladenia tentaculata* plantlets grown under photoautotrophic conditions developed red-violet pigmentation (V).
Rhizoids (R) and shoot (S) are indicated

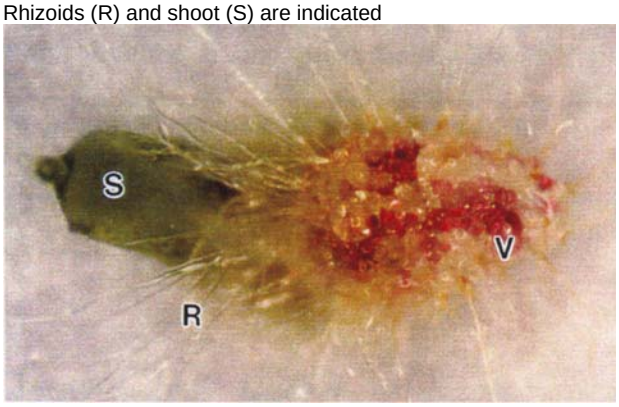


Figure 4: Cells of the protocorm of *Caladenia entaculata* plantlets grown under non- photoautotrophic conditions remain without pigmentation. Rhizoids (R) and shoot (S) are indicated.

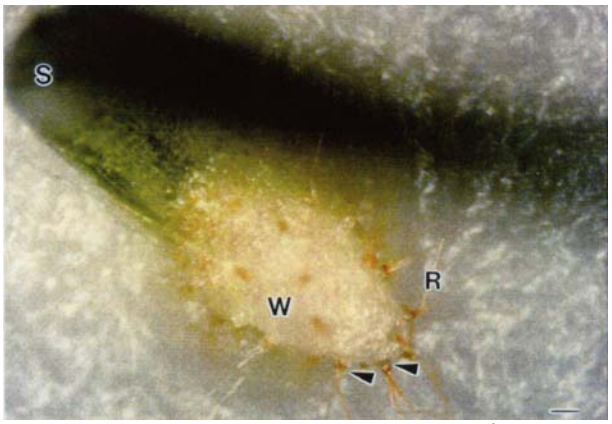


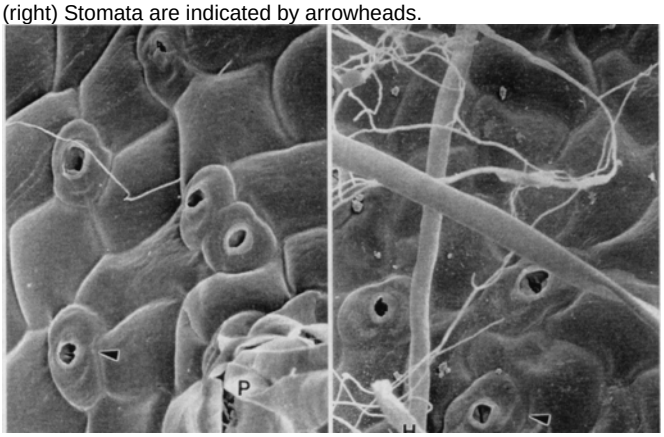
Figure 6: Scanning electron micrograph of a *Caladeniaentaculata* plantlet. The leaf arose from a sheathing leaf base.

Figure 7: Scanning electron micrograph of a *Caladenia tentaculata* plantlet. The hairs on the leaf surface.

parenchymal tissue of non-photoautotrophically grown plantlets remained white without pigment (Figure 4).

Stomata were numerous on the surface of the protocorms of both photoautotrophically and non- photoautotrophically incubated plantlets, and their structure appeared similar in both treatments (Figure 5).

Figure 5: Scanning electron micrographs of plantlet protocorms indicating similar stomatal structure for plantlets grown under photoautotrophic conditions(left) and non-photoautotrophic conditions (right) Stomata are indicated by arrowheads.



Leaves of the plantlets arose from a sheathing leaf base of the protocorm (Figure 6).

Hairs were observed on the leaf surface (Figure 7).

The cuticle of the leaf had a regularly patterned surface (Figure 8). Closed stomata were present for both non-photoautotrophic and photoautotrophic treatments at the tip, on the ventral surface, and on the sides of the leaves (Figure 9), but were absent on the dorsal surface.

Figure 8: Scanning electron micrograph of a *Caladenia tentaculata* plantlet leaf cuticle.

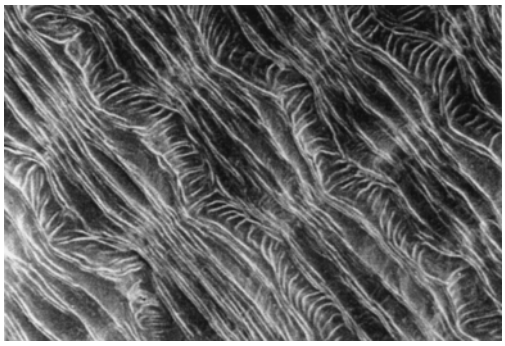
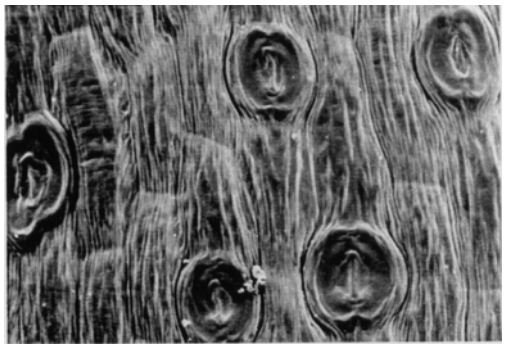


Figure 9: Scanning electron micrograph of a *Caladenia tentaculata* plantlet leaf side with closed stomata.



3.4 Effect of photoautotrophic conditions on the growth of mycorrhizal fungi

The fastest mycorrhizal growth, as measured by colony diameter, was in sealed flasks incubated without light, where the mean colony diameter reached 65 mm in 42 days. Introducing high light (230 µmol.$m^{-2}s^{-1}$) to sealed flasks or to unsealed flasks resulted in a reduced mean colony diameters of 44 mm for sealed flasks (68% smaller than sealed flasks incubated without light) and 28 mm for unsealed flasks (74% smaller than colonies incubated in unsealed flasks without light) (Figure 10).

The inhibitory effect of light on colony growth is also clearly indicated in the second trial where the mycorrhizal fungus was incubated with a raised $C0_2$ atmosphere (1000 ppm) and with no, low (30 µmol.$m^{-2}s^{-1}$) or high light levels (230 µmol.$m^{-2}s^{-1}$). After 28 days the mean colony diameter of the high light treatment (230 µmol.$m^{-2}s^{-1}$) was less than 15% that of the dark treatment (Figure 11).

Allowing gaseous exchange between the flask and the normal atmosphere reduced mycorrhizal growth. For flasks incubated in either the light or the dark, mean colony diameters were smaller when flasks were unsealed (Figure 10). For flasks incubated in the dark, the mean colony diameter of sealed flasks was 1.7 times larger than for the unsealed flasks. For flasks incubated in the light (230 µmol.$m^{-2}s^{-1}$), the mean colony diameter for sealed flasks was 1.6 times larger than for the unsealed flasks.

Figure 10: Growth of orchid mycorrhizal fungi under different *in vitro* environmental conditions

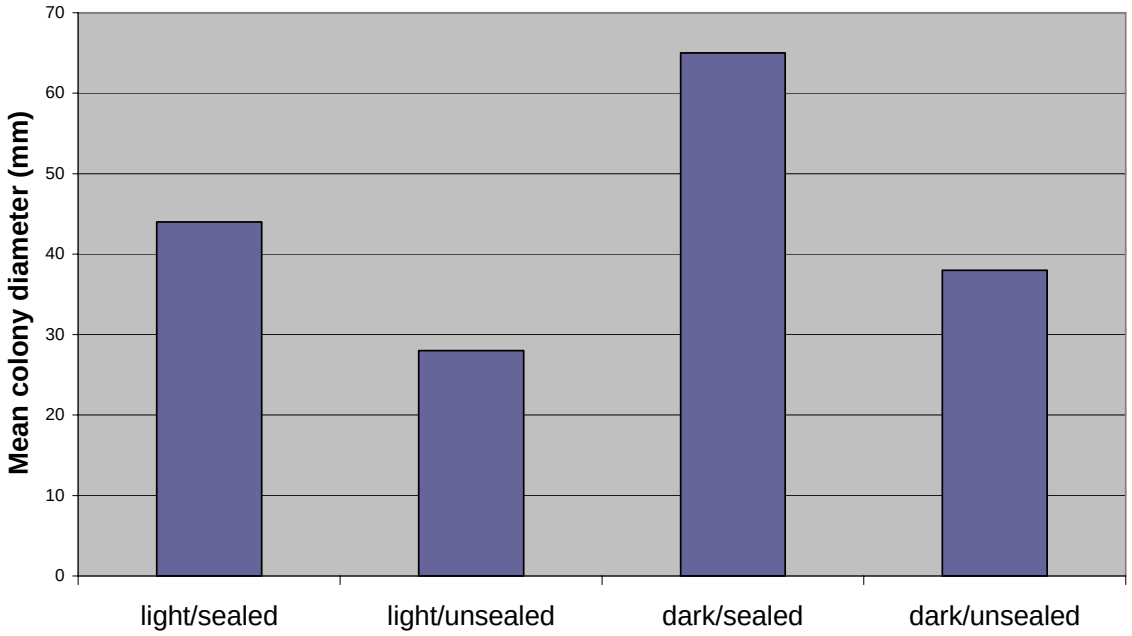
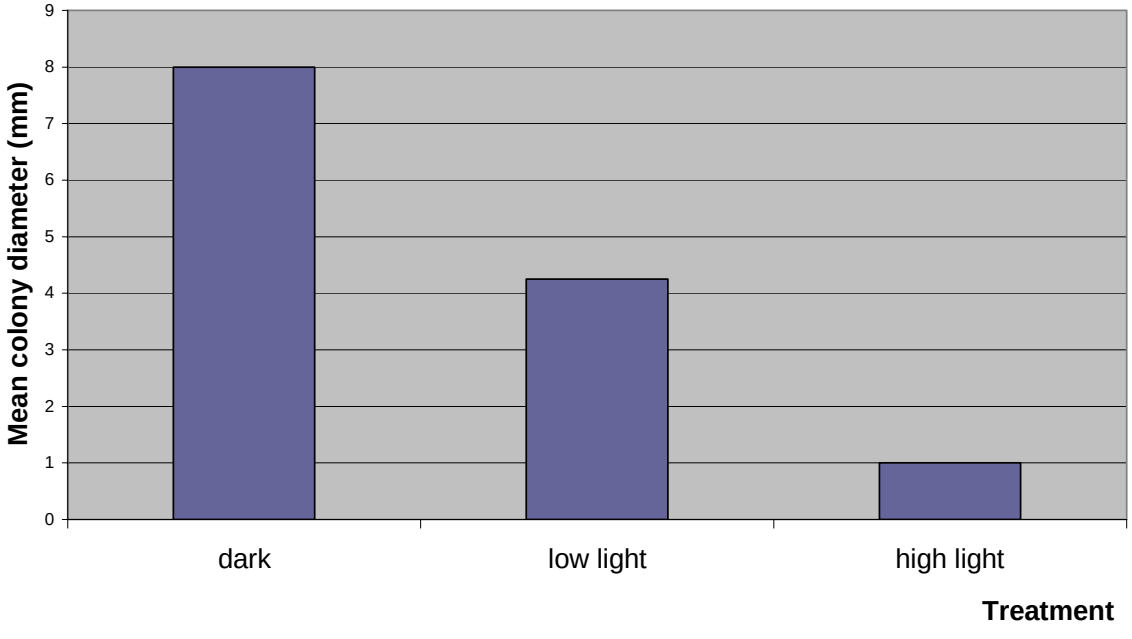


Figure 11: Growth of orchid mycorhizal fungi in the presence of elevated carbon dioxide and different light levels



4. Discussion

It has been shown that photoautotrophic *in vitro* conditions provide an improved micropropagation environment and thus increased growth rates for the epiphytic orchid, *Cymbidium* (Kozai *et al*., 1987). Growth rates have also been increased for non-orchid species such as *Eucalyptus camaldulensis* in photoautotrophic *in vitro* conditions, and the improved plantlet quality has also increased survival rates during the acclimatisation period after deflasking (Kirdmanee et al., 1995).

Improved germination rates and in vitro growth of the Australian terrestrial orchids belonging to the genus *Caladenia* would assist the conservation program for a number of threatened species in the genus by providing an increased number of plants that can be used for reintroduction into natural habitats. However, the trials undertaken in the current project did not indicate that photoautotrophic conditions can successfully be applied to the common orchid species *Caladenia tentaculata,* and therefore may not be suitable for threatened *Caladenia* species. It was found with *C. tentaculata* that

- optimum germination occurred with low light and ambient carbon dioxide levels
- the growth rate of seedlings was slightly greater in non-photoautotrophic conditions
- light inhibited the growth of the Caladenia-associated mycorrhiza, and
- the growth rate of the Caladenia-associated mycorrhizal fungus was greater in sealed flasks with non-photoautotrophic conditions than in aerated flasks.

The special relationship that Australian terrestrial orchids have with mycorrhizal fungi dictates that the *in vitro* conditions provided need to be suitable for the growth of the fungus as well as the orchid plant itself. It is clear from these trials that photoautotrophic conditions do not favour the growth of the *Caladenia tentaculata*-associated fungus. The growth rate of the fungus slows with increasing light intensity, and therefore the high light of photoautotrophic conditions means slower mycorrhizal growth (Figure 11). This may explain, at least in part, the poor response of *Caladenia tentaculata* to photoautotrophic conditions, although it is not possible to determine from the current trials to what extent the effect of high light levels on fungal growth, causes lower germination and growth rates.

In addition to higher light intensities, elevated carbon dioxide levels also appear to play a negative role in the *in vitro* symbiotic germination of *Caladenia tentaculata*. Carbon dioxide levels elevated above normal atmospheric conditions, reduced the in vitro percentage germination of *C. tentaculata* seeds compared with those in aerated flasks where carbon dioxide levels remain closer to ambient atmospheric carbon dioxide concentrations (Figure 2). In sealed flasks too, where respiring explants can raise carbon dioxide concentrations above normal atmospheric levels during dark periods (Fujiwara, 1987), a lower percentage germination was observed compared with the aerated flasks, although the effect was not as marked. Aerating the flasks, that is providing ambient atmospheric carbon dioxide concentrations, resulted in the highest germination.

The red-violet pigmentation that developed in protocorm tissue of plantlets placed into photoautotrophic conditions may be an indication that these conditions cause stress to the orchid plant. UV light is known to cause the synthesis of pigments such as anthocyanins that help protect plant tissue from the harmful effects of the UV rays, and it has been shown (Rudat & Goring 1995) that the formation of betacyanin-containing red-violet pigments can be induced in *Chenopodium album* in vitro by exposing the explants with UV

light. UV light emitted from the metal halide lights used for these trials is a likely cause for the pigmentation that developed in the *Caladenia tentaculata* protocorms in the high light photoautotrophic conditions. Using alternative low UV emitting light sources or UV filters may be more appropriate for the *in vitro* symbiotic germination and growth of C. *tentaculata.*

Implications for conservation

These trials indicate that photoautotrophic micropropagation is not an appropriate method for the terrestrial orchid *Caladenia tentaculata,* and is perhaps unsuitable for other species of Australian terrestrial orchids including those that are threatened. Both raised light and carbon dioxide levels adversely affected the germination of the *C. tentaculata* seed, and the growth of the associated mycorrhizal fungus.

However, the trials also indicate that some modifications to the usual non-photoautotrophic *in vitro* conditions may improve germination and growth of symbiotically germinated *Caladenia* species. In particular, it is appropriate to aerate the flasks to provide ambient atmospheric carbon dioxide concentrations in the flask rather than the variable atmospheric carbon dioxide levels of sealed flasks.

Light levels need to be kept low for the fungus, but not necessarily for the *Caladenia* itself, although using low UV-emitting light sources and/or protecting the protocorm from light may be important. In nature, the fungi associated with orchids naturally grow through the soil, and are exposed to little or no light. An *in vitro* system where the fungus and seed are protected from high light levels initially, but allows the leaf of an older *Caladenia* protocorm to grow into higher light levels, may stimulate higher photosynthetic levels and improved growth without negatively impacting on germination and growth. A thin layer of sterile coco-peat or similar material covering the seed, fungus and protocorms on the growth substrate may be suitable for this purpose.

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Appendix

Fungal Isolating Medium (FIM)

Modified from the method of Clements & Ellyard (1979)

Chemical		Mass per litre
Calcium nitrate	$Ca(N0_3)_2 \cdot 4H_2O$	0.5g
Potassium phosphate	KH_2P0_4	0.2g
Potassium chloride	KCI	0.1g
Magnesium sulphate	$MgSO_4 \cdot 7H_2O$	0.1g
Yeast extract		0.1g
Sucrose	$C_6H_{12}0_6$	5.Og
Streptomycin sulphate		0.05g
Agar		8g
pH		4-5